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Three-dimensional approach for identification of red grape cultivars by fingerprint of wine anthocyanins

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Summary

The authenticity of grape cultivars is an important tool for wine controlling systems. The identification of red grape cultivars by wine anthocyanin spectra is a well-known method, but the analysis of applied parameters does not produce exact results. This work aimed at identifying substantive parameters, which, if applied simultaneously, will allow identifying numerous cultivars with high certainty. Liquid chromatography has been applied to obtain anthocyanin profiles of single-cultivar red wines made of various cultivars. Ratios of the peak areas of individual anthocyanins were identified. The proportions of anthocyanins depend on many factors, but their constancy is predetermined genetically. Certain ratios of peak areas of individual anthocyanins can be estimated as constant and typical for concrete cultivars. Revealed combinations of three ratios were estimated as unique for each cultivar. Moreover, different clones of certain cultivars have different combinations of constant ratios. Analysed samples were distributed in groups according to the combination of constancies of three ratios manifested in chromatographic peaks. The three-dimensional model produced allows data distribution in different groups. Each group corresponds to individual cultivars or different clones of one cultivar. Their belonging to concrete groups makes it possible to efficiently identify unknown single-cultivar wines and detect forgery.

K e y w o r d s : Liquid chromatography; authenticity; red wine; clones; 'Saperavi'.

Introduction

Among other known methods, the evaluation of anthocyan compositions in red wines is the important approach to the identification of red grape cultivars. The anthocyanins are an important quality parameter of red grapes due to their ability to give red colour to wines by the passage of anthocyanins from grapes into wine during maceration and winemaking.

Nine major anthocyanins are identified in red wines. They are mostly responsible for colour and other properties of wines due to anthocyanins. There are monoglucosides of delphynidin (De-3-gl), cyanidin (Cy-3-gl), petunidin (Pt-3-gl), peonidin (Pe-3-gl), and malvidin (Mv-3-gl), as well as acetylated and coumarylated glucosides of peonidin (Pe-3-acgl, Pe-3-cugl) and malvidin (Mv-3-acgl, Mv-3cugl), of which the amount of malvidin-3-glucoside reaches in most cases up to 40 % of the total composition of anthocyanins (Fei He *et al.* 2012).

Composition of anthocyanins are genetically predetermined in red grape cultivars (Holton and Cornish 1995, Fei He *et al.* 2010, Azuma 2017) and are typical of certain varieties of grapes. Although the total amount of anthocyanins in wines depends on weather conditions, vintage, vineyard treatment, and vinification methods, all these variables have little effect on the composition of anthocyanins in wines (Eder 2002). Correspondingly, the relative proportions of individual anthocyanins are typical for concrete grape cultivars (Arozarena *et al.* 2000 a, b). On the other hand, some authors report that oxidation, hydrolysis, condensation, and other reactions take place from the beginning of wine production. In addition, ageing also leads to a decrease in the amount of anthocyanins in wines (Cheynier *et al.* 1994, Dallas *et al.* 1995, Fulcrand *et al.* 1996, Monagas *et al.* 2005). In spite of this, interaction between anthocyanins can be used for characterising grape cultivars. Roggero *et al.* (1988) and Larice *et al.* (1989) wrote about several approaches to identifying varieties through relative ratios of individual anthocyanins. The main approach is the ratio between acetylated and coumarylated monoglucosides of peonidin and malvidin. Shikimic acid (Holbach *et al.* 2001) has been applied as additional variables besides the ratio of acetylated peonidin to coumarylated one for identification and classification of cultivars. Discriminant Canonical Analysis (Otteneder *et al.* 2002, 2003, 2004, Von Baer *et al.* 2007, González-Neves *et al.* 2007, Kumšta *et al.* 2014) and Principal Component Analysis (Holbach *et al.* 1997, Berente *et al.* 2000, De Andrade *et al.* 2013) has been used to allow distributing of unknown samples in appropriate groups of cultivars. It is important to choose the constants of certain ratios, since in this case the absolute content of individual anthocyanins in wine is not an important value which in turn depends on such factors as squeezing, maceration, stabilization, filtering, etc., as mentioned above. In our case, the factor is

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the biosynthesis of anthocyanins due to genetic factors that directly links with a variety of cultivars. Consequently, the aim of this work was to identify and select at least three such ratios (variables) of individual peak areas of anthocyanins that are constant (in reality, they are scattered in a small range within the limits of a statistical error) and can be used in combination for the creation of a three-dimensional model. Correspondingly, it becomes possible to characterise many red grape cultivars through analysing single-cultivar wines with a high level of confidence. Interest of our investigation spans only the wines produced exclusively from cultivars of *Vitis vinifera*.

The combinations of three variables have been selected in our work and used for the purpose of creating three-dimensional models.

Material and Methods

Chemicals and reagents: Acetonitrile (HPLC grade) and formic acid (HPLC grade) were obtained from Merck (Germany). Commercially available Standard substances of Delphinidin-3-glucoside chloride, Cyanidin-3-glucoside chloride, Petunidin-3-glucoside chloride, Peonidin-3-glucoside chloride and Malvidin-3-glucoside chloride were purchased from Roth (Germany). HPLC water was purified with a Milli-Q system (Millipore, USA).

Standard solutions: The stock solutions of standard substances were prepared by the dilution of 1 mg powdered standard with water/acetonitrile (50/50) to a final concentration of 500 $mg \cdot L^{-1}$. These solutions were stored under refrigeration at -18 °C until they were analysed.

Samples: A total of 191 samples of dry red wines have been analysed. The main test material were commercially available bottled wines of different years from different regions and climatic zones of Georgia produced by different wine companies, as well as some home-made wines. Among them, 172 samples were vinified from popular and widespread indigenous cultivar 'Saperavi', including several clones of this cultivar. To show the difference, wines produced from other Georgian indigenous cultivars: 'Usakhelouri' (6 samples), 'Alexandrouli' (1 sample), and Ockhanuri Sapere (8 samples) have also been analysed. Several imported wine samples from non-Georgian cultivars have been analysed, too: 'Blauer Spätburgunder' (1 sample) (from Germany), 'Cabernet Sauvignon' (1 sample) (from Chile) 'Shiraz' (1 sample) (from Chile) and 'Montepulciano' (1 sample) (from Italy). All wines were stored in a dark room at 11 °C, until analysis. All analysed wines were no older than 7 years by the time they were analysed. At least five HPLC analytical replications of each sample were performed. Average values were taken into account for data analysis.

Sample preparation: Wine samples were filtered through 0.45 μm membrane filters (MF-Millipore™, USA), transferred to vials and 20 μL of each solution was injected into the HPLC equipment.

Equipment: Knauer (Berlin, Germany) system with gradient pump WellChrom K-1001; DAD detector WellChrom UV-Vis K-2501 with 190-740 nm range; Autosampler Smartline 3800; Solvent Organizer K-1500 WellChrom; on-line degasser and thermostat. Chromatographic separation was performed in an LiChroCart 250-4 with LiChrospher 100 RP 18 (5 μm) (Merck, Darmstadt, Germany) column (250 mm × 4.6 mm). The column temperature was set at 50 °C. Operating software - ChromGate V.3.1.

HPLC analysis: The chromatographic separation of the anthocyanins was achieved using a mobile phase containing solvent A of water/formic acid/acetonitrile (87:10:3, v/v/v) and solvent B of water/formic acid/acetonitrile (40:10:50, v/v/v). The flow rate was set as 0.8 $mL \cdot min^{-1}$ and the detection was performed at 518 nm. Gradient elution listed below was used. Anthocyanins were identified by comparison of their spectra with commercial standards and by their elution order with previously published separations (Holbach *et al.* 1997, Berente *et al.* 2000).

Gradient Programme

Time solvent B min (v/v)	Solvent % (v/v)	%
0	94	6
1	94	6
15	92	8
18	75	25
21	70	30
24	60	40
35	50	50
37	20	80
44	20	80
46	94	6
57	94	6

Plots: 3D Plots were done with SciDAVis 2D/3D Plotting Software.

Results and Discussion

The HPLC method was used to detect the fingerprints of anthocyan profiles in wine samples. The method reported by the OIV - Compendium of International Methods of Wine and Must Analysis 2006 was taken as basis. We introduced changes in its gradient programme of the mobile phase to achieve baseline separation of all components presented. Another reason for changing the gradient was to achieve better rinsing and equilibration of the chromatographic column for the next analysis in series. Fig. 1 shows a typical chromatogram for wine produced from *V. vinifera* 'Saperavi'.

Ratios of individual anthocyanin components were calculated by dividing the peak areas expressed in AUs. For this purpose, peak areas of nine major anthocyanins were summed up and set as 100 %. The peak areas of unidentified compounds have not been taken into account. A total of 191 single-cultivar wine samples have been analysed. Taking into account that biosynthesis of anthocya-

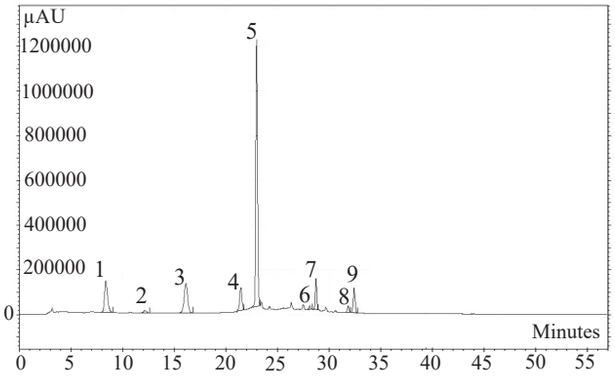

Fig 1: Chromatogram for wine produced from *V. vinifera,* 'Saperavi'. Peak numbers define appropriate anthocyanins: **1**: Delphinidin-3-glucoside; **2**: Cyanidin-3-glucoside; **3**: Petunidin-3-glucoside; **4**: Peonidin-3-glucoside; **5**: Malvidin-3-glucoside; **6**: Peonidin-3-acetylglucoside; **7**: Malvidin-3-acetylglucoside; **8**: Peonidin-3-coumaroylglucoside; **9**: Malvidin-3-coumaroylglucoside.

nins is genetically predetermined and that relative ratios of anthocyanin contents are weakly influenced by vinification technique, the main idea of this work was to select the constant ratios of individual anthocyanins, with this constancy being unique only for certain grape cultivars. However, it should be noted that a wide variety of self-associations and polymerisation reactions of monomeric anthocyanins occur in wines as well as modification reactions with other compounds. As a result, concentration of monomeric anthocyanins in red wines steadily decreases during the aging of wines. After a number of years, the content of monomeric anthocyanins significantly decreases, but the wines can remain red or mostly red. Of course, the processes of the aging of wines significantly differ, depending on many parameters of particular wines. To achieve reliable results, the age of all analysed wines was limited to seven years.

The object of our research were the wines prepared by different methods of maceration and vinification, undergone to different fermentation time and aging period. This means different enzymatic activity in the biosynthesis of anthocyanins and that their overall profile varies depending on all of the factors listed above. Based on this, we searched for such factors where not the overall profile is important, but the ratios of separate components are constant.

As mentioned before, nine major anthocyanins were identified, but eventually seven peak areas were used as data (variations) for the next evaluation. The sum of Pe-3-acgl and Mv-3-acgl was set as one variation and the sum of coumarylated glucosides of Pe-3-cugl and Mv-3-cugl was set as another. The variations of the ratios of the peak areas between separate anthocyanins and the aforementioned two sums and also ratios of the peak areas between separate anthocyanins and the aforementioned two sums and all possible combinations of monoglucosides of De-3-gl, Cy-3-gl, Pt-3-gl, Pe-3-gl and Mv-3-gl, as well as the sums of acetylated glucosides of Pe-3-acgl and Mv-3-acgl and the sums of coumarylated glucosides of Pe-3-cugl and Mv-3-cugl, were also evaluated. First, we applied combinatorial analysis. Using the basic formulas of combinatorics, using the method of combinations without repetitions (combinations refer to various combinations of m objects that are selected from a set of different n objects and which differ from each other by at least one object. In other words, a single combination is a unique sample of m elements in which their order (location) is not important). Specifically, for our case, we calculate the total number of such unique combinations using the formula

$$C_m^n = \frac{n!}{(n-m)!\,m!}$$

where C_m^n is the number of combinations of n elements by m. Thus, in our case, n = 7, and m = 2, as the combinations start from two elements. The number of relationships like a/b can be calculated by a direct search method, which gives 21 variants for n = 7. Given the fact that the number of objects (peak areas) is 7, the generalised combination formula takes the shape of

$$N = 21 + 7 \cdot \sum_{m=0}^{7} \frac{n!}{(n-m)!\,m!}$$

and gives 861 combinations for each analysis. Then, a table is created in the Excel format with the appropriate formulas for all combinations. Further data processing for all analyses is carried out in this format.

A regularity (recurrent data) was observed in three cases. In particular, constancies of three different ratios were detected. One of them is well known and has been firstly substantially described by authors Holbach *et al.* 2001 – ratio of acetylated to coumarylated anthocyanins (the sum of acetylated glucosides of peonidin and malvidin and the sum of coumarylated glucosides of peonidin and malvidin). The second constancy observed was the ratio of Pt-3-gl to De-3-gl. The third constancy was the ratio of Mv-3-gl to sum of De-3-gl, Cy-3-gl, Pt-3-gl and Pe-3-gl. These observed constancies can be defined as variables unique for each different cultivar and thus used for the identification of grape cultivars. Applying three variables simultaneously, it is possible to build a 3D distribution and in this way, make correct identification of grape cultivars highly reliable, making the likelihood of a mistake negligible.

A majority of analysed wines of different names, types, places of origin, yields, and wine-makers were produced from 'Saperavi' which is most popular and widespread in Georgia.

A total of 161 such wine samples were analysed. Obtained results such as numerical data for the aforementioned variables were analysed and divided in several groups according to different combinations of these three variables. Finally, all 161 samples were divided into 12 groups with different numbers of samples. Each group corresponds to one unique combination. In contrast to the conventional ratio $R_{ac/cum}$, we used in our work $R_{cum/ac}$ to make 3D image more perceptible. Twelve groups for the exception of one 'Saperavi' indicate different clones of this variety of grapes. Thus, our approach is appropriate for the identification of not only different varieties of grapes, but also different clones. Statistical data are given in Tab 1. Among these 161 samples, several wines were defined as produced from undeclared red grape varieties, but they were positively

Table 1

Statistical data for the identified peak area ratios of different clones of 'Saperavi'. Statistical data for the ratio of the peak area of Pt-3-gl to the peak area of De-3-gl ($R_{Pt/De}$), for the ratio of the peak area of Mv-3-gl to the sum of peak areas of De-3-gl, Cy-3-gl, Pt-3-gl and Pe-3-gl ($R_{Mv/DeCyPtPe}$) and the ratio of the sum of peak areas of cumarylated glucosides of peonidin (Pe-3-cugl) and malvidin (Mv-3-cugl) to the sum of peak areas of acetylated ones ($R_{cum/ac}$). Data are divided in 12 groups of clones of 'Saperavi'. S1 - S12 designate 12 different clones of 'Saperavi'

Cultivar group		S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
Number of samples per group		32	30	20	14	10	4	12	15	6	5	7	6
Ratio													
$R_{Pt/De}$	Min	1.18	1.04	1.03	1.13	1.28	0.89	1.09	1.15	0.94	1.32	1.15	1.17
	Max	1.36	1.16	1.16	1.25	1.40	1.09	1.21	1.35	1.10	1.42	1.24	1.29
	Mean	1.28	1.09	1.10	1.18	1.34	1.00	1.17	1.24	1.01	1.37	1.20	1.22
$R_{Mv/DeCyPtPe}$	Min	2.42	1.67	1.75	2.01	2.69	1.41	1.76	2.04	1.43	3.18	1.93	2.23
	Max	2.61	1.85	1.95	2.16	2.82	1.51	1.93	2.20	1.58	3.30	2.09	2.41
	Mean	2.51	1.76	1.82	2.11	2.74	1.46	1.86	2.13	1.51	3.23	2.00	2.32
$R_{cum/ac}$	Min	1.10	1.38	1.07	0.84	0.84	1.98	0.71	1.20	1.23	0.79	1.82	1.62
	Max	1.25	1.55	1,.23	0.93	0.98	2.04	0.81	1.36	1.32	0.85	1.94	1.70
	Mean	1.17	1.47	1.15	0.88	0.90	1.99	0.76	1.27	1.27	0.81	1.88	1.67

distributed into the 'Saperavi' groups in accordance with the combinations of proper variables. These samples were home-made wines and were provided by peasants from their own small old vineyards.

Some groups with four or five samples are not statistically sufficiently reliable, but vineyards such wines were produced from, belong to 'Saperavi', which was confirmed by ampelographic analyses carried out by Institute of Viticulture and Oenology of Agricultural University of Georgia. The characteristics were reordered visually through quick and precise characterisation of grape varieties in accordance with primary descriptor priority list recommended for use by OIV and given in the 2nd edition of the OIV descriptor list for grape varieties and *Vitis* species (2009) and also in accordance with the official list of 84 ampelographic characteristics providing a sufficiently accurate description of varieties given in the OIV Code.

To ensure the reliability of results, Grubbs' test has been used to detect outliers in the set of each variable in all 12 groups. No outliers were detected.

Some wines produced from other Georgian authentic grape cultivars such as 'Usakhelauri' (6 samples), 'Alexandrouli' (1 sample), and 'Ochkhanuri Sapere' (8 samples) were analysed. All of them show different combinations in comparison with all clones of 'Saperavi'. Four imported wines produced from 'Blauer Spätburgunder' (Germany), 'Cabernet Sauvignon' (Chile), 'Shiraz' (Chile) and 'Montepulciano' (Italy) were also analysed. Obtained combinations of variables were different in all cases. Statistical data are given in Tab. 2.

The combinations of three variables have been selected in our work. A three-dimensional model of the obtained data (variables) for all samples is shown in Fig. 2.

To identify varieties by analyzing anthocyanins in wine, a number of authors applied statistical methods of data processing, including Discriminant Canonical Analysis and Principal Component Analysis. In some cases, as object of the study the authors used freshly made wines specifically for research. In other cases, the object was bottled commercially available wine. However, according to publications, in all cases two factors were taken as the basis for conclusions. It were either the ratio of the sum of acetylated and coumarylated anthocyanins ($V_{ac/cum}$) and the sum of acetylated and the remaining five major anthocyanins (Otteneder *et al.* 2002), or $V_{ac/cum}$ and the sum of acetylated anthocyanins (Otteneder *et al.* 2003, 2004), or different individual anthocyanins and acetylated anthocyanins depending on the varieties (González-Neves *et al.* 2007), or different individual anthocyanins and acetylated and coumarylated anthocyanins depending on the regions

Table 2

Defined peak area ratios $R_{Pt/De}$, $R_{Mv/DeCyPtPe}$ and $R_{cum/ac}$ for listed varieties

Varieties	$R_{Pt/De}$	$R_{Mv/DeCyPtPe}$	$R_{cum/ac}$
Alexandrouli	0.97	1.33	3.27
Usakhelouri (sample I)	1.20	2.94	0.68
Usakhelouri (sample II)	1.15	3.06	0.64
Usakhelouri (sample III)	1.20	2.93	0.71
Usakhelouri (sample IV)	1.22	2.98	0.66
Usakhelouri (sample V)	1.14	3.02	0.65
Usakhelouri (sample VI)	1.17	3.04	0.70
Ochkhanuri Sapere (sample I)	1.22	1.64	0.42
Ochkhanuri Sapere (sample II)	1.19	1.61	0.39
Ochkhanuri Sapere (sample III)	1.16	1.59	0.45
Ochkhanuri Sapere (sample IV)	1.14	1.59	0.45
Ochkhanuri Sapere (sample V)	1.19	1.70	0.39
Ochkhanuri Sapere (sample VI)	1.21	1.60	0.46
Ochkhanuri Sapere (sample VII)	1.17	1.66	0.38
Ochkhanuri Sapere (sample VIII)	1.14	1.60	0.38
Montepulciano	1.88	5.11	0.40
Blauer Spaetburgunder	2.09	2.07	-
Shiraz	1.85	6.67	0.27
Cabernet Sauvignon	1.06	3.30	0.28

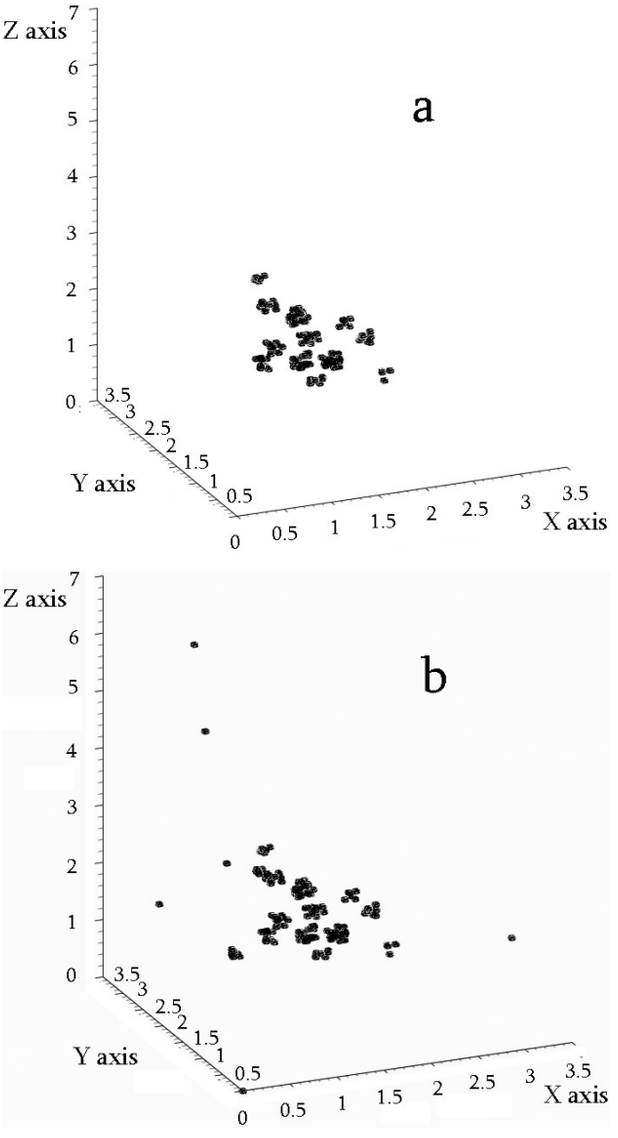

Fig 2: 3D-images for all combinations of variables of all analysed samples pooled in different groups. **a**: image of 12 groups of wines produced from 'Saperavi' variety and listed in Tab. 1; **b**: image of 12 groups listed in Tab. 1 and of other varieties given in Tab. 2. X axis: Ratio of peak areas of cumarylated anthocyans to acylated ones ($R_{cum/ac}$); Y axis: Ratio of peak areas of Pt-3-gl to De-3-gl ($R_{Pt/De}$); Z axis: Ratio of peak area of Mv-3-gl to the sum of De-3-gl, Cy-3-gl, Pt-3-gl and Pe-3-gl ($R_{Mv/DeCyPtPe}$)

(De Andrade *et al.* 2013). Also it were either $V_{ac/cum}$ and shikimic acid as a parameter, as well as $V_{ac/cum}$ and in contrast among malvidin, acetylated glucosides of malvidin and peonidin, acetylated glucosides of peonidin, petunidin, coumarylated glucosides of peonidin, delphynidin, cyanidin, as well as log peonidin % and ratio of total Quercetin and Myricetin content (Von Baer *et al.* 2007), or acetylated anthocyanins and free anthocyanins (Berente *et al.* 2000), or different individual anthocyanins (Kumšta *et al.* 2014). These approaches provide an acceptable level of simultaneous identification of varieties for a relatively small number of them. Classification of varieties by $V_{ac/cum}$ ratio and shikimic acid content as a parameter is also described in the work of the authors (Holbach *et al.* 2001). Ultimately, all these approaches give us a planar (2D) image of the distribution by variety with a noticeable fluctuation and consequently the overlapping of the results. It should also be noted that in the studies cited, a limited number of varieties were analyzed simultaneously.

The more varieties analysed at the same time, the more homogeneous the matrix should look. However, images below give a full impression of individual groups of cultivars, because combinations of variables for each cultivar build separate unique three-dimensional groups. Each group corresponds to one concrete grape cultivar or clone. Thus, the 3D method is suitable for the classification of grape cultivars as seen in Fig 2. Discrete points that stand out in the main group correspond to wines those are produced from other Georgian endemic varieties (not 'Saperavi') and also from other well-known worldwide varieties listed in Tab 2. These samples were included in our study to show that points in the 3D model are grouped closer to each other for the clones of any variety, and groups of different varieties are noticeably distant and scattered. This provision is one more argument in favor of the correctness of the presented 3D model.

Finally, it must be noted that our approach is effective for wines produced from one definite grape cultivar (or clone), hence for single-cultivar wines. Our approach to the identification of varieties and their clones has not previously been encountered in the literature and is original.

The amount of fixed clones of 'Saperavi' is not surprising since 'Saperavi' population mostly emerged long ago ('Saperavi' - an ancient grape that traces its origin from the Kakheti region of Georgia and the nearby regions as far back as 6000 B.C.). The local Georgian cultivar represents the collection of clones that preserve local signs and adaptability to certain conditions. 'Saperavi' is one of the main technical cultivars for the production of different types of wines. Besides, active breeding and clone development was carried out during Soviet times in order to increase yields and adapt the cultivar to different climatic conditions to enhance resistance to diseases and pests, as before regaining independence in 1990, Georgia was one of the main suppliers of wine in the former Soviet Union.

Conclusions

Thus, identified combinations of three variables - the ratio of Pt-3-gl to De-3-gl ($R_{Pt/De}$), the ratio of Mv-3-gl to the sum of De-3-gl, Cy-3-gl, Pt-3-gl and Pe-3-gl ($R_{Mv/DeCyPtPe}$), and the ratio of cumarylated anthocyanins to acetylated ones ($R_{cum/ac}$) are unique for each grape cultivar. Moreover, different clones of grape cultivars have their own typical combinations of these variables.

According to the combination of constancy of specified anthocyanin ratios, 180 of 191 wine samples were distributed in groups. Eleven samples could not be classified. Obviously, they belong to wines produced from different not systematised cultivars or their clones or mixtures of cultivars. The identification of cultivars by presented 3D model becomes impossible in this last case.

The three-dimensional model created from obtained data makes it possible to form groups that correspond to

different cultivars or different clones of one cultivar in space and by doing so, determine and classify high numbers of grape cultivars and/or clones by concrete groups. In this way, it becomes possible to efficiently determine the belonging of unknown single-cultivar wines to particular groups and to detect forgery.

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