

Research on the reduction of SO₂ doses in winemaking using lysozyme and oenological tannin

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ABSTRACT

The effect of reducing the dose of sulfur dioxide on the physico-chemical and organoleptic properties of wines with Murfatlar controlled designation of origin was studied in this paper, experimenting with different prefermentative oenological treatments. For this purpose, a series of experimental variants was carried out on two Romanian grape cultivars for white wines ('Fetească regală') and red wines ('Fetească neagră'), using two yeast strains that produce low amounts of SO₂ (*Saccharomyces cerevisiae* var. *bayanus* and *Metschnikowia pulcherrima*, a Non-*Saccharomyces* strain) and four prefermentative treatments with different doses of sulfur dioxide, lysozyme and oenological tannin. The obtained results highlighted the fact that reducing the dose of sulfur dioxide using lysozyme and oenological tannin can provide oxidative protection during the alcoholic fermentation stage, improving the organoleptic characteristics of white and red wines. From the physico-chemical point of view, the wines did not show statistically significant differences, except for the color intensity, which was higher in the case of the variants supplemented with 500 mg/L lysozymes, for both of the yeast strains used for fermentation. This study encourages the continuation of research regarding the experimentation of alternative products, to reduce the sulfur dioxide normally used in the winemaking process, in order to obtain healthy products, in accordance with current consumer requirements.

Keywords: oenology, prefermentative treatments, yeast strains, low sulphites, oxidative protection

INTRODUCTION

In the winemaking process, during the processing and conditioning stages, sulfur dioxide is the most frequently used additive, being one of the most versatile and effective due to its antiseptic and antioxidant properties. Sulfur dioxide induces multiple effects, on the one hand, on the must: it prevents prefermentative oxidation; solubilizes the coloring substances in the skins, favoring the diffusion of anthocyanins; inhibits the development of some microorganisms; and on the other hand, on the wine: it prevents the development of microorganisms; delays the onset of alcoholic fermentation, which allows a longer period of prefermentative maceration (for certain wine types), also having the role of improving or maintaining wine aromas (Ribéreau-Gayon *et al.*, 2006; Giacosa *et al.*, 2019). The positive properties of sulfur dioxide outweigh the negative ones, which makes its use acceptable in organic wineries, as it is an active element in the winemaking and preservation technology. However, too much sulfur dioxide can lead to organoleptic changes in the wine, as well as

unwanted effects on human health. Allergies caused by sulfur dioxide derivative compounds, (sulfites), are becoming more common, causing headaches, nausea, gastric irritation, and breathing difficulties in asthma patients (Santos *et al.*, 2012). For these reasons, the International Organization of Vine and Wine has gradually lowered the maximum level of total sulfur dioxide allowed in wine. In the European Union, the allowed limit for conventional wines is 150 mg/L for red and 200 mg/L for white and rosé wines (Guerrero *et al.*, 2015). The recommended dose of free SO₂ to prevent wine oxidation is 20-40 mg/L, depending on the wine cultivar, aging conditions, and estimated shelf life (Waterhouse *et al.*, 2016).

The current consumer trend involves a healthier lifestyle, which leads to an increased interest in healthy, high-quality products. Due to the growing demand for products with low sulphite content, oenologists are facing one of the biggest challenges of modern winemaking, namely finding alternative treatments that could replace sulfur dioxide or experimenting with combined treatments and low doses of SO₂ to protect the chemical and sensory properties of the wine (Jagatić Korenika *et al.*, 2020). Thus, reducing the content of sulfur dioxide in wines has gained particular importance for research in the field of oenology. Obtaining wines without sulfur dioxide is not possible, because this compound is an intermediate metabolite in the biosynthesis process of amino acids, necessary for the multiplication of yeasts during alcoholic fermentation (Ljungdahl and Daignan-Fornier, 2012). Physical (application of high hydrostatic pressure, ultrasounds, UV light), chemical (lysozyme, chitosan, colloidal silver, dimethyl dicarbonate), and biological methods (application of fermentative strains with a bioprotective role), but also plant extracts (polyphenols from grapes or vine canes) have been proposed in order to replace sulfur dioxide treatments (Yildirim *et al.*, 2020).

By-products obtained from winemaking, such as skins, are very rich in phenolic compounds (Stănciuc *et al.*, 2017). These biomolecules, if extracted properly, could constitute valuable sources of natural preservatives as alternatives to reduce sulfur dioxide (Esparza *et al.*, 2020). The use of oenological tannins is a practice authorized by the International Organization of Vine and Wine (OIV) to facilitate the clarification of wines and musts (Vignault *et al.*, 2019) and color stabilization of red wines (Bautista *et al.*, 2007). Also, oenological tannins can inhibit the enzymatic oxidation processes of must and wine (Bellachioma *et al.*, 2008). Another additive for wines and musts is lysozyme, an enzymatic protein that can degrade the cell wall of Gram-positive bacteria, controlling the proliferation of lactic acid bacteria during the alcoholic fermentation of wines, among others (Gerbaux *et al.*, 1997).

The purpose of this work was to study the effect of reducing the dose of sulfur dioxide on the physico-chemical and organoleptic properties of wines with Murfatlar controlled designation of origin. In order to achieve this objective, experimental variants were created using a grape cultivar for white wines ('Fetească regală') and one for red wines ('Fetească neagră'), two yeast strains that produce low amounts of SO₂ (*Saccharomyces cerevisiae* var. *bayanus* and the non-*Saccharomyces* strain *Metschnikowia pulcherrima*) and four prefermentative treatments with different doses of sulfur dioxide, lysozyme and oenological tannin.

MATERIALS AND METHODS

'Fetească regală' and 'Fetească neagră' are two Romanian grapevines (*Vitis vinifera*) cultivars that have gained more and more recognition for the quality wines they produce. The grapes from the 'Fetească regală' cultivar were harvested at a sugar concentration of 23.0° Brix on September 14, 2021, and the grapes of the 'Fetească neagră' cultivar were harvested at 21.9° Brix on September 10, 2021. For each cultivar, a grape quantity of approximately 300-400 kg was transported to the winemaking microstation within the Murfatlar Research Station for Viticulture and Oenology, and subjected to the classic process of obtaining white and red wines. The technological operations for the 'Fetească regală' cultivar consisted of crushing and

de-stemming, after which the must be pressed with a hydraulic press and stored for fermentation in 25-liter demijohns, in which the fermentation strains and oenological treatments were applied: *Saccharomyces cerevisiae* var. *bayanus* strain (Viniferm BY, Agrovin, Spain); *Metschnikowia pulcherrima* strain (Primaflora VB BIO, AEB, Italy); lysozyme (Lysocid W, AEB, Italy); and oenological tannin (Finitan, Agrovin, Spain), according to the protocol presented in figure 1. a). In the case of the 'Fetească neagră' cultivar, after crushing and de-stemming, the grape mash was transferred to containers for maceration-fermentation, applying, in this case, the protocol presented in figure 1. b). The homogenization of the grape mash was carried out 3 times a day, monitoring the density and color intensity daily. The fermenting must was separated from the pomace after 8 days, using a hydraulic press, after which the must was stored in 25-liter demijohns, where the alcoholic and malolactic fermentation continued. The latter was evaluated by monitoring malic acid degradation using the FTIR wine Analyzer Lyza 5000 (Anton Paar, Austria). When the alcoholic (for white and red wines) and malolactic (red wines) fermentation stages were finished, the wines were racked off the sediment, filtered, bottled, and stored at 15°C.

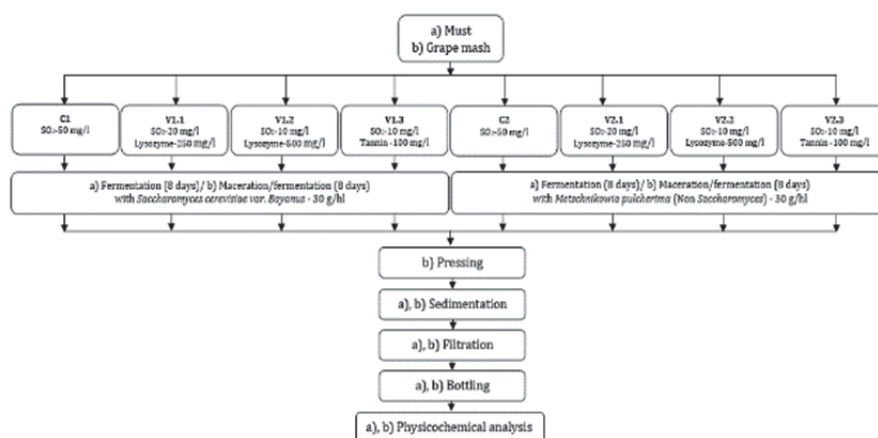


Figure 1. Scheme for obtaining wines from the a) 'Fetească regală' and b) 'Fetească neagră' cultivars

Must and wines physico-chemical analyses

The density of the must in fermentation was determined aerometrically, using densimeters with a measuring range from 0.9600-1.1000 g/cm³. To assess the quality of the wines and compliance with the regulations, the main physicochemical parameters have been determined in accordance with the methods provided by ASRO (2012) in their standards. The tested parameters included the alcoholic strength (% vol.), reducing sugar content (g/L), total acidity (g/L tartaric acid), volatile acidity (g/L acetic acid), total extract (g/L), non-reducing extract (g/L), total SO₂ (mg/L) and free SO₂ (mg/L). Total polyphenols were determined with the Folin-Ciocalteu reagent by the Singleton-Rosi method (1965). The color intensity, IC (420 + 520 + 620 nm) of the must in fermentation and the wines, was determined using the Helios Alpha UV-VIS spectrophotometer (ThermoScientific, USA). The contribution of each color (yellow, red, blue) to the color of the wine was calculated by dividing the absorbance at 420 nm (yellow), 520 nm (red), and respectively 620 nm (blue) by the color intensity. The proportion of the red color, produced by free and bound anthocyanins in the form of flavylum cations, was calculated using the following formula, described by Glories in 1984:

$$dA\% = \left[1 - \frac{\text{Abs } 420 + \text{Abs } 620}{2 \times \text{Abs } 520} \right] \times 100$$

Sensory analyses

For the sensory appreciation of the wines, the evaluation method proposed by the International Union of Oenologists (UIO) was applied, which consists in the organoleptic evaluation of the aromatic parameters through "blind tasting". The purpose of this method is to characterize the wines from the point of view of specific olfactory and gustatory aromas and to achieve a sensorial determined aromatic profile. For this, a number of 7-9 olfactory characteristics were chosen - fruit aromas: citrus fruits (grapefruit), forest fruits (strawberries, raspberries, blackberries, rose hips, black currants), pineapple, sweet cherries, apples, pears, lychee, dried fruits (plums); spices: pepper; and 7-9 relevant taste characteristics: acidity, sweetness, bitterness, unctuousness, tannic intensity (components that determine the harshness, aggressiveness and hardness of wines) and the aromatic persistence, determined by the duration of aroma retention after the evacuation of the wine from the oral cavity. These characteristics were recorded in the tasting sheets used by the evaluators during the tasting; the 3 members of the jury (2 women and 1 man), members of the Association of Authorized Tasters from Romania, rated each characteristic with marks from 0 to 9, and the results were mathematically processed (arithmetic mean).

The analyzed data represent averages of three replication. To determine if significant influences on the analyzed indicators are incurred by yeast and oenological treatment, an analysis of variance (One-way ANOVA) was performed and the mean differences of the various samples were compared by a post-hoc test (Duncan multiple mean comparison test).

RESULTS AND DISCUSSION

The results regarding the daily monitoring of the density and coloring intensity of the 'Fetească neagră' cultivar are presented in figure 2. Alcoholic fermentation started in all variants approximately 72 hours after grape crushing. Density decrease was slow, with yeasts consuming on average 0.03 g/cm³ in a 24-hour interval, and regardless of the used strain, the fermentation process lasted 8 days. Regarding visual characteristics, the color intensity increased rapidly, displaying ascending curves. Maximum values were reached by day 6, while in the following two days, the color intensity measured a slight decrease.

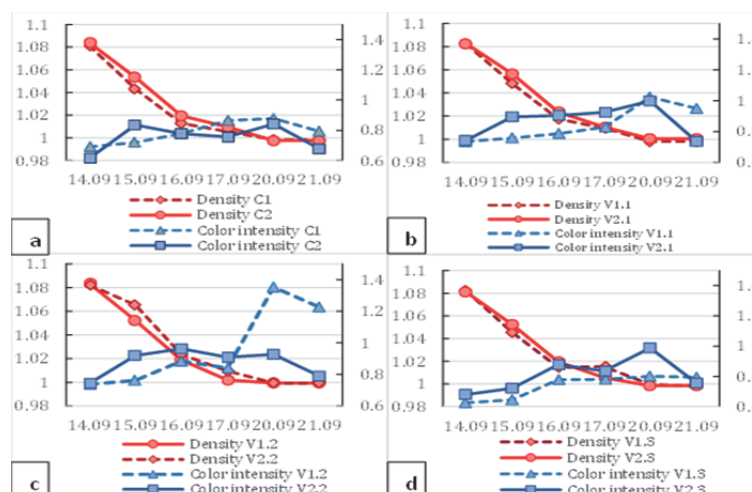


Figure 2 - Density and color intensity monitoring during alcoholic fermentation by *Saccharomyces cerevisiae* var. *bayanus* (1) and *Metschnikowia pulcherrima* (2) yeasts for the 'Fetească neagră' cultivar: a. Control variants; b. Variants with 250mg/L lysozyme; c. Variants with 500 mg/L lysozyme; d. Variants with 100mg/L tannin.

At the end of the maceration period, the color intensity of the obtained wines was determined (Figure 3). The variants fermented by the *Saccharomyces* strain resulted in a color intensity higher on average by 27.2% than in the case of the variants fermented by the non-*Saccharomyces* strain. In the case of both yeasts, the highest value of the color intensity was reached in the case of treatment with 500 mg/L lysozymes (variants V1.2 and V2.2). Similar results regarding the accumulation and preservation of coloring intensity following the application of lysozyme were also confirmed by other authors (Liburdi *et al.*, 2014).

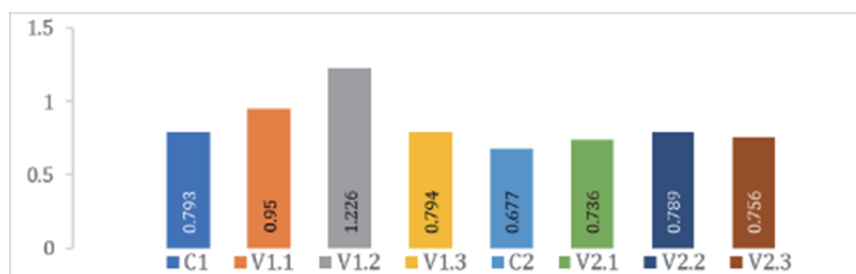


Figure 3. The color intensity of the 'Fetească neagră' experimental variants, after pressing

The wines obtained from the two studied cultivars, 'Fetească neagră' and 'Fetească regală', were analyzed from a physico-chemical point of view 3 months after bottling, performing analyses such as alcohol concentration (% vol), total acidity (g/L tartaric acid), volatile acidity (g/L acetic acid), reducing sugars (g/L), total and non-reducing extract (g/L) and free and total sulfur dioxide (mg/L). In the case of the 'Fetească neagră' cultivar, the color intensity and total polyphenols were determined spectrophotometrically. As it can be seen, the applied treatments did not significantly influence the physico-chemical quality of the wines; the alcohol concentration is specific to wines with Murfatlar controlled designation of origin, the values being situated between 11.70-11.89% vol. for the 'Fetească neagră' cultivar (Table 1), and between 12.25-12.49% vol. for the 'Fetească regală' cultivar (Table 2). The wines fermented until the sugars were depleted, becoming dry (sugar content of less than 4.0 g/L). The total acidity was measured in the range of 6.16-6.53 g/L tartaric acid for the variants of the 'Fetească neagră' cultivar, which proves that the wines underwent malolactic fermentation quite quickly. The volatile acidity values ranged between 0.51- 0.58 g/L acetic acid, which confirms that volatile acids were formed only during the alcoholic and malolactic fermentation stages. For the 'Fetească regală' cultivar, the total acidity varied in the range of 6.92-7.64 g/L tartaric acid, while the volatile acidity values were 0.59-0.63 g/L acetic acid. Volatile acidity values are very important because they indicate the health status of the wines and condition the quality category in which they can fall.–Regarding color intensity, in the case of the 'Fetească neagră' cultivar, the wines were significantly influenced from a statistical point of view by the treatments. Higher values were recorded in the variants fermented by the *Saccharomyces* yeast, the V1.2 variant standing out with values higher by 35.6% when compared to the control. The total content of polyphenols recorded values in the range of 1.27-1.47 g/L, higher values being recorded in the case of the V1.2 variant, although with statistically insignificant differences. In the case of the 'Fetească regală' cultivar, the coloring intensity showed significant differences, and higher values, denoting oxidation being recorded in the case of the variants fermented with the non-*Saccharomyces* strain. The lysozyme and oenological tannin treatments had an antioxidant effect during the fermentation period, with the color intensity reaching values 77.2% lower when compared to the control variants.

Table 1 – Physico-chemical analyses for the experimental variants of the 'Fetească neagră' cultivar

Variant/ Analytic parameters	<i>Saccharomyces cerevisiae</i> var. <i>bayanus</i>				Non- <i>Saccharomyces</i> (<i>Metschnikowia pulcherrima</i>)				ANOVA		
	C1	V1.1	V1.2	V1.3	C2	V2.1	V2.2	V2.3	Y	T	Y*T
Alcoholic strength (%vol)	11.89±0.2a	11.80±0.1a	11.79±0.2a	11.87±0.2a	11.70±0.1a	11.74±0.1a	11.81±0.2a	11.73±0.2a	ns	ns	ns
Reducing sugars (g/L)	1.0±0.1d	1.2±0.2cd	1.1±0.1d	1.0±0.1d	1.5±0.2ab	1.4±0.2bc	1.7±0.1a	1.5±0.1ab	***	ns	ns
Total acidity (g/L C ₄ H ₆ O ₆)	6.16±0.1b	6.40±0.2ab	6.53±0.3a	6.30±0.2ab	6.52±0.2ab	6.38±0.1ab	6.26±0.1ab	6.47±0.2ab	ns	ns	ns
Volatile acidity (g/L CH ₃ COOH)	0.51±0.01a	0.53±0.04a	0.54±0.03a	0.53±0.04a	0.54±0.06a	0.58±0.02a	0.55±0.01a	0.54±0.05a	ns	ns	ns
Total extract (g/L)	26.9±1.0a	27.6±1.0a	27.4±1.1a	27.0±1.2a	26.8±1.2a	28.7±1.1a	28.7±1.1a	27.8±1.2a	ns	ns	ns
Non-reducing extract (g/L)	25.9±1.2a	26.4±1.1a	26.3±1.2a	26.0±1.0a	25.3±1.1a	27.3±1.3a	27.0±1.2a	26.3±1.2a	ns	ns	ns
Color intensity	8.22±1.1bc	9.70±1.3ab	11.15±1.3a	9.07±1.5abc	7.82±1.0bc	7.42±1.1bc	7.10±1.3c	7.44±1.1bc	**	ns	ns
dA%	92.32±6.8a	89.06±7.2a	84.90±6.6a	90.19±8.1a	93.09±7.8a	93.98±9.9a	94.81±8.5a	93.74±7.8a	ns	ns	ns
d420%	29.93±2.1a	30.2±3.6a	31.7±3.1a	32.5±2.8a	29.8±3.1a	28.71±3.6a	26.76±2.9a	30.65±3.5a	ns	ns	ns
d520%	65.09±4.5abc	63.20±4.1abc	58.39±3.9c	60.75±5.0bc	65.47±4.2abc	67.65±3.8ab	70.99±5.5a	65.46±4.9abc	*	ns	ns
d620%	4.99±1.2bc	6.60±1.8b	9.87±1.4a	6.73±1.5b	4.73±1.1bc	3.64±1.3cd	2.25±1.0d	3.90±1.1cd	***	**	*
Total polyphenols (g/L)	1.27±0.2a	1.38±0.5a	1.47±0.3a	1.47±0.4a	1.42±0.2a	1.37±0.1a	1.33±0.2a	1.38±0.1a	ns	ns	ns
Free SO ₂ (mg/L)	1.8±0.1e	6.2±0.8bc	8.8±0.6a	7.5±2.2ab	3.6±0.4d	4.8±0.7cd	4.6±0.9cd	5.9±1.0bc	*	***	***
Total SO ₂ (mg/L)	40.4±2.7a	35.6±2.9bc	33.7±2.5cd	38.4±2.2ab	22.4±2.5f	28.5±1.8e	30.2±2.2de	39.8±3.1ab	***	***	***

Average values ± standard errors (n=3). The letters in the brackets show the statistical difference among results for p<0.05. For the same compound, a common letter for 2 or more variants shows no significant difference among them, Y=yeast, T=treatment

Table 2 – Physico-chemical analyses for the experimental variants of the 'Fetească regală' cultivar

Variant/Analytic parameters	<i>Saccharomyces cerevisiae</i> var. <i>bayanus</i>				Non- <i>Saccharomyces</i> (<i>Metschnikowia pulcherrima</i>)				ANOVA		
	C1	V1.1	V1.2	V1.3	C2	V2.1	V2.2	V2.3	Y	T	Y*T
Alcoholic strength (% vol)	12.44±0.2a	12.49±0.4a	12.41±0.5a	12.37±0.9a	12.25±0.1a	12.26±0.6a	12.29±0.8a	12.33±0.2a	ns	ns	ns
Reducing sugar (g/L)	1.6±0.2bcd	1.3±0.1a	1.4±0.4cde	1.0±0.1e	2.1±0.5ab	2.2±0.4a	2.1±0.2ab	1.9±0.3abc	***	ns	ns
Total acidity (g/L C ₄ H ₆ O ₆)	6.92±0.5a	7.14±0.7a	7.14±0.3a	7.04±0.3a	7.50±0.2a	7.63±0.5a	7.64±0.3a	7.62±0.4a	*	ns	ns
Volatile acidity (g/L CH ₃ COOH)	0.60±0.02a	0.59±0.04a	0.63±0.01a	0.63±0.02a	0.63±0.01a	0.63±0.03a	0.61±0.02a	0.61±0.01a	ns	ns	ns
Total extract (g/L)	24.8±1.1a	26.4±1.4a	27.2±1.1a	26.3±1.3a	28.9±1.3a	30.1±1.4a	30.6±1.5a	30.0±1.4a	***	*	ns
Non-reducing extract (g/L)	23.2±1.2a	25.1±1.0a	25.8±1.3a	25.3±1.1a	26.8±1.2a	27.9±1.3a	28.5±1.3a	28.1±1.1a	***	*	ns
Color intensity	0.4±0.01c	0.8±0.02bc	0.6±0.01c	0.5±0.01c	4.4±0.1a	1.0±0.05bc	1.0±0.03bc	1.5±0.02b	***	***	***
Free SO ₂ (mg/L)	2.4±0.2c	7.2±0.4a	2.4±0.1c	4.8±0.2b	4.8±0.3b	4.8±0.1b	4.8±0.2b	1.0±0.1d	***	***	***
Total SO ₂ (mg/L)	45.7±3.1a	26.4±1.8a	24.0±1.5a	19.2±1.3d	40.9±2.9b	12.0±1.2e	12.0±1.4e	16.8±1.7d	***	***	**

Average values ± standard errors (n=3). The letters in the brackets show the statistical difference among results for p<0.05. For the same compound, a common letter for 2 or more variants shows no significant difference among them, Y=yeast, T=treatment

The olfactory sensations of the 'Fetească neagră' cultivar, specific to forest fruits (strawberries, raspberries, black currants, and blackberries), were appreciated depending on the yeast strains and the applied treatments (Figure 4 a, b). It can be observed from the perspective of the points assigned to each sensation, that the variants fermented by the *Saccharomyces* yeast present a greater complexity of aromas, with more intense notes of black currants and blackberries, Variant 1.3, accumulating the highest score, followed by V2.1. The variant that stands out from a taste point of view is V1.1, the wine being balanced, mellow, and round, with a usual alcoholic strength, and balanced tannin content, favorable to quality.

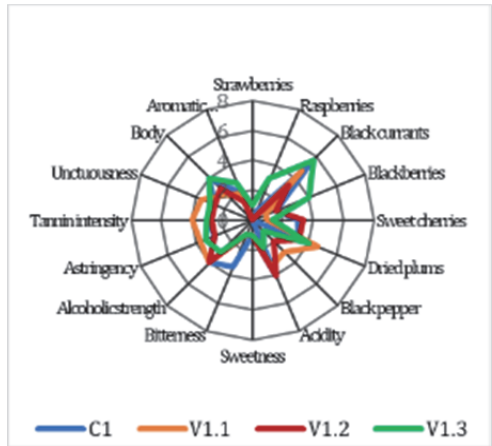


Figure 4.a The organoleptic profile of 'Fetească neagră' wines fermented by *Saccharomyces*

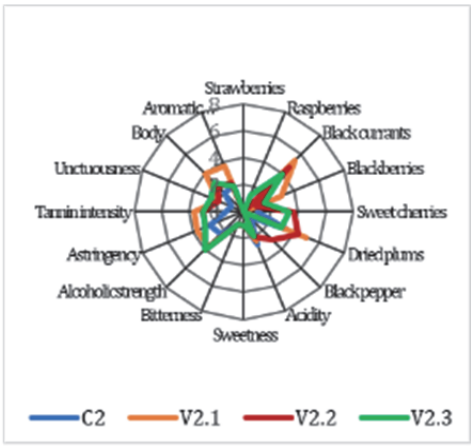


Figure 4.b The organoleptic profile of 'Fetească neagră' wines fermented by *Metschnikowia*

The general olfactory character of 'Fetească regală' wine is fruity, given by the intense aromas of lemon, grapefruit, pineapple, and pear. In the case of the variants fermented with *Saccharomyces* yeast, the smell of grapefruit and pineapple was more dominant in the V1.2 variant, and in terms of extractives and aromatic persistence, the V1.3 variant stood out. Honey aromas were also present, due mainly to the overripe grapes, particularly perceived in the V1.1 variant (Figure 5). From a taste point of view, the variant that stood out was V1.3, the wine was full, balanced, with a fairly good alcoholic strength. It can be concluded that the wines obtained using the *Saccharomyces* strain present superior organoleptic characteristics.

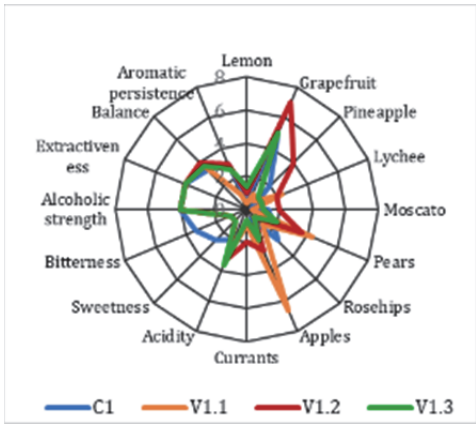


Figure 5. a. The organoleptic profile of 'Fetească regală' wines fermented by *Saccharomyces*

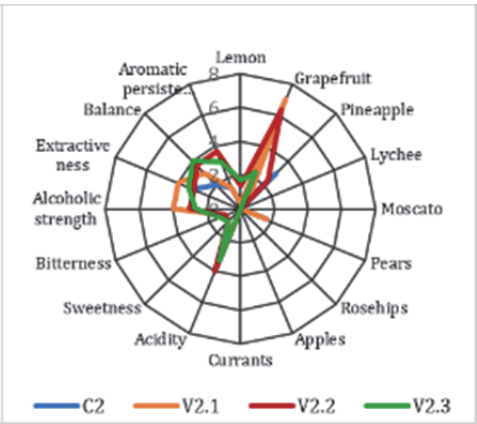


Figure 5. b. The organoleptic profile of 'Fetească regală' wines fermented by *Metschnikowia*

CONCLUSIONS

This study consolidates previous research and validates the fact that the application of treatments with lysozyme and oenological tannin ensures oxidative protection during the fermentation period, preserving the aromas and improving the color intensity in the case of the use of some *Saccharomyces* yeasts. The research carried out encourages the continuation of research regarding the experimentation of alternative products in order to reduce or eliminate the sulfur dioxide used in winemaking, to obtain healthy products following current consumer requirements.

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