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DEVELOPMENT OF METHODS FOR IDENTIFICATION AND QUANTIFICATION OF PHENOLIC COMPOUNDS IN WINE AND GRAPE USING SPECTROPHOTOMETRY, LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY

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ABSTRACT

Spectrohotometric assays of total phenolics, total anthocyanins, total catechins, total flavonoids, color intesity and hue were performed on twenty four red Vranec and Merlot wines obtained with 3, 6 and 10 days of maceration, containing 30 and 70 mg/L SO_2, and eight white Smederevka and Charodonnay wines, with 50 and 100 mg/L SO_2. Macedonian yeast, Vinalco and French yeast Levuline were used for fermentation for the red and white wines. Changes of phenolic contents were observed during four stages of storage of the wines: after maceration (for the red wines) or after fermentation (for the white wines), after 2, 6 and 16 months in order to check the effect of maceration time (for red wines), SO_2 and time of wine storage. Wines were stored at low and higher temperature to check also the influence of storage temperature on the studied parameters. Folin-Ciocalteu method was used for total phenolics determination, p-DMACA reagent was used for measurements of total falvan-3-ols in the wines and colorimetric method with aluminium chloride was applied for determination of flavonoids. It was found that maceration and storage time and SO_2 influence the content of antocyanins, phenolics, flavan-3-ols and flavonoids in the analysed wines, and the yeast did not have significant influence on phenolics contents.

An HPLC-DAD-MS study of anthocyanins, phenolic acids, flavan-3-ols and flavonols in Vranec and Merlot wines obtained under different vinification is reported here. The percentage of formic acid in the mobile phase and pH value of the wine samples were optimized in order to transform the chalcone forms of anthocyanins into their flavylium red cations, to increase sensitivity and perform a simultaneous MS identification of different groups of phenolic compounds in wine. Quantification of compounds was performed by direct injection of the wines into HPLC, using mobile phase consisted of water/formic acid (95:5; solution A) and acetonitrile/water/formic acid (80:15:5; solution B) with flow rate of 0.25 mL/min at 38 °C.

The proanthocyanidin composition, mean degree of polymerization and concentration of tannins of the same Vranec and Merlot wines, were determined by reversed-phase HPLC after acid-catalysis in the presence of excess of phloroglucinol. Two procedures for preparation of wine samples, solid-phase extraction and precipitation with methanol, were compared. Precipitation with methanol was chosen for sample preparation before phloroglucinol treatment and HPLC-DAD-FLD quantification. An HPLC-MS analysis was used for identification purposes of monomers and phloroglucinol adducts.

Principal component analysis was performed in order to check the possible grouping of the wine samples from both, red and white varieties accoriding to spectrophotometric and HPLC data.

Matrix-assisted laser desorption/ionization mass spectrometry is a new valuable screening technique for the presence and identification of anthocyanins in wine and grape samples. The MALDI matrices: α-cyano-4-hydroxycinnamic acid (CHCA), sinapic acid (SA), 2,5- hydroxybenzoic acid (2,5-DHB) and C70 fullerene were tested in this study. 2,5-DHB was superior with respect to all the matrices used. Fullerene was used for the first time as a possible matrix for the MALDI TOF MS analysis of anthocyanins.

Keywords: anthocyanins, phenolic acids, flavan-3-ols, flavonols, dihidroflavonols, phenolic alcohols, HPLC-DAD-MS, identification, vinification, maceration time, yeast, SO_2 influence, color intensity, hue, wine-making, spectrophotometry, Vranec, Merlot, Smederevka, Chardonnay, wine, grape.

1. INTRODUCTION

The quality of wines depends mostly on the polyphenolic composition – the most important components which determine the color, mouthfeel, astrigency and bitterness of the wine. They are responsible for the differences between red and white wines, especially for the color and taste of red wines. Those substances are present in different parts of the grape and they are extracted during the vinification. The structure of polyphenolic compounds varies when wine ages in the barrel and in bottle, according to the conditions of storage. Polyphenolics possess bactericide and antioxidant properties that protect the consumers from cardiovascular disease.

Taking into account the great importance of phenolic components, on one hand, and the fact that there are no data for the phenolic content of Macedonian wines and grapes, on the other hand, the aim of this doctoral dissertation was to study the polyphenolic components, in direction of development and application of methods for their identification and quantification in grapes at different ripening stages and wines obtained with different wine-making techniques. For those purposes, the following techniques were used:

1 **Spectrophotometry**,
2 **High performance liquid chromatography with UV-Vis detection and mass spectrometry (HPLC-DAD-MS), and**
3 **Matrix assisted laser desorption/ionization mass spectrometry-time of flight (MALDI-TOF-MS).**

Namely, in the first phase of the research work, existing spectrophotometric methods were utilized for determination of total phenolics, total flavonoids, total anthocyanins and total catechins, color intensity and hue. The total content of phenolics was determined with the Folin-Ciocalteu method, whereas, the influence of the temperature at the time of reaction was checked. Colorimetric assay with aluminium chloride was carried out for analysis of total flavonoids in wine and grape; total anthocyanins were analyzed after dissolution of the samples with solvent consisted of C_2H_5OH, H_2O and HCl (37 %) in appropriate ratio, while the content of total catechins was measured with *p*-(dimethylamino)cinnamaldehyde assay. The spectrophotometric methods were applied for quantitative determination of phenolic composition of Macedonian wines and grapes and the changes of phenolic components from different groups were followed during the grape ripening at three stages for Vranec, Merlot, Smederevka and Chardonnay varieties, as well as for the wines produced form the same grape varieties, obtained with different technological processes. Thus, the experimental wines from the red varieties Vranec and Merlot and white varieties, Smederevka and Charodannay, were produced with two doses of SO_2 and two yeasts for fermentation and for red wine production, 3, 6 and 10 days of maceration were applied in order to check the influence of those parameters on extraction of phenolics.

During the second phase, series of investigations were performed in order to develop suitable HPLC methods for qualitative and quantitative simultaneous determination of phenolic components.

The used HPLC methods were developed:

- ✓ at the **Department of Analytical Chemistry and Environmental Chemisty, Faculty of Natural Sciences, Univeristy of Pecs, Pecs, Hungary**, and
- ✓ at the **Science for Enology, INRA, French National Institute of Agricultural Research, Montpellier, France**.

Taking into account that several different forms of anthocyanins exist at wine pH, high acidity of the mobile phase is needed for chromatographic analysis of anthocyanins in their flavylium red forms, which on other hand, is incompatible for MS detection of phenolic acids and the intensity in the electro spray source. Thus, the effect of pH of the sample and mobile phase were explored using anthocyanin extract from grape skins and it was shown that 1 % formic acid in the mobile phase allows proper separation and detection of anthocyanins if the sample is acidified at pH < 2. The stability of the other phenolic components present in wine was tested by storage of the wine samples with adjusted pH 1.1 at room temperature for 30 min, 2 h and 3.5 h and compared with the initial conditions after immediate injection into HPLC. The optimized conditions were applied for MS identification of 68 compounds from different phenolic groups present in the wine.

Quantification of 28 phenolic components from different groups, by direct injection of the wines was performed. The tannins in wines, terminal and extensional units, as well as the mean degree of polymerisation after acid depolymerisation with phloroglucinol were determined by HPLC.

The third aim of the research in this PhD was application of MALDI-TOF-MS for wine analyses. In this study, different MALDI matrices for anthocyanin identification were tested in order to find the best possible matrix. Fullerene was introduced as a possible new matrix, used for the first time for MALDI wine and grape analyses.

The developed methods are significant for scientific and practical purposes and the obtained results are important for setting the optimal conditions for production of wine rich with phenolic components.

2. EXPERIMENTAL

2.1. Grapes and wines

2.1.1. Red Vranec and Merlot wines produced with different technological procedures

Grapes from *Vitis vinifera* L., Vranec and Merlot variety, cultivated in Skopje region (2007 vintage), were harvested at optimal maturity (22 and 20 °Brix, respectively) and separately transported to the experimental cellar of the Wine Department, Institute of Agriculture in Skopje, R. Macedonia. Grapes from both varieties were divided into 12 lots (11 kg for each lot) and using mechanical crusher/destemmer, they were processed separately in the same way, and crushed grapes were collected in 25 L plastic fermentation tanks.

Two doses of aqueous solution of potassium metabisulfite were added to the Vranec grapes mashes and mixed to give six tanks having 30 mg/L SO_2 (V30) and six tanks with 70 mg/L SO_2 (V70). The same procedure was performed for the grape mashes of Merlot variety, obtaining the lots, M30 and M70.

Two yeasts (*Saccharomyces cerevisiae*) were used for fermentation: Vinalco (Bitola, R. Macedonia) and Levuline CHP (Bordeaux, France), kindly supplied from Vinea winery-Štip and Tikveš-winery-Kavadarci, respectively, both from R. Macedonia. The yeast were prepared by rehydrating (20 g/100 L for Vinalco and 30 g/100 L for Levuline) in water and added to the musts. Macedonian yeast (Mac) was applied to three lots of both varieties, containing 30 mg/L SO_2 (V30-Mac and M30-Mac) and 70 mg/L SO_2 (V70-Mac and M70-Mac). French yeast (Fr) was applied to the other three lots of both varieties with 30 mg/L SO_2 (V30-Fr and M30-Fr) and 70 mg/L SO_2 (V70-Fr and M70-Fr).

After addition of SO_2 and yeasts, set of wines was obtained applying maceration times of 3, 6 and 10. After maceration, the wines were separated from the pomace, stabilized at -4 °C for a period of two weeks for tartarate stabilization and bottled. The labelling of the wine samples is given in Table 1.

Table 1. Labels for Vranec and Merlot wine samples prepared under different vinifications

Vinification conditions	30 mg/L SO_2 Macedonian yeast		30 mg/L SO_2 French yeast		70 mg/L SO_2 Macedonian yeast		70 mg/L SO_2 French yeast	
	Vranec	**Merlot**	**Vranec**	**Merlot**	**Vranec**	**Merlot**	**Vranec**	**Merlot**
3 days of maceration	V30-Mac-3d	M30-Mac-3d	V30-Fr-3d	V30-Fr-3d	V70-Mac-3d	M70-Mac-3d	V70-Fr-3d	M70-Fr-3d
6 days of maceration	V30-Mac-6d	M30-Mac-6d	V30-F-6d	V30-Fr-6d	V70-Mac-6d	M70-Mac-6d	V70-Fr-6d	M70-Fr-6d
10 days of macacration	V30-Mac-10d	M30-Mac-10d	V30-F-10d	V30-Fr-10d	V70-Mac-10d	M70-Mac-10d	V70-Fr-10d	M70-Fr-10d

2.1.2. White wines (Smederevka and Chardonnay) produced with different technological procedures

Grapes from Smederevka and Chardonnay varieties, cultivated in Skopje region (vintage 2007), were harvested at ~ 20 and ~22 °Brix, respectively, and transported to the experimental winery. Grapes of both varieties were divided into four lots, processed with mechanical crusher/destemmer and immediately, a liquid solution of potassium metabisulfite was added, giving two lots of both varieties with 50 mg/L SO_2 (Sm50 and Ch50) and other two lots with 100 mg/L SO_2 (Sm100 and Ch100).

The two yeasts (Vinalco and Levuline) were applied to start the fermentation of the white musts. After the rehydration, Macedonian yeast (Mac) was applied to the lots of both varieties containing 50 mg/L SO_2 (Sm50-Mac and Ch50-Mac) and 100 mg/L SO_2 (Sm100-Mac and Ch100-Mac). French yeast (Fr) was applied to the other two lots with 50 mg/L SO_2 (Sm50-Fr and Ch50-Fr) and 100 mg/L SO_2 (Sm100-Fr and Ch100-Fr).

After finishing the fermentation, wines were treated with 1 g/L bentonite and cold stabilised at -4 °C for 2 weeks before bottling. Bottles were stored in a winery, at temperature between 12-15 °C. The labels for the obtained lots are shown in Table 2.

Table 2. Labels for Smederevka and Chardonnay wine samples prepared under different vinifications

Vinification conditions	50 mg/L SO_2		100 mg/L SO_2	
	Smederevka	**Chardonnay**	**Smederevka**	**Chardonnay**
Macedonian yeast	Sm50-Mac	Ch50-Mac	Sm100-Mac	Ch100-Mac
French yeast	Sm50-Fr	Ch50-Fr	Sm100-Fr	Ch100-Fr

2.2. Chemicals and standards

The reagent *p*-(dimethylamino)cinnamaldehyde (*p*-DMACA), gallic acid and (+)-catechin were from Fluka (Switzerland), and the Folin-Ciocalteu reagent was from Merck (Germany). Acetic acid (puriss. p.a. grade, eluent additive for LC–MS) was from Fluka, methanol (HPLC-grade) was purchased from Scharlau Chemie S.A., acetonitrile and methanol with HPLC grade were purchased from Sigma (St. Louis, MO). The following standards were purchased from the company LGC Promochem GmbH, Szentendre, Hungary: gallic, caffeic and ferulic acids, malvidin chloride, quercetin, resveratrol, resveratrol-3-glucoside and rutin and dissolved in methanol. Malvidin-3-glucoside was purchased from Extrasynthese (France), while, the following standards were from Sigma (St. Louis, MO): (+)-catechin, (-)-epicatechin, tyrosol and acids: sinapic, protocatechuic, p-hydroxybenzoic, chlorogenic, syringic, coumaric, cinammic, caffeic and ferulic. Reagents phloroglucinol and L-ascorbic acid were also from Sigma.

The MALDI matrices: α-cyano-4-hydroxycinnamic acid (CHCA), 2,5-dihydroxybenzoic acid (2,5-DHB), sinapic acid (SA) and C70 fullerene (Gold grade) were purchased from Hoechst AG (Frankfurt, Germany).

Water was purified and deionised with a PURELAB Option-R system (ELGA Lab Water) before use. The other reagents were with analytical purity grade. All the other used reagents were of analytical purity grade.

2.3. Preparation of grape skins, seeds and pulps for analyses of phenolics

Skins (1 g), seeds (1 g) and pulp (1 g) were extracted twice for 15 min with 10 mL acetone/water (80/20, *V*/*V*) containing 0.1 % HCl, in an ultrasonic bath and then stirred for 30 min on a magnetic stirrer. After centrifugation (3000 rpm for 10 min), the supernatants from both extractions were collected, joined and brought to a final volume of 25 mL with distilled H_2O.

2.4. Spectrophotometric analyses

Spectrophotometric measurements of phenolic components were carried out with a HP 8452 and Agilent 8453 UV-Vis spectrophotometers.

2.4.1. Total phenolics determination

The Folin-Ciocalteu method (Slinkard & Singleton, 1977) was used for total phenolics determination. The measurements were performed after 16 min storage of the samples at 50 °C and the absorbance was measured at 765 nm. The concentration of total phenolics was expressed as gallic acid equivalent (GAE) per 1 g of fresh sample.

2.4.2. Total anthocyanins determination

Determination of total anthocyanins was performed by the method proposed by Di Stefano, et al. (1989) and expressed as malvidin-3-glucoside equivalents.

2.4.3. Total flavonoids determination

Total flavonoids were determined using the colorimetric assay with aluminium chloride and (+)-catechin as standard for calibration according to Zhishen et al. (1995).

2.4.4. Total flavan-3-ols determination

The concentration of total flavan-3-ols was measured using the *p*-(dimethylamino)cinamaldehyde (*p*-DMACA) method (Di Stefano, et al.,1989; Vivas, et al., 1994) and content was expressed as catechin equivalenths (mg/L) of the wines.

2.4.5. Color intensity, hue, color composition and brilliance of wines

A direct measurement of wine absorbance at 420, 520 and 620 nm was carried out using a 2 mm optical path and the color intensity (CI), hue or tint (T), proportion of red color (% Rd), proportion of blue color (% Bl), proportion of yellow color (% Ye) and brilliance of wine were calculated (dA) (Ribéreau-Gayon, et al. 2000). The data were adjusted to 1 cm length path.

2.5. HPLC ANALYSIS

2.5.1. Adjustment of pH of the samples

The working solutions with pH values: 0.2; 2.2; 4 and 6 (distilled water) were used for acidification of the antbocyanin extract obtained from the Alicante Bouché grape variety (INRA UE, Pech-Rouge, Grussan, France). The grape solutions were injected into HPLC after 2 h of storage. Additionally, one wine sample was diluted with solution pH 0.2 and analyzed immidiatelly, after 30 min, 2 h and 3.5 h storage at room temperature.

2.5.2. Determination of tannins in wine

The wine samples were prepared by the method of Preys, et al. (2006). Wines (1 mL) were concentrated under vacuum (Genevac) and redissolved in 1 mL methanol containing 0.5 % HCl (*V/V*). After centrifugation (8 min, 10 000 tr/min), an aliquot of 100 μL was subjected to reaction with phloroglucinol described below.

2.5.3. Phloroglucinolysis procedure

The reaction with phloroglucinol was performed as described by Kennedy and Jones (2001). Dried extracts of the analyzed wines were redissolved in 100 µL of MeOH containing 0.2 N HCl, 50 g/L phloroglucinol, and 10 g/L ascorbic acid and heated for 20 min at 50 °C. Then, an equivalent volume of aqueous sodium acetate (200 mM) was added to stop the reaction.

2.5.4. HPLC instrumentation

Four HPLC instruments were used for analysis of phenolic components and tannins in the wines.

I - An **Agilent Series 1100** LC System combined with an Agilent 6300 Series Ion Trap LC-MS system, consisting of a binary pump, a degasser, an autosampler, a column thermostat and UV–Vis diode-array detector (DAD). A Phenomenex RP C18 column (60 x 4.6 mm, 3µm) was used at 25 ºC for the separations. The flow rate of the mobile phase was 0.2 mL/min. A multi-step gradient method was applied, using 1 % (*V/V*) acetic acid in water as solvent A and 1 % (*V/V*) acetic acid in methanol as solvent B. For the elution program, the following proportions of solvent B were used: 0–10 min, 5–20 %; 10–45 min, 20–50 %; 45–50 min, 50–80 %; 50–60 min, 80–90 %. The HPLC system was connected to the mass spectrometer equipped with electrospray ion source (ESI), operated in alternating (positive and negative) mode. Nitrogen was used as drying gas at 325 ºC, with a flow rate of 5 L/min; the pressure of the nebulizer was set at 15 psi. The scanning mass to charge range of the ion trap was 50–1200 *m/z* with a maximum accumulation time of 200 ms.

II and **III** – Two HPLC systems, **Waters 2690** equipped with an autosampler, Waters 2996 photodiode array detector (DAD), Waters fluorimetric detector 2475 (FLD) and Empower 2 chromatography manager software

were used for identification and quantification of phenolic acids, flavan-3-ols and anthocyanins, as well as tannin analysis after phloroglucinolysis.

Separation of the components by direct injection was performed with reversed-phase Atlantis dC18 column (Waters, Milford, MA; 5 μm, 250 mm x 2.1 mm i.d.) protected by a guard column of the same material and mobile phase consisting of water/formic acid (95:5; solvent A), and acetonitrile/water/formic acid (80:15:5; solvent B) at a flow rate of 0.25 mL/min at 38 °C. Proportions of solvent B were as follows: isocratic for 2 min with 0 %; 2-5 min, 0-2 %; 5-12 min, isocratic with 2 %; 12-15 min, 2-3 %; 15-25 min, 3-8 %; 25-40 min, 8-20 %; 40-45 min, 20-25 %; 45-55 isocratic with 25 %, followed with washing and reconditioning of the column.

Released terminal subunits and phloroglucinol adducts were analyzed on a reversed-phase Atlantis dC18 column (Waters, Milford, MA; 5 μm, 250 mm x 4.6 mm i.d.) protected by a guard column of the same material. The mobile phase was a linear gradient of water/formic acid (98:2; solvent A) and acetonitrile/water/formic acid (80:18:2; solvent B), at a flow rate of 1 mL/min at 30 °C. Proportions of solvent B were as follows: isocratic for 5 min with 0 %; 5-35 min, 0-10- %; 35-70 min, 10-20 %; 70-75 min, 20-100 %; and 75-80 min, 100-0 %. The proanthocyanidins were detected by two successive detectors, DAD and fluorimetric detector (FLD).

IV - HPLC-DAD-MS, Waters 2690 system equipped with an autosampler, a Waters 2996 photodiode array detector and Empower 2 chromatography manager software was used for analysis. Atlantis column (dC18) was used and the mobile phase consisted of water/formic acid (99:1; solvent A), and acetonitrile/water/formic acid (80:19:1; solvent B) at a flow rate of 0.25 mL/min at 38 °C for identification of phenolic acids, flavonols and anthocyanins. The elution profile was the following: isocratic for 2 min with 0 %; 2-5 min, 0-2 %; 5-12 min, isocratic with 2 %; 12-15 min, 2-3 %; 15-25 min, 3-8 %; 25-40 min, 8-20 %; 40-45 min, 20-25 %; 45-55 min isocratic with 25 %; 55-70 min, 25-65; 70-75 min, isocratic with 65 %, followed by washing and reconditioning of the column.

The MS ion trap detector was ThermoFinnigan LCQ Advantage (San Jose, CA) operated in positive and negative mode, at a source voltage of 3.81 kV in positive and 3.5 kV in negative mode, capillary voltage, 11 V in positive and –5 V in negative mode; capillary temperature of 250 °C, sheath gas flow (N_2) of 50 unit; collision energy for fragmentation 35 % for MS2. ESI-MS data ranging from *m/z* 200 to 1200 were taken in negative and positive mode.

2.5.5. Statistical analysis

Statistical treatment including calculations of means and standard deviations was performed applying Excel (Microsoft Office, 2007). Cluster analysis and Principal Component Analyses were performed using the PC software packages STATISTICA 6.0 (StatSoft, Tylsa, USA) and TANAGRA 1.4.28 (Lyon, France), respectively.

3. RESULTS AND DISCUSSION

3.1. SPECTROHOTOMETRIC ANALYSIS OF GRAPE

3.1.1. Evaluation of polyphenols during the different stages of grape ripening

Many factors influence the phenolic synthesis: high temperatures, low water availability, sun exposure of the clusters, which may inhibit the phenolic synthesis, in particular anthocyanin synthesis. In this work, the changes of the polyphenolic contents in skins, seeds and pulp of two red grape varieties, Vranec and Merlot, and two white varieties, Smederevka and Chardonnay were followed in three ripening phases: veraison, technological ripeness and late harvest.

The results from the spectrophotometric determinations of total phenolics, total flavonoids, total catechins and total anthocyanins (only for red grapes) in pulp, seeds and skin, are presented at Fig. 1, 2, 3 and 4.

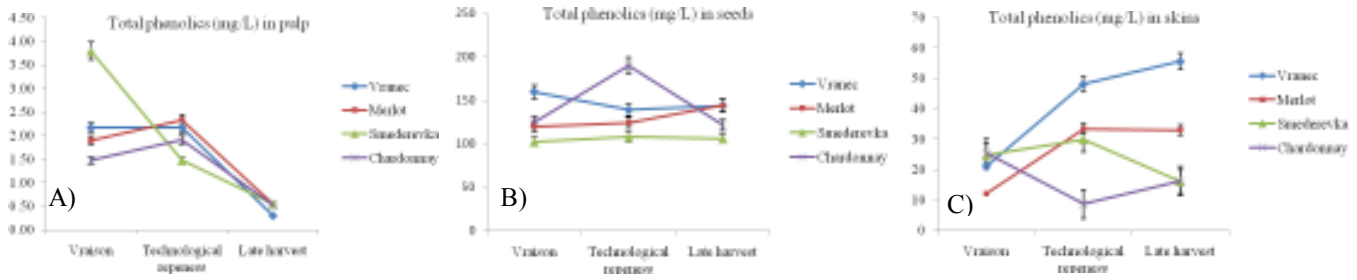


Fig. 1. Changes of total phenolics content in pulp (A), seeds (B) and skins (C) of Vranec, Merlot, Smederevka and Chardonnay grapes

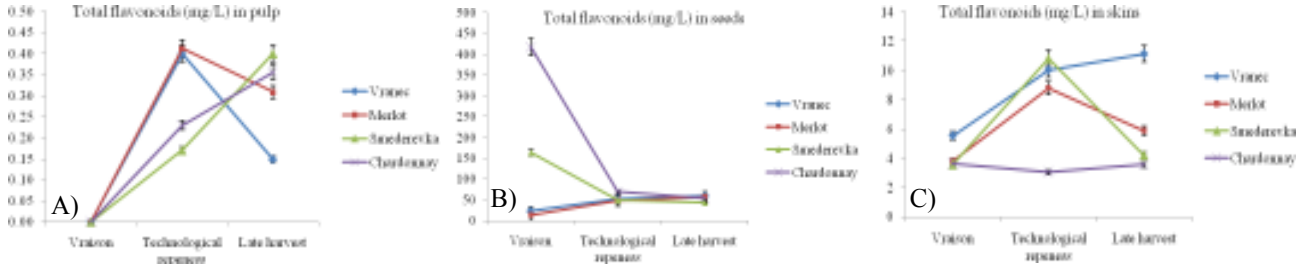


Fig. 2. Changes of total flavonoids content in pulp (A), seeds (B) and skins (C) of Vranec, Merlot, Smederevka and Chardonnay grapes

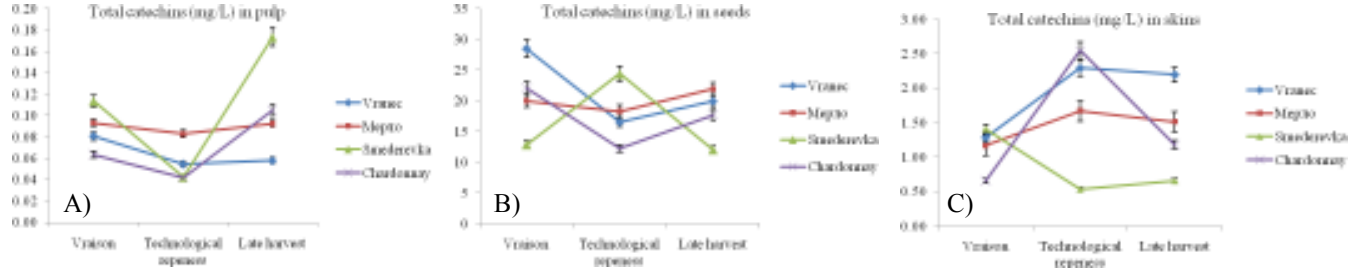


Fig. 3. Changes of total catechins content in pulp (A), seeds (B) and skins (C) of Vranec, Merlot, Smederevka and Chardonnay grapes

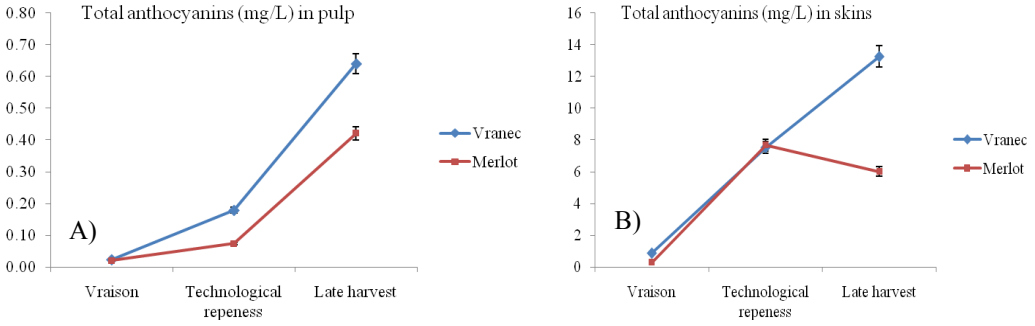


Fig. 4. Changes of total anthocyanins content in pulp (A) and skins (B) of Vranec and Merlot grapes

When the grape berries start to grow, the synthesis of proanthocyanidins and hydroxycinnamic acids begins and goes on until the beginnig of the veraison, when synthesis of (+)-catechin, (-)- epicatechin and (-)-epicatechin gallate slows down or, in some cases, stops. At verasion, the synthesis of anthocyanins begins and continues until the end of the technological ripeness of the grape. At the end of verasion, the anthocyanin profile is well defined, and after, different changes occur during the ripening. From the results of the spectrophotometric measurements (Fig. 1) it can be concluded that the total phenolic content in the pulp reaches the maximum values at the technological ripeness of Vranec, Merlot and Chardonnay grapes, and for Smederevka, the highest concentrations of phenolics were measured when grapes were at veraison, decreasing during ripening. Vranec seed extracts contained highest total phenolic content, decreasing until the technological ripeness, followed with slight increasing when grapes were late harvested. For Vranec skins, total phenolics increased during the ripeness, reached higest content at late harvest, in contrast to Merlot skins, where highest phenolic concentrations were measured for grapes at technological ripeness. Merlot seeds contained highest content of total phenolics when grapes were late harvested. For white grape varieties, Smederevka and Chardonnay, the total phenolics content in seeds had maximum concentrations at technological ripeness, and for their skins, total phenolic contents were different; Smederevka skins contained highest concentrations at technological ripeness and Chardonnay skins had lowest total phenolic contents at this stage.

Flavonols belong to the group of flavonoids and their production begins before verasion. It was noticed that flavonoids in pulp increased during ripening, reaching maximum values at technological ripeness, observing decrease after this phase for the red varieties (Vranec and Merlot) and increase for the white varieties (Smederevka and Chardonnay). Highest contents of total flavonoids were found in the seeds of late harvested Vranec and Merlot grapes, and for white varieties, grapes at technological ripeness contained highest contents of flavonoids (Fig. 2).

The content of total catechins in the seeds of both red varieties and seeds of Chardonnay grapes were highest at veraison, as expected, followed by decreasing during the ripeness and slight increasing during late harvest. In Smederevka seeds, increase of catechins was observed at technological ripeness, followed by decrease at late harvest (Fig. 3).

From the results for total anthocyanins (Fig. 4), it can be noticed that, for Vranec grapes, anthocyanin contents increased continuously, from veraison, when their production begins, across the technological ripeness, till the moment of late harvested grape. For Merlot grapes, it was noticed that total anthocyanins reached the maximum value at technological ripeness, and then they decreased.

The obtained results were in concordance with the literature data published for phenolic components in grape extracts (Downey, et al., 2003; Poudel, et al., 2008; Seddon and Downey, 2008).

3.1.2. SPECTROHOTOMETRIC ANALYSIS OF RED WINES

3.1.2.1. VRANEC and MERLOT

"Vranec" is the most widely cultivated and the most important variety in Republic of Macedonia for production of quality red wines. **"Merlot"** is widely cultivated variety in the world and also grown in R. Macedonia, in Tikveš, Skopje, Ovče Pole, Ohrid and Kumanovo vineyard areas.

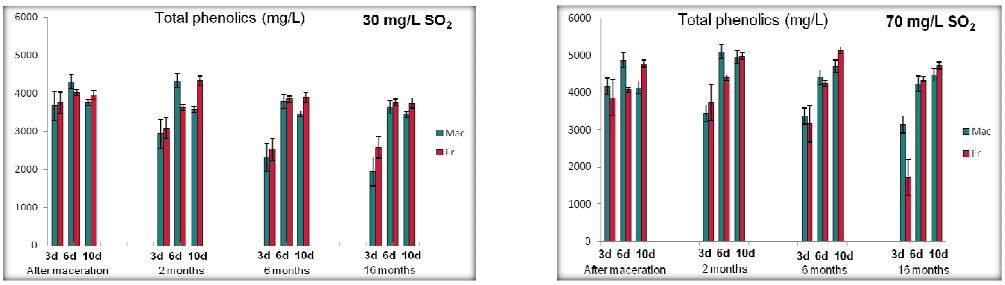
Spectrophotometric methods for determination of total phenols, total anthocyanins, total catechins, total flavonoids, color intensity and hue were applied for analysis of Vranec and Merlot wines produced with different technological procedures:

- ✓ different maceration time (3, 6 and 10 days),
- ✓ two doses of SO_2 (30 and 70 mg/L SO_2),
- ✓ two yeasts for fermentation (Macedonian, Vinalco and French, Levuline)

Changes of polyphenols were followed during the four phases:

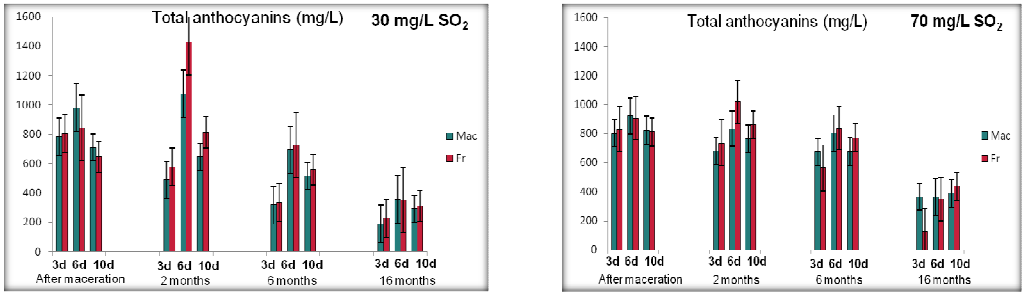
- ✓ after the period of maceration (3, 6 and 10 days),
- ✓ after 2 months,
- ✓ after 6 months, and
- ✓ after 16 months of storage of the wines at low temperature in the winery.

Measurements were performed in order to check the influence of the time of maceration, doses of SO_2 and yeasts on extraction of phenolic components from grape, and also to check how phenolic components are changing during aging of the wines in bottles. Concentrations of TP, TA, TF and TC for Vranec wines are presented in Fig. 5, 6, 7, and 8, respectively. Additionaly, twelve wines, after maceration and separation of the wines from the pomace, were stored at higher temperature (~ 25 °C) and they were analyzed after period of 6 months of storage.



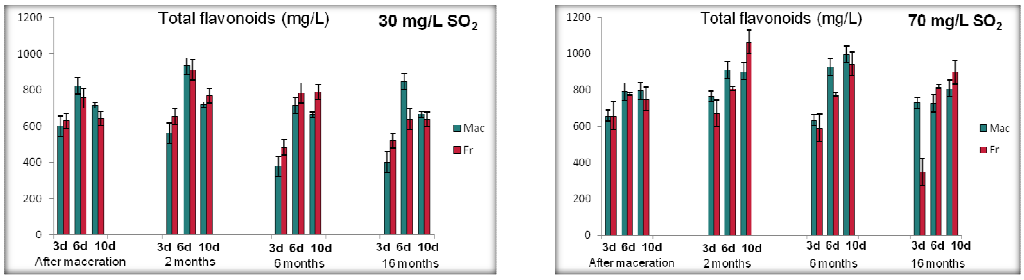
A B

Fig. 5. Changes of concentrations of total phenolics in Vranec wines fermented with Macedonian (Mac) and French (Fr) yeast with: (A) **30 mg/L SO_2**, and (B) **70 mg/L SO_2** followed in four phases (after maceration, after 2, 6 and 16 months of storage)



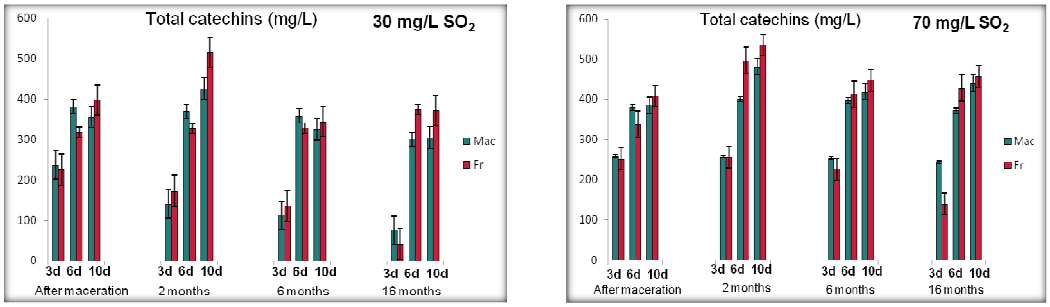
A B

Fig. 6. Changes of concentrations of total anthocyanins in Vranec wines fermented with Macedonian (Mac) and French (Fr) yeast with: (A) **30 mg/L SO_2**, and (B) **70 mg/L SO_2** in the four phases (after maceration, after 2, 6 and 16 months of storage)



A B

Fig. 7. Changes of concentrations of total flavonoids in Vranec wines fermented with Macedonian (Mac) and French (Fr) yeast with: (A) **30 mg/L SO_2**, and (B) **70 mg/L SO_2** followed in four phases (after maceration, after 2, 6 and 16 months of storage)



A B

Fig. 8. Changes of concentrations of total catechins in Vranec wines fermented with Macedonian (Mac) and French (Fr) yeast with: (A) **30 mg/L SO_2**, and (B) **70 mg/L SO_2** followed in four phases (after maceration, after 2, 6 and 16 months of storage)

Influence of maceration time. According to the previous knowledge for anthocyanins and tannins in wine, longer maceration time can prompt higher extraction of tannins from skins and seeds, but not of anthocyanins (Sacchi, et al., 2005). Gomez-Plaza et al. (2001) observed the most intensive increasing of anthocyanins between the fourth and fifth day with slight decreasing after the tenth day of maceration.

In our investigation, it was noticed that the concentrations of total phenolics in Vranec wines, after the performed maceration, were highest in the wines obtained with 6 days of maceration. Similar results were obtained for the contents of anthocyanins and flavonoids, which were also highest for the wines macerated for 6 days, followed with decreasing of their content with increased maceration time. Those results are in accordance with previous investigations (Gil-Muñoz, et al., 1999; Bautista-Ortín, et al., 2004). Decreasing of the total phenolics and total flavonoid content can be a result of their precipitation. For Merlot wines, it was observed that total phenolic contents were continuasly increased reaching highest values for the wines macerated for 10 days (Fig. 9A and 9B). As for SO_2 influence, concentrations of total anthocyanins had highest values in the wines containing 70 mg/L SO_2 and macerated for 6 days, and wines with 30 mg/L SO_2 macerated for 10 days.

Longer maceration time means higher contents of catechins (Kennedy, 2008), as was observed for the wines from both varieties. Thus, the lowest concentration was observed for the wines macerated for 3 days, followed with increasing as the maceration times increased, whereas the highest content was measured for the wines macerated for 10 days (Fig. 8 and 12). The results were expected because the catechins in seeds which are protected with lipidic layer will be disrupted when appropriate content of alcohol will be formed, allowing extraction of tannis from grape at the latest phase of vinification. The results are in accordance with previous studies (Sun et al., 1998; Bautista-Ortín, 2004).

Highest values for the color intensity and hue were measured for the wines from both varieties, macerated for 3 days, followed with decreasing with increased maceration time.

Influence of time of aging. Time of wine aging in bottles has influence on the polyphenolic content, causing changes of their contents with time. From the results for total phenolics for Vranec and Merlot wines, highest decrease of the contents was noticed for the wines macerated for 3 days after the period of 2 months of storage. For those wines, the most intensive decrease of total phenolics was observed after 16 months of storage in the cellar. For

the Vranec wines macerated for 10 days, total phenolics increased after 2 months, while for the other Vranec and Melot wines the total phenolics slightly decreased, possibly as a result of precipitation (Gómez-Plaza, et al., 2000).

Intensive decrease, for the first two months, was observed for the total anthocyanin contents, again for the wines macerated for 3 days. During the time, decreasing of anthocyanins was noticed for all wines, and after the period of 16 months, the decreasing was highest for the wines macerated for 3 days. Those results were exptected, taking into accout that with 3 days of maceration skin anthocyanins are extracted, but not seed tannins followed by decreasing of anthocyanins because they can not form stable pigments. Also, one part of anthocyanins can precipitate, or adsorb at dead yeasts. For the wines macerated for 10 days, lower decrease of the anthocyanin content was noticed because they are more stabilized (involved in complexes, polymers with flavan-3-ols, pyruvic acid, caffeic acid).

Similar like anthocyanins, the color intensity decreased during the wine storage, and again, the most intensive decresing was registered for the wines macerated for 3 days. The hue values for the wines from both varieties increased after storage of 16 months, confirming that the color, depends also, on the presence of the other pigments formed during the period of aging.

In the case of total flavonoids, increasing of their concentration after the period of 2 months was observed, but during the wine aging, decreasing of the contents was found. For the wines containing 70 mg/L SO_2, macerated for 10 days, highest concentrations were measured after 16 months of storage. Increasing was observed for the Merlot wines macerated for 10 days, while wines macerated for 3 days contained lower flavonoids concentrations compared with the initial stages.

The bigest decrease of total catechins was noticed for the wines macerated for 3 days which was most intensive after 16 months because for those wines lowest contents of catechins were extracted and probably most of them participated in polymers formation.

Vranec wines are rich in phenolics, with intensive color that can be attained after 6 days of maceration. With regard to the limited capacities of the wineries, shorter maceration time could abbreviate the time of wine production, save time and enable to use the equipement for production of wines from other varieties. From Merlo variety, the quality wines rich with polyphenols can be produced with 10 days of maceration.

Influence of the yeast. The choice of the yeast for fermentation can influence the phenolic content of the wines, but, its role in the evaluation of phenolic components during the maceration time is not enough studied. Interactions between the tannins and manoproteins released from different yeasts can generate condensation between anthocyanins and tannins, decreasing the astringency of the wine. In this work, two yeasts, *Saccharomyces cerevisiae*, from two different manufacturers: Vinalco, from Macedonian manufacturer and Levuline from French manufacturer, were used for fermentation.

Comparing the results for Vranec wines with same amount of SO_2, but different yeasts, obtained after maceration, it can be concluded that highest concentrations of total phenolics were measured for the wines fermented with Macedonian yeast macerated for 6 days (30 mg/L SO_2), and wines macerated for 3 and 6 days containing 70 mg/L SO_2, but the differences were small. Higher contents of anthocyanins, flavonoids and catechins were obtained for the wines fermented with Macedonian yeast, macerated for 6 and 10 days, while the contents of those components were similar for the wines macerated for 3 days and fermented with both yeasts. For the wines that contained higher amounts of SO_2 (70 mg/L SO_2), influence of the yeast was not significant on the extraction of phenolics and their changes during the wine storage.

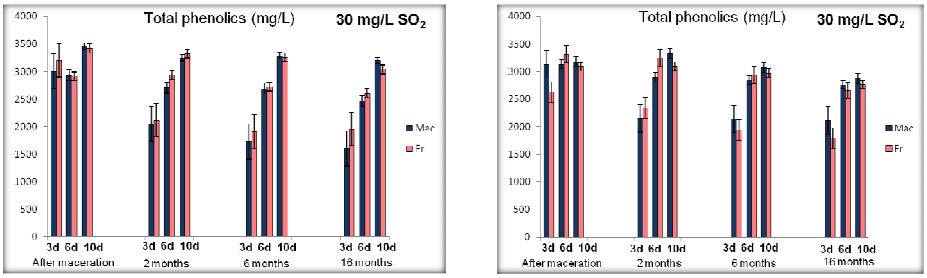
For Merlot wines with 30 mg/L SO_2, analyzed after the maceration, no significant differences were found for the concentrations of the phenolics from the different groups, except for the contents of total flavonoids and total catechins (for 6 days macerated wines), as well as for the values of color intensity which were higher for the wines fermented with French yeast and macerated for 3 and 6 days. During aging, significantly lower values for phenolics, anthocyanins, flavonoids, catechins and color intensity were measured for the wines fermented with French yeast (70 mg/L SO_2), macerated for 3 days, compared with the same wine macerated with Macedonian yeast. For the other wines macerated for 6 days, no significant influences of the yeasts were observed.

Influence of the SO_2 content. Addition of sulphur dioxide is usually performed after the grape crushing in order to protect the must from oxidation at the beginning of fermentation. Higher doses of SO_2 in the must can lead to faster and more efficient precipitation, especially SO_2 in free form contributes to better extraction of phenolic components from grape skins and seeds. In this research, two doses of SO_2 were used, 30 and 70 mg/L SO_2. Thus, the results for Vranec wines imply that SO_2 has influence on the polyphenolic extraction, and higher concentration of phenolics, flavonoids, anthocyanins and catechins were measured in the wines with higher doses of SO_2. During aging, after 16 months of storage, it was noticed that wines fermented with French yeast and macerated for 3 days, treated with lower dose of SO_2, contained higher amounts of total phenolics, total anthocyanins and flavnonoids.

On the other hand, no clear tendency of the influence of SO_2 on polyphenolic extraction was observed for Merlot wines. It was found that higher dose of SO_2 leads to higher extraction of catechins after 3 days of maceration and anthocyanins after 6 days of maceration.

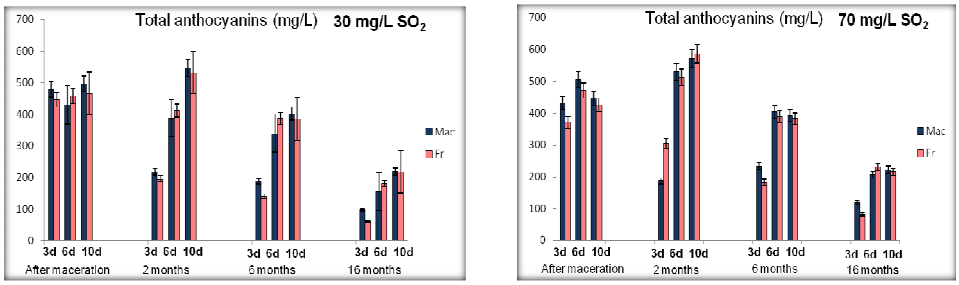
Influence of temperature of storage. The differences found for the phenolics, anthocyanins, flavonoids and catechins in the wines stored at different temperatures (~15 °C and ~ 25 °C) are slight and not significant, except for the anthocyanins. Significant decrease of their content was observed and it was highest for the wines macerated for 3 days. The reason for the small differences probably is the small range of the temperatures which is not so big to give significant differences.

The results for total phenolics, anthocyanins, flavonoids and catechins for the Merlot wines after maceration and storage are presented in Fig. 9, 10, 11 and 12, respectively.



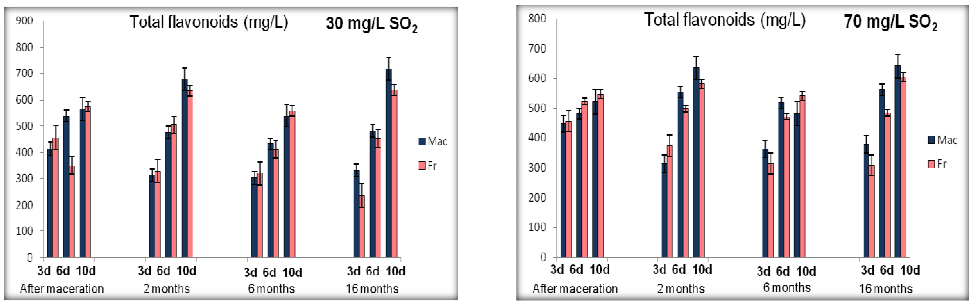
A) B)

Fig. 9. Changes in concentrations of total phenolics in Merlot wines fermented with Macedonian (Mac) and French (Fr) yeast, with (A) **30 mg/L SO_2** and (B) **70 mg/L SO_2** followed in four phases (after maceration, after 2, 6 and 16 months of storage)



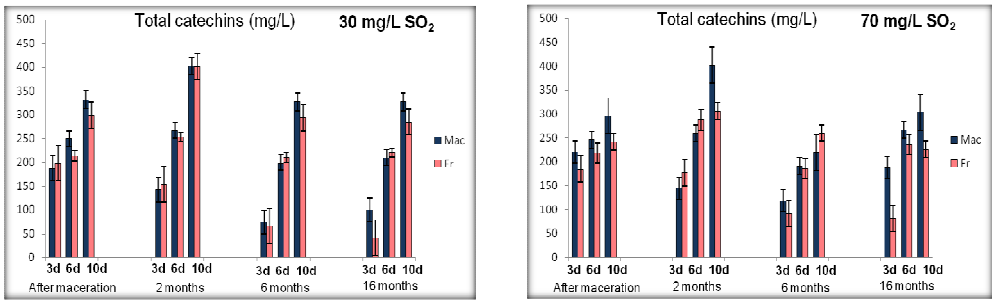
A) B)

Fig. 10. Changes in concentrations of total anthocyanins in Merlot wines fermented with Macedonian (Mac) and French (Fr) yeast, with (A) **30 mg/L SO_2** and (B) **70 mg/L SO_2** in four phases (after maceration, after 2, 6 and 16 months of storage)



A) B)

Fig. 11. Changes in concentrations of total flavonoids in Merlot wines fermented with Macedonian (Mac) and French (Fr) yeast, with (A) **30 mg/L SO_2** and (B) **70 mg/L SO_2** followed in four phases (after maceration, after 2, 6 and 16 months of storage)



A) B)

Fig. 12. Changes in concentrations of total flavonoids in Merlot wines fermented with Macedonian (Mac) and French (Fr) yeast, with (A) **30 mg/L SO_2** and (B) **70 mg/L SO_2** followed in four phases (after maceration, after 2, 6 and 16 months of storage)

Principal component analysis. PCA was applied separately on the parameters for Vranec and Merlot wine:

- Obtained with different wine-making technologies applying: maceration times of 3, 6 and 10 days, two doses of SO_2 and two yeasts for fermentation.
- Analyzed during the four phases of storage: after maceration (3, 6 and 10 days), after 2, 6 and 16 months of wine aging.

PCA was performed in order to evaluate according to which parameter/s (maceration time, time of wine aging, SO_2 or yeast) the wines can be distinguished, and for that purpose, the spectrophotometric results for total phenols, total anthocyanins, total flavonoids, total catechins, color intensity and hue were used.

From the correlation score plot at Fig. 13 for Vranec wines, it can be seen that grouping of the samples is obtained according to the time of storage and maceration time. Thus, wines analyzed after 16 months of storage, were clearly separated from the other samples and mainly located in the positive part of PC2. On the other hand, grouping of the samples, according to the time of maceration was noticed where 3 days macerated wines were separated from the other samples. Also, wines analyzed after store period of 2 and 6 months were grouped according to the maceration time.

Separation of Merlot samples (Fig. 14), which was not fully clear, was made according to the maceration time and aging time. Wines macerated for 3 days and analyzed after the maceration were well separated and located in the positive part of PC2. Although not clear enough, grouping of the samples according to maceration time still can be observed, whereupon, the wines macerated for 3 days and analyzed after 2, 6 and 16 months are separated from the other samples and located in the negative part of PC1.

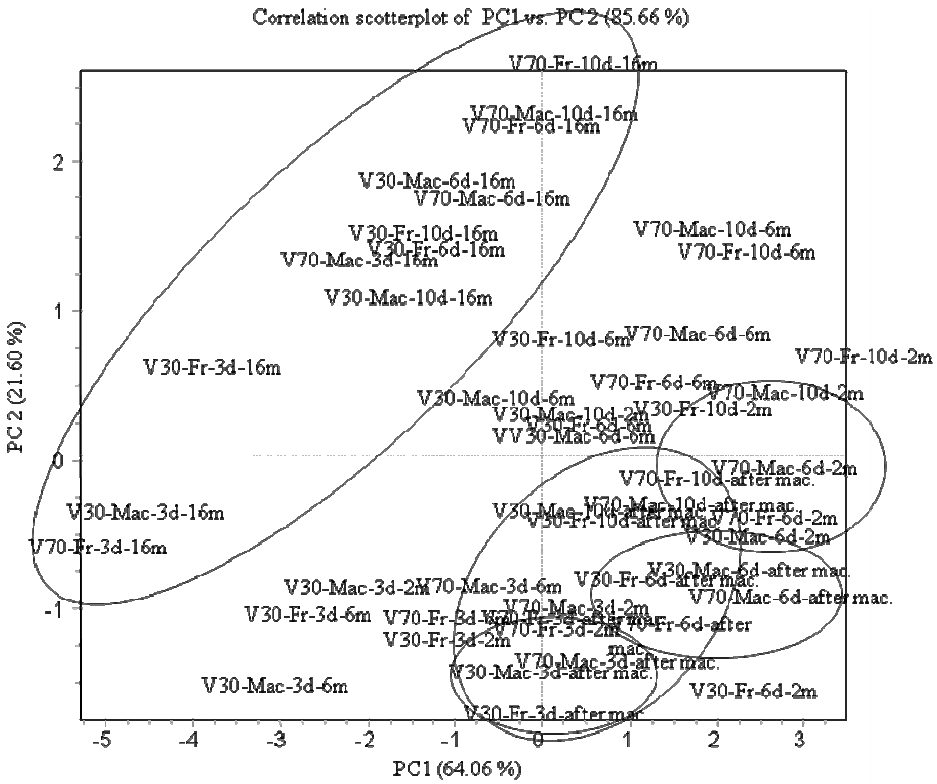


Fig. 13. Principal Component score plot with PC1 and PC2 of the variables based of spectrophotometric data for total phenolics, total anthocyanins, total flavonoids, total catechins, color intensity and hue, and grouping of the Vranec wines according to the aging and maceration time

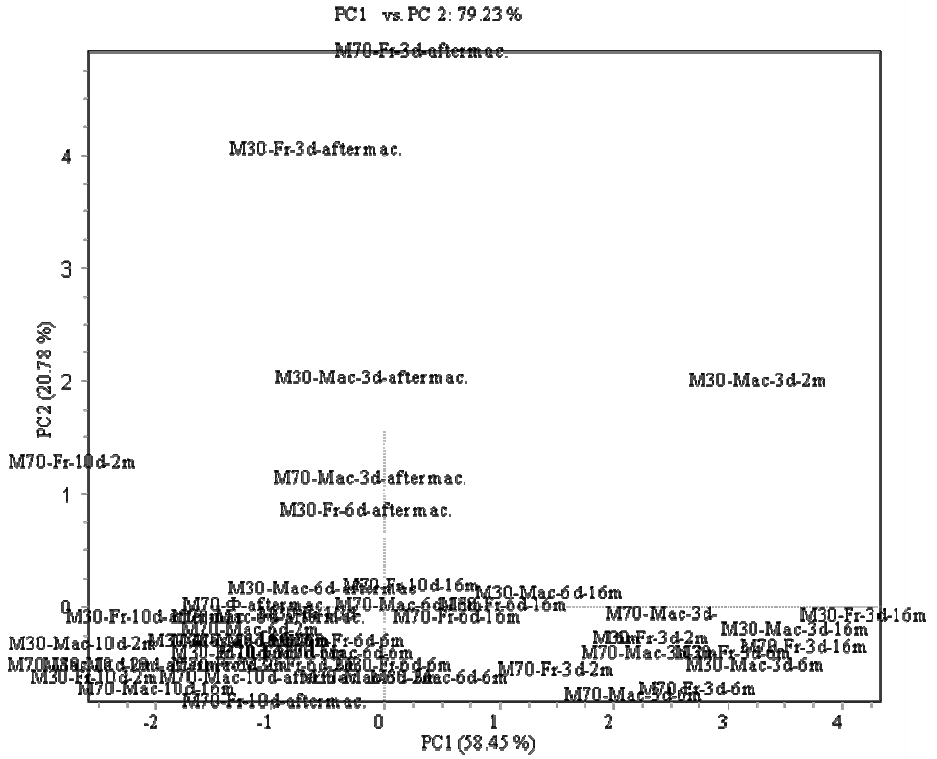


Fig. 14. Principal Component score plot with PC 1 and PC 2 of the variables based of spectrophotometric data for total phenols, total anthocyanins, total flavonoids, total catechins, color intensity and hue, and grouping of Merlot wines according to the aging and maceration time

3.1.3. SPECTROHOTOMETRIC ANALYSIS OF WHITE WINES

3.1.3.1. SMEDEREVKA and CHARDONNAY

"Smederevka" is most widely cultivated white grape variety in R. Macedonia, planted in all vineyard areas of Povardarie wine region. **"Chardonnay"** is a widely cultivated variety in the world, and in R. Macedonia, it is planted at small areas, at Tikveš, Skopje, Veles vineyard areas, with tendency for further spreading.

Total phenols and the individual groups - total flavonoids and total catechins were determined in these wines obtained with:

- two doses of SO_2 (50 and 100 mg/L SO_2) and two yeasts for fermentation (Macedonian and French)
- All wines were analyzed in four phases: after fermentation, after 2, 6 and 16 months of aging.

Set of wines from both varieties, stored at higher temperature (~25 °C) were analyzed after 6 months of storage and the results were compared with the corresponding wines stored at lower temperature (~15 °C).

The changes of polyphenolic compounds in Smederevka and Chardonnay wines, during storage, are shown in Fig. 15 and 16. It could be noticed that phenolic contents decreased gradually up to the sixth month of storage, followed with a subsequent increase to the sixteenth month. The final total phenolic content of Smederevka wines fermented with French yeast was higher in the wine with higher level of SO_2, despite of the wines fermented with Macedonian yeast, whereas higher phenolic content was observed in the wine containing 50 mg/L SO_2. Chardonnay wines showed high contents of total phenolics at the initial phase and during the subsequent stages of wine storage, a significant gradual reduction was observed.

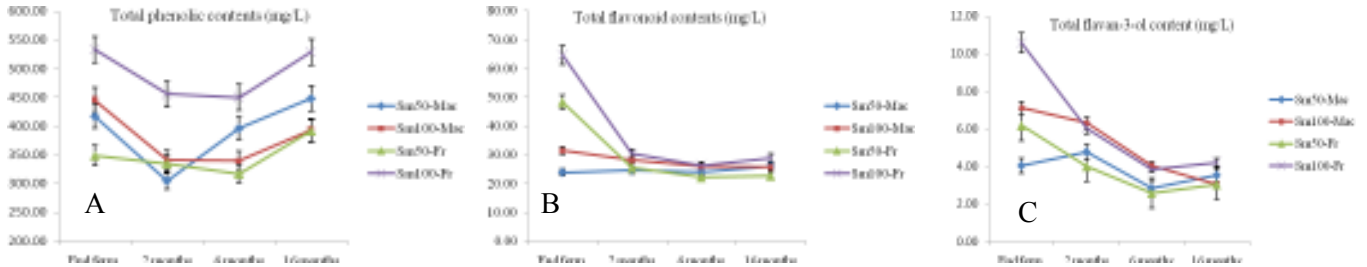


Fig. 15. Changes in total phenolics (A), total flavonoids (B) and total flavan-3-ols (C) contents of Smederevka wines during storage: after fermentation, 2, 6 and 16 months (Sm – Smederevka)

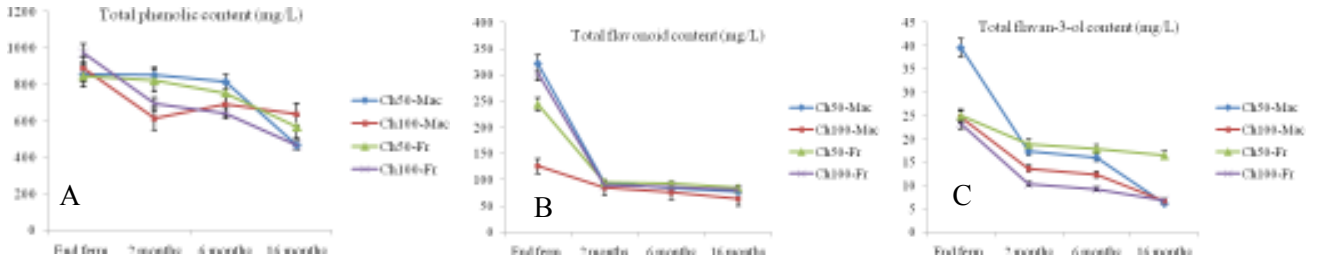


Fig. 16. Changes in total phenolics (A), total flavonoids (B) and total flavan-3-ols (C) contents of Chardonnay wines during storage: after fermentation, 2, 6 and 16 months (Ch - Chardonnay)

Hydroxycinnamic acids are the most important group of phenolics in white wines and present in ester forms in the grapes. They can increase in quantity during storage as a result of hydrolysis of tartaric esters, increasing the free forms influencing the total phenolic content (Betés-Saura, et al., 1996; Ibern-Gómez, et al., 2000; Kallithraka, et al., 2009). This could be the reason for the higher concentration of total phenolics observed for Smederevka wines. Oxidation processes can decrease the phenolic content during the aging as it was observed for Chardonnay wines.

Changes of total flavonoid contents of Smederevka and Chardonnay wines during storage are shown in Fig. 15B and 16B. The flavonoid content decreased for all wines from both varieties, except for the wine Sm50-Mac. The decreased flavonoid content in both wines is probably a result of oxidative degradation or hydrolysis of flavonols and as a result of hydrolysis, flavonol glucosides content decreases producing higher flavonoid aglycone content (Zafrilla, et al. 2003).

Wines showed significantly higher contents of flavan-3-ols for the wines at the initial phase, which gradually decreased during storage because those compounds are easily subjected to oxidation or polymerisation (Zafrilla, et al. 2003).

Influence of SO_2. The obtained results for the total phenolics, total flavonoids and total catechins were compared for the wines made with two doses of SO_2, and higher phenolics extraction was noticed during the short time of contact between the grape juice and grapes in presence of higher dose of SO_2. Thus, after the fermentation, higher contents of TP, TF and TC were measured for Smederevka wines with 100 mg/L SO_2. For Chardonnay wines, higher concentrations of TP and TF were also measured for the wines containing higher content of SO_2, while concentration of TC was higher in the wines with lower dose of SO_2.

Influence of yeast. For Smederevka wines higher contents of TP, TF and TC were measured in the wines fermented with French yeast. For Chardonnay wines, higher contents of phenolics were measured for the wines fermented with French yeast after the fermentation, but the contents of catechins were higher for the wines fermented with Macedonian yeast.

Influence of temperature. It was noticed that all wines stored at higher temperature contained higher concentrations of total phenolics, except the wine Ch100-Mac which had higher phenolic content, stored at lower temperature. The contents of catechins were lower in the wines stored at room temperature, confirming that rapid reactions of oxidation occur at higher temperatures.

Principal component analysis. PCA was applied to the parameters from the spectrophotometric analyses of Smederevka and Chardonnay wines:

- Obtained with different technologies: two doses of SO_2 and two yeasts for fermentation,
- Analyzed during four phases of storage: after fermentation, after 2, 6 and 16 months of wine aging.

Grouping of the samples was observed mainly according to the variety, whereupon Smederevka wines are separated from Chardonnay wines. Smederevka wines were located in the positive part of the PC 2, except the wines Sm50-Fr-6m and Sm100-Fr-16m, which were located in the negative part of PC 2. Grouping of those wines, was not observed (Fig. 17).

Although not very clear, grouping of Chardonnay wines was observed, mainly, according to the storage time, after fermentation, after 2, 6 and 16 months and partly, according to the SO_2 content.

In general, differences in polyphenolic content between Smederevka and Chardonnay wines were observed. Higher concentrations of total phenols, total flavonoids and total flavan-3-ols were measured for Chardonnay wines in all stages of storage. This is probably due to the cultivar-related differences.

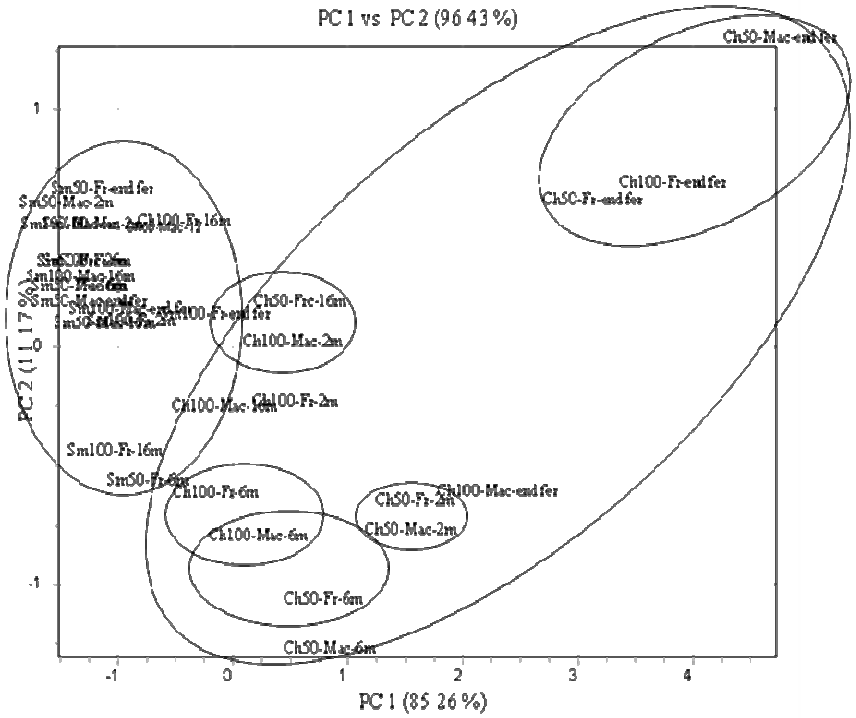


Fig. 17. Principal Component score plots of the variables with PC1 and PC2 based on total phenols, total flavonoids and total flavan-3-ols spectrophotometric data for the analyzed Smederevka (A) and Chardonnay (B) wines

3.2. HPLC ANALYSIS OF WINES

3.2.1. Adjustment of pH of the sample

Different forms of anthocyanins exist at wine pH: the flavylium cations, the quinoidal anhydrobase, the carbinol pseudobase and the chalcone forms. High acidity of the mobile phase is needed for chromatographic analysis of anthocyanins to keep them in their flavylium red forms, which on the other hand, is incompatible for the intensity in the electrospray source and MS detection of phenolic acids. Thus, the effect of pH of the sample and mobile phase was studied using anthocyanin extract from grape skins and the stability of the other classes of phenolic compounds in the acidified sample after storage at room temperature was examined.

The anthocyanin extract was dissolved in solutions with the following pH: 0.2, 2, 4 and 6 (distilled water) and the amount of formic acid in the HPLC solvent was 0.5, 1 and 2 %. The influence of sample pH and mobile phase pH was checked with calculation of the peak areas of chalcone (*m/z* 481) and flavylium forms (*m/z* 463) of peonidin-3-glucoside. According to the results, suitable conditions can be achieved applying 2 % formic acid in the HPLC mobile phase, but also, 1 % formic acid allowed proper separation and detection of anthocyanins and, at the same time, detection of phenolic acids, compensated by acidifying the sample below pH 2. The stability of phenolic components was checked in acidified wine, pH 1.1, analyzed immediately, after 30 min, 2 h and 3.5 h storage at room temperature. The well known wine components from each phenolic group were chosen, such as malvidin-3-glucoside from the group of anthocyanins; quercetin-3-glucoside and quercetin-3-glucuronide from the group of flavonols; caftaric acid, gallic acid and GRP from the group of phenolic acids and derivatives; and procyanidin B2 from the group of flavan-3-ols, and no changes of the peak areas were observed, except for malvidin-3-glucoside which increased in the first 30 min, and after, it remained stable. This showed that phenolic compounds are stable in highly acidic media and they can be analyzed at least 30 min after the preparation or after few hours, which is convenient if large number of samples should be analyzed. It was concluded that suitable conditions for analysis of

phenolic compounds can be established with acidification of the sample at pH 1.1 and elution with 1 % formic acid in the mobile phase.

3.2.2. HPLC identification and quantification of phenolic compounds by direct injection of wines and grape extracts

In this doctoral dissertation, 68 phenolic compounds have been identified in the wines, applying HPLC-DAD-MS. Identification was performed with comparison of the UV-Vis spectra and retention times of the standards available and most of the components were identified with ESI/MS data.

Phenolic acids. Gallic, protocatechuic and syringic acids were identified in Vranec and Merlot wines. In negative ion mode, hydroxybenzoic acids produced a deprotonated ion $[M–H]^-$ and a fragment ion $[M–H–44]^-$ corresponding to the loss of CO_2 group from the carboxylic acid moiety, as was observed for the detected acids in this study. Hydroxycinnamic acids also produce deprotonated molecular $[M–H]^-$ and $[M–H–44]^-$ fragment ion corresponding to elimination of CO_2, as was observed for the hydroxybenzoic acids (Monagas, et al. 2005). Caffeoyl tartaric (caftaric) acid, *p*-coumaroyl tartaric (coutaric) acid and ferulyl tartaric (fertaric) acid have also been identified in Vranec and Merlot wines (Baranowski and Nabel, 1981, Baderschneider and Winterhalter, 2001). For these compounds, a fragment ion $[M-H-132]^-$ corresponding to the free acid after the cleavage of the ester bond was observed (Monagas, et al., 2005). GRP (2-*S*-glutationyl caffeoyl tartaric acid) was detected in the wines, which is formed as a result of enzymatic reactions catalysed by polyphenoloxidase enzymes (Singleton, et al. 1985, 1986).

Stilbenes. *cis*/*trans*-resveratrol-glucosides were detected in the analyzed wines. The quasi-molecular ion of *trans/cis*-resveratrol glucoside (*trans/cis*-piceid) $[M–H]^-$ at *m/z* 389 gave a fragment ion $[M–H]^-$ at *m/z* 227 corresponding to free resveratrol by loss of the glucoside moiety (Vitrac, et al., 2002; Sun, et al., 2007).

Flavan-3-ols. Flavan-3-ol monomers, (+)-catechin and (-)-epicatechin ($[M-H]^+$ = *m/z* 291), and dimmers ($[M-H]^-$ = *m/z* 577) were detected in the wines (Bourzeix, et al. 1986; Da Silva, et al. 1991a; Da Silva, et al. 1991b). Identification of flavan-3-ols was performed under positive and negative ESI mode, observing higher intensity of the peaks of monomers in positive mode, allowing fragmentation of the ions. Thus, the *m/z* 291 ion in positive mode produces fragment ions at *m/z* 273, 165, 139, 123. Fragment ion at *m/z* 273 corresponds to elimination of water molecule, while fragment ion with *m/z* 165 corresponds to loss of a phloroglucinol molecule ($[M-H-126]^-$). The obtained ion with *m/z* 139 results from Retro-Diels-Alder (RDA) rearrangement on the C-ring of catechin and epicatechin derivatives and as a result of that cleavage, two possible fragment ions are formed: *m/z* 139 by a loss of 152 Da and *m/z* 123 by a 168 Da removal of the B-ring.

Flavonols. The aglycone quercetin ($[M-H]^+$ = *m/z* 303) was detected in the Vranec and Merlot wines. The glucoside derivatives of myricetin, quercetin, laricitrin and syringetin were identified and fragment ions ($[M-H-162]^-$) corresponding to elimination of glucose molecule were detected (Mattivi, et al. 2006; Castillo-Muñoz, et al., 2007). Myricetin-3-*O*-glucuronide and quercetin-3-*O*-glucuronide were also present in the wines, identified by the loss of 176 Da ($[M-H-176]^-$), corresponding to elimination of glucuronide (Cheynier and Rigaud, 1986).

Phenolic alcohols. Tyrosol (*p*-hydoxyphenylethanol) with $[M-H]^-$ = *m/z* 137, UV max at 274.2 nm and retention time of 15.6 min, was detected in the wines, which is formed from tyrosine (3-(4-hydroxyphenyl)-alanine) during yeast fermentation (Sapis and Ribereau-Gayon, 1969).

Dihydroflavonols. Astilbin (dihydroquercetin-3-*O*-rhamnoside), engeletin (dihydrokaempferol-3-*O*-rhamnoside) (Trousdale and Singleton, 1983) and dihydromyricetin-3-*O*-rhamnoside with molecular ions *m/z* 449, 433, 465, respectively, were detected in the wines. Their molecular ions produce same characteristic fragment by elimination of rhamnoside molecule with *m/z* 164 (Souquet, et al. 2000).

Antocyanins and pigments. The presence of glucoside, acetylglucoside and *p*-coumaroylglucoside derivatives of delphinidin, cyanidin, petunidin, peonidin and malvidin was confirmed in the Vranec and Merlot wines (Vivar-Quintana, et al., 2002; Alcalde-Eon, et al., 2004, De Villers, et al., 2004, Wu and Prior, 2005; Alcalde-Eon, et al., 2006; Kelebek, et al., 2007; Rubilar, et al., 2007; Chinnici, et al., 2009). Molecular and fragment ions, as well as λ_{max} values are presented in Table 3.

Pyranoanthocyanidins. Pyranoanthocyanidins are formed in reaction of anthocyanins with different compounds, such as vinylphenol derivatives (Cameira dos Santos, et al., 1996), acetaldehyde (Fulcrand, et al. 1996), pyruvic acid (Fulcrand et al., 1998), and they have been detected in wines and wine model solutions (Mateus, et. al, 2001; Salas, et al., 2004; de Villiers, et al., 2004; Chinnici, et al. 2009).

Pyranoanthocyanins resulting from reaction of cycloaddition between anthocyanins and pyruvic acid are named A-type vitisins (Bakker and Timberlake, 1997; Fulcrand, et al. 1998), while B-type vitisins result from cycloaddition of acetaldehyde with anthocyanins (Bakker and Timberlake, 1997).

A-type and B-type vitisins were detected in the analyzed Macedonian wines. Carboxy-pyrano-malvidin-3-glucoside (vitisin A), carboxy-pyrano-malvidin-3-acetylglucoside (acetylvitisin A) and carboxy-pyrano-malvidin-3-*p*-coumaroylglucoside (*p*-coumaroylvitisin A) were identified according to their molecular ions (M^+), and the main fragments in their mass spectra. These 3 compounds have the same characteristic fragment $[M+H]^+$=399 which corresponds to carboxy-pyrano-malvidin aglycone (Bakker & Timberlake, 1997). In the group of A-type vitisins, carboxy-pyrano-petunidin-3-glucoside with *m/z* 547 and carboxy-pyrano-petunidin-3-*p*-coumaroylglucoside with *m/z* 677 (Alcalde-Eon, et al. 2004) were detected in the wines.

Vitisin B (pyrano-malvidin-3-glucoside), acetylvitisin B (pyrano-malvidin-3-acetylglucoside) and *p*-coumaroylvitisin B (pyrano-malvidin-3-*p*-coumaroylglucoside) were detected. They produced $[M-H]^+$ at 517, 559 and 663 mass units, respectively, and a fragment ion at 355, by a loss of 162 Da, 204 Da and 308 Da corresponding to elimination of glucoside, acetylglucoside and *p*-coumarylglucoside molecules, respectively (Bakker and Timberlake, 1997; Fulcrand, et al. 1998; Morata, et al., 2007). Compounds with *m/z* 487 and 529 were identified as B-type vitisins: pyrano-peonidin-3-glucoside and pyrano-peonidin-3-acetylglucoside, respectively. The compound with *m/z* 805 was identified as (epi)catechin-pyrano-malvidin-3-glucoside (Mateus et al. 2003). In addition, the components with *m/z* 1093 (fragment ions: *m/z* 913, 803) and 1135 (fragment ions: *m/z* 913, 845) have been identified as pyrano-malvidin-3-glucoside-dimer B and pyrano-malvidin-3-acetylglucoside-dimer, respectively, according to He et al. (2006). Fragmentaion of the ions is given in Table 3.

Dimeric compounds resulting from acid-catalyzed cleavage of proanthocyanidins between anthocyanins and flavan-3-ols that takes place spontaneously in wine, were also detected. Thus, (epi)catechin adducts of malvidin-3-glucoside and peonidin-3-glucoside, displaying molecular ions at *m/z* 781 (fragment ions: *m/z* 619, 467 and 373) and *m/z* 751 (fragment ions: *m/z* 589, 437 and 343) were identified as (epi)catechin-malvidin-3-glucoside and (epi)catechin-peonidin-3-glucoside, respectively (Remy, et al. 2000; Salas, 2004).

The signal at *m/z* 1069 was assigned to the molecular ion of the flavylium form of the trimer (epi)catechin-(epi)catechin-malvidin-3-glucoside, according to Salas et al. (2004) and the fragment ions at *m/z* 907 and 619 are reported here for the first time. The first ion corresponds to elimination of a glucose moiety ($[M-162]^+$) and the second ion (*m/z* 619) is obtained with fragmentation of the ion with *m/z* 907 by loss of quinone methide (*m/z* 288) molecule. No further fragmentation was performed due to the low peak intensity.

As previously reported, acetaldehyde-mediated condensation between anthocyanins and (epi)catechin leads to ethyl-bridged pigments (Timberlake and Bridle, 1976). Compounds (epi)catechin-ethyl-malvidin-3-glucoside and (epi)catechin-ethyl-malvidin-3-*p*-coumaroylglucoside were detected with molecular ions at *m/z* 809 and *m/z* 955, respectively. The compounds with *m/z* 779, 925 and 795 were identified as (epi)catechin-ethyl-peonidin-3-glucoside, (epi)catechin-ethyl-peonidin-3-*p*-coumaroylglucoside and (epi)catechin-ethyl-petunidin-3-glucoside, respectively.

Other ethyl bridged compounds were detected at *m/z* 1029 (and $[M+2H]^{2+}$ = 506 corresponding to double charged form of 1029) and at *m/z* 1097, and identified as malvidin-3-glucoside-ethyl-malvidin-3-glucoside ((epi)catechin$_2$-ethyl-malvidin-3-glucoside, respectively (Francia-Aricha et al. 1997).

Table 3. Phenolic compounds identified with MS

t_R/min	Phenolic acids	λ max/ nm	$[M-H]^-$	Fragments (*m/z*)
7.15	Gallic acid	272.3	169	
11.25	Protocatechuic acid	293.7; 255.8	153	
18.2	Caftaric acid	328	311	179, 149
21.7	*cis*-Coutaric acid	310.4	295	163,
25.1	*trans*-Coutaric acid	310	295	163
25.1	GRP		616	
32.5	Fertaric acid	312.8	325	193
33.17	Syringic acid	272.3	197	
t_R/min	**Flavan-3-ols**	**λ max/ nm**	**$[M-H]^-$**	**Fragments (*m/z*)**
26.8	Procyanidin B3	284.2	577	559; 451; 425; 407; 299; 245
28.4	Catechin	276.5	289	245, 205, 179
30.5	Procyanidin B1	264.7	577	559; 451; 425; 407; 299; 245
36.3	Procyanidin B4	264.7	577	559; 451; 425; 407; 299; 245
37	Epicatechin	276.5	289	245, 205, 179
41.1	Procyanidin B2	264.7	577	559; 451; 425; 407; 299; 245
t_R/min	**Flavan-3-ols**	**λ max/ nm**	**$[M-H]^+$**	**Fragments (*m/z*)**
28.4	Catechin	276.5	291	273, 165, 139, 123
37	Epicatechin	276.5	291	273, 165, 139, 123
t_R/min	**Phenolic alcohols**	**λ max/ nm**	**$[M-H]^-$**	**Fragments (*m/z*)**
15.6	Tyrosol	274.2	137	
t_R/min	**Dihydroflavonols**	**λ max/ nm**	**$[M-H]^-$**	**Fragments (*m/z*)**
41.96	Dihydromyricetin 3-*O*-rham		465	339, 301
48.5	Astilbin		449	303, 285
52.1	Engeletin		422	287, 269
t_R/min	**Flavonols**	**λ max/ nm**	**$[M-H]^+$**	**Fragments (*m/z*)**
46.8	Myricetin-3-glcr	343.5	495	319
46.8	Myricetin-3-glc	343.5	481	319
50.1	Quercetin-3-glcr	353.4	479	303
50.1	Quercetin-3-glc	353.4	465	303
52.1	Laricitrin-3-glc	364.2	495	333
55.2	Syringetin-3-glc	358.2	509	481; 392; 347
61.6	Quercetin	368.7	303	
t_R/min	**Anthocyanins, derived pigments**	**λ max/ nm**	**M^+**	**Fragments (*m/z*)**
40.4	(epi)cat-Pn-3-glc	526	751	
40.8	(epi)cat- Mv-3-glc	521	781	619; 601; 373; 242
41.5	Dp-3-glc	525	465	303
41.7	(epi)cat-(epi)cat -Mv-3-glc		1069	907, 619
43.1	Cy-3-glc	517	491	287
45.1	Pt-3-glc	525	479	317
46.8	Pn-3-glc	516	463	301
47.4	carboxy-pyrano- Pt-3-glc		547	385
48.1	Mv-3-glc	528	493	331
49.2	carboxy-pyrano- Pn-3-glc		531	369
50.0	pyrano- Pn-3-glc		487	325
50.5	Vitisin A	513	561	399
51.1	Vitisin B	491	517	355
52	Procyanidin B (2)-ethyl-Mv-3-glc		1097	
52.4	pyrano- Pn-3-Acglc		529	325
52.4	AcVitisin A	516	603	399
53.4	Pyrano-Mv-3-glc-dimer		1093	931, 803
53.5	AcVitisin B	494	559	355
53.5	(epi)cat-ethyl-Mv-3-glc	535	809	647; 519; 358; 357; 341
53.7	Mv-3-Gglc-ethyl-Mv-3-glc		1029	357
54.5	Pyrano-Mv-3-glc-dimer		1093	931, 803

54.7	(epi)cat-ethyl-Mv-3-glc		809	647; 519; 358; 357; 341
54.9	Dp-3-Acglc	530	507	303
55	Cy-3-Acglc	532	491	287
55.3	Pyrano-Mv-3-Acglc-dimer		1135	931, 845, 641, 435
55.4	Pt-3-Acglc	535	521	317
56.2	Pn-3-Acglc	525	505	301
57.5	Mv-3-Acglc	528	535	331
56.6	Pyrano-Mv-3-Acglc-dimer		1135	931, 845, 641, 435
57.8	Carboxy-pyrano- Pt-3-*p*-coumglc		677	369
58.6	CoumVitisin A		707	399
62.8	CoumVitisin B	494	663	355
63.4	Dp-3-*p*-coumglc	530	611	303
63.6	Cy-3-*p*-coumglc		595	287
63.9	Mv-3- *cis*-*p*-coumglc	530	639	331
64	Mv-caffeoyl-3-glc		655	493, 331
64.7	Pt-3-*p*-coumglc	528	625	317
65.3	(epi)cat-ethyl-Mv-3-*p*-coumglc		955	665; 495, 357
65.9	(epi)cat-ethyl-Pn-3-*p*-coumglc		925	
65.9	Pn-3-*p*-coumglc	523	609	301
66.05	Cat-pyrano-Mv-3-glc		805	
66.4	*trans* -Mv-3-*p*-coumglc	530	639	331
67.3	Epicat-pyrano-Mv-3-glc		805	

Labels: Dp: delphinidin, Cy: cyanidin, Pt: petunidin, Pn: peonidin, Mv: malvidin, glc: glucose, Aclglc: acetylglucoside, *p*-coumglc: *p*-coumaroylglucoside, glcr: glucuronide, (epi)cat: (epi)catechin, AcVitisin:acetyl vitisin, CoumVitisin: coumaroylvitisin, rham: rhamnoside

3.2.3. Quantification of phenolic acids, catechins, anthocyanins and pigments

The results for the quantified components are presented in Tables 4, 5, 6 and 7.

Influence of the variety. In agreement with literature data (Wulf and Nagel, 1978), the most abundant anthocyanins found in the Macedonian wines were the monoglucoside derivatives and malvidin-3-glucoside was the predominant compound regardless of variety and wine-making conditions (Bakker and Timberlake, 1985). Results indicated that Vranec wines were richer in monoglucosides and *p*-coumaroylglucosides, while Merlot wines contained higher amounts of acetylglucosides compared to the content of those components present in Vranec wines. The cultivar differences can be attributed to differences of the anthocyanin content (Bakker and Timberlake, 1985); and compared to literature data for Merlot and other red varieties (Mazza, et al. 1999; Suárez, et al. 2007; González-Neves, et al. 2007; Cadahía, et al. 2009), the anthocyanin content of Vranec wines does not differ from the common *Vitis vinifera* L. varieties.

With regards to hydroxicinnamic acid and derivatives content, Vranec wines were richer than Merlot wines, irrespective of the applied wine-making techniques. In general, concentrations of *trans*-caftaric and coutaric acids are around 100 mg/L and 55 mg/L, respectively, (Ong and Nagel, 1978; Nagel and Wulf, 1979; Herrick and Nagel, 1985; Singleton et al. 1986; Cheynier et al. 1988) as was observed for the Vranec wines. Tyrosol was the phenolic alcohol identified in the wines, with contents in accordance with the published data (Ibern-Gómez, et al. 2002). Vranec and Merlot wines had remarkably high levels of (+)-catechin and (-)-epicatechin, indicating that Vranec wines contained higher levels of condensed tannins compared to Merlot wines obtained with the same technological procedure. The results were in accordance with published data for tannins in different red varieties (Fulcrand et al. 1999; Monagas et al. 2003; Morel-Salmi et al. 2006).

Influence of the maceration time. Three maceration times, 3, 6 and 10 days were applied for production of Vranec and Merlot wines. For Vranec wines, the highest concentration of glucosides was reached in the wines macerated for 6 days, only insignificant difference was noticed between the wines V70-Fr-6d and V70-Fr-10. For Merlot wines, highest content of anthocyanins was found in the wines macerated for 6 days containing higher amount of SO_2, while, wines with lower dose of SO_2, macerated for 10 days, contained highest amounts of

anthocyanins. Usually, longer maceration time leads to higher contents of anthocyanins, but also, longer contact of must with the grape skins can decrease their content, as was eveident for the analyzed wines. Decreasing can be a result of their conversion to other pigments (or non pigmented compounds) including direct reactions between anthocyanins and flavanols or reactions between anthocyanins and flavanols through ethyl bridges (Bakker, et al., 1997; Fulcrand, et al., 1998; Schwarz, et al., 2004) forming stable pigments which stabilize the wine color. The obtained results suggest that changes of anthocyanins, which are easily involved in degradation or polymerization processes, start early in the wine-making process and then go on very slowly in the wine, which is in agreement with previously published data (Morel-Salmi, et al. 2006). Vitisin A was formed in higher quantities in the wines macerated for 6 days and consistent with the yeast product release, pyrivic acid, which is formed in the earlier stages of fermentation (Morata, et al. 2006).

The content of hydroxycinnamic acids and their derivatives was not much different in the wines obtained with different maceration time. The concentration of caftaric acid, the most abundant tartaric ester in grape, reached highest values in Vranec wines macerated for 6 days and fermented with Macedonian yeast, while wines fermented with French yeast contained highest content after maceration of 3 days, observing gradual decrease of the content in the wines obtained with longer maceration time. For Merlot wines, caftaric acid was extracted in highest amount during the period of maceration for 6 days, and then decreased. Decreasing can be attributed to the hydrolysis of hydroxycinnamic tartaric esters during the fermentation and maceration process and, as a result of this process, caffeic acid, which was identified and quantified, slightly increased in wines (Singleton, et al. 1985).

With regard to the results of tyrosine for both varieties, no significant differences were observed when wines with same maceration time, fermented with Macedonian and French yeast were compared, since the used yeasts were from the same specie (*S.cerevisiae*). The conversion of tyrosine into tyrosol is possibly carried out when cells grow, in the earlier stages of the fermentation when the medium is rich in nutrients and higher concentration of tyrosine is expected to be formed. That can be the reason for the higher contents of tyrosol in the wines macerated for 3 days, compared to the wines macerated for 10 days, which contained lower amounts.

The total catechin concentrations in the analyzed wines increased with maceration time. Concentration of monomeric flavan-3-ols reached highest values in the wines macerated for 10 days as expected, taking into account that catechins, which are less hydrophilic than other flavonoids, are extracted from the seeds in the later stages of fermentation when the alcohol content increases. For Vranec wines, it was observed that the mDP slightly increased from 3 days to 6 days macerated wines and remained almost the same in the wines macerated for 10 days, and for the Merlot wines, mDP increased with maceration time.

Influence of the yeast strain.. Manoproteins which determine the most of the surface properties of the cell wall, can adsorb the anthocyanins and other phenolic compounds (Mazauric and Salmon, 2005). Differences in polyphenol concentrations in wines fermented with both yeasts were found, and it was observed that wines fermented with French yeast contained lower tannin content, probably as a result of its higher ability to adsorb those compounds compared to the Macedonian yeast (a weaker adsorber of tannins). This was evident for all Merlot and Vranec wines that contained higher level of SO_2 indicating that Macedonian yeast is more resistant at higher levels of SO_2. With regard to caffeic acid and hydroxycinnamic esters, no clear tendency of the effect of yeast strain used for fermentation was observed. Also, the efficiency of the yeast to adsorb the anthocyanins was not very clear and no significant differences of phenolic compounds in the wines fermented either with Macedonian and French yeast were observed. That can be a result of the similar structure of the cell walls of both yeasts.

Table 4. Phenolic contents of Vranec and Merlot wines quantified at 320 and 280 nm

Wines		320 nm					280 nm				
VRANEC		*trans*-Caftaric acid*	*trans*-Coutaric acid*	*cis*-Coutaric acid*	Caffeic acid*	**Σ***	(+)-Catechin*	(-)-Epicatechin*	Procyanidin B2*	**Σ***	Tyrosol*
30 mg/L SO_2, **Macedonian** yeast	3 days	106.47	50.06	5.39	1.38	**163.3**	7.52	21.56	10.58	**39.66**	16.11
	6 days	157.04	76.29	6.38	1.61	**241.32**	11.22	17.11	31.80	**60.13**	17.75
	10 days	91.99	42.87	4.79	1.19	**140.84**	13.82	5.69	40.07	**59.58**	10.75
70 mg/L SO_2, **Macedonian** yeast	3 days	159.98	69.82	7.32	1.63	**238.75**	7.17	10.43	19.39	**36.99**	20.13
	6 days	163.66	70.47	5.79	1.31	**180.23**	13.31	25.83	48.01	**87.15**	14.76
	10 days	112.59	53.85	4.62	1.64	**172.7**	26.67	14.52	26.99	**68.18**	10.42
30 mg/L SO_2, **French** yeast	3 days	134.33	61.70	5.73	1.35	**203.11**	7.79	8.04	14.95	**30.78**	19.00
	6 days	124.81	63.38	5.61	1.59	**195.39**	11.73	22.02	40.92	**74.67**	14.07
	10 days	102.54	48.92	5.33	1.64	**158.43**	14.73	23.13	42.99	**80.85**	11.55
70 mg/L SO_2, **French** yeast	3 days	164.08	67.40	7.99	1.42	**240.89**	7.17	10.43	19.39	**36.99**	20.13
	6 days	139.25	70.89	6.02	1.82	**217.98**	14.79	30.38	56.46	**101.63**	10.71
	10 days	140.53	68.54	4.92	2.11	**216.1**	18.07	35.21	65.45	**118.73**	10.88
MERLOT											
30 mg/L SO_2, **Macedonian** yeast	3 days	44.54	17.74	2.59	0.75	**65.62**	11.37	9.43	17.52	**38.32**	27.19
	6 days	72.69	28.14	4.36	0.95	**106.14**	16.22	19.22	35.72	**71.16**	23.64
	10 days	15.58	22.28	2.54	0.96	**41.36**	19.32	18.23	33.89	**71.44**	17.99
70 mg/L SO_2, **Macedonian** yeast	3 days	50.66	27.81	4.13	0.91	**83.51**	11.90	13.05	24.25	**49.2**	31.89
	6 days	66.82	26.75	3.59	1.01	**98.17**	15.37	16.88	31.37	**63.62**	25.06
	10 days	52.34	22.68	2.96	0.99	**78.97**	15.12	15.86	29.49	**60.47**	16.09
30 mg/L SO_2, **French** yeast	3 days	38.35	23.14	3.50	0.70	**65.69**	6.69	7.42	13.79	**27.9**	36.84
	6 days	58.54	26.99	3.27	0.98	**89.78**	11.19	11.57	21.50	**44.26**	29.55
	10 days	58.89	27.24	3.20	0.98	**90.31**	17.68	21.68	40.30	**79.66**	25.05
70 mg/L SO_2, **French** yeast	3 days	26.17	24.09	3.42	0.74	**53.68**	8.39	9.52	17.69	**35.6**	41.02
	6 days	62.49	29.74	3.09	0.88	**96.06**	12.80	13.91	25.86	**52.57**	32.97
	10 days	51.14	24.00	2.66	0.88	**78.68**	13.30	12.60	23.41	**49.31**	25.67

*Concentration of the components expressed in mg/L

Table 5. Red pigments content in Vranec and Merlot wines

Wines		3-monoglucosides*						3-acetylglucosides*				3-cumaroyl glucosides*					
VRANEC	**Days of macer.**	Dp	Cy	Pt	Pn	Mv	**Σ**	Pt	Pn	Mv	**Σ**	Cy	Pt	Pn	*trans*-Mv	*cis*-Mv	**Σ**
30 mg/L SO_2 **Macedonian** yeast	3 days	0.10	0.02	0.27	0.33	2.87	**3.59**	0.86	0.27	0.32	**1.45**	0.08	0.13	0.24	0.34	0.05	**0.84**
	6 days	4.38	0.37	6.58	4.98	47.93	**64.24**	1.13	2.32	4.29	**7.74**	0.44	0.96	1.08	5.08	0.29	**7.85**
	10 days	1.70	0.17	2.68	2.36	25.82	**32.73**	2.05	0.73	2.62	**5.4**	0.23	0.37	0.57	1.95	0.19	**3.31**
70 mg/L SO_2 **Macedonian** yeast	3 days	2.21	0.14	3.46	1.82	24.16	**31.79**	1.97	0.73	2.12	**4.82**	0.46	0.41	0.58	2.33	0.09	**3.87**
	6 days	7.67	0.65	13.04	8.75	86.74	**116.85**	2.32	1.78	7.34	**11.44**	0.46	1.99	2.00	8.54	0.42	**13.41**
	10 days	4.94	0.50	8.36	6.19	61.64	**81.63**	2.43	1.34	5.69	**9.46**	0.37	1.18	1.31	6.29	0.48	**9.63**
30 mg/L SO_2 **French** yeast	3 days	0.20	0.04	0.25	0.35	2.68	**3.52**	1.16	0.32	0.29	**1.77**	0.25	0.16	0.17	0.32	0.05	**0.95**
	6 days	4.40	0.38	7.51	5.75	55.17	**73.21**	2.30	1.21	4.53	**8.04**	0.40	1.08	1.20	5.33	0.31	**8.32**
	10 days	2.50	0.25	3.57	2.89	29.24	**38.45**	2.26	0.63	2.16	**5.05**	0.35	0.37	0.63	2.12	0.19	**3.66**
70 mg/L SO_2, **French** yeast	3 days	0.55	0.06	1.09	0.85	9.02	**11.57**	2.13	0.26	0.97	**3.36**	0.31	0.19	0.40	0.54	0.07	**1.51**
	6 days	7.79	0.67	14.15	9.48	99.05	**131.14**	2.00	1.90	8.73	**12.63**	0.90	2.18	2.62	10.37	0.48	**16.55**
	10 days	9.39	1.11	15.55	11.01	98.76	**135.82**	2.99	1.86	8.27	**13.12**	0.36	2.46	2.60	10.97	0.66	**17.05**
MERLOT																	
30 mg/L SO_2 **Macedonian** yeast	3 days	0.08	0.03	0.35	0.34	26.89	**27.69**	0.87	0.25	2.09	**3.21**	0.20	0.13	0.19	0.42	0.08	**1.02**
	6 days	1.06	0.05	1.60	1.48	39.08	**43.27**	1.13	0.98	7.15	**9.26**	0.30	0.26	0.67	2.40	0.19	**3.82**
	10 days	1.90	0.11	2.80	1.67	16.67	**23.15**	1.73	1.09	11.09	**13.91**	0.20	0.31	0.88	3.37	0.44	**5.2**
70 mg/L SO_2 **Macedonian** yeast	3 days	0.40	0.04	0.76	0.90	41.46	**43.56**	0.89	0.63	4.22	**5.74**	0.16	0.18	0.46	1.22	0.12	**2.14**
	6 days	1.66	0.06	2.67	1.87	24.13	**30.39**	1.21	1.27	11.34	**13.82**	0.15	0.32	0.61	3.64	0.39	**5.11**
	10 days	0.93	0.05	1.43	1.08	0.44	**3.93**	1.53	0.58	6.47	**8.58**	0.12	0.19	0.44	0.11	0.26	**1.12**
30 mg/L SO_2 **French** yeast	3 days	0.00	0.00	0.00	0.14	16.64	**16.78**	0.49	0.35	0.27	**1.11**	0.06	0.16	0.19	0.11	0.04	**0.56**
	6 days	0.58	0.03	0.82	1.20	41.69	**44.32**	1.20	0.36	3.78	**5.34**	0.22	0.15	0.30	1.03	0.14	**1.84**
	10 days	2.10	0.16	2.89	3.46	2.1	**10.71**	1.30	1.72	10.22	**13.24**	0.86	0.40	0.98	4.13	0.50	**6.87**
70 mg/L SO_2, **French** yeast	3 days	0.04	0.02	0.07	0.30	32.86	**33.29**	0.75	0.07	0.42	**1.24**	0.13	0.11	0.21	0.11	0.04	**0.6**
	6 days	1.50	0.12	2.12	2.09	9.2	**15.03**	1.36	0.85	7.85	**10.06**	0.25	0.26	0.59	2.63	0.23	**3.96**
	10 days	0.33	0.08	0.45	0.61	8.38	**9.85**	0.86	0.56	4.20	**5.62**	0.23	0.91	1.07	3.95	0.29	**6.45**

Labels- Dp: delphinidin, Cy: cyanidin, Pt: petunidin, Pn: peonidin, Mv: malvidin

*Concentration of the components expressed in mg/L

Table 6. Red pigments content in Vranec and Merlot wines

Wines	Days of macer.	Vitisin A*	Ac Vitisin A*	Σ*	Vitisin B*	Ac VitisinB*	Coum Vitisin B*	Σ*	Mv-caff-glc*	flav-pyr-mv-3-glc*	Mv/Pn	Acglc/Coumglc
VRANEC												
30 mg/L SO_2, **Macedonian** yeast	3 days	2.51	0.37	**2.88**	2.14	0.13	0.54	**2.81**	0.12	0.00	8.77	1.73
	6 days	9.31	1.47	**10.78**	1.69	0.17	1.09	**2.95**	0.46	0.08	9.63	0.99
	10 days	3.97	0.67	**4.64**	1.88	0.40	0.74	**3.02**	0.47	0.10	10.95	1.63
70 mg/L SO_2, **Macedonian** yeast	3 days	3.27	0.45	**3.72**	2.80	0.29	1.32	**4.41**	0.35	0.15	13.25	1.25
	6 days	9.37	1.57	**10.94**	1.12	0.25	0.93	**2.3**	0.33	0.23	9.91	0.85
	10 days	5.52	0.98	**6.5**	1.23	0.34	0.44	**2.01**	0.49	0.18	9.96	0.98
30 mg/L SO_2, **French** yeast	3 days	2.93	0.38	**3.31**	1.52	0.13	0.68	**2.33**	0.15	0.00	7.67	1.86
	6 days	8.14	1.28	**9.42**	2.02	0.43	1.16	**3.61**	0.45	0.13	9.59	0.97
	10 days	4.84	0.71	**5.55**	1.91	0.40	0.85	**3.16**	0.36	0.11	10.12	1.38
70 mg/L SO_2, **French** yeast	3 days	4.14	0.58	**4.72**	1.44	0.14	0.38	**1.96**	0.22	0.10	10.67	2.23
	6 days	4.36	0.94	**5.3**	1.16	0.25	0.59	**2**	0.29	0.28	10.45	0.76
	10 days	5.38	1.06	**6.44**	1.27	0.35	0.58	**2.2**	0.48	0.15	8.97	0.77
MERLOT												
30 mg/L SO_2, **Macedonian** yeast	3 days	4.40	1.95	**6.35**	1.14	0.08	0.47	**1.69**	0.13	0.21	22.50	3.15
	6 days	6.67	3.27	**9.94**	0.87	0.22	0.70	**1.79**	0.40	0.29	15.29	2.42
	10 days	8.07	3.95	**12.02**	0.42	0.09	0.50	**1.01**	0.34	0.14	19.52	2.68
70 mg/L SO_2, **Macedonian** yeast	3 days	6.87	3.06	**9.93**	0.90	0.12	0.48	**1.5**	0.20	0.37	16.25	2.68
	6 days	10.56	4.76	**15.32**	0.77	0.14	0.67	**1.58**	0.35	0.36	18.85	2.70
	10 days	8.37	4.30	**12.67**	0.85	0.16	0.33	**1.34**	0.22	0.21	19.18	7.66
30 mg/L SO_2, **French** yeast	3 days	2.60	1.07	**3.67**	0.16	0.03	0.26	**0.45**	0.10	0.00	2.15	1.98
	6 days	10.65	4.62	**15.27**	1.02	0.08	0.51	**1.61**	0.18	0.12	11.72	2.90
	10 days	7.14	3.82	**10.96**	0.73	0.15	1.07	**1.95**	0.19	0.14	9.57	1.93
70 mg/L SO_2, **French** yeast	3 days	4.50	1.90	**6.4**	0.31	0.08	0.37	**0.76**	0.15	0.06	5.59	2.07
	6 days	13.06	5.92	**18.98**	1.69	0.20	0.90	**2.79**	0.33	0.26	12.95	2.54
	10 days	6.68	3.18	**9.86**	1.01	0.14	0.48	**1.63**	0.33	0.14	12.57	0.87

Labels : AcVitisin A (B) : acetylvitisin A (B), Coumvitisin B : coumaroylvitisin B, Mv-caff-glc: malvidin-3-caffeoyl-glucoside, flav-pyr-mv-3-glc: flavanyl-pyrano-malvidin-3-glucoside, Mv/Pn: ration between malvidin-3-glucoside and peonidin-3-glucoside, Acglc/Coumglc: ratio between the total contents of acetylglucoside and coumaroylglucoside anthocyanins

*Concentration of the components expressed in mg/L

Table 7. Tannin content in Vranec and Merlot wines

VRANEC		mDP	% Gal	% EGC	Total tannins
30 mg/L SO_2 **Macedonian** yeast	3 days	2.49	11.56	13.61	58.21
	6 days	4.45	5.23	8.13	316.04
	10 days	4.45	5.79	18.50	317.19
70 mg/L SO_2 **Macedonian** yeast	3 days	4.23	4.86	9.90	20.25
	6 days	5.31	5.02	12.01	58.50
	10 days	5.26	6.13	12.97	68.40
30 mg/L SO_2 **French** yeast	3 days	3.42	6.30	10.50	10.01
	6 days	4.52	5.04	11.31	36.82
	10 days	4.61	5.62	12.69	50.37
70 mg/L SO_2 **French** yeast	3 days	4.09	7.76	8.61	12.96
	6 days	4.95	5.13	11.33	49.00
	10 days	5.34	5.53	14.10	85.93
MERLO					
30 mg/L SO_2 **Macedonian** yeast	3 days	2.19	15.86	3.50	2.14
	6 days	2.45	4.89	7.47	15.77
	10 days	3.60	4.95	11.25	42.49
70 mg/L SO_2 **Macedonian** yeast	3 days	2.53	9.55	3.82	3.34
	6 days	2.39	4.80	8.15	18.11
	10 days	3.48	5.79	9.55	27.43
30 mg/L SO_2 **French** yeast	3 days	1.52	14.09	0.00	0.00
	6 days	3.12	7.84	13.12	20.12
	10 days	3.29	4.27	11.04	33.72
70 mg/L SO_2 **French** yeast	3 days	1.58	12.37	0.00	0.00
	6 days	2.89	4.73	10.85	20.72
	10 days	2.98	6.18	7.47	15.69

Lables: [a] - %, percentage content;
[b] – concentration (mg/L)
Gal-Gallocatehin; Egc-Epigallocatechin;

Influence of SO_2. SO_2, as an antioxidant (free SO_2) and antimicrobial (molecular SO_2) agent, is commonly used before the fermentation (Amerine, et al. 1967). Higher concentrations of anthocyanin monoglucosides, acetylglucosides and *p*-coumaroyl derivatives were found in Vranec wines with higher content of SO_2, which was in accordance with the data published by Berg and Akiyoshi (1962), implying that SO_2 aids the extraction of pigments. For Vranec and Merlot wines fermented with either yeast (Macedonian and French), formation of vitisin A and acetylvitisin A was slightly increased with increasing of SO_2 dose, regardless of maceration time, which was in accordance with the data of Asenstorfer et al. (2003). On the other hand, the concentration of vitisin B in Vranec wines was lower in wines with higher doses of SO_2 which is due to the ability of SO_2 to limit the acetaldehyde formation resulting in reduced production of B-type vitisins, including the acetylvitisin B and *p*-coumaroylvitisin B (Morata et al. 2006).

Regarding to concentrations of hydroxycinnamic acid derivatives, the content of *trans*-caftaric acid was highest in Vranec wines with 70 mg/L SO_2, which was expected because addition of higher doses of sulphur dioxide can limit the oxidation of those phenols (Singleton et al., 1985). On the other hand, no clear trend of this component in Merlot wines was observed.

Cluster analysis (CA). CA was performed in order to reveal possible similarities or affinities among the Merlot and Vranec wine samples taking the Euclidean distance as metric and the Ward's method as amalgamation rule. In the dendrogram obtained from the cluster analysis of the variables (phenolic acids, anthocyanins and flavan-3-ols), two main clusters can be observed (Fig. 18). To the left, Merlot wines formed a cluster (A) which is divided in two sub-clusters (A1 and A2). To the right, the second cluster (B) comprised the Vranec wines and it is also divided in additional sub-clusters (B1 and B2). In general, separation of the wine samples in clusters was performed according to the variety (Merlot and Vranec) and separation in each cluster was made by the period of maceration and content of SO_2.

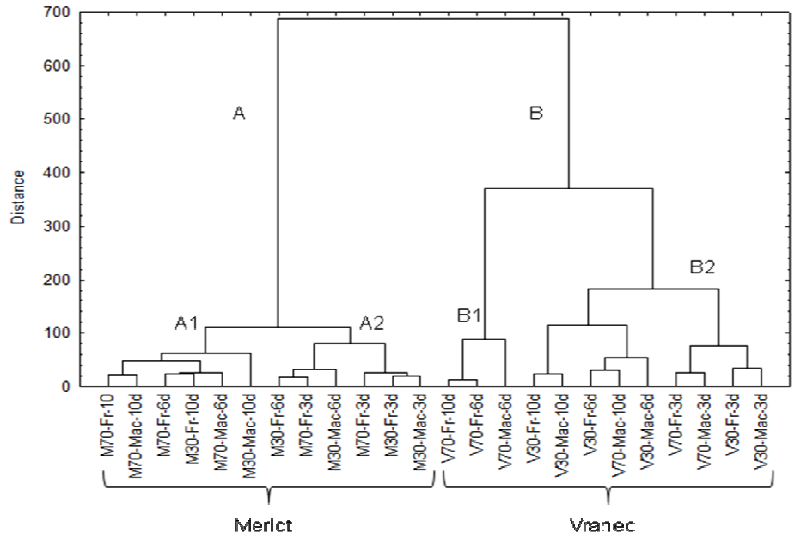


Fig. 18. Dendrogram obtained after agglomerative CA performed on phenolic compounds of all samples studied: Merlot and Vranec

Principal Component Analysis (PCA). PCA was employed in order to check if the studied wine samples obtained from two varieties, with different maceration times, SO_2 and yeasts can be distinguished according to the obtained results for anthocyanins, phenolic acids and flavan-3-ols.

Vranec wines macerated for 3 days were grouped (Fig. 19) and located in the positive part of the principal component 2 (PC2, accounting for 18.08 % of the total variability). For the other Vranec wines located in the first principal component (PC1, accounting for 49.08 % of the total variability), separation according to the content of SO_2 was observed, not depending on maceration time and used yeast for fermentation. The PCA results agree with those obtained by CA, since a clear clustering trend in the samples macerated for 3 days was observed, as well as grouping according to the SO_2 content.

Separation of Merlot wines was not clear, that also agree with the CA results, whereas the separation for the wines macerated for 6 and 10 days was not observed.

It was noticed that anthocyanins (glucosides, acetyl- and *p*-coumaroyl derivatives) prevail in the first principal component and among them Pn-3-glc, Pn-3-acglc, Pn-3-*p*-coumgluc, Mv-glc Mv-coumglc, as well as procyanidin B2 and epicatechin are dominant components in the first principal component. Terminal units of proanthocyanidins, gallate units and tyrosol were shown as dominant in the second principal component. Therefore, the separation of the samples can be attributed mainly to the anthocyanin content of the wines.

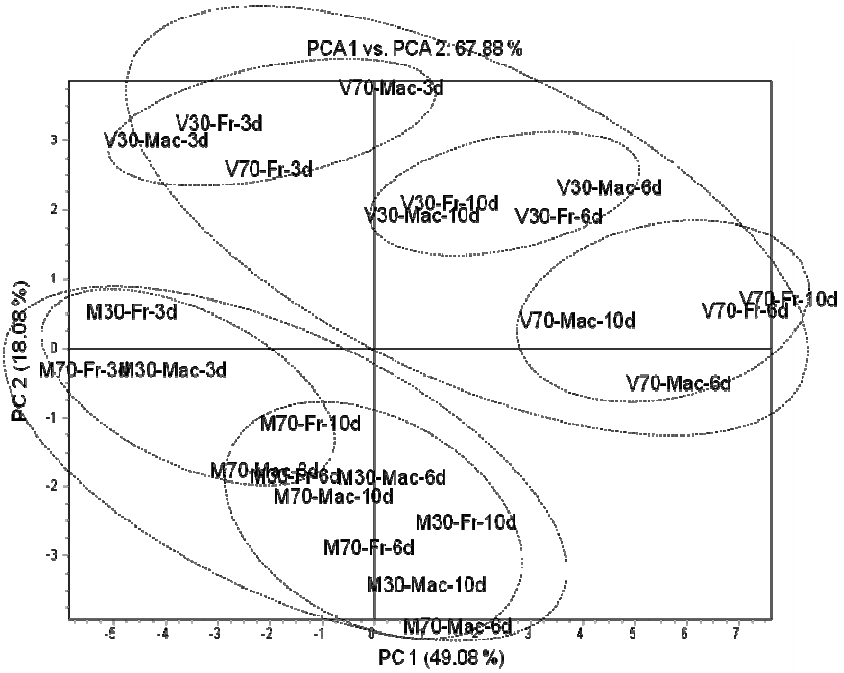


Fig. 19. Principal Component score plot of the variables with PC1 and PC2 based on phenolic acids, anthocyanins and proanthocyanidins for the anthocyanins for the analyzed Vranec and Merlot wines

3.2.4. HPLC quantification of phenolic acids in white wines

The main phenolic components in white wines are nonflavonoids, i.e. hydroxycinnamic acid derivatives: caftaric acid (caffeoyl tartaric acid) and coutaric acid (coumaroyl tartaric acid), and the corresponding acids caffeic and coumaric.

The HPLC results for the white wines Smederevka and Chardonnay, produced with different technological processes, applying two doses of SO_2 (50 and 100 mg/L SO_2) and two yeasts for fermentation (Macedonian yeast, Vinalco and French yeast, Levuline), are presented in Table 8.

As can be seen from the table, *trans*-coutaric acid dominates in Smederevka wines, while, for Chardonnay wines, *trans*-caftaric acid is the dominant component. Caffeic acid was present in lower concentration in Smederevka wines, compared to Chardonnay wines. Smederevka wines with lower content of SO_2 (50 mg/L SO_2) contained higher concentrations of *trans*-caftaric acid and *trans*-coutaric acid, despite of Chardonnay where higher content of both acids was measured for the wines with 100 mg/L SO_2.

Table 8. Phenolic acids (mg/L) determined by HPLC analysis of Smederevka and Chardonnay wines

Components	*trans*-Caftaric acid	*cis*-Coutaric acid	*trans*- Coutaric acid	Caffeic acid	Tyrosol	Total acids
Sm50Mac	7.17	-	26.87	2.08	36.75	**36.11**
Sm100Mac	3.62	-	11.43	1.8	29.1	**16.85**
Sm50Fr	11.78	-	27.81	1.9	38.39	**41.49**
Sm100Fr	3.33	-	13.77	1.68	29.84	**18.78**
Ch50Mac	132.18	31.37	47.03	3.97	32.88	**117.95**
Ch100Mac	57.14	25.71	30.9	4.21	40.33	**214.56**
Ch50 Fr	81	33.1	28.08	3.95	36.41	**146.12**
Ch100Fr	142.18	32.35	51.35	3.65	40.41	**229.53**

The concentrations of tyrosol were measured for the wines from both varieties. This component, similar like phenolic acids, was present in higher concentrations in Smederevka wines with 50 mg/L SO_2 and Chardonnay wines with 100 mg/L SO_2. Tyrosol was present in concentration range from 29.10 to 40.41 mg/L in the white wines, contents that could influence the bitterness of the wines.

3.3. MALDI-TOF-MS ANALYSIS OF ANTHOCYANINS IN WINE AND GRAPE SAMPLES USING DIFFERENT MATRICES

MALDI operates in positive and negative modes followed by generation of anions, $[M-H]^-$ and $[M+Cl]^-$, or cations, $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$. Recent investigations demonstrated that MALDI as a sensitive and efficient technique could be used for characterization of different molecules: for analysis of anthocyanins in red wine and fruit juice (Wang and Sporns, 1999), analysis of carotenoids in crop plants (Fraser, et al. 2007), and it has become the routine method of choice to assess peptides and proteins (Schiller et al. 2004; Sheoran et al. 2007; Piraino et al. 2007; Joss et al. 2006; Zhang et al. 2008).

The primary purpose of this research was application of MALDI-TOF-MS method for analysis of polyphenols in wine and grape samples. Furthermore, different MALDI matrices were tested in order to compare their efficiency for identification of anthocyanins in grape extracts and wine samples. The following matrices were tested:

- ✓ α-cyano-4-hydroxycinnamic acid (CHCA);
- ✓ Sinapic acid (SA);
- ✓ 2,5-dihydroxybenzoic acid (2,5-DHB), и
- ✓ C70 fullerene

The obtained quasimolecular, fragment and adduct ion peaks for CHCA, SA and 2,5-DHB have relatively high intensity in the range of *m/z* 100-700 which means that those matrix peaks can interfere in identification of anthocyanins with masses in the range *m/z* 300-700. On the other hand, the peaks of C70 fullerene matrix had very low intensity in this region. However, all those matrices were tested on wine and grape sample and the relative intensities of anthocyanins identified in skin extract and two Merlot wines (M30-Mac-6d and M30-Mac-10d) are presented in Table 9. It was observed that 2,5-DHB matrix was superior for identification of anthocyanins present in the analyzed samples, with respect to all the matrices tested. This matrix allowed confirmation of 3-glucosides, 3-acetylglucosides and 3-*p*-coumaroylglucosides of petunidin, peonidin and malvidin, as well as identification of the five anglycones, which can be attributed to its facility for both homogeneous sample preparation and higher ionization efficiency. MALDI-TOF-MS spectra of Vranec skin extract in the presence of different MALDI matrices are presented in Fig. 20.

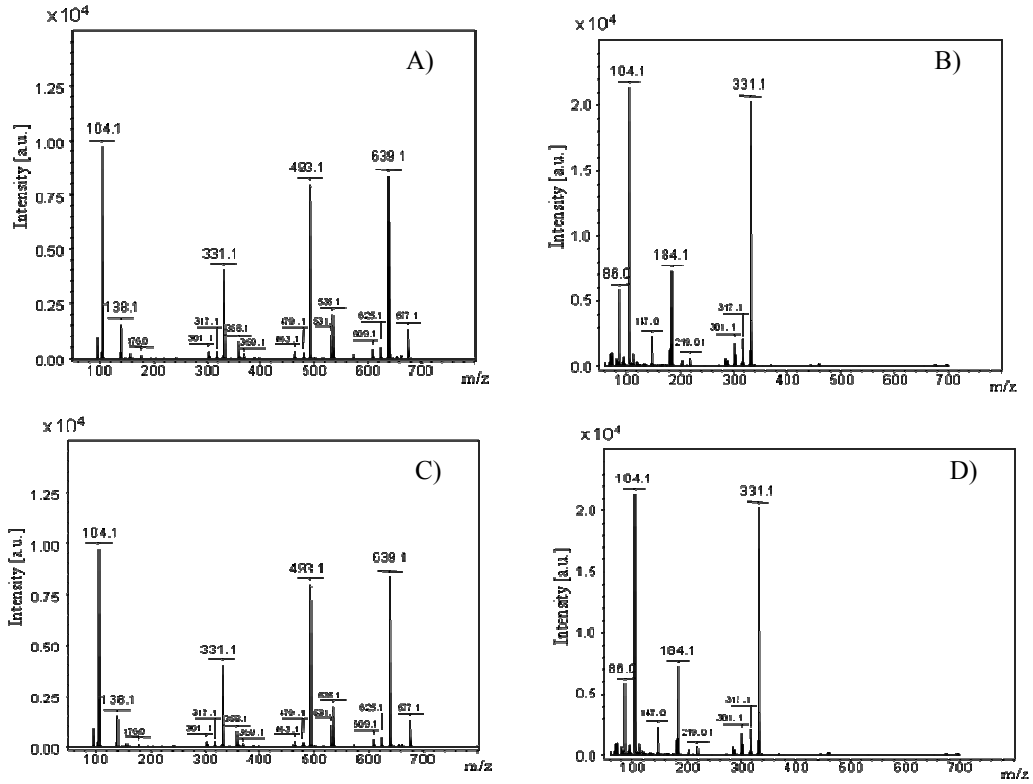


Fig. 20. MALDI TOF MS spectra of Vranec skin extract in presence of different matrices: (A) CHCA, (B) SA, (C) 2,5-DHB, (D) C70 fulleren "sandwich" method under positive and reflective mode

The positively charged flavylium ion of malvidin (*m/z* 331) was detected in all samples under all experimental conditions. Other peaks in the mass spectrum, *m/z* 493, 535 and 639 corresponded to malvidin-3-glucoside, malvidin-3-acetylglucoside and malvidin-3-*p*-coumaroylglucoside, respectively. MALDI-TOF-MS analyses of Vranec skin extract applying 2,5-DHB matrix, exhibited peaks at *m/z* 317, 479 and 625, consistent with petunidin, petunidin-3-glucoside and petunidin-3-*p*-coumaroylglucoside, respectively. The peaks at *m/z* 301, 463 and 609 represented to peonidin, peonidin-3-glucoside and peonidin-3-*p*-coumaroylglucoside, respectively. The peaks with *m/z* 303 and 287 were attributed to delphinidin and cyanidin cations observed in wine samples ionized with 2,5-DHB matrix. The polyphenol content of grape pulp, skins and seeds are presented in Fig. 21. Mass spectra are obtained with 2,5-DHB matrix under the equal parametars of analysis.

The molecular ions of anthocyanins were compared and agreed with previously published data obtained by HPLC-MS (Wang & Sporns, 1999; Heier ,et al. 2002; Vivar-Quintana, et al., 2002; Kelebek, 2007). According to literature HPLC-MS data, but also from the obtained results from the MS analysis of anthocyanins in this work, the mass spectra of those compounds contain two signals, the molecular ion, M^+ and the fragments $[M-162]^+$, $[M-204]^+$ and $[M-308]^+$ resulting from the elimination of glucose, acetylglucose and p-coumaroylglucose moiety, respectively.

Table 9. Relative intensities (%) of anthocyanin peaks identified with MALDI TOF MS in Vranec grape and Merlot wines applying different matrices

	Aglycone [c]						3-glucoside [d]						3-acetylglucoside [e]						3-coumaroylglucosides [f]				
	Cy	**Pn**	**Dp**	**Pt**	**Mv**		**Cy**	**Pn**	**Dp**	**Pt**	**Mv**		**Cy**	**Pn**	**Dp**	**Pt**	**Mv**		**Cy**	**Pn**	**Dp**	**Pt**	**Mv**
Skin extract																							
CHCA	<1	4	<1	5	100		–	–	–	–	2		–	–	–	–	<1		–	<1	–	<1	1
SA	–	<1	<1	<1	10		1	<1	–	<1	11		–	–	–	–	2		–	1	–	1	27
2,5-DHB	<1	3.1	<1	3.3	41		–	3	–	2	78		–	<1	–	<1	19		–	4	–	5	83
C70 sendwich	2	8	3	9	90		–	–	–	–	–		–	–	–	–	–		–	–	–	–	–
ne (M30-Mac-6d) [a]																							
CHCA	<1	5	1	4	72		–	<1	–	–	9		–	–	–	–	1		–	–	–	–	<1
SA	–	<1	<1	<1	8		–	–	–	–	19		–	–	–	–	3		–	<1	–	<1	14
2,5-DHB	–	1	1	1	14		–	2	–	1	30		–	<1	–	–	6		<1	1	–	<1	10
C70 sendwich	<1	2	<1	1	35		–	–	–	–	–		–	–	–	–	–		–	–	–	–	–
ne (M30-Mac-10d) [b]																							
CHCA	–	2	<1	1	43		–	<1	–	–	4		–	–	–	–	<1		–	–	–	–	<1
SA	–	1	1	1	25		–	<1	–	<1	20		–	–	–	–	4		–	<1	–	<1	18
2,5-DHB	–	<1	1	<1	5		–	1	<1	<1	13		–	<1	–	–	2		–	<1	–	<1	4
C70 sendwich	<1	5	1	2	100		–	–	–	–	–		–	–	–	–	–		–	–	–	–	–

[b] Fermentation performed with Macedonian yeast, applied of 10 days maceration, containing 30 mg/L SO_2

[c] Cy: Cyanidin (*m/z* 287), Pn: Peonidin (*m/z* 301), Dp: Delphinidin (*m/z* 303), Pt: Petunidin (*m/z* 317), Mv: Malvidin (*m/z* 331)

[d] Cy-3-Glc: Cyanidin-3-glucoside (*m/z* 449), Pn-3-Glc: Peonidin-3-glucoside (*m/z* 463), Dp-3-Glc: Delphinidin-3-glucoside (*m/z* 465), Pt-3-Glc: Petunidin-3-glucoside (*m/z* 479), Mv-3-Glc: Malvidin-3-glucoside (*m/z* 493)

[e] Cy-3-AcGlc: Cyanidin-3-acetylglucoside (*m/z* 491), Pn-3-AcGlc: Peonidin-3-acetylglucoside (*m/z* 505), Dp-3-AcGlc: Delphinidin-3-acetylglucoside (*m/z* 507), Pt-3-AcGlc: Petunidin-3-acetylglucoside (*m/z* 521), Mv-3-AcGlc: Malvidin-3-acetylglucoside (*m/z* 535)

[f] Cy-3-CoumGlc: Cyanidin-3-*p*-coumarpylglucoside (*m/z* 595), Pn-3-CoumGlc: Peonidin-3-*p*-coumaroylglucoside (*m/z* 609), Dp-3-CoumGlc: Delphinidin-3-*p*-coumaroylglucoside (*m/z* 611), Pt-3-CoumGlc: Petunidin-3-*p*-coumaroylglucoside (*m/z* 625), Mv-3-CoumGlc: Malvidin-3-*p*-coumaroylglucoside (*m/z* 639)

[a] Fermentation performed with Macedonian yeast, applied 6 days of maceration, containing 30 mg/L SO_2

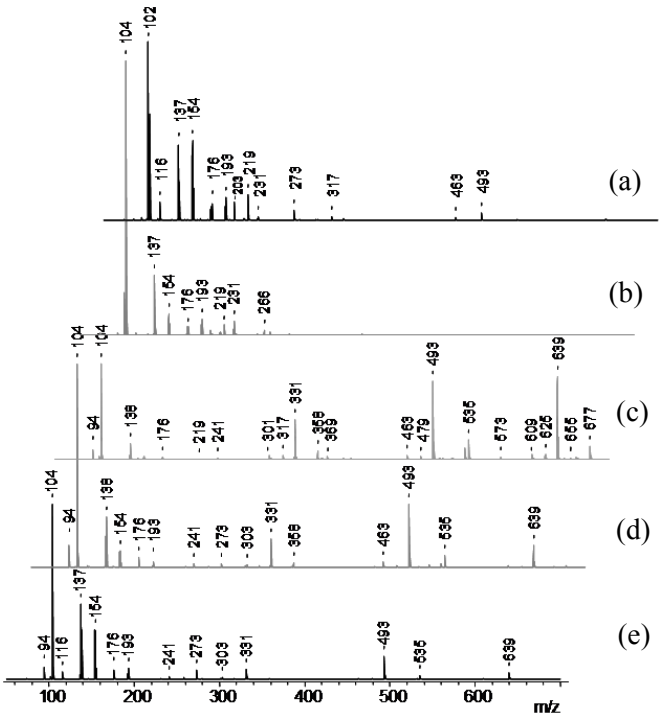


Fig. 21. MALDI-TOF-MS spectra of Vranec grape and wine samples, applying 2,5-DHB for ionization, under positive reflective mode: (a) pulp, (b) seeds, (c) skins, (d) wine: V30-Mac-6d, (e) wine: V30- Mac-10d

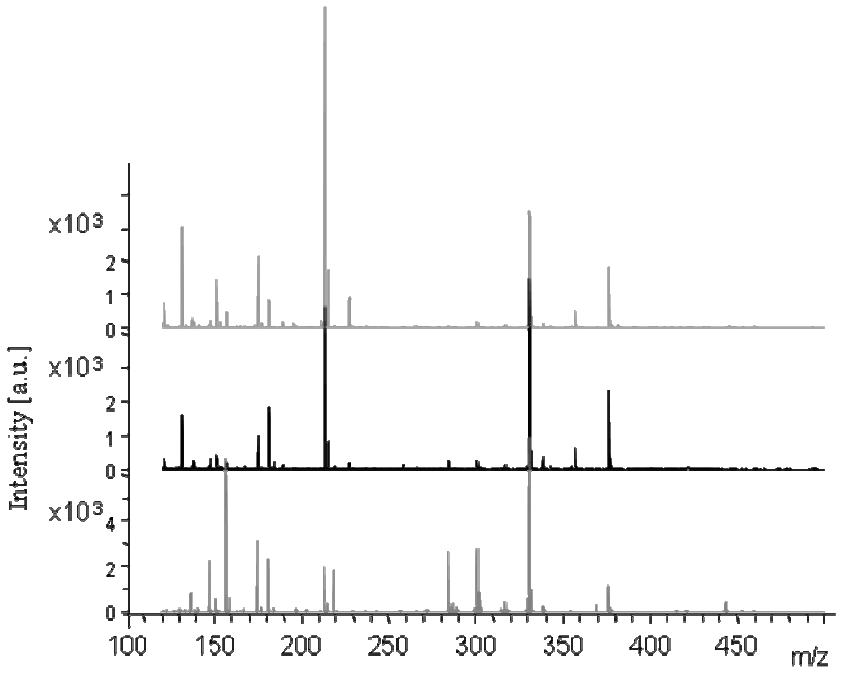


Fig 22. MALDI-TOF-MS „fingerprints" of the analyzed wines and grapes: (A) Merlot wine, M30-Mac-6d (B) Merlot wine, M30-Mac-10d (C) Merlot grape skin extract, applying C70 fulleren "sandwich" for ionization

In our research, best results were obtained applying the matrix 2,5-DHB, whereupon, most of the anthocyanins present in the samples were identified. However, the purpose of this study was to make an attempt for introducing a new

MALDI matrix for analysis of anthocyanins. Therefore, fullerene was tested as a matrix, and because of its high molecular mass, it was expected that peaks of fullerene will not interfere with the peaks of the sample compounds. Indeed, the molecular mass of fullerene is *m/z* 840, and the obtained peaks of this matrix were with very low intensity in the mass range of *m/z* 100 to 700, i.e. the mass of fullerene is outside of the mass range of the anthocyanins and can not influence their identification. The sandwich method was applied for anthocyanin analyses of wine and grape samples, with fullerene as a new matrix. Under those conditions, all five anthocyanins (delphinidin, cyanidin, petunidin, peonidin and malvidin) were identified only in their aglycone forms since applying higher laser energy for ionization, which is necessary for the fullerene, caused fragmentation of the glucosides.

On the basis of the obtained data from the investigation with MALDI, it can be concluded that for better identification of anthocyanin 3-glucosides, acetylglucosides and *p*-coumaroylgucosides, and for the other pigments present in the wine, further investigations are needed in order to test the effect of the derivatized and/or acidified fullerenes as possible matrices. The ability to use lower laser power is thus one advantage of these matrices. There are a number of other advantages that this new class of matrices possess: high analyte ionization efficiency, small molar ratios (less than 1) of matrix/analyte, and a broader optical absorption spectrum, which should obviate specific wavelength lasers for MALDI acquisitions (Ugarov, et al., 2004).

5. CONCLUSIONS

1) Spectrophotometric methods for determination of total phenolics, total flavonoids, total anthocyanins, total catechins, color intensity and hue were applied for analyses of red grape varieties (Vranec and Merlot) and white grape varieties (Smederevka and Chardonnay) which were used for wine production appplying different vinifications. It was noticed that skins and seeds of Merlot and Smederevka reached the maximal phenolic values when grape was at technological ripeness. Total flavonoids decreased from the veraison to the technological ripeness of the grape (except for the Chardonnay variety). Total catechin contents increased in skins, from veraison to the technological ripeness, and decreased in seeds. The contents of anthocyanins increased during ripening, reaching highest values at late harvested Vranec grapes and grapes in technological ripeness for Merlot grapes.

The red wines (Vranec and Merlot) were produced with two doses of SO_2, fermented with two yeasts, *Saccharomyces cerevisiae*, supplied from different manufacturers, Vinalco-Bitola and Levuline-Borodeaux. Wines were obtained with maceration of 3, 6 and 10 days, and the changes in the polyphenolic content were followed at four phases: after maceration, after 2, 6 and 16 months of storage in bottles. White wines were produced with two doses of SO_2 and fermented with the same two yeasts. From the obtained results it can be concluded that the concentrations of total phenolics and other subgrous (total flavonoids, total anthocyanins and total catechins) were changing during the storage period, i.e. decreased with the aging. Maceration time influenced the phenolics extraction from the grapes into the wine. Thus, for Vranec wines, highest concentrations of phenolic components were obtained in the wines produced with 6 days of maceration, except for the catechins which were present in highest amounts in the wines macerated for 10 days. For the Merlot wines, 10 days of maceration leaded to highest extraction of polyphenols. Influence of SO_2 and yeast was not considerable even though higher concentrations of phenolics were noticed for the wines with higher amount of sulphur dioxide.

2) HPLC-DAD-MS analysis was performed for simultaneous identification of the phenolic components from the different groups. It was observed that acidifying the samples at pH 1 and their storage for 30 min to equilibrate is sufficient to convert all anthocyanins to their flavylium cations and makes it possible to carry out the elution with solvents containing only 1 % formic acid in the mobile phase, which is also compatible with ESI-MS detection of phenolic acids. The stability of the other compounds was tested by storage of the wine samples at room temperature for 30 min, 2 h and 3.5 h compared with the initial conditions after immediate injection, and no degradation was observed for any of the

phenolic compounds. The optimized conditions including previous acidification of wine samples and HPLC conditions for elution provided successful MS identification of 68 phenolic components from different gropus of in wines. Fragmentation ions of the trimer (epi)catechin-(epi)catechin-malvidin-3-glucoside were reported for the first time in this study.

Quantification of the phenolic components was performed by direct injection of the wines into the HPLC system, without filtration. It was noticed that caftaric acid was the dominant hydroxycinnamic acid derivatives in both red wines (Vranec and Merlot), as well as for Chardonnay wines too, while, for Smederevka wines, *trans*-coutaric acid was dominant component. In the group of catechins, highest concentrations were measured for procyanidin B2, while, malvidin 3-glucoside was the dominant component for the group of anthocyanins and pigments, for the wines from both red varieties.

For determination of proanthocyanidins, terminal and extension subunits, as well as for determination of the mean degree of polymerization of red wines, the sample preparation was performed with recovering with methanol after the evaporation of the wine, and than, acid depolymerization was performed using phloroglucinol. After the phloroglucinolysis, the samples were anaysed with HPLC-MS for identification, and HPLC-DAD-FLD for quantification of the terminal and extension monomers and phloroglucinol adducts. It was noticed that the total concentrations of proanthocyanidins, catechin and epicatechin were highest for the wines obtained with maceration of 10 days, while the values for the mean degree of polymerization were close for the wines macerated for 6 and 10 days.

3) Rapid and simple MALDI-TOF-MS method was used for identification of anthocyanins, testing different matrices:

- ✓ α-cyano-4-hydroxycinnamic acid (CHCA);
- ✓ Sinapic acid (SA);
- ✓ 2,5-dihydroxybenzoic acid (2,5-DHB), и
- ✓ C70 fulleren

The MALDI-TOF-MS spectra of the wine and grapes samples, using 2,5-DHB matrix for ionization, confirmed the presence of the dominant colored component in wine and grapes, malvidin and its derivatives: malvidin-3-glucoside, malvidin-3-acetylglucoside and malvidin-3-*p*-coumaroylglucoside. Grape extracts and wine samples were analyzed without additional purification confirming the ability of MALDI-TOF-MS to analyze crude samples. Fullerene was used for the first time as a matrix for MALDI-TOF-MS analysis of anthocyanins in wine and grape, detecting the anthocyanins in their aglycon form, since the high ionization energy needed for fullerene, was applied and caused fragmentation of the molecules. The "sandwich" method was applied, appointed as suitable for wine and grape analyses. Further investigations should be performed for testing of the effect of the derivatized fullerenes in order to find appropriate substance as a matrix for MALDI TOF MS wine analysis.

6. REFERENCES

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List of publications from the PhD Thesis

I. Publications published in journals with impact factor:

1. **Ivanova V**., Vojnoski B., Stefova M. (2012). Effect of winemaking treatment and wine aging on phenolic content in Vranec wines, DOI: 10.1007/s13197-011-0279-2, *Journal of Food Science and Technology*, 49(2) 161-172, (**IF=1.123**).
2. **Ivanova V**., Stefova M., Vojnoski B., Dörnyei Á., Márk L., Dimovska V., Stafilov T., Kilár F. (2011). Identification of polyphenolic compounds in red and white grape varieties grown in R. Macedonia and changes of their content during ripening, *Food Research International*, DOI:10.1016/J.FOODRES.2011.06.046, 44, 2851-2869, 44 (9) 2851–2860, (**IF=2.416**).
3. **Ivanova V**., Vojnoski B., Stefova M. (2011). Effect of the winemaking practices and aging on phenolic content of Smederevka and Chardonnay wines, DOI: 10.1007/s11947-011-0566-y, *Food and Bioprocess Technology*, 4(8) 1512-1518, (**IF=3.576**).
4. **Ivanova V**., Dörnyei Á, Stefova M., Stafilov T., Vojnoski B., Kilár B., Márk L. (2011). Rapid MALDI-TOF-MS Detection of Anthocyanins in Wine and Grape Using Different Matrices. *Food Analytical Methods* 4, 108-115, (**IF=1.400**), DOI: 10.1007/s12161-010-9143-7.
5. **Ivanova V**., Dörnyei Á, Márk L., Vojnoski B., Stafilov T., Stefova M., Kilár F. (2011). Polyphenolic content of Vranec wines produced by different vinification conditions, *Food Chemistry,* 124(1) 316-325, (**IF=3.146**), 2011.
6. **Ivanova V**., Stefova M., Chinnici F. (2010). Determination of polyphenol contents in Macedonian grapes and wines assessed by standardized spectrophotometric methods*. Journal of the Serbian Chemical Society,* 75:45-59, (**IF=0.820**).
7. **Ivanova V**., Stefova M., Vojnoski B. (2009). Assay of the phenolic profile of Merlot wines from Macedonia: effect of maceration time, storage, SO_2 and temperature of storage. *Macedonian Journal of Chemistry and Chemical Engineering*, 28, 141-149 , (**IF=0.200**).

II. Publications presented at scientific manifestations:

1. **Ivanova V.**, Stefova M., Vojnoski B., Beleski K., Dimovska V. Phenolic content of Vranec grapes during ripening, XXII Congress of Chemists and Technologists of Macedonia, 5-9 September, Ohrid, Macedonia, 2012, p. 137, **poster**.
2. **Ivanova V**., Stefova M., Stafilov T., Hermosín-Gutiérrez I., Phenolic composition of red wines from Republic of Macedonia, IX Studentski kongres na hemicari i tehnolozi na Makedonija so megunarodno ucestvo, Skopje, 2011, **plenary lecture.**
3. **Ivanova V**., Boros B., Hermosín-Gutiérrez I., Stefova M., Stafilov T., Vojnoski B., Dimovska V., Dörnyei Á., Kilár F., Phenolic composition, colour and antioxidant activity of Vranec, Merlot and Cabernet Sauvignon wines from R. Macedonia, International Symposium and Summer School on Bioanalysis, Graz, Austria, 2011, **oral lecture**.
4. **Ivanova V.**, Meudec E., Souquet J-M., Vojsnoski B., Cheynier V., Stefova M., HPLC analysis of hydroxycinnamic acid derivatives in Smederevka and Chardonnay wines, XXI Congress of Chemists and Technologists of Macedonia, Ohrid, p. 126, 2010, **poster**.
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