

OPTIMIZATION AND COMPARISON OF DIFFERENT TECHNIQUES FOR EXTRACTION OF FLAVONOIDS OF BULGARIAN MAVRUD GRAPE BY-PRODUCTS

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ABSTRACT

In recent years, the interest in antioxidants in grape by-products has increased significantly as scientific research for the extraction and quantification of these compounds. In the present investigation was optimized the antioxidant yield of the grape pomace and seeds discharged as waste from the Bulgarian Mavrud winery, near to Trakiets village. There is a lack of information about extraction optimization and utilization of the selected grape by-products. The aim of the paper was studying the influence of some process parameters: effect of the degreasing pre-treatment of the material, effect of the extraction method and the extraction time on the yield of total flavonoid and anthocyanin contents and Trolox equivalent antioxidant capacity (TEAC). For this propose the extraction kinetics were obtained by using conventional and non-conventional extraction methods - magnetic stirring and ultrasound-assisted extraction (UAE) and using of degreased and non-degreased plant material. Degreasing was done by conventional Soxhlet extraction with n-hexane for an hour. All analyses were done using ultraviolet-visible (UV-vis) spectrophotometry. The results obtained show that the applied ultrasound power after the degreasing pre-treatment promoted optimal anthocyanin and flavonoid recovery for 180 minutes extraction time of both - grape pomace and seeds. The TEAC is not affected by applying UAE and degreasing pre-treatment technique. The optimization of the extraction conditions is important because it could alter an economic impact on the extraction evaluation of the Mavrud grape by-products. The grape pomace and seeds from Bulgarian Mavrud wine-making can be used to obtain secondary derivatives with high-added antioxidant value.

Keywords: Bulgarian Mavrud, grape pomace, grape seeds, flavonoids, anthocyanins, antioxidant capacity, ultrasound-assisted extraction

INTRODUCTION

Wine consumption has increased over the years and, along with that, the concomitant increase in grape pomace production became an important subject of research. Solid waste produced after winemaking might account for over 30 % (w/w) of the grapes used. Grape pomace is the main solid organic waste from winery industries; resulting from the pressing and/or fermentation processes it is generated in large amounts in many parts of the world (Abarghuei *et al.*, 2010; Christ *et al.*, 2013)

The main components of grape pomace are seeds and skin. Studies have shown the potential of phenolics and antioxidant fibers recovery from the skin (Brenes *et al.*, 2016), as well as oil recovery from the seeds (Fiori *et al.*, 2014). However, there is still a long way to go until all these residues gain a real recovery pathway, thus making the winemaking process a more sustainable activity.

Wine production generates solid wastes as grape marcs and liquids called "wine by-products" (Martinez-carrasco *et al.*, 2005). These by-products are also of interest to the cosmetics industry. Indeed, the wine contains many phenolic compounds that possess antioxidant properties. These compounds play an essential role in the mechanism of skin rejuvenation. Thus, in recent years, the interest in antioxidants has increased significantly as scientific research for the extraction and quantification of these compounds.

Grape pomace consists of the skin, stems, and seeds that remain after grapes processing in the wine industry, where large amounts of bagasse are generated. However, grape pomace may become a product with a potential economic return since it is the source of bioactive compounds (phenolic compounds, fatty acids, pectins, etc.) that may be used in the manufacturing of food products. Furthermore, grape pomace may be used in ethanol production and in the recovery of organic acids, while the grape seeds are a source of several types of oil (Krishnaswamy *et al.*, 2012). Specifically, grape skin is a source of anthocyanins, natural pigments with antioxidant properties and may be used to recover hydrocolloids and phenolic compounds (Rodríguez *et al.*, 2010). Generally, grape skin has antimutagenic activities (Pedreschi *et al.*, 2006).

Grape seeds are known to be rich in procyanidins (Tounsi *et al.*, 2009) which have been reported for cardioprotective effects (Bagchi *et al.*, 2003), cataract prevention (Yamakoshi *et al.*, 2002), antihyperglycemic effects (Pinent *et al.*, 2004), anti-inflammatory effects (Terra *et al.*, 2007) as well as anticancer efficacy (Veluri *et al.*, 2006).

Recovery of the antioxidant components in the grape pomace and seeds is commonly performed in the literature and a lot of data is available. Moreover, the results are difficult to be compared even because the different extraction procedures are used, or the different grape varieties by-products are extracted. There is a lack in the literature concerning the systematic approaches to optimize the extraction process aiming to maximize the extraction yield and antioxidant power of the Bulgarian grape by-products. The extraction optimization of the Bulgarian Mavrud grape pomace and seeds is important because both low-cost wine-making wastes can be used to obtain secondary derivatives with high antioxidant value.

MATERIAL AND METHODS

Plant materials

In the present study were used grape pomace and grape seeds recovered as a residue from a vinification process in the Mavrud winery „Lozev wine Ltd.“, Bulgaria. The grape for wine production was collected from 2016 harvest at the winery in the „Trakiets” village.

Chemicals

Ethanol (96 %) was supplied by Valerus (Sofia, Bulgaria). Methanol (HPLC grade), sodium nitrite (NaNO₂), sodium acetate (CH₃COONa), potassium chloride (KCL), n-hexane (≥99 %) and aluminum chloride hexahydrate (AlCl₃ x 6H₂O) was supplied by Merck (Sofia, Bulgaria). Folin-Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), rutin hydrate, quercetin hydrate (≥95 %), (-) -

catechin hydrate ($\geq 96\%$), sodium hydroxide, malvidin 3-glucoside (97%), cyanidin 3-glucoside (97%) and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (97%) were supplied by Sigma Aldrich (Sofia, Bulgaria). Ammonia iron alum was supplied by Scharlau (Sofia, Bulgaria). Deionized water was from an "Elix70C Gulfstream" water deionizer supplied by Merck (Germany).

Sample preparation and extraction procedure

The grape pomace and the grape seeds were kept frozen in the freezer ($-18\text{ }^{\circ}\text{C}$) for six months. Before examinations, the frozen samples were left at room temperature for 24h after that were dried using an air conventional drier at $57\text{ }^{\circ}\text{C}$ for 4 hours.

It is well established that the phenolic compounds are heat sensitive substances, therefore temperatures lower than $60\text{ }^{\circ}\text{C}$ are considered suitable for the pre-treatment of grape pomace in order to keep its bioactive compounds (Bocco *et al.*, 1998).

The water content of the pomace and seeds was measured. The values found were 10.50% for the grape pomace and 8.24% - for the seeds. For the experimental runs were used grape pomace (skins: seeds, 90:10) and grape seeds. The grape seeds were chopped with a particles fraction size from 0.5 - 1.0 mm and the grape pomace was left whole. The stems accompanying usual the pomace was separate and non-used. All experiments on extraction kinetics were carried out at constant parameters: RT (room temperature approx. $25\text{ }^{\circ}\text{C}$), 50% ethanol in water solution, and 0.1 $\text{g}\cdot\text{L}^{-1}$ solid to the solvent ratio (1 g grape pomace or seeds to 10 ml solvent). Part of the pomace and the seeds were extracted with n-hexane through conventional Soxhlet apparatus. Afterthought was performed extraction kinetics applying constant ultrasound power of 280 W and frequency of 25 kHz on an ultrasound bath (AU-32, ARGOLAB, Italy) and by applying conventional extraction at 1411 RCF (relative centrifugal force) through magnetic stirrer (MS-H-Pro+, Dragon Laboratory Instruments, China). At certain previously determined times (5, 10, 15, 20, 30, 50, 80, 120, 180, 220) samples of the extracts were taken, filtered and analyzed immediately for total flavonoid content (TFC), total anthocyanin content (TAC) and TEAC. For the UAE all samples in each single extraction run were placed in the center of the ultrasound bath with the dept of the 3 centimeters and each batch of samples to be sonicated was kept the same position. Besides this, all samples of each experiment were sonicated simultaneously in the ultrasonic device.

Soxhlet extraction was conducted by the following manner: plant material (30.0 g) - homogenized pomace or seeds was placed in Whatman No. 1 (Watchmen, GE Healthcare, UK) filter paper and transferred to a reflux type Soxhlet extractor. The Soxhlet extractions were carried out for an hour using n-hexane as a solvent (300 mL) and heated under reflux. The long pre-treatment could lead to degradation of flavonoids and anthocyanins during refluxing. That is why a minimum time for degreasing was chosen. Thereafter the extracted plant materials were air dried and kept in a tightly closed dark glass tube. All extraction kinetics were performed in duplicate and the results were expressed as mean value \pm standard deviation (SD).

Total flavonoid content

The formation of a flavonoid-aluminum complex was employed to determine the flavonoid content (Patel *et al.*, 2010). An aliquot of 1 mL previously diluted extract (0.3 mL of the extract was added to a 10 mL volumetric flask containing deionized water), 4 mL of deionized water, and 0.3 mL of 5% NaNO_2 was dropped into a 10 mL volumetric flask. After 5 minutes, 0.3 mL AlCl_3 (10%) and 2 mL of 1M NaOH were added. Finally, a volume of 10 mL was adjusted with deionized water.

A standard curve of quercetin was drowned ($(0.5-5.0)\text{ g}\cdot\text{L}^{-1}$, $y=0.2175\cdot x$, $R^2=0.9936$) and the results were expressed as quercetin equivalents per gram dry weight (mg QE/g dw) and calculated by the following formula:

$$\text{TFC} = C \times V \times F / M$$

where TFC is the total flavonoids content, mg QE/g dw; C is the concentration of quercetin, $\text{g}\cdot\text{L}^{-1}$; V is the volume of the used solvent, L; F is the dilution coefficient of the sample; M is the mass of the sample, g.

Total anthocyanin content

This method makes possible to determine the total anthocyanins' content and is based on the change in the structure of this compound between pH 1.0 and 4.5. Depending on the pH of the solution in which they are present, these compounds are either in flavylium form colored at pH 1.0 or in colorless hemiacetal form at pH 4.5. The absorbance was measured spectrophotometrically at 510 nm and 700 nm respectively. So, the difference in absorbance is proportional to the concentration of anthocyanins and was calculated using the following equation:

$$\text{Abs} = (\text{A}_{510} - \text{A}_{700})_{\text{pH } 1.0} - (\text{A}_{510} - \text{A}_{700})_{\text{pH } 4.5}$$

where A_{510} - absorbance of the sample measured at 510 nm with buffers pH 1.0 and pH 4.5, respectively; A_{700} - absorbance of the sample measured at 700 nm with buffers pH 1.0 and pH 4.5, respectively (Lee *et al.*, 2005).

The determination of total anthocyanins was carried out as follows. For the preparation of the buffer solution with pH 1.0, an aliquot of 1.86 g of KCL was dissolved in 980 mL deionized water. Then, for the preparation of the buffer solution with pH 4.5, an aliquot of 53.43g CH_3COONa was dissolved in 960 mL deionized water. After that, an aliquot of 0.6 mL of the extract was introduced into a microcuvette with 2.4 mL buffer solution. The absorbance was measured at 510 nm and 700 nm respectively. The TAC was calculated using the following equation:

$$\text{TAC} = (\text{A} \times \text{MW} \times \text{DF} \times \text{V} \times 1000) / (\text{E} \times \text{L} \times \text{M})$$

where: TAC - the equivalent of cyanidin-3-glucoside (CGE) or malvidin-3-glucoside (MGE), $\text{mg}\cdot\text{L}^{-1}$, MW molecule mass of cyanidin-3-glucoside ($449.2\text{ g}\cdot\text{mol}^{-1}$) or molecule mass of malvidin glucoside ($493\text{ g}\cdot\text{mol}^{-1}$), DF - dilution factor; E - the molar absorbance of cyanidin-3-glucoside ($(26900\text{ (L}\cdot\text{cm)}\cdot\text{mol}^{-1})$) or the molar absorbance of malvidin glucoside ($(20200\text{ (L}\cdot\text{cm)}\cdot\text{mol}^{-1})$), M - the mass of the sample, g; L - the cell path length (1 cm).

The results were expressed as mg cyanidin and respectively malvidin glucoside equivalent per 100 grams dry weight (mg CGE, MGE/100 dw).

TEAC

The chemical 2,2-diphenyl-1-picrylhydrazyl, known as DPPH, is one of the first free radicals used to study the antioxidant activity of phenolic compounds. DPPH solutions show high absorption at 517 nm due to the dark blue color. The decrease in the blue color is measurable spectrophotometrically which allows estimating the effectiveness of the antioxidant's presence (Loizzo *et al.*, 2010).

The determination of antioxidant capacity was carried out as follows:

- Preparation of the DPPH solution: an aliquot of 0.0040 g of DPPH, was dissolved in 100 mL of methanol.
- Sample preparations: An aliquot of 1 mL of the extracts (previously diluted 10 times) were added to 4 mL of DPPH solution.

The samples were stored in the dark for 30 min. The absorbance was measured at 517 nm. Free radical scavenging ability or inhibition capacity of the tested samples was calculated using the formula (Yen *et al.*, 1994):

$$\text{IC} = (\text{A}_0 - \text{A}_a) / \text{A}_0 \times 100$$

where: IC - inhibition capacity, %; A_0 - the average value of absorbance blank; A_a - the average value of absorbance sample;

The TEAC assay is often used to measure the antioxidant capacity of foods and nutritional supplements. The Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) is an antioxidant vitamin E derivative [20]. The results obtained were performed using the Trolox concentration linearity range of 2.5–175 $\mu\text{mol}\cdot\text{L}^{-1}$. The obtained equation of rights was $y=1.332\cdot x + 0.5634$, where the „y“ is the concentration of the Trolox solution (plotted on the ordinate), and the „x“ is the absorbance (plotted on the abscise). The TEAC was calculated using the formula:

$$\text{TEAC} = \text{IC sample} - 1.332/0.5634$$

where: TEAC - Trolox equivalent antioxidant capacity, $\mu\text{mol TEAC}\cdot\text{L}^{-1}$; IC - inhibition capacity of sample, %; 1.332 - the slope of the Trolox calibration curve; 0.5634 - the intercept from the Trolox calibration curve.

The data obtained were expressed in $\mu\text{mol Trolox equivalent per gram dry weight}$ ($\mu\text{mol TEAC/g dw}$) after using the mass of the samples.

Water content

The water content of the selected samples was evaluated gravimetrically following the corresponding European pharmacopeia's method. About 2.0 g of the grape pomace or seeds were dried at $105\text{ }^{\circ}\text{C}$ in an oven to a constant mass (Loss on drying 2.8.17., Eur.Ph.).

RESULTS AND DISCUSSION

There are no results on the literature about extraction optimization of the Mavrud by-products discharged from the winery in the region of Trakiets village except the study of Dimcheva *et al.*, (2018). The polyphenol composition of grape pomace depends on many factors, not only the environmental factors in which the grapes are matured but also on the type of solvent used, the analytical technique, extraction manner, the extraction time as well as the presence of interfering substances (Dimcheva *et al.*, 2018). The extraction efficiency may be significantly influenced by operating parameters, not only by changing the extraction time and solvent composition but by applying an ultrasound power and

temperature (Gironi et al., 2011; Ma et al., 2009). The mechanical effects of ultrasound induce a greater penetration of solvent into cellular materials and improve mass transfer. Ultrasound extraction can also disrupt biological cell walls, facilitating the discharge of contents (Mason et al., 1996).

In the present investigation all kinetical experiments were done with 50 % ethanol in water solution because was earlier found as optimal with the same plant material by Dimcheva et al., (2018). Likewise, 50 % of ethanol was chosen as the most adequate conditions for phenolic compounds recovery of the grape skins reported by Caldas et al., (2018). Methanol seems to be the most effective, followed by ethanol than water according to Pinelo et al., (2005). Nevertheless, for food application, ethanol and water are preferred because of hygiene, low

cost, and health compatibility (Moire et al., 2001). Ethanol concentration has been reported to affect the phenolic compounds extraction since it diminishes the boiling point and influences the polarity of the mixed solvent (Wijngaard et al., 2009).

Recently, along with the variables in the optimization of the plant extraction, namely solvent/sample ratio, type of solvent, time and temperature, a method of extraction and pre-treatment of the sample such as degreasing is commonly used. In Table 1 provided below are presented total monomeric anthocyanin kinetic study obtained by UAE of previously degreased and non-degreased separately grape pomace and grape seeds.

Table 1 Total monomeric anthocyanins calculated as cyanidin and malvidin glucoside equivalents per 100 g/dw obtained by UAE before and after degreasing with n-hexane of grape pomace and grape seeds

Time, min	Without degreasing		After degreasing		Without degreasing		After degreasing	
	CGE ± SD*	MGE ± SD*	CGE ± SD*	MGE ± SD*	CGE ± SD*	MGE ± SD*	CGE ± SD*	MGE ± SD*
5	1.00 ± 1.25	1.46 ± 1.78	1.30 ± 1.09	1.90 ± 0.98	16.83 ± 1.65	24.60 ± 1.12	39.24 ± 2.10	57.35 ± 0.56
10	2.00 ± 0.87	2.93 ± 1.78	1.60 ± 1.29	2.34 ± 1.34	17.63 ± 2.07	25.77 ± 2.35	42.42 ± 0.67	61.99 ± 2.00
15	2.40 ± 1.28	3.51 ± 0.78	1.70 ± 2.45	2.49 ± 1.23	26.18 ± 1.78	38.27 ± 0.56	74.31 ± 1.89	108.61 ± 2.01
20	2.50 ± 2.00	3.66 ± 1.89	2.00 ± 0.67	2.93 ± 1.76	34.47 ± 0.98	50.37 ± 1.89	120.40 ± 1.09	175.97 ± 1.56
30	2.91 ± 1.76	4.25 ± 1.96	2.10 ± 1.55	3.08 ± 1.33	32.86 ± 0.87	48.03 ± 1.89	151.96 ± 1.76	222.09 ± 0.78
50	3.21 ± 0.76	4.69 ± 2.15	3.11 ± 1.78	4.54 ± 0.68	53.57 ± 1.76	78.29 ± 1.23	166.99 ± 1.13	244.06 ± 0.67
80	3.41 ± 1.68	4.98 ± 1.23	4.81 ± 1.12	7.03 ± 1.26	74.28 ± 2.15	108.56 ± 2.08	168.83 ± 1.98	246.74 ± 1.60
120	4.91 ± 0.90	7.18 ± 1.65	8.00 ± 0.56	11.69 ± 1.34	95.92 ± 1.23	140.19 ± 1.89	176.17 ± 1.90	257.48 ± 1.76
180	5.51 ± 1.26	8.15 ± 1.66	9.22 ± 0.79	13.47 ± 0.45	101.66 ± 0.79	148.58 ± 1.68	204.34 ± 1.87	298.66 ± 1.26
220	5.61 ± 1.56	8.24 ± 2.34	9.23 ± 1.25	13.62 ± 1.57	102.00 ± 1.25	149.08 ± 1.80	205.00 ± 1.12	299.62 ± 1.96
Grape seeds					Grape pomace			

Legend: CGE - Cyanidin glucoside equivalent, MGE - Malvidin glucoside equivalent, SD - standard deviation,

*Extractions were performed in duplicate and the results are expressed as the mean ± standard deviation (SD).

Based on the kinetic results it can be concluded that the optimal yield of the desired components is achieved after 180 minutes. Or, it is evident, that UAE after degreasing with n-hexane increases the yield of the anthocyanins almost twice for the grape pomace and for the grape seeds. For the grape seeds, the anthocyanins reached before decreasing from 8.15 to 13.47 mg MGE/100 g dw, respectively, after it. For the grape pomace, the MGE anthocyanins are increased from 148.58 to value of 298.66 mg MGE/100 g dw which is almost 2 times larger after an hour degreasing in comparison with of the case without degreasing. The non-polar nature of the chosen solvent n-hexane is interacting with the non-polar compounds which are content permanently in all plant matrices. Therefore, is achieving a higher concentration of the polar constituents in the grape by-products such as polyphenols and anthocyanins. The best results obtained in the kinetics shown above are from 2 to 4 times less than total anthocyanins in the

best red wines (Brenes et al., 2016) which demonstrates the possibility to use the by-products.

Lapornik et al., (2005) was proposed a potential alternative for an industrial solid-liquid extraction process of antioxidant compounds from grape pomace. The incubation temperature was increased up to 93 °C, the time of the process was reduced to 93 minutes and was used 66 % ethanol/water as a solvent mixture to be obtained an optimal value of 70.3 mg CGE/100g dw total monomeric anthocyanins. The received result by Lapornik et al., (2005) is almost like our value obtained from non-degreased pomace at 80th minute – 74.28 mg CGE/100g dw but is 2.4 lower than obtained from degreased grape pomace - 168.83 mg CGE/100g dw (Table 1).

In Table 2 and Table 3 was carried out the total flavonoid's kinetics obtained by UAE of the degreased and non-degreased grape by-products.

Table 2 Total flavonoid content calculated as quercetin, rutin and (-) catechin equivalents per g/dw, obtained by UAE before and after degreasing with n-hexane of grape seeds

Time, min	Without degreasing			After degreasing		
	QE ± SD*	RE ± SD*	CE ± SD*	QE ± SD*	RE ± SD*	CE ± SD*
5	5.52 ± 1.11	2.51 ± 1.04	0.75 ± 0.95	7.76 ± 0.56	3.53 ± 1.78	1.05 ± 1.13
10	5.92 ± 1.10	2.69 ± 1.07	0.80 ± 1.26	12.80 ± 1.36	5.82 ± 0.67	1.73 ± 0.79
15	6.48 ± 1.29	2.95 ± 1.45	0.88 ± 0.87	15.52 ± 0.78	7.05 ± 1.58	2.10 ± 1.89
20	6.80 ± 1.01	3.09 ± 1.40	0.92 ± 0.55	16.64 ± 0.45	7.56 ± 0.12	2.25 ± 0.98
30	7.84 ± 1.48	3.56 ± 1.22	1.06 ± 0.15	21.28 ± 1.49	9.67 ± 1.78	2.88 ± 1.21
50	7.28 ± 1.31	3.31 ± 1.45	0.98 ± 1.20	26.00 ± 0.12	11.82 ± 0.29	3.51 ± 0.35
80	9.76 ± 0.29	4.44 ± 0.75	1.32 ± 0.85	35.84 ± 0.76	16.29 ± 0.57	4.84 ± 1.29
120	13.12 ± 1.14	5.96 ± 1.29	1.77 ± 0.45	44.00 ± 1.08	20.00 ± 0.96	5.95 ± 0.78
180	20.40 ± 0.78	9.27 ± 1.57	2.76 ± 1.85	53.20 ± 0.84	24.18 ± 0.57	7.19 ± 0.69
220	20.50 ± 1.14	9.42 ± 1.45	2.92 ± 1.28	53.50 ± 0.48	24.25 ± 1.27	7.27 ± 1.89
Grape seeds						

Legend: QE - quercetin equivalent, RE - rutin equivalent, CE - (-) catechin equivalent SD - standard deviation

*Extractions were performed in duplicate and the results are expressed as the mean ± standard deviation (SD).

Degreased grape seeds extracted by UAE show 2.3 times better yield than non-degreased ones (Table 2).

For the grape pomace, the obtained results are much better than the grape seeds – the flavonoid yield in the degreased grape pomace is increased 3.5 times more than non-degreased ones as shown in Table 3. For the total flavonoids' kinetics, the optimal extraction time was found as a 180th minutes, like the anthocyanin kinetic study.

In Table 4 is shown the kinetic study by TAC of the same degreased and non-degreased by-products extracted by conventional extraction method – magnetic stirring.

Table 3 Total flavonoid content calculated as quercetin, rutin and (-) catechin equivalents per g/dw, obtained by UAE before and after degreasing with n-hexane of grape pomace

Time, min	Without degreasing			After degreasing		
	QE ± SD*	RE ± SD*	CE ± SD*	QE ± SD*	RE ± SD*	CE ± SD*
5	5.36 ± 1.25	2.44 ± 1.29	0.72 ± 0.80	9.28 ± 0.59	4.22 ± 1.44	1.25 ± 1.31
10	7.12 ± 1.05	3.24 ± 1.25	0.96 ± 0.79	11.52 ± 1.36	5.24 ± 0.89	1.56 ± 0.65
15	7.84 ± 1.18	3.56 ± 1.12	1.06 ± 0.95	12.96 ± 0.78	7.89 ± 1.18	1.75 ± 1.49
20	8.56 ± 1.58	3.89 ± 1.47	1.16 ± 0.98	16.00 ± 0.63	7.27 ± 0.79	2.16 ± 1.58
30	13.28 ± 0.36	6.04 ± 1.05	1.79 ± 1.25	17.44 ± 1.11	7.93 ± 1.13	2.36 ± 1.58
50	15.76 ± 1.69	7.16 ± 1.58	2.13 ± 1.06	24.40 ± 1.44	11.09 ± 1.08	3.30 ± 1.43
80	23.44 ± 1.45	10.65 ± 1.36	3.17 ± 0.36	64.00 ± 0.11	29.09 ± 1.69	8.65 ± 1.76
120	28.64 ± 1.28	13.02 ± 1.45	3.87 ± 0.89	88.00 ± 1.55	40.00 ± 0.36	11.89 ± 1.06
180	30.40 ± 1.30	13.82 ± 1.11	4.11 ± 0.36	108.00 ± 0.63	49.09 ± 1.00	14.59 ± 0.47
220	30.80 ± 1.14	14.02 ± 0.25	4.27 ± 0.98	108.05 ± 1.82	49.51 ± 1.47	15.00 ± 0.89

Grape pomace

Legend: QE - quercetin equivalent, RE - rutin equivalent, CE - (-) catechin equivalent SD - standard deviation
*Extractions were performed in duplicate and the results are expressed as the mean ± standard deviation (SD).

Table 4 Total monomeric anthocyanins calculated as cyanidin and malvidin glucoside equivalents per 100 g/dw, obtained through magnetic stirrer before and after degreasing with n-hexane of grape pomace and grape seeds

Time, min	Without degreasing		After degreasing		Without degreasing		After degreasing	
	CGE ± SD*	MGE ± SD*	CGE ± SD*	MGE ± SD*	CGE ± SD*	MGE ± SD*	CGE ± SD*	MGE ± SD*
5	0.20 ± 0.58	0.29 ± 1.89	1.10 ± 1.18	1.61 ± 1.28	38.24 ± 1.80	55.89 ± 1.48	32.06 ± 1.08	46.86 ± 1.45
10	0.30 ± 1.08	0.44 ± 1.13	1.60 ± 1.12	2.35 ± 1.12	39.08 ± 1.13	57.11 ± 2.11	42.48 ± 1.89	62.09 ± 1.25
15	1.50 ± 2.25	2.20 ± 1.08	1.70 ± 1.29	2.49 ± 1.45	53.94 ± 1.00	78.83 ± 1.46	55.84 ± 2.28	81.61 ± 1.12
20	1.90 ± 1.25	2.79 ± 0.25	2.71 ± 1.15	3.96 ± 1.08	55.11 ± 0.56	80.54 ± 1.08	59.31 ± 1.55	86.69 ± 1.78
30	2.61 ± 1.48	3.81 ± 1.25	2.91 ± 0.69	4.25 ± 1.95	58.78 ± 1.89	85.91 ± 1.55	69.87 ± 1.45	102.11 ± 1.15
50	3.31 ± 1.13	4.84 ± 1.13	3.11 ± 0.58	4.55 ± 1.59	69.13 ± 0.25	101.04 ± 1.59	74.41 ± 1.12	108.75 ± 1.13
80	3.61 ± 1.26	5.28 ± 1.12	3.61 ± 1.16	5.28 ± 1.13	69.30 ± 1.25	101.28 ± 2.28	98.46 ± 1.09	143.90 ± 1.07
120	4.21 ± 1.45	6.16 ± 1.09	4.81 ± 1.14	7.04 ± 1.09	71.30 ± 1.09	104.21 ± 1.19	112.00 ± 1.19	163.69 ± 1.89
180	4.71 ± 1.89	6.90 ± 1.55	5.01 ± 1.09	7.34 ± 1.48	84.83 ± 0.58	123.98 ± 1.25	126.78 ± 1.25	185.29 ± 1.45
220	4.81 ± 1.95	7.01 ± 1.89	5.11 ± 1.12	7.35 ± 1.08	84.93 ± 1.18	124.10 ± 0.58	127.10 ± 1.18	185.98 ± 1.55

Grape seeds

Grape pomace

Legend: CGE - Cyanidin glucoside equivalent, MGE - Malvidin glucoside equivalent, SD - standard deviation
*Extractions were performed in duplicate and the results are expressed as the mean ± standard deviation (SD).

It can be seen that the values of the monomeric anthocyanins are lower than previous TAC^o kinetic. Nevertheless, the UAE kinetics the optimal extraction time when applying magnetic stirring is again about a 180th minute. It can be concluded that the UAE does not decrease the extraction time of the grape pomace and seeds at the expense of higher yields for the same time. In Table 4 again are compared the kinetic before and after Soxhlet extraction but the increase of anthocyanin yield under magnetic stirring is negligible unlike when using the UAE. The MGE anthocyanins increasing only from 6.90 to 7.34 mg MGE/100g dw for the seeds and from 123.98 to 185.29 mg MGE/100g dw for the pomace. From comparing the kinetics conducted through magnetic stirring and UAE, can be concluded that the UAE, almost doubles the anthocyanins yield from 185.98 to 298.66 mg MGE/100g dw when is extracted grape pomace and from 7.35 to 13.47 mg MGE/100g dw when the grape seeds are extracted. This can be explained with the hard nature of the grape seeds and ungrounded pomace

that release more useful components when is applied ultrasound power and mass transfer is improved.

In Table 5 and Table 6 are shown the kinetic studies performed through magnetic stirrer by total flavonoids of the degreased and non-degreased grape seeds and pomace. Likewise, the kinetics by total anthocyanins extracted from the same plant material through magnetic stirrer the optimal extraction time is again about the 180th minute. The values of the total flavonoids in the presented kinetics are lower than flavonoids obtained by UAE. The QE flavonoids before and after degreasing are increased from 12.56 to 16.16 mg QE/g dw for the seeds and from 25.04 to 36.24 mg QE/g dw for the pomace. Can be concluded that UAE increases almost twice the flavonoids' yields of grape by-products but do not decrease the extraction time. There are bioactive substances which need more time for discharging, nevertheless applying ultrasonic power has a positive effect on the yields of anthocyanins and flavonoids in the Mavrud grape by-products.

Table 5 Total flavonoid content calculated as quercetin, rutin and (-) catechin equivalents per g/dw, obtained by magnetic stirring before and after degreasing with n-hexane of grape seeds

Time, min	Without degreasing			After degreasing		
	QE ± SD*	RE ± SD*	CE ± SD*	QE ± SD*	RE ± SD*	CE ± SD*
5	7.20 ± 1.18	3.27 ± 1.27	0.97 ± 1.14	7.92 ± 1.49	3.60 ± 1.18	1.07 ± 1.45
10	7.20 ± 1.44	3.27 ± 1.77	0.97 ± 1.78	8.16 ± 1.18	3.71 ± 1.67	1.10 ± 0.58
15	8.08 ± 0.58	3.67 ± 1.10	1.09 ± 1.75	9.76 ± 1.98	4.44 ± 2.18	1.32 ± 1.56
20	8.96 ± 1.46	4.07 ± 0.23	1.21 ± 1.18	9.76 ± 0.58	4.44 ± 1.49	1.32 ± 1.55
30	9.12 ± 1.17	4.15 ± 1.14	1.23 ± 1.48	10.00 ± 1.67	4.55 ± 1.77	1.35 ± 1.11
50	9.76 ± 1.12	4.44 ± 1.11	1.32 ± 1.88	11.20 ± 1.18	5.09 ± 1.47	1.51 ± 1.78
80	11.84 ± 0.58	5.38 ± 1.51	1.60 ± 1.18	13.12 ± 1.15	5.96 ± 0.58	1.77 ± 1.16
120	12.00 ± 0.18	5.45 ± 1.58	1.62 ± 2.28	14.88 ± 1.13	6.76 ± 1.47	2.01 ± 1.25
180	12.56 ± 1.89	5.71 ± 1.77	1.70 ± 0.58	16.16 ± 1.19	7.35 ± 1.45	2.18 ± 1.68
220	12.68 ± 1.07	5.96 ± 1.48	1.77 ± 1.18	16.96 ± 1.47	7.71 ± 1.58	2.29 ± 1.78

Grape seeds

Legend: QE - quercetin equivalent, RE - rutin equivalent, CE - (-) catechin equivalent SD - standard deviation
*Extractions were performed in duplicate and the results are expressed as the mean ± standard deviation (SD).

Table 6 Total flavonoid content calculated as quercetin, rutin and (-) catechin equivalents per g/dw, obtained by magnetic stirring before and after degreasing with n-hexane of grape pomace

Time, min	Without degreasing			After degreasing		
	QE ± SD*	RE ± SD*	CE ± SD*	QE ± SD*	RE ± SD*	CE ± SD*
5	6.40 ± 1.27	2.91 ± 1.36	0.86 ± 1.89	6.96 ± 1.23	3.16 ± 1.36	0.94 ± 1.63
10	7.04 ± 1.65	3.20 ± 1.78	0.95 ± 1.27	8.32 ± 1.29	3.78 ± 1.66	1.12 ± 1.36
15	7.84 ± 1.29	3.56 ± 1.27	1.06 ± 0.78	10.64 ± 1.22	4.84 ± 1.54	1.44 ± 1.23
20	8.80 ± 1.66	4.00 ± 1.42	1.19 ± 1.48	12.24 ± 1.65	5.56 ± 1.16	1.65 ± 1.45
30	10.96 ± 0.47	4.98 ± 1.05	1.48 ± 1.06	14.48 ± 1.54	6.58 ± 1.27	1.96 ± 1.78
50	14.56 ± 1.27	6.62 ± 1.27	1.97 ± 0.36	17.04 ± 1.27	7.75 ± 1.27	2.30 ± 1.35
80	17.84 ± 1.98	8.11 ± 1.24	2.41 ± 1.27	19.44 ± 0.79	8.84 ± 0.46	2.63 ± 1.49
120	21.28 ± 1.45	9.67 ± 1.21	2.88 ± 1.69	25.36 ± 1.65	11.53 ± 1.32	3.43 ± 1.27
180	25.04 ± 1.29	11.38 ± 1.78	3.38 ± 1.63	36.24 ± 1.78	16.47 ± 1.89	4.90 ± 1.43
220	25.08 ± 1.32	11.44 ± 1.45	3.48 ± 1.98	37.00 ± 1.56	16.57 ± 2.27	5.04 ± 1.27

Grape pomace

Legend: QE - quercetin equivalent, RE - rutin equivalent, CE - (-) catechin equivalent, SD - standard deviation
*Extractions were performed in duplicate and the results are expressed as the mean ± standard deviation (SD).

The TEAC kinetics of the grape seeds and pomace also was done. The antioxidant power of the degreased and non-degreased by-products extracted by UAE and by magnetic stirring are shown in Figure 7 and Figure 8, respectively. In comparison with other previous kinetics, where after degreasing of the samples, the flavonoids' and anthocyanins' yields are increasing almost from 2 to 3 times here in TEAC' kinetics there is no sensible increase. The UAE in

comparison than stirring increase the TEAC with 1.5 times for the seeds. For the grape pomace, there is no difference when using the UAE and conventional extraction. The values obtained after degreasing are 30.52 µmol TEAC/g dw by UAE and 31.36 µmol TEAC/g dw by stirring at the 180th minute. In the presented kinetics the optimal extraction time again can be evaluated after 180th minute.

Table 7 TEAC calculated as µmol TEAC/g dw, obtained by UAE before and after degreasing with n-hexane of grape pomace and grape seeds

Time, min	Without degreasing	After degreasing	Without degreasing	After degreasing
	TEAC ± SD*	TEAC ± SD*	TEAC ± SD*	TEAC ± SD*
5	12.38 ± 1.78	15.49 ± 1.22	11.46 ± 1.21	18.16 ± 0.98
10	13.14 ± 1.45	21.13 ± 1.79	14.13 ± 0.23	21.58 ± 1.80
15	13.33 ± 1.49	25.96 ± 0.98	15.88 ± 1.28	22.30 ± 1.18
20	13.59 ± 1.25	25.54 ± 1.09	18.46 ± 0.78	23.37 ± 1.75
30	14.39 ± 1.14	29.61 ± 1.44	21.20 ± 1.96	25.80 ± 1.77
50	18.99 ± 1.47	30.90 ± 1.24	24.55 ± 1.22	30.33 ± 1.58
80	22.42 ± 1.09	31.97 ± 1.08	27.78 ± 1.65	31.28 ± 1.36
120	29.38 ± 1.65	32.16 ± 1.05	31.09 ± 2.03	31.36 ± 1.90
180	32.16 ± 1.40	32.54 ± 1.04	31.36 ± 1.71	31.59 ± 1.46
220	32.35 ± 1.74	32.73 ± 1.25	31.21 ± 1.55	32.88 ± 1.61

Grape seeds

Grape pomace

Legend: TEAC - Trolox equivalent antioxidant capacity, SD - standard deviation

*Extractions were performed in duplicate and the results are expressed as the mean ± standard deviation (SD).

Table 8 TEAC calculated as µmol TEAC/g dw, obtained by magnetic stirring before and after degreasing with n-hexane of grape pomace and grape seeds

Time, min	Without degreasing	After degreasing	Without degreasing	After degreasing
	TEAC ± SD*	TEAC ± SD*	TEAC ± SD*	TEAC ± SD*
5	7.03 ± 0.36	10.24 ± 1.67	9.59 ± 1.49	11.03 ± 1.45
10	11.23 ± 1.40	13.21 ± 1.87	10.70 ± 1.51	16.10 ± 1.34
15	10.29 ± 1.02	14.61 ± 1.44	14.78 ± 1.47	19.27 ± 1.40
20	14.41 ± 1.34	15.81 ± 1.63	15.64 ± 2.00	20.55 ± 1.17
30	15.11 ± 1.30	17.17 ± 0.98	18.94 ± 1.40	22.89 ± 1.13
50	17.99 ± 1.46	18.82 ± 1.12	25.70 ± 1.46	28.13 ± 1.10
80	18.02 ± 1.20	20.34 ± 1.42	26.27 ± 1.40	29.28 ± 1.47
120	18.16 ± 1.41	21.27 ± 1.78	28.66 ± 1.36	31.13 ± 1.55
180	20.55 ± 1.67	22.89 ± 2.02	30.52 ± 1.23	31.63 ± 1.78
220	20.59 ± 1.05	22.94 ± 0.25	31.01 ± 1.56	32.05 ± 1.12

Grape seeds

Grape pomace

Legend: TEAC - Trolox equivalent antioxidant capacity, SD - standard deviation

*Extractions were performed in duplicate and the results are expressed as the mean ± standard deviation (SD).

The UAE is alternative to conventional extraction methods because it may improve the mass transfer but not in all cases is the best extraction technique. As concerns the influence of pre-treatment of the sample, it was shown in the literature that degreasing pre-treatment reduces total phenols yield, increasing only slightly the phenolic content of the extracts according to **Spigno et al., (2007)**. However, in our study, degreasing increases only the yields of the total flavonoids and the anthocyanins without affecting of the TEAC.

CONCLUSION

There is increasing interest in the antioxidants of foods and nutraceuticals because of health benefits and the economical recovering the wastes containing bioactive molecules. In the present investigation, the results obtained show that the applied pre-treatment degreasing of the grape by-products and applied subsequent UAE for 180 minutes extraction time give optimal flavonoids' and anthocyanins' yields. The quantity of the total anthocyanin content after degreasing increase from 101.66 to 298.66 mg MGE/100 g dw and the quantity

of the total flavonoids from 30.40 to 108.05 mg QE/g dw for the grape pomace. For the grape seeds at the same extraction conditions the increasing after degreasing for the total anthocyanins is from 8.15 to 13.47 mg MGE/100 g dw and for the total flavonoids is from 20.40 to 53.20 mg QE/ g dw. From the present investigation can be concluded that the proper choice of extraction technique influences the extraction efficiency. In general, the degreasing with n-hexane is a useful pre-treatment technique with a positive effect on the flavonoids' and anthocyanins' yields of the Mavrud pomace and seeds discharged from Bulgarian Mavrud winemaking.

The application of the UAE to the extraction of plant samples has increased in the last years. The UAE is an alternative to conventional extraction methods because it may improve the mass transfer and reducing the extraction time. However, in our investigation UAE do not reduce the extraction time but increases twice even three times the bioactive antioxidants yield (flavonoids and anthocyanins) in comparison with the used conventional extraction. The TEAC of the grape by-products is not affected by applying UAE and degreasing pre-treatment technique. Likewise, a mathematical model describing the mass transfer mechanism for both extraction methods should be developed in the future. A complete characterization of the extract's composition will help to understand the different nature of the Bulgarian Mavrud pomace and grape. Therefore, further extraction optimization is required for productive and substantial economic use of such valuable wastes to obtain high-value by-products, such as natural health remedies, food supplements, and novel food ingredients.

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