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Biotechnology

Dependence of Phenolic Compound Content on Geographical Distribution of Chkhaveri Grapes in Adjara

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ABSTRACT. Technical characterization and chemical analyses, including total content of biologically active compounds and their antioxidant activities in grape, juice and wine of "Chkhaveri", the western Georgian endemic variety, have been carried out. Amount of phenolic compounds were determined using Folin–Ciocalteu reagent. Existence of flavonoids, catechins and anthocyanidins has been detected by spectral method; antioxidant activity by DPPH method. The following research involves technical characterization and chemical analyses of grape, juice, and wine of "Chkhaveri", western Georgian endemic grapes. The study involves determination of the total amount of biologically active compounds and their antioxidant activity. The quantity of phenols was identified by Folin-Ciocalteu reagent. Flavonoids, catechins and anthocyanins are also present and confirmed by spectral method. The antioxidant activity was analysed with DPPH method. The dependence of phenol, anthocyanins, flavonoids, and catechin content and the antioxidant activity on geographical distribution of grapes was established. In the Chkhaveri grape the dominant anthocyanins (HPLC-Prep column, UPLC-MS) malvidin 3-O-glucoside m/z 493, peonidin 3-O-glucoside m/z 463, cyanidin-3-O-glucoside m/z 449, petunidin 3-O-glucoside m/z 479, delphinidin-3-glucoside m/z465, flavon-3-ols -catechin m/z 291, phenolic acid - gallic acid m/z 171 are studied. *© 2019 Bull. Georg. Natl. Acad. Sci.*

Key words: Chkhaveri, wine, bioactive content

Cultivation of vine in Georgia is known for more than 8000 years [1]. Only in one region of Georgia - Adjara more than 44 varieties of vine can be found, including 34 local and 16 introduced ones. Among them there are 27 kinds of red grapes, 16 kinds of white grapes and one variety of pink grapes [2].

During wine-making process, the number of extracted phenolic compounds grows in the conditions of increasing fermentation quality and a long-term maceration [3-13]. Among wine phenols there is a group of flavonoids, which includes catechins, flavonoids, anthocyanins and tannins [14-17]. Most of these substances are

found in all types of wines, although some of them characterize only one particular type of wine. Their number in wine depends on the kind of vine, its quality, its age, wine making technology, methods and processes of wine-making such as maceration, use of enzymes, boiling temperature and other environmental conditions [18,19].

The goal of our research is to define the qualitative and quantitative composition of biologically active compounds, their antioxidant activity and technical characteristics of semi-dry pink wine made by European technology and juice taken from the oldest vine varieties of the Black Sea basin – the autochthonous promising and popular varieties of Chkhaveri (planted at different heights of the sea level). We aim to identify individual phenolic compounds in fruit, juice and semi sweet rose wine (processed by European technology) and evaluate their antioxidant properties.

Materials and Methods

Grape berries of Chkhaveri were collected in November from different regions of Adjara, western Georgia. The study was conducted for three years (2016-2018). Samples were collected during the period of technical maturity. Chkhaveri is a grape variety of the late period.

The samples were collected at 5 m above the sea level (Kobuleti), 300 m (Koromkheti), 360 m (Erketi), 380 m (Vaio), 400 m (Ortsva), 780 m (Jalabashvilebi). Grape bunches were washed and dried at room temperature and three-fold extraction of 80% ethanol (100 ml per gram of the sample) was conducted, then the extracts were combined, freezed in the freezer at -30°C and filtered, Cartridge Solid Phase Extraction (SPE-Waters Sep-Pak C18 500 mg).

Juice was obtained directly from the peeled grapes. To make wine 5 kg of Chkhaveri grapes with skin were put in glassvessels and fermented with enzymatic yeast (*Saccaromyces cerevisiae*). The fermentation lasted for 5 days, during which the fermenting mass was systematically mixed in the glassware and protected from air through the valve. Then we released wine from the pulp, filtered and continued maturation. The obtained wine was further stored in the refrigerator at temperature of 8°C [20].

For the qualitative analysis extraction was performed only on the skin, extract was then put into vacuum distilator. Afterwards it was filtered using Cartridge Solid Phase Extraction (SPE) Waters Sep-Pak C18 (500 mg).

Biochemical analysis was conducted using different physico-chemical and instrumental methods. Separation-identification and quantative analysis was conducted using UPLC-MS (Waters Acquity QDa detector), HPLC (Waters Breeze 1525, UV-Vis 2489 detectors), pH-meters (Mettler Toledo), refractrometer – Misco, spectrometer – Cuvette Changer (Mettler Toledo UV5A), chemicals – stability radical-2,2-diphenil-1-picrilhydrazyl (Aldrich-Germany), aluminum chloride ($AlCl_3$), Folin-Ciocalteu reagent (preparation), standards – gallic acid, rutin. C18 Cartridge Solid Phase Extraction (SPE) Waters Sep-Pak C18 (500 mg).

The determination of dry substance is done with refractometer in juice, while water and dry matter are determined by a standard thermogravimetric method (digital refractometer # PA202 Palm Abbe MISCO); determination of pH and titrated acidity is done with potentimeter (METTLER TOLEDO) by AOAC method; Folin-Ciocalteu's spectrophotometric method is used in determination of phenols. Extraction of the samples was conducted using 80% ethylalcohol, at temperature of 70-75^{0}C. Extract of 0.5 or 1.0 is poured into 25 ml volumetric flask, and 5.0 ml of water with 1.0 ml of folin-ciocalteu reagent is added. In 8 minutes at 25^{0}C, 10.0 ml of 7% Na_2CO_3, flask is then filled with water and left at room temperature for 2 hours. Determination is conducted at 750 nm. As control 1 ml of extragent is used. After the

values are obtained the calculations are performed using calibration curve of gallic acid. The formula used to determine phenols is provided X = (D K V F) x 1000 / mX – amount of phenols mg/kg; D – optical density; K – coefficient; F – factor of dilution; V – volume of extract in ml; m – mass of the raw material used for extraction in grams. Folin-Ciocalteu's reagent is prepared by adding 10 grams of sodium volphramate and 2.5 grams of sodium molibdate to 70 ml of water. Also, 5 ml of 85% phosphoric acid and 10 ml of hydrochloric acid are added to solution. Solution is left for 10 hours. Then 15 grams of lithium sulfate and 1 drop of bromium are added with 5 ml of water. In 15 minutes 100 ml of water is added [9,10].

Antioxidant activity. Determination of antioxidant activity using DPPH method. In general, the methods for determination of antioxidant activity can be divided in several groups like, photometric, methods. One of the most popular methods is DPPH (DPPH – ALDRICH, LOT#STBD4147V (product of Germany) free radical colorimetric with 50% of radical inhibition. This method was first described in 1958 by Blois and then it was modified for several times. DPPH method is known for being fast, easy and precise test-method. It is widely used not only for determining the capability of retaining free radicals but also determining antioxidant activity in food products and juices. DPPH – ($C_{18}H_{12}N_5O_6$ M=394.33) is a stable free radical with maximum absorbance at 515-517 nm. The extract that is dissolved in methanol is initially violet but after the reduction it becomes yellow. Reaction is provided:

DPPH + AH $\rightarrow$ DPPH-H + A; DPPH + R $\rightarrow$ DPPH-R. AH is antioxidant and R is free radical. For determination of antioxidant activity – radical retention to the 1 ml of the sample 3 ml of DPPH extract (0.1 mM DPPH-0.004 g/100mL in ethyl alcohol) and after 30 minutes optical density was evaluated on spectrophotometer. DPPH and 96% ethyl alcohol were used as blanks. Formula used to determine activity of free radical inhibition (DPPH) is provided below:

$$In\ \% = A_C - A_S/A_C\ x100\%$$

Ac indicates absorbtion of DPPH/Alcohol solution, and As indicates absorption of the extract [17,19, 21]

Determination of total monomeric anthocyanin pigment content by the pH differential method [22]. The quantitative determination of the common flavonoids by a spectral method: the extraction of a sample taken for analysis was carried out by 80% ethyl spirit; 1 ml of extract, taken from the total volume, was placed in 10 ml flask, then 5 ml of H_2O was added, 0.3 ml of 5% $NaNO_2$ was aged for 5 minutes; then we added 0.3 ml of 10% $AlCl_3$, which was aged for 6 minutes, then 2 ml was added to 1N NaOH, and the determination occured at 510 nm. 1 ml of the appropriate extract, taken for control, underwent the same process. The data, received as a result of the determination, was calculated on the Routine Fibre Curve. Composition of common flavonoids was calculated by the following formula:

$$X = (\ D\ K\ V\ F)\ x\ 1000\ /\ m,$$

where, X is a composition of common flavonoids, measured in mg/kg;
D is an optical density;
K – Routine Calculation Coefficient;
F – dilution factor;
V – total volume of extract, (ml);
m – the mass of the raw materials extracted, (g).

Anthocyanin HPLC analysis: C18 analytical and preparative columns. Solvent A: Water/Formic acid/Acetonitrile 87:10:3 (v/v/v)

Solvent B: Water/Formic acid/Acetonitrile 40:10:50 (v/v/v) gradient (0-15 min- 6%-from 30% B, 30 min 50% B, 35 min 60% B, 41-45 min 6% B). Detection of anthocyanin 518 nm, flavonoid glycoside 370 nm. UPLC-MS analysis BEN C18, 1.7µm, BEN Amide 1.7µm, columns. Eluents acetonitrile formic acid, formic acid (gradient), Flow 0.4 ml/min, column temp 50^0C, MS- scan 40-1200 da, Probe 500^0C, Positive 0.8 kV, Capilari 1.5 kV, CV -15, all samples before chromatography filtration (Waters Acrodisc LC PVDF Filter 13 mm 0.45 µm).

Results and Discussion

Chkhaveri is a medium-yield (5.5-8 t/ha) variety, characterized by volatile productivity, especially in the high mountainous region of Adjara. The bunches are medium or less than medium size. Their length is 13.0-15.8 cm and the width is 8.0-16.0 cm. The number of berry grapes on bunches reaches 90-100. The mass of bunches varies between 126.0-383.6 grams.

All of the analyzed Chkhaveri's samples were characterized by dark-red color of bunches, the round grains and sweet taste; only in Jalabashvilebi Chkhaveri berries have sweet-sour taste. The fruit of the plant in Erketi (360 m above sea level) had large bunch mass (383.6 g) and high sugar content (20.1%), while Chkhaveri in Kobuleti (5 m from sea level) territory was characterized by high acidic resistance – 0.95%. The individuality of Chkhaveri wine is relatively low level of sugar and titrated acid concentration unlike other grape varieties.

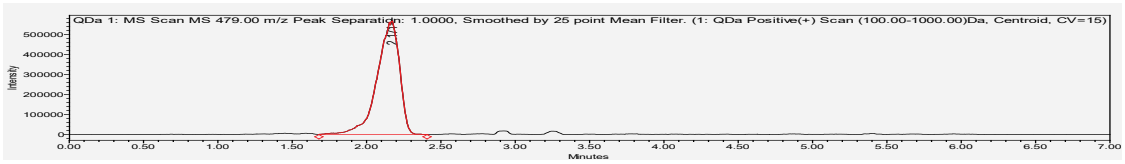
The common phenols, flavonoids and anthocyanins were identified in the grapes and their amount was compared according to the location. The data showed that in 2018 the grape harvest was the lowest.

The amount of total phenols in samples of 2016 fruit varies from 976.7 to 1767.0 mg/kg. Amount of anthocyans is 721.2-1630.2 mg/kg and the amount of flavonoids varies from 300.6 to 825.5 mg/kg. Relatively high amount of anthocyans was found in the Chkhaveri at 780-meter altitude – 1630.2 mg/kg. Accordingly, the amount of phenols and flavonoids is high – 1767.9 mg/kg and 825.5 mg/kg. These characteristics are low in samples gathered from 5 meters above sea level, anthocyans-721.2 mg/kg, total phenols-976.7 mg/kg, and flavonoids-300.6 mg/kg.

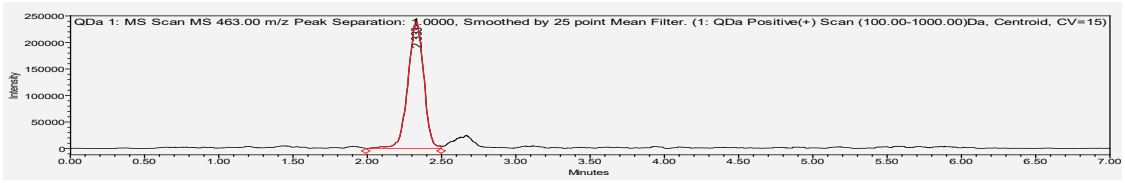
The intensity of phenolic compounds accumulation occurs at the highest altitude above the sea level and its number is much larger due to the relatively strict climatic conditions (low temperatures), with which the plant struggles with the help of the intensity of biologically active compounds accumulation, allowing to cope with the adverse conditions.

Researches continued monitoring the juice obtained from grapes. In contrast to grapes, anthocyanins were not detected in juice, while the number of common phenols was almost halved, and the number of flavonoids was changed from 501.16 mg/l to 305.7 mg / l.

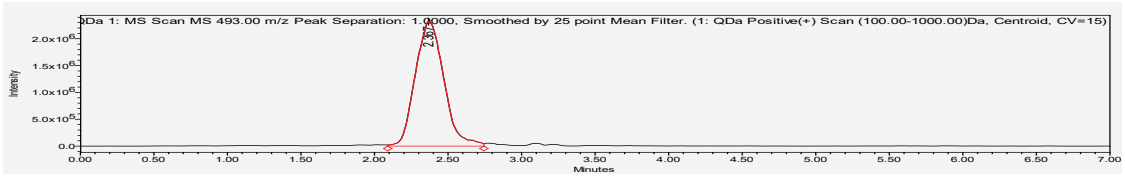
In the study of individual compounds of Chkhaveri, it became possible to obtain the individual fractions of the dominant anthocyanins HPLC-prep column, to study their spectral UPLC-MS, it also became possible to obtain malvidin 3-O-glucoside m/z 493 (fragment 331), peonidin 3-O-glucoside m/z 463 (fragment 301), cyanidin-3-O-glucoside m/z 449 (fragment 287), petunidin 3-O-glucoside m/z479 (fragment 317), delphiin-3-glucoside m/z465 (fragment 303), Flavon-3-ols – catechin m/z 291, phenolic acid-gallic acid m/z 171.



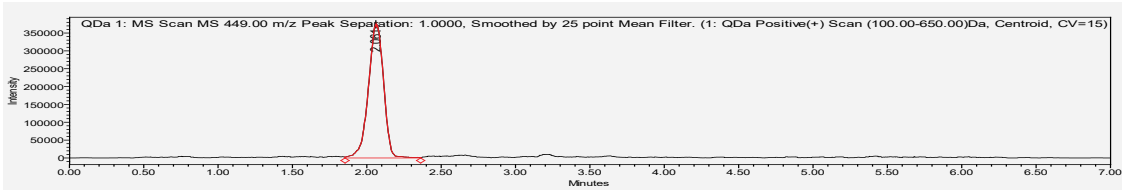
UPLC-MS Grape Chkhaveri petunidin 3-O-glucoside m/z479 (fragment 317)



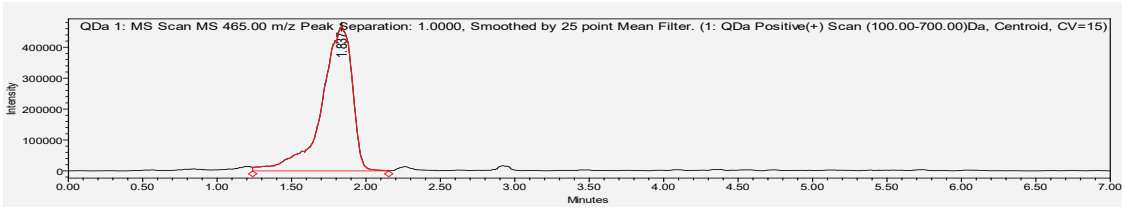
UPLC-MS Grape Chkhaveri peonidin 3-O-glucoside m/z 463 (fragment 301)



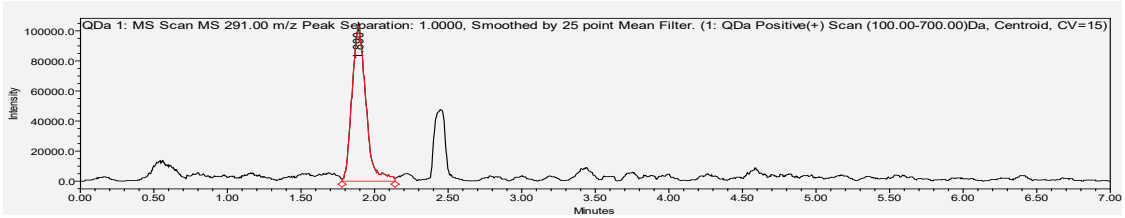
UPLC-MS Grape Chkhaveri malvidin 3-O-glucoside m/z 493 (fragment 331)



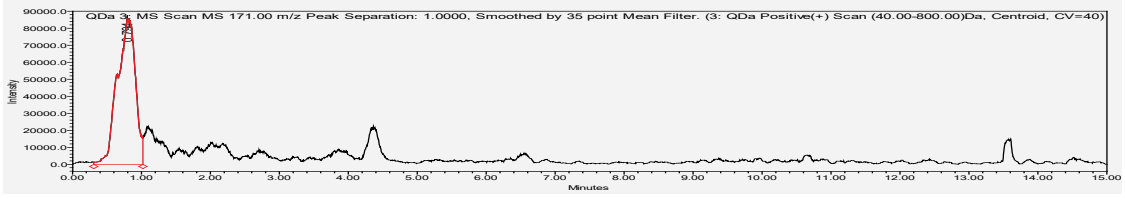
UPLC-MS Grape Chkhaveri Cyanidin-3-O-glucoside m/z 449 (fragment 287)



UPLC-MS Grape Chkhaveri Delphinidin-3-glucoside m/z465 (fragment 303)



UPLC-MS Grape Chkhaveri Catechin m/z 291



UPLC-MS Grape Chkhaveri Gallic acid m/z 171.

Fig. Individual phenolic compounds according to UPLC-MS.

As a rule, phenolic compounds are mainly localized in the grape skin, seeds and stems. The wine making technology significantly determines their content in the final product (Table 1,2). Maceration is one of the technological processes of wine making and it involves the interaction between the solid and liquid phase of the grapes for certain period of time, to obtain more extract and color. In the process of developing the wine-making technology from Chkhaveri, one of the most important tasks is to maintain a pleasant, typical pink color in the process of maceration and maturation.

Table 1. Quantity analysis of common phenols, flavonoids and anthocyanins of Chkhaveri grapes, juice and wine

Samplers name Chkhaveri	Monomeric anthocyanins by cyanidin-3-O-glucoside (mg/l)	Total phenols by gallic acid (mg/l)	Total flavonoids by ruthin (mg/l)
Grapes	939.8	1322.3	501.16
Juice	–	799.98	305.7
Wine	521.3	1057.7	23.5

More over, during the maceration the excessive or insufficient amount of extractive components may worsen the wine color, taste and other indicators of its quality; therefore, the procedures for the implementation of this process should be determined individually for each specific case, taking into consideration the grape variety and the parameters of the final product obtained from the macerated sweetener or wine material. The modes of maceration and maturation processes have been established in the process of the development of pink wine making technology from Chkhaveri. As we have already mentioned, anthocyanins have not been fixed at the time of Chkhaveri filtration. Its number increases during the maceration.

During the process, the total concentration of the anthocyanins was defined every day in order to determine the optimal period. At the same time, the pulp was pre-treated with enzyme preparations to provide equal fermentation. The 5th day turned out to be optimal to get pink wine. Indeed, color intensity increases after this day, it gains darker tones, however, the number of monomeric anthocyanins decreases and the polymerization of the anthocyanins is in progress correspondingly.

Table 2. Quantity content of common phenols, flavonoids and anthocyanins in Chkhaveri wine and antioxidant activity

Samplers name – Wine Chkhaveri	Monomeric anthocyanins by cyanidin-3-O-glucoside (mg/l)	Antioxidant activity DPPH 50% inactivation, ml wine
Vaio	318.61	0.031
Ortsva	356.14	0.025
Koromkheti	414.67	0.021
Jalabashvilebi	663.48	0.019
Kobuleti	309.11	0.033
Erketi	324.19	0.028

Nevertheless, the maceration contributes to increasing the amount of antioxidants to 521.3 mg/l, if we compare the grains, half of their amount turns into wine, while in juice they are not observed. The number of common phenols is slightly less than in the grains – 1057.7 mg/l, while the number of flavonoids is relatively small – 23.5 mg/l and is almost 20 times less than in juice. The antioxidant

activity has been calculated using 50% inhibition of samples. The antioxidant activity is quite high at an altitude of 780 m above sea level, it comprises 0.019 ml, correspondingly, and the activity decreases as we approach to the sea level. Therefore, there is a correlation between the antioxidant activity and the number of anthocyanins.

Conclusion

The fruits of the vine Chkhaveri, spread over various altitudes, differ in the total acidity of sugar as well as the content of common phenols, monomeric anthocyanins and flavonoids, what is caused by the climatic conditions. Among the analyzed 6 samples, the fruits, collected on the highest territory (780 m) from the sea level are distinguished by high content of the studied compounds. Chkhaveri grape juice does not contain anthocyanins unlike the grape skin. In the process of maceration its number increases in wine produced by the Imeretian technology. The most optimal time of maceration is 5 days. There was determined the directly proportional coloration between antioxidant activity and monomeric anthocyanins.

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ბიოტექნოლოგია

აჭარაში გავრცელებული ვაზის ჯიშის – ჩხავერის ყურძნისა და ღვინის ფენოლური ნაერთების დამოკიდებულება გავრცელების ადგილმდებარეობაზე

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**ბათუმის შოთა რუსთაველის სახელმწიფო უნივერსიტეტი, ბათუმი, საქართველო

(წარმოდგენილია აკადემიის წევრის თ. სადუნიშვილის მიერ)

შესწავლილია აჭარის რეგიონში (დასავლეთ საქართველო) გავრცელებული ვაზის ენდემური ჯიშის, ჩხავერის ყურძნის ტექნიკური მახასიათებლები, ყურძნის მარცვლის, წვენისა და ღვინის ქიმიური შედგენილობა. განსაზღვრულ იქნა ბიოლოგიურად აქტიურ ნაერთთა რაოდენობრივი შემცველობა და ანტიოქსიდანტური აქტივობა. საერთო ფენოლები განისაზღვრა ფოლინ-სიოქალთეუს მეთოდით, ფლავონოიდების, კატექინების და ანტოციანების პექტრალური მეთოდით, ხოლო ანტიოქსიდანტური აქტივობა DPPH მეთოდით. დადგენილ იქნა დამოკიდებულება საერთო ფენოლების, ანტოციანების, ფლავონოიდებისა და კატექინების შემცველობას, ანტიოქსიდანტურ აქტივობასა და გავრცელების ადგილმდებარეობას შორის. ჩხავერის ჯიშის ყურძენში იდენტიფიცირებულია დომინანტი ანტოციანები (HPLC-პრეპარატული სვეტი, UPLC-MS) მალვიდინ 3-O-გლუკოზიდი m/z 493, პეონიდინ 3-O-გლუკოზიდი m/z 463, ციანიდინ-3-O-გლუკოზიდი m/z 449, პეტუნიდინ 3-O-გლუკოზიდი m/z 479, დელფინიდინ-3-გლუკოზიდი m/z 465, ფლავონოლ-3-ოლი-კატექინი m/z 291 და ფენოლკარბონმჟავა – გალის მჟავა m/z 171.

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