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Clone candidates differentiation of grapevine *Vitis vinifera*'Škrlet bijeli' using aroma compounds detected by gas chromatography-mass spectrometry

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ABSTRACT

The aim of this work was to investigate existence presence and stability of must specific aroma compounds (monoterpenes C_{13}-norisoprenoids, C_6-alcohols, alcohols, esters and carbonyl compounds) and which can be used to establish differences among clone candidates of 'Škrlet bijeli' (*Vitis vinifera* L.) grapevine variety. The compounds responsible for the varietal aroma profile were determined by gas chromatography- mass spectrometry (GC-MS), in must samples of ten clone candidates grown on two vineyard sites for three consecutive years. Significant variation among clone candidates is shown in 22 out of the total 35 identified aroma compounds. Significant impact of the vineyard site on the clone candidate's aroma profile was identified. Differences in primary aroma compounds responsible for flavour of 'Škrlet bijeli' variety, linalool, terpinolen, nerol and α-terpineol, were not significant among clone candidates, while remarkable differences were established for β-damascenone. Contrary to expectation, monoterpene geraniol was not detected. Other identified aroma compounds (*trans*-ocimene, 2-methyl-1-butanol, myrcene, α-phelandrene, *cis*-ocimene and 3-methyl-1-butanol) noticeably less participate in total flavour description, but they still enable notable clone candidates discrimination.

Key words: clonal selection, must, aroma compounds, gas chromatography-mass spectrometry (GC-MS), multivariate statistical analysis

IZVLEČEK

RAZNOLIKOST KLONSKIH KANDIDATOV *Vitis vinifera* 'ŠKRLET BIJELI' V AROMATIČNIH SNOVEH, DOLOČENIH S PLINSKO KROMATOGRAFIJO-MASNO SPEKTROSKOPIJO

Namen tega dela je bil ugotoviti prisotnost in stabilnost specifičnih aromatičnih spojin mošta (monoterpeni C13-norizoprenoidi, C6-alkoholi, alkoholi, estri in karbonilne spojine) ter katere od teh spojin se lahko uporabljajo za razlikovanje klonskih kandidatov sorte *Vitis vinifera* 'Škrlet bijeli'. V ta namen smo spojine, ki so odgovorne za sortni aromatični profil, identificirali s plinsko kromatografijo-masno spektrometrijo (GC-MS), v vzorcih mošta desetih klonskih kandidatov, ki rastejo na dveh lokacijah, v treh zaporednih letnikih. Za določanje razlik med kloni in opredelitev spojin, ki so odgovorne za te razlike, smo uporabili multivariatne statistične metode (analizo glavnih komponent in linearno diskriminantno analizo). Značilne razlike med klonskimi kandidati so se pokazale v 22 od skupno 35 identificiranih aromatičnih spojin. Za aromatski profil klona smo ugotovili prevladujoč vpliv lokacije vinograda. Razlike v primarnih aromatičnih spojinah, odgovornih za aromo 'Škrlet Bijeli', linaloola, terpinolena, nerola in α-terpineola, niso bile statistično značilne med klonskimi kandidati, medtem ko so bile določene pomembne razlike v β-damascenonu. V nasprotju s pričakovanji, monoterpena geraniola nismo določili. Druge določene aromatične spojine (*trans*-ocimen, 2-metil-1-butanol, mircen, α-felandren, *cis*-ocimen in 3-metil-1-butanol) občutno manj sodelujejo pri skupnem opisu sortne arome, vendar še vedno omogočajo razlikovanje klonskih kandidatov.

Ključne besede: klonska selekcija, mošt, aromatične spojine, plinska kromatografija-masna spektrometrija (GC-MS), multivariatna statistična analiza

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1 INTRODUCTION

Individual clonal selection is the method most commonly used for genetic improvement of autochthonous grapevine varieties, which results by divergent clones. Usually the selection is performed on grape quality parameters such as sugars and acids content, while aroma profile is checked in late selection stages. How the wine aroma profile is important for wine identity and quality and as aroma precursors originated from must, it is very important to include monitoring of must aroma compounds in early stages of clonal selection. Specific varietal wine aroma originates from volatile compounds such as monoterpenes, norisoprenoids, aliphatic compounds, phenylpropanoids, methoxypyrazine and volatile sulfur compounds synthetized in grapes, which in numerous combinations make a unique, distinctive, typical varietal aroma (Coombe and McCarthy, 1997; Ebeler and Thorngate, 2009). Listed compounds can be used for varietal identification (Marais, 1983; Rapp and Mandery, 1986) because characteristic wine aroma of specific variety is attributed to the aroma compounds of grape.

The most important group of grape compounds with the greatest contribution to distinctiveness of aroma variety are terpenes (Marais, 1983; Câmara et al., 2007) to which belongs groups of monoterpene and C_{13} norisoprenoids (Mateo and Jiménez, 2000). The biggest impact on wine primary aroma provide monoterpenes that are present in wine in free form, while they are present in grape in both free and bound glycoside forms. Monoterpens have very important contribution on white wines aroma of Muscat varieties, but also on other aromatic varieties (Mateo and Jiménez, 2000; del Caro et al., 2012). Free monoterpenes are present in less aromatic and non-aromatic varieties in significantly lower concentration (Iyer et al., 2010; Genovese et al., 2013). Aroma precursor's analysis has been used as a strategy to determine the aroma potential of grape both from aromatic and non-aromatic varieties (Loscos et al., 2009). Monoterpenes have strong impact on wine flavor character that is verified by strong correlation of linalool and α-terpineol content and floral description of wine (Komes et al., 2006; Skinkis et al., 2008; Sánchez-Palomo et al., 2012).

Aroma profile is extremely important in clonal selection procedure despite that main subject is always wine aroma profile but not grape aroma. Koch et al. (2010) highlighted a significance of aroma compound 2-methoxy-3-isobutylpyrazine in clonal selection of 'Cabernet Sauvignon' variety as characteristic compound of primary aroma, while Boidron (1995) quoteed that wine of two clones of 'Chardonnay' variety have pronounced Muscat aroma tint in comparison to other studied clones and recognizes this as positive and desirable clone characteristic. Versini et al. (1990) stated that it is for quality of clone grapes comparison necessary to study specific aroma compounds on which content in grapes the effect of environmental is at minimum, they are monoterpenes and by them were defined differences between clones of 'Traminer Red' and 'Chardonnay' variety. Marais and Rapp (1991) concluded that it is possible to distinguish clones based on terpene content. They proved that clones 457/48, 14Gm D35, 925/643 and FR46/106 of 'Gewiirztraminer' variety appeared to have a greater potential to produce aroma-rich and variety-typical wines than N20 Kieselberg. With respect to 'Weisser Riesling', two clones, namely 37 and 327, could possibly be selected as more flavorful than the others. McCarthy (1992) studied grapes of 10 clones 'Muscat à petite grains blanc' variety and found that there was no difference in the free volatile terpene concentration between clones, but there were significant differences in bounded form of monoterpene concentration. In order to assess the suitability of some genotypes for functional genomics studies on terpenol synthesis in grapevine, Duchene et al. (2009) studied two varieties differing in their aromatic pattern: 'Gewurztraminer' and 'Sauvignon Rose' and two clones of 'Chardonnay' (76 and 809). There are evidences that clonal variation, through somatic mutations, can modify the aromatic profile of fruits. Genovese et al. (2013) reported results that showed different aroma profile (free and bound volatile compounds) in 'Aglianica' and 'Uva di Troia' grapes.

Various authors are using different methods for determination of terpenes in grape and wines. Setkova et al. (2007) developed a rapid headspace solid-phase micro extraction-gas chromatographic–

time-of-flight mass spectrometric method for qualitative profiling volatile fraction of wines. Volatile compounds of grapes are responsible of varietal aroma. In order to obtain an appropriate technique to study grape volatile compounds in pulp and skins of 'Muscat' grapes, Sánchez-Palomo et al., (2005) have developed HS-SPME method coupled with GC-MC. Sixteen volatile compounds have been quantified. Prosen et al. (2007) using synthetic solution developed an extraction procedure for the aroma compounds from musts and wines, using solid-phase micro extraction. The method was suitable for analyzing free aroma compounds in must of different varieties and for monitoring of their release after enzymatic or acidic hydrolysis. Coelho et al. (2007) propose headspace-solid phase micro extraction (HS-SPME) for the variety- and pre-fermentation-related volatile compounds of 'Fernão-Pires' (FP) white grape berries. Two C_{13} norisoprenoids, two aromatic alcohols, two C6 aldehydes, and three C6 alcohols were identified by gas chromatography-quadruple mass spectrometry (GC-qMS). Bordiga et al. (2013) suggest using combination of HS-SPME technique with GCxGC/TOF-MS system for the analysis of wine volatile compounds.

In a last few years, not only on Croatian but also on European wine market, there is a growing interest for wines made of local grapevine varieties with distinguish quality. These wines contribute in raising a regional wine identity and tourism potential. Therefore, efficiency of individual clone selection is very important to provide high quality propagating material and revitalization of forgotten local varieties. 'Škrlet bijeli' is autochthonous variety that grows on very limited areas in continental region of Croatia, in Pokuplje, Vukomeričke gorice and Moslavina. Vineyards planted with 'Škrljet bijeli' represent only 0.3 % of all Croation vineyard area or 66 ha. It is characterized by discrete aroma and freshness, which consumers recognize and prices. Up to now on propagating material market for this particular variety was available only material of the lowest category (CAC; Conformitas Agraria Communitatis). Final phase of individual clonal selection, where is studying the most perspective clone candidates is in progress. The aim of this work was (1) to investigate the presence and stability of specific aroma compounds (monoterpenes, C_{13}-norisoprenoids, benzenoids, alcohols C_6, alcohols, esters and carbonyl compounds) in must of 10 clone candidates of 'Škrlet bijeli' variety applying GC-MS method, with intention of (2) their mutual distinction and identification of clones by multivariate analysis.

2 MATERIALS AND METHODS

2.1 Samples

Ten clone candidates of 'Škrlet bijeli' variety produced by propagation of elite vines, selected in process of mass positive clonal selection, and planted in two sites, Popovača and Repušnica, during three vintages were investigated. Both locations are in viticulture region of continental Croatia, sub-region Moslavina. Production viticulture zone is B (Winkler et al., 1974). Both field trails were planted in period from year 2001 to 2004.

Clone candidates coded as ŠK-07, ŠK-11, ŠK-29, ŠK-32, ŠK-33, ŠK-57, ŠK-60, ŠK-69, ŠK-74, ŠK-77 represent progeny of individual elite vine selected from old vineyards. All clone candidates originated from mass clonal selection of 'Škrlet bijeli' which was performed on agricultural traits (yield and sugar accumulation) as well as on good vigour of mother plants. In the field trails, each clone candidate was represented with 3-5 vines planted in same row, that were grafted, at the place, with gem originated from elite vine by method „green on green" on virus-free rootstock: Leaf Roll Virusis.(LR1 and LR3) and Raspberry Ringspot Virus (RRSV). Harvest date was determined based on phenotype evaluation and refractometry tracking of sugar accumulation dynamics and was harvested at the same date for all clone candidates at about 85 ºOe as it is common sugar content at harvest of 'Škrlet bijeli'. The basic agricultural traits (yield and sugar accumulation) are listed in Annexes 1-2.

Sampling for aroma compound analysis carried out according to the plan: first vintage year 2006, the average samples of must for four clone candidates ŠK-29, ŠK-33, ŠK-57 and ŠK-69; second (2007) and third (2008) vintage year, the average samples of must for ten clone candidates ŠK-29, ŠK-32, ŠK-33, ŠK-60, ŠK-69, ŠK-74, ŠK-77, ŠK-07, ŠK-11, ŠK-57 were prepared from both location. Must samples were frozen at – 28 °C and defrost right before analysis.

2.2 Head-space SPME extraction

The SPME extraction conditions were 10 ml of sample that was spiked with internal standard of 3-octanol in concentration of 0,0844 µg l^{-1} (Sigma-Aldrich, St. Louis, MO, USA) in a 20 ml glass headspace vials, with addition of 1,5 g NaCl, extraction time of 45 min and extraction temperature of 50 °C under stirring at 350 r min^{-1}. The headspace was sampled using a 50/30 µm divinylbenzene-carboxen-poly(dimethylsiloxane) (DVB-CAR-PDMS) coated fiber in a Supelco fiber holder (Bellefonte, PA, USA). After equilibration, the fiber was removed from the sample and the analytes were thermally desorbed in the injector port of the GC.

2.3 GC-MS

A multiPurpose autosampler MPS2 (Gerstel GmbH, Germany) with an agitator and SPME fiber conditioning station was used to extract the volatiles from sample vial headspace. All chromatographic analysis was performed using an Agilent 7890A Series GC system with an Agilent 5975C Mass Selective Detector (Agilent, Palo Alto, USA). The apparatus used was equipped with split/split less injector, J&W DB-Wax column (60 m length x 0.32 i.d. x 0.25 µm film thickness (J&W Scientific, Folsom, CA, USA). The temperature program used was 40 °C for 5 min; 4 °C min^{-1} to 230 °C; 20 min at maximum temperature. Carrier gas (He) flow was 1.2 ml min^{-1}. Injections of 1 µl were performed in split less mode while the injector port and the ion source were maintained at 230 °C and 250 °C, respectively. Positive electron impact spectra were recorded at 70 eV in a range *m/z* 30 – 250. Mass spectrometric information of each chromatographic peak was compared to NIST (National Institute for Standards and Technology, USA) mass spectra library. Data is given as relative peak area (RPA) ± standard deviation (SD) presented ratio of area peak of identified compound and peak area of internal standard.

2.4 Statistical analysis

Significant differences between clones of 'Šklet bijeli' were determined on the basis of the most abundant aroma compounds: linalool, β-damascenone, terpinolen, nerol and α-terpineol by one-way analysis of variance using software package Statistics (version 8.0, Statsoft Inc., Tulsa, USA), while differences between averages of RPA by Student-Newman-Keul test. Principle component analysis (PCA) and linear discriminant analysis (LDA) were performed to classify the grapevine clone candidates regarding to vineyard site and vintage. A total of 35 major aroma compounds were included in analysis. PCA was performed to provide a data structure study over a reduced dimension, covering the maximum amount of the information present in the basic data. It was conducted using software Statistical Package for the Social Sciences (version 15.0 for Windows; SPSS Inc., Chicago, USA) and statistics software (version 8.0; Statsoft Inc., Tulsa, USA).

Annex 1: Yield (kg of grape per stock) of clone candidates in investigated vintages and vineyard sites
Priloga 1: Pridelek (kg grozdja po trsu) klonskih kandidatov v proučevanih letnikih in lokacijah

	2006		2007		2008	
Clone candidate	Popovača	Repušnica	Popovača	Repušnica	Popovača	Repušnica
ŠK-07	3.40	2.21	5.88	2.38	3.70	3.95
ŠK-11	2.87	0.76	3.82	2.91	3.76	3.39
ŠK-29	4.04	1.40	4.63	3.73	2.79	3.30
ŠK-32	3.34	1.78	2.34	1.40	3.67	1.67
ŠK-33	2.20	1.55	4.04	3.62	3.00	2.10
ŠK-57	2.82	1.41	2.32	2.91	3.09	2.13
ŠK-60	3.89	1.37	2.29	2.62	3.57	3.32
ŠK-69	2.44	1.62	3.80	4.95	3.27	4.09
ŠK-74	4.20	1.58	2.25	4.33	2.96	2.13
ŠK-77	4.20	1.50	2.87	4.29	3.89	3.84
Average	3.34	1.50	3.32	3.22	3.33	3.02

Annex 2: Sugar content (g l^{-1}) of clone candidates in investigated vintages and vineyard sites
Priloga 2: Vsebnost sladkorja (g l^{-1}) klonskih kandidatov v proučevanih letnikih in lokacijah

	2006		2007		2008	
Clone candidate	Popovača	Repušnica	Popovača	Repušnica	Popovača	Repušnica
ŠK-07	205.0	186.8	187.4	229.7	179.4	179.8
ŠK-11	224.3	205.2	224.5	191.2	216.4	221.0
ŠK-29	217.0	188.0	206.9	224.6	223.4	168.2
ŠK-32	215.3	170.7	245.1	215.5	211.5	188.7
ŠK-33	215.8	194.2	206.8	206.0	207.6	208.2
ŠK-57	217.7	185.0	215.3	200.3	189.5	205.7
ŠK-60	196.3	162.8	222.3	186.4	168.3	153.5
ŠK-69	213.0	181.3	192.5	196.1	207.0	173.4
ŠK-74	196.0	190.3	221.5	201.7	209.0	182.4
ŠK-77	186.7	196.3	201.9	194.5	217.7	177.4
Average	208.8	186.3	213.5	204.6	204.8	188.6

3 RESULTS AND DISCUSSION

3.1 Free terpenes content in grape must

In grape must of ten clone candidates 'Škrlet bijeli', the most abundant aroma compounds were as followed: linalool, β-damascenone, terpinolen, nerol and α-terpineol. However, monoterpene geraniol was not identified.

According to the literature, the most usually analyzed aroma compounds in grape must generally are linalool, geraniol, nerol, α-terpineol and β-damascenone (Sánchez-Palomo et al., 2012; Gómez García-Carpintero et al., 2011).

In order to determine if there are differences among clones on the level of free terpene compounds linalool, β-damascenone, terpinolen, nerol and α-terpineol expressed as mean RPA values for all ten clones, the belonging rank of significant differences was determined by analysis of variance. The results are presented in Table 1.

Table 1 The influence of clone candidates of 'Škrlet bijeli' on the content of linalool, β-damascenone, terpinolen, nerol and α-terpineol (mean value of RPA ± SD) with belonging rank of significant differences, for both vineyard sites through two (n = 4) or three (n = 6) years, respectively.

Preglednica 1: Vpliv klonskih kandidatov 'Škrlet bijeli' na vsebnosti linaloola, β-damascenona, terpinolena, nerola in α-terpineola (povprečna vrednost relativne ploščine vrha ± SD) s pripadajočim rangom značilnih razlik, za obe lokaciji tekom dveh (n = 4) oziroma treh (n = 6) let.

Clone	n	Linalool		β-Damascenon		Terpinolen		Nerol		α-Terpineol	
						(RPA / 10^6)					
ŠK-07	4	57440 ± 36976	a*	10621 ± 4117	ab	1863 ± 557	a	1599 ± 2137	a	1257 ± 1126	a
ŠK-11	4	32705 ± 38096	a	12198 ± 7591	ab	1522 ± 1311	a	869 ± 1645	a	754 ± 1144	a
ŠK-29	6	61376 ± 88124	a	8479 ± 4461	a	2436 ± 2744	a	2543 ± 4030	a	1523 ± 2311	a
ŠK-32	4	98760 ± 91769	a	14019 ± 7740	ab	6518 ± 6917	a	3168 ± 3799	a	2696 ± 2929	a
ŠK-33	6	96225 ± 108533	a	11924 ± 3845	ab	5593 ± 4995	a	2627 ± 4793	a	2270 ± 3104	a
ŠK-57	6	94418 ± 90699	a	16135 ± 6800	b	9573 ± 12949	a	2396 ± 4452	a	2836 ± 4030	a
ŠK-60	4	89147 ± 95225	a	16826 ± 9992	b	7078 ± 7568	a	2033 ± 3101	a	2983 ± 3279	a
ŠK-69	6	56035 ± 37655	a	9442 ± 2654	a	4693 ± 4096	a	1152 ± 1597	a	1356 ± 1404	a
ŠK-74	4	61547 ± 54192	a	13340 ± 5299	ab	4455 ± 4217	a	1656 ± 2006	a	1779 ± 2001	a
ŠK-77	4	52773 ± 36890	a	10821 ± 3911	ab	4373 ± 4546	a	1092 ± 1194	a	1726 ± 1970	a

*Student-Newman-Keul test; values in column marked by the same letter are not significantly different ($p \leq 0.05$)

Neither linalool, with the highest RPA values which ranged from 32705 to 98760, nor terpinolen (RPA ranged from 1522 to 9573), nerol (RPP ranged from 869 to 3168) nor α-terpineol (RPP ranged from 754 to 2983) content were not statisticaly significantly different among clone candidates. RPA values of norisoprenoid compound β-damascenone were statistically different and clone candidats ŠK-29 and ŠK-69 had lower RPA values then clones ŠK-57 and ŠK-60). These results were expected because the study was done on progeny obtained by vegetative propagation of the same plant, and selection of ten clone candidates that were subject of this study based only on phenotype selection of clones from population, selection was not include preliminary aroma compounds analysis.

The impact of vintage and vineyard site on the RPA values of linalool, β-damascenone, terpinolen, nerol and α-terpineol in grape must of clone candidates of 'Škrlet bijeli' with the belonging rank of significant differences was determined by analysis of variance. The results were presented in Table 2 and 3.

Table 2: The influence of vintage on the content of linalool, β-damascenone, terpinolen, nerol and α-terpineol (mean value of RPA ± SD) in must with belonging rank of significant differences for 10 clone candidates of 'Škrlet bijeli' for both sites.

Preglednica 2: Vpliv letnika trgatve na vsebnosti linaloola, β-damascenona, terpinolena, nerola in α-terpineola (povprečna vrednost relativne ploščine vrha ± SD) v moštu s pripadajočim rangom značilnih razlik za 10 klonskih kandidatov 'Škrlet bijeli' za obe lokaciji.

		Linalool		β-Damascenon		Terpinolen		Nerol		α-Terpineol	
Year	n	(RPA / 10^6)									
2006	8	58837 ± 31215	a*	9362 ± 2961	a	4701 ± 2467	a	246 ± 134	a	938 ± 544	a
2007	20	19730 ± 12942	a	9765 ± 4492	a	1073 ± 622	a	141 ± 338	a	310 ± 385	a
2008	20	127626 ± 77314	b	15849 ± 6167	b	8898 ± 7756	b	4460 ± 3445	b	3950 ± 2631	b
F exp		24.23		8.29		13.67		23.41		2711	
Pr > F		< 0.0001		0.0009		< 0.0001		< 0.0001		< 0.0001	

*Student-Newman-Keul test; values in column marked by the same letter are not significantly different ($p \leq 0.05$); p-value is the significance; Pr > F - This is the p-value associated with the F-statistic. It is used in testing the null hypothesis that all of the model coefficients are 0; F - This is the F-statistic is the mean square model divided by the mean square error; F exp – this is the variance between treatments devided by the variance within treatments

Table 3: The influence of vineyard site on the content of linalool, β-damascenone, terpinolen, nerol and α-terpineol (mean value of RPA ± SD) with belonging rank of significant differences for 10 clone candidates of 'Škrlet bijeli', for both vineyard sites through three years.

Preglednica 3: Vpliv lokacije vinograda na vsebnosti linaloola, β-damascenona, terpinolena, nerola in α-terpineola (povprečna vrednost relativne ploščine vrha ± SD) s pripadajočim rangom značilnih razlik za 10 klonskih kandidatov 'Škrlet bijeli' za obe lokaciji.

		Linalool		β-Damascenon		Terpinolen		Nerol		α-Terpineol	
Location	n	(RPA / 10^6)									
Popovača	24	78129 ± 90991	a*	11826 ± 6206	a	6288 ± 8190	a	2539 ± 3921	a	2469 ± 3082	a
Repušnica	24	59333 ± 42874	a	12639 ± 5606	a	3587 ± 2764	a	1377 ± 1778	a	1393 ± 1414	a
F exp		1.45		0.11		1.74		1.85		2.97	
Pr > F		0.235		0.739		0.194		0.182		0.092	

*Student-Newman-Keul test; values in column marked by the same letter are not significantly different ($p \leq 0.05$); p-value - this is the significance; Pr > F - This is the p-value associated with the F-statistic. It is used in testing the null hypothesis that all of the model coefficients are 0; F - This is the F-statistic is the Mean Square Model divided by the Mean Square Error; F exp – this is the variance between treatments/variance within treatments

Results of vintage year and vineyard site influence on RPA values of five the most abundant terpene compounds linalool, β-damascenone, terpinolen, nerol and α-terpineol in must (Table 2 and 3), show during year 2008, significantly higher RPA values for all five dominant aroma compounds which indicates favourable climatological conditions for their individual synthesis. At the same time, RPA values for all five the most abundant terpene compounds were not significantly influenced by vineyard site.

It is not possible to make a real judgment on must aroma based on processing data of individual aroma compounds because different combination of compound concentration at the end bring different wine olfactory experience (Robinson, 2011; Botelho, 2008). To determine if there is stable correlation between clones, vintage years and location and all 35 detected aroma compounds, PCA method was used.

Results of PCA analysis imply that the loading values of the variables associated with the first five principal components were as followed: *cis*-ocimene, *trans*-ocimene, myrcene, limonene, geranic oxide and hotrienol, were the dominant variables in the first principal component, which accounted for 32 % of the total variance. The 2-methyl-1-butanol, 3-methyl-1-butanol, ethyl acetate, hexyl acetate, isoamyl acetate, acetaldehyde, 2-hexenal and 2-hexene-1-ol (E) dominated the second principal component that explained up to 26 % of the total variance. The first five principal components thus accounted for 83 % (PC3 14 %, PC4 8 % and PC5 5 %) of the variation among the samples analyzed. Out of these 35 parameters, 12 were recognized by PCA as being less important. Therefore, the remaining 22 parameters were included in the LDA test (Table 4).

Table 4: Content of aroma compounds present in grape musts of 10 clone candidates of 'Škrlet bijeli'
Preglednica 4: Vsebnosti aromatičnih spojin, prisotnih v moštu 10 klonskih kandidatov 'Škrlet bijeli'

	Aromatic compound*																				
Sample	A**	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V
P200629***	432	9285	177	n.d.	810	44		6	76	1878	2722	5	19	34	300	60	142	21	49090	591	24	156
P200633	355	2946	134	96	83	474	24	40	697	424	727	59	141	368	2693	67	83	29	38395	1352	105	697
P200657	373	7243	235	105	122	442	21	36	685	506	837	60	148	328	2361	45	119	25	48568	1396	115	728
P200669	491	11215	178	110	217	536	29	57	815	979	2180	76	133	421	3232	57	136	21	46481	621	138	918
P200707	328	2002	3732	81	86	414	47	52	571	298	551	115	1299	159	1182	6	80	42	61337	12181	n.d.	474
P200711	237	2242	2004	3	56	60	n.d.	n.d.	72	65	145	9	1049	23	117	5	46	26	52399	17743	n.d.	217
P200729	413	25950	2319	n.d.	1364	16	n.d.	n.d.	31	539	1120	n.d.	725	4	22	3	420	26	77309	8422	n.d.	80
P200732	393	8537	4659	6	103	112	4	7	93	291	590	18	1475	38	246	4	185	32	80501	7636	n.d.	341
P200733	465	38886	1751	35	2854	175	9	16	235	1154	1810	32	339	79	484	4	648	24	65414	1070	45	544
P200757	450	3459	2668	53	155	185	10	16	242	486	826	35	741	83	503	3	155	32	67941	2637	53	526
P200760	392	12400	1733	17	1221	152	7	10	193	1001	2152	24	368	68	387	7	245	34	58110	440	n.d.	322
P200769	245	1328	980	19	118	90	2	6	142	279	574	14	426	43	223	6	80	16	47649	2976	n.d.	263
P200774	402	9047	2701	7	159	96	3	7	115	366	694	15	915	42	243	6	194	28	62514	3801	n.d.	227
P200777	226	2191	1786	37	67	304	19	26	326	196	384	51	912	132	862	4	80	32	54642	11820	46	590
P200807	975	72796	59	12	4385	223	14	22	190	671	1483	33	156	93	756	73	2050	15	56091	3964	26	344
P200811	142	2249	272	14	45	219	94	66	204	22	64	161	1588	89	650	10	85	9	36842	19595	26	418
P200829	588	9445	553	93	675	2067	170	179	1386	914	1471	344	439	571	5238	30	379	34	123024	3368	31	276
P200832	428	6993	44	162	143	1305	128	185	1905	219	509	263	351	692	6338	19	196	12	59320	10002	42	612
P200833	884	92124	120	176	1602	2569	251	304	2390	766	1389	541	190	885	8033	338	929	53	84514	3376	74	919
P200857	295	10949	423	578	133	3320	263	432	4905	83	463	534	1157	1613	12311	34	132	70	42657	17773	67	672
P200860	203	1654	707	349	46	2318	178	243	2522	35	235	362	1174	935	7460	13	83	42	34643	18186	42	407
P200869	136	1746	716	136	21	817	82	133	1380	26	144	146	1686	452	4067	10	63	8	29348	13378	41	518
P200874	193	2127	448	164	36	915	95	130	1184	72	220	198	1189	384	3581	26	162	18	61264	21516	52	760
P200877	151	2039	813	203	28	928	88	128	1559	32	160	179	1938	499	4139	8	64	7	26104	13920	58	870
R200629	360	7802	411	9	542	108	91	62	140	1065	2007	132	156	78	669	64	234	11	59173	2626	n.d.	330
R200633	538	78267	145	51	4485	543	36	69	687	1731	2985	82	25	412	3791	67	828	8	41660	526	91	853
R200657	833	69384	154	85	4046	783	59	82	918	1767	2771	134	28	533	4702	113	1054	11	48843	1000	119	1007
R200669	518	90488	260	36	7057	499	28	56	538	1987	3779	66	22	327	3078	146	1624	23	32974	882	110	900
R200707	415	62791	914	19	4099	180	210	189	157	1153	1900	294	487	86	1095	36	1513	24	64440	3738	50	639
R200711	224	13746	909	8	1861	57	21	22	73	873	1195	33	475	35	378	12	1376	53	63692	3819	22	203
R200729	195	26649	1334	n.d.	722	39	1515	782	40	418	725	1775	862	17	271	19	472	22	65137	10472	n.d.	221
R200732	468	97154	1834	6	7985	146	1952	1025	120	1326	2109	2433	666	73	841	103	3489	31	74030	1305	33	380
R200733	400	38595	1285	4	2517	101	40	33	97	663	1323	54	433	56	518	21	2002	24	59930	3558	21	297
R200757	386	60035	840	7	4709	146	108	79	129	1570	2030	129	246	78	748	17	2036	25	80490	1710	27	351
R200760	297	29803	922	17	1588	140	39	34	123	468	904	60	525	73	611	12	1860	25	66148	11042	30	460
R200769	196	2093	837	4	394	69	3	9	68	876	1200	10	365	35	331	9	431	21	92184	4165	21	180
R200774	295	11357	1045	18	1087	139	9	20	156	492	787	23	972	80	699	10	764	19	65886	12623	38	313
R200777	300	21365	2484	4	1422	75	4	7	79	1026	1350	16	957	32	227	13	516	26	74599	10441	18	202
R200807	1522	103793	173	28	6620	635	127	92	398	1936	2760	239	361	161	1595	81	2693	24	79533	2899	40	418
R200811	1166	49889	109	66	2100	568	139	112	549	1231	1993	251	78	174	1734	30	1103	23	83628	2525	35	425
R200829	715	136871	75	38	22703	811	1194	813	656	2814	4301	1725	145	228	2398	59	2050	18	53826	1804	58	326
R200832	1935	164594	73	103	7920	1184	188	205	1026	1910	3172	354	51	431	4276	40	3388	45	86157	1857	97	1430
R200833	730	24316	75	85	976	676	480	370	811	532	1062	805	231	263	2605	35	866	27	73882	9542	57	612
R200857	350	4684	996	106	269	705	110	135	975	863	1103	211	752	315	2938	117	278	23	83945	7493	69	850
R200860	1403	68263	162	396	3655	757	70	100	1830	1050	1745	164	81	424	3486	19	826	34	66365	4360	107	1121
R200869	188	2782	988	42	198	534	61	68	637	298	487	121	1059	228	1985	7	126	14	41303	15781	35	259
R200874	427	13648	99	87	835	721	172	164	933	761	1204	306	220	336	2803	18	426	17	65548	9222	64	703
R200877	305	22093	63	56	1805	492	54	68	695	948	1281	110	23	236	2020	11	410	10	40714	505	42	370

*mean values of aroma compounds expressed in relative peak area (x10^6) calculated for three replicates; ** aroma compounds: A=acetaldehyde, B=ethyl acetate, C=hexanal, D=geranic oxide, E=isoamyl acetate, F=myrcene, G=α-felandren, H=α-terpinene, I=limonene, J=2-methyl-1-butanol, K=3-methyl-1-butanol, L=β-phellandrene, M=2-hexenal, N=*trans*-ocimene, O=*cis*-ocimene, P=cinnamen, Q=hexyl acetate, R=6-methyl-5-hepten-2-one, S=1- hexanol, T=2-hexene-1-ol (E), U=Z-linalool oxide, V= hotrienol; ***sample code: location (P-Popovača, R-Repušnica), vintage year (2006-2008), code of clones; n.d.=indication that aroma compound is at level lower to detection limit

3.2 Influence of vineyard site and clone candidates on aroma compounds content

Using LDA method, six parameters were selected as the most discriminating variables: *trans*-ocimene, 2-methyl-1-butanol, myrcene, α-phelandrene, *cis*-ocimene and 3-methyl-1-butanol. The other five parameters isoamyl acetate, acetaldehyde, Z-linalool oxide, ethyl acetate and limonene also contribute significantly to better separation among the samples. When the LDA was applied to the data (48 samples, 22 variables), three discriminate functions explained 80 % of the total variance. Function 1 explains 43.8 % of the total variance, function 2 explains 23.4 %. The scores of the samples and parameters for these first two functions are plotted on Figure 1. As it can be seen, the samples are well separated depending on vineyard site and clone candidates. The accuracy of the placement of each sample into 20 groups was 100 %.

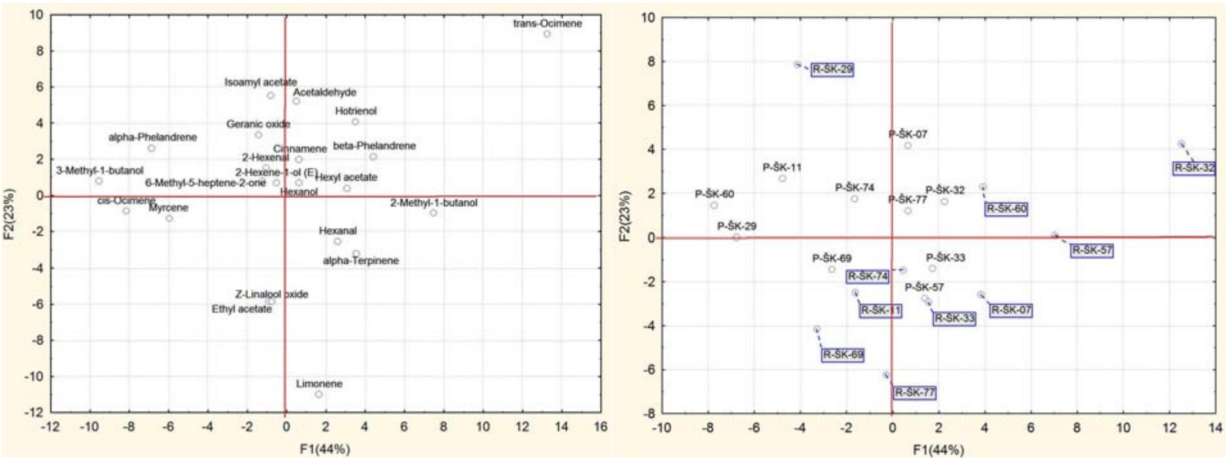

Figure 1: Projection of the scores of the samples (right) and parameters (left) for 48 must samples depending on vineyard site and clone candidates in the plane defined by the two standardized canonical discriminant function coefficients.

Slika 1: Projekcija rezultatov vzorcev (desno) in parametrov (levo) za 48 vzorcev mošta v odvisnosti od lokacije vinograda in klonskih kandidatov v ravnini, ki ju določata standardizirani funkciji diskriminantnih koeficientov.

From Figure 1 it can be seen, that only two clone candidates (ŠK-69 and ŠK-33) on two vineyard sites (Popovača and Repušnica) do not show differences in analyzed parameters.

3.3 Influence of vintage year on aroma compounds in vineyard site Popovača

Using LDA method, four parameters were selected for vintage year of grape production and clone candidate as the most discriminating variables: myrcene and α-phelandrene, as well as trans-ocimene and cis-ocimene. The other ten parameters: (2-hexene-1-ol (E), α-terpinene, acetaldehyde, 2-hexenal, 3-methyl-1-butanol, 2-methyl-1-butanol, isoamyl acetate, hexanal, ethyl acetate and geranic oxide also contribute significantly to better separation among the samples. When the LDA was applied to the data (24 samples, 22 variables), three discriminate functions explained 84.6 % of the total variance. Function 1 explains 48.0 % of the total variance, function 2 explains 26.1 % and function 3 10.5 %. The scores of the samples and parameters for these first two functions are plotted on Figure 2. As it can be seen, the samples are well separated depending on grape production year. The accuracy of the placement of each sample into 10 groups was 1007%. Clone candidates' ŠK-60, ŠK-69, ŠK-77, ŠK-29, ŠK-11 and ŠK-07 during three or two vintage years did not show differences in analyzed parameters.

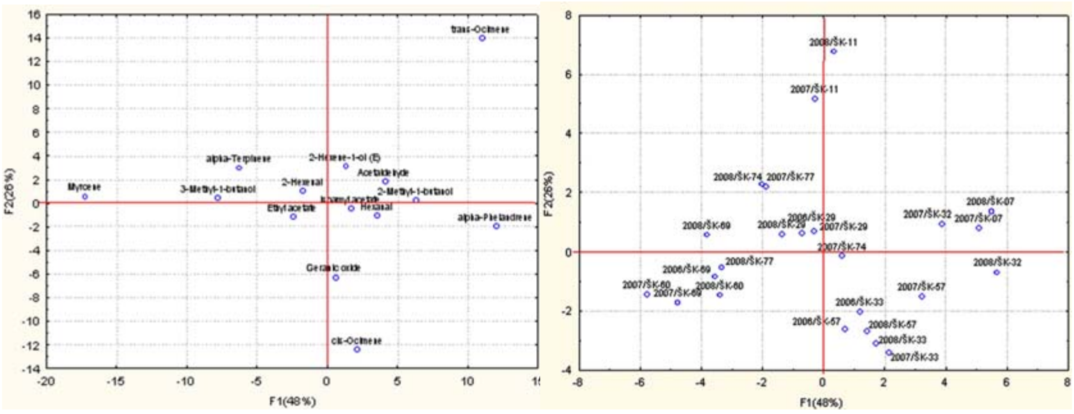

Figure 2: Projection of the scores of the samples (right) and parameters (left) for 24 must samples from vineyard site Popovača depending on vintage year and clone candidates used in the plane defined by the two standardized canonical discriminant function coefficients.

Slika 2: Projekcija rezultatov vzorcev (desno) in parametrov (levo) za 24 vzorcev mošta iz lokacije vinograda Popovača v odvisnosti od letnika trgatve in klonskih kandidatov v ravnini, ki ju določata standardizirani funkciji diskriminantnih koeficientov.

3.4 Influence of vintage year on aroma compounds in vineyard site Repušnica

Using LDA method, six parameters were selected for vintage year of grape production and clone candidate as the most discriminating variables: 3-methyl-1-butanol, ethyl acetate and trans-ocimene, as well as 2-methyl-1-butanol, α-phelandrene and α-terpinene. The other eight parameters: 2-methyl-1-butanol, acetaldehyde, hexanal, geranic oxid, isomyl acetate, myrcene, 2-hexanal and hexyl acetate also contribute significantly to better separation among the samples. When the LDA was applied to the data (24 samples, 22 variables), two discriminant functions explained 82.7 % of the total variance. Function 1 explains 60.8 % of the total variance, function 2 explains 21.9 %. The scores of the samples and parameters for these first two functions are plotted on Figure 3. As it can be seen, the samples from site Repušnica are well separated depending on vintage year. The accuracy of the placement of each sample into 10 groups was 100 %. Clones ŠK-32, ŠK-57, ŠK-77 and ŠK-29 during three or two production years did not show differences in analyzed parameters.

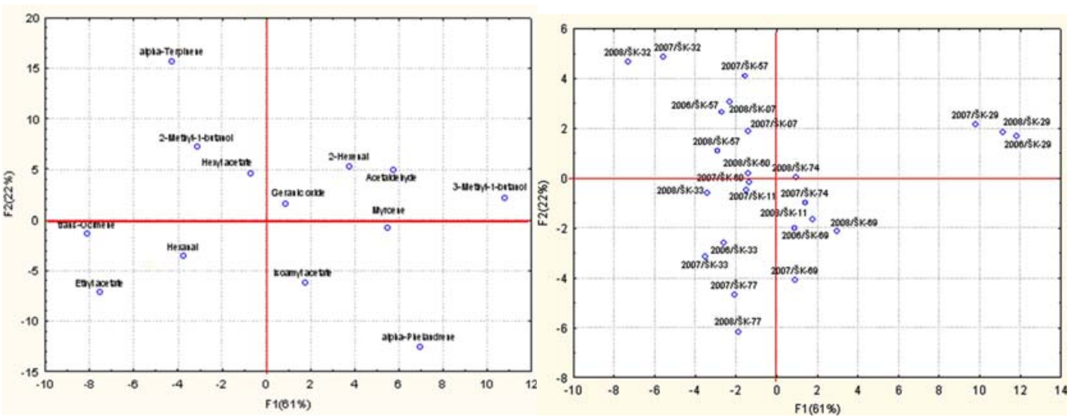

Figure 3: Projection of the scores of the samples (right) and parameters (left) for 24 must samples from vineyard site Repušnica depending on vintage and clone candidates used in the plane defined by the two standardized canonical discriminant function coefficients.

Slika 3: Projekcija rezultatov vzorcev (desno) in parametrov (levo) za 24 vzorcev mošta iz lokacije vinograda Repušnica v odvisnosti od letnika trgatve in klonskih kandidatov v ravnini, ki ju določata standardizirani funkciji diskriminantnih koeficientov.

If compared both vineyard sites it can be concluded that only two clones ŠK-77 and ŠK-29 during investigated vintage years did not show differences in analyzed parameters.

4 CONCLUSION

Analyzing free volatile terpene compounds responsible for primary aroma compounds responsible for flavour of 'Škrlet bijeli' such as linalool, terpinolen, nerol, α-terpineol and β-damascenone were detected, while contrary to expectation, monoterpene geraniol was not detected. Differences of RPA values for the first four compounds were not significant among clone candidates. However, remarkable differences of RPA values among clone candidates were established for some less represented compounds, as eg. norisoprenoid compound β-damascenone. It is noteworthy that significantly higher RPA values for all five dominant aroma compounds were established in the 2008 vintage, which indicates favourable climatological conditions for their synthesis and they can be used as quantitate indicator for prediction of wine aroma intensity. At the same time, RPA values for all five the most abundant aroma compounds were not significantly influenced by vineyard site and therefore could not effectively discriminated among them.

Meanwhile, other aroma compounds were identified (*trans*-ocimene, 2-methyl-1-butanol, myrcene, α-phelandrene, *cis*-ocimene and 3-methyl-1-butanol) that noticeably less participate in total flavour description, but they still enable notable clone candidates discrimination using LDA method according to the individual compounds (only within individual vineyard site). Results of these aroma compounds showed that influence of vineyard site (soil, climate, fertilization and other) was dominant over clone genetic potential when it is placed in other environment (or ecological) conditions, that their RPA values for individual clones and their order (rank) were not consistent on different location. However, within individual site, clones were through three very different vintages retained their mutual relations regarding to aroma compounds synthesis and it was possible to differentiate them.

Analysis of must aroma compounds enabled positive discrimination of two clones ŠK-32 and ŠK-57 in comparison to others. It is necessary to initiate additional comparative study on must and further the wines of these clones in order to find answer what is must aroma profile analysis can help in clonal selection procedure.

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