CHARACTERIZATION OF SELECTED GRAPE SEEDS BASED ON SOME CHEMICAL AND BIOCHEMICAL PARAMETERS

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***Abstract*— A large amount of seeds is produced as a by-product after grape processing. In general, mainly due to fatty acid composition, the seeds oil is extracted to be used as a potential ingredient for food applications. In the present study, some important chemical and biochemical characteristics of grape seeds from some Iranian varieties were assessed. To this end, protein concentration, fatty acid profile, antioxidant activity, β-carotene content, and concentration of carbohydrates were determined using standard methods. The results showed that the protein concentration of the seeds ranged from 53.15 to 81.75 mg/g. Major fatty acid identified in all the studied seeds was linoleic acid followed by palmitic acid and oleic acid. Antioxidant activity of the seeds varied from 68.31 to 92.64%. The content of β-carotene in the seeds was obtained to be in the range of 5.47–12.83 µg/g. The soluble and insoluble sugars in the seeds varied in the range of 99.45–306.79 mg/g and 60.12–123.81 mg/g, respectively. For all the varieties, soluble sugar content was significantly ($P < 0.05$) higher than insoluble sugar. The variance analysis revealed significant ($P < 0.05$) difference of the variety factor on the studied characteristics of the seeds.**

***Keywords*— Grape seed, Protein content, Fatty acid profile, Antioxidant activity, β-carotene, Carbohydrates**

I. INTRODUCTION

For centuries, grapes have been cultivated and revered by several ancient civilizations. There are many types of grapes, which grow in clusters and come in seeded and seedless varieties. Grapes offer a wealth of health benefits due to their high nutrient and antioxidant contents.

The fruit and vegetable juice is one of the most important product in the food and drinks industry. It is also the most competitive section in the beverage sector. In this regard, production of different types of grape juice is of particular importance since its by-products contain valuable amounts of bioactive compounds. Grape seeds present about 5% of total weight of the berries and 38–52% of the pomace (Tița *et al.,* 2021), and contain fiber (40% w/w), lipids (16% w/w), protein (11% w/w), carbohydrates, and minerals (Silva *et al.,* 2021).

Polyphenols and oils are the two major compounds' groups in grape seeds. Significant concentration (about 7% w/w) of specific polyphenolic compounds (such as proanthocyanidins) could be found in grape seeds. Flavonoids, phenolic acids, anthocyanins and carotenoids have antioxidant role helping to control blood pressure, protect the heart, blood vessels and brain (Sano, 2017). In the Alzheimer's disease pathology and its inhibition, and improvement in cognitive decline because of utilization of extract from grape seeds has been claimed by some researchers. They have stated that in the seeds' polyphenols increase the antioxidants levels as well as prevent the protein mutations. The phenomena results in a reduction in induced damage by free radicals in the hippocampus (Wang et *al.,* 2009). In addition to antioxidant capacity, through their provitamin A activity, carotenoids play important roles in human nutrition (Bunea et *al.,* 2012).

Grape seed oil also contains fatty acids, tannins, sterols, unsaponifiable lipids, and triglycerides. Due to the high content in polyunsaturated fatty acids, the oil is known as a high-quality nutritional element exhibiting inhibition capability of thrombosis and cardiovascular diseases, reduction in cholesterol serum and cancer as well as autonomic nerves regulation (Lucarini *et al.,* 2020). In the open literature, relatively a few scientific works have been reported on chemical and biochemical properties of grape seeds. Therefore, knowing that several factors such as variety, location, harvest time, *etc.*, affect the composition of grape seeds; the aim of this work was to assess the some important chemical and biochemical properties of seven well-known Iranian grape varieties. To this end, protein content, fatty acid profile, antioxidant capacity, β-carotene content, and concentration of carbohydrates were determined.

II. METHODS

A. Grapes

Seven well-known grape Iranian varieties twitched off in September 2020 at physiological maturation from 10 years old vineyard in Gijali Village, Borujerd City, Lorestan Province (Western Iran). The products identity were confirmed by the Cultivation and Development Group of the Tehran Institute of Medicinal Plants. Figure 1 represents the vineyard and the studied grapes with their local names.

The collected fresh grapes were transported to the laboratory, washed carefully and the seeds removed manually from pulp. After washing with distillated water, the

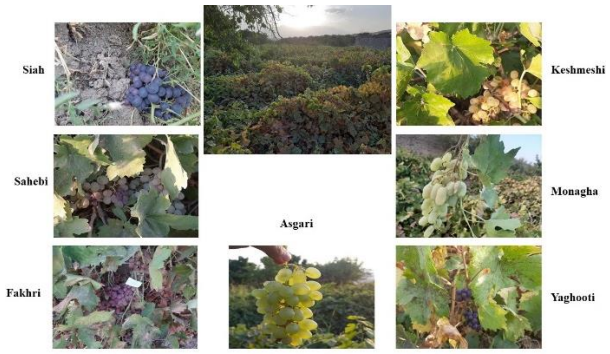

Figure 1. The studied vineyard and grape species.

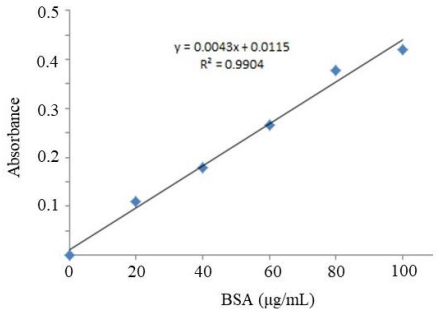

Figure 2. The BSA standard curve absorbance at 595 nm.

seeds were dried at temperature of 50 °C for 30 min in a vacuum oven (Samadi Varedesara *et al.*, 2021). Using a mechanical blender, the dried seeds were completely grounded, packed in plastic bags and stored in a refrigerator at 4±2 °C in a refrigerator until the further experiments were started.

B. Extraction and determination of protein concentration

TCA-acetone method was practiced to obtain protein extracts from the samples according to the protocol described by Pavoković *et al.* (2012) with little modifications. About 5 g of the powder was dissolved in 5 mL of ice-cold extraction buffer and extracted with acetone containing 10% TCA (w/v) and 1% DTT (w/v). Removing of the cell debris was conducted by centrifuging at 500×*g* for 15 min at 4 °C. the supernatant was transferred into a new tube, and proteins were precipitated by adding four volumes of ice-cold acetone. The samples were kept at -20 °C for 2 h. By centrifuging at 15000×*g* for 20 min at 4 °C, the pellets were obtained. The pellets were washed three times using an ice-cold solution of 2-mercaptoethanol in water/acetone (80:20, v/v). The pellets were centrifuged at 15000×*g* for 45 min at 4 °C between the washes. Supernatants were separated and discarded, and pellets were dried at room temperature.

The obtained protein pellets were dissolved in isoelectric focusing buffer and incubated at room temperature for at about 2 h; and sonicated three times for 15 s in an ice bath and centrifuged at 14000×*g* for 5 min. To determine protein concentration, the Bradford assay was conducted according to the method described by Bradford and Williams (1976). Briefly, 100 mg Coomassie Brilliant Blue G-250 was dissolved in 50 mL 95% ethanol (C_2H_5OH). Then, 100 mL of 85% phosphoric acid (H_3PO_4) was carefully added under stirring, before H_2O was added to a total volume of 1 L. The solution was filtered and kept at 4 °C. For the measurements, 100 µL extract and 5 mL Bradford solution were mixed and incubated for 5 min. A standard curve was made of bovine serum albumin (BSA, 0–100 µg/mL) and absorbance was read at 595 nm. The curve is shown in Fig. 2.

C. Oil preparation and fatty acid profile analysis

The seeds was extracted practicing the well-known Soxhlet method as described by Hewavitharana *et al.* (2020) using a porous thimble (mainly included flask, extraction chamber and condensing system) with some modifications. 5 g of the grounded seeds were weighed accurate to 0.001 g using a digital balance (ViBRA, model EG 620-3NM, Japan) was stirred with 160 mL *n*-hexane into the flask and heated for 8 h at temperature of 50–60 °C. Subsequently, the extracts were filtered by Whatman 1 filter paper, evaporated under reduced pressure (at temperature of 40 °C for 30 min) (Daneshzadeh *et al.*, 2020). The obtained extracts were submitted to liquid partition with water and ethyl acetate (Tomaz *et al.*, 2012), and fatty acid profile analysis was performed using a gas chromatography/mass spectrometry (GC/MS). To this end, fatty acid methyl esters were analyzed using Agilent Technologies 7890 gas chromatograph coupled to Agilent 5975 C mass selective detector and quadruple EI mass analyzer, and practicing the following procedure: After remaining constant for 1 min, the oven temperature was programmed to increase from 150 to 200 °C (15 °C/min). Then, the temperature increased from 200 to 225 °C with a ramp rate of 4 °C/min and maintained for 2 min. The nitrogen flow rate, injection temperature and detector temperature was 1.6 mL/min, 250 and 275 °C, respectively. Finally, identification of fatty acid methyl esters were conducted by refereeing to standard chromatograms (Moufida and Marzouk, 2003).

D. Determination of antioxidant activity

The antioxidant capacity of the extracts was assessed by evaluating the free radical-scavenging effect on the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical based on the method proposed by Do *et al.* (2014). To prepare the extracts, the procedure described by Jayaprakasha et al. (2001) was used. Practicing a Soxhlet extractor and hexane, the grape seeds powders were extracted for 6h to remove the fatty matter. For each experiment, 50 g of defatted powders was stirred with 150 mL methanol (80%) into Soxhlet and extracted for 10 h at temperature of 60–70 °C (Jayaprakasha *et al.*, 2001). The obtained extracts were passed twice through filter paper (Whatman no. 1), concentrated in a rotary evaporator at reduced pressure of 100 psi and controlled temperature of 40 °C (Rezaei *et al.*, 2019).

By dissolving 6 mg DPPH in 50 mL methanol, a fresh 0.3 mM DPPH methanol solution was prepared. 2.5 mL of the extract was mixed with 2.5 mL of the DPPH solution in a test tube. The mixture was incubated in the dark and room temperature. The decrement in absorbance after 30 min was measured at 517 nm using the UV-VIS spectrophotometer. Finally, percentage of inhibition of

the DPPH radical was calculated as follow (Budaraju *et al.*, 2018):

$$Inhabition\ of\ DPPH\ (\%) = \left(\frac{Abs_{control} - Abs_{sample}}{Abs_{control}}\right) \times 100, \quad (1)$$

where, $Abs_{control}$ and is the absorbance of DPPH solution without extracts and Abs_{sample} is the sample absorbance with DPPH solution.

E. Extraction and measurement of β-carotene

To determine β-carotene content, the spectrophotometric method described by Grulichová *et al.* (2018) was practiced. 500 mg of the seeds powder was weighed using the digital balance and mixed with 2 mL of a 1:1 mixture of pure petroleum ether (PE, approximately 70 °C) and tetrahydrofuran (THF). The process was followed by addition of a total of 3 mL of PE to the solution, rinsing twice with 3 mL of PE:THF (4:1) mixture and filtering through a syringe containing polyamide filters (pore size of 0.45 μm). Finally, β-carotene was measured using a spectrophotometer (Perkin–Elmer Lambda, US) at wavelength of 480 nm.

F. Carbohydrates

To quantify the total soluble sugars (TSS), the procedure described by Irigoyen *et al.* (1992) was practiced. Based on the method, 95% ethanol extracts of the samples were used. To this end, 500 mg of the grounded seeds was extracted with 5 mL of ethanol (concentration of 95%, v/v). Using 5 mL of 70% ethanol, the insoluble fraction of the extract was washed twice, and all soluble fractions were at 3500 g for 10 min. About 0.1 mL of the alcoholic extract was reacted with 3 mL freshly prepared anthrone (150 mg anthrone+100 mL H_2SO_4 (72%, w/w)) and placed in a water bath at temperature of 100 °C for 10 min. After cooling, the TSS was measured by determining the absorbance at 625 nm in the spectrophotometer.

To determine the concentration of insoluble carbohydrates in the seeds, the standard colorimetric procedure described by DuBois *et al.* (1956) with some minor modifications was used. The method is principally based on production of furfural derivatives by during dehydration of carbohydrates through reaction with concentrated sulfuric acid (Albalasmeh *et al.*, 2013). 500 mg of the seeds flour was boiled with 80% ethanol for 10 minutes. After filtering using Wattman paper, the extract was allowed to be evaporated. Then, it was washed with distillated water at 60-70 °C and its volume reached to 50 mL. About 2 mL the solution was mixed in a test tube with 1 mL of 5 % aqueous solution of phenol; and immediately, 5 mL of concentrated sulfuric acid was added to the mixture. For color development, after 10 min standing, the test tubes vortexed for 30 s and placed for 20 min in a water bath at temperature of 25 °C. Finally, the absorption was recorded at 485 nm on the spectrophotometer.

F. Statistical analysis

To assess the influence of variety on the studied properties of the grape cultivars, the obtained experimental data were analyzed using analysis of variance (ANOVA) by SPSS software (version 21). Data is presented as mean values ± standard deviation (SD) and the differences among the means were compared for significance at $P < 0.05$ using Duncan's multiple range tests.

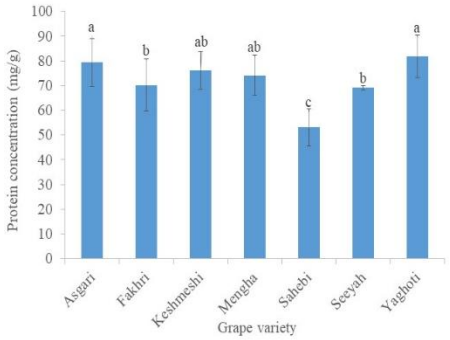

Figure 3. Protein concentration (mg/g) in the investigated grape seeds.

III. RESULTS

A. Protein concentration

Despite of several research works conducted to identify and characterize the proteins in grape pulp and skin, there are few studies reported on grape seed proteins in the literature (Gazzola *et al.*, 2014). Protein content in the assessed grape seeds is presented in Figure 3. Based on the results obtained through the experiments, the protein concentration of the seeds ranged from 53.15 to 81.75 mg/g. The values obtained in this study could be compared with the findings reported by Abdrabba and Hussein (2015) for Libyan red grape (188.9 mg/g), Costa *et al.* (2019) for Alicante Bouschet grape seed pomace (96.5 mg/g), Maman and Yu (2019) for seeds from Muscadine Carlos and Noble grapes (mean value of 150 and 132 mg/g, respectively), and Iuga *et al.* (2019) for white and red wine grapes' seeds (mean value of 97 and 127 mg/g, respectively).

B. Fatty acid

The proportional fatty acid compositions determined for the studied grape seeds are shown in Table 1.

Table 1. Identified components of fatty acids in the total lipids (%) from the investigated grape seeds.

Fatty acid	Myristic acid	Pentadecanoic acid	Palmitic acid	Oleic acid	Linoleic acid	Stearic acid	Phthalic acid	Linolenic acid
Asgari	0.99^{c}±0.16	n.d	18.69^{a}±0.91	10.14^{bc}±1.01	53.37^{b}±1.98	4.15^{b}±0.27	1.14^{b}±0.11	n.d
Fakhri	0.139^{d}±0.08	n.d	15.25^{b}±0.29	8.05^{c}±0.32	66.01^{a}±2.01	8.67^{a}±0.93	0.04^{b}±0.01	n.d
Keshmeshi	4.31^{a}±0.22	5.38^{a}±0.42	20.03^{a}±1.02	4.96^{d}±0.44	6.17^{d}±0.16	1.23^{c}±0.46	22.72^{a}±1.02	n.d
Mengha	n.d	n.d	11.51^{c}±0.64	24.57^{a}±0.63	54.83^{b}±1.39	5.54^{b}±0.33	n.d	n.d
Sahebi	0.08^{d}±0.01	n.d	13.30^{bc}±0.75	12.84^{b}±0.74	70.42^{a}±2.64	2.39^{c}±0.48	n.d	n.d
Seeyah	0.30^{d}±0.05	n.d	9.32^{c}±0.56	14.03^{b}±0.52	69.45^{a}±3.42	6.43^{ab}±0.71	n.d	n.d
Yaghoti	2.01^{b}±0.31	n.d	5.82^{d}±0.21	3.82^{d}±0.19	35.90^{c}±0.89	4.02^{b}±0.57	0.43^{b}±0.13	7.80^{a}±0.25

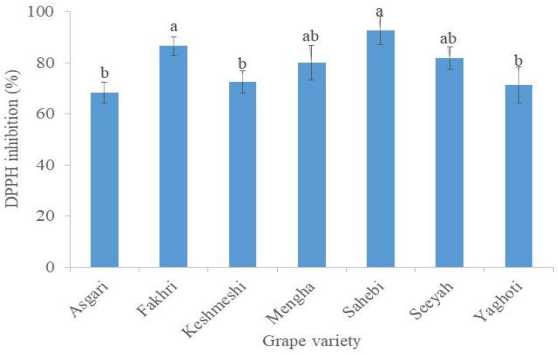

Figure 4. Antioxidant activity (%) in the investigated grape seed extracts.

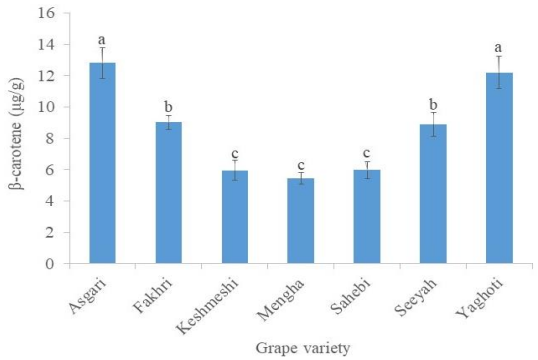

Figure 5. β-carotene concentration (µg/g) in the investigated grape seeds.

Based on the free fatty acid composition analysis, significant ($P < 0.05$) differences are evident among the different varieties. As shown in Table 1, for all the grape cultivars investigated in the present work, the major fatty acid was linoleic acid followed by palmitic acid and oleic acid. The observations in cases of the important influence of cultivar on fatty acids composition of as well as the most abundant fatty acids in grape seeds are well supported by the findings reported by Kapcsándi *et al.* (2021) and Pérez-Navarro *et al.* (2019). Lucarini *et al.* (2020) studied the profile of fatty acid for seeds of three *Vitis vinifera* (L.) cultivars (white and red grapes). To this end, they used the procedure described by Bligh and Dyer (1959) to extract the fat and gas chromatographic to analyze an aliquot of the extract. According to their findings, linoleic acid, oleic acid, palmitic acid, and stearic acid were the main components in the profile; adding up to about 98% of the total fatty acids. Milinčić *et al.* (2020) examined fatty acid profile for grapes seed flour from different varieties, and identified twelve fatty acids in the samples. They found linoleic, oleic, stearic and palmitic in all the studied grapes. As the dominant, the linoleic acid was reported to be in the range of 61.15–83.47% depending on the variety. Ohnishi *et al.* (1990) evaluated seeds from five grape varieties, and found six fatty acid components in the total lipids from the seeds. Among the components, linoleic acid was chief (69-81 % of the whole depending on the variety) followed by oleic, palmitic and stearic acids.

C. Antioxidant activity

DPPH radical scavenging assay is a standard method usually practiced to measure the free radical scavenging capacity of an antioxidant molecule. The procedure is also used for colorimetric assessment of antioxidant characteristics of pure compounds (de Oliveira *et al.*, 2014). DPPH shows a strong absorption band at 517 nm due to its odd electron and solution appears a deep violet color. By accepting a hydrogen atom from the scavenger molecule i.e. antioxidant, the electron becomes paired off. Subsequently, the absorption fades and the resulting decolorization is stoichiometric with respect to the number of electrons taken up (Viacava *et al*., 2015).

The values obtained for DPPH radical scavenging activity for the extracts from the investigated grape seeds in the present study are shown in Fig. 4.

From the results, the maximum (92.64%) and minimum (68.31%) activity belonged to Keshmeshi and Fakhri varieties, respectively. The obtained range for antioxidant activity of the extracts are comparable with the findings reported by some researchers for grape seed. For example, Farhadi *et al*. (2016) investigated antioxidant activity in seed of five Iranian native cultivars as well as one international grape variety. They observed a significant activity in methanol extract of the samples ranged from 88.10–94.46%. The activity for ethanol extract from different varieties of the seeds was reported to be the range of 41.9–73.3% (Choi *et al.*, 2011). The analysis of variance revealed significant ($P < 0.05$) influence of variety on the antioxidant activity. The finding is supported by the reported observations of in the open literature. Mandić *et al.* (2009) reported that individual cultivars of grape could affect the antioxidant capacity in grape seed. They also, found the activity might be influenced by cultivation condition, maturation stage, and seasonal variations. In addition, Kupe *et al.* (2021) studied the antioxidant activity of grape seed extract and reported significant ($P<0.05$) differences among different studied Clones for DPPH scavenging activity.

D. β-carotene content

β-carotene is a precursor of vitamin A, and the human body converts β-carotene into vitamin A. Vitamin A is needed for healthy skin and mucus membranes, immune system as well as good eye health and vision. β-carotene in itself is not an essential nutrient, but vitamin A is. Although vitamin A can be sourced from the eating food, the advantage of dietary β-carotene is that the body only converts as much as it needs.

β-carotene and lutein, are the most abundant of carotenoids' species exist in grapes are, although at detectable levels, violaxanthin and neoxanthin are also available. In general, during maturation and before veraison, grapes are rich in β-carotene and several xanthophylls. However, the levels decrease during ripening (de Pinho *et al.*, 2001). The content of β-carotene in the seeds was determined based on measuring the spectrophotometric properties and obtained to be in the range of 5.47–12.83 µg/g (Fig. 5). Furthermore, the statistical analysis revealed three different groups ($P < 0.05$) of the seeds in case of β-carotene content.

Table 2. Soluble and insoluble sugar contents (mg/g) in the investigated grape seeds.

Carbohydrates (mg/g)	Soluble	Insoluble
Asgari	$306.79^{a}\pm16.68$	$102.89^{ab}\pm19.42$
Fakhri	$150.02^{b}\pm13.59$	$115.47^{a}\pm14.54$
Keshmeshi	$189.67^{b}\pm16.31$	$60.12^{c}\pm3.77$
Mengha	$99.45^{c}\pm9.13$	$91.77^{b}\pm14.03$
Sahebi	$136.96^{bc}\pm15.76$	$91.74^{b}\pm4.53$
Seeyah	$253.26^{ab}\pm10.43$	$123.81^{a}\pm2.28$
Yaghoti	$301.90^{a}\pm17.11$	$67.21^{c}\pm3.45$

Although some researchers have focused on β-carotene content of different products, to our best knowledge and based on the open literature review, no work has been reported on β-carotene content of grape seeds. de Pinho *et al.* (2001) determined the carotenoid in pulp and skin of grapes from five cultivars using two different identification methods including chromatographic procedure and HPLC quantification. The chromatographic procedure revealed a very different profile between skin and pulp in grapes, where skin contributed to about 65% of carotenoids (lutein, monoesters of xantophylls, and β-carotene) while the pulp portion was observed to be about 35%. The HPLC determination showed a different profile from skin and pulp. In skin, β-carotene existed in much higher levels, while β-carotene and lutein existed in the same quantities in the pulp. Bunea *et al.* (2012) used high performance liquid chromatography to identify the main carotenoids in different grape varieties under two types of cultural practices including organic and conventional. They practiced mixture of methanol/ethyl acetate/petroleum ether (1:1:1, v/v/v) to extract the total carotenoids from fresh grapes skin. Following the lutein, for the both cultural practices, β-carotene was found to be the second major carotenoid in the samples. β-carotene was reported to be in the range of 273.5–504.9 μg/kg and 229.6–593.2 μg/kg for organic and conventional cultivation systems, respectively. They also reported that both the variety and cultivating practice factors significantly (P<0.05) influenced the carotene content.

E. Sugars

The content of carbohydrates extracted from the grapes' seeds are presented in Table 2.

As shown, the soluble and insoluble sugars in the seeds varied in the range of 99.45–306.79 mg/g and 60.12–123.81 mg/g, respectively depending on the grapes variety. Based on the obtained results through the experiments in this study, for all the varieties, soluble sugar content was higher than insoluble one where the difference was statistically significant (P<0.05). Furthermore, the performed variance analysis revealed significant (P<0.05) differences among the studied grape varieties in case of both the soluble and insoluble sugars contents. Water soluble and water insoluble structural polysaccharides are the main residual carbohydrates in the grapes pomace. In the literature, different studies conducted on total soluble sugar concentration in grape pomace could be found (Milinčić *et al.*, 2020). However, until now, rare research works have been reported on sugar concentration in grapes seed. Total soluble sugar content in seven varieties of grape was reported to be in the range of 40.59–91.32 mg/g by Milinčić *et al.* (2020). The researchers reported that the sugar content was influence significantly (P<0.05) by the variety factor. They also stated that the quantities of dominant detected monosaccharides (such as glucose or fructose), as typical energy components, are not negligible. From the results of the present work, significant amounts of carbohydrates in grape seeds and therefore, besides fatty acid profiles, determination of sugars content in the seeds is useful to enrich the food products.

V. CONCLUSIONS

This work aimed at characterization of grape seeds from seven different varieties based on protein content, fatty acid profile, β-carotene content as well as soluble and insoluble sugars concentrations. From the obtained results, in general, all the evaluated properties were significantly influenced by variety. Relatively considerable amounts of protein were found in the seeds makes them as a potential nutrient component since, in addition to healthy muscles and tissues, plant protein may be beneficial for weight loss. Major fatty acids were determined to be linoleic acid, palmitic acid and oleic acid. These compounds could effectively affect the sensory characteristics of wines since they are precursor of C_6 and C_9 aroma compounds. Considerable antioxidant activity (68.31–92.64%%) was observed in the seeds. The content of β-carotene in the seeds was obtained to be in the range of 5.47–12.83 μg/g indicates the usefulness of the seeds for immune system as well as good eye health and vision. For all the varieties, soluble sugar content was significantly higher than insoluble sugar.

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