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Research Article

Biologically Active Compounds and Antioxidant Activity of "Zeibel 5455" Grape-Seed Superfluid Extracts

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Abstract

"Zeibel 5455" is one of the hybrid varieties of colored grapes that does not contain diglycosidic forms of anthocyanins. It also provides raw material of ecologically clean grape, as chemical fertilizers and toxicants are not used during its cultivation.

The grape-seed dried up to 7 - 9% humidity by vacuum-sublimation method, was fragmented to MM-10 in a micromill on average of 50 - 100 mcm. Fraction and biologically active compounds were extracted from the grape-seed by two different methods.

In the first stage, vegetable fat was extracted by Waters Corporation's Supercritical Fluid Extractor SFE - 100-2-C10. In the second stage, fluid extraction of microdispersed micropowder of the grape-seed with ethyl alcohol was performed, namely optimal parameters of extraction were experimentally determined: pressure 100 bar, CO_2 delivery rate 7.5 kg/h. The quality of extraction was also affected by 75% ethyl alcohol in the form of solvent, the ratio of which to CO_2 was 21 - 22%.

A coupage of both extracts of Zeibel 5455 with 1:1 relationship and the concentration were made in the first stage with vacuum-rotary evaporator until the consistence of 61 - 63% dry substances and in the second stage by vacuum sublimation or lyophilized method till the consistence of 74 - 75% dry substances. Biologically active compounds of the received liquid concentrate and the antioxidant activity were estimated.

More than 81% of the fatty acids existed in the composition of the received liquid bioflavanoid concentrate is unsaturated, rich in polyphenolic complex and characterized by high antioxidant activity - 58.62% (F = 100).

Keywords: *"Zeibel 5455"; Carbonic Acid; Colored Grape; Phenolic Compounds; Antioxidant Activity*

Introduction

Herbal extracts of therapeutic-preventive potential have been used in folk medicine since ancient times. But, since the second half of the past 20th century powerful drug remedies of synthetic origin have been produced and used, which has not slowed interest not only towards bioflavonoid micropowders of ecologically clean colored grape solid parts, but also towards the extracts and concentrates - the composition of which is characterized by powerful antioxidant effect and therapeutic-preventive potential [1-3].

The selection of hybrid varieties of ecologically clean, colored grape «Zeibel 5455» was caused by the absence of diglycosidic forms of anthocyanidins, as well as malvidin-3,5-diglycoside among them. As it is established, large quantities of diglycosidic forms of anthocyanidins are found in American varieties of *Vitis labrusca*-derived vines and European-American clones and hybrids received by its selection. The total amount of diglycosidic forms of anthocyanidins in some grape varieties derived from *Vitis labrusca* can reach 90% of

the total summary amount of anthocyanidins. Zeibel 5455, however, is one of the ecologically clean, hybrid varieties of vines, the grape of which do not contain diglycosidic forms of anthocyanins [4,5].

The raw material of the *Vitis labrusca* hybrid vine "Zeibel 5455" cultivated in the Imereti viticulture zone, as well as its grape-seed is ecologically clean, as chemical fertilizers and toxicants are not used in the process of it's cultivation [6].

Superfluid extraction of biologically active compounds from solid parts of the grape (seed and skin) is one of the best methods for separating vegetable fats.

Supercritical fluid is a state of substance when the boundary between liquid and gaseous conditions vanishes under certain pressure and temperature conditions [7].

CO_2-supercritical fluid is the best solvent for the extraction of nonpolar and medium polar substances from the vegetable raw materials to be extracted. However, it is one of the safest extragent for both human and ecological environment and is therefore successfully used in the extraction of substances of vegetable fats, fat soluble vitamins, saturated and unsaturated fatty acids, tocopherols, etc. Hereby, at the end of CO_2 extraction, it is removed from the extract by itself without any additional process [8].

Depending on the place of cultivation and environmental factors, each kilogram of colored grapes contains up to 10 - 15 grams of phenolic compounds, most of which, up to 90% (13 - 13.5 grams) are localized in the seed and skin, whilst the total number of seed and skin equals 12 - 20% of the grape. It follows from simple arithmetical calculation that 1g. grape-seed collects more than 68 mg phenolic compounds. Grape-seed is also rich in other biologically active compounds, including unsaturated fatty acids, mineral compounds, C-vitamin, etc [9,10].

Aim of the Study

The aim of this work is to determine optimal parameters of the superfluid extraction of "Zeibel 5455" red grape seed and skin, which is cultivated in Imereti region and has't been studied yet and to research biologically active substances of the received extracts and their antioxidant activity.

Materials and Methods

The grape seed of "Zeibel 5455" vine, cultivated in Imereti (Georgia) viticulture zone, in particular in Baghdati micro zone, its microdispersed powder and biologically active compounds of superfluid extracts received from that powder and their antioxidant activity was the object of study.

The object of the study was also to determine the optimal parameters to maximally scrutinize bioflavanoid compounds from the grape seed of "Zeibel 5455".

Gravimetric, extractive, spectral and chromatographic methods were used for the study [11-18].

In the study samples, we determined: The content of moisture and dry substances by thermogravitational (ГОСТ 28561- 90) and refractometric methods (digital refractometer PA202 Palm Abbe MISCO); determination of pH and titrated acidity is done with potentimeter (METTLER TOLEDO) by AOAC method.

Biochemical analysis was conducted using different physico-chemical and instrumental methods. Separation-identification and quantitative analysis was conducted using UPLC-MS (WatersAcquityQDa detector), HPLC (Waters Breeze 1525, UV-Vis 2489 detectors), pH-meters (MettlerToledo), refractometer - Misco, spectrometer-Cuvette Changer (Mettler Toledo UV5A), chemicals - stability radical-2,2-di-

phenil-1picrilhydrazyl (Aldrich-Germany), aluminum chloride ($AlCl_3$), Folin-Ciocalteu reagent (preparation), standards -gallic acid, rutin. C18Cartridge Solid Phase Extraction (SPE) Waters Sep-Pak C18 (500 mg).

Total quantitative analysis of phenols was conducted by Folin-Ciocalteu reagent spectrophotometrical method. In particular, the extraction of crushed samples taken for analysis is performed by using 75 - 81% ethanol at temperatures of 72 - 75°C and in the condition of periodical stirring for 6 - 7 hours. 1 ml of the received extract was placed in the 25 ml volumetric flask, the reagent of 0.5 ml. H_20, 1 ml Folin-Ciocalteu were added, and was delayed at room temperature for 8 minutes, then 10 ml of 7% Na_2CO_3 was added, flask was filled with H_2O and was delayed at room temperature for 2 hours.

Analysis was conducted at 750 nm. We take 1 ml of the appropriate extragent as control and go through the same process. Calculation of the obtained data is conducted on the calibration curve of gallic acid.

The total phenolic content is calculated by the formula:

X = (DKVF)1000/m

Where: X- total phenolic composition, in mg/kg; D- optical density; K- calculated coefficient for gallic acid;

F- mixing factor; V- total capacity of extract, ml; m- mass of raw material to be extracted, g.

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 1drop of bromium are added with 5 ml of water. In 15 minutes 100 ml of water is added.

Quantitative determination of total flavonoids is performed by $AlCl_3$ - reagent spectral method-extraction of sample taken for analysis is performed by using 80% ethanol in the conditions of 70 - 75°C temperature. 1 ml of the total extract is placed in a 10 ml volumetric flask, 5 ml H_2O is added, 0.3 ml 5% $NaNO_2$ is delayed for 5 minutes, then 0.3 ml 10% $AlCl_3$ is added and delayed for 6 minutes, then 2 ml 1N NaOH is added and determination is performed at 510 nm. 1 ml of the extract is taken for control and go through the same process.

The data received as a result of determination are calculated on the Ruthin calibration curve. The total flavonoid content is calculated by the formula:

X = (DKVF)·1000/m

For quantitative determination of leukoanthocyanins, leukoanthocyanidin reagent was used, vanillin reagent and spectral method for flavan-3-ols. Extraction of the samples taken for analysis was performed with 75% ethanol, in the condition of 72 - 75°C temperature. 1 ml of the received extract was added 3 ml. vanillin reagent, we determined the optical density of the red-coloured sample after 3 minutes (λ = 500 nm). The obtained results were calculated on the (+) catechin calibration curve. The calculations were performed using the formula:

X = (DKVF)·1000/m

To determine total monomeric anthocyanins, a pH-differentiated method was used and extraction from the samples used for the analysis was performed by using 45% ethanol.

In the studing samples antioxidant activity is determined by one of the widely used DPPH methods. One of the most popular methods is DPPH free radical colorimetric with 50% of radical inhibition. The DPPH method is a fast, simple and accurate test method for determining antioxidant activity.

DPPH - ($C_{18}H_{12}N_5O_6M$ = 394.33) is a stable free radical with a maximum absorbance at 515 - 517 nm, the strong purple color of extract including methanol changes into the pale yellow as a result of restoring. The reaction proceeds as follows:

DPPH. + AH DPPH-H + A. DPPH. + R. DPPH-R,

Where AH is an antioxidant, and R. -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/100 mL in ethylalcohol) 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 x 100%

A_c indicates absorption of DPPH/Alcohol solution, and A_s indicates absorption of the extract.

Chromatographic methods were used for studying the compounds. Liquid chromatographic mass detection (UPLC-MS) method of high pressure (HPLC) and ultra-high pressure, air-liquid gas chromatographic (GS) and near-infrared spectrophotometric (NIRS) methods were used.

Waters Corporation's supercritical superfluid extractor SFE - 100-2-C10 was used for extraction, on which extracts rich with biologically active compounds and vegetable fat were received from the grape seed of "Zeibel 5455".

Results and Discussion

It has been studied that the composition of phenolic complex in the parts grapevine berry reaches its maximum at the beginning of ripening period and when it reaches its full maturity there is 40 - 80% of soluble phenolic compounds left in the water and alkaline region and 25 - 40% in the grape seeds. Therefore, "Zeibel 5455" grapes were harvested at the beginning of technical maturity on August 30, 2018, when the composition of sugar reached 16.0 - 16.5% (Figure 1) in it.

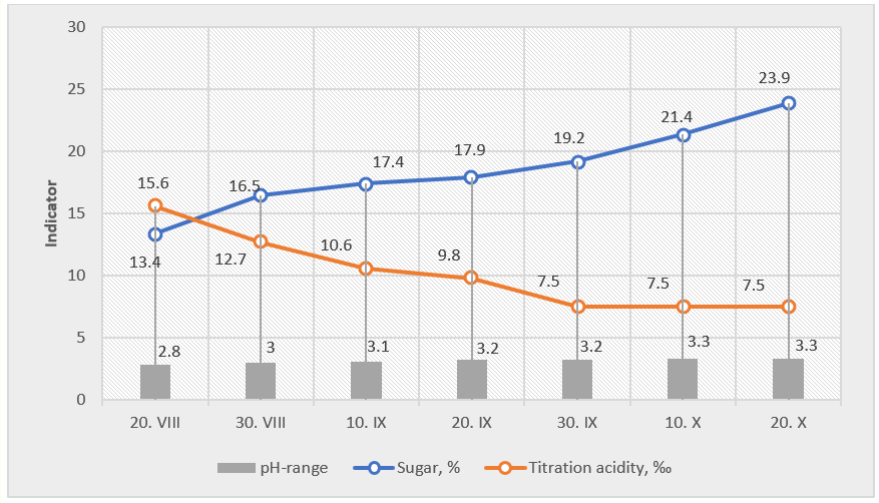

Figure 1: *Dynamics of change of sugars, titratable acids and pH in the berry "Ziebell 5455".*

As it is shown in the figure 1, the composition of sugars and titratable acids is in a disproportional relationship in the grape during the ripening period. As for the pH range, it partly varies.

The uvological characteristics of "Zeibel 5455" grape cluster were studied (Table 1).

Indicators of "Zeibel 5455" grape bunch 30.8.2018		Harvest dates	
		15.9.2018	
Parts of the grape bunch, %	Juice and pulp	75,98	76,94
	Stem	4,67	4,12
	Grape skin	14,69	14,51
	Seed	4,66	4,43
Number of seeds in the berry		2	2
The sum of solid parts of grape %		24,02	23,06
Structural indicator of grape		3,2	3.4
Phenolic compounds in the clusters, mg/100g.		468,9	327,5

Table 1: *Uvological characteristics of "Zeibel 5455" grape bunch.*

Composition of phenolic compounds decreases in parallel with the increase of structural indicator (the ratio of juice and softness to the sum of solid parts).

Raw material of colored grapes of «Zeibel 5455» was processed in 3 - 4 hours after harvesting with the help of the following technological scheme:

- Determining qualitative indicators of the grape's raw material;
- Processing the raw material of grape in DMCSI-type crusher and stem remover;
- Stem-removed, dreg is pressed in the press machine and the juice is removed;
- Vacuum-sublimation drying of juice-removed, sweet husks (Выжимка) of grape with 45 - 65% moisture content up to 7 - 9% ultimate moisture level.
- Separating skin and seeds of "Zeibel 5455" grape, dried up to 7 - 9% moisture level with the help of tea sorting machine designed by G.Lominadze;
- Crushing the skin in the MM-10 micromillon average of 50 - 100 mkm. fraction.
- Delivering the crushed seeds for extraction.

Microdispersed powder extraction was carried out by two different methods.

In the first stage, we extracted vegetable fat by the Super critical Super Fluid Extractor SFE - 100-2-C10 of Waters Corporation.

The optimum extraction parameters were experimentally selected, pressure 270 bar, temperature 33°C, carbon dioxide delivery rate 1.5 kg/h and duration of the extraction 3 hours. The main objective was to maximize the productivity of vegetable fat.

The average productivity of the fat fraction from the "Zeibel 5455" grape seed equals to 13.6 - 16.4% and the refractive index is 1.4759.

It is known that Ascorbic acid is a well-known, vital natural substance belonging to the derivatives of polyoxy-γ-lactones of unsaturated carbon acids. The human body is not able to synthesize this compound, it comes from the outside only, mainly with food products of raw origin or herbal agents [19]. According to this, the fractional composition of carbonic acids was interesting (Table 2).

Peak No	Component Name	Pk End Time (min)	Area, (%)
1	Butyric acid methyl ester (C4:0)	3.375	0.010
2	Lauric acid methyl ester (C12:0)	10.393	0.011
3	Myristic acid methyl ester (C14:0)	12.118	0.070
4	cis-10-Pentadecenoic acid methyl ester (C15:1)	13.025	0.010
5	Pentadecanoic acid methyl ester (15:0)	13.195	0.015
6	Palmitoleic acid methyl ester (C16:1)	14.292	0.141
7	Palmitic acid methyl ester (C16:0)	14.650	8.407
8	Linoleic acid methyl ester (C18:2n6c)	18.007	66.801
9	Oleic acid methyl ester (C18:1n9c)	18.240	19.779
10	Elaidic acid methyl ester (C18:1n9t)	18.205	0.656
11	Stearic acid methyl ester (C18:0)	18.692	3.451
12	cis-11,14-Eicosadienoic acid methyl ester (C20:2)	19.063	0.004
13	cis-11-Eicosenoic acid methyl ester (C20:1)	19.298	0.006
14	Arachidic acid methyl ester (C20:0)	19.763	0.012

Table 2: *Carbon acids of «Zeibel 5455» vegetable fat.*

For determining carbonic acids in the samples of grape-seed oil, we have prepared the samples to be analysed (etherification).

The sample to be analysed was filtered to purify it from mechanical impurities. 1 ml of the filtered sample was taken in a centrifuge tube, 0.5 ml of 2 normal KOH 99.8% methanol solution was added (ethanol can be used). Then, 10 ml hexane (total volume 11.5 ml) was added. It was shaken until it was completely dissolved (at least 30 seconds) and centrifuged for 10 minutes at 1000 rotations. 1 mkl was taken from the upper fraction of the sample and injected it in the chromatograph. The quantitative composition of carbonic acids is determined by a peak ratio with 0.01% accuracy.

Identification of components obtained by chromatography was realized in comparison with sample data of known composition and specific composition of carbonic acid in the oil of grape seed was identified (Figure 2).

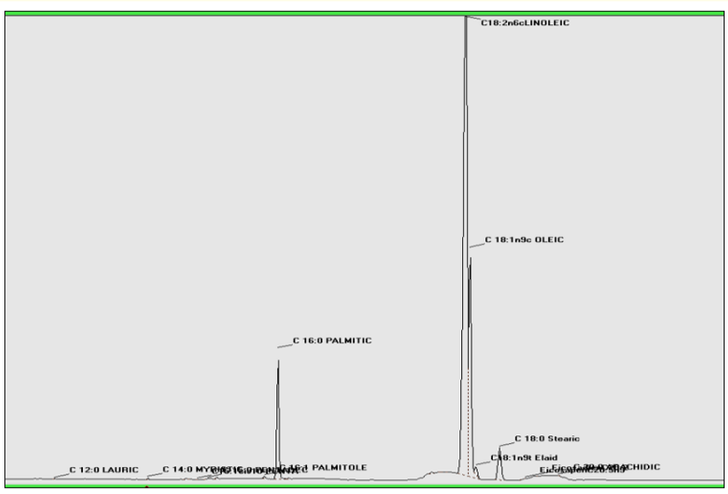

Figure 2: *Fatty Acid Methyl Ester Chromatogram.*

Chromatographic study showed that the oil received from hybrid grape (Zeibel-5455) seed contains five dominant carbonic acids.

In particular, their distribution in the oil is as follows:

- Linoleic acid methyl ester(C18:2n6c) 66.801%
- Oleic acid methyl ester (C18:1n9c) 19.779%
- Palmitic acid methyl ester (C16:0) 8.407%
- Stearic acid methyl ester (C18:0) 3.451%
- Elaidic acid methyl ester (C18:1n9t) 0.656%.

In the second stage, fluid extraction of microdispersed micropowder was performed with ethyl alcohol, namely we have experimentally determined the optimal parameters of the extraction: pressure 100 bar, CO_2 delivery rate of 7.5 kg/h. At the same time, quality of the extraction was affected by 75% ethyl alcohol in the form of cosolvent, the ratio of which to CO_2 was 21 - 22%.

Fluid extract of the grape seed was precipitated in the condition of 4 - 5°C for 7-9 hours, removed from sediment and filtrated with wine lamella filter. The study of biologically active compounds of superfluid extract from the grape seed is given in table 3.

Compounds mg/100g on dry mass	Stages of super fluid extraction								Total
	1	2	3	4	5	6	7	8	
Phenolic compounds	198,3	1115,8	828.7	496,2	462,4	275,6	371,9	274,9	4023,8
Flavonoids	341,1	523,8	436,1	328,8	272,2	154.1	251,9	143,9	2451,19
Flavan-3-ols	129,9	368,2	436,1	322,5	252,3	121,5	181,0	94,3	1795,8
Leucoantho-cyanins	-	143,7	276,1	164,42	-	-	-	-	584,22

Table 3: *Fluid extraction stages of grape seed micropowder in the presence of 75% ethyl alcohol (21 - 22%).*

Extraction of bioflavonoid compounds is influenced by pressure, temperature, dilution rate of cosolvent or ethyl alcohol and its relation to CO_2. The maximum amount of phenolic compounds are extracted. The pressure and the quality of extraction are also influenced by cosolvent ethyl alcohol concentration in relation to carbon dioxide, namely in the case of 16% ethyl alcohol the content of phenolic compounds reaches 3245 units and in the case of 22% ethyl alcohol their content is much higher (4023 mg/100g).

The coupage of both types of "Zeibel 5455" grape seed extracts was made with 1:1 ratio and concentration on the first stage by vacuum-rotary evaporator to 61 - 63% composition of dry substances and on the second stage by vacuum sublimation or lyophilized method to 74 - 75% composition of dry substances. Biologically active compounds and antioxidant activity of the received liquid concentrate were estimated (Table 4).

Biologically active compounds of "Zeibel 5455" liquid concentrate, mg/100g. on dry mass and AOA, %	Quantitative significance
Phenolic compounds	4896,5
Flavonoids	2534.3
Flavan -3- ols	1975,2
Leucoanthocyanins	625,8
Dry substances %	74 - 75
AOA, (F = 100), In, %	58,62

Table 4: *Biologically active compounds and antioxidant activity of bioflavanoid concentrate.*

More than 81% of the fatty acids existed in the composition of the received liquid bioflavanoid concentrate are unsaturated, rich in polyphenolic complex and characterized by high antioxidant activity - 58.62% (F = 100).

Conclusion

Biologically active compounds and antioxidant activity of the superfluid extracts composition of the seed of "Zeibel 5455" colored grape, dried by lyophilized method up to 7 - 8% moisture level, cultivated in individual micro-zones of Imeretian viticulture and winemaking have been studied.

Chromatographic study showed that the oil received from hybrid grape (Zeibel-5455) seed contains five dominant carbonic acids.

In particular, their distribution in oil is as follows:

- Linoleic acid methyl ester (C18:2n6c) 66.801%
- Oleic acid methyl ester (C18:1n9c) 19.779%
- Palmitic acid methyl ester (C16:0) 8.407%
- Stearic acid methyl ester (C18:0) 3.451%
- Elaidic acid methyl ester (C18:1n9t) 0.656%.

Liquid bioflavonoid concentrate of extracts thickened on two stages till the consistence of 74 - 75% dry substances is characterized by strong antioxidant activity - 58.62% (F = 100).

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