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RECOVERY OF PHENOLIC COMPOUNDS FROM WILD BILBERRY, BLACKCURRANT AND BLACKBERRY POMACES BY MACERATION AND ULTRASOUND-ASSISTED EXTRACTION

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ABSTRACT. Wild bilberry, blackcurrant and blackberry fruit pomaces obtained after industrial juice processing were extracted in water, 1% citric acid, 40%, 60% and 80% (v/v) aqueous ethanol using two extraction methods: maceration and ultrasound-assisted extraction. The total phenolics content (TPC), total anthocyanins content (TAC), and DPPH radical scavenging activity (RSA) were quantified in the extracts. TPC was about 2.3-3.2 times higher in ethanolic extracts as compared with the water extracts. The extracts made in 60% aqueous ethanol showed the highest values of TPC, TAC and RSA irrespective of extraction method and pomace matrix while water and 1% aqueous citric acid were very little effective in recovering anthocyanins and phenolic compounds. Bilberry pomace extracts made in 60% ethanol using maceration presented the highest TAC (585.21 mg CGE/L), TPC (3381.82 mg GAE/L) and RSA (2.05 mmol Trolox/l). The results showed that bilberry, blackcurrant and blackberry fruit pomaces can be a valuable source of bioactive compounds to be used in food supplements and functional foods.

Keywords: *fruit pomaces, extraction, total phenolics content, total anthocyanins content, DPPH radical scavenging activity, correlations*

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INTRODUCTION

Nowadays berries are widely consumed because they represent an important source of bioactive natural compounds and antioxidants [1] with immense health benefits and medicinal properties [2]. Their production and processing into juices, jams, and jellies, have led to an increase in food waste in the form of skins, pulp, and seeds [3, 4], called by-products that also possess valuable bioactive compounds [5, 6]. Therefore, the valorization of these by-products has become of interest in the research field.

Bilberry (*Vaccinium myrtillus*), also known as the European blueberry, is a wild berry extremely rich in bioactive compounds, mainly polyphenols, with high antioxidant properties. Several potential health benefits have been associated with their consumption including amelioration of type 2 diabetes by reducing glycemia and neuroprotective properties [7, 8]. The pomace generated after bilberry processing in juices, which is mainly composed of seeds and peels, is still abundant in fibers, beneficial phenolic compounds, and other antioxidants [9, 10].

Both wild and cultivated blackcurrant (*Ribes nigrum* L.) represent a rich source of phenolics, especially phenolic acids, anthocyanins, flavonols, condensed tannins, and hydrolyzable tannins [11]. Because of their astringency, blackcurrants are mainly processed in juices, jams, jellies, and alcoholic beverages. Post-processing, a high amount of pomace is generated, which is extremely valuable due to its abundance in fiber and anthocyanins which are known to work in the prevention of cardiovascular illnesses, diabetes, and cancer [12, 13].

Blackberry (*Rubus* spp.) is a berry with increasing consumption in recent years, due to its high content of nutritional and bioactive compounds and its health benefits [14, 15]. Blackberries are consumed either fresh or processed into jam, syrups, wine, tea, and desserts. The pomace resulting from blackberry juice processing is a valuable source of fiber and antioxidants [16,17].

The bioactive substances that are preponderant in berry pomaces are phenolic compounds, fatty acids, and tocopherols [18]. The amount of bioactive compounds in the by-products of berries varies based on berry species, cultivar, genetic factors, growth season and cultivation conditions, ripening stage, and extraction methods [18, 19].

The recovery of high-valuable compounds from fruit by-products, as an alternative to the food waste problem [20-23], has become of major interest in the research field. For these compounds to be delivered in the highest amount with less degradation [24], the extraction operation plays an important role [25].

The selection of the extraction method depends on several factors such as fruit by-product matrices, physicochemical properties of the desired compound, purity of the extract, and economic value [23]. Because the wastes resulting from the processing of fruits are in the form of wet residues (pomace) with a high degree of moisture that can lead to degradation caused by different microorganisms, the preparation of the samples by dehydration and milling also plays a crucial role in the extraction process [26, 27].

For an extended period, conventional methods have been used for the extraction of bioactive compounds from fruits and pomaces [28]. The most utilized conventional methods are maceration, distillation and Soxhlet extraction [25, 26]. These conventional extraction technologies present some drawbacks like prolonged extraction time, potentially harmful solvent residue, low yields, and inferior purity of the target compounds [27, 29]. Therefore, nowadays the focus is on novel, innovative extraction technologies that have emerged as fast and efficient, safe for the environment [30], based on green chemistry principles [31].

For extraction technologies to be considered efficient and eco-friendly, they have to meet the criteria for reduced solvent consumption and energy [22], shorter operation time that can lead to less or no damage to the bioactive molecules, and high surface area of the desired compound that comes in contact with the chosen solvent [32]. Among these new technologies, pulsed electric field, ultrasound, enzyme-assisted extraction, microwave-assisted extraction, and supercritical fluid extraction [33-35] are the most commonly used for the extraction of bioactive compounds from fruits and pomaces.

Ultrasound-assisted extraction (UAE) principle works on cavitation, a phenomenon based on the ability of ultrasonic waves to produce energy dissipation by compression and rarefaction in a liquid medium, that is the extraction solvent, with the generation and subsequent collapse of vapor bubbles or cavities that transform the acoustic energy into thermal energy and cause mechanical agitation [35, 36]. This leads to the amplification of surface area contact between solid and liquid phases [37] and the rupture of plant cell structures enhancing the release and extraction of intracellular bioactive components [38].

When choosing an extraction method, several parameters must be adjusted for optimal operation, including liquid-solid ratio, extraction temperature, pH, extraction time, type and concentration of the solvent [4, 39]. Our study aimed to investigate the influence of extraction solvent (water, 1% aqueous citric acid, 40%, 60% and 80% aqueous ethanol) on conventional maceration and ultrasound-assisted recovery of bioactive phenolics from wild bilberry, blackcurrant, and blackberry pomaces. The resulting extracts were compared concerning their total phenolics content, total anthocyanins content, and DPPH radical scavenging activity.

RESULTS AND DISCUSSION

Extraction of phenolic compounds

Results on the total phenolic content of berry pomace extracts are presented in Figure 1 (a-c). In bilberry pomace extracts, the maximum and minimum TPC values were 3381.82±146.67 GAE/l (in 60% ethanol) and 981.82±38.14 mg GAE/l (in water) after maceration while 2245.45±75.56 mg GAE/l (in 60% ethanol) and 872.73±24.45 mg GAE/l (in water) after ultrasound-assisted extraction. In a previous study, Aaby et al. [40] reported a total phenolic content between 249 and 1153 mg GAE/l of extract made using hot water from the press residue obtained after industrial bilberry juice processing.

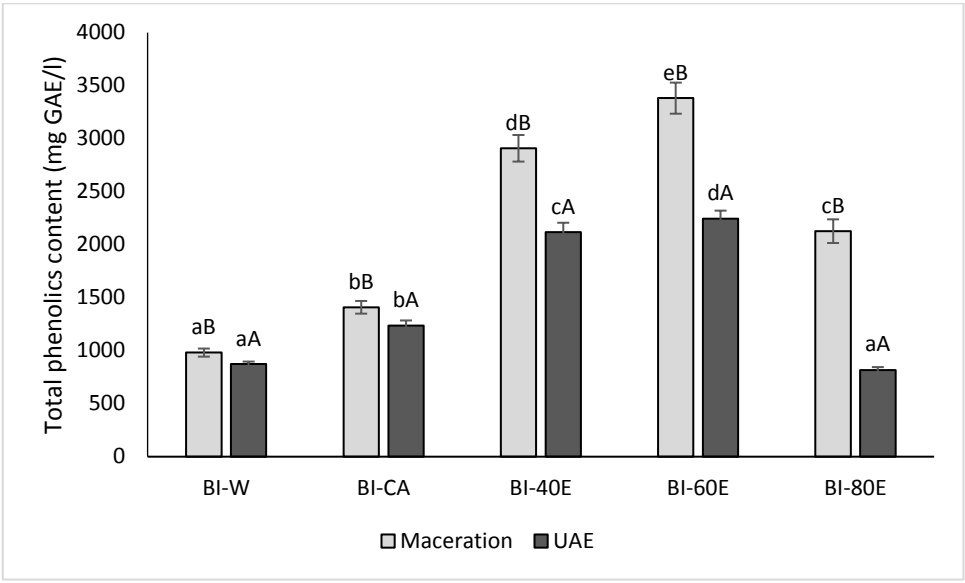
In blackcurrant pomace extracts, the total phenolic content ranged from 500.09±18.33 to 2227.27±88.56 mg of gallic acid equivalents per liter. The highest total phenolic content was found in the extracts using 60% alcohol as a solvent (2227.27±88.56 mg GAE/l), followed by those made in 40% ethanol (2036.36±96.23 mg GAE/L). Nour et al. [41] reported only 1371.1-1665.1 mg GAE/l in blackcurrant fruit extracts made in 60% ethanol and 1261.7-1646.5 mg GAE/l in those made in 40% ethanol. The increase of ethanol concentration to 80% resulted in a significantly lower extraction of phenolics from blackcurrant pomace as compared with the extraction in 60% ethanol, in agreement with the findings of Cacace and Mazza [42] who found that the content of total phenolics extracted from black currants with aqueous ethanol increased with ethanol concentration up to a maximum at about 60% and then decreased with further increase in ethanol concentration. In contrast to these results, Pompeu et al. [43] reported that 70-80% aqueous ethanol maximized the yields of total phenolics and total anthocyanins extracted from fruits of *Euterpe oleracea* (Açai palm).

In blackberry extracts, total phenolic content ranged between 754.55±28.32 and 2454.55±112.21 mg GAE/l after maceration and between 336.36±14.22 and 1827.27±77.11 mg GAE/l as a result of UAE extraction. In a study on ultrasound-assisted recovery of phenolics from blueberry pomace, Bamba et al. [44] reported a total phenolic content ranging from 5.84 to 6.31 mg GAE/g DM in water extracts obtained at a solid/liquid ratio of 1/20 at 40 °C and noticed that extraction duration significantly affected TAC, but not, TPC. In agreement with our results, Bamba et al. [44] found that TPC, TFC and TAC were about 5, 3 and 1.5 times greater in the ethanolic extracts, respectively, than in the water extracts.

The lower concentration of phenolics in water and 1% aqueous citric acid extracts as compared with the ethanolic extracts may be attributed to the nonpolar portions of the polyphenols, including aromatic rings, which limit their solubility in a highly polar solvent such as water.

The higher extraction yields of polyphenols in binary solvents of ethanol and water have been previously reported and they were related to the contribution of ethanol to increase their solubility by reducing the dielectric constant of the aqueous solvent and by increasing the diffusion of polyphenols in the solvent, and again of water to enhance their desorption from plant matrices [45]. Previous studies concluded also that water is an important co-solvent for the extraction of anthocyanins and total polyphenols from blackcurrant [1] and elderberry pomace [46].

A similar variation was found in the present study during maceration. In agreement with these findings, Ćujić et al. [47] reported also higher concentrations of TPC and TAC in chokeberry extracts made with 50% ethanol as compared to those made with water.



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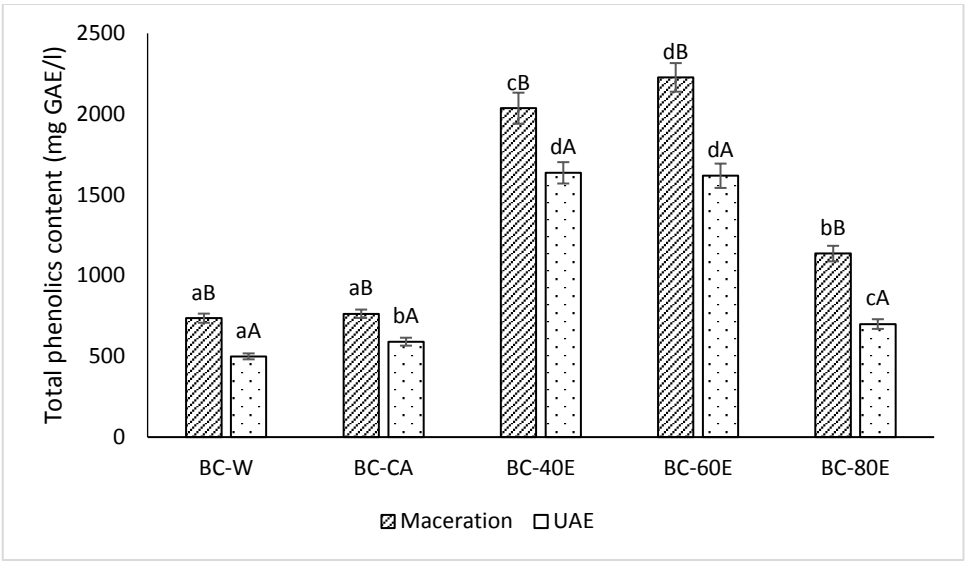

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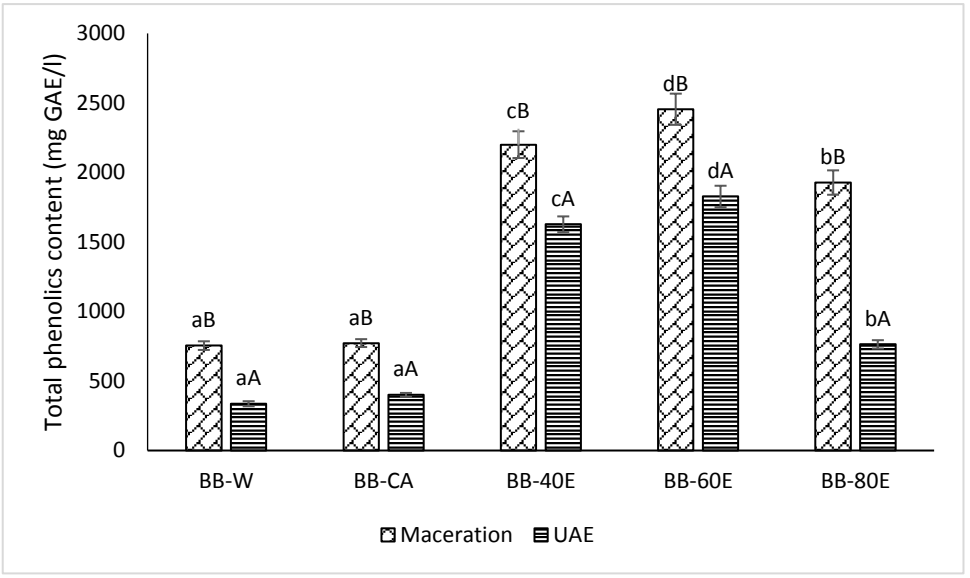


(c)

Figure 1. Effect of solvent on the total phenolic content of (a) bilberry (BI), (b) blackcurrant (BC) and (c) blackberry (BB) pomace extract made in water (W), 1% aqueous citric acid (CA), 40% aqueous ethanol (40E), 60% aqueous ethanol (60E), 80% aqueous ethanol (80E). Different uppercase letters indicate significant differences between extraction methods ($p < 0.05$) for the same solvent, while different lowercase letters are indicative of significant differences between solvents for the same extraction method ($p < 0.05$).

In the chokeberry extracts obtained through maceration, the phenolic content was about 1.5-2 times higher in ethanolic extracts as compared with the water extracts [47] while in our study the magnitude of the difference in phenolic contents between ethanolic and water extracts was about 2.5–3.2 times. Lapornik et al. [48] reported also that the phenolic content of ethanol extracts made without sonication was 2 times greater in red and black currant 70% ethanolic extracts as compared with water extract and concluded that the extraction yields vary greatly with plant material and extraction conditions.

In addition to being recognized as safe for food applications, ethanol is a suitable solvent for polyphenol extraction. However, Bamba et al. [44] found significantly higher TPC, TFC, TAC and DPPH free radical scavenging activity in the extracts made in 50% aqueous ethanol compared to 90% ethanol. They found also that increasing the concentration of ethanol from 50% to 90% resulted in significantly lower values of TPC, TAC and DPPH free radical scavenging activity than those obtained with 50% ethanol and attributed this behavior to the dehydration effect of ethanol on the plant cells which could prevent the diffusion of polyphenols from the plant material to the solvent. In blueberry leaves, Wang et al. [49] also found that phenolic extraction yield from blueberry leaves increased when raising ethanol concentration from 40% to 70%, while the further increase of ethanol concentration up to 90% decreased the extraction yield.

The addition of 1% citric acid to the water used as a solvent caused an increase in the amount of phenolic compounds extracted from all pomaces, however, the increase was not significant except for the bilberry pomace. The available literature shows that pH is an important parameter affecting the extractability of polyphenolic compounds. Moreover, the different phenolic fractions are differently affected by the pH decrease.

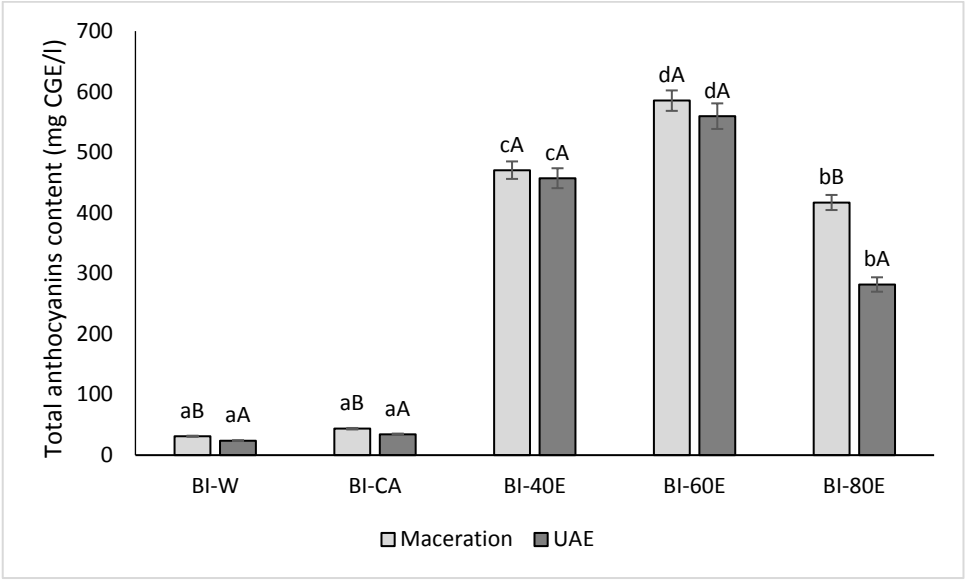
Maceration for 10 days proved to be more effective in extracting phenolic compounds than ultrasound-assisted extraction for 60 min for all three analyzed pomaces. Previously, Varo et al. [50] compared UAE combined with stirring versus conventional maceration on the anthocyanins and flavonol profile, as well as on the antioxidant capacity extraction from freeze-dried bilberry juice by-products using water as a green extraction solvent. They found higher total phenolic content and antioxidant activity when ultrasound was used. The highest total phenolic content was found in bilberry extracts regardless of the method or solvent used. The total phenolic content in bilberry extracts was higher than in blackberry extracts made in water and 1% citric acid, while it reversed in the extracts made in the ethanol/water mixture.

Extraction of anthocyanins

Bilberry pomace is a rich source of anthocyanins and several methods have been carried out to improve their extraction [13]. The use of 60% ethanol

as a solvent achieved the efficient recovery of anthocyanins from bilberry pomace (585.21±16.87 mg CGE/l) followed by 40% ethanol (470.45±14.44 mg CGE/l) and 80% ethanol (417.09±12.45 mg CGE/l) (Figure 2, a-c). Water and 1% aqueous citric acid were very little effective in extracting anthocyanins (31.20±0.89 and 43.64±1.22 mg CGE/l, respectively). Lapornik et al. [48] demonstrated that long extraction times in water (1-24 h) can lead to a decrease in TPC and TAC of red currant and black currant by-product extracts made without sonication and related these results to the degradation of anthocyanin compounds during the excessively long extraction. In good agreement with our results, Aaby et al. [40] reported 95 to 625 mg CGE/l of extract made in hot water from the press residue obtained after industrial bilberry juice processing. The same as for phenolic compounds, the 60% aqueous ethanol provided the best anthocyanins extraction yield from all three pomace matrices.

Ultrasound achieved higher extraction of anthocyanins from blackcurrant pomaces as compared with maceration, while for bilberry pomace maceration was more effective. Bamba et al. [44] previously concluded that an extraction time between 30 and 60 min would be a good compromise for the ultrasound-assisted extraction of anthocyanins to avoid longer processing times while Varo et al. [50] found that monomeric anthocyanin content decreased after 60 min of ultrasound-assisted extraction from bilberry juice by-products, revealing a degradation phenomenon induced by the technology.



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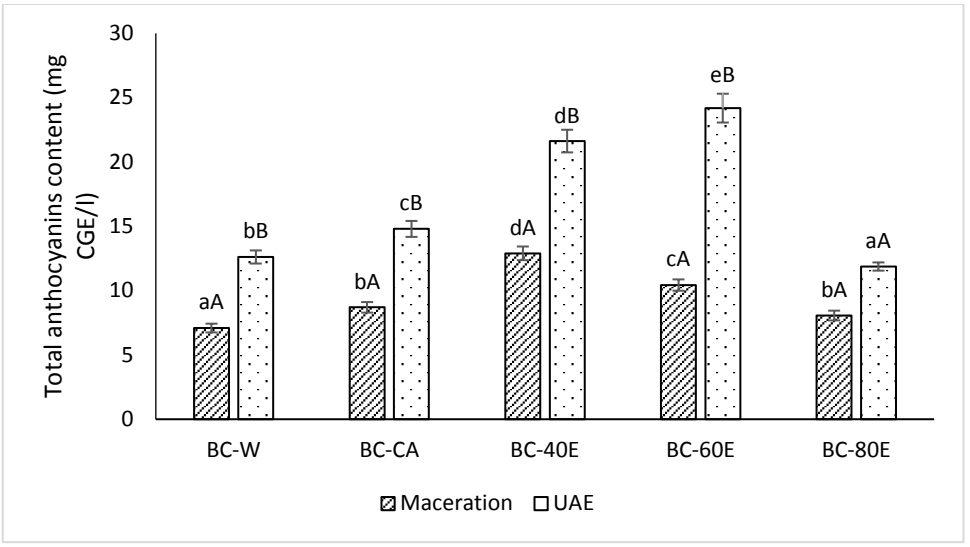

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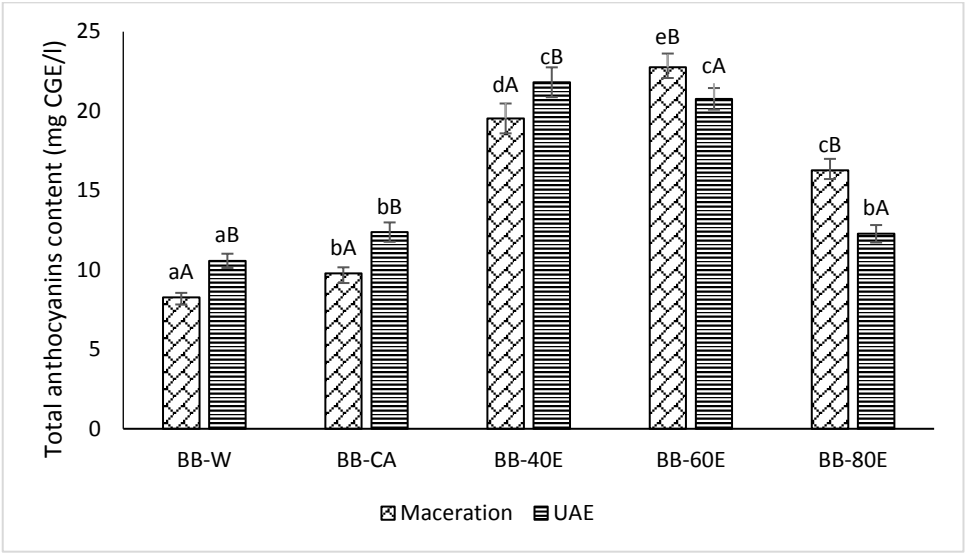

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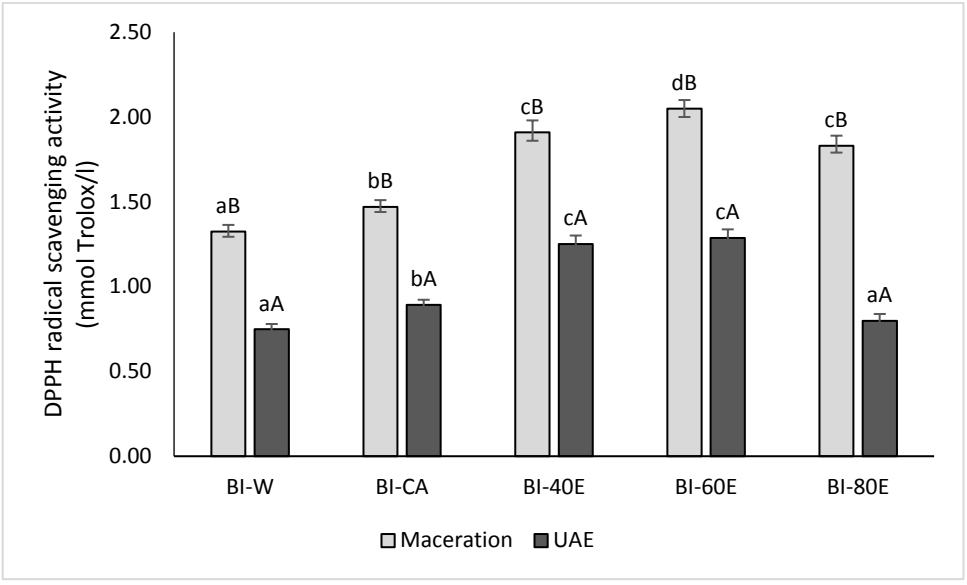
Figure 2. Effect of solvent on the total anthocyanin content of (a) bilberry (BI), (b) blackcurrant (BC) and (c) blackberry (BB) pomace extract made in water (W), 1% aqueous citric acid (CA), 40% aqueous ethanol (40E), 60% aqueous ethanol (60E), 80% aqueous ethanol (80E). Different uppercase letters indicate significant differences between extraction methods ($p < 0.05$) for the same solvent, while different lowercase letters are indicative of significant differences between solvents for the same extraction method ($p < 0.05$).

As for blackberry pomace, ultrasound was more effective than maceration when using water, 1% citric acid and 40% ethanol and less effective when using 60% and 80% ethanol as a solvent. Zafra-Rojas et al. [51] found a lower extraction yield of anthocyanins from blackberry (*Rubus fructicosus*) residues using ultrasound as compared with conventional extraction methods.

Antioxidant activity

The antioxidant capacity of bilberry, blackcurrant and blackberry extracts obtained by maceration and UAE were assessed by DPPH radical scavenging activity evaluation assay (Figure 3, a-c).

DPPH radical scavenging activity values in the extracts performed with water both in maceration and UAE were lower compared to the extracts made in polar mixtures of ethanol/water. The highest levels of radical scavenging activity were found in bilberry pomace extracts, ranging from 0.75±0.03 to 2.05±0.07 mmol Trolox/l, depending on the solvent used. As expected, the maximum radical scavenging activity of pomace extracts was achieved in 60% ethanol. Except for blackcurrant, in our study we found very strong correlations (r>0.9) between total phenolics content and radical scavenging activity of the pomaces extracts, in agreement with several previous studies on berry and berry pomaces extracts [52-54].



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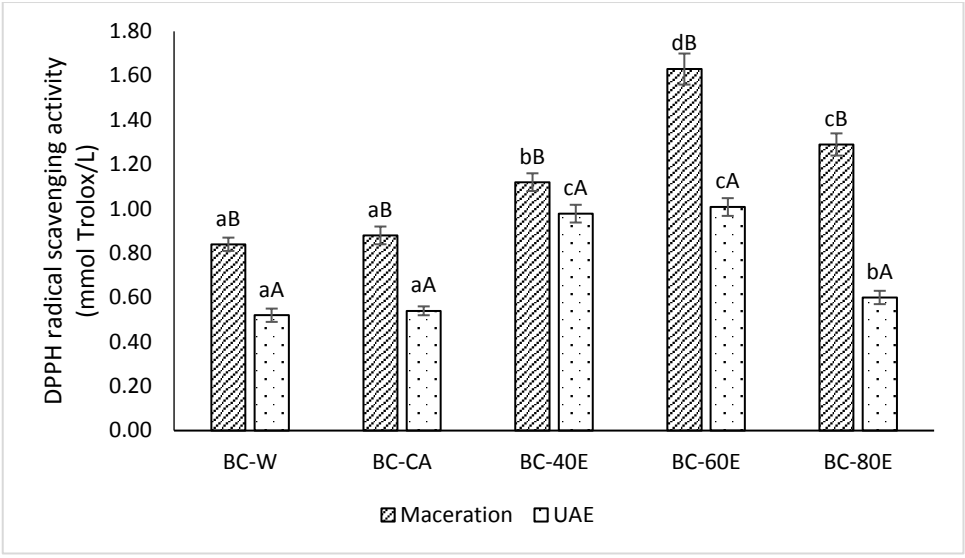

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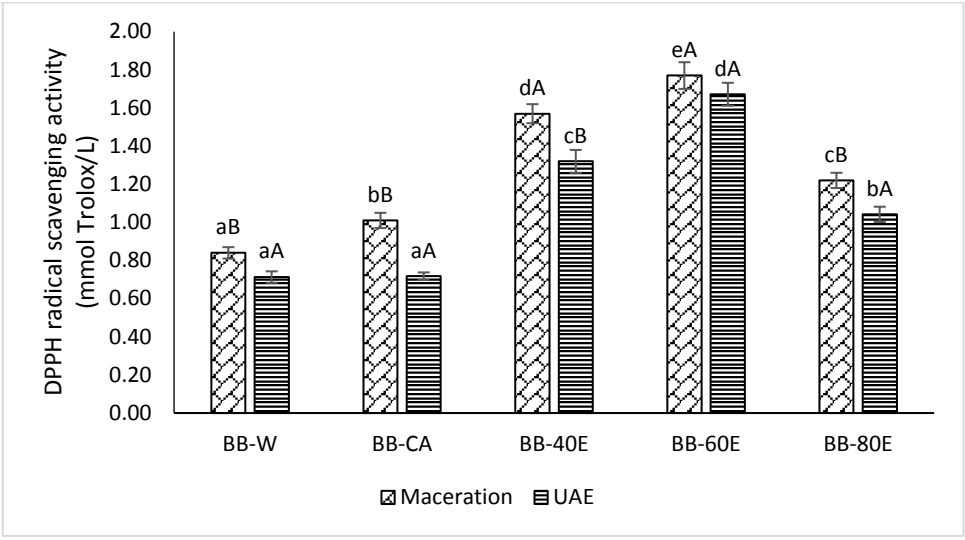

(c)

Figure 3. Effect of solvent on the DPPH radical scavenging activity of (a) bilberry (BI), (b) blackcurrant (BC) and (c) blackberry (BB) pomace extract made in water (W), 1% aqueous citric acid (CA), 40% aqueous ethanol (40E), 60% aqueous ethanol (60E), 80% aqueous ethanol (80E). Different uppercase letters indicate significant differences between extraction methods ($p < 0.05$) for the same solvent, while different lowercase letters are indicative of significant differences between solvents for the same extraction method ($p < 0.05$).

Total anthocyanin content was also highly correlated with antioxidant activity in blackcurrant UAE extracts (Table 1). Nour et al. [41] found also good correlations between antioxidant activity and the sum of anthocyanins (r=0.85) and only a moderate correlation (r=0.78) between total phenolics content and antioxidant activity of blackcurrant alcoholic extracts. However, Vulić et al. [55] found a high linear correlation between the IC50 and the content of anthocyanins (r^2=0.98) and polyphenols (r^2=0.85) of four berry (bilberry, blackberry, strawberry and raspberry) fruits pomaces, indicating the great importance of these compounds in the radical scavenging activity.

Very strong correlations were found between DPPH radical scavenging activity, total phenolic content and total anthocyanins content in blackberry extracts.

Table 1. Correlation matrix (Pearson coefficients and p values in brackets; n=45) for TAC, TPC and RSA of bilberry, blackcurrant and blackberry pomace extracts

Pomace		Maceration			UAE		
		TAC	TPC	RSA	TAC	TPC	RSA
Bilberry	TAC	1	0.9585** (0.0101)	0.9875*** (0.0017)	1	0.8178 (0.0907)	0.8714 (0.0543)
	TPC		1	0.9738** (0.0044)		1	0.9938*** (0.0006)
	RSA			1			1
Blackcurrant	TAC	1	0.8365 (0.0774)	0.3733 (0.5360)	1	0.9509** (0.0130)	0.9547** (0.0115)
	TPC		1	0.7839 (0.1165)		1	0.9981*** (0.0001)
	RSA			1			1
Blackberry	TAC	1	0.9862*** (0.0019)	0.9822*** (0.0028)	1	0.9666*** (0.0073)	0.8862** (0.0453)
	TPC		1	0.9408** (0.0171)		1	0.9731*** (0.0053)
	RSA			1			1

*** The significance level is 0.1% or less
** The significance level is 1% or less

Zafra-Rojas et al. [51] reported also a good correlation between antioxidant activity by ABTS and phenolic compounds and anthocyanins (r^2=0.824, r^2=0.893, respectively) in blackberry residues extracts, but not in

the case of DPPH. These findings have been attributed to the presence of other antioxidants such as ascorbic acid or other nonphenolic components present in berries' seed oil.

Regarding the extraction method, the results show the highest radical scavenging activity in the extracts made through maceration as compared with UAE in all three matrices and using all investigated solvents.

CONCLUSIONS

The results showed that bilberry, blackcurrant and blackberry pomaces contain considerable amounts of polyphenols and anthocyanins which deserve to be recovered and used to formulate food supplements and functional foods. The total phenolic content was about 2.3-3.2 times higher in ethanolic extracts as compared with the water extracts, showing that the presence and concentration of ethanol in the aqueous environment had significant effects on the extraction yields of these antioxidant compounds. The extracts made in 60% aqueous ethanol presented the highest values for TPC, TAC and RDA irrespective of extraction method and pomace matrix while water and 1% aqueous citric acid were very little effective in recovering anthocyanins or even phenolic compounds. Among the three pomace matrices, the highest TAC, TPC and RSA were found in bilberry pomace extracts made both using maceration and UAE. Correlation analyses revealed strong correlations between DPPH radical scavenging activity, total phenolic content and total anthocyanin content in bilberry and blackberry extracts and lower correlations in blackcurrant pomace extracts. Under the conditions of this experiment, the extracts obtained by maceration presented higher TPC values and were more antioxidant than the extracts obtained by UAE.

EXPERIMENTAL SECTION

Materials

Wild bilberries (*Vaccinium myrtillus* L.), blackcurrants (*Ribes nigrum* L.) and blackberries (*Rubus fructicosus* L.) harvested from the wild flora of Valcea county (South-West Oltenia Region, Romania) were subjected to industrial juice processing at a commercial juice manufacturer from Vaideeni (Vâlcea county, Romania). The berries were processed without enzyme treatment and samples of 5 kg fresh pomaces, consisting of peels, seeds, and residual pulp, were collected for each species. The pomaces were stored frozen (at −18 °C)

in sealed polyethylene sacks until further use. When needed, aliquots of the frozen pomaces were thawed in the air at room temperature and dried at 57°C in a convective laboratory dryer (Deca +SS Design, Profimatic, Romania). The dried pomaces were ground in a household electric grinder, sieved using a 0.5 mm screen, and stored in closed containers at 20 °C in the dark until use.

Chemicals and reagents

DPPH (2,2-diphenyl−1-picrylhydrazyl), methanol (Merck) and 6-hydroxy−2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) purchased from Sigma-Aldrich (Steinheim, Germany) were employed for the determination of the radical scavenging activity. Folin-Ciocalteu's phenol reagent (2N, Sigma-Aldrich), gallic acid (99% purity, Sigma-Aldrich) and anhydrous sodium carbonate (Merk) were used for the determination of the total phenolic content. Hydrochloric acid, potassium chloride and sodium acetate provided by Merck (Darmstadt, Germany) were of reagent grade and used without further purification.

Preparation of the extracts

The berry pomaces (BI – bilberry, BC – blackcurrant, BB – blackberry) were subjected to conventional maceration and ultrasound-assisted extraction using five different extraction solvents: water (W), 1% (w/v) aqueous citric acid (CA), 40% (40E), 60% (60E) and 80% (v/v) (80E) aqueous ethanol. Three grams of powdered pomace were extracted with 30 ml solvent. The total time for ultrasound-assisted extraction was 60 min while conventional maceration was carried out without stirring for ten days at room temperature. Ultrasound-assisted extraction was performed in a DK 102 p Bandelin ultrasonic bath (Bandelin Electronic GmbH, Berlin, Germany). Extracts were then filtered using Whatman membrane filters 0.45 μm and stored at 4 ◦C until analysis. Each experiment was done in triplicate.

Total anthocyanin content

Total anthocyanin content was determined according to the pH differential method proposed by Lee et al. [56]. Briefly, the extracts were properly diluted in 0.025 M potassium chloride buffer at pH 1.0 and 0.04 M sodium acetate buffer at pH 4.5 insomuch that their absorbances, measured after 30 min of incubation at room temperature on a Varian Cary 50 spectrophotometer (Varian Co., USA) at 510 and 700 nm, were lower than 1000. The total anthocyanin content was calculated using the following formula:

$$\text{Total anthocyanins (mg CGE/L)} = (A \times MW \times DF \times 1{,}000)/(\varepsilon \times l)$$

where $A = (A_{510\ nm} - A_{700\ nm})pH\ 1.0 - (A_{510\ nm} - A_{700\ nm})pH\ 4.5$; MW is the molecular weight of cyanidin-3-O-glucoside (449.2 g/mol); DF is dilution factor of the samples; ε is the molar absorbtivity of cyanidin 3-*O*-glucoside [29,600 L/(mol•cm)]; l is the cuvette path length (1 cm). The results were expressed in milligrams of cyanidin-3-O-glucoside equivalents per liter of extract (mg CGE/L).

Total phenolic content

The total phenolic content of the extracts was assessed by the Folin–Ciocalteu spectrophotometric method described by Singleton et al. [57]. Briefly, an aliquot of extract (0.1 mL) diluted tenfold with distilled water was mixed with 5 mL of distilled water and 0.5 mL of Folin-Ciocalteu reagent (diluted 1:1 with distilled water). After 3 min, 1.5 mL of sodium carbonate solution (20% w/v) was added and the mixture was made up to 10 mL with distilled water. The solution was mixed thoroughly and incubated at 40 °C for 30 minutes in the dark. Further, the absorbance was measured at 765 nm using a Varian Cary 50 UV spectrophotometer (Varian Co., USA). A standard curve of gallic acid was prepared (50–250 mg/L) and the results were expressed in milligrams of gallic acid equivalents per liter of extract (mg GAE/L).

DPPH radical-scavenging activity

The pomace extracts were evaluated for their capability to scavenge the DPPH radical using the spectrophotometric method previously described by Oliveira et al. [58]. Briefly, aliquots of 50 µL extract were mixed with 3 mL of DPPH methanolic solution (0.004%). After shaking and incubation in the dark for 30 min, the absorbance of the reaction mixture was read at 517 nm with a Varian Cary 50 UV-VIS spectrophotometer (Varian Co., USA). The inhibition of the DPPH radical by the sample was calculated as follows:

$$\text{DPPH scavenging activity (\%)} = (1 - \text{absorbance of sample/absorbance of blank}) \times 100$$

Trolox was used as a standard reference and results were expressed in millimoles of Trolox per liter of extract (mmol Trolox/L).

Statistical analysis

Extraction experiments and composition analyses were carried out in triplicate and the results were expressed as mean ± standard deviation. Data were evaluated by one-way analysis of variance (ANOVA) using Statgraphics Centurion XVI software (StatPoint Technologies, Warrenton, VA). The significance

of differences was analyzed using the Multiple Range Test based on Fisher's least significant difference (LSD) procedure at $p < 0.05$. The correlation coefficients and their significance were calculated by Pearson's test.

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