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ULTRASOUND AND ENZYMATIC ASSISTED EXTRACTIONS OF BIOACTIVE COMPOUNDS FOUND IN RED GRAPE SKINS BĂBEASCĂ NEAGRĂ (*VITIS VINIFERA*) VARIETY

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Abstract

The phenolic composition of the skin of the Băbească neagră grapes grown in south-eastern Romania was studied by two extraction techniques, i. e., ultrasound-assisted extraction (UAE) and enzyme-assisted extraction (EAE). This study aimed to analyze the grape skin bioactive compounds and the antioxidant potential using ethanol of three different concentrations 50%, 70%, and 96% and acidified with acetic, citric acid, or hydrochloric acid. This research aimed to maximize the extraction of bioactive compounds from red grape skin by using commercially available oenological enzyme preparations, varying the enzyme dosage, pH, temperature and extraction time. The results indicated that the highest total anthocyanin content was obtained when using 70 % ethanol extract acidified with 0.1 N hydrochloric acid, characterized by 4.29±0.04 mg C3G/g DW for ultrasound extraction and 2.54±0.13 for enzyme-assisted extraction. The results of antioxidant potential of the extracts investigated showed that the 96% ethanol extract had the highest antioxidant activity (18.76±0.24 mM of Trolox/g DW), followed by the 50% ethanol extract and 70% ethanol extract (16.35±0.79 and 16.48±0.54 mM of Trolox/g DW, respectively). The results disclosed that the highest extraction yield for antioxidant activity quantification was obtained by cellulase (61.48±1.19 mMol Trolox / g DW) after only one hour of extraction.

Keywords: red grape, skin, anthocyanins, flavonoids, phenolic compounds, antioxidant activity, ultrasound-assisted extraction, enzyme-assisted extraction

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Introduction

In Romania, the use of grapes, and red grapes, in particular, has a special place in the winemaking industry. Grapes are considered an essential source of manganese, and vitamins, such as B6, B1 and C vitamins (Corrales *et al.*, 2008; Kammerer *et al.*, 2004), and also an abundant source of phenolic compounds in terms of flavonoids, phenolic acids, anthocyanins, proanthocyanidins and stilbenes (Rodríguez Montealegre *et al.*, 2006; Singh *et al.*, 2019). Of these compounds, anthocyanins and proanthocyanidins are responsible for the red, blue, or purple colors of grapes and are found primarily in the grape skin (Singh *et al.*, 2019), the amount and composition of anthocyanins in grape depending on the variety and cultivation conditions (Huang *et al.*, 2009; Liang *et al.*, 2008).

The Băbească neagră variety (Engl. grandmother's black) is an old native red grape variety cultivated in the south-eastern part of Romania at the Dealu Bujorului vineyard used mainly to produce light and fruit wines with an alcohol content of 12-12.5% (v/v) (Constantin *et al.*, 2015).

Due to its beneficial health properties and lipid antioxidant properties, the recovery of phenolic compounds from different parts of grapes in the wine industry has attracted particular interest in recent years (González-Centeno *et al.*, 2014; Pinelo *et al.*, 2005). For bioactive compounds recovery from red grape by-products, several extraction methods have been used, such as: conventional extraction (CE), ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), supercritical fluid extraction (SFE), pulsed electric field (PEF), and pressurized liquid extraction (PLE) (Zia *et al.*, 2020). Compared to the conventional extraction methods, ultrasound-assisted extraction presents many advantages, including shorter extraction time, less solvent, and higher extraction yields (Ma *et al.*, 2009).

The extraction of bioactive compounds from vegetal materials using ultrasound treatments was lately explored mainly due to its productivity and simplicity. The UAE efficiency in increasing the extraction yield in bioactive compounds may be the result of ultrasound capacity to break the plant cells to facilitate the contact surface between the solvent used and the plant material (Cheok *et al.*, 2013; Garcia-Salas *et al.*, 2010; Zou *et al.*, 2011). Also, EAE is considered a potential substitute for CE methods, mainly because of its efficiency and sustainability (Nadar *et al.*, 2018; Puri *et al.*, 2012). The application of EAE in both food and pharmaceutical industries proved to be a useful 'green' extraction technique for obtaining high product yields, primarily because of the use of specific enzymes to disrupt the cell wall of the plant (Marathe *et al.*, 2017). The UAE and EAE combined with a solvent acidified with acetic acid, citric acid, or hydrochloric acid have been commonly used for the acidification of anthocyanin extraction solvents (Anuar *et al.*, 2013; Borges *et al.*, 2011; Fan *et al.*, 2008; Pompeu *et al.*, 2009; Xavier *et al.*, 2008).

The wine industry's main research direction is to select and settle treatments and methods to measure the quality and functional characteristics of bioactive compounds recovered from the grape parts to comply with European requirements and consumer demands. The use of remaining products resulting from the wine

technology is a developed practice with the primary goal of bioactive compounds extraction from the residual pomace.

The present study aimed to extract biological active compounds from red grape skins (Băbească neagră variety) using UAE. Further, their characterization in terms of phenolic content, flavonoids, individual anthocyanins, and antioxidant activity was performed.

Materials and methods

Chemicals and enzymes

2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), Folin-Ciocalteu reagent, ethanol, glacial acetic acid, citric acid, hydrochloric acid (HCl), and xylanase extracted from *Thermomyces lanuginosus* were supplied by Sigma Aldrich Steinheim, Germany. Cellulase extracted from *Trichoderma viride* was purchased by Merck, Germany, and Zymorouge EG produced by *Aspergillus niger* was achieved by Sodinal, Romania. Standards used delphinidin chloride, delphinidin 3-glucoside, malvidin chloride, malvidin 3-glucoside, cyanidin 3-glucoside, cyanidin chloride, pelargonidin chloride, pelargonidin 3-glucoside, peonidin chloride were obtained from Extrasynthese, GenayCedex (France).

Red grape skins powder preparation

Red grapes from the Băbească neagră variety were acquired from Galati area, Romania (from the 2019 harvest). The grape skins were separated, washed and rinsed with distilled water in a 1:2 ratio (w/w). Finally, for the removing any residual pulp, the skins were pressed using on paper towels. The collected skins were lyophilized using a CHRIST Alpha 1-4 LD plus lyophilizer (Germany) and then were grounded and maintained at 4°C until analysis.

Extraction procedures

Ultrasound-assisted extraction

The extraction was accomplished using an ultrasonic bath (MRC Scientific Instruments, LTD, Israel). Grounded freeze-dried powder of red grape skins was mixed with the designated volume of ethanol of varying concentrations and placed in the ultrasonic bath. The parameters for ultrasound-assisted extraction were: frequency of 40 kHz and power of 100 W. For maintaining the temperature constant cold-water was added in the ultrasonic bath.

Four variables were tested to evaluate their influence on the extraction of total anthocyanins and total phenolic compounds: number of extractions (1 to 3), extraction temperature (25 and 50ºC), solvent concentration (50% to 96% v/v), and extraction time (25 min to 55 min). The extraction was carried out as follows: 1 g powder was mixed with 9 mL solvent (50%, 70%, or 96% ethanol acidified with glacial acetic acid, 99.5% citric acid, or 0.1 N HCl) and then sonicated at different temperature (25 or 50°C) and extraction times (25 min or 55 min). Two successive extractions were afterwards done using the residue collected after centrifugation at

5000 rpm, at 4 °C for 10 minutes. The supernatants separated from the three extraction steps were pooled together and were further characterized.

Enzyme -assisted extraction

Three enzymes were used to extract anthocyanins from the grape skin: Cellulase extracted from *Trichoderma viride* (Merck, Germany), Zymorouge (Sodinal, Romania) which is a mixture of pectintranseliminase, polygalacturonase, pectinesterase and small amounts of hemicelluloses and cellulose produced by Aspergillus niger, and xylanase produced by *Thermomyces lanuginosus* (Sigma Aldrich, Germany). The enzymes were solubilized in sodium acetate at pH of 3.5 for cellulase, pH of 5.0 for Zymorouge and pH of 6.5 for xylanase, such as to obtain a final concentration of 10%. In order to extract the bioactive compounds, the grape skin was homogenized with sodium acetate 0.2 M such as to get a ratio of 1 to 28. The enzyme solution (2 mL/100 mL mixture) was added after adjusting the pH to the optimum value corresponding to each enzyme used in the study (pH of 3.5 for cellulase, pH of 5.0 for zymorouge and pH of 6.5 for xylanase). Incubation took place on an orbital shaker at 40ºC, 120 rpm for 1, 2 and 3 hours. The enzyme was finally inactivated by treating the extracts in a water bath at 100ºC for 5 minutes. The extract obtained through centrifugation for 10 minutes at 14000 rpm and 4ºC was analyzed in terms of phytochemicals content.

Determination of bioactive compounds and antioxidant activity

Total monomeric anthocyanins content

In order to estimate the total monomeric anthocyanins content (TMA), a modified pH differential method was used (Turturica *et al.*, 2016). The absorbance of diluted extracts using different buffer solutions at pH 1.0 and 4.5 was measured at 520 nm and 700 nm. The results were expressed as milligrams of cyanidin-3-glucoside (C3G) per gram of dry weight (DW).

Total flavonoids determination

The total flavonoid content (TFC) was determined using Dewanto *et al.* (2002) modified method. Briefly, a mixture obtained by incorporating each red grape skin extract (250 µL) with ultrapure water (1250 µL) and of 5% $NaNO_2$ solution (75 µL) was allowed to react for 5 minutes. Then, were added 10% aluminum chloride solution (150 µL), NaOH solution 1 M (500 µL), and ultrapure water to a final volume of 3000 µL. The mixture absorbance was measured at a wavelength of 510 nm. The results were expressed as mg catechin equivalents (CE) /g DW.

Total polyphenols determination

The *total polyphenols content (TPC)* was achieved using Dewanto *et al.* (2002) modified method. Briefly, a mixture was obtained by adding 200 µL extract, 15.8 mL ultrapure water, and 1 mL of Folin-Ciocalteau reagent. After 10 minutes, a volume of 3 mL of 20% Na_2CO_3 was added, and the mixture was maintained in a dark place for 60 min, at 25°C. The mixture absorbance was measured at a wavelength of 765 nm. The results obtained were expressed as mg GAE/ g DW.

Antioxidant activity - DPPH assay

The antiradical activity (AA) of red grape skin extracts was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) according to Castro-Vargas *et al.* (2010) and Turturica *et al.* (2016). Briefly, a mixture was obtained by adding 200 µL extract and 3.9 mL DPPH solution 0.1M. The mixture was maintained in a dark place for 90 min, at 25°C. The mixture absorbance was measured at a wavelength of 515 nm. A control was prepared by using adding 200 µL methanol and 3.9 mL DPPH solution 0.1M, and the absorbance mixture was also measured. The results obtained were expressed as mM Trolox/ g DW.

High-performance liquid chromatography (HPLC) analysis of anthocyanins

The anthocyanins identification from the red grape skin extracts were determined using the protocol described by Turturica *et al*. (2016), using a Thermo Finnigan Surveyor HPLC system and an Xcalibur software (Finnigan Surveyor LC, Thermo Scientific, USA).

Statistical analysis of data

The results are expressed as a mean of triplicate measurements ± standard deviation. In order to identify the significant differences between values, the analysis of variance (ANOVA) and Tukey test ($p < 0.05$) were performed.

Results and discussion

The phytochemical compounds determination

In this study, the two extraction methods (UAE and EAE) were used to evaluate the influence of four independent variables on the extraction yield of bioactive compounds present in the skin of Băbească neagră grapes. Extraction of phenolic compounds from grape skin was performed using ultrasound using ethanol treatment in three different combinations (50%, 70%, and 96% ethanol) at 25 and 50°C for 25, 40, and 55 minutes. Selecting a suitable extraction solvent combination is one of the essential steps in ensuring a successful extraction, and was based on previous studies that aimed to extract the bioactive compounds found in red grape skins (*Vitis vinifera*) (Burin *et al.*, 2011; Constantin *et al.*, 2015; Moreno-Montoro *et al.*, 2015). Moreover, the ethanol was proven to be the most appropriate solution for the extraction of various phenolic compounds from different plant materials (González-Centeno *et al.*, 2014; He and Giusti, 2010). In most cases, the ratio of solvent to the matrix used, temperature, and solvent concentration were investigated to recover bioactive components. The results indicated that the extraction depends on several factors specific from one plant material to another.

In this study, all concentration of ethanol extractions provided high extraction yields of bioactive compounds from red grape skins. Table 1 shows the anthocyanin content obtained from red grape skin using UAE by varying the extraction parameters. Ultrasound-assisted ethanolic extraction allowed the extraction of maximum anthocyanin content of 4.29±0.04 mg C3G/g DW when using 96% ethanol acidified with 0.1 N HCl after 55 minutes of extraction at 50 °C.

Table 1. Total monomeric anthocyanins (TMA) and polyphenol content (TPC) of red grape skin extracts using ultrasound-assisted extractions.

TMA, mg C3G/g DW

Solvent	Extraction time	50% Ethanol		70% Ethanol		96% Ethanol	
		25°C	50°C	25°C	50°C	25°C	50°C
Glacial acetic acid	25 min	2.54±0.17^{abB}	4.06±0.07^{aA}	3.21±0.54^{aA}	2.44±0.06^{aB}	0.83±0.16^{bB}	2.84±0.10^{aA}
	40 min	2.27±0.14^{bB}	3.96±0.10^{Aab}	2.83±0.13^{aA}	2.15±0.37^{aB}	1.08±0.10^{aB}	3.06±0.51^{aA}
	55 min	2.64±0.32^{aB}	3.84±0.16^{Ab}	3.05±0.42^{aB}	4.00±0.37^{bA}	1.00±0.07^{abB}	2.84±0.06^{aA}
99.5% citric acid	25 min	3.05±0.99^{aA}	3.99±0.13^{aA}	2.66±0.20^{aB}	3.87±0.04^{abA}	1.60±0.14^{aB}	3.67±0.46^{aA}
	40 min	3.78±0.25^{aA}	3.81±0.21^{aA}	2.78±0.58^{aB}	3.92±0.02^{aA}	1.64±0.29^{aA}	3.67±0.43^{aB}
	55 min	3.91±0.12^{aA}	3.97±0.10^{aA}	3.13±0.54^{aB}	3.77±0.14^{bA}	1.36±0.14^{aB}	3.70±0.18^{aA}
0.1N hydrochloric acid	25 min	3.70±0.37^{bB}	4.17±0.07^{aA}	3.02±0.90^{bB}	4.10±0.05^{aA}	2.69±0.55^{bB}	3.56±0.29^{bA}
	40 min	3.78±0.12^{abA}	3.88±0.53^{aA}	4.01±0.07^{aA}	3.98±0.04^{bA}	3.08±0.19^{abB}	4.03±0.09^{aA}
	55 min	4.12±0.19^{aA}	3.93±0.23^{aA}	3.88±0.07^{aB}	4.16±0.04^{aA}	3.36±0.25^{aB}	4.29±0.04^{aA}

TPC, mg GAE/g DW

Solvent	Extraction time	50% Ethanol		70% Ethanol		96% Ethanol	
		25°C	50°C	25°C	50°C	25°C	50°C
Glacial acetic acid	25 min	28.56±1.83^{aB}	41.52±4.01^{aA}	30.42±5.54^{aB}	39.86±1.24^{aA}	12.45±1.30^{aB}	23.18±1.53^{aA}
	40 min	26.21±1.70^{aB}	36.97±3.83^{aA}	27.46±2.81^{aA}	28.80±8.15^{bA}	13.69±0.82^{aB}	22.69±1.18^{aA}
	55 min	26.70±2.40^{aB}	40.68±4.04^{aA}	29.05±4.21^{aA}	34.24±6.98^{abA}	12.96±1.22^{aB}	22.46±3.16^{aA}
99.5% citric acid	25 min	38.01±8.83^{bB}	47.43±1.38^{aA}	35.77±3.22^{aB}	48.35±3.11^{aA}	20.23±1.64^{aB}	31.06±2.85^{aA}
	40 min	48.95±4.75^{aA}	48.32±3.04^{aA}	38.28±8.00^{aA}	45.04±3.52^{aA}	19.15±2.37^{aB}	30.01±1.55^{aA}
	55 min	46.79±4.11^{abA}	49.01±5.08^{aA}	42.04±5.26^{aB}	47.41±1.03^{aA}	16.51±3.20^{aB}	30.42±1.91^{aA}
0.1N hydrochloric acid	25 min	40.03±2.84^{bA}	44.67±5.08^{aA}	43.19±3.84^{aA}	45.94±4.67^{aA}	22.28±2.96^{aB}	25.60±1.22^{bA}
	40 min	45.74±3.52^{aA}	41.00±6.46^{aA}	46.58±6.13^{aA}	45.14±4.83^{aA}	26.84±3.34^{aB}	30.43±2.09^{aA}
	55 min	41.19±3.91^{abA}	40.45±9.83^{aA}	40.4±5.86^{aA}	40.76±2.77^{aA}	25.47±4.65^{aA}	31.74±3.33^{abA}

DW-dry weight. Results are expressed as mean ± SD of three parallel measurements. Within an experimental set, means that on the same row do not share a lowercase letter (a, b) are significantly different at $p<0.05$. Means that on the same column do not share an uppercase letter (A, B) are significantly different at $p<0.05$.

The extraction with 70% and 50% ethanol, both acidified with 0.1 N HCl allowed the extraction of 4.16±0.04 mg C3G/g DW after 55 minutes, respectively 4.17±0.07 mg C3G/g DW after 25 minutes at 50°C. In this study, the extraction with 96% ethanol and glacial acetic acid performed for 25 minutes at 25°C led to the extraction of the lowest amount of anthocyanins of 0.83±0.16 mg C3G/g DW. Like other plant materials, the composition of red grape skins in anthocyanins is affected by cultivation, soil properties, maturity, harvesting sites, extraction conditions, actors that may explain the significant variability of anthocyanins. For example, Rockenbach *et al.* (2011) reported a value of the total anthocyanin content found in four red grape pomace (Cabernet Sauvignon, Merlot, Bordeaux, and Isabel) grown in Brazil between 1.84-11.22 mg malvidin-3-glucoside /g DW.

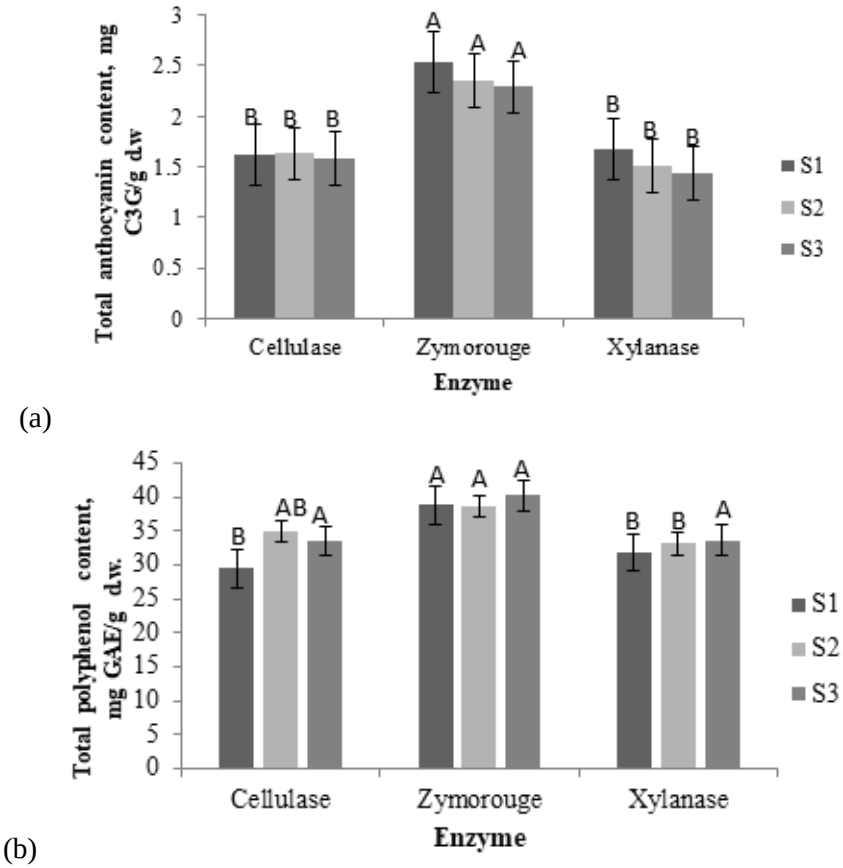


Figure 1. Total monomeric anthocyanin (a) and total polyphenol (b) contents obtained by extraction with the addition of enzymatic preparations by varying the extraction time **(**S1 - 1h of hydrolysis; S2 - 2h of hydrolysis; S3 - 3h of hydrolysis). Different uppercase letters show significant differences, using $p<0.05$, between samples obtained with different enzymes and the same extraction time

For enzyme-assisted extraction, a high TMA content (2.54±0.13 mg C3G/g DW) was found after one hour of hydrolysis using the Zymorouge pectolytic enzyme (Figure 1a). As the extraction time increased, the anthocyanins began to degrade, with a value of (2.29±0.30 mg C3G/ g DW) after 3 hours of hydrolysis. In the same time, after 3 hours of hydrolysis xylanase extracted the lowest amount of anthocyanins from the cell walls (1.44±0.12 mg C3G/g DW). The higher TMA yields obtained by UAE can be the results of the ultrasound waves that facilitate the solvent dispersal in the plant matrix mainly because of the breaking down of the plant wall, which leads to the transfer increasing of the anthocyanins to the solvent (Avhad and Rathod, 2015; Ramić *et al.*, 2015). Different solvent combinations were used to extract polyphenols from the grape skin, the results being collected in Table 1.

The highest recovery of TPC (49.01±5.08 mg GAE/g DW) from red grape skin was identified at 50 °C, after 55 minutes of treatment at a temperature of 50 °C, when using 50 % ethanol acidified with 99.5% citric acid (Table 1). On the other hand, the lowest TPC, was observed for the extraction with 96% ethanol acidified with glacial acetic acid using the following extraction parameters: 25 minutes and 25 °C. He *et al.* (2016) stated that the ultrasonic treatment applied to blueberry vinification by-products extracted content of 16.41 mg GAE/g DW using acidified water as a solvent. In another study Santos *et al.* (2011) reported a phenolic content between 0.04 and 122.35 mg GAE /g DW found in different parts (pulp and peel) from four grape varieties such as Brazil and Benitaka (*Vitis vinifera*), and Isabel and Niagara (*Vitis labrusca*). Other researchers González-Centeno *et al.* (2014) stated that ultrasound treatment for 30 minutes extracted 5.37 to 31.87 mg gallic acid/100 g of fresh grapes. The differences in extraction efficiency and antioxidant activity of natural extracts depended on the solvent used for extraction (Dranca and Oroian, 2016).

For extraction of the polyphenols from plants, the polar solvents are commonly in aqueous mixtures with ethanol, methanol, acetone, and ethyl acetate (Do *et al.*, 2014). In this study, ethanol was chosen as a solvent for polyphenol extraction mainly because it is safe for human consumption, and by mixing the solvent with water leads to the increase of the polarity index and the improvement of the extraction (Razali *et al.*, 2012). Several authors reported that the bioactive compounds extraction yield was higher as the solvents' polarity increased (Alothman *et al.*, 2009; Musa *et al.*, 2011; Razali *et al.*, 2012). In the present study, the selected solvent had a significant influence on the TPC ($p<0.05$). For enzyme-assisted extraction, in terms of the amount of polyphenols extracted, Zymorouge rich in pectolytic enzymes extracted the most considerable amount of total polyphenols than cellulase, and xylanase assisted extraction, the highest polyphenols content being 38.85±1.82 mg GAE/g DW after one hour of hydrolysis (Figure 1b).

In Table 2, the content of TFC and antioxidant activity (AA) of the grape skins extracts by using ultrasound-assisted extraction are presented.

The highest TFC of 11.34±1.45 mg CE/g DW was found for extraction with 96% ethanol acidified with citric acid, after 25 minutes of extraction at 25°C. An equally high content was obtained for the extraction with 70% ethanol acidified with citric acid, after 55 minutes of extraction at 50°C. The lowest flavonoid content was calculated after 25 minutes of extraction, with 96% ethanol acidified with glacial acetic acid at 25°C.

Ivanova *et al.* (2010) obtained similar grape skin extract results and reported a value of 6.90±0.42 mg CE/g DW after extraction with 80% ethanol. Novak *et al.* (2008) obtained by extraction with methanol acidified with 1% hydrochloric acid, a value of 4.99±0.07 mg CE/g lyophilized grape skin extract. (Burin *et al.*, 2014) obtained for red and white grapes extracts from six *V. vinifera* varieties and five *V. labrusca* varieties harvested in Brazil, macerated under agitation (100 rpm) for 24 h, a total flavonol content between 139.2 and 421.4 μg/100 g grapes (fresh weight).

The values of the antioxidant activity of extracts using UAE were between 3.70±1.39 and 18.76±0.24 mMol Trolox/g DW. The lowest value was obtained for 70% ethanol combined with 99.5% citric acid after 55 minutes of extraction at 50 °C. Pure ethanol (96%) with glacial acetic acid at 25°C allowed from the first 25 minutes of extraction to obtain the highest antioxidant activity (18.76±0.24 mMol Trolox/g DW).

Constantin *et al.* (2015) evaluated the content of the antioxidant activity of red grape skins in the case of Fetească neagră and Băbească neagră varieties and reported a value of 4.89±0.02 μg Trolox/g DW, respectively 3.06±0.04 μg Trolox/g DW. Rockenbach *et al.* (2011) determined an inhibition value of 41.13% in the Bordeaux grape variety using the ß-carotene/linoleic acid method. They demonstrated that Cabernet Sauvignon and Bordeaux red grape varieties showed the highest potential as a source of antioxidant compounds and natural dyes, respectively.

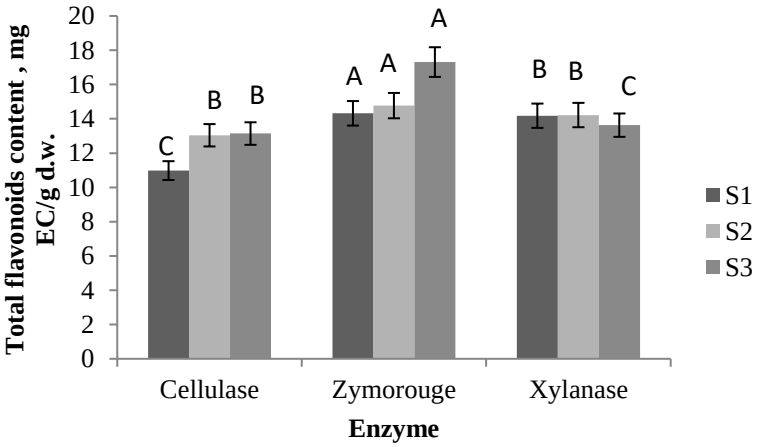
Regarding extraction with enzymes, the most significant amount of total flavonoids was quantified by using Zymorouge addition after three hours of extraction (17.31±8.42 mg CE/g DW). However, cellulase was the one that extracted the lowest flavonoid concentration (10.98±3.02 mg CE/g DW) after one hour of extraction. Other authors that used 1% hydrochloric acidified methanol extraction reported a value of 4.99±0.07mg CE/g frozen grape skin extract (Novak *et al.*, 2008). Tomaz *et al.* (2016) reported in grape skin a total flavonoid concentration of 3718 ± 5 mg EC / kg DW using pectinase-assisted extraction at a temperature of 45° C after 3 hours after extraction.

Enzyme assisted extraction by cellulase addition in the case of antioxidant activity, gave the best extraction yield. After only one hour of extraction, an extract with antioxidant activity of 61.48±1.19 mMol Trolox/g DW was obtained. The lowest value of antioxidant activity (44.41±1.91 mMol Trolox/g DW) was obtained after 3 hours of hydrolysis by using Zymorouge addition. According to Constantin *et al* (2015), the content found for the antioxidant activity of grape skin extracts from Fetească neagră and Băbească neagră varieties recorded values of 4.89±0.02 μg Trolox/g DW, respectively 3.06±0.04 μg Trolox/g DW.

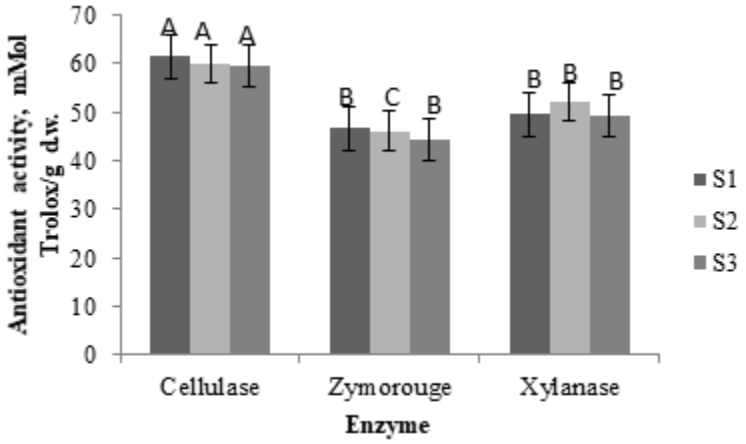
Table 2. Total flavonoids (TFC) and the DPPH radical-scavenging activity (AA) content of red grape skins extracts using ultrasound - assisted extraction.

TFC, mg CE/g DW							
Solvent	**Extraction time**	**50% Ethanol**		**70% Ethanol**		**96% Ethanol**	
		25°C	**50°C**	**25°C**	**50°C**	**25°C**	**50°C**
Glacial acetic acid	**25 min**	4.81±0.45^{aB}	6.04±1.14^{aA}	5.40±1.00^{bB}	6.72±0.71^{aA}	4.29±0.35^{bB}	6.18±0.62^{aA}
	40 min	4.90±0.40^{aA}	5.45±1.34^{aA}	5.62±0.60^{abA}	6.21±0.99^{aA}	4.31±0.25^{bB}	5.92±0.99^{aA}
	55 min	4.98±0.30^{aB}	6.54±1.32^{aA}	7.02±1.23^{aA}	5.90±0.37^{aA}	5.00±0.45^{aB}	6.22±0.38^{aA}
99.5% citric acid	**25 min**	5.22±1.31^{aA}	5.76±1.62^{aA}	5.66±0.68^{bA}	6.15±0.97^{cA}	11.34±1.45^{aA}	6.30±1.08^{aB}
	40 min	4.81±1.01^{aB}	7.12±0.51^{aA}	6.48±0.44^{abB}	7.66±0.60^{bA}	9.16±0.77^{bA}	6.57±1.32^{aB}
	55min	6.11±1.31^{aA}	7.76±1.94^{aA}	7.42±0.76^{aB}	9.30±1.03^{aA}	10.61±0.82^{abA}	6.43±0.68^{aB}
0.1N hydrochloric acid	**25 min**	6.61±0.59^{bA}	7.04±1.03^{aA}	6.56±0.48^{aB}	8.40±0.24^{aA}	5.90±0.52^{aA}	5.87±0.92^{aA}
	40 min	4.82±0.78^{bB}	6.66±0.94^{aA}	6.46±0.53^{abB}	8.04±1.12^{aA}	5.14±1.29^{aA}	6.22±1.33^{aA}
	55 min	6.39±1.22^{bA}	6.08±0.67^{aA}	5.26±1.18^{bB}	8.32±1.66^{aA}	6.20±0.46^{aA}	6.89±0.81^{aA}
AA, mMol Trolox /g DW							
Solvent	**Extraction time**	**50% Ethanol**		**70% Ethanol**		**96% Ethanol**	
		25°C	**50°C**	**25°C**	**50°C**	**25°C**	**50°C**
Glacial acetic acid	**25 min**	16.35±0.79^{aA}	11.76±0.56^{aB}	14.39±0.41^{aA}	12.60±0.48^{aB}	18.76±0.24^{aA}	17.79±0.23^{aB}
	40 min	16.25±0.35^{aA}	11.76±1.12^{aB}	14.67±0.35^{aA}	12.14±0.33^{aB}	18.67±0.38^{aA}	17.66±0.51^{aB}
	55 min	16.11±0.29^{aA}	10.70±0.78^{aB}	14.16±0.52^{aA}	12.54±0.29^{aB}	18.45±0.39^{aA}	17.74±0.20^{aB}
99.5% citric acid	**25 min**	11.69±3.36^{aA}	9.34±2.63^{aA}	10.83±1.10^{aA}	4.23±0.72^{aB}	14.85±0.64^{aA}	9.32±0.77^{aB}
	40 min	10.54±0.68^{aA}	6.13±2.35^{aB}	10.30±0.69^{aA}	4.78±1.08^{aB}	14.23±0.71^{aA}	10.32±2.08^{aB}
	55min	9.72±1.17^{aA}	6.99±2.46^{aB}	10.16±1.43^{aA}	3.70±1.39^{aB}	15.20±0.70^{aA}	8.8±0.59^{aB}
0.1N hydrochloric acid	**25 min**	13.61±1.17^{aA}	12.67±0.95^{aA}	16.36±1.17^{aA}	14.50±0.68^{aB}	13.70±1.65^{aA}	9.85±1.51^{bB}
	40 min	13.48±0.72^{aA}	13.59±1.43^{aA}	15.23±1.17^{aA}	14.36±0.92^{aA}	14.07±1.03^{aA}	12.10±0.90^{aB}
	55 min	13.7±0.47^{aA}	12.07±0.84^{aB}	16.48±0.54^{aA}	13.37±1.11^{aB}	13.07^{aA}±1.16	11.68^{aA}±1.09

DW- dry weight. Results are expressed as mean ± SD of three parallel measurements. Within an experimental set, means that on the same row do not share a lowercase letter (a, b, c) are significantly different at $p<0.05$. Means that on the same column do not share an uppercase letter (A, B) are significantly different at $p<0.05$.



(a)



(b)

Figure 2. Total flavonoids (a) and total antioxidant activity (b) contents obtained by extraction with the addition of enzymatic preparations by varying the extraction time (S1 - 1h of hydrolysis; S2 - 2h of hydrolysis; S3 - 3h of hydrolysis). Different uppercase letters shows significant differences, using $p<0.05$, between samples obtained with different enzymes and the same extraction time.

HPLC anthocyanins profile determination

The red grape skins ethanolic UAE extraction at 50°C with 70% Ethanol and 0.1N hydrochloric acid for 55 min was analyzed and a profile that contains nine anthocyanins was obtained. Figure 3 illustrates the chromatographic profile of anthocyanins in the Băbească neagră grape variety, in which nine anthocyanins were separated, namely: 1 - delphinidin-3-glucoside, 2 - cyanidin-3-glucoside, 3 - petunidin-3-glucoside, 4 - pelargonidin-3-glucoside, 5 - malvidin-3-glucoside, 6 - cyanidin, 7 - peonidin-3-coumarylglucoside, 8 - peonidin.

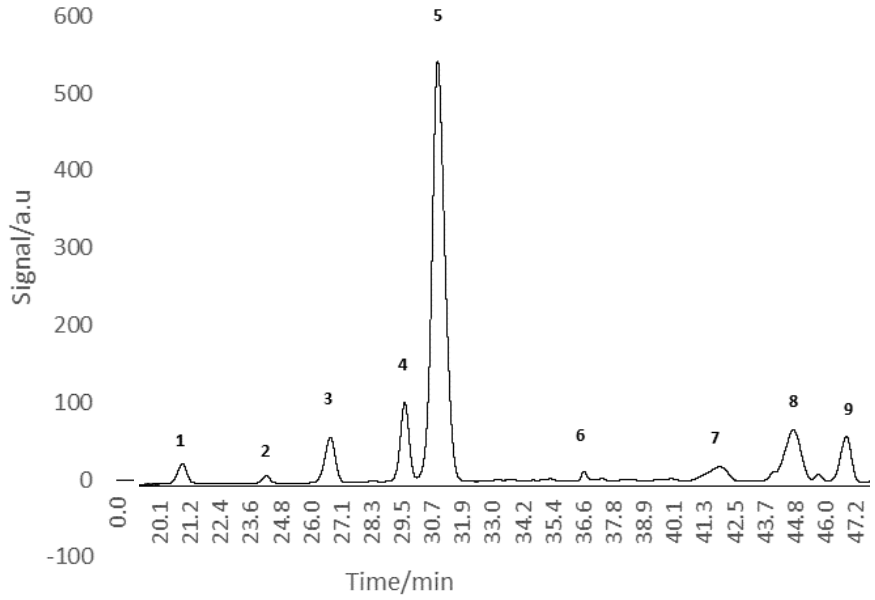


Figure. 3. HPLC–DAD chromatograms of anthocyanin/anthocyanidin profile of Băbească neagră grape skin at 520 nm. Compounds identified: 1 - delphinidin-3-glucoside, 2 - cyanidin-3-glucoside, 3 - petunidin-3-glucoside, 4 - pelargonidin-3-glucoside, 5 - malvidin-3-glucoside, 6 - cyanidin, 7 - peonidin-3-coumarylglucoside, 8 – peonidin.

The highest peak was registered for the compound malvidin-3-O-glucoside, 19.02 ± 0.15 mg C3G/g DW (Table 3). The results are similar to those obtained by Stănciuc *et al.* (2017), who reported 14 anthocyanins, malvidin-3-O-glucoside being also the major anthocyanin(18.46 mg C3G/g DW).

In a study conducted by Silva and Queiroz (2016) on Touriga Nacional red grapes from the Dão region (Portugal), the major anthocyanin reported was malvidin-3-O-glucoside, having a content of 2.80±0.12 mg/g. Also, 14 anthocyanins were identified by Budić-Leto *et al.* (2018) in the G1, IJK 92, IJK 96 and Merlot grape samples, namely: delphinidin 3-monoglucoside, cyanidin 3-monoglucoside, petunidin 3-monoglucoside, peonidin 3-monoglucoside, malvidin 3-monoglucoside, delphinidin 3-monoglucoside-acetate, cyanidin 3-monoglucoside-acetate, petunidin 3-glucoside-acetate, peonidin 3-monoglucoside-acetate, malvidin 3-monoglucoside-acetate, delphinidin 3-monoglucoside-p-cumarate, cyanidin3-monoglucoside-p-cumarate, petunidine 3- monoglucoside-p-cumarate, peonidine 3-monoglucoside-p-coumarate and malvidin 3-monoglucoside-p-coumarate.

Similar results regarding anthocyanins' presence were also related in studies by Lima *et al.* (2015) who identified in grape juice delphinidin-3-glucoside, cyanidin-3, 5-diglucoside, and cyanidin-3-glucoside. Natividade *et al.* (2013) determined a similar anthocyanin profile, with malvidin-3-glucoside and delphinidin-3-glucoside found in the highest amount.

Table 3. The compounds detected in extracts of grape skins by HPLC–MS using by UAE extraction at 50°C with 70% Ethanol and 0.1N hydrochloric acid for 55 min.

Peak	Compound	t_r, min	Anthocyanins/Anthocyanidins mg/g
1	Delphinidin-3-glucoside	21.10 ± 0.02	1.73 ± 0.03
2	Cyanidin-3-glucoside	24.60 ± 0.01	0.81 ± 0.0
3	Petunidin-3-glucoside	26.90 ± 0.01	NQ
4	Pelargonidin-3-glucoside	29.70 ± 0.02	4.01 ± 0.06
5	Malvidin-3-glucoside	30.09 ± 0.01	19.02 ± 0.15
6	Cyanidin	37.50 ± 0.03	0.84 ± 0.01
7	Peonidin-3-coumarylglucoside	42.30 ± 0.04	NQ
8	Peonidin	44.90 ± 0.04	3.11 ± 0.09
9	Malvidin	46.90 ± 0.06	2.95 ± 0.01

*NQ -not quantified

The composition of anthocyanins may differ depending on the grape variety. For example, Huang *et al.* (2009) reported six anthocyanins in *Vitis rotundifolia* (Muscadine) grapes.

Conclusions

Grapes contain many bioactive compounds such as polyphenols, flavonoids, minerals and vitamins that have many health benefits. Due to a limited number of studies regarding the reuse of by-products from the vinification of red grapes, the present study brings new opportunities to exploit these biologically active compounds extracted from grape skin. Two extraction techniques (ultrasound and enzyme assisted extraction) were used to improve the recovery of total anthocyanins and total phenolic compounds from the skin of Băbească variety. The profile of the bioactive compounds extracted from the red grape skins varied with the extraction method. In case of the ultrasound assisted extraction the results showed that ethanol 96% was the best solvent for the phenolic, flavonoids, and anthocyanins extraction, followed by the ethanol 70% and ethanol 50% extracts. In the case of the enzymes assisted extraction of bioactive compounds from red grape skin, the Zymoruge allowed the best extraction yield, followed by cellulase and xylanase extract.

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1.2 – Institutional Performance – Projects for financing excellence in Research, Development and Innovation, Contract no. 14PFE/17.10.2018".

References

Alothman, M., Bhat, R., Karim, A.A. 2009. Antioxidant capacity and phenolic content of selected tropical fruits from Malaysia, extracted with different solvents. *Food Chemistry*, **115**(3), 785-788.

Anuar, N., Mohd Adnan, A. F., Saat, N., Aziz, N., & Mat Taha, R. (2013). Optimization of Extraction Parameters by Using Response Surface Methodology, Purification, and Identification of Anthocyanin Pigments inMelastoma malabathricumFruit. *The Scientific World Journal*, 1–10.

Avhad, D.N., Rathod, V.K. 2015. Ultrasound assisted production of a fibrinolytic enzyme in a bioreactor. *Ultrasonics Sonochemistry*, **22**, 257-264.

Borges, G. D. S. C., Vieira, F. G. K., Copetti, C., Gonzaga, L. V., & Fett, R. (2011). Optimization of the extraction of flavanols and anthocyanins from the fruit pulp of Euterpe edulis using the response surface methodology. *Food Research International*, **44**(3), 708–715.

Budić-Leto, I., Mucalo, A., Ljubenkov, I., Zdunić, G. 2018. Anthocyanin profile of wild grape Vitis vinifera in the eastern Adriatic region. *Scientia horticulturae*, **238**, 32-37.

Burin, V.M, Rossa, P.N., Ferreira-Lima, N.E., Hillmann, M.C.R., Boirdignon-Luiz, M.T. 2011. Anthocyanins: optimisation of extraction from Cabernet Sauvignon grapes, microcapsulation and stability in soft drink. *International Journal of Food Science and Technology*, **46**(1), 186–193.

Burin, V.M., Ferreira-Lima, N.E., Panceri, C.P., Bordignon-Luiz, M.T. 2014. Bioactive compounds and antioxidant activity of Vitis vinifera and Vitis labrusca grapes: Evaluation of different extraction methods. *Microchemical Journal*. **114**, 155-163.

Castro-Vargas, H.I., Rodríguez-Varela, L.I., Ferreira, S.R.S., Parada-Alfonso, F. 2010. Extraction of phenolic fraction from guava seeds Psidium guajava L. using supercritical carbon dioxide and co-solvents. *Journal of Supercritical Fluids*. **51**(3), 319-324.

Cheok, C.Y., Chin, N.L., Yusof, Y.A., Talib, R.A., Law, C.L. 2013. Optimization of total monomeric anthocyanin (TMA) and total phenolic content (TPC) extractions from mangosteen Garcinia mangostana Linn. hull using ultrasonic treatments. *Industrial Crops and Products*, **50**, 1–7.

Constantin, O.E., Skrt, M., Poklar Ulrih, N., Râpeanu, G. 2015. Anthocyanins profile, total phenolics and antioxidant activity of two Romanian red grape varieties: Feteasca neagra and Babeasca neagra Vitis vinifera. *Chemical Papers* ,**12**, 69.

Corrales, M., Toepfl, S., Butz, P., Knorr, D., Tauscher, B. 2008. Extraction of anthocyanins from grape by-products assisted by ultrasonics, high hydrostatic pressure or pulsed electric fields: A comparison. *Innovative Food Science and Emerging Technologies*, **9**(1), 85–91.

Dewanto, V., Wu, X.K. Adom, K., Hai Liu, R. 2002. Thermal Processing Enhances the Nutritional Value of Tomatoes by Increasing Total Antioxidant Activity. *Journal of Agricultural and Food Chemistry*, **50**(10), 3010–3014.

Do, Q.D., Angkawijaya, A.E., Tran-Nguyen, P.L., Huynh, L.H., Soetaredjo, F.E., Ismadji, S., Ju, Y.H. 2014. Effect of extraction solvent on total phenol content, total flavonoid

content, and antioxidant activity of Limnophila aromatica. *Journal of Food and Drug Analysis*. **22**(3), 296-302.

Dranca, F., Oroian, M. 2016. Optimization of ultrasound-assisted extraction of total monomeric anthocyanin (TMA) and total phenolic content (TPC) from eggplant Solanum melongena L. peel. *Ultrasonics Sonochemistry*. **31**, 637-646.

Fan, G., Han, Y., Gu, Z., & Gu, F. (2008). Composition and colour stability of anthocyanins extracted from fermented purple sweet potato culture. *LWT - Food Science and Technology*, **41**(8), 1412–1416.

Garcia-Salas, P., Morales-Soto, A., Segura-Carretero, A., Fernández-Gutiérrez, A. 2010. Phenolic-Compound-Extraction Systems for Fruit and Vegetable Samples. *Molecules*, **15**(12), 8813–8826.

González-Centeno, M.R., Knoerzer, K., Sabarez, H., Simal, S., Rosselló, C., Femenia, A. 2014. Effect of acoustic frequency and power density on the aqueous ultrasonic-assisted extraction of grape pomace Vitis vinifera L. - A response surface approach. *Ultrasonics Sonochemistry*, **21**(6), 2176–2184.

He, B., Zhang, L.L., Yue, X.Y., Liang, J., Jiang, J., Gao, X.L., Yue, P.X. 2016. Optimization of Ultrasound-Assisted Extraction of phenolic compounds and anthocyanins from blueberry Vaccinium ashei wine pomace. *Food Chemistry*, **204**, 70-76.

He, J., Giusti, M. 2010. Anthocyanins: Natural colorants with health-promoting properties. Annual Review of *Food Science and Technology*. **1**, 163-187.

Huang, Z., Wang, B., Williams, P., Pace, R.D. 2009. Identification of anthocyanins in muscadine grapes with HPLC-ESI-MS. LWT - *Food Science and Technology*, **42**(4), 819–824.

Ivanova, V., Stefova, M., Chinnici, F. 2010. Determination of the polyphenol contents in Macedonian grapes and wines by standardized spectrophotometric methods. *Journal of the Serbian Chemical Society*. **75**(1), 45-59.

Kammerer, D., Claus, A., Carle, R., Schieber, A. 2004. Polyphenol Screening of Pomace from Red and White Grape Varieties Vitis vinifera L. by HPLC-DAD-MS/MS. *Journal of Agricultural and Food Chemistry*, **52**(14), 4360–4367.

Liang, Z., Wu, B., Fan, P., Yang, C., Duan, W., Zheng, X., Liu, C., Li, S. 2008. Anthocyanin composition and content in grape berry skin in Vitis germplasm. *Food Chemistry*, **111**(4), 837–844.

Lima, M.D.S., da Conceição Prudêncio Dutra, M., Toaldo, I.M., Corrêa, L.C., Pereira, G.E., de Oliveira, D., Bordignon-Luiz, M.T., Ninow, J.L. 2015. Phenolic compounds, organic acids and antioxidant activity of grape juices produced in industrial scale by different processes of maceration. *Food Chemistry*, **188**, 384-392.

Ma, W., Lu, Y., Dai, X., Liu, R., Hu, R., Pan, Y. 2009. Determination of anti-tumor constitute mollugin from traditional Chinese medicine Rubia cordifolia: Comparative study of classical and microwave extraction techniques. *Separation Science and Technology*, **44**(4), 995–1006.

Marathe, S.J., Jadhav, S.B., Bankar, S.B., Singhal, R.S. 2017. Enzyme-Assisted Extraction of Bioactives. In Food Bioactives: Extraction and Biotechnology Applications. *Springer International Publishing* 171–201.

Moreno-Montoro, M., Olalla-Herrera, M., Gimenez-Martinez, R., Navarro-Alarcon, M., Rufián-Henares, J.A. 2015. Phenolic compounds and antioxidant activity of Spanish commercial grape juices. *Journal of Food Composition and Analysis*, **38**, 19–26.

Musa, K.H., Abdullah, A., Jusoh, K., Subramaniam, V. 2011. Antioxidant Activity of Pink-Flesh Guava Psidium guajava L. : Effect of Extraction Techniques and Solvents. *Food Analytical Methods*. **4**(1), 100-107.

Nadar, S.S., Rao, P., Rathod, V.K. 2018. Enzyme assisted extraction of biomolecules as an approach to novel extraction technology: A review. *In Food Research International* **108**, 309–330. Elsevier Ltd.

Natividade, M.M.P., Corrêa, L.C., de Souza, S.V.C., Pereira, G.E., de Oliveira Lima, L.C. 2013. Simultaneous analysis of 25 phenolic compounds in grape juice for HPLC: Method validation and characterization of São Francisco Valley samples. Microchemical Journal, **110**, 665-674.

Novak, I., Janeiro, P., Seruga, M., Oliveira-Brett, A.M. 2008. Ultrasound extracted flavonoids from four varieties of Portuguese red grape skins determined by reverse-phase high-performance liquid chromatography with electrochemical detection. *Analytica Chimica Acta*. **630**(2), 107-115.

Pinelo, M., del Fabbro, P., Manzocco, L., Nuñez, M.J., Nicoli, M.C. 2005. Optimization of continuous phenol extraction from Vitis vinifera byproducts. *Food Chemistry*, **92**(1), 109–117.

Pompeu, D. R., Silva, E. M., & Rogez, H. (2009). Optimisation of the solvent extraction of phenolic antioxidants from fruits of Euterpe oleracea using Response Surface Methodology. *Bioresource Technology*, **100**(23), 6076–6082.

Puri, M., Sharma, D., Barrow, C.J. 2012. Enzyme-assisted extraction of bioactives from plants. *In Trends in Biotechnology*, **30**(1), 37–44).

Ramić, M., Vidović, S., Zeković, Z., Vladić, J., Cvejin, A., Pavlić, B. 2015. Modeling and optimization of ultrasound-assisted extraction of polyphenolic compounds from Aronia melanocarpa by-products from filter-tea factory. *Ultrasonics Sonochemistry*. **23**, 360-368.

Razali, N., Mat-Junit, S., Abdul-Muthalib, A.F., Subramaniam, S., Abdul-Aziz, A. 2012. Effects of various solvents on the extraction of antioxidant phenolics from the leaves, seeds, veins and skins of Tamarindus indica L. *Food Chemistry*. **131**(2), 441-448.

Rockenbach, I.I., Rodrigues, E., Gonzaga, L.V., Caliari, V., Genovese, M.I., Gonalves, A.E.D.S.S., Fett, R. 2011. Phenolic compounds content and antioxidant activity in pomace from selected red grapes Vitis vinifera L. and Vitis labrusca L. widely produced in Brazil. *Food Chemistry*. **127**(1), 174-179.

Rodríguez Montealegre, R., Romero Peces, R., Chacón Vozmediano, J.L., Martínez Gascueña, J., García Romero, E. 2006. Phenolic compounds in skins and seeds of ten grape Vitis vinifera varieties grown in a warm climate. *Journal of Food Composition and Analysis*, **19**(6–7), 687–693.

Santos, L. P., Morais, D. R., Souza, N. E., Cottica, S. M., Boroski, M., & Visentainer, J. V. (2011). Phenolic compounds and fatty acids in different parts of Vitis labrusca and V. vinifera grapes. *Food Research International*, **44**(5), 1414–1418.

Silva, L.R., Queiroz, M. 2016. Bioactive compounds of red grapes from Dão region Portugal: Evaluation of phenolic and organic profile. *Asian Pacific Journal of Tropical Biomedicine*, **6**(4), 315-321.

Singh, K., Kumar, T., Vishvavidyalaya, K., Kumar, V., Sharma, S. 2019. A review on conversion of food wastes and by - products into value added products. *International Journal of Chemical Studies*, **7**(2), 2068–2073.

Stănciuc, N., Turturică, M., Oancea, A.M., Barbu, V., Ioniță, E., Aprodu, I., Râpeanu, G. 2017. Microencapsulation of anthocyanins from grape skins by whey protein isolates and different polymers. *Food and Bioprocess Technology*, **10**(9), 1715-1726.

Tomaz, I., Maslov, L., Stupić, D., Preiner, D., Ašperger, D., Kontić, J.K. 2016. Recovery of flavonoids from grape skins by enzyme-assisted extraction. *Separation Science and Technology Philadelphia*, **51**(2), 255-268.

Turturica, M., Stanciuc, N., Bahrim, G., Rapeanu, G. 2016. Effect of thermal treatment on phenolic compounds from plum (prunus domestica) extracts - A kinetic study. *Journal of Food Engineering*, **171**, 200–207.

Xavier, M. F., Lopes, T. J., Quadri, M. G. N., & Quadri, M. B. (2008). Extraction of red cabbage anthocyanins: optimization of the operation conditions of the column process. *Brazilian Archives of Biology and Technology*, **51**(1), 143–152.

Zia, S., Khan, M.R., Shabbir, M.A., Aslam Maan, A., Khan, M.K.I., Nadeem, M., Khalil, A.A., Din, A., Aadil, R.M. 2020. An Inclusive Overview of Advanced Thermal and Nonthermal Extraction Techniques for Bioactive Compounds in Food and Food-related Matrices. *In Food Reviews International*. Taylor and Francis Inc.

Zou, T.B., Wang, M., Gan, R.Y., Ling, W.H. 2011. Optimization of Ultrasound-Assisted Extraction of Anthocyanins from Mulberry, Using Response Surface Methodology. *International Journal of Molecular Sciences*, **12**(5), 3006–3017.