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Original research paper

EFFECTS OF SPONTANEOUS AND INOCULATED FERMENTATION ON THE TOTAL PHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY OF CABERNET SAUVIGNON WINES AND FERMENTED POMACE

Nikolina M. Živković[1], Uroš D. Čakar[2], Aleksandar V. Petrović[*1]

[1]University of Belgrade, Faculty of Agriculture, Department of Food Technology and Biochemistry, 11080 Zemun, Nemanjina 6, Serbia
[2]University of Belgrade, Faculty of Pharmacy, Department of Bromatology, 11000 Belgrade, Vojvode Stepe 450, Serbia

Abstract: The total phenolic content and antioxidant activity of wine and fermented pomace (FP) from Cabernet Sauvignon grapes harvested at the stage of full ripeness were evaluated by spectrophotometric analysis. Wine and pomace were obtained after prolonged maceration during spontaneous and inoculated fermentation of fully ripe grapes. Three individual vinifications were inoculated with the following commercial yeasts: BDX (Lallemand, Montréal, QC, Canada), FX10 (Laffort, Bordeaux, France) and Qa23 (Lallemand, Montréal, QC, Canada). For each vinification, maceration lasted 0, 3, 5, 7, 14 and 21 days, respectively. The total phenolic content was determined spectrophotometrically using the Folin-Ciocalteu method. Two different methods were used to evaluate the antioxidant activity of the wine and pomace samples: the Ferric Reducing Activity of Plasma (FRAP) and the Trolox Equivalent Antioxidant Capacity (TEAC). The use of a winemaking process that included different maceration times and inoculation with yeasts, as well as spontaneous fermentation, significantly modulated the total phenolic content of the obtained wines and FP. This study could provide a good basis for the practical application and obtaining wines with a high content of phenolic compounds and antioxidant properties.

Key words: *phenols, fermentation, yeasts, red wine, grape pomace, antioxidant activity*

INTRODUCTION

Polyphenols represent a diverse group of very important molecules in red wines that are responsible for wine quality and depend on their content in the grapes from which the wines are made, on the extraction from the grapes during vinification and on the reactions that take place during the maturation of the wine. Winemaking techniques have a major influence on polyphenols, as they can lead to very different final levels of polyphenols in the wine (Budic-Leto, Garcin, Lovrić & Vrhovsek, 2008).

Quality wines usually contain a high content of phenolic compounds, which make an important contribution to the taste, colour, mouthfeel (astringency and bitterness) and health-promoting

Corresponding author: Phone: +381641660637 Fax: +381112199711
E-mail address: aleksandar.petrovic@agrif.bg.ac.rs

properties (antioxidants, anti-inflammatory substances, etc.) of the wine (Gao, Zietsman, Vivier & Moore, 2019). The polyphenols are released when the cells of the grapes are physically broken (grape crushing) or the cell walls are degraded during maceration, but even with prolonged maceration, only a fraction of the total polyphenols present in the grapes are extracted (Bindon, Madani, Pendleton, Smith & Kennedy, 2014; Lisov et al., 2020).

It is very important to point out appropriate winemaking protocols that include the use of different yeast strains. During alcoholic fermentation, yeasts carry out the biotransformation of grapes into wine components by converting sugars into ethanol and other metabolites. Numerous studies have discussed the effects of yeast on phenolic composition (Caridi, Cufari, Lovino, Palumbo & Tedesco, 2004), primarily through adsorption to the cell wall. This is particularly true for anthocyanins (Morata et al., 2003) and for changes in the antioxidant capacity of wines (Brandolini, Fioure, Maietti, Tedeschi & Romano, 2007). The addition of commercial yeast strains (*Saccharomyces cerevisiae*) makes fermentation safer and easier to control and is the most commonly chosen yeast.

On the other hand, there is spontaneous fermentation, the dynamics of which are often unpredictable and can introduce undesirable characteristics into the wine that can even lead to spoilage. However, wild yeasts are capable of producing high-quality wines with unique aromas (Samoticha, Wojdyło, Chmielewska & Nofer, 2019). In addition to the selection of yeasts, the maceration process is an important step in the production of red wine, the effects of which influence the quality of the wine (Francesca et al., 2014).

Besides the phenols in wine, grape pomace has also been investigated in recent years as a by-product that still contains considerable amounts of polyphenolic compounds. The ability to extract polyphenols from the grape skin varies from variety to variety.

The extraction of polyphenols from the berry into the wine is essentially a diffusion process and therefore depends on the molecular size and type of polyphenol, maceration time, concentration gradient, cell permeability, ethanol production and surface area of the concentration gradient (Gao et al., 2019).

In particular, the phenolic compounds contained in all parts of the grapes, which are extracted into red wines during vinification, and t he fermented grape marc are a source of antioxidants. Thus, the "bioactive" properties of wine phenols can help to reduce the incidence of atherosclerosis, cancer, neurodegenerative and heart diseases (Brandolini et al., 2007).

This study aimed to provide technical and experimental evidence of the influence of the dynamics of extraction of phenolic compounds on the total phenolic content and antioxidant properties of wine and FP. The wines and FP analyzed in this study were obtained after spontaneous and inoculated fermentation during which prolonged maceration was carried out.

MATERIAL AND METHODS

Plant Material

The Cabernet Sauvignon grape variety used for this study comes from the Belgrade wine-growing region, the experimental vineyard “Radmilovac” of the Faculty of Agriculture of the University of Belgrade (44°45′23.8″ N 20°34′57″ E). In terms of phytosanitary state, 100% healthy grapes were used for all experiments.

Winemaking

The experiment focused on vinification from fully ripe grapes during spontaneous and inoculated fermentation using different maceration times (Fig. 1). During alcoholic fermentation, maceration was carried out by vinification at a temperature of 25 ± 2 °C, with the grapes being mechanically punched down twice a day. Before alcoholic fermentation, the grapes were inoculated with the *Saccharomyces cerevisiae* yeast strain and $K_2S_2O_5$ (10 g per 100 kg) was added. Three individual vinifications were inoculated with the following commercial yeasts: BDX (Lallemand, Montréal, QC, Canada), FX10 (Laffort, Bordeaux, France) and Qa23 (Lallemand, Montréal, QC, Canada). For each vinification, maceration lasted 0, 3, 5, 7, 14 and 21 days, respectively (Fig. 1). In addition to these vinifications, wine samples were also obtained after spontaneous fermentation (SF) without the addition of yeast strains, as well as the same maceration duration as inoculated fermentation (0, 3, 5, 7, 14 and 21 days).

The control wine samples were those obtained according to the technology of white wines

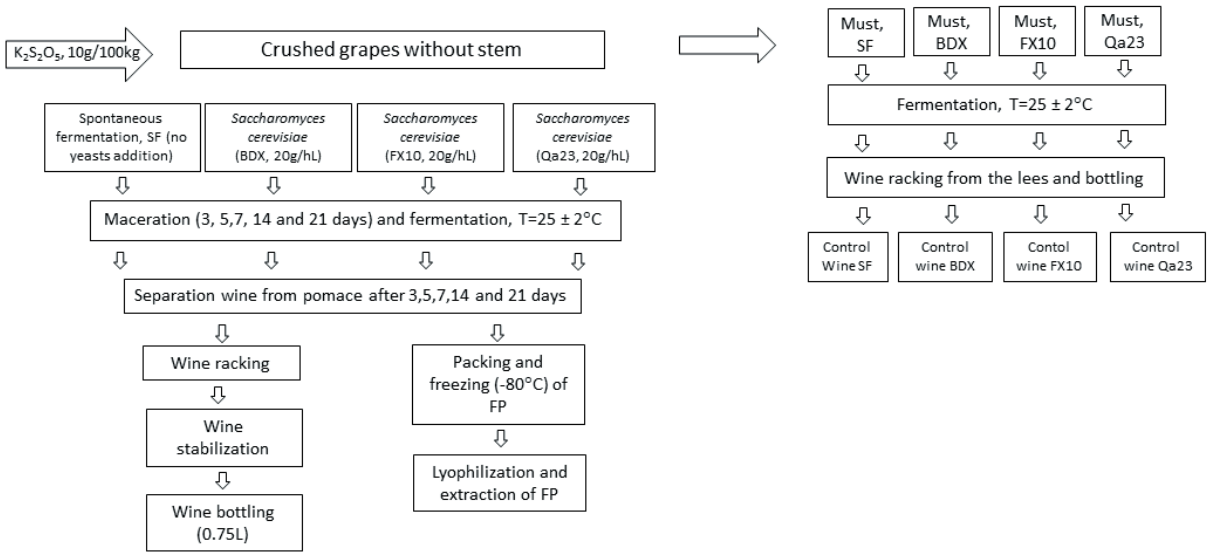


Figure 1. Description of winemaking

(without maceration) for inoculated and spontaneous fermentation at 0 days. Wine and FP samples were obtained from all four vinifications (three with inoculated yeast and SF) and used for further analysis.The pomace sample obtained after pressing the grapes (not fermented) served as a control for all four vinifycations in which the phenolic compound content was analyzed. After a certain maceration time, the wine samples were separated from the solid components of the grapes and stored in glass jars for stabilization. After one week, the wines were racked and bottled in 0.75-litre glass bottles. The wines obtained were stored in a wine cellar at a temperature of 14 °C until they were prepared for analysis.

Sample Preparation

The wine samples were filtered through 0.45 µm filters before analysis. Fermented pomace samples were prepared according to the following procedure: The fermented pomace samples were lyophilized; The lyophilization process was carried out using a Christ Alpha 2-4 LD plus laboratory freeze dryer (Osterode am Harz, Germany) for 72 hours at a temperature of −80 °C and a pressure of 0.008 mbar; For further extraction, 2.5 g of each lyophilized sample was used; The extraction was carried out with a methanol/water mixture (1:1) for 45 minutes in a Bandelin SONOREX SUPER ultrasonic bath RK 514 BH (Berlin, Germany). The filtrates obtained were evaporated in a rotary vacuum evaporator IKA RV 10 basic (Staufen, Germany) until dryness was reached. The temperature during evaporation was up to 35 °C to preserve the thermolabile phenolic compounds of the sample. The rotation speed of the vacuum evaporator was adjusted by decreasing the filtrate volume. The obtained dry extracts were weighed and dissolved in 12% ethanol (imitation wine) and used for further analysis.

Total Phenolic Content (TPC)

The total phenolic content (TPC) in wine and pomace samples was determined by the Folin–Ciocalteu (FC) method using gallic acid as a standard (Tanner & Brunner, 1979). Firstly, all wine and pomace samples were diluted with distilled water, 1:5 and 1:10. Briefly, 1 ml of diluted sample mixed with 75 ml of distilled water, was added in a volumetric flask (100 ml). After 3 min, 5 ml of FC reagent was added. Then, 10 ml of saturated sodium carbonate solution was added, followed by filling the volumetric flask with distilled water to the mark.

The reaction mixture was incubated at room temperature for 60 min, and its absorbance was measured at 720 nm using a UV-Vis double beam spectrophotometer (HALO DB-20; Dynamica GmbH). The TPC was determined using a calibration curve prepared with gallic acid. The values were reported as mg of gallic acid equivalent (GAE), referring to the gallic acid standard curve, and the results were expressed in mg of GAEs per litre of wine (mg GAE/L) and mg GAE/kg of fermented pomace. All samples were measured in triplicate.

Ferric Reducing Activity of Plasma (FRAP)

The antioxidant activity of the wine samples and the pomace extract was determined using the FRAP method. The FRAP assay depends on the conversion of the ferric tripyridyltriazine (Fe(III)-TPTZ) complex to ferrous tripyridyltriazine (Fe(II)-TPTZ) by a reductant at low pH. Fe(II)-TPTZ had an intense blue colour and was monitored at 593 nm. The FRAP working solution was prepared as a mixture with acetate buffer (pH = 3.6), TPTZ solution, and $FeCl_3 \cdot 6H_2O$ solution (10:1:1). Furthermore, 75 µl of diluted wine/ pomace samples were mixed with 2.25 ml of FRAP working solution. After reacting for 6 min at 37°C, the absorbance was measured at 593 nm using an Ultraviolet-Visible double beam spectrophotometer (HALO DB-20; Dynamica GmbH). The results are expressed in mmol Fe^{2+}/L for wine and mmol Fe^{2+}/kg for FP, according to a pre-made calibration curve (100–1000 µmol/l were concentrations of Fe(II)) (Benzie & Strain, 1996). All samples were measured in triplicate.

Trolox Equivalent Antioxidant Capacity (TEAC)

The TEAC test is based on the reduction of the ABTS cation radical and was performed according to the method described by Re et al. (1999), but with slight modifications. In brief, ABTS radical cations were generated by reacting aqueous ABTS with potassium persulfate and maintaining the mixture in the dark at room temperature for at least 12 h before use. Afterwards, the absorbance of the ABTS solution was set to 0.70 ± 0.02 at 734 nm by adding phosphate buffer. Then, 30 µl of appropriately diluted wine samples were mixed with 3 mL of ABTS solution.

After reacting for 7 min, the absorbance was measured at 734 nm using a UV-Vis double beam spectrophotometer (HALO DB20; Dynamica GmbH). Antioxidant activity was calculated by decreasing absorbance and expressed as mmol Trolox equivalents per liter of wine or kg of fermented pomace (Trolox mmol/L or mmol/kg). All samples were measured in triplicate.

Statistical analysis

Independent samples t-test was used to test differences between groups. All p values less than 0.05 were considered significant. Statistical data analysis was performed using IBM SPSS Statistics 22 (IBM Corporation, Armonk, NY, USA). Linear regression correlation analysis was obtained with Origin Pro 8 (OriginLab, Northampton, MA, USA; 2008).

RESULTS AND DISCUSSION

TPC during spontaneous and inoculated fermentation in wine

During spontaneous fermentation, an increase in the total phenol content was observed up to the 21st day of maceration (Fig. 2). When inoculated with the selected wine yeast Qa23, the total phenolic content increased until the 19th day of maceration and decreased thereafter. The use of two other wine yeasts, BDX and FX10, resulted in maximum extraction on the 16th day of maceration and fermentation. This behaviour of total phenolic content during prolonged maceration and inoculated fermentation was caused by condensation, polymerization, degradation and adsorption on the yeast cells. The lowest concentration of total phenolic content was found in the control wine samples. Damijanić, Staver, Kovačević Ganić, Bubola and Palman (2012) analysed the dynamic extraction of phenolic compounds from the Teran grape variety during the 21-day maceration and found the highest content on day 17.

The maceration time had a significant influence on the total phenolic content of all the wine yeasts used. A difference was found between the content in all control samples (SF, BDX, FX10 and Qa23) and the wines with longer maceration ($p \leq 0.05$). The effect of maceration duration on total phenolic content in Karaoglan wine was significant ($p < 0.05$) and the concentration increased from the 5th to the 15th day of maceration, which is consistent with our results (Kocabey, Yilmaztekin & Hayaloglu, 2016). Another study confirmed that the longer the maceration time, the higher the total phenolic content (Budić-Leto et al., 2008). In contrast, according to Muñoz-Bernal et al. (2020), the total phenolic content increased during the 15-day maceration period, but these changes were not significant.

The use of different yeasts had no significant effect on the total phenolic content of the wines tested ($p > 0.05$), nor did the use of SF. Similar findings were reported by Ivanova, Vojnoski and Stefova (2012), who found the same effect of two different yeasts (Levuline, France and Vinalco, North Macedonia) on the total phe-

nolic content in wine. This could be explained by the use of yeasts of the same species *Saccharomyces cerevisiae*. Then again, in another study by Ivanova-Petropulos et al. (2015), it was shown that using Lallemand yeasts, to which BDX belongs, reduced the total phenolic content compared to Vinalco yeast, which is probably due to possible adsorption on the yeast cells. This is consistent with our results that wine produced without the addition of selected yeasts (SF) had the highest phenolic content.

It is important to note that the Folin-Ciocalteu reagent (FCR) is nonspecific to phenolic compounds as it can be reduced by many nonphenolic compounds (e.g., vitamin C, Cu(I), etc.). Phenolic compounds react with FCR only under basic conditions (adjusted by a sodium carbonate solution to pH ~10). Despite the undefined chemical nature of FCR, the total phenols assay by FCR is convenient, simple, and reproducible.

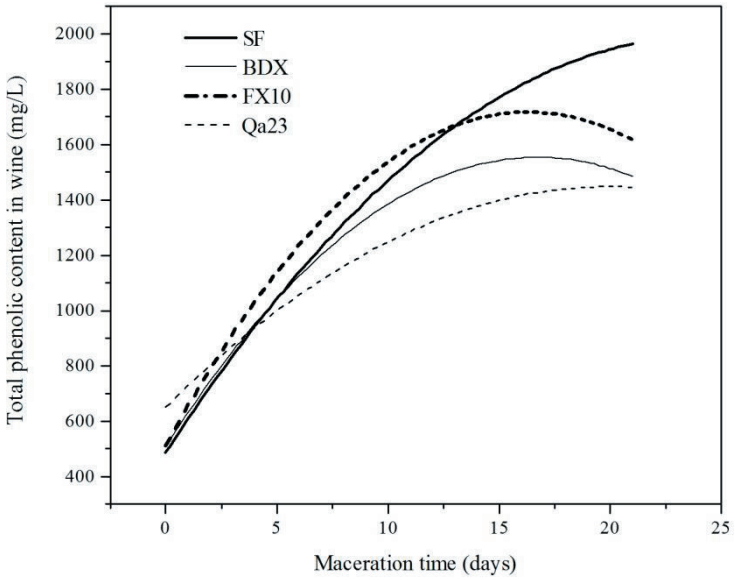


Figure 2. Total phenolic content in wine samples

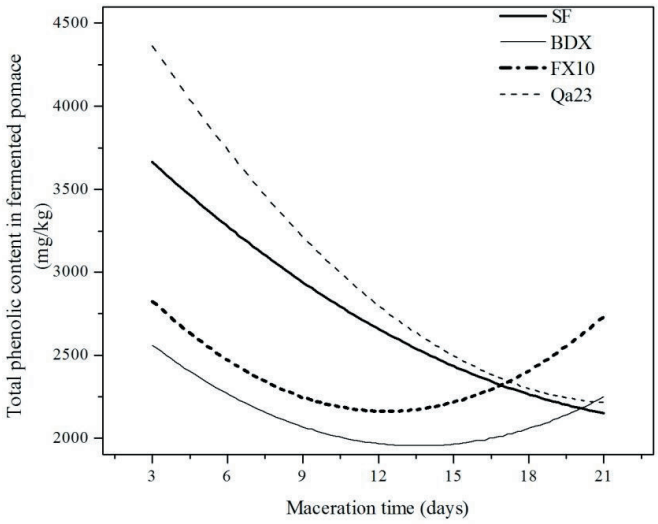


Figure 3. Total phenolic content in fermented pomace during 21 days of maceration

As a result, a large body of data has been accumulated, and it has become a routine assay in studying phenolic antioxidants (Huang, Ou & Prior, 2005).

Influence of spontaneous and inoculated fermentation on TPC in FP

Comparing the total phenolic content in fermented pomace obtained after spontaneous and inoculated fermentation (BDX, FX10 and Qa23), a statistically significant difference was found only for BDX yeast compared to spontaneous fermentation ($p < 0.05$). The highest total phenolic content was found in the fermented pomace after spontaneous fermentation with 3-day maceration (3719.75±96.0 mg/kg). During maceration, an exponential decrease in TPC content was observed up to day 21 for spontaneous and Qa23-inoculated fer-mentation (Fig. 3). In contrast, in maceration with FX10 and BDX, the minimum TPC content was observed on days 12 and 13, respectively. Thereafter, TPC increased until day 21. In this case, mainly desorption reactions of phenolic compounds from the pomace into the wine and adsorption reactions on solid components of the grape pomace or yeast cellstook placed up to the minimum (Setford, Jeffery, Grbin & Muhlack, 2017; Bautista-Ortín, Busse-Valverde, Fernández-Fernández, Gómez-Plaza & Gil-Muñoz, 2016).

Besides desorption, degradation of com-plexes polyphenols may occur, for example, condensed tannins and proanthocyanidins can degrade to simple phenols which could also cause an increase in TPC (Ribéreau-Gayon, Glories, Maujean & Dubordieu, 2006). A pronounced adsorption of phenolic compounds also depends on the properties of the grape cell walls and the binding activity of the phenols (Gao et al., 2019). In contrast, the extraction of phenolic compounds from the pomace in the wine during spontaneous fermentation occurred continuously up to the 21st day of maceration.

Antioxidant activity of wine samples measured by TEAC test

The antioxidant activity of the spontaneously fermented wines did not differ significantly from that of the inoculated wines ($p > 0.05$). In spontaneous fermentation, the highest value of antioxidant activity (15.7 mmol TE/L) was observed in wines with a maceration period of 15 days (Fig. 4). The authors Muñoz-Bernal et al. (2020) also demonstrated such a tendency to increase the antioxidant activity of the wine during the 16-day maceration of the same grape variety using the TEAC test. According to Plavša, Jurinjak, Antunović, Peršurić and Kovačević Ganić (2012), who also used BDX yeast for the production of Teran wines, a higher value for antioxidant activity measured by the TEAC test was found. After 20 days of maceration, it was 33.12 mmol TE/L (Plavša et al., 2012). According to Fernández-Pachón, Villaño, García-Parrilla and Troncoso (2004), the fraction of extracted flavan-3-ols and anthocyanins is responsible for about 40% of the antioxidant activity of red wines determined by the TEAC test.

The antioxidant activity of wine is not only based on the properties of individual phytochemical compounds but also on the multitude and synergistic mechanism of phenolic compounds. Easily oxidizing phenols are regenerated by less active phenols (Jordão, Correia, & Gonçalves, 2012). For example, Jordão et al. (2012), found a negative correlation between individual anthocyanins and ABTS antioxidant test.

But, no single method is sufficient so the FRAP test was applied too. The antioxidant activity could also be altered by effects caused by interactions of polyphenolic principles and the presence of other (non-phenolic) compounds. More than one type of antioxidant capacity measurement needs to be performed to take into account various modes of action of antioxidants (Huang et al., 2005).

This assay is more related to the total constituent levels than to the concentration of any individual compound even though some compounds may contribute more than others (for example, compounds like a galloylated flavanols have a higher antioxidant activity than their non-galloylated homologues). Also, polymeric fraction exhibited a higher antioxidant than the monomeric and oligomeric fraction (Ky, Lorrain, Kolbas, Crozier & Teissedre, 2014; Rockenbach et al., 2011).

Antioxidant activity of wine samples measured by FRAP test

Regarding the results for FRAP values, the antioxidant activity of the spontaneously fermented

wines differed significantly from that of all wines produced by inoculated fermentation ($p < 0.05$). The lowest antioxidant activities were determined in spontaneously fermented wines (Fig. 5). Application of Qa23 yeast gave the highest value after 21 days of maceration and amounted to 31.58 mmol Fe^{2+}/L.

Damijanic et al. (2012), who also used *Saccharomyces cerevisiae* but „Premium Zinfandel“ (Verona, Italy), obtained a lower value for antioxidant activity after the same maceration time, which amounted to 28.6 mmol Fe^{2+}/L. The FRAP values of the control wine samples in our study differed significantly from the FRAP values of the wines macerated for longer than 5 days. This is in line with the study by Damijanic et al. (2012). It can be related to the higher phenolic content; however, not only phenolic compounds contribute to antioxidant activity.

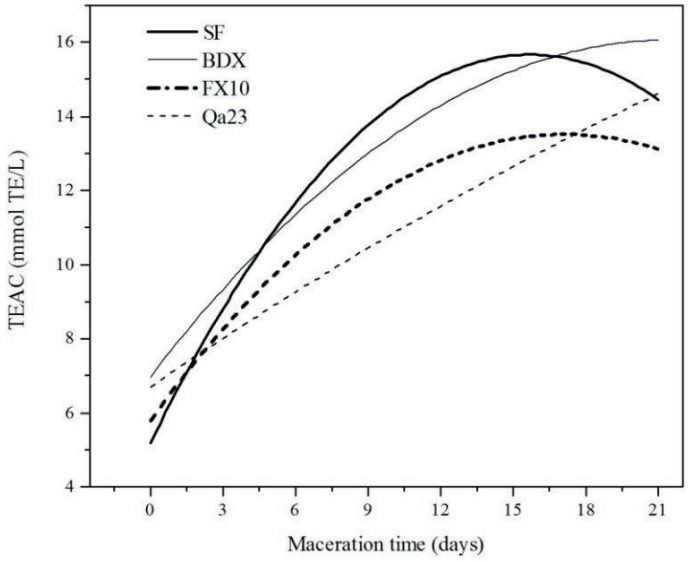


Figure 4. Antioxidant activity of wines produced by spontaneous and inoculated fermentation

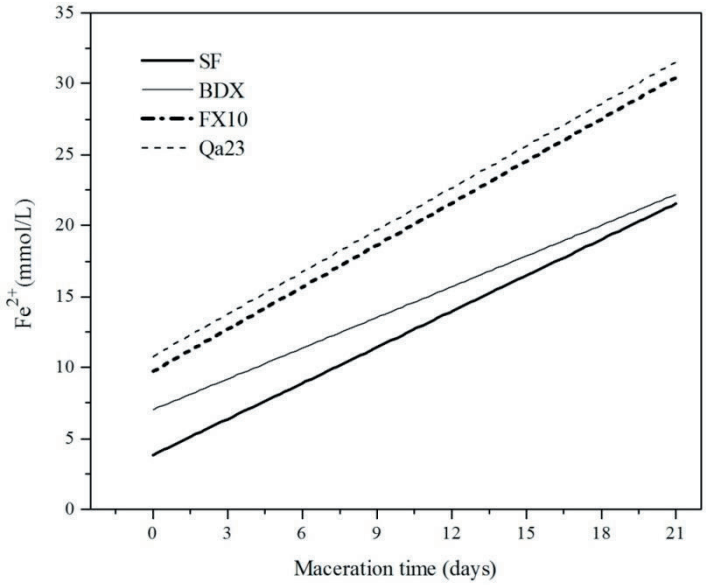
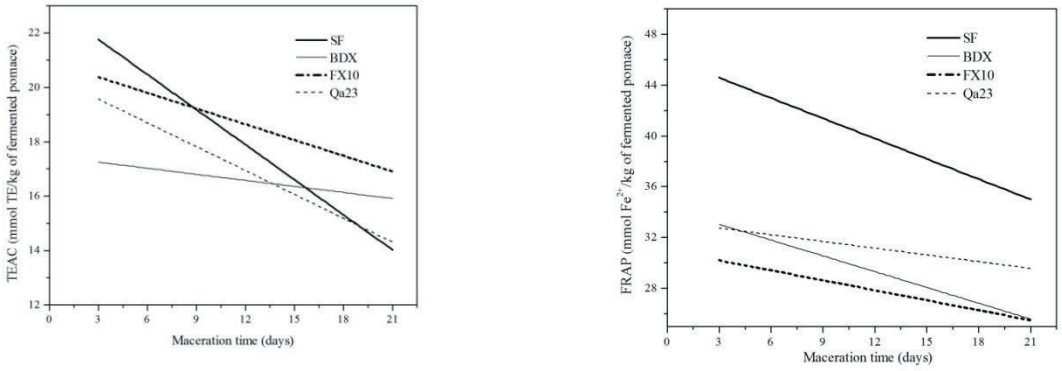


Figure 5. FRAP values of wines produced by spontaneous and inoculated fermentation



a) b)

Figure 6. Antioxidant activity of fermented pomace measured by TEAC (a) and FRAP (b)

Other constituents of wine, such as polysaccharides, peptides, and yeasts could contribute to antioxidant activity, too. Peptides released by winemaking exert multifunctional antihypertensive and antioxidant activities (Zhou et al., 2021).

Antioxidant activity of fermented pomace

As already described, the total phenolic content decreased almost completely during the prolonged maceration.

This phenomenon was the result of the extraction of phenolic compounds into the wine. However, these interactions are determined by a variety of factors, the most important of which are the physicochemical properties of the substances: their morphology (surface area and porosity), and their chemical composition (sugar content, solubility) (Liu, Le Bourvellec & Renard, 2020). As the total phenolic content decreased, the antioxidant activity in the fermented pomace also declined.

Inoculated and spontaneous fermentation had no significant effect on the FRAP and TEAC values of the fermented pomace ($p > 0.05$). Higher antioxidant activity was observed in the pomace samples that underwent shorter maceration (Fig. 6), meaning that most of the phenolic compounds remained in this pomace and therefore had higher antioxidant activity. The reason for this was probably the combination or adsorption of these phenolic compounds with solids, proteins or even yeasts and lactic acid bacteria, polymerization or oxidation with other phenolic or non-phenolic compounds during this period, thus altering or precipitating an important part of the phenols (Sun et al., 2011). The total content of phenolic compounds in the pomace samples and the antioxidant activity measured during the 21-day maceration were well correlated, which is consistent with the literature (Jara-Palacios, Hernanz, Escudero-Gilete & Heredia, 2014). When comparing the different maceration times of two identical varieties (Syrah and Grenache), better antioxidant activity was found in samples with a shorter maceration time (Ky et al., 2014). The research results of de la Cerda-Carrasco, López-Solís, Nuñez-Kalasic, Peña-Neira and Obreque-Slier (2015), who compared the antioxidant activity of two white and two red varieties, including Cabernet Sauvignon, concluded that the antioxidant activity of the pomace of white grapes was better because no maceration was carried out. The pomace of red grape varieties that were macerated for 3 days had lower measured antioxidant activity, but also lower total phenolic content (Ky et al., 2014). The French red varieties Petit Verdot and Syrah showed higher values for antioxidant activity of the pomace measured with the TEAC assay compared to our results (Melo et al., 2015).

CONCLUSION

Winemakers face a series of challenges to satisfy consumers and facilitate the protocol of wine production so that it does not impair the quality characteristics of the wine. By observing the dynamics of polyphenol extraction during the prolonged maceration (21 days) of the Cabernet Sauvignon grape variety, it was found

that the maceration process significantly modulates the total phenolic content of the wines obtained. Comparing the winemaking techniques, which included both yeast inoculation and spontaneous fermentation, no significant differrence in total phenolic content was found, although spontaneous fermentation had the highest TPC content. In addition, the studied samples of FP showed a rich source of phenolic compounds, especially after a short maceration time. The highest total phenolic content was found in fermented pomace obtained after spontaneous fermentation (3-day maceration). The dynamics of extraction of phenolic compounds helped to find the most suitable winemaking conditions for the highest total phenolic content and antioxidant properties. The antioxidant properties of the spontaneously fermented wines and the fermented pomace samples were not significantly different from those of the inoculated samples, while the pomace samples subjected to shorter maceration showed higher antioxidant activity. Concerning that phenolic compounds possess health-promoting properties as well as contribute to wine quality, such experiments could find application, apart from the wine industry, for the development of a functional food or in the pharmaceutical industry. This study gives an insight into the behaviour and content of phenolic compounds under different vinification conditions. This could be significant for future researchers as well as for the development of food with added value and enrichment food by extracting the phenolic compounds originating from wine and fermented grape pomace.

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EFEKTI SPONTANE I INOKULISANE FERMENTACIJE NA SADRŽAJ UKUPNIH FENOLNIH JEDINJENJA I ANTIOKSIDATIVNU AKTIVNOST VINA CABERNET SAUVIGNON I PREVRELE KOMINE

Nikolina M. Živković[1], Uroš D. Čakar[2], Aleksandar V. Petrović[*1]

[1]Univerzitet u Beogradu, Poljoprivredni fakultet, Katedra za prehrambenu tehnologiju i biohemiju, 11080 Zemun, Nemanjina 6, Srbija
[2]Univerzitet u Beogradu, Farmaceutski fakultet, Katedra za bromatologiju, 11000 Beograd, Vojvode Stepe 450, Srbija.

Sažetak: Sadržaj ukupnih fenolnih jedinjenja i antioksidativna aktivnost vina i prevrele komine grožđa sorte vinove loze Cabernet Sauvignon, ubranog u fenofazi pune zrelosti, su određeni spektofotometrijskom analizom. Vino i komina su dobijeni posle produženog perioda maceracije tokom spontane i inokulisane fermentacije grožđa pune zrelosti. Tri posebne vinifikacije su sprovedene koristeći sledeće komercijalne kvasce: BDX (Lallemand, Montréal, QC, Canada), FX10 (Laffort, Bordeaux, France) and Qa23 (Lallemand, Montréal, QC, Canada). Za svaku vinifikaciju maceracija je trajala 0, 3, 5, 7, 14 i 21 dan, respektivno. Sadržaj ukupnih fenolnih jedinjenja je određen spektrofotometrijski Folin-Ciocalteu metodom. Antioksidativna aktivnost vina i uzoraka komine je određena sledećim metodama: Ferric Reducing Activity of Plasma (FRAP) i Trolox Equivalent Antioxidant Capacity (TEAC). Upotreba procesa proizvodnje vina koji je uključivao različita vremena maceracije i inokulacije kvascima, kao i spontanu fermentaciju, značajno je menjao ukupan sadržaj fenola dobijenih vina i prevrele komine. Ova studija bi mogla biti dobra osnova za praktičnu primenu i dobijanje vina sa visokim sadržajem fenolnih jedinjenja i antioksidativnim svojstvima.

Ključne reči: *fenoli, fermentacija, kvasci, crveno vino, komina grožđa, antioksidativna aktivnost*

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