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ANTHOCYANINS LEVELS MODIFICATION ON WINES WITHOUT SULFUR DIOXIDE. NEW PERSPECTIVE.

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Abstract

Anthocyanins are considered important compounds in wine production. Their role is important in wine color in case of red and rose wines. Anthocyanins are involved all stages of wine production from grapes, fermentation, treatment and aging. Wine evolution produce modification of pH media, color or other physico-chemical parameters. Principal characteristics as aromas are determined by several reactions as acido-basic and redox which are related to modifications of wine composition. An UFLC method was involved in evaluation of several compounds as Cyanidol-3-glucoside (Cy3gl), delphidin (Dp), peonidin 3-glucoside (Po3gl), malvidin 3-glucoside (Mv3gl), petunidin (Pt) and malvidin (Mv) which were separated and analyzed in case of several wine samples from Cabernet Sauvignon and Cabernet Sauvignon rose. Comparative profiles showed significant differences in levels of these compounds in case of the wines treated with sulphur dioxide and without sulphur dioxide. Conclusion is that use of natural compounds as Pichia kluyveri yeasts not only prevented oxidation with implications in anthocyanins variation but showed potency against bacterial contamination.

Key words*: wines, sulphur dioxide, anthocyanins, UFLC.*

INTRODUCTION

Through Pichia variants, *P. kluyveri* is the most studied and is the only available on commercial level. *P. kluyveri* has the potential to produce aromatic compounds as thiols, terpenes and several esters (Vicente et al., 2021). Among the commercial products, Frootzen® (Hansen®, Hoersholm, Danemarck) is recognized with the capacity of improve varietal and thiols aromas (Vejarano and Gil-Calderón, 2021). There is a concern regarding the wine alteration in presence of non-*Saccharomices* species, thus special consideration should be taken in order to be efficient and viable for inhibition of fermentation–yeasts (Vejarano, 2020). For this purpose, at wide scale sulphur dioxide is used for antimicrobial effect, but the extended use may produce allergic reaction and other health disorders.

Several reactions are encountered during wine aging, one of most importance is oxidation which is produced at the levels of compounds susceptible to be sensible and in this category are included also anthocyanins (Deshaies et al., 2020). The stability of monomeric anthocyanin in red wines is influenced by grape variety, fermentation techniques or wine management (Baiano et al., 2016). Over influences are related to pH value, temperature, oxidation state, exposure to light, presence of other substances such as ascorbic acid, sugars, sulphites and metal ions as cofactors (He et al., 2012). Normally, anthocyanins appear to be more stable in acidic media at lower pH values than in alkaline solutions with higher pH values.

Involved in wine colour, anthocyanins are influenced by the presence of other compounds as flavanols or tannins. As function of their structure, anthocyanins are classified in more groups: anthocyanins non-acylated, acetylated anthocyanins, proanthocyanins, anthocyanins – flavanol, acetyl mediated condensation flavanol anthocyanins (Pervaiz et al., 2017).

The objective of this study is to evaluate the chemical substances and sensory impact of ome commercial yeast product of *non-Saccharomyces* yeasts used for bio-protection.

MATERIALS AND METHODS

Wine preparation procedures

The study is based on Cabernet Sauvignon grape sort from Panciu wine region with the following coordinates 45°52'52.3"N and 27°03'23.4"E. Two classical wine types were included in the study, each with samples without sulphur dioxide and control samples that had added SO_2 in doses of up 50 mg/L.

Namely, Cabernet Sauvignon rose (CSr) and Cabernet Sauvignon red (CS) were vinified in two wine making procedures. Representative samples were taken in 2018, 2019, 2020 and 2021 and stored in wine cellar for aging and codified in the following manner: CS - Cabernet Sauvignon, CSr - Cabernet Sauvignon rose, 0-(-SO_2 samples), 1-(+SO_2 samples), year - (18, 19, 20, 21) → (2018, 2019, 2020, 2021).

In every of each year, vinification of CS0 and CSr0 started with grape treatment with *P. kluyveri* in doses of 1.3 kg/T while for CS1 and CSr1 wines grape treatment consisted in use of potassium metabisulphite (6 g/100 kg), ascorbic acid (3 g/100 kg) and gallic acid (1 g/100 kg).

Maceration was produced for maximum 3 hours for CSr0 and CSr1, followed by must separation and destemming using pectolytic enzymes (3 g/hL). Alcoholic fermentation used promotors in two stages and doses of 25 g/L followed by *S. cerevisiae* (20 g/hL). CSr0 had malolactic fermentation 2 days after alcoholic debut using standard *O. Oeni. yeast* with nutrient (20 g/hL) to avoid any refermentation of wine.

CS0 and CS1 wines had alcoholic (*S. cerevisiae* - 20 g/hL) and malolactic fermentation (*O. Oeni.* in presence of fermentation nutrient - 20 g/hL) concomitant with maceration (pectolytic enzymes - 3.0 g/hL), inactivated yeasts and autolysates - 25 g/hL)). All the wines had discontinuous press and decanting for separation from deposits. Sulfur dioxide variants had SO_2 correction (up to 40 mg/L free SO_2) while samples without SO_2 had an antioxidant treatment with oenological tannins 5 g/hL. Both type of samples had acidity correction with lactic and tartaric acid up to 6.1-6.3 g tartaric acid/L. CSr0 and CSr1 samples had polyphenols reduction by using PVPP (15 g/hL) and clarification with bentonite (100 g/hL), and fish clay (25 ml/hL). Tartaric stabilization was made with potassium polyaspartate (200 ml/hL) for CS while CSr wines were treated with carboxymethylcellulose (100 g/hL) and arabic gum (100 g/hL). Final aromatic complexity was assured using mannoproteins (3 g/hL) (CS) and condensed tannins (0.5 g/hL) (CS and CSr), while CS1 and CSr1 had final SO_2 correction before bottling.

Reagents and reference materials

For analysis of anthocyanins, formic acid, acetonitrile (LC grade) were used and purchased from Merck KGaA - Germany. Cyanidin 3-glucoside chloride (koumarin hydrochloride) (Cy3gl - CAS 7084-24-4), delphidin chloride (Dp - CAS 208-437-0), peonidin 3-glucoside chloride (Po3gl - CAS 6906-39-4), malvidin 3-glucoside chloride (Mv3gl - CAS 7228-78-6), petunidin chloride (Pt - CAS 1429-30-7) and malvidin chloride (Mv - CAS 643-84-5) were used as standards and procured from Extrasyntesis France. For UFLC analysis a type I water produced by a Thermo Scientific GenPure UV-TOC system.

Instrumental methods

A high-performance chromatography method was applied using an Agilent 1220 series. As chromatographic column a C18 Phenomenex with 150 mm length, 4.6 mm internal diameter and 2.6 µm was used. Stationary phase consisted in superficial porous particles with 100Å size (SPP - Kinetex XB - C18, 00F-4496-E0) pre-column (AJ0-8768). Chromatographic elution was gradient and used a mixture of Water/Formic acid/Acetonitrile. in the following parts-per-volume 87:10:3 (v/v/v) (Solvent A) and 40:10:50 (v/v/v) (Solvent B). Flowrate was 1.85 mL/min, temperature 50°C and injected volume was 10 µL. Detection was at 525 nm, 1 nm slit and 80 Hz (0.13 s) data sampling rate.

Statistical analysis

All the statistical analysis were performed using StatSoft Tibco Statistica. Univariate analysis of variance was applied to make a comparison between the levels of biogenic amines in the case of different wine varieties,

but also between the wines which are treated with and without SO_2. When different values of the concentrations were registered, LSD (Least Significant Difference) test was applied to determine which mean values have significant differences.

RESULTS AND DISCUSSIONS

Instrumental evaluation

Chromatographic method followed the conditions from reference methodologies (OIV-MA-AS315-11), but with slight modifications in terms of elution and flowrate. Reference method used a flowrate of 0.8 mL/min on a SPP chromatographic column with a particle size of 2.6 µm (0.35 µm porous shell and 1.9 µm solid core) and achieved a 41-minute gradient program. Significant improvement was produced using our conditions in terms of lower chromatography time and optimum resolution for every compound. In terms of gradient, composition started with solvent A:B 94:6 (v/v), changed to 70:30 (v/v) in 4.5 minutes and 40:60 (v/v) in 7 minutes. Re-equilibration was achieved in 2 minutes and total chromatography time was 9 minutes. Retention times (min) were Cy3gl (2.26), Dp (2.72), Po3gl (3.39), Mv3gl (3.91), Pt (4.26), Pg (4.64), and Mv (5.77). Correlation coefficient for every slope was of no less than 0.99. Limit of quantification ranged between 3.2 µM for Dp and 43.5µM for Mv.

Sample evaluation during the study

Representative samples were identified as previously mentioned. After sampling all the specimens were stored in cellar, in conditions which assured minimization of any supplementary oxidation to the samples. Also, representative analysis was taken to verify the levels of fixed acidity and content of sulphur dioxide. For the CS1 and CSr1 wines, the levels of free sulphur dioxide ranged between 40-58 mg/L. samples without added sulphur dioxide as CS0 and CSr0 the quantities were not higher than 6 mg/L and originated from fermentation stage (Pezley 2015). All the samples were stored for specified time interval (2018-2021) and the final analysis for the anthocyanins was performed on 2021.

pH was important for stability of wines and maintained between 3.1 and 3.3 during winemaking. pH, acidity, alcoholic content, volatile acidity, sugar content were monitored to verify the primary conditions that could lead to anthocyanins alterations. Regarding pH, CS showed values between 3.0 and 3.74 for CS0 and 2.91 to 3.72 for CS1 samples. Same evaluation was done for CSr: variations of 3.12-3.51 were registered for CSr0 samples and 3.38-3.82 for CSr1 samples. pH modification is determined by organic acids variation. Increase of pH which was correlated with slight decrease of fixed acidity and is determined by precipitation of tartaric acid which is considered unstable (Vicente et al., 2022). Variation of pH did not show any statistical differences between these samples. Anthocyanins are be more stable in acidic media at lower pH values than in alkaline solutions. Redox reactions are favored by modification of oxidation state directly related with change of ionization status (Wahyuningsih et al., 2016). In these conditions, no wine samples showed conditions for important oxidative reactions. Although stabilization was considered efficient, some changes in concentrations of anthocyanins were produced for CS and CSr samples. Variations are related with polymerization and slight oxidative condensation (Cotea et al., 2009). Data is presented in the Tables 1a and 1b.

Table 1a. Levels of anthocyanins for Cabernet Sauvignon rose (CSr) (#1 - CSr018, #2 - CSr019, #3 - CSr020, #4 - CSr021, #5 - CSr118, #6 - CSr119, #7 - CSr120, #8 - CSr121; Av (CSr0 and CSr1) - average value, St - standard deviation)

Anthocyanins (µmols/L) Cabernet Sauvignon rose (CSr)							
#	*Cy3gl*	*Dp*	*Po3gl*	*Mv3gl*	*Pt*	*Mv*	*Total*
1	12.5	6.2	1228	1503	11.7	51.3	2813
2	12.2	4.2	1252	1524	12.9	43.4	2849
3	13.1	3.3	1251	1671	14.2	44.1	2997
4	15.2	6.3	1229	1454	7.4	85.0	2797
Av	***13.3***	***5.0***	***1240***	***1538***	***11.6***	***56.0***	***2864***
St	***1.4***	***1.5***	***13.3***	***93.4***	***2.9***	***19.7***	***91.2***
5	8.5	3.3	1250	1399	6.8	58.2	2726
6	9.1	4.4	1253	1423	8.9	62.1	2761
7	9.2	6.2	1258	1443	12.0	58.8	2787
8	9.3	4.5	1261	1429	9.8	62.0	2776
Av	***9.0***	***4.6***	***1256***	***1424***	***9.4***	***60.3***	***2762***
St	***0.4***	***1.2***	***4.9***	***18.4***	***2.2***	***2.1***	***26.7***

Table 1b. Levels of anthocyanins for Cabernet Sauvignon (CS) (#1' - CS018, #2' - CS019, #3' - CS020, #4' - CS021, #5' - CS118, #6' - CS119, #7' - CS120, #8' - CS121; Av (CS0 and CS1) - average value, St - standard deviation)

Anthocyanins (mmols/L) Cabernet Sauvignon (CS)							
#	***Cy3gl***	***Dp***	***Po3gl***	***Mv3gl***	***Pt***	***Mv***	***Total***
1'	0.33	0.59	2.1	9.2	0.35	0.73	13.3
2'	0.33	0.59	2.2	13.5	0.41	0.83	17.9
3'	0.34	1.15	2.1	23.5	0.44	2.39	29.9
4'	0.35	1.33	2.2	22.9	0.53	2.42	29.7
Av	***0.34***	***0.91***	***2.1***	***17.3***	***0.43***	***1.59***	***22.7***
Sd	***0.01***	***0.38***	***0.06***	***7.05***	***0.07***	***0.94***	***8.40***
5'	0.25	0.41	2.0	8.6	0.39	0.54	12.2
6'	0.26	0.45	1.9	10.7	0.41	0.54	14.3
7'	0.49	1.31	2.5	25.0	0.49	1.63	31.4
8'	0.50	1.36	2.5	25.5	0.52	1.67	32.0
Av	***0.37***	***0.88***	***2.2***	***17.5***	***0.45***	***1.10***	***22.5***
st	***0.14***	***0.52***	***0.29***	***9.04***	***0.06***	***0.64***	***10.68***

According to previous data, in terms of Cabernet Sauvignon rose, no statistical differences were registered for any compound included in the study at the comparison between treatments. Only for Cy3gl the mean value was 13.3 ± 1.4 µmol/L for CSr0, while CSr1 samples showed a significant lower value of 9.0 ± 0.4 µmol/L.

The other anthocyanins ranged between 1424 ± 18.4 µmol/L (Mv3gl; CSr1) and 4.6 ± 1.2 µmol/L (Dp, CSr1). As in other studies, Po3gl and Mv3gl are the major constituents as pigmentation compounds in red wines.

CS varieties had similar characterization but with higher levels and relative content (Fernandes et al., 2017). Mv3gl and Po3gl were the major constituents in red wine pigmentation, followed by Pt, but also Mv and glycolysis form as Mv3gl (Table 1b).

Since the spectra of color compounds is the same and the statistical differences between (CS0, CSr0) and control samples (CS1, CSr1) are not statistically significant, the influence and impact of antioxidants are verified as function of variation during wine aging (Obreque-Slier et al., 2023).

For CS, modification of color was more intense than the CSr, aspect that was confirmed by the slightest variation of the anthocyanins, as showed in Figure 2b.

For CSr, several compounds had a better stability, showed by the Figure 2a where the trendline have correlation coefficients of maximum 0.832. Cy3gl and Po3gl showed a clear evolution during aging period of wine from 2018 and 2021 with r (coefficient of correlation) values of 0.913 and 0.995. Statistical differences between samples at treatment comparison did not show any relevance for every compound in the study.

Continuous evolution of compounds showed statistical differences at comparison using year of production (from 2018 → 2021) as criteria. Only Cy3gl, Po3gl had constant values for which Pearson coefficients have values higher than 0.05.

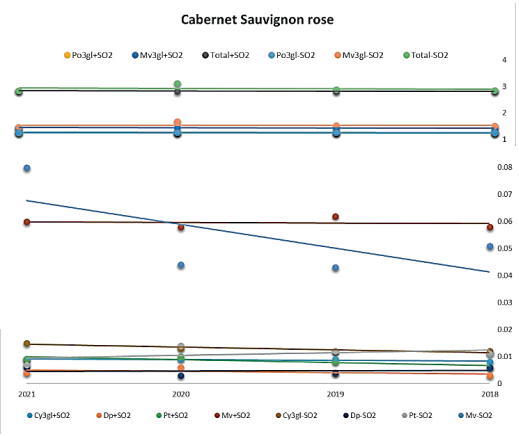

Figure 2. a) Evolution of anthocyanin content for CSr samples

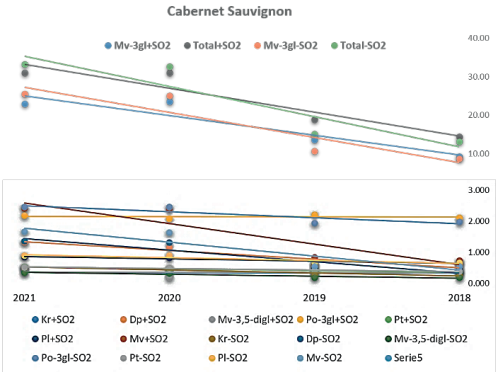

Figure 2b) Evolution of anthocyanin content for CS samples

In direct correlation with low values of correlation coefficients, there are also the mean values of anthocyanins concentrations at treatment comparison, which were not significant different, as mentioned previously, exception was Cy3gl with average value was

13.3 ± 1.4 µmol/L CSr0 against 9.0 ± 0.4 µmol/L (CSr1) which is significant lower. For CS samples the coefficient of determinations have positive values which show a decrease concentration of some anthocyanins as Cy3gl, Dp, Mv3gl, Pt, Mv or total content because the highest values were registered for 2021 and the lowest values were found for 2018 sample, indifferent of treatment (Table 2).

Table 2. Correlation coefficients between levels of anthocyanins and year of production (aging period) (marked values corresponds to p<0.05)

Treatment	-SO_2		+SO_2	
Sample	**CS0**	**CSr0**	**CS1**	**CSr1**
Cy3gl	0.905	0.913	0.924	0.775
Dp	0.914	**-0.086**	0.940	**0.513**
Po3gl	0.872	**0.019**	0.158	0.995
Mv3gl	0.925	**0.000**	0.933	0.774
Pt	0.979	**-0.439**	0.983	0.832
Mv	0.902	0.654	0.912	0.135
Total	0.923	0.154	0.943	0.914

CONCLUSIONS

Present study created the premises for evaluation of wine stability and evolution during aging period in case of red and rose Cabernet Sauvignon wines. Most important compounds showed clear behavior during wine storage and produced two different evolutions. Some of the compounds as Po3gl, Mv3gl, Cy3gl and Dp had a higher stability in terms of concentration modification, especially for rose wine as confirmed by other studies used as reference. In exchange, glycosides forms presented an instability in terms of decrease, fact that was confirmed by structural modification determined by their functions and biosynthetic pathways.

For red wines, the levels were superior to rose samples and had a different behavior in terms of decrease of anthocyanins during monitorization time. Important is that the samples without sulphur dioxide presented a slight superior stability since Po3gl had a stable concentration. From the perspective of treatment comparison, the two types of wine presented similar behavior, so the vinification technology achieved the goal of producing reliable wines from the perspective of minimization the oxidative reactions that could alter wine quality. Further studies are required in case of new technology to produce wines without added sulphur dioxide. Considering the modification of anthocyanins during wine aging, evaluations for compounds as condensation and polymerization forms will be realized.

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