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Original Article

Assessment of Major Volatile and Phenolic Compounds from 'Fetească Regală' Wine Samples after Pre-fermentative Treatments using GC-MS Analysis and HPLC Analysis

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Abstract

'Fetească regală', the most cultivated white Romanian grape variety produces elegant wines with flowery notes and white fruits (especially apricot and citrus) hues, with a good acidity. Its volatile profile (esters, terpenes, higher alcohols) and phenolic compounds are of great interest to the wine industry, especially regarding the influence pre-fermentative stages have on their extraction, as most producers choose to follow this path. This study analyses how different pre-fermentative treatments (addition of bentonite, glutathione, tannins, clarifying enzymes) change the final profile of the product. The four variants are the most used prefermentative methods. A sensorial analysis of the obtained wines was performed and the obtained data was correlated with GC-MS and HPLC analysis. 25 volatile compounds were identified and quantified through headspace analysis, using different compound libraries, while through HPLC the presence and quantity of 14 major phenolic compounds was determined. Statistical analysis (One Way Anova) was applied to establish the influence of the pre-fermentative treatments on the quantitative presence of the analysed groups of compounds. The applied treatments bring changes to the final wines' profile by increasing the quantity of some phenolic compounds (gallic, protocatechic, p-hydroxybenzoic, gentisic, vanillic and caffeic acid). From a sensorial point of view, all prefermentative treatments bring a smoother feel of obtained wines, with lower acidities and less of a 'green' character. The final results can be used by industry by adding or removing from their technological process certain steps that have influence on aroma compounds of 'Fetească regală' wines.

Keywords: alcohols, esters, phenolic compounds, Romanian grape variety, terpenes

Introduction

Fragrance plays a key role in the food and beverage quality, since the interaction of aromatic substances with the senses of smell and taste leads to consumer acceptance or rejection. The aroma of wine is a junction between the volatile compounds of different origins: grapes, yeasts, bacterial metabolism, winemaking practices and eventually oak when used.

'Fetească regală' is a relatively new Romanian grape variety and it represents a hybrid between 'Fetească albă' and 'Grasă de Cotnari' (ancient traditional Romanian grape varieties). This variety is a semi-aromatic one and the wines obtained are characterized by elegant rose hues, with notes of wild flowers, dried apricots and almonds (Antoce and Cojocaru, 2015). Furthermore the wines are characterized by freshness, a pleasant acidity with harmonious notes of mineral and fruity flavours, as well as honey flavour (Antoce, 2012). Still, the development of the aroma and various aromatic notes depends on the management of the winemaking process.

The great variety of volatile compounds with different polarities, volatilities and wide range of concentrations, such as: esters, terpenes, aldehydes, alcohols, etc. ensure the complexity of a wine, its character and finally helps in the process of differentiating one wine from another (Jiang and Zhang, 2010). Better understanding the chemical nature of a wine aroma demands the quantitative determination of quite a large number of different odorous active compounds (Ferreira *et al.*, 2001). In this case, gas chromatography analysis of volatile compounds becomes an important tool

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useful for wine classification (Guth, 1997a, 1997b; Ferreira, 2001), quality control (Blank *et al*., 1997; Tominaga *et al*., 1998a; Mestres *et al*., 1998; Mestres *et al*., 2000) or for understanding the sensorial proprieties of a wine (Allen *et al*., 1994; Ebeler and Spaulding, 1998).

The phenolic compounds are secondary metabolites that are derivates of pentose phosphate, shikimate and phenylpropanoid pathways in plants (Randhir *et al*., 2004). Referring strictly to grapes and wines, these compounds are responsible for the development of major organoleptic proprieties, in particular: colour, astringency, flavour and the body and texture of a wine (Minussi *et al*., 2003). In this regard the phenolic composition of wines depends on the variety of grapes, on the winemaking process and also on the aging conditions (Cheynier *et al.,* 1997).

Usually the literature approaches the presence and dynamic of phenolic compounds in red wines and less in white wines due to the fact that here the presence of the phenolic compounds is lower. For example the presence of catechin is more notable in red wines where this compound was identified in average amounts of 6.81 mg $(100\ mL)^{-1}$ and maximum amounts of 39 mg $(100\ mL)^{-1}$ (Frankel *et al*., 1995). On the other hand the presence of this compound in white wines is lower (average amount 1.08 mg $(100\ mL)^{-1}$) a maximum content being identified in Californian Chardonnay wines, respectively 4.60 mg $(100\ mL)^{-1}$ (Frankel *et al*., 1995). Another example is the presence of gallic acid that varies from an average amount of 0.22 mg $(100\ mL)^{-1}$ in white wines (Minussi *et al*., 2003) to an average amount of 3.59 mg mL^{-1} in red wines (Kilink and Kalkan, 2003). However, the presence and the variability of these compounds in the white wines should not be disregarded.

Phenolic aldehydes (eg. vanillin), hydroxycinamic acids (e.g. caffeic, ferullic and p-coumaric acid) and their esters, flavonols, flavanols, benzoic acids (e.g. gallic acid) and anthocyanins are extracted from grapes during the winemaking process. Furthermore, in wines can be identified also the presence of flavon-3-ols, hydrolysable tannins (Martin *et al*., 1992; Guichard *et al*., 1993), stilbenes (Tominaga *et al*., 1998b; Ortega *et al*., 2001).

The style of a wine strongly depends on the intrinsic factors such as: cultivar, year of harvest, climate conditions, terroir, but also on the extrinsic factors of which the most important is the winemaking process.

The importance of this study results from a natural necessity and also from the consumer's requirements. Thus, pre-fermentative treatments are critical when the nature does not help due to the fact that grapes may not have ripened optimally or simply when personalized wines are requested according to consumer's demands.

The aim of this research was to quantify the presence of major volatile compounds and the phenolic compounds from the experimental variants of white wines after applying pre-fermentative treatments such as: bentonite, glutathione, tannins and clarifying enzymes in certain predetermined conditions and to evaluate their impact on the chemical substrate and also on the sensorial palette.

Materials and Methods

Grape samples and reagents for the pre-fermentative treatments

In order to conduct the present study a Romanian grape variety was used: 'Fetească regală'. This grape variety represents one of the most popular wine grape varieties of Romania (Robinson and Harding, 2015). Generally, this indigenous grape variety is used to obtain dry white wines with a great aging potential.

The grapes were manually harvested from the Ampelographic Collection of USAMV Iasi, Copou vineyard (the NE part of Romania, latitude: 47.19487; longitude: 27.552405; altitude: 137 meters), in 2014. The raw material was processed using the classical fermentation technology for white wines as follows: after crushing and destemming, the marc was homogenized. Then a hydraulic press was used for pressing. The general physical-chemical characteristics of the 'Fetească regală' grapes and must are presented in Table 1.

In the pre-fermentative stage, the grape must variants were submitted to different treatments, as follows: FRV0 - no treatment was applied, FRV1 - treatment with bentonite – clay on the must, FRV2 - treatment with glutathione on the must in fermentation, FRV3 -treatment with tannins on the marc and a short maceration applied (24 hours), FRV4 - clarifying enzymes treatment on the marc and a short maceration applied (24 hours). The pomace obtained after crushing the mark was homogenized and divided and added back in the five 15 L demi-johns for the rest of the alcoholic fermentation process.

In the case of the control sample, the marc obtained from 'Fetească regală' grapes was pressed using a hydraulic press, at a pressure of two bars.

In the initial stage, fermentation activators were added in a quantity of 7 g at 15 L of must and the addition of selected yeasts was then carried out in amount of 5 g at 15 L of must. The yeasts were rehydrated before using.

The obtained must was stored in 15 L demi-johns, ensuring the necessary headspace for the fermentation. The fermentation lasted 10 days under controlled temperature conditions of 15-18 °C, avoiding direct contact with air.

The wines were racked, conditioned and filtered using the Grifo with cellulose filter plates (SA−995, Sodinal, France). In the last stage, 1 mL of SO_2 (conc. 5%) was added at 750 mL of wine before bottling with a semiautomatic bottling machine.

In the case of FRV1, the wine samples were obtained using an identical process to the one described above. The addition of bentonite was made within 24 hours after the must started its fermentation. Bentonite was added as bentonite milk in quantity of 210 ml at 15 L of must in fermentation, thereby providing a greater clarification and deproteinization. The must was homogenized to provide the best possible dispersion of bentonite in the medium.

For the sample FRV2, the addition of glutathione was done after must settling. 5.25 g of glutathione were added after a pre-dilution of the product in the must for a more efficient homogenization. The product that was used is mixture of glutathione and gallic tannin, one gram of product producing 6 mg of glutathione. The product favored the development of aromatic expression and increased structure and volume, and by its oxygen affinity

glutathione protects the aromatic notes of young wines and prevents their early oxidation.

For the sample FRV3, the marc resulting from the destemming and the partial crushing was introduced into 30 L vessels, with the addition of 5 g oenological tannin per 25 L of marc. The sample was subjected to maceration process for 24 hours and then pressed.

For the sample FRV4, marc was introduced into 30 L vessels and treated with clarifying enzymes. In order to carry out the proposed treatment, a standardized product was used, adding 0.75 g of enzymes to 25 liters of marc. The product contained a high concentration of pectolytic enzymes that provides fast static clarification of the marc, even under low temperature conditions, by hydrolyzing pectins and reducing the viscosity of the marc. The marc was homogenized and left to macerate for 24 hours, then pressed. The resulting must was transferred into 15 L glass vessels.

Before analysis, the samples were then submitted to the decarbonatation process and were analysed regarding the alcoholic strength, total acidity, volatile acidity, non-reductive extract, reductive extract, free and total SO_2, according to the OIV methods.

Gas-chromatographic quantification of volatile compounds

In order to assess the aroma fraction, a HS20-GC-MS (head space extractor coupled to gas chromatography with mass spectrometer) was used. The system used as carrier gas the helium and the adsorption material for the stationary phase was a resin 2, 6-diphenylene oxide (TENAX).

A headspace method was used: 7 mL of wine were added in a vial. All samples were heated up to 70 °C and shaken (25 rpm for 5 minutes). The vials were pressurized to 60 KPa with helium to remove condense. The volatile fraction was transferred into a Tenax trap at -10 °C by a transfer line at 150 °C. Before injection, for half a minute the trap was dry purged and then the volatiles were transferred at 280 °C with a split of 1/50 into the analytic column.

The separation column used was a Phenomenex Zorbax FFAP type 50 m × 0.32 mm ID × 0.5 μm which was kept at a starting temperature of 82 °C. After 3 min of isothermal condition (transfer load and equilibration of pressure between head space device and GC oven), the temperature increased by 3 °C $min.^{-1}$ up to 135 °C, maintaining this plateau for 1 minute, then by 7.5 °C $min.^{-1}$ up to 160 °C, maintained for 1 minute and finally by 27 °C min^{-1} to 240 °C, remaining constant for 8 minutes (total temperature program 37 minutes). Separation was carried-out at a constant linear velocity of 35 cm s^{-1} (column flow 1.5 mL min^{-1} at start-up).

The 8040 MS triple quadrupole was used in Q3 (third quadrupole) scan mode (TIC) from 30-400 Da at 0.1 s event time (5000 scan sec^{-1}). The ion source interface was maintained at 230 °C and source at 200 °C. The molecules were ionized by electron impact (EI) at 70 eV.

The results were processed qualitatively comparing them to different MS databases available: NIST 14, Wiley 10, FFNSC and SZTERP. For the quantitative results, all samples were processed by relative response to an internal standard of 4-methyl-pentan-2-ol. The chromatograms of the blank sample and of the wine samples analysed are presented in Fig. 6 and the fragmentation for the compounds is presented in Table 6.

HPLC-analysis of the phenolic compounds

The analysis of the phenolic acids was carried out using a high performance liquid chromatography technique. In this sense a liquid chromatography Shimadzu series Proeminence LC20 was used.

The chromatographic column presented has a pre-column Phenomenex Security- Guard Ultra cartridge UHPLC C18 for the column that has a 4.6 mm diameter, same as the analytical column.

The particles in the column were made by Fused-Core type technology presenting a solid silica core of 2.25 μm and adsorption layer of 0.35 μm that were with 85% more efficient with high resolution and low counter pressure system. Separation was achieved through a Kinetex column produced by Phenomenex Inc. having the following characteristics: 150 × 4.6 mm stainless steel, loaded with particles of silan grafted with an octadodecil (C18), 96 Å pores diameter and having a graft capacity of 3 μmol $(m^2)^{-1}$.

The separation of compounds was obtained with a mixture of solvents:

- eluent A: 0.1% solution of methanol (CH_3OH) acidified with trifluoracetic acid (TFA) at a pH of 2.17;
- eluent B: 50% solution of methanol (CH_3OH) acidified with trifluoracetic acid (TFA) at a pH of 2.22.

The autosampler was kept at 0 °C and 10μL of sample previously filtered through a nylon filter with pore size of 0.45 μm was injected into the separation system.

The elution was done in the column oven at 50 °C following a gradient program: 0% B at the start maintained for 3 minutes, linear increase to 18% B until minute 11, then 30% B until minute 19, 35% B until minute 25, 60% B until minute 33, maintained at 60% B until minute 38, linear increased to 80% B until minute 43, 100% B until minute 46, maintained until minute 50, then linear recovery to 0% B in 3 minutes and re-equilibration for 7 minutes. The system flow is 0.85 mL min^{-1}, which developed back–pressure ranging from 175 bar to 290 bar.

The system detector was a DAD (diode array detector) with a speed scan of 6.25 Hz between 190-600 nm for an 8 nm slit aperture. The analysis for protocatehic acid, p-hydroxybenzoic acid, vanilic acid was done at 256 nm. In the case of gallic acid, syringic acid, epicatechin, catechin, ferulic acid the 280 nm wavelength was chosen. Finally for the: gentisic acid, caffeic acid, clorogenic acid, p-coumaric acid, sinapic acid, trans-resveratrol and quercetin, the majority of maximum spectral absorbance is around 324 nm. In the Fig. 7 are presented the chromatograms for the blank sample and for the wine samples.

Reagents

Reagents for the pre-fermentative treatments

In what concerns the pre-fermentative treatments the following reagents were used: Bentonite Clarit 360 from Sodinal (France) (for the sample treated with bentonite), Mannoblanc from Agrovin (Spain) (for the glutathione treatment), Taniblanc from Sodinal (France) (for the tannin treatment) and Zymoclaire CG from Sodinal (France) (for clarifying enzymes treatment). Furthermore, in the winemaking process there were used: fermentation

activators – Fermoplus Integrateur from Sodinal (France) and selected yeasts Fermactive AP from Sodinal (France).

Reagents for GC-MS analysis

In this case, a standard solution 4-methyl-2-pentanol (assay 98%) from Merk Chemicals GmBH (Germany) was used. All the others commercial substances and reagents were provided by Sigma Aldrich (USA), as in detailed in the Annex 1.

Reagents for the HPLC analysis

The mixture of solvents used for the separation of compounds was provided from Sigma Aldrich (USA), respectively methanol (assay- 99.8%) and trifluoracetic acid (assay- ≥ 99%). The other reagents for the HPLC analysis were also provided from Sigma Aldrich (USA) and were presented bellow in Annex 1.

Sensorial analysis

The wine samples were assessed for aroma and flavor acceptability by 10 tasters according with the evaluation method originally proposed by the International Union of Oenologists - organoleptic evaluation of aroma parameters through the "closed" tasting method and recording them on a chart tasting. The tasters evaluated specific characteristics of the wines with ratings from 0 to 10 (Annex 2). The final score was calculated as the mean of all the results, taking into account the evaluation of each taster.

Statistical analysis

In order to determine the influence of the applied treatments on the analysed compounds (esters, terpenes, alcohols and phenolic compounds) a one way ANOVA test was applied. The test was done with the SPSS17 with the aim to determine whether there are any statistically significant differences between the means of the groups. The independent variable was represented by the applied treatments and the phenolic compounds identified were considered as the dependent variable. The reference significance α value of the test was 0.05 with a confidence level of 95% and two hypotheses were created: the null hypothesis that the treatments didn`t affected the chemical and sensorial structure of the wine sample and the alternative one that in fact the treatments did affect the structure of the analysed wines.

Results and Discussion

Basic physic-chemical parameters of the analysed wine samples

The basic physic-chemical parameters of the analysed wines are presented in Table 2 and it is immediately noticed that the wine cannot be considered a quality wine. A possible cause might be the grapes, whose qualitative characteristics show that the raw material was not harvested at technological maturity (Table 1).

The alcoholic strength has varied from a minimum of 7.7% in the control sample and a maximum of 9.58% for the sample treated with bentonite-clay. The values of the total acidity were high for all the samples, the maximum value being in the sample treated with glutathione: 9.13 g L^{-1} tartaric acid (Table 2).

Regarding the volatile acidity the limits of variability were rather small, between a minimum of 0.16 g L^{-1} acetic acid for the sample treated with bentonite) and a maximum of 0.26 g L^{-1} for the control sample (Table 2). The reductive substances showed a rather high dynamic, the values of this parameter varying from a minimum of 0.44 g L^{-1} for the bentonite sample to a maximum of 32.11 g L^{-1} for the control sample. In this case, the high value recorded for the control sample suggested an incomplete metabolism of the sugars by the yeasts, so an incomplete fermentation process. As expected, the values of non-reductive extracts and reductive extracts were higher for the control sample and lower for the other samples. Relative density followed the pattern of variation of the reductive substances, the maximum value being registered in the control sample (1.0672).

GC-MS analysis of esters, terpenes and alcohols

Taking into account that aldehydes and ketones are present in particular in grape must during fermentation and are subjected to oxidation and converted into acids at the end of the fermentation process, but also that the analyzes were performed on the final product (wine after the fermentation process), these groups of compounds were not considered in the present study.

The esters are flavour compounds that occur widely in a variety of food products (Gatfield, 1992). In the fermented beverages such as wine, as a group, these compounds might be considered major constituents after water, ethanol and

Table 1. Basic physico-chemical parameters of the 'Fetească regală' grapes and must

Grape variety	Total acidity (g L^{-1} $C_4H_6O_6$)	Sugars (g L^{-1})	Volumetric mass at 20 °C	Gluco-acidic index	Potential alcoholic strength (%vol. alc.)
'Fetească regală'	10.92	158.1	1.0685	14.47	9.3

Table 2. Parameters of wines obtained through different pre-fermentative treatments

Sample	1	2	3	4	5	6	7	8	9	10	11
FRV0	7.7	8.6	0.26	32.11	202.8	170.6	20.43	91.97	1.0672	2.91	1639
FRV1	9.58	8.36	0.16	0.44	13.7	13.26	18.27	59.76	0.9923	2.97	1688
FRV2	9.25	9.13	0.17	1.58	19.8	18.22	38.39	87.32	0.9950	2.90	1707
FRV3	9.18	8.48	0.16	1.71	19.3	17.59	52.02	111.79	0.9957	2.99	1794
FRV4	9.34	8.48	0.19	2.01	19.6	17.52	29.72	75.87	0.9958	3.14	1670

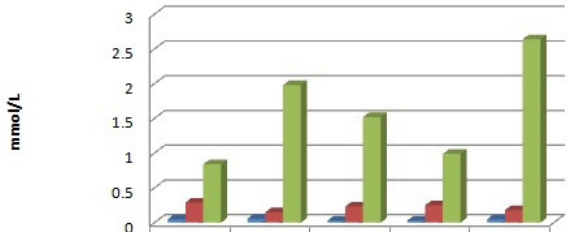
Note 1- Alcoholic concentration (% vol. alc.); 2- Total acidity (g tartaric acid L^{-1}); 3- Volatile acidity (g acetic acid L^{-1}); 4- Reductive substances (g L^{-1}); 5- Total extract (g L^{-1}); 6- Non reductive extract (g L^{-1}); 7-Free SO_2 (mg L^{-1}); 8- Total SO_2 (mg L^{-1}); 9- Relative density; 10- pH; 11- Conductivity (μS cm^{-1})

fusel alcohols and are the primary source of fruity aromas (Etievant, 1991).

The esters present in wine can be classified in two groups, those formed enzymatically and those formed during wine aging, by chemical esterification between alcohol and acids at low pH (Margalit, 1997). The present study focused on the research of the first group of esters, those formed enzymatically. As expected in young wines, the fruity-flowery aroma esters have prevailed. Among the esters identified using headspace analysis are ethyl hexanoate, ethyl benzoate, ethyl octanoate, ethyl myristate, ethyl palmitate.

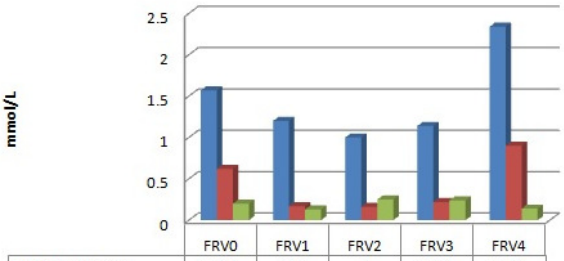
The ethyl hexanoate, a volatile ester produced during fermentation process by yeasts, varied from a minimum of 0.03 mmol L^{-1} in the samples treated with tannin and glutathione to a maximum of 0.05 mmol L^{-1} for the control sample and the sample treated with clarifying enzymes. Ethyl caprate, an ester specific for the alcoholic beverages characterized by the fruity, apple notes (Mosciano, 1990) revealed an interesting variability in the analysed samples. As it can be observed from figure 3 the maximum quantity was identified in the control sample (7.29 mmol L^{-1}), but in samples subjected to the pre-fermentative treatments, the amount of this compound decreased by half.

The ethyl octanoate, an ester resulting from the fatty acid degradation process and characterized by soapy, floral notes (Berger, 2007) has reached maximum concentrations in the samples treated with bentonite and clarifying enzymes, the lower concentrations being registered in the sample treated with glutathione (6.55 mmol L^{-1}). The presence of ethyl caprate and ethyl octanoate in higher concentrations compared to other identified esters might be explained by the temperatures during fermentation.



	FRV0	FRV1	FRV2	FRV3	FRV4
Ethyl hexanoate	0.05	0.06	0.03	0.03	0.05
Ethyl benzoate	0.29	0.15	0.23	0.25	0.18
Ethyl palmitate	0.84	1.98	1.52	0.99	2.64

Fig. 1. Ethyl hexanoate, ethyl benzoate and ethyl palmitate in the analyzed wine samples



	FRV0	FRV1	FRV2	FRV3	FRV4
Ethyl myristate	1.57	1.2	1	1.14	2.34
Ethyl pentadecanoate	0.62	0.17	0.16	0.22	0.9
Diethyl succinate	0.2	0.13	0.25	0.24	0.14

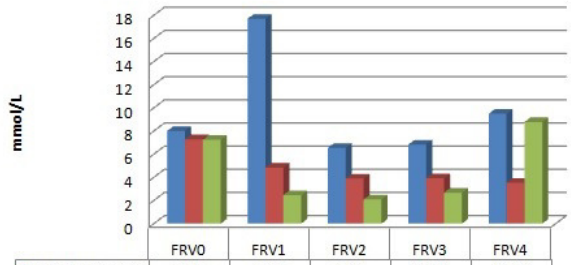
Fig. 2. Ethyl myristate, ethyl pentadecanoate and diethyl succinate in the analyzed wine samples

The ethyl lactate, an ester resulting from the cellular carbon metabolism (Hui, 2007) was identified in all the analysed samples. The pre-fermentative treatments induced an important decrease in the concentration of this compound (Fig. 3), the only exception being the sample treated with clarifying enzymes. In this case, an increase relative to the control sample was recorded. Following the dynamics of ethyl myristate in the experimental samples, the same variation model can be observed as in the case of ethyl lactate.

The analysis of the studied wine samples revealed also the presence of other esters such as: ethyl pentadecanoate, ethyl palmitate, diethyl succinate. Diethyl succinate is an ester characterized by aromatic notes of apple, ylang, cooked-like aroma and it is often present in the fermented beverages that have aged a long time (Dragone *et al*., 2008). The presence of this compound in the studied samples suggested the presence of a premature aging process, atypical aging.

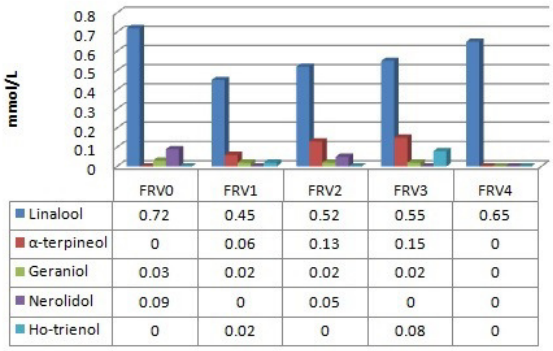
Literature underlines the fact that the terpenoid compounds play a significant role in varietal wine aroma because of their characteristic fruity flowery odour (Camara *et al*., 2004; Feng *et al*., 2015). The presence of these odoriferous compounds depends mainly on the grapes variety and also on the winemaking process. Even if some terpenoid compounds can be found in larger quantities in some grapes varieties, it can be implied that each grape variety has specific volatile compounds (Slegers *et al*., 2015). Moreover the personality of each wine is due to the infinitely varied combinations and concentrations of the various volatile compounds (Ribéreau-Gayon *et al*., 2006). Referring to 'Fetească regală' wine samples, several terpenoid compounds were identified: linalool, α-terpineol, geraniol, nerolidol, ho-trienol. As regard to linalool, nerolidol and geraniol the application of pre-fermentative treatments had a negative impact and decreased the concentration of these compounds (Fig.4).

Alcohols and especially higher alcohols (those containing four to ten carbon atoms) are quantitatively the largest group of aroma compounds in alcoholic beverages and are secondary products of alcoholic fermentation (Lambrechts and Pretorius, 2000). These alcohols are also known as fusel alcohols and have a strong, pungent smell and taste with an important influence on the character of a wine and in the right concentration contributes definitely to the complexity of a wine (Moss, 2015).



	FRV0	FRV1	FRV2	FRV3	FRV4
Ethyl octanoate	8.01	17.7	6.55	6.83	9.49
Ethyl caprate	7.29	4.84	3.89	3.92	3.52
Ethyl lactate	7.25	2.46	2.09	2.67	8.78

Fig. 3. Ethyl octanoate, ethyl caprate and ethyl lactate in the analyzed wine samples



	FRV0	FRV1	FRV2	FRV3	FRV4
Linalool	0.72	0.45	0.52	0.55	0.65
α-terpineol	0	0.06	0.13	0.15	0
Geraniol	0.03	0.02	0.02	0.02	0
Nerolidol	0.09	0	0.05	0	0
Ho-trienol	0	0.02	0	0.08	0

Fig. 4. Terpenoid compounds in the analyzed wine samples

Table 3. Alcohols identified in the analyzed wine samples

Compound (mmol L^{-1})	FRV0	FRV1	FRV2	FRV3	FRV4
Propan-1-ol	0.15	0.31	0.29	0.24	0.40
Pentan-1-ol	0.28	0.10	0.04	0.04	0.09
Hexan-1-ol	1.73	0.00	0.69	1.03	0.00
Hexan-3-ol	0.05	0.05	0.07	0.08	0.00
Glycerol	1.07	0.79	0.37	0.25	0.32
Dodecan-1-ol	0.11	0.04	0.07	0.03	0.18
Hexandecan-1-ol	0.93	0.65	0.72	0.80	2.01
Decan-1-ol	0.08	0.06	0.07	0.08	0.07
Octan-1-ol	0.09	0.18	0.08	0.12	0.09
2,3-butandiol	0.05	0.08	0.03	0.01	0.02
Izobutanol	1.40	1.22	1.03	1.13	0.93

The quantitative presence of the alcohols in the wines depends on the yeast strains used and also on the raw material (grapes) and by default on the viticulture conditions (Giudici *et al*., 1990).

Regarding the studied samples, the qualitative and quantitative alcohols are presented in Table 3. Propan-1-ol is an alcohol whose presence in wine is directly dependent on the action of the yeast strain, the incapacity of yeasts to produce hydrogen sulfides and also on the metabolism of methionine and threonine (Lambrechts and Pretorius, 2000). In the present study, it can be observed that the pre-fermentative treatments didn`t affect the presence of propanol in the samples, but on the contrary the application of these treatments determined a quantitative increase of this compound. On the other hand, the presence of 1-pentanol was strongly influenced by the treatments since their application caused an important decrease from a maximum of 0.28 mmol L^{-1} for the control sample to a minimum of 0.04 mmol L^{-1} for the samples treated with glutathione and tannins. This variation might be explained by the fact that the production of higher alcohols is somehow increased by the media in which solid suspensions are present. In addition it might be possible of having large suspensions in larger quantities which facilitated the formation of higher alcohols.

Another important group of higher alcohols is the group of C6 alcohols which derives from the grape polyunsaturated fatty acids (linoleic acid) and gives the herbaceous smell of wines and they are a marker for wine origin (Nykanen, 1986). At concentrations below 300 mg L^{-1}, higher alcohols can contribute to the desirable complexity of wine, but when their concentrations exceed 400 mg L^{-1}, they are regarded as having a negative effect on quality (Falqué *et al*., 2001). In the studied samples the presence of hexan-1-ol and hexan-3-ol was recorded. The importance of these two alcohols results from the fact that they are considered markers for assessment of wine origin (Oliveira *et al*., 2006) and they are formed during pre-fermentative stages including harvesting, crushing and pressing, as well as during must heating or grape maceration (Oliveira *et al*., 2006). In the present study hexan-1-ol ranged from a minimum of 0.69 mmol L^{-1} for the sample treated with glutathione to a maximum of 1.93 mmol L^{-1} in the control sample, while, in the samples treated with bentonite and clarifying enzymes, it was under the detection limit. On the other hand the presence of hexan-3-ol was detected almost in every sample, except in the sample treated with clarifying enzymes. The quantitative presence of this compound varied from a minimum of 0.05 mmol L^{-1} in the control sample and the sample treated with bentonite and a maximum of 0.08 mmol L^{-1} in the sample treated with tannins.

Glycerol is another important alcohol, a non-volatile compound which has no aromatic proprieties, but which significantly contributes to wine quality by providing sweetness and fullness (Orlic *et al*., 2009). This compound is one of the most important by-products of alcoholic fermentation. Referring to the studied wine samples the largest amount was identified in the control sample (1.07 mmol L^{-1}), the pre-fermentative treatments applied resulting in a quantitative decrease of more than a half of the initial amount. However, in this case glycerol was a product that has resulted from the catalytic oxidation from the analytical column.

HPLC analysis of phenolic compounds

More than 10 phenolic compounds have been identified, both non-flavonoids and flavonoids. Non-flavanoids compounds include: benzoic acids (vanillic acid, gallic acid, protocatechuic acid, gentisic acid, syringic acid), cinnamic acids (*p*-coumaric acid, ferulic acid, caffeic acid). Regarding the flavonoid compounds in the studied samples, the following groups of compounds were identified: flavonols (quercitin), flavan-3-ols (epicatechin). In addition trans- resveratrol was identified.

Gallic acid, a phenolic compound largely derived from grape seeds and stems as well as contact with oak during fermentation process (Jordão *et al*., 2001; Cheynier, 2005) in the analysed samples has varied from a minimum of 19.36 mg L^{-1} for the sample treated with glutathione to a maximum of 79.88 mg L^{-1} in the sample treated with clarifying enzymes. This variation might be explained by the fact that the sample FRV4 (clarifying enzymes) was submitted to a short maceration (24 hours) that permitted the extraction of this compound from seeds and stems. In terms of p-hydroxybenzoic acid, it decreased by half with the application of pre-fermentative treatments compared with the control sample.

The pre-fermentative treatments with tannins and clarifying enzymes and short maceration resulted in a significant increase in gentisic acid content, even double compared to its concentration in the control sample (from 45.37 mg L^{-1} to 89.75 mg L^{-1}). The same pattern of variation could also be identified in the case of vanillic acid, tannins and clarifying enzymes treatments, all resulting in

an increase of up to five times the content of this compound relative to the control sample.

Caffeic acid, a compound that reaches modest levels in red wines (18.8 mg L^{-1}) (Schwarz *et al*., 2004) has recorded an important increase after applying the tannin treatment, from a value of 19.49 mg L^{-1} in the control sample to a value of 233.46 mg L^{-1} in the tannin treatment sample. The bentonite treatment and clarifying enzymes treatment determined a decrease in half. Ferulic acid and p-coumaric acids show a slight increase in concentration after the application of pre-fermentative treatments. Glutathione and clarifying enzymes treatments have led to an increase in quercitin while, for the samples with bentonite treatment the presence of this compound was under the detection limit. From the group of flavon-3-ols only epicatechin was identified. This compound did not undergo major variations after applying pre-fermentative treatments and compared with the control sample.

Sensorial analysis

Fig. 5 shows that all the wines samples had a high level of acidity, a statement sustained by the total acidity values of the analyzed samples that were presented in Table 2. In the organoleptic charts it can be observed that in all wine samples prevailed the green/vegetable, green fruits and citric flavours. Compared with the control sample the mineral flavour was better expressed in the sample treated with glutathione and the bitter taste was expressed more intensely in the sample treated with clarifying enzymes. This is usual since the using of enzymes in the modern winemaking determines the increase of phenolic compounds. The idea is supported by the HPLC analysis of the phenolic compounds presented in Table 4, with higher values for gallic acid, protocatechuic acid, gentisic acid and vanillic acid. The higher values of ethyl myristate could have caused the prevalence of wild flowers flavour in the sample treated with clarifying enzymes.

Statistical analysis

Concerning the statistical analysis of the esters, there were obtained alpha (α) values lower than the reference value 0.05 (Table 5) in the case of ethyl benzoate, ethyl palmitate, ethyl myristate, ethyl octanoate, ethyl caprate and ethyl lactate. Therefore, in this case the null hypothesis is rejected, the results being statically significant.

In the case of terpenes a higher value of alpha criteria than the reference value (0.05) (Table 5) was obtained for the linalool, so the null hypothesis is accepted and the result was not statically significant. On the other hand for the others terpenes identified the values of alpha criteria were lower than 0.05 meaning that the null hypothesis is accepted and the results are statistically significant.

Regarding the alcohols higher values than 0.05 (Table 5) were obtained for pentan-1-ol and decan-1-ol. In these cases the null hypothesis is accepted. For the others alcohols identified, the values of α were lower than 0.05, so the null hypothesis is rejected and the results are statistically significant.

In what concerns the phenolic compounds, the null hypothesis is accepted in the case of ferulic acid, trans-resveratrol and quercitin ($\alpha > 0.05$) (Table 5). For the gallic acid, protocatechuic acid, p-hidroxibenzoic acid, caffeic acid, clorogenic acid, syringic acid, epicatechin, p-coumaric acid, sinapic acid the values of alpha (α) were lower than 0.05, therefore the null hypothesis is rejected, being accepted the alternative hypothesis.

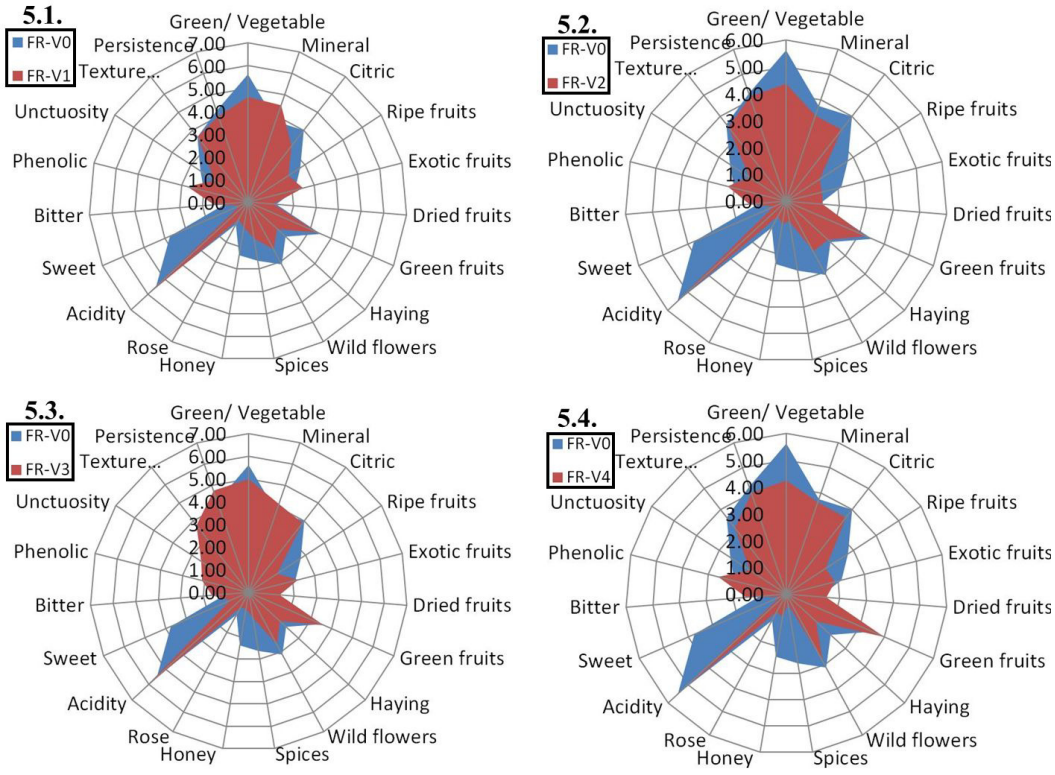


Fig. 5. Comparative organoleptic charts of the analysed samples (organoleptic chart of the control sample and the sample treated with: 5.1. bentonite; 5.2. glutathione; 5.3. tannins; 5.4. clarifying enzymes)

Table 5. Results of Anova statistical analysis

Compounds	Type III Sum of Squares	df	Mean Square	F	Significance	Rsquared	Rsquared adjusted
Ethyl hexanoate	0.02[a]	4	5.733	2.324	0.127	0.482	0.275
Ethyl benzoate	0.35[a]	4	0.08	21.900	0.000	0.898	0.875
Ethyl palmitate	6.573[a]	4	1.643	145.774	0.000	0.983	0.976
Ethyl myristate	3.483[a]	4	0.870	81.573	0.000	0.970	0.958
Diethyl succinate	0.41[a]	4	0.010	3.018	0.071	0.547	0.366
Ethyl octanoate	255.203[a]	4	63.801	113.922	0.000	0.979	0.970
Ethyl caprate	28.153[a]	4	7.038	31.787	0.000	0.927	0.898
Ethyl lactate	117.619[a]	4	29.315	1406.682	0.000	0.998	0.998
Linalool	0.136[a]	4	0.034	2.614	0.099	0.511	0.316
α-terpineol	0.060[a]	4	0.015	19.618	0.000	0.887	0.842
Geraniol	0.001[a]	4	0.000	4.115	0.032	0.622	0.471
Nerolidol	0.020[a]	4	0.005	33.455	0.000	0.930	0.903
Ho-trienol	0.014[a]	4	0.004	180.000	0.000	0.986	0.981
Propan-1-ol	0.102[a]	4	0.025	11.048	0.001	0.815	0.742
Pentan-1-ol	2.83[a]	4	0.746	1.500	0.274	0.375	0.125
Hexan-1-ol	6.448[a]	4	1.612	155.603	0.000	0.984	0.978
Hexan-3-ol	0.011[a]	4	0.003	4.704	0.021	0.653	0.514
Dodecan-1-ol	0.046[a]	4	0.011	19.477	0.000	0.886	0.841
Hexandecan-1-ol	3.790[a]	4	0.948	45.081	0.000	0.947	0.926
Decan-1-ol	0.001[a]	4	0.000	0.225	0.919	0.082	-0.285
Octan-1-ol	0.021[a]	4	0.005	5.804	0.011	0.699	0.579
2,3-butandiol	0.008[a]	4	0.002	14.364	0.000	0.852	0.792
Isobutanol	0.391[a]	4	0.098	8.211	0.003	0.767	0.673
Gallic acid	8523.516[a]	4	2130.879	2065.419	0.000	0.999	0.998
Protocatechuic acid	459.449[a]	4	114.862	51.018	0.000	0.953	0.935
p-hydroxybenzoic acid	2123.071[a]	4	530.768	66.326	0.000	0.964	0.949
Gentisic acid	5791.319[a]	4	1447.830	2087.814	0.000	0.999	0.998
Vanillic acid	79076.505[a]	4	19769.126	54.676	0.000	0.956	0.939
Caffeic acid	105205.014[a]	4	26301.254	383.473	0.000	0.994	0.991
Clorogenic acid	1.654[a]	4	0.413	20.277	0.000	0.890	0.846
Syringic acid	4.007[a]	4	1.002	100.316	0.000	0.976	0.966
Epicatechin	2.378[a]	4	0.595	6.677	0.007	0.778	0.619
p-coumaric acid	0.756[a]	4	0.189	4.906	0.019	0.662	0.527
Ferulic acid	153.348[a]	4	38.337	2.971	0.074	0.543	0.360
Sinapic acid	0.117[a]	4	0.029	5.991	0.010	0.706	0.588
Trans-resveratrol	6657.113[a]	4	1664.278	3.162	0.064	0.558	0.382
Quercitin	385.709[a]	4	96.427	3.142	0.065	0.557	0.338

Table 6. Fragmentation table for the compounds identified in the analysed samples

Chemical name	m/z (ion molar mass)	quantifier ion (m/z)	1st qualifier ion (m/z)	2nd qualifier ion (m/z)
ethyl hexanoate	144	88	43	99
ethyl benzoate	150	105	77	122
ethyl palmitate	284	88	101	43
ethyl myristate	256	88	101	43
ethyl pentadecanoate	270	88	101	43
diethyl succinate	174	101	129	55
ethyl octanoate	172	88	101	57
ethyl caprate	200	88	101	43
ethyl lactate	103	45	43	75
linanol	154	71	93	55
α-terpineol	136	59	93	121
geraniol	154	69	41	68
nerolidol	204	41	69	43
hotrienol	91	71	82	43
Propan-1-ol	60	31	42	59
Pentan-1-ol	87	42	55	41
Hexan-1-ol	102	56	43	41
Hexan-3-ol	101	59	55	73
Dodecan-1-ol	168	55	43	69
Decan-1-ol	157	70	55	56
Octan-1-ol	112	56	55	41
2,3-butandiol	90	45	43	57
Isobutanol	74	43	41	42

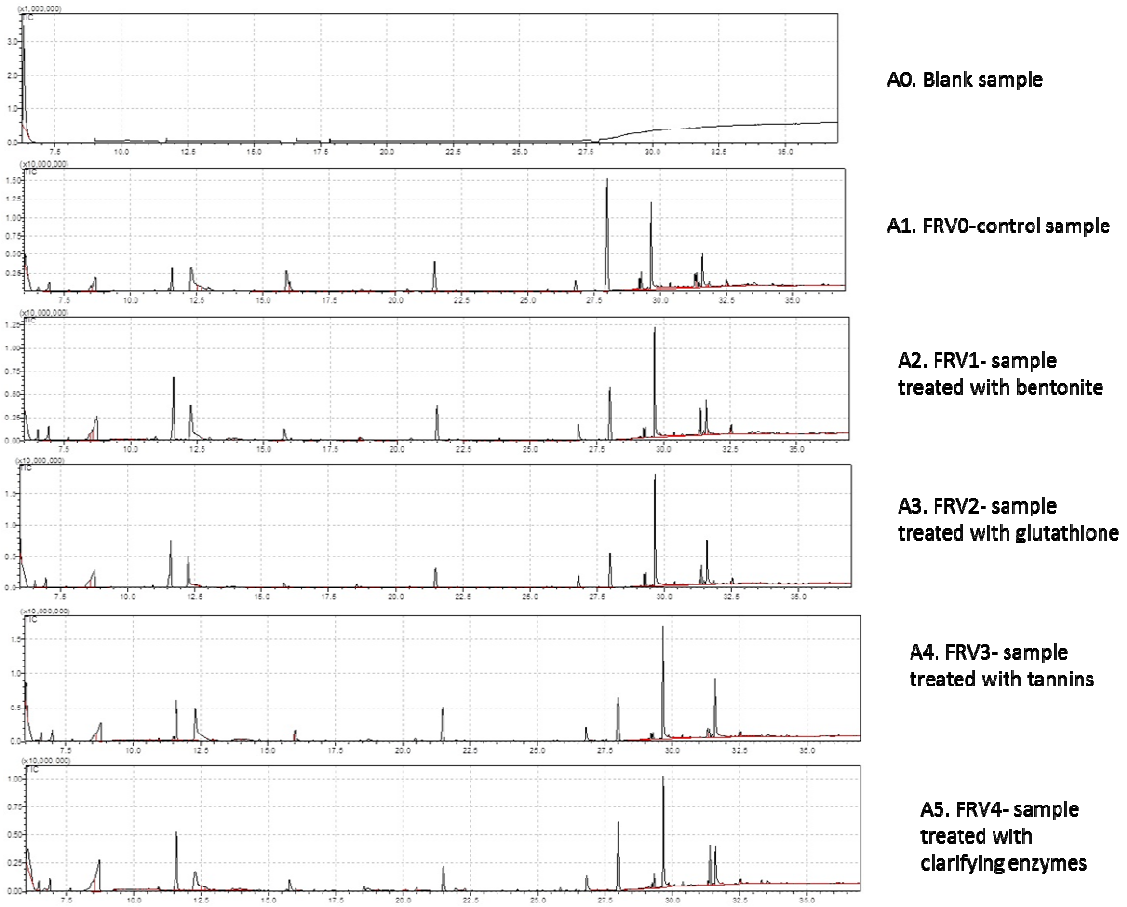
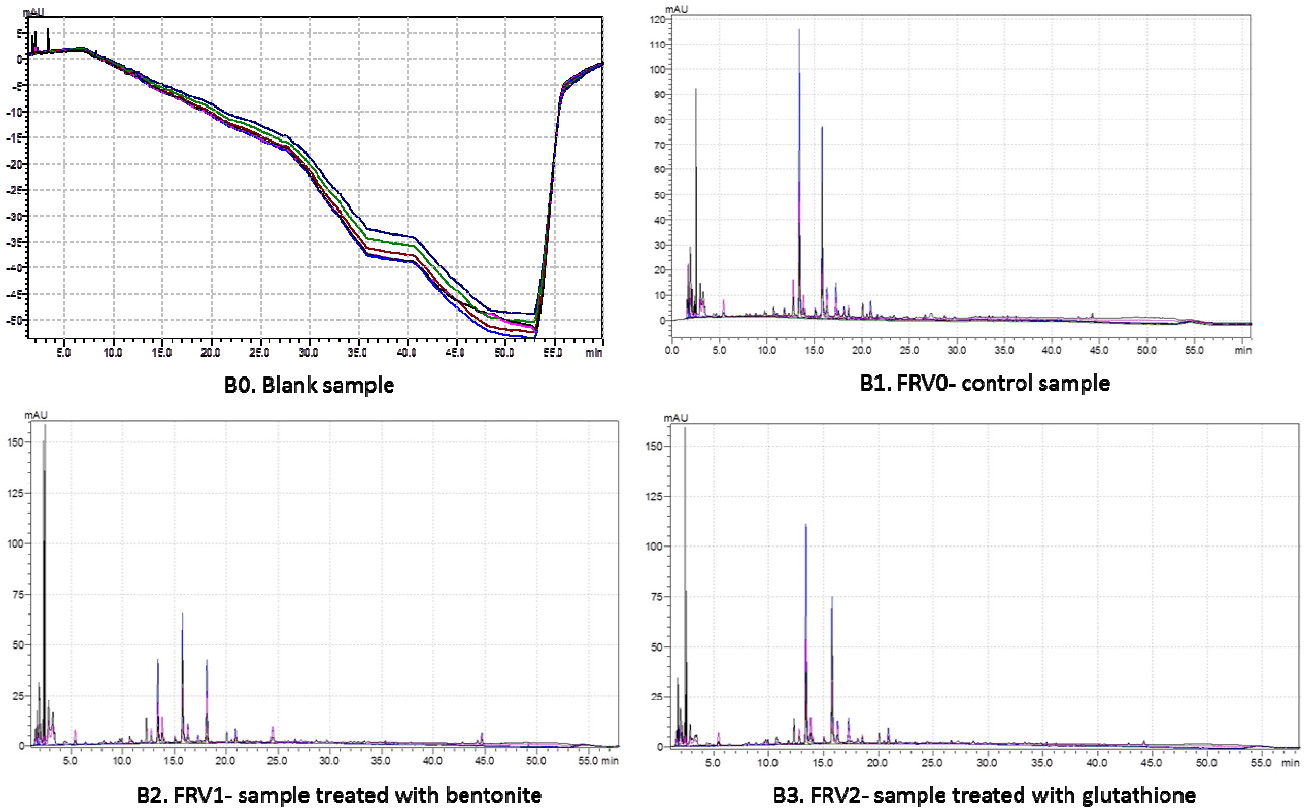


Fig. 6. Chromatograms of GC-MS analysis of the standard (A0) and wine samples (A1, A2, A3, A4, A5)



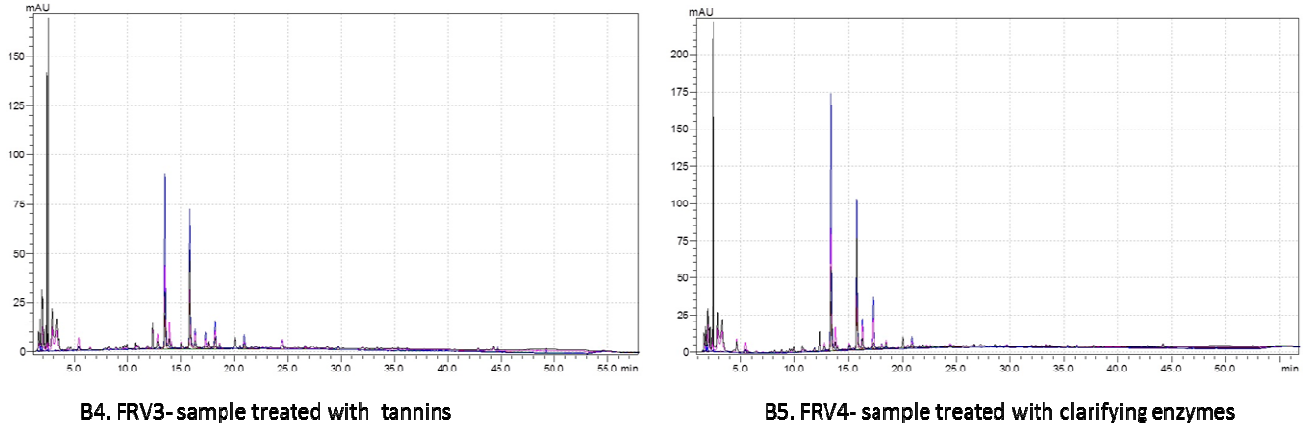


Fig. 7. Chromatograms of HPLC analysis of standard (B0) and wine samples (B1, B2, B3, B4, B5)

Conclusions

Thirty-eight compounds have been identified in the wine samples of 'Fetească regală' with the help of GC-MS analysis and HPLC analysis. According to the statistical analysis applied there were found results that were statistically significant. So the application of pre-fermentative treatments affected the quantitative variation of the alcohols, especially: ethyl benzoate, ethyl octanoate, ethyl palmitate, ethyl myristate, ethyl caprate and ethyl lactate. Also, the quantitative variation of some varietal aroma compounds such as: α−terpineol, geraniol, nerolidol, ho-trienol, was influenced significantly by the treatments applied. The presence of alcohols it was influenced by the treatments applied, the exceptions would be pentan-1-ol and decan-1-ol. The phenolic compounds identified were influenced by the treatments, with the exception of ferulic acid, trans-resveratrol and quercitin were no significantly statistic results were found.

Referring to the sensorial analysis, differences following the application of pre-fermentative treatments were observed. Thereby, the application of glutathione treatments and clarifying enzymes determined the accentuation of green/vegetable flavours and also the green fruits flavours. Probably this effect is due to antioxidant activity of the glutathione that prevented the presence of oxidative processes. In the case of the clarifying enzymes treatments the amplification of the green/vegetable and green fruits flavours was probably due to the maceration process that allowed an intimate contact between seeds, skins and must. The mineral flavour has been intensified by the bentonite, glutathione and clarifying enzymes treatments. As expected the phenolic taste was better expressed in the last sample due to the fact that enzymes generally determine the quantitative increase of phenolic compounds. The acidity of the wines was in concordance with the values of the total acidity determined.

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Annex 1. Aroma compounds identified in the analysed samples

No		Compound name	CAS	Odor descriptor
1	Esters	Ethyl hexanoate	123-66-0	Sweet, fruity, pineapple, fatty, green banana
2		Ethyl benzoate	93-89-0	Sweet, wintergreen, fruity, medicinal, cherry grape
3		Ethyl palmitate	628-97-7	Fruity, creamy, milky, balsamic
4		Ethyl myristate	124-06-1	Sweet, violet, orris
5		Diethyl succinate	123-25-1	Fruity, apple, cooked apple, ylang
6		Ethyl octanoate	106-32-1	Fruity, winey, sweet, apricot, banana, brandy, pear
7		Ethyl caprate	110-38-3	Sweet, fruity, apple, grape, oily, brandy
8		Ethyl lactate	97-64-3	Sweet, fruity, acidic, etherial with a brown nuance
9	Terpenes	Linalool	78-70-6	Citrus, floral, sweet, bois de rose, woody, green, blueberry
10		α-terpineol	98-55-5	Pine, terpenic, lilac, citrus, woody, floral
11		Geraniol	106-24-1	Sweet, floral, fruity, rose, citrus
12		Nerolidol	7212-44-4	Floral, green, waxy, citrus, woody
13		Ho-trienol	20053-88-7	Sweet, tropical, floral, fennel, ginger, spicy
14	Alcohols	Propan-1-ol	71-23-8	alcoholic fermented fusel tequila musty yeasty sweet fruity apple pear
15		Pentan-1-ol	71-41-0	Fusel, oily, sweet, balsamic
16		Hexan-1-ol	111-27-3	Ethereal, fusel, oily, fruity, alcoholic, sweet, green
17		Hexan-3-ol	623-37-0	Alcoholic solvent like, fusel notes of rum, egg nogg and whiskey, green fruity nuances of guava and apple
18		Glycerol	56-81-5	–
19		Dodecan-1-ol	112-53-8	Earthy, soapy, waxy, fatty, honey, coconut
20		Hexandecan-1-ol	36653-82-4	Waxy, floral
21		Decan-1-ol	112-30-1	Fatty, floral, orange, sweet, clean, watery
22		Octan-1-ol	111-87-5	Waxy, green, citrus, aldehydic and floral with a sweet, fatty, coconut nuance
23		2,3-butandiol	513-85-9	Fruity, creamy, buttery
24		Isobutanol	78-83-1	Fusel, whiskey
25	Phenolic compounds	Gallic acid	149-91-7	–
26		Protocatechuic acid	99-50-3	Phenolic, balsamic
27		p-hydroxybenzoic acid	99-96-7	Phenolic, nutty
28		Gentisic acid	490-79-9	–
29		Vanillic acid	121-34-6	Sweet creamy, phenolic, brown and powdery with vanilla beany nuances
30		Caffeic acid	331-39-5	–
31		Clorogenic acid	327-97-9	–
32		Syringic acid	530-57-4	–
33		Epicatechin	490-46-0	–
34		p-coumaric acid	501-98-4	Balsamic
35		Ferulic acid	537-98-4	–
36		Sinapic acid	530-59-6	–
37		Trans-resveratrol	501-36-0	–
38		Quercitin	117-39-5	–

Annex 2. Wine tasting chart example

	Name	Sample:										
	Characteristics	Notes										
		0	1	2	3	4	5	6	7	8	9	10
Olfactory Sensations	Green / Vegetable											
	Mineral											
	Citric											
	Ripe fruits											
	Exotic fruits											
	Dried fruits											
	Green fruits											
	Haying											
	Wild flowers											
	Spices											
	Honey											
	Rose											
Gustatory Sensations	Acidity											
	Sweet											
	Bitter											
	Phenolic											
	Onctuositaty											
	Texture (structure)											