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ORIGINAL RESEARCH PAPER

SPECTROPHOTOMETRIC DETERMINATION OF POLYPHENOLIC AND PROTEIN CONTENT OF YOUNG WHITE WINES IN THE MOLDOVA AREA

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Abstract: In this paper, the processes of protein stability were treated in order to identify the level of protein substances as well as the polyphenolic compounds in white wines. For an optimal determination of the oxidation degree, respectively of the protein stability, a sample of 6 types of white wine from different grape varieties was chosen for analysis. For the heat treatment of protein stability has not yet materialized and published a working protocol in the literature, because many researchers in the field of vinification use different time intervals and temperature. In this paper, the method of thermal stability of proteins was used in two different working environments, respecting the same temperature and time: in the water bath and thermostat. By this method can be deduced the optimal amount of stabilizing agent for the removal of unstable proteins at high temperatures, being less affected by the presence of other polyphenolic compounds, metal cations and the *p*H of wine. The polyphenolic oxidation method was used to determine the degree of oxidability of the wines.

Keywords: *hydroxycinnamic acids, protein, polyphenolic oxidation, stabilization, vinification*

INTRODUCTION

White wines contain a wide variety of proteins, some of which are unstable, naturally forming an opalescent precipitate, so that the wine will have an unpleasant appearance, not being marketable. Obtaining a protein-stable finished product can be achieved by removing proteins [1].

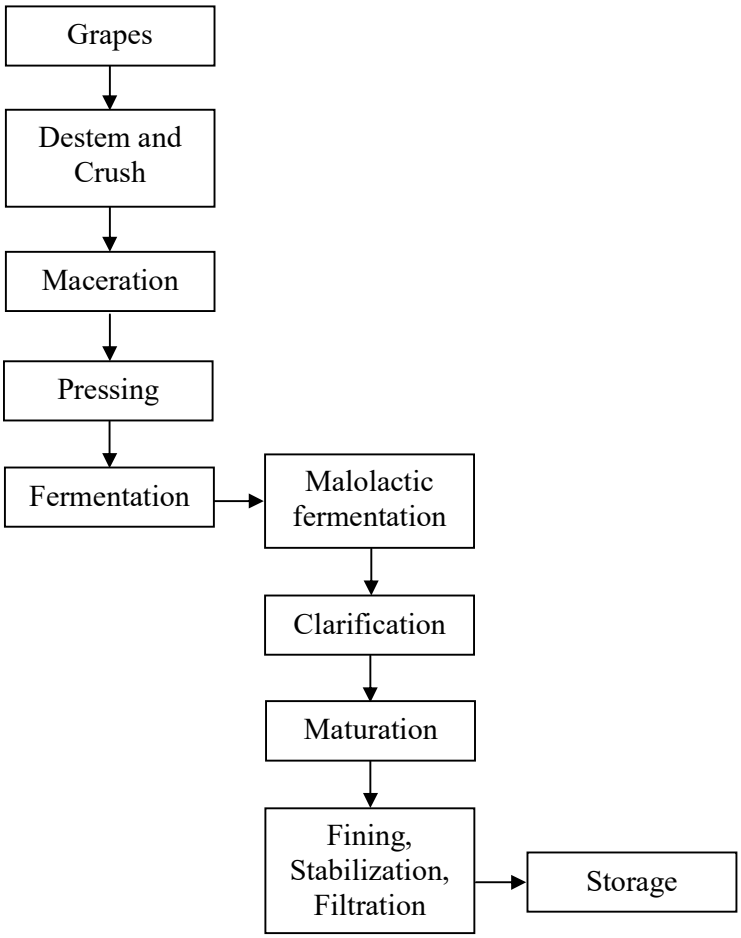


Figure 1. *Specific steps in the technological process of obtaining white wines [2]*

From an oenological point of view, the process of protein stability is shown as the state of equilibrium, its role being to maintain clarity, specific physico-chemical parameters in winemaking and organoleptic properties particular to each type of raw material [3]. The tests used to determine the level of instability of the proteins known and applied so far also include those with heat treatment [4 - 8].

In this paper, six types of white wine from the area of Moldova, obtained by the classical technological process specific to white wine (Figure 1), were used for analysis, which were subjected to the thermal test of protein stability, at the same temperature and heating time, but in different working environments. The purpose of this type of test is to check which of them indicates better the protein content. Thus, before and after the heat stability tests, the degree of turbidity of each type of wine was measured by

nephelometric and spectrophotometric methods [7, 9]. Another type of experiment was performed to determine the degree of oxidability using the POM (Polyphenols Oxidative Medium) test [9, 10]. In the experiments performed the oxidability levels for each type of wine were recorded, which showed a massive decrease in hydroxycinnamic acids for local white wine varieties. In the case of the determination of the total polyphenolic index (IPT) [9, 10], performed before and after the protein stability test and it was determined which is the highest index.

MATERIALS AND METHODS

Wine samples and chemicals

The selected wines used for the experimental analysis were produced in 2019 and stored at the Institute of Oenology of TUM (Technical University of Moldova, Chisinau) in the Republic of Moldova. The wines were produced from 2 European grape varieties: Aligote and Sauvignon Blanc and 4 local grape varieties: Viorica, Alb de Onitcani, Feteasca Alba and Feteasca Regala. Hydrogen peroxide (H_2O_2) 3 % solution, phosphoric acid (H_3PO_4) 25 % solution, sodium hydroxide (NaOH) 0.01 N and sulfur dioxide (SO_2) purchased from Sigma Aldrich were used in the experiments.

Physico-chemical parameters in vinification

A series of tests were performed to determine certain indices of physico-chemical parameters, using the following apparatus such as: Gibertini DEE automatic distiller, Densimat CE hydrostatic scale with Alcomat-2 module (Gibertini Elettronica, purchased from Italy), for the content of volume of ethyl alcohol and dry residues; GlassChem SO_2 (GlassChem, purchased from the Republic of South Africa), for the free and total sulfur dioxide content; respectively, electronic titrator type Titrette (Brand, Germany), pH meter type WTW Inolab 7110 (WTW, Germany), for the *p*H and conductivity values of the wine samples [9, 11].

Stability test

Protein precipitation occurs in wines with a high protein content, which can come from grapes, and in a lesser extent from yeast cells released during maturation on yeast [12]. Protein precipitation takes place according to the mechanism of flocculation of hydrophilic colloids. Another factor responsible for the precipitation of proteins in already bottled white wines is the presence of oxygen; the presence of this element attracts the modification of the redox potential in the mass of the finished product [12]. Various tests indicate the level of instability requiring different doses of stabilizers to clarify the finished product depending on the results of each type of test [7]. Protein stability test is not closely related to the final concentration of bio-polymeric compounds in the finished product [13] and total protein analyzes are limited to predicting the stability of proteins in wine and omit the role of other white wine-specific compounds responsible for this type of defect. The thermal test [14, 15] is the most used method in winemaking to predict the level of wine disorder and to determine the

level of protein instability. It is the simplest method to observe the level of sediment in the finished product during storage. In the literature, a concrete working protocol for performing the thermal test has not yet been published, because many researchers in the field of vinification use different time intervals and temperature (Table 1) [7]. In order to generalize a working protocol is needed more profound insights.

Table 1. *Different ways of thermal testing [7]*

Heating temperature	Working time	References
60 °C	4 days	[16]
80 °C	2 hours	[6, 8, 17-20]
	3 hours	[21, 22]
	6 hours	[23-25]
	30 minutes	[4, 26]
90 °C	1 hour	[16, 27]

In order to perform the stability test, in this part of the research the temperature of 80˚C for one hour on the selected white wine samples was used. The purpose of this test is represented by the denaturation of proteins in the presence of heat. By this method can be deduced the optimal amount of stabilizing agent for the removal of unstable proteins at high temperatures, being less affected by the presence of other polyphenolic compounds, metal cations and the *p*H of wine [7].

POM-test

Polyphenols Oxidative Medium (POM [9 - 11, 28 - 30]), this method is used for assessing the oxidative capacity of white wine after increasing light absorption at 420 nm [31] with spectrophotometer, caused by oxidation induced by hydrogen peroxide of 3 % concentration in wine samples [9, 10, 32]. Oxidation of wine is often associated with denaturation of certain phenolic compounds and organoleptic degradation leading to wine browning and unpleasant odor [33]. Phenolic compounds are responsible for oxidative browning [9]. This complex biochemical process is catalyzed by the enzyme type polyphenol oxidase [9] or by ions and metals with variable valence (such as Fe and Cu, as intermediate oxidants) [12, 34]. Phenolic compounds [35, 36] are oxidized by polyphenol type oxidase during alcoholic fermentation, while the oxidation of polyphenolic compounds of ions and transition metals takes place after the fermentation process or during storage of the finished product [11]. After performing the oxidability test on the polyphenolic compounds of the wine, a major change was observed, in terms of intensification of the color of each wine. The value identified at wavelengths of 420, 520 and 620 nm is compared with the absorbance of the original wine at the same wavelengths, using the following equation (Equation 1):

$$POM_{test} = \frac{A\,420_{(wine\ +H2O2)} - A420_{(wine\ )}}{A420_{(wine\ )}} \quad (1)[11]$$

Each wine sample was analyzed in the visible field, the absorbance for the initially untreated wine, the mulled wine, respectively the wine treated with peroxide in 3 replications. The wine was filtered using a 0.45 μm microfilter for a better reading of the absorbance.

Spectrophotometric analysis

The white wine samples were filtered through a 0.45 μm filter in order to be analyzed spectrophotometrically [35 - 38], such as: absorption spectrum, Total Polyphenolic Index (IPT), the level of flavonoid and non-flavonoid polyphenolic substances, the degree of improvement of white wine- before and after POM-test). Spectrophotometric analyzes were performed using a Specord 250 Plus double beam spectrophotometer (Analytik Jena, Germany) [10, 11]. All tests were performed in triplicate. The range of wavelengths measured were in the range 250-400 nm. In Table 2, are presented the main polyphenolic compounds identified at certain wavelengths reported in literature.

Table 2. *The main polyphenolic compounds identified at certain wavelengths*

Polyphenolic compounds of wine			**Reference absorbent, [nm]**	**References**
Polyphenols	Total		280	[37]
	Cinnamic	Trans-2-L-glutathoylcafeolitartric acid	329-280	[11]
		Trans-caffeyltartaric acid	329-263	
		Trans-coumaril tartaric acid glucoside	311-248	
		Trans-coumaril tartaric acid	315-250	
		Tartaric trans-ferulic acid	330-258	
		Trans-caffeic acid	326-260	
		Trans-p-coumaric acid	311-248	
		Trans-diethylcafeoyl tartrate	332-264	
		Trans-ethylcafeat	327-263	
		Trans-ethyl-p-cumarate	312-262	
	Flavonoids		320	[11]
	Flavones		365	[38]

In order to be able to determine certain polyphenolic compounds, the specialized literature has proposed the reading of certain absorbents specific to hydroxycinnamates, polyphenols and flavonoids, according to well-established (Equations 2, 3 and 4) [11, 39]. At the wavelength of 280 nm the absorption of phenolic compounds takes place, and in the interval of 300-320 nm the cinnamic substances from white wine are found [30].

$$D_{SFT} = (G \cdot A_{280} - 4) \cdot 29.5 \quad (2)[11]$$

$$D_{SFC} = (G \cdot A_{320} - 1.4) \cdot 10 \quad (3)[11]$$

$$D_{SFF} = (G \cdot A_{280} - 4) - 2/3(G \cdot A_{320} - 1.4) \quad (4)[11]$$

where G is the degree of dilution of white wine samples, SFT-total phenolic substances, SFC-cinnamic phenolic substances, SFF-flavonoid substances.

To record the absorbance value at 280 nm specific to polyphenolic substances, a correction coefficient equal to 4 is applied. The correction coefficient of 1.4 is also appreciated for the hydroxycinnamic ones identifiable at the wavelength of 320 nm [11, 39]. In general, UV spectra for white wines have a maximum absorption in the wavelength range of 260-280 nm, being a characteristic area of phenolic substances

(absorption of the aromatic ring, to which adhere various radicals able to influence the position of the maximum) [10]. In white wines the main phenolic substances are the hydroxycinnamic ones together with their esters (Table 2). In the absorption spectra can be found inflection bridges or even maximums highlighted at the characteristic wavelengths (310-350 nm). More often, a complex, combined spectrum of the overlap and additivity of the individual absorption spectra according to Firordt's law is observed [40].

RESULTS AND DISCUSSION

Table 3 summarizes the main characteristics performed for the white wine samples used in this paper. The total SO_2 content varies between 43-73 $mg \cdot L^{-1}$. This assumes that this series of white wine is not stable which means that it is necessary to perform the hot protein stability test. In order to concretize this aspect, nephelometric tests were also performed. In the case of ethyl alcohol content (TAV), the alcohol content is 10.43-13.16 % volume measured at 20 °C, these values being corresponding to dry white wines, respectively, for the density of the wine samples tested. It can be seen that the *p*H of wines is optimal, ideal even for inhibiting unwanted microorganisms.

Table 3. *Determination of the characteristic physico-chemical parameters performed on the selected white wine series before treatment*

White wine	TAV % vol	Density wine	Total dry extract [$g \cdot L^{-1}$]	Conductivity, [$mS \cdot cm^{-1}$]	*p*H	SO_2 [$mg \cdot L^{-1}$]		
						Total	Free	Molecular
Aligote	13.16	0.9895	18.5	1.634	3.62	67	14	0.21
Alb de Onitcani	12.58	0.9915	20.2	1.563	3.06	43	8	0.43
Feteasca Alba	12.00	0.9908	17.2	1.518	3.43	56	9	0.21
Feteasca Regala	12.54	0.9913	20.6	1.512	3.36	59	10	0.27
Sauvignon Blanc	10.43	0.9908	17.7	1.464	3.31	73	15	0.46
Viorica	13.10	0.9911	20.6	1.835	3.11	60	11	0.52

In Table 4, are presented the results of the concentration of total phenolic substances (gallic acid, $mg \cdot L^{-1}$), cinnamic substances (caffeic acid, $mg \cdot L^{-1}$) and flavonoids (catechins, $mg \cdot L^{-1}$). After performing the hot protein stability test, significant changes in the content of cinnamic phenolic compounds and flavonoids were observed in almost every type of white wine selected. There are increases of over 25 % for the content of total phenolic compounds found in Feteasca Albă wine samples (30 %), followed by Aligote and Viorica. Also, an increase for cinnamic phenolic compounds was observed for the same types of wine.

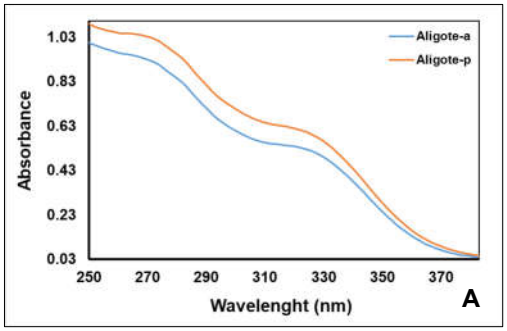
In the case of flavonoid content there are massive decreases (85 %) in Feteasca Albă wine followed by Sauvignon Blanc. Of all the white wine samples that were tested, only

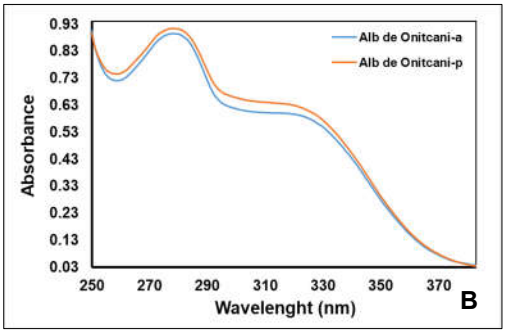
the type of white wine from Onitcani does not show significant changes, according to the data presented in Table 4.

Table 4. *Concentrations of the main phenolic compounds, total polyphenols in white wines*

White wine	SFT (Galic Acid Eq., mg·L^{-1})		SFC (Caffeic Acid Eq., mg·L^{-1})		SFF (Catechin Eq., mg·L^{-1})		IPT		R^2
	Before	After	Before	After	Before	After	Before	After	
Alb de Onitcani	145.38	151.1	45.65	48.91	1.888	1.861	8.93	9.12	0.9991
Aligote	130.95	163	39.77	47.88	1.788	2.333	8.44	9.53	0.9996
Feteasca Alba	58.44	75.23	31.92	37.29	-0.147	0.064	5.98	6.55	0.9978
Feteasca Regala	61.33	72.04	21.69	24.97	0.633	0.777	6.08	6.44	0.9987
Sauvignon Blanc	46.10	56.23	26.81	29.08	-0.224	-0.033	5.56	5.91	0.9958
Viorica	96.08	118.47	27.94	32.62	1.394	1.841	7.26	8.02	0.9997

Following the analysis performed with the spectrophotometer in the ultraviolet range, in the wavelength range 250-400 nm, only the adsorption values at 280 nm were highlighted, because here can be determined exactly the quantities of flavonoids present in each selected wine type. According to the results in Table 4, the highest amount of total phenolic substances (hydroxycinnamates and their esters) determined before the protein stability test was found in the Alb de Onitcani wine sample, followed by Aligote wine. This result indicates the formation of oxidized compounds, with spectrophotometric characteristics different from those of non-oxidized compounds. But, after performing the hot test, it is observed that the Sauvignon Blanc, Viorica and Aligote wine samples have a high content of total phenolic substances, which means that they are prone to wine browning, through the premature formation of the pinking phenomenon.





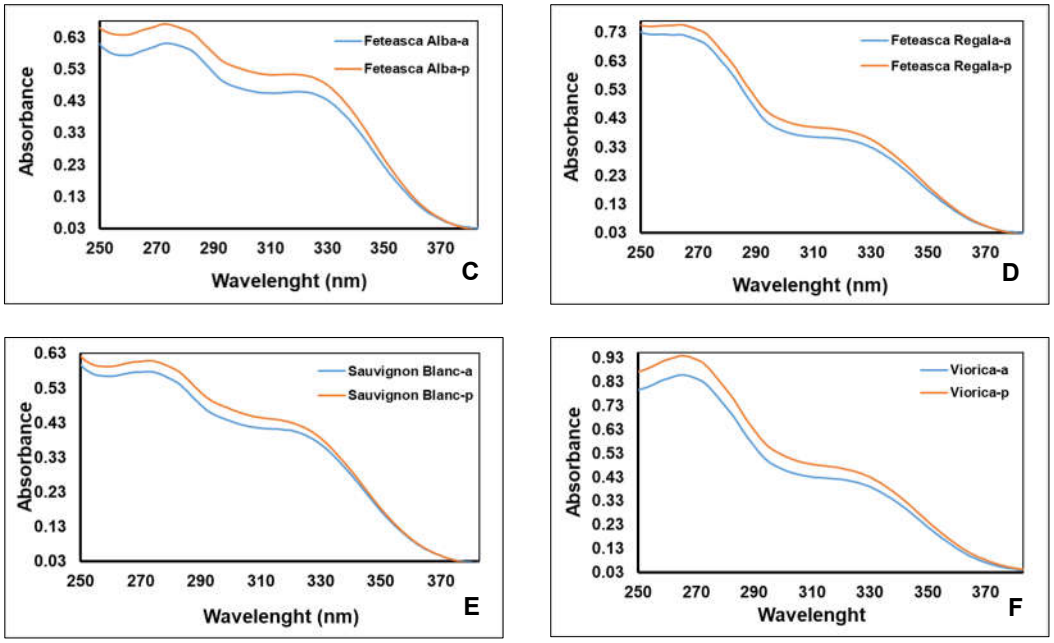


Figure 2. *Absorption spectra of white wine samples performed in the ultraviolet range before (a) and after (p) the protein stability test: A-Aligote, B-Alb de Onițcani, C-Feteasca Albă, D-Feteasca Regală, E-Sauvignon Blanc, F-Viorica*

The absorption spectra obtained by UV-VIS type analyzes for the studied wine samples were measured in the range of 250-380 nm. The range of 260-280 nm is the absorption zone characteristic of the benzene cycles of most phenolic compounds that can be found, more precisely at A = 280 nm. Hydroxycinnamic substances (such as phenolic C6-C3) have a maximum absorption with a bathochromic displacement in the wavelength range of 310-350 nm, found in the wine varieties Aligote and Feteasca Regală (Figures 2A and 2D), 300-350 nm for Alb de Onițcani and Viorica (Figures 2A and 2F) and 315-340 nm for Feteasca Alba and Sauvignon Blanc (Figures 2C and 2E). These hydroxycinnamic substances are the main non-flavonoid compounds present in grapes and white wine, and their absorption spectra may differ from those of a red wine up to wavelengths of 260-280 nm and 300-350 nm respectively. Following the hot protein stability test of Aligote wine and the absorption spectra, the occurrence of the hyperchromic effect was found between 290-310 nm and 370-380 nm (Figure 2A). The same effect is observed for Feteasca Alba in the range 255-265 nm and 290-320 nm (Figure 2C), respectively, Viorica in the range 295-305 nm (Figure 2F). In the case of White Onitcani wine (Figure 2B), simple and warm, there are minor changes in the spectra at different wavelengths compared to other wine samples. In the range 275-290 nm, young white wines have absorption maxima at 275 nm. The studied wines move their absorption maxima at 286 nm, which indicates the formation of oxidized phenolic compounds, with spectrophotometric characteristics different from those of non-oxidized compounds. This bathochromic shift is evident for each sample of wine indicated in Figure 2. But, among the samples studied, Aligote, Feteasca Alba and Viorica seem to be more prone to the formation of protein colloids and oxidation.

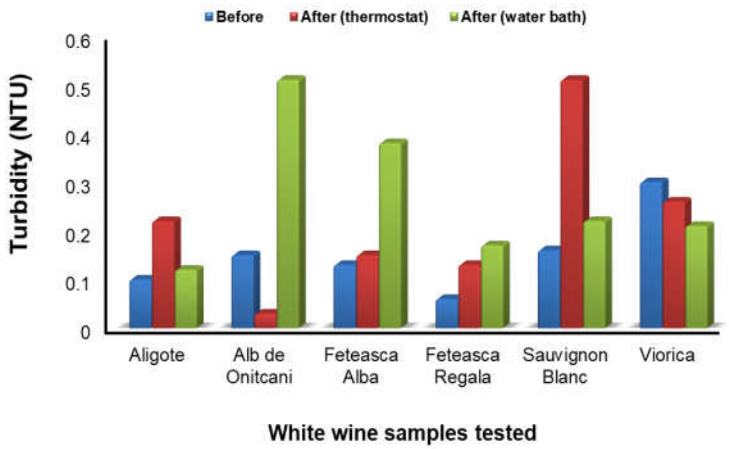


Figure 3. *Measurement of the degree of turbidity for the selected white wine samples, performed before and after the protein stability test (in thermostat and water bath)*

Figure 3 shows the nephelometric levels of each type of white wine tested before and in the initial phase, respectively after their hot treatment but in different environments: thermostat and water bath, at the same temperature and working time. Before exposing the samples to heat treatment, a high degree of turbidity was observed (in the range of 0.16-0.10 nephelometric units (NTU)) for wine samples from the Viorica variety, followed by Alb de Onitcani, Feteasca Alba and Aligote. However, after applying the protein test to the thermostat, it was found that the highest degree of turbidity was recorded for wine samples of Sauvignon Blanc with 0.51 NTU (twice higher than the initial, unheated) followed by Aligote and Fetească Regală with values of 0.26-0.15 NTU. In contrast to these results, in the Alb de Onitcani wine sample there was a decrease (80 %) of the degree of turbidity, compared to the untreated wine sample. In the case of the same sample of wine treated in the water bath there were very large increases (about twice) in the nephelometric degree. This increase was observed in the Feteasca Alba and the Regal samples, and a decrease of the Viorica type.

However, from an organoleptic point of view, the wine samples tested in the water bath compared to those on the thermostat had a higher amount of amorphous sediment in their composition, which means that it is more efficient to perform the protein stability test on the thermostat, as it is much easier to analyze in the next stage at UV-VIS, even if no wine sample exceeds the permissible value of 1NTU.

According to the diagrams presented in Figure 4, regarding the results obtained both before and after performing the oxidability test specific to white wine, it can be said that the influence of various conditions applied to the selected young white wine sample was highlighted, of white wine tested indicates its oxidative capacity.

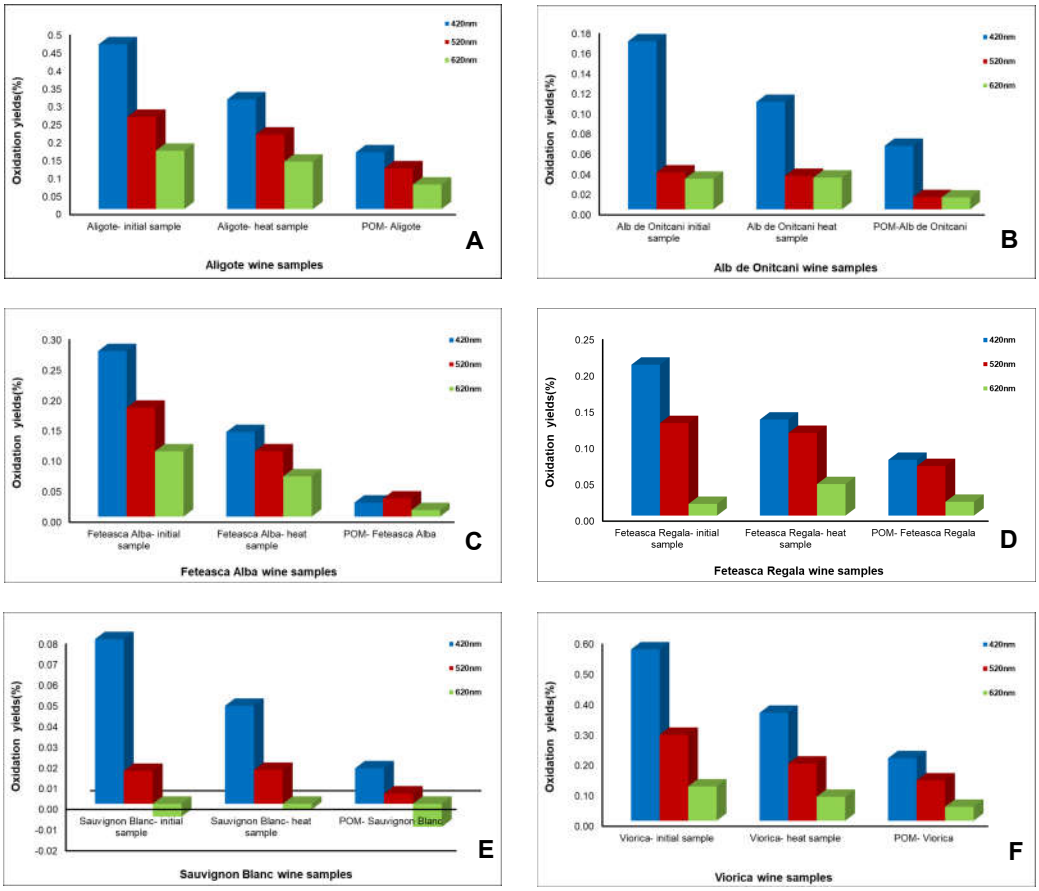


Figure 4. *POM test oxidation type on the tested white wine samples: A-Aligote, B-Alb de Onitcani, C-Feteasca Albă, D-Feteasca Regală, E-Sauviognon Blanc, F-Viorica*

The minimum value is given by Sauvignon Blanc (Figure 4E), respectively the maximum by Viorica (Figure 4F); this may indicate the premature formation of the pinking phenomenon. After oxidative treatment, without the addition of H_2O_2, the differences are significant. Thus, at the absorbance of 420 nm, where the oxidation of polyphenols takes place, major decreases of up to 76 % were recorded for Alb de Onitcani (Figure 4B), followed by Viorica and Feteasca Albă by 50 % and 48.8 %, respectively (Figures 4F and 4C).

For a better highlighting of the degree of oxidability for each type of wine, certain wavelengths were taken into account: 520 nm and 620 nm. For the wavelength of 520 nm, a decrease of absorbance of up to 58 % was observed for Alb de Oniţcani followed by Viorica with 47 % and Feteasca Alba 40 %. However, for the same wavelength, an increase of over 61 % was found in the case of Sauvignon Blanc wine, respectively twice compared to the initial value at 620 nm, which means that this wine sample is already oxidized (Figure 4E). Also for 620 nm a decrease of up to 80 % is observed in Alb de Onitcani, and for the other wine samples the decrease is below 18 %. In the second part of the POM test, the one with the addition of H_2O_2, the differences are much more pronounced compared to the untreated variant. A 92 % decrease in oxidability can

be observed for Feteasca Regala and Feteasca Alba (Figures 4D and 4C) followed by Alb de Onitcani and Viorica by approximately 80 % at the wavelength specific to this typ As it was found from the results presented above, both from the difference between the initial value of the unmodified wine with the variant only heated (in the first part of the test) and then with the addition of hydrogen peroxide (the second part of the test) it can be said that the analyzed sample highlights the wine sample from the Aligote variety (Figure 4A) with the lowest level of polyphenolic content e of test.
This variety of white wine shows small changes when subjected to antioxidant processes because both after the heat treatment and addition of H_2O_2 had a high level of turbidity. This result can be explained by the presence of the respective biopolymer particles, with sediment formation. This aspect was also found in the other selected wine samples, but in insignificant quantities, even imperceptible.

CONCLUSIONS

Among the defects of white wines, those of biochemical and physico-chemical nature were chosen for testing, ie the oxidability and protein disturbance of the wine. According to the data obtained after determining the basic parameters of the white wine sample, it was found that the wine samples tested have an alcohol content corresponding to white wines, and their *p*H is optimal (3.06-3.62).
Following the stability test, an increase of absorption of 15 % was observed in the case of Aligote wine (in the wavelength range- λ = 290-310 nm) -21 % (370-380 nm). This indicates that it is more prone to oxidation due to the higher amount of hydroxycinnamic compounds. After the analysis performed with the UV spectrophotometer, the level of IPT in each type of white wine tested was determined. Thus, the largest amount of total polyphenolic compounds, before the protein stability test, was found in the white wine of Onitcani followed by the Aligote wine variety. However, after performing the thermal stability test, it is observed that the Sauvignon Blanc, Viorica and Aligote white wine varieties indicated a high level of total polyphenolic compounds-IPT, which means that they are prone to wine browning.
The POM type test applied on the 6 types of white wine offers the possibility to predict the risk of browning of white wine. This type of test showed an increased oxidation behavior in the case of Aligote wine followed by Feteasca Regala. This can be explained by the presence in the respective white wine samples of the non-oxidized forms of hydroxycinnamic acids and their derivatives. The lowest value was identified in the white wine samples from Onitcani.

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