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From the Autochthonous Grape Varieties of the Kastav Region (Croatia) to the Belica Wine

Running head: Characterization of Autochthonous Grapes of the Kastav Region and the Corresponding Wine

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SUMMARY

Research background. Coastal region of Croatia is rich in autochthonous grape varieties. Many of them have been almost abandoned such as the autochthonous varieties of Kastav (Croatia), used for the production of the Kastavska Belica wine. Therefore, the rationale of the presented study was to characterize autochthonous varieties 'Verdić', 'Mejsko belo', 'Jarbola', 'Divjaka' and 'Brajkovac' grape varieties. In addition, we performed a molecular characterization of the corresponding Belica wines.

Experimental approach. Firstly, for five autochthons grape varieties, their genetic origin and ampelographic and economic characteristics were determined. Standard physico-chemical profiles and phenolic components by use of liquid chromatography coupled to triple quadrupole mass

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spectrometer (LC-QQQ) were determined for 12 corresponding wines from different producers. Fourier-transform infrared spectroscopy (FTIR) was used for determination of standard physico-chemical parameters

Results and conclusions. Ampelographic analysis, which includes the data on producing characteristics and cluster and berry composition of varieties, revealed significant differences between analysed grape varieties. Results of physico-chemical analysis of the Belica wine showed that all wines met the requirements needed for the production of quality and top quality wines labelled with protected designation of origin (PDO) in Croatian coastal region. The LC-QQQ analysis confirmed the presence of different phenolic components in the Belica wines, where the most prominent phenols were flavonoids from the flavan-3-ol group. Overall, these results showed that autochthonous grapes from the Kastav region can be used for production of wines with added market value due to a growing demand for autochthonous products on the global market.

Novelty and scientific contribution. The presented results represent scientific insight and a basis for further determination of the optimal cultivation technology aimed to take advantage of the best characteristics of each variety for production of a wine with desirable features.

Key words: Belica wine; autochthonous wines; autochthonous grape varieties; polyphenols; FTIR

INTRODUCTION

A significant number of grape varieties deserve revitalization, due to their varietal characteristics that may also include resilient properties in the context of climate changes. In addition, the global market recognizes typical and autochthonous products, such as wines of indigenous grape varieties, often through high prices. In particular, EU recognized the importance of traditional products sector, not only as a way to strengthen the local economy, but also as a way to generally develop a sustainability system (*1*).

Croatia is rich in indigenous grape assortment. Unfortunately, many of them are still neglected or scientifically uncharacterized. Successful example of a revitalized, nearly forgotten variety, is 'Tribidrag' (*syn*. 'Crljenak kaštelanski'/'Primitivo'/'Zinfandel'/'Kratošija'), for which only 22 wines were found (*2*) near Kaštela. After revelation of its true identity, links to Plavac mali and eastern Adriatic origin (*2*), the renaissance and the resurgence of its production in Croatia began and increased demand for its planting material has been documented (*3*).

'Malvazija istarska', 'Mejsko belo', 'Divjaka', and 'Jarbola' are also unique varieties, grown exclusively in Croatia, whilst synonyms of 'Verdić' are spread over the wider area of the Northern Adriatic coast (*4*) under names of 'Teran bijeli' (*5*), Glera', 'Prosecco' (*6*) and 'Beli Teran' (*7*) in Slovenia and 'Prosecco tondo' (*8*) in Italy. Apart in vineyards, they are also conserved in the National Collection of Autochthonous Varieties at the University of Zagreb, Faculty of Agriculture (AGR). However, that is not the case with 'Brajkovac', a variety mentioned back in 1853. (*9*) whose varietal status in terms of its uniqueness and possible synonyms/homonyms has not been evaluated before.

With these background data as a rationale for our study, we set the unique interdisciplinary approach for characterization of the autochthonous grape varieties and the corresponding wine. We tested our approach on the autochthonous varieties of the Kastav region and corresponding Belica wine. Belica wine is a mixture of 'Mejsko belo', 'Verdić', 'Divjaka', 'Jarbola', and 'Brajkovac' grape varieties and belongs to the group of wine made from neglected and somewhat endangered varieties. Some of the varieties indeed, have been on the verge of extinction. The 'Brajkovac' variety occurs sporadically in some older vineyards and is used in a small percentage in Belica wine. The 'Jarbola' variety is also less present. Varieties 'Divjaka' and 'Mejsko belo' are very important for production of Belica wines and can be found exclusively in the vineyards of the Kastav region.

In this interdisciplinary approach the (a) ampelographic and economic analysis of varieties that can usually be found in the Belica wine, (b) genetic background of tested varieties and (c) molecular components of the corresponding Belica wine will be determined. Standard wine chemical parameters were evaluated as well. The Fourier-transform infrared spectroscopy (FTIR) analysis was done as a confirmation of the results along with major phenolic compounds evaluation by use of liquid chromatography coupled to triple-quadrupole mass spectrometry (LC-QQQ).

MATERIALS AND METHODS

Samples

The ampelographic and genetic research was conducted in the fall of 2017 at the time of harvest. For wine analysis, total of 12 Belica wines (year 2017) were obtained from the local producers of the Kastav area (Croatia) (Fig. S1). The total number of wines ranges from 108-2050 pieces per vineyard of an individual producer. All producers use selected yeast (EC 1118, Saccharomyces cerevisiae) for the Belica wine production. The total area of vineyards and number of wines on which these twelve producers produce grapes for Belica wine is 13442 m² and 7285 pieces, respectively. The total annual production of Belica wine from these twelve producers is 6470 L. The predominant grape growing system (cultivation form) is single-legged or double-legged Guyot and the substrates are Kober 5BB and SO4. The maximum yield per hectare for quality wines is 12,000 kg or 8,400 L of wine per hectare, or for premium wines 11,000 kg or 6,600 L of wine per hectare. Since each producer has different proportions of varieties in the vineyard and no standard guideline has been agreed so far, it was not possible to determine the exact share of each variety in the Belica wine.

Ampelographic and economic analysis of the varieties

Ampelographic analysis include many parameters for grape variety characterization (morphology, phoenology, production characteristic, *etc*.). This study includes only parameters important for producing characteristics of varieties. In time of the harvest five healthy and vigorous vines were selected. Total yield and number of clusters were measured by picking, numbering and weighing of clusters for each vine. From the total mass of grapes, ten clusters were randomly sampled for further ampelographic analysis for cluster and berry composition. Cluster composition analysis encompass measuring dimensions and mass of cluster according to Maletic *et al.* (*10*) and basic chemical composition of must. Berry composition parameters were chosen according to research of Rustioni *et al*. (*11*). A total of thirteen berries samples were included for each variety. Berry composition analysis encompassed measurement of dimensions and mass of berry and skin and seed mass. Skin and seed mass of each sample was crushed, skins and seeds were placed on papers. After drying on room temperature for two weeks, seeds and skins were finally weighted.

Dimensions of clusters and berries were measured on graph paper. Mass of clusters and berries samples were measured with precision laboratory balance (Radwag PS 4500.R2.M, Radom, Poland). The basic chemical analysis of must comprised analysis of sugar content (Brix scale, Atago 2352 MASTER-53T refractometer, Tokyo, Japan), total acid content (g/L expressed as tartaric acid equivalents) and pH value (Schott Model Lab 850 pH meter, Mainz, Germany) according to OIV international standards (*12*). Those three parameters represent most important quality parameters in wine production. Data obtained by weighing berries and berries' parts were used to calculate mass of flash and share of skin and seeds in berry composition. Parameter of yield per vine was used to calculate economical value of grape production. First, grape production is expressed by yield per hectare and multiplied with average price for one kilogram of grape. Economic analysis is expressed by total revenue, which included variable costs and gross margin.

Genetic analysis

For variety identification and confirmation, eight vines were sampled. DNA extraction was performed using the peqGOLD Plant DNA Mini Kit (PEQLAB Biotechnologie GmbH, Erlangen, Germany) according to the manufacturer's instructions. Nine microsatellite (SSR) primers recommended for routine variety distinction of grapevine were used (*13*). PCR amplifications were carried out in a VeritiTM Thermal Cycler (Applied Biosystems, Foster City, California, USA). List and information about primers used, as well as detailed information on multiplex PCR reactions performed, are described in Žulj Mihaljević *et al.* (*4*). Amplified products were separated using an ABI3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) with GeneScan- 500 LIZ™ size standard. Sizing of the fragments was performed using GeneMapper 4.0 software (*13*). Obtained SSR profiles were compared to internal microsatellite database comprising profiles on 9 common loci from European *Vitis* database (*14)* as well as published SSR profiles from various other research (*4*). Data standardization and comparison were conducted as described previously (*4*).

Reagents and materials

l(+) potassium sodium tartarate tetrahydrate was obtained from VWR Chemicals (Vienna, Austria). Potassium iodide was obtained from BDH Prolabo Chemicals (Leuven, Belgium). Iodine, sodium hydroxide (1M and 0.1M), sodium thiosuplhate (0.1M) and sodium hydroxide pellets were obtained from Gram-mol (Zagreb, Croatia). Sulphuric acid (96 %), starch (soluble, p.a. indicator) and phenolphthalein indicator were obtained from Kemika (Zagreb, Croatia). Bromothymol blue indicator was obtained from Merck (Darmstadt, Germany). (+)-catechin, (-)-epicatechin, 2,5-dihydroxybenzoic acid (2,5-DHBA), 3,4-dihydroxybenzoic acid (3,4-DHBA), 3-hydroxytyrosol, caffeic acid, ellagic acid, quercetin, naringenin, luteolin-7-O-glucoside, pinobanksin, *p*-coumaric acid and syringic acid were obtained from Sigma Aldrich (St. Louis, MO, USA). Gallic acid was obtained from Alfa Aesar (Thermo Fischer Scientific, Tewksbury, Massachusetts, USA). Ferulic acid and resveratrol were obtained from Extrasynthese (Genay, France). Honeywell research chemicals (Charlotte, North Carolina, USA) supplied ethanol (HPLC grade) and acetonitrile (LC-MS grade). Sigma Aldrich (St. Louis, MO, USA) supplied formic acid (LC-MS grade), ultrapure water (LC-MS grade) and ethanol (96 %).

Standard wine analysis

Standard chemical parameters determined for the Belica wine samples were as follows: alcohol content, reducing sugars, ash content, pH, total titratable acids, volatile acidity and free and total sulphur dioxide. Total alcohol content was determined by use of the electric Ebulliometer (Exacta+Optech Labcenter S.p.A.,S.Prospero, Modena, Italy) (*15*). For the determination of pH (OIV-MA-AS313-15:R2011), pH meter (Lab 860, SI Analytics GmbH, Mainz, Germany) was used (*16*). The total acidity in the samples was determined according to modified OIV-MA-AS313-01:R2015 method (*17*). The only modification in method was usage of the ultrasonic bath for the elimination of carbon dioxide from wines instead of a vacuum flask and water pump. The results are expressed as tartaric acid equivalents. The determination of reducing sugars in wine samples was carried out according to the method developed by H. Rebelein (*18*). This is shortened iodometrical method based on the difference of consumption rates of sodium thiosulphate on the titration of the copper cation. The volatile acid in the samples (expressed as acetic acid equivalents) was determined according to the modified OIV-MA-AS313-02:R2015 method (*19*). The only modification in method was usage of the ultrasonic bath for the elimination of carbon dioxide from wines instead of a vacuum flask and water pump. The ash in the wine samples was determined according to the OIV-MA-AS2-04:R2009 method (*20*). Free and total sulphur dioxide were determined by the titration with standard solution of iodine. Methods were developed according to the OIV-MA-AS323-04B:R2009 (*21*) method and the rapid method by the Ripper, M (*22*). For free SO_2 determination, 5 ml of diluted H_2SO4 (1:3) with a 2 ml of 1% starch was added to 50 ml of the sample and titrated with 0.01 M solution of J_2 until a blue color appeared. The titration consumption was multiplied by a factor of 12.8 and the results are expressed as the mg/L of free SO_2 in the sample. For total SO_2 determination, 25 ml of 1 M soluble NaOH was added to the 50 mL of the sample, left to stand for 15 minutes. Afterwards, 10 ml of diluted H_2SO4 (1:3) and 2 ml of 1 % starch were added and titrated with 0.01 M J_2 solution until a blue color appeared. The titration consumption was multiplied by a factor of 12.8 and the results are expressed as the mg/L of total SO_2 in the sample.

Fourier-transform infrared spectroscopy (FTIR) analysis

Infrared spectral measurements were performed using WineScan^{TM} FTIR spectrometer (FOSS, Hillerød, Denmark) within mid-IR (1000-5000 cm^{-1} wavenumber) range. The samples used were directly collected from the bottle without any kind of pre-treatment. Calibrations that are part of the WineScan^{TM} FTIR allowed simultaneous analysis of major wine quality parameters.

LC-QQQ analysis

Wine samples were diluted two times with 10 % ethanol solution, filtrated with Chromafil cellulose acetate microfilters (0.45 µm, 25 mm) (Macherey-Nagel, Düren, Germany) and analysed.

LC-QQQ analysis was obtained by use of an Agilent 1260 series HPLC chromatograph equipped with a degasser, binary pump, auto-sampler and column oven coupled to an Agilent 6460 triple quadrupole mass spectrometer equipped with jet stream electrospray (AJS ESI) source (Agilent Technologies, Palo Alto, CA, USA). For chromatographic separation, Zorbax SB-C18, Rapid resolution HT, 600 bar column (2.1 mm x 50 mm I.D, 1.8 µm, Agilent Technologies, Palo Alto, CA, USA) was used. The mobile phases were (A) 0.1 % formic acid in LC-MS grade water and (B) 0.1 % formic acid in acetonitrile. Details of the method used for quantification of flavonoids and phenolic acids are described in our previous publication (*23*). Parameters of calibration curves of the analysed phenolic compounds (linearity, limit of detection (LOD), limit of quantification (LOQ) and coefficient of determination (R^2) used for quantification of phenolic compounds are given in Table S1.

Statistical analysis

The obtained data was statistically processed by using the statistical software SAS System Software, v. 9.3. (SAS Institute Inc., Cary, NC, USA, 2012) (*24*). Statistical analysis included descriptive statistics (average, minimum and maximum value), analysis of variance (One-way ANOVA) and comparison of mean values (Duncan's multiple-range test). Principal component analysis (PCA) was constructed by use of Phyton programming software. Implementation in the Python library Scikit-learn version 0.20.3 (*25*) was used for both classifiers.

RESULTS AND DISCUSSION

Ampelographic analysis of varieties

All analysed varieties are white skin varieties with specific morphological characteristics. One-way ANOVA for producing characteristics showed significant differences in a most of parameters except of cluster mass, skin mass and share of skin and seeds (Table 1). 'Verdić' had the largest cluster by dimensions (average length 187.56±232.10 mm, average width 115.98±15.34 mm), while the 'Jarbola' had smaller clusters by dimension (average length 125.61±22.07 mm, average width 79.4±14.28 mm) (Table 2). Even though cluster mass was not significant, the ANOVA comparison of the mean value has shown that a difference between varieties exists. Greatest cluster mass was obtained for "Mejsko belo" and the smallest one also "Jarbola". Our analysis of the berry composition showed that "Mejsko belo" had the largest berry (16.61±1.56 mm average length and 14.94±1.96 mm average width) while "Jarbola had the smallest one (13.9±1.32 mm average length and 12.51±1.24 mm average width) (Table 3). Variety 'Verdić' had also the highest values for majority of other parameters: berry mass, flesh, skin and seed mass, and share of skin. All average values of analysed varieties are very close to average values of 22383 data from analysed samples in the study of Rustioni *et al*. (*11*). For example, the value of berry length/width ratio for "Verdić" and Brajkovac" are the same as the average in the mentioned study. In this parameter, varieties "Mejsko belo" and "Brajkovac" have the highest value, which confirms characteristic morphological ovoid shape of the berry.

Considering the results on producing characteristics of varieties, these were also varying (Table 4). According to the cluster and berry analysis, 'Verdić' shown to be the variety of large cluster, due to lower load (ten bunches per vine) and yield per vine (2.66 kg) and the best quality of must. Additionally, 'Verdić' variety together with the 'Mejsko belo' variety showed the highest sugar content (17.4 °Brix) and thus can be confirmed as the variety with the best qualitative potential. Total acid concentration is very important quality parameter because it affects biochemical stability and organoleptic character of wine. Low acid concentration in must (3 to 5 g/L like in 'Verdić' and 'Mejsko belo') is thankless by technological aspect and means artificial acidification of wine.

Varieties 'Brajkovac' and 'Divjaka' in the observed year had the highest number of clusters and the highest yield per vine, over 3 kg. Such a high yield also affected the quality of the must, so both varieties had the lowest concentration of sugar and the highest concentration of total acids (Table 4). On the other hand, high concentration of total acid, especially in 'Divjaka' had a great variety potential for natural correction of acid concentration in 'Belica wine' as a variety blend wine. In order to test the potential grouping of the samples and get deep insight into the differences between Belica wines, we performed PCA analysis (Fig. 1). Figs. 1a and 1b shows the PCA projection of all analysed varieties and their potential grouping in the space of major components. The first two components (PC1 and PC2) describe 97.4 % of the total variability. These results confirm a large difference between grape varieties in terms of standard chemical parameters

Overall, from a technological point of view, more attention should be put in the future to the reduction of yields for all varieties in order to achieve a higher concentration of sugar in the must but keep optimal acid concentration. Yield reduction in all varieties should be achieved by a combination of stronger pruning to maturity (leaving a smaller number of buds) and subsequent thinning of the clusters (after flowering or before the beginning of the pattern) and early defoliation. In varieties that show higher yield potential in different years of research, yield reduction can only be achieved by stronger pruning in the spring. The results showed better insight into the agrobiological properties of these varieties, unexplored so far. However, we need to emphasize that this is a one-year study, and that results were expectedly slightly different for every year. Therefore, for the final confirmation of the obtained results multi-year research should be carried out in the above environmental conditions. The analysed varieties differ in their production characteristics (yields per vine and hectare), but also in economic characteristics (sales price, revenue and gross margin). The relationship between yield and selling price shows the highest profitability of growing varieties Brajkovac and Divjaka. Despite the relatively high yield of the variety Mejsko belo, it achieves lower revenues and coverage contribution due to the lowest selling price.

It can be assumed that the market design (branding) of Kastav Belica wine will contribute to the increase of the total income of wines of a mixture of autochthonous varieties, as well as individual varieties wines.

Genetic analysis

Belica is a blend (cuvee - mixture) of several grape varieties. The following grape varieties can be found in Belica, most of which are found only in the Kastav region: 'Mejsko belo', 'Verdić', 'Divjaka', 'Jarbola', 'Malvazija istarska' and 'Brajkovac'. Among them, four (Mejsko belo, Verdić, Istrian Malvasia and Divjaka) are the most represented. 'Malvazija istarska' was not analysed in this paper, as it is not autochthonous variety of Kastav area, but of Istrian peninsula. All eight accessions were successfully amplified (Table 5). As expected, true to typness of 'Mejsko belo', 'Verdić', 'Divjaka', 'Jarbola' was confirmed after comparison with internal, previously harmonized SSR database (*4*) containing more than 2000 nonredundant grape genotypes. Four vines were sampled and assumed under the name 'Brajkovac', however three different genetic profiles were obtained. Accessions labelled RAJ_ORIG1 and BRAJ_ORIG2 showed to be identical to 'Duranija' and 'Mejsko bijelo', respectively. Nevertheless, two accessions (BRAJ A and BRAJ B) showed identical profiles on all 9 SSR markers analysed. The obtained genetic profile did not match any other previously known variety/genotype from SSR database nor has this name appeared in foreign literature thus confirming the unique status of this variety. Those two accessions were further considered as true 'Brajkovac' and were subject of further analysis. This result enables next conservational steps needed for preservation of this variety, like its inclusion in AGR collection and further propagation.

Belica wine analysis

According to physico-chemical parameters, all analysed Belica wines, meet the requirements for quality wine produced in PDO (protected designation of origin) on the Croatian coast (Table 6). FTIR analysis confirmed these results (Table S2). Particularly, the alcohol levels in Belica wine samples were between 11.4 and 13.5 % (Table 6). As for the sugar content, the results show that all the analysed samples are within the limits of dry wine production (1.05-3.89). Ash content was in the range 0.8 to 1.9 g/L. According to regulation established for PDO Croatian Coastal region (*26,27*), the minimum amount of ash for white wines in the category of quality wine with controlled geographical origin is 1.4 g/L. From the obtained results, it is evident that all, except one (sample B10) analysed wine meet the given criteria. Results of the analysis of total acids in wine showed that all the analysed samples are above the minimum legal limit of wine production (3.5 g/L), whereas the lowest measured value of total acid in Belica wine samples was 4.50 g/L. Furthermore, the upper limit for the volatile acid concentrations was 1.1 g/L. It is evident that all analysed wines meet the given criteria. Results of the concentration of the free sulphur in wine showed that in samples no.1-4, 6 and 12-13, the measured amount of free sulphur was too low, and the wines were in a state of oxidation. After the analysis of total sulphur in wine, all relevant samples of Belica wine complied with the regulation on wine, whereas the upper limit for white wines and rosé wines are 200 mg/L of free SO_2 (Table 6). The PCA projection of the standard physico-chemical parameters (Figs. 1c and 1d) has shown moderate variability among Belica wine samples. The first two components (PC1 and PC2) describe 61.6 % of the total variability. These results show that a more uniform production of Belica wine should be pursued.

During the winemaking process, a number of chemical modifications that occur significantly affect the final phenolic profile of the wine. For example, grape ripeness, processing methods and environmental factors. Therefore, a systematic quantitative analysis of phenolic components in wine can provide reliable data on their quantity and type. During the handling and ripening of the grapes, the composition of the polyphenols in the wine may change. Phenolic analysis is therefore crucial to draw conclusions about the winemaking process, as well as about the final wine quality.

Phenolic content in Belica wines was analysed by use of LC-QQQ. The results showed that the most common groups of polyphenols are hydroxycinnamic acids and flavan-3-ols, which is in line with previous research on white wines (*28–30*). Among all hydroxycinnamic acids, caffeic acid was present at highest concentrations ranging from (0.75±0.44) to (9.12±0.10) mg/L (Table 7). Lukić *et al*. conducted qualitative research of phenols in different white wines and reported different trends (*31*). Namely, in their research, the phenolic acid with the highest concentration was the gallic acid, with concentrations up to (16.68±15.30) mg/L in Muscat Blanc wine. Our results report significantly lower concentrations for this phenolic acid in analysed samples ((0.39±0.00) to (1.93±1.11) mg/L). However, results of the caffeic acid and ferulic acid concentrations were similar. Rochetti *et al.* reported lower concentrations of caffeic acid in Chardonnay wines (up to (0.26±0.15) mg/L), that are more in the line with our results (*32*). Along with ferulic acid, their results of syringic acid and ferulic acid concentrations were also in the line with our data.

As for flavan-3-ols, catechins were found in the highest concentrations, whereas (+)catechin was dominant ((4.38±0.11) to (17.02±2.25) mg/L) (Table 7). It is the catechins that are responsible for the bitterness in wine (*33*-*35*) but also for wine health properties (*36*). In addition to sensory properties, they are also important as antioxidants, *i.e.* factors that protect wine from oxidation during maturation (*37*-*38*). These results are in line with work from Lukić *et al.* that reported concentrations of epicatechin and catechin in white wines in the range from (3.10±2.60) to (17.92±13.10) and from (1.51±1.82) to (3.54±2.99), respectively (*31*). Rochetti *et al.* reported lower concentrations of catechin in Chardonnay wines, ranging from (1.19±0.49) to (6.81±2.53) mg/L. In addition, we also reported higher hydroxytyrosol content in Belica wines, up to (2.26±0.31) mg/L, while the highest observed result for Chardonnay wines was 0.87±0.17 mg/L (*32*).

Figs. 1e and 1f shows the PCA projection of LC-QQQ quantitative phenolic analysis where all analysed phenols were used as variables. The first two components describe a 47.3 % of the total variability. Although most wine samples are grouped centrally, samples B5, B6, B10, and B12 contribute to a larger variability of the system. Large differences may be observed in single polyphenol concentrations among samples, showing a need for development of a more uniform method of Belica wine production.

CONCLUSIONS

The tested interdisciplinary approach for characterization of chosen autochthonous grape varieties and corresponding wine samples proved useful in assessment of important parameters for branding and quality assessment. Analyses of ampelographic characteristics of 'Verdić', 'Mejsko belo', 'Jarbola', 'Divjaka' and 'Brajkovac' varieties used for production of the Belica wine in the region of Kastav (Croatia), showed high genetic variability between confirmed grape varieties. Confirmed variability certainly impacts the productivity and economical aspect of the Belica wine production. Results of this study are crucial in determination of an optimal cultivation technology as its required by modern trends in grape and wine production. Microsatellite genetic profile and its uniqueness was confirmed the new variety 'Brajkovac'. Knowledge on standard wine characteristics coupled to molecular analyses may be used to evaluate best characteristics of each variety and establish production of a wine with desirable characteristics. Recording and monitoring of typical molecular composition of wine through different years, often called 'molecular profiling' may be an important tool for standardization and/or monitoring of the technological process of Belica wine production, protection and branding of this autochthonous product. Current global market trends indeed, emphasize local, specific and autochthonous products, increasing the demand for such products. Currently, there are large differences in the production characteristics of tested varieties, which is determined by differences in yield and selling prices. Further work on branding the Kastavska Belica wine brand might increase this specific wine quality and provide benefits to the producers.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

SUPPLEMENTARY MATERIALS

All supplementary materials are available at www.ftb.com.hr

AUTHORS' CONTRIBUTION

TP, LSM and SKP manuscript drafting and literature search; SKP, TP and EM conceived and designed the study; TP preformed standard wine analysis and FTIR analysis; ŽP and LSM preformed MS analysis; EM conceived the original idea and supervised the ampelographic analysis; DS and ZA planned and carried out ampelographic analysis and statistical analysis. MZM performed DNA analysis and interpretation; ZG and TP economic analysis; SKP secured part of the project funding and performed final write-up and revision of the manuscript.

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Table 1. Results of One-way ANOVA for cluster and berry composition parameters

Parameter	Pr > F(Model)	Significant
l(cluster length)/(mm)	<0.0001	Yes
l(cluster width)/(mm)	0.004	Yes
m(cluster mass)/(g)	0.176	No
l(berry length)/(mm)	<0.0001	Yes
l(berry width)/(mm)	<0.0001	Yes
Length/width	0.005	Yes
m(berry mass)/(g)	0.005	Yes
m(flesh mass)/(g)	0.011	Yes
m(skin mass)/(g)	0.440	No
m(seed mass)/(g)	0.004	Yes
w(skin)/%	0.794	No
w(seed)/%	0.075	No

Table 2. Results of descriptive statistics (mean values with standard deviation) and comparison of mean values (Duncan's multiple-range test) for cluster parameters of five autochthonous grape varieties of Kastav region*

Grape variety	*l*(cluster length)/(mm)	*l*(cluster width)/(mm)	*m*(cluster mass)/(g)
Verdić	187.56±23.10 [a**]	115.97±15.34 [a]	261.55±95.76 [ab]
Mejsko belo	164.0±32.53 [b]	112.71±30.79 [a]	279.91±117.85 [a]
Brajkovac	146.02±18 [bc]	104.21±31.56 [a]	233.34±72.85 [ab]
Divjaka	144.7±19.68 [bc]	88.05±20.7 [b]	237.38±87.88 [ab]
Jarbola	125.61±22.07 [c]	79.39±14.28 [c]	183.8±59.3 [b]

*Sample of 10 clusters. **The different letters show a statistically significant difference in mean values between varieties at $p < 0.05$ (Duncan's multiple range test)

Table 3. Results of descriptive statistics (mean values with standard deviation) and comparison of mean values (Duncan's multiple-range test) for berry dimension parameters of five autochthonous grape varieties of Kastav region*

Grape variety	*l*(berry length)/(mm)	*l*(berry width)/(mm)	Length/width	*m*(berry mass)/(g)	*m*(flesh mass)/(g)	*m*(skin mass)/(g)	*m*(seed mass)/(g)	*w*(skin)/%	*w*(seed)/%
Verdić	15.55±1.5 [b*]	14.76±1.41[a]	1.05±0.09 [b]	63.32±9.06 [a**]	59.21±9.56 [a]	2.67±0.83 [a]	1.43±0.17 [a]	4.34±1.6 [a]	2.30±0.51 [ab]
Mejsko belo	16.61±1.56 [a]	14.94±1.96 [a]	1.12±0.1[a]	62.09±2.35 [a]	58.88±1.83 [a]	2.14±0.89 [a]	1.06±0.07 [b]	3.42±1.37 [a]	1.72±0.17 [ab]
Brajkovac	13.96±1.51 [d]	13.16±1.26 [b]	1.06±0.10 [b]	56.05±1.71 [ab]	52.74±2.19 [ab]	1.91±0.27 [a]	1.39±0.29 [ab]	3.42±0.57 [a]	2.49±0.6 [a]
Divjaka	14.71±1.44 [c]	13.52±1.56 [b]	1.09±0.09 [ab]	48.84±4.88 [b]	46.19±4.84 [b]	1.57±0.54 [a]	1.08±0.07 [b]	3.22±1.15 [a]	2.23±0.26 [ab]
Jarbola	13.89±1.32 [d]	12.51±1.24 [c]	1.11±0.12 [a]	47.07±0.29 [b]	44.42±0.7 [b]	1.93±0.74 [a]	0.71±0.17 [c]	4.11±1.59 [a]	1.51±0.37 [b]

*Sample of thirteen berry. **The different letters show a statistically significant difference in mean values between varieties at $p < 0.05$ (Duncan's multiple range test)

Table 4. Producing and economical characteristics of five autochthonous grape varieties of the Kastav region (Croatia)

Grape variety	Average number of bunches per vine	Total sugar/(°Brix)	γ(total acids)/(g/L)	pH	Average yield per vine/(kg)	Average price/(€ per kg)	Revenue/(€ per ha)	Variable costs/(€ per ha)	Gross margin/(€ per ha)
Divjaka	15.00	13.8	8.05	3.04	3.36	1.20	23889.60	5606.36	18283.24
Jarbola	12.20	15.8	5.27	3.05	2.47	0.93	13610.32	5150.84	8003.96
Mejsko belo	12.20	17.4	3.51	3.22	3.14	0.66	12278.97	5493.76	6672.61
Verdić	10.00	17.4	4.19	3.13	2.66	0.73	11505.17	5248.08	5898.81
Brajkovac	16.00	15.2	5.86	3.15	3.56	1.20	25311.60	5708.72	19705.24

Table 5. Microsatellite profiles and their genetic match on 9 SSR loci for eight analyzed accessions. Alleles are presented as base pairs

Accession name	VVS2		VVMD7		MD27		VrZAG62		VrZAG79		VVMD5		VVMD25		VVMD28		VVMD32		Matches
BRAJ_A	141	151	245	261	175	177	193	203	234	256	224	232	253	261	232	276	272	272	BrajA=BrajB
BRAJ_B	141	151	245	261	175	177	193	203	234	256	224	232	253	261	232	276	272	272	BrajA=BrajB
BRAJ_ORIG1	131	143	237	245	175	177	187	203	234	256	222	224	237	239	256	276	272	272	Duranija
BRAJ_ORIG2	141	143	237	245	175	177	187	193	234	256	222	224	239	253	247	256	272	272	Mejsko bijelo
Mejsko belo	141	143	237	245	175	177	187	193	234	256	222	224	239	253	247	256	272	272	Mejsko belo
Divjaka	141	149	245	261	175	177	193	203	234	256	224	232	253	261	232	276	272	272	Divjaka
Verdić	131	141	237	245	175	190	187	203	246	256	222	242	237	241	234	242	262	264	Verdić
Jarbola	141	153	245	247	175	177	201	203	240	256	232	237	241	253	234	244	250	272	Jarbola

Table 6. Results of standard wine analyses of Belica wine samples from the Kastav region (Croatia)

Wine sample	Alcoholic strength/(% vol)	γ(sugar)/(g/L)	γ(ash)/(g/L)	pH	γ(total acidity)/(g/L)	γ(volatile acidity)/(g/L)	γ(free SO_2)/(mg/L)	γ(total SO_2)/(mg/L)
B1	11.4	1.30	1.5	3.48	4.50	0.22	5	98
B2	13.5	1.20	1.8	3.13	5.70	0.28	6	66
B3	12.0	1.74	1.6	3.35	5.10	0.20	7	78
B4	12.5	1.09	1.8	3.30	5.77	0.20	4	56
B5	11.7	1.66	1.4	3.15	4.87	0.22	25	141
B6	12.2	1.47	1.5	3.19	5.02	0.25	6	133
B7	12.6	3.89	1.8	3.10	5.92	0.15	20	128
B8	12.1	3.15	1.9	2.97	7.12	0.25	32	190
B9	12.3	2.36	1.4	3.18	5.17	0.30	23	87
B10	11.9	3.06	0.8	2.98	6.82	0.20	20	106
B11	12.9	1.60	1.6	3.19	5.10	0.15	16	170
B12	12.9	1.05	1.5	3.22	4.57	0.24	6	95

Table 7. Mass fractions of specific phenolic acids and flavonoids in Belica wine samples obtained by use of LC-QQQ method

Wine sample	*w*/%															
	2,5-DHBA	3,4-DHBA	caffeic acid	ellagic acid	ferulic acid	gallic acid	*p*-coumaric acid	syringic acid	(+)-catechin	(-)-epicatechin	3-hydroxytyrosol	quercetin	luteolin-7-O-glucoside	naringenin	pinobanksin	resveratrol
B1	0.58±0.05	2.18±0.10	2.42±1.15	1.98±0.13	0.04±0.04	1.02±0.14	0.52±0.01	0.41±0.00	7.83±6.01	2.68±2.09	1.60±0.01	-	-	0.06±0.00	0.15±0.01	0.04±0.00
B2	0.32±0.02	1.03±0.15	0.75±0.44	0.13±0.02	0.05±0.01	0.39±0.00	0.11±0.06	0.40±0.01	9.70±0.03	1.22±0.77	0.49±0.01	0.03±0.00	0.04±0.03	0.05±0.01	0.20±0.01	0.04±0.01
B3	0.43±0.03	2.62±0.02	7.01±0.08	0.23±0.02	0.05±0.01	0.85±0.08	0.69±0.06	0.43±0.03	10.17±0.32	2.02±0.43	1.04±0.08	-	0.02±0.00	0.11±0.01	0.30±0.00	0.05±0.00
B4	0.46±0.01	1.53±0.02	8.21±0.03	0.27±0.17	0.05±0.00	0.64±0.03	0.72±0.49	-	8.64±1.99	3.72±0.01	1.89±0.10	0.01±0.00	0.01±0.00	0.07±0.00	0.19±0.00	0.04±0.00
B5	0.99±0.01	1.18±0.02	1.15±0.77	0.25±0.06	0.11±0.02	0.50±0.03	0.43±.032	0.45±0.00	6.36±4.21	1.39±0.71	1.20±0.17	-	0.04±0.00	0.12±0.01	0.28±0.04	0.04±0.00
B6	0.68±0.00	1.21±0.09	1.79±0.05	0.30±0.09	0.17±0.05	0.50±0.04	0.40±0.28	0.48±0.03	9.03±0.34	1.51±0.45	2.26±0.31	0.20±0.07	0.04±0.00	0.10±0.02	0.22±0.07	0.03±0.00
B7	0.34±0.03	0.73±0.05	3.97±0.21	0.10±0.01	0.10±0.01	0.41±0.01	0.64±0.01	0.22±0.31	11.33±0.04	3.38±0.03	0.62±0.16	-	0.02±0.00	0.05±0.01	0.21±0.02	0.04±0.00
B8	0.14±0.01	3.04±0.12	2.23±0.02	0.05±0.01	0.07±0.00	1.00±0.10	0.40±0.01	0.44±0.06	14.67±0.90	3.93±0.22	0.50±0.10	0.05±0.01	-	0.08±0.00	0.25±0.01	0.03±0.00
B9	0.25±0.01	1.17±0.01	1.29±0.00	0.20±0.07	0.07±0.01	0.45±0.04	0.20±0.02	0.21±0.30	7.88±0.58	1.82±0.51	0.45±0.12	-	0.03±0.00	0.04±0.00	0.10±0.01	0.03±0.01

B10	0.30±0.00	2.27±0.06	0.90±0.05	0.21±0.01	0.03±0.04	0.63±0.03	0.19±0.02	0.41±0.02	13.19±7.66	4.70±0.18	0.32±0.00	-	0.01±0.00	0.07±0.01	0.25±0.01	0.07±0.01
B11	0.21±0.00	1.11±0.01	1.15±0.05	0.02±0.00	0.09±0.02	0.49±0.02	0.49±0.02	0.40±0.00	17.02±2.25	2.81±0.32	0.54±0.05	0.01±0.00	0.04±0.01	0.07±0.00	0.18±0.03	0.02±0.00
B12	0.34±0.23	0.62±0.01	9.12±0.10	0.12±0.01	0.14±0.06	1.93±0.11	0.77±0.24	0.42±0.00	4.38±0.11	1.44±0.12	0.95±0.35	0.04±0.01	0.03±0.00	0.03±0.01	0.12±0.00	0.01±0.00

Results are expressed as a mean value of mass concentration γ/(mg/L) ± standard deviation (SD)

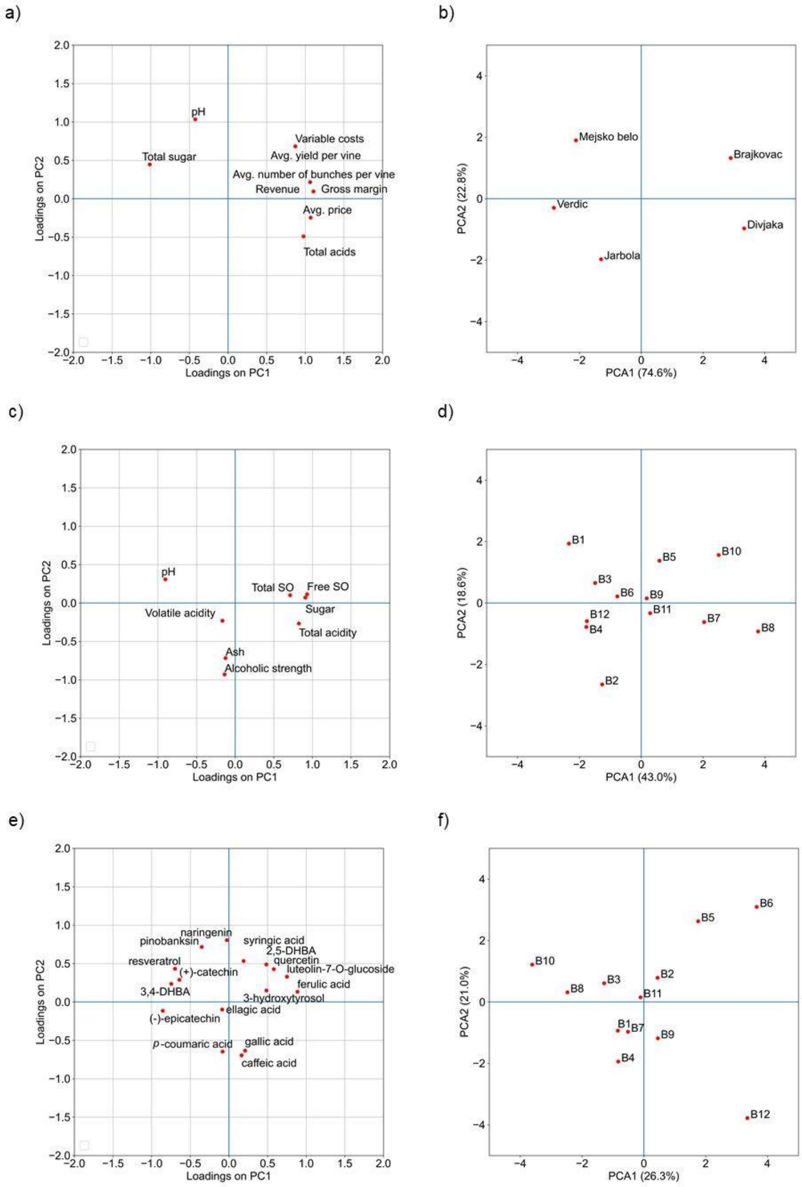

Fig. 1. Distribution of elements (variables and samples) in the space of principal component 1 (PC1) and principal component 2 (PC2) when a, b) standard physico-chemical parameters of grape varieties as variables were used, c, d) standard physico-chemical parameters of Belica wines as variables were used, e, f) phenolic compounds (LC-QQQ) in Belica wines as variables were used

Supplementary material

Table S1. LC-QQQ method parameters of phenolic acids and flavonoids analysis of Belica wine samples

Compound	*t*/min	Polarity	m/z precursor ion	*m/z* product ion*	Collision energy/V	Y=ax+b		Linearity range/(µg/mL)	R^2	LOD/(µg/mL)	LOQ/(µg/mL)
						Slope (a)	Intercept (b)				
Phenolic acids											
2,5-DHBA	4.42	-	152.8	108.0 81.8 53.0	20 16 20	15806.66	33.47	0.001-7.5	0.995	0.007	0.021
3,4-DHBA	1.70	-	152.9	108.0 81.0 53.0	20 16 18	3761.51	15.01	0.005-7.5	0.998	0.013	0.040
caffeic acid	5.35	-	178.8	135.0 116.9 88.9	12 24 34	27277.16	206.79	0.005-5.0	0.991	0.025	0.076
ellagic acid	6.70	-	301.0	283.7 228.4 244.6	28 30 32	898.41	291.00	1.0-5.0	0.997	1.069	3.239
ferulic acid	6.70	-	192.9	177.9 149.0 134.0	8 6 12	2289.69	4.91	0.01-10.0	0.997	0.007	0.021
gallic acid	0.86	-	168.8	125.0 78.9	10 20	11611.28	58.78	0.01-7.5	0.998	0.017	0.051
p-coumaric acid	6.26	-	162.9	119.0 92.8 64.9	12 36 48	30897.42	769.42	0.025-2.5	0.991	0.082	0.249
syringic acid	5.59	-	197.0	181.8 166.9 122.6	8 16 22	463.54	-89.15	0.25-7.5	0.992	0.6347	1.923
Flavonoids											
(+)-catechin	5.20	-	298.0	244.8 204.9	10 12	1753.68	7.32	0.1-5.0	0.997	0.014	0.042

				122.7 108.8	30 26						
(-)-epicatechin	5.70	-	298.1	244.9 108.9	10 26	2893.55	-0.61	0.01-2.5	0.999	0.001	0.002
3-hydroxytyrosol	1.73	-	152.9	95.0 94.8	18 20	756.90	18.41	0.1-2.5	0.994	0.080	0.243
quercetin	9.15	-	300.9	178.8 151.0 120.9	14 18 24	17670.76	-23.67	0.1-1.0	0.992	0.004	0.013
luteolin-7-O-glucoside	6.80	+	449.1	287.0	14	122734.19	117.66	0.001-2.5	0.999	0.003	0.010
naringenin	10.16	-	270.9	151.0 118.9	12 24	33448.04	10.74	0.001-0.5	0.998	0.001	0.003
pinobanksin	10.10	-	271.0	252.9 225.0 196.6 160.7	18 18 24 24	10928.37	27.17	0.01-1.0	0.998	0.008	0.025
resveratrol	8.31	+	228.8	163.3 107.2 135.0	26 8 20	7089.69	-3.93	0.001-10.0	0.995	0.002	0.005

*Quantifier ions are underlined

Table S2. Results of the Fourier-transform infrared spectroscopy (FTIR) analysis of Belica wine samples from Kastav region (Croatia)

Wine sample	*φ(alcohol)/%*	*γ*(glucose and fructose)/(g /L)	*γ*(total acidity)/ (g/L)	*γ(*malic acid)/ (g /L)	*γ(*lactic acid)/ (g /L)	*γ*(volatile acidity)/(g /L)	pH	specific gravity
B1	11.5	2.6	4.7	0.6	1.4	0.20	3.39	0.9915
B2	12.5	2.0	5.9	1.7	0.2	0.25	3.15	0.9907
B3	12.9	1.9	5.6	2.5	0	0.18	3.32	0.991
B4	11.6	1.9	6.1	2.6	0	0.23	3.35	0.9922
B5	12.3	2.5	5.2	1.9	0	0.21	3.25	0.9904
B6	12.3	2.2	5.5	2.2	0	0.23	3.27	0.9909
B7	13.0	4.4	6.3	2.6	0	0.16	3.09	0.9915
B8	11.4	3.3	7.0	2.9	0	0.24	3.15	0.9924
B9	12.9	2.3	5.6	1.6	0	0.29	3.10	0.9913
B10	13.5	3.4	6.7	2.3	0	0.20	3.33	0.9907
B11	11.2	2.0	5.5	2.2	0	0.12	3.48	0.9913
B12	12.3	1.9	5.0	1.7	0	0.18	3.27	0.9908

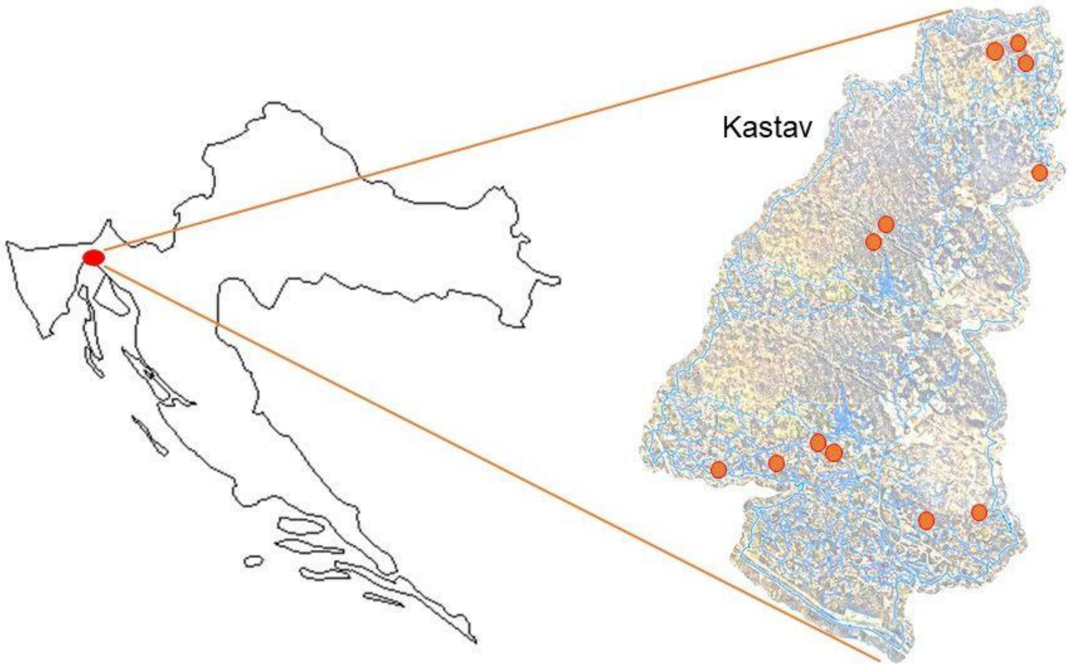

Fig. S1. Map of the Kastav area (Croatia). Red circles indicate the locations of the vineyards from which Belica grapes were collected