Elaboration of Novel Extraction Procedure to Reveal Bioactive Component Profile of Anthocyanin-Rich Plants

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Abstract

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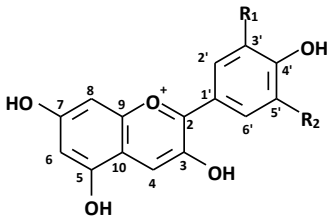
The content of anthocyanin derivatives, antioxidant activity, and phenolic content were determined in black elderberry (*Sambucus nigra* L.), sweet cherry (*Prunus avium* L.), blackberry (*Rubus fruticosus* L.), black currant (*Ribes nigrum* L.), and blackthorn (*Prunus Spinosa* L.). The extraction efficiency was examined of several solvents including hot water, 2% phosphoric acid, ethanol and acetone. A new sequential (cascade) extraction procedure was developed in order to improve the efficiency of the conventional methods. This novel extraction protocol consists of 3 different steps with the prevalence of low pH extraction conditions. When comparing the effectiveness of the conventional and presently improved procedures, it was stated that significantly increased anthocyanin yields had been observed. The highest anthocyanin content, determined with HPLC method, was found in the case of sweet cherry (222.7 mg/kg) on using the three step extraction procedure. The highest antioxidant activity determined with DPPH method was also assigned to the sweet cherry sample (5272 mg/kg). The highest phenolic content was found in blackberry (434 mg/kg).

Keywords: anthocyanins; antioxidant activity; phenolic content, fruits

Anthocyanin derivatives belong to the flavonoid family carrying an aromatic benzopyran structural moiety and being biosynthesised by the phenylpropanoid pathway in nature (Hahlbrock & Scheel 1989). As a result of the existing permanent positive charge and a fully conjugated system (flavylium cation), these molecules usually carry an intense colour (Braverman 1963) strongly depending on the pH of the environment and substitution pattern of the skeleton. The highly substituted skeleton and permanent charge result in pronounced water solubility, and possible proton transfer, isomerisation, and tautomerisation processes (Brouillard 1980).

The above described properties explain the wide appearance of the anthocyanin derivatives in higher plant tissues as colouring materials (flowers, fruits, and leaves). The most important aglycones of the compound family are cyanidin, peonidin, pelargonidin, delphinidin, malvidin, and petunidin (Figure 1) differing only in the substituents at C-3' and C-5' positions of the phenyl ring. Natural anthocyanins are frequently glycosylated (Harborne 1998), mostly



Name	R1	R2
Pelargonidin (Pg)	-H	-H
Cyanidin (Cy)	-OH	-H
Delphinidin (Dp)	-OH	-OH
Peonidin (Pn)	-OMe	-H
Petunidin (Pt)	-OMe	-OH
Malvidin (Mv)	-OMe	-OMe

Figure 1. The general structure of some anthocianin skeleton

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at C-3, with glucose (Prior & Wu 2006), however arabinose, galactose, rhamnose, or xylose derivatives might appear along with di- and trisaccharide substitutions as well. Rarely, glycosylation may appear at C-5, C-7, C-3', C-5', and even C-4' positions (Harborne 1998). Furthermore, acylation (Dougall 1997) may take place to form more stable derivatives with acetic, malonic, malic, oxalic, succinic, *p*-hydroxybenzoic or hydroxycinnamic acids mostly on the carbohydrate component attached to the C-3 position.

Anthocyanins represent highly important natural food colour components playing crucial role in metabolism and tissue protection (Strack & Wray 1994; Bohm 1998), and also versatile beneficial health effects are attributed to them. These positive biological impacts include high antioxidant activity (Tsuda *et al*. 1994, 1998; Wang *et al*. 1997), the reduction of the risk of cancer (Hou 2003) and on cardiovascular diseases. Moreover, significant anti-inflammatory effects (Wang *et al*. 1999) has been observed for anthocyanin derivatives.

Several qualitative and quantitative analytical protocols have been developed to obtain anthocyanins from natural resources including advanced extraction, separation, detection, and structure determination methods (DaCosta *et al*. 2000; Takeoka *et al*. 2002; Andersen & Fossen 2003). The published methods usually apply the freeze-drying step to produce moisture free plant parts, blender or mill protocols or liquid nitrogen to yield fine powders, the prepared parts undergoing extraction procedures regularly with moderate acidic organic solvents as a rule. However, the published methods use only one-step extraction procedures and the yield of these protocols is usually low.

Our intention was to improve the efficiency of the previously described simple extraction methods in respect of yielding antioxidants, polyphenols, and anthocyanins from the selected fruit variants: black elderberry (*Sambucus nigra* L.), sweet cherry (*Prunus avium* L.), blackberry (*Rubus fruticosus* L.), black currant (*Ribes nigrum* L.), and blackthorn (*Prunus spinosa* L.). A novel three-step sequential extraction method in a cascade arrangement has been elaborated and introduced by optimising the order and quality of the applied solvents. Acidified ethanol, methanol, and acetone solutions have been applied to reach maximum obtained yields of the bioactive compounds. The efficiency of the investigated procedures has been compared in terms of antioxidant activity, and polyphenolic and anthocyanin contents of the obtained extracts.

MATERIAL AND METHODS

Fruit samples were collected from the Eger region, Hungary in 2009, dried at 20°C and kept at ambient temperature. Single-solvent extraction methods were accomplished with 3 g of the samples mixed with 30 ml solvent using Ultra Turrax T25 (9500 rpm). Different solvents were investigated as the extraction media: hot water, 2% phosphoric acid, ethanol, ethyl-acetate, acetone, and *n*-hexane. The samples were placed in an ultrasound bath for 10 minutes. The solid material was subjected to the same procedure twice more after filtration and the resulted extracts were combined and filtered through 0.25 µm membrane filter.

The sequential extraction procedure in cascade arrangement included the application of 70% ethanol with 0.01% HCl (e), 70% methanol with 0.01% HCl (m), and 70% acetone with 0.01% HCl (a) in various compositions (single solvent in three consecutive extraction steps, or two distinctive solvents in different sequences, or sequential three-step cascade extraction in various orders of the specific solvents). The efficiency of this new, complex system applying sequential extractions with one, two, or three solvents was studied on three fruits with the highest anthocyanin contents, namely elderberry, sweet cherry, and blackberry. The fruit parts were chopped and 1 g was mixed with 10 ml solvent, the extraction taking place in an orbital shaker for 30 min and in an ultrasound bath for 10 minutes. The mixtures were centrifuged at 6000 rpm for 10 min and filtered through a 0.25 µm membrane filter.

The antioxidant activity (Wootton-Beard *et al*. 2011) was measured using the DPPH method and the activity was given in ascorbic acid units. 0.1 mM stock solution was prepared with DPPH (A ~1 at 514.5 nm). The concentration of the standard ascorbic acid solutions varied between 1–20 mg/kg. The volume of both the standard and the measured solutions was 50 µl. Mixture with 3 ml of the DPPH solution were prepared, the reaction time was set to 30 min and the absorbance was measured at 514.5 nm. The measured absorbance values were converted to ascorbic acid units.

The polyphenolic content (Wootton-Beard *et al*. 2011) of the solutions was determined using Folin-Ciocalteau method and the activity was given in Gallic acid units. The concentration of the Folin-Ciocalteu reagent was 2N and 0.5 ml was used for the measurements in a diluted form (with 9.7 ml distilled water). The sample (0.1 ml) was mixed with

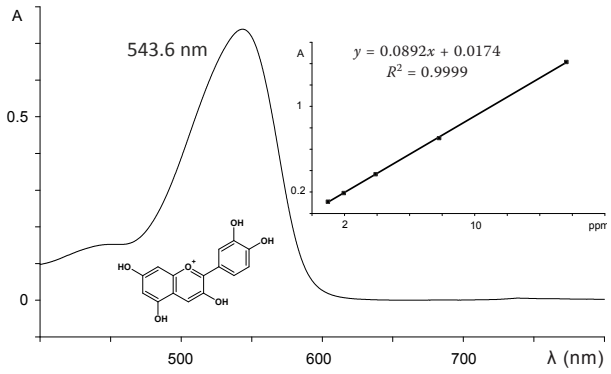

Figure 2. The UV/VIS spectrum of the cyanidin-chloride used as reference material in the 400–800 nm range with the absorption maximum at 543.6 nm, inset: the calibration curve in the 1–15 mg/kg concentration region

the reagent and after 5 min 1.5 ml sodium-carbonate (60 g/l) solution was added. The mixture was kept at room temperature for 2 h and the photometric measurement was carried out at 765 nm.

Cyanidin chloride (Sigma, St. Louis, USA) was used as the standard material. The quantitative determination took place at 543.6 nm, and the anthocyanin content was given in cyanidin units. The calibration curve was determined in the 1–15 mg/kg concentration range with the detection limit of 0.2 mg/kg (Figure 2).

RESULTS AND DISCUSSION

Single solvent extraction

Extraction of anthocyanin derivatives. The application of ethyl-alcohol and acetone led to the highest yield of extracted anthocyanins, 20–138 mg/kg. In contrast to that hot water extraction provided reasonably good efficiency, however, its wide application may be limited by the heat sensitive nature of anthocyanins. An analogous statement applied also to phosphoric acid solution, as the strong acidic character may be considered as a factor restraining the applicability. Apolar solvents such as *n*-hexane and ethyl-acetate displayed reduced extraction capabilities compared to the previously mentioned agents.

The highest anthocyanin level was found in the case of sweet cherry, while the least one with blackthorn.

Extraction of polyphenolic derivatives. The phenolic content of black elderberry varied between 60 and 620 mg/kg gallic acid. The low phenolic content indicates that the main active components in the sample belong to the anthocyanin derivatives. The blackberry samples could be characterised by polyphenol content between 180 and 420 mg/kg gallic acid. Phenolic content of the black currant samples was found to be between 720 mg/kg and 1160 mg/kg gallic acid. The phenolic content of sweet cherry varied from 180 to 420 mg/kg gallic acid. Hot water extracts showed the highest antioxidant activity, while ethanolic solutions contained the highest levels of anthocyanin derivatives. Blackthorn can be characterised by an average phenolic content (350–560 mg/kg gallic acid).

Extraction of antioxidant compounds. A high antioxidant activity was obtained with black elderberry samples (3090–4760 mg/kg ascorbic acid equivalent). In this case the water extracts showed the highest antioxidant activity. The antioxidant activity of the blackberry varied between 60–3650 mg/kg ascorbic acid. The most effective solvent for the extraction of antioxidants was acetone, while ethanol displayed the highest efficiency for anthocyanin extraction. The antioxidant activity of the black currant varied between 80–2440 mg/kg ascorbic acid. The antioxidant activity of sweet cherry was found to be between 50 and 4550 mg/kg ascorbic acid, depending on the solvent. The blackthorn samples possessed a high antioxidant activity, i.e. between 320–7270 mg/kg ascorbic acid.

Three-step extraction procedures

Anthocyanins yield. The efficiency of the three-step sequential extraction was studied on three fruits with the highest anthocyanin contents: elderberry, sweet cherry, and blackberry. The anthocyanin amounts obtained by each sequential step are illustrated in Table 1. In the case of sweet cherry, the highest total amount obtained was found to be 222.7 mg/kg with ethanol, while lower anthocyanin amounts yields were achieved with acetone (176.4 mg/kg) and methanol (180.7 mg/kg). Table 1 illustrates the quantities extracted in the three-step extraction procedure. In the first step, the highest amount of anthocyanins (161.7 mg/kg) could be achieved by the extraction with ethanol. In the second and third steps of the extraction procedure, significantly lower anthocyanin amounts (45–56 and 7–21.2 mg/kg, respectively) were gained.

In the case of blackberry, the anthocyanin content was below 200 mg/kg. In this case the highest total amount could be obtained by the extraction with ethanol (146.9 mg/kg). The first extraction step was found to be the most efficient with acetone (117.1 mg/kg),

Table 1. The anthocyanin content of sweet cherry, blackberry, and elderberry obtained with the three step extraction procedure with three different solvents: acetone, ethanol, and methanol in different variations. The first three rows show the results with pure solvents, the following 12 raws contain results with two different solvents in different variations, the results with three different solvents in different sequences are indicated in the last 6 rows

Solvent	Sweet cherry				Solvent	Blackberry				Solvent	Elderberry			
	1st step	2nd step	3rd step	sum		1st step	2nd step	3rd step	sum		1st step	2nd step	3rd step	sum
aaa	122.7	45.9	7.8	176.4	aaa	117.1	22.7	3.3	143.2	aaa	153.2	48.4	9.1	210.7
eee	161.7	53.4	7.6	222.7	eee	100.5	40.5	5.9	146.9	eee	155.8	49.5	9.3	214.6
mmm	103.6	55.9	21.2	180.7	mmm	86.1	40.0	8.4	134.5	mmm	100.6	60.4	21.0	182.1
aae	122.3	45.8	5.9	174.0	aae	116.7	22.7	5.9	145.3	aae	152.8	48.3	9.1	210.2
aee	122.5	40.5	5.8	168.7	aee	116.9	34.8	6.4	158.1	aee	153.0	48.6	9.1	210.6
aam	122.5	45.8	22.4	190.7	aam	116.9	22.7	9.2	148.9	aam	153.0	48.4	16.8	218.1
amm	122.6	47.2	19.9	189.7	amm	116.6	29.5	7.2	153.4	amm	153.1	39.7	17.6	210.3
eea	161.5	53.3	6.1	221.0	eea	100.3	40.4	3.3	144.0	eea	155.6	49.4	9.0	214.0
eaa	161.6	34.9	6.7	203.1	eaa	100.4	26.5	3.6	130.5	eaa	155.7	47.6	9.0	212.4
eem	161.5	53.3	5.6	220.5	eem	100.3	40.4	7.5	148.2	eem	155.6	49.4	7.2	212.2
emm	161.6	35.8	17.1	214.6	emm	100.4	34.3	7.8	142.5	emm	155.7	39.0	17.4	212.2
mma	103.2	55.7	7.4	166.2	mma	85.7	39.8	3.0	128.5	mma	100.2	60.2	7.2	167.6
maa	103.3	38.6	6.6	148.5	maa	85.8	16.7	2.4	104.9	maa	100.3	31.7	6.0	138.0
mme	103.4	55.8	5.6	164.8	mme	85.9	39.9	5.3	131.1	mme	100.4	60.3	7.3	168.0
mee	103.5	34.2	18.3	156.0	mee	86.0	34.7	8.0	128.7	mee	100.5	31.9	17.3	149.7
aem	122.5	40.5	21.7	184.6	aem	116.3	16.8	8.8	142.0	aem	153.0	48.6	11.6	213.1
ame	122.6	47.2	6.0	175.8	ame	117.0	16.7	5.6	139.4	ame	153.1	39.7	8.7	201.5
eam	161.5	34.9	26.1	222.5	eam	100.3	19.5	7.9	127.7	eam	155.6	47.7	7.3	210.7
ema	161.4	35.9	9.1	206.4	ema	100.2	34.4	3.2	137.7	ema	155.5	39.1	8.8	203.4
mae	94.4	35.3	4.6	134.3	mae	76.9	14.9	3.9	95.7	mae	91.4	28.9	5.4	125.7
mea	103.5	34.2	6.4	144.0	mea	86.0	34.7	2.8	123.5	mea	100.5	31.9	6.0	138.4

a – 70% acetone with 0.01% HCl; e – 70% ethanol with 0.01% HCl; m – 70% methanol with 0.01% HCl

while the application of methanol resulted in the least amount of anthocyanins (86.1 mg/kg). In the third step, less than 10 mg/kg of anthocyanins could be obtained with all the three solvents. In the case of elderberry, the implementation of the three extraction steps resulted in similar results in terms of the three solvents (the yields obtained ranged from 182.1 mg/kg to 214.6 mg/kg). In the first extraction step, around 150 mg/kg anthocyanin content was detected, while in the third step it was below 20 mg/kg.

The sequential extraction procedure was carried out with different solvent systems in different orders as well. However, in the case of sweet cherry, the highest total yield extracted was achieved by means of the application of ethanol for three sequential steps (eee). Slightly lower extraction efficiency was achieved with the ethanol-ethanol-methanol (eem) three step extraction procedure and the ethanol-acetone-methanol (eam) system. The efficiency of single solvent extraction of blackberry was represented by 130–140 mg/kg extracted amounts. The mixed extraction with two solvents in three steps, however, resulted in higher yields (aee and amm). The three step extraction of elderberry with one specific solvent demonstrated similar efficiency as in the case of sweet cherry (180–210 mg/kg), and the application of some of the two solvent system extraction procedures led to similar results (aae, aee, aam, amm, eea, eaa, eem, and emm).

Polyphenols yield. Sequential extraction of the three fruits (Table 2) led to an intense decrease of the phenolic materials amount in the remaining pulp. In the case of sweet cherry, the highest polyphenolic content was measured when methanol was used as the extracting agent (347.2 mg/kg for mmm). The binary solvent extraction provided results similar to one another with of combinations of the two alcohols and acetone (eem, emm, mma, maa, mme,

Table 2. The polyphenol content of sweet cherry, blackberry, and elderberry obtained with the three-step extraction procedure with three different solvents: acetone, ethanol, and methanol in different variations. The first three rows show the results with pure solvents, the following 12 raws contain results with two different solvents in different variations, the results with three different solvents in different sequences are indicated in the last 6 rows

Solvent	Sweet cherry				Solvent	Blackberry				Solvent	Elderberry			
	1st step	2nd step	3rd step	sum		1st step	2nd step	3rd step	sum		1st step	2nd step	3rd step	sum
aaa	146.0	53.6	9.3	208.9	aaa	137.2	43.2	8.2	188.6	aaa	73.6	13.5	3.2	90.3
eee	239.6	79.6	7.6	326.8	eee	256.4	112.6	15.3	384.3	eee	86.6	32.3	6.3	125.2
mmm	253.6	72.4	21.2	347.2	mmm	268.9	143.5	22.3	434.7	mmm	88.3	36.1	5.2	129.6
aae	145.6	53.5	4.7	203.8	aae	136.8	43.1	7.5	187.3	aae	73.2	13.4	4.6	91.2
aee	145.8	48.4	4.6	198.9	aee	137.0	210.7	14.4	362.2	aee	73.4	27.4	5.3	106.1
aam	145.8	53.5	13.0	212.3	aam	137.0	43.1	9.7	189.9	aam	73.4	13.5	7.4	94.3
amm	145.9	125.8	25.4	297.2	amm	136.7	282.3	21.9	440.9	amm	73.5	43.4	5.5	122.4
eea	239.4	79.5	5.8	324.8	eea	256.2	112.5	4.0	372.7	eea	86.4	32.2	2.3	121.0
eaa	239.5	32.7	6.8	279.0	eaa	256.3	23.1	5.3	284.7	eaa	86.5	11.5	2.8	100.8
eem	239.4	79.5	7.8	326.7	eem	256.2	112.5	24.9	393.7	eem	86.4	32.2	3.4	122.0
emm	239.5	76.7	21.9	338.0	emm	256.3	150.6	22.6	429.5	emm	86.5	36.9	5.2	128.6
mma	253.2	72.3	15.2	340.7	mma	268.5	143.3	18.7	430.5	mma	87.9	35.9	4.5	128.4
maa	253.3	93.0	16.1	362.4	maa	268.6	84.6	16.1	369.2	maa	88.0	16.1	3.8	108.0
mme	253.4	72.3	7.8	333.5	mme	268.7	143.4	17.1	429.2	mme	88.1	36.0	6.6	130.7
mee	253.5	84.2	22.0	359.7	mee	268.8	118.0	20.9	407.8	mee	88.2	32.9	5.1	126.2
aem	145.8	48.4	12.6	206.9	aem	136.4	85.2	12.0	233.5	aem	73.4	27.4	5.1	105.9
ame	145.9	125.8	6.5	278.2	ame	137.1	84.7	9.2	231.0	ame	73.5	43.4	6.2	123.1
eam	239.4	32.7	17.7	289.8	eam	256.2	80.7	18.2	355.1	eam	86.4	11.5	8.0	105.9
ema	239.3	76.7	14.7	330.8	ema	256.1	150.7	18.5	425.3	ema	86.3	36.9	4.5	127.8
mae	244.4	89.7	8.0	342.1	mae	259.7	81.8	14.2	355.6	mae	79.1	14.5	5.0	98.6
mea	253.5	84.2	15.7	353.5	mea	268.8	118.0	17.6	404.4	mea	88.2	32.9	4.4	125.5

a – 70% acetone with 0.01% HCl; e – 70% ethanol with 0.01% HCl; m – 70% methanol with 0.01% HCl

and mee). The sequential application of the 3 different solvents provided a high yield (353.5 mg/kg). The phenolic content of the blackberry sample varied between 188.6 and 434.7 mg/kg, while the highest values were found with methanolic (434.7 mg/kg) and ethanolic (384.3 mg/kg) extractions. In the case of the three solvent systems, the use of two solvent combinations (ema and mea) resulted in the polyphenolic content above 400 mg/kg. The phenolic content of elderberry samples fell into the 90–130 mg/kg rang. The accomplishment of sequential extraction just with ethanol and methanol, respectively, resulted in similar yields of polyphenols (125.2 and 129.6 mg/kg, respectively), while significantly lower efficiency was observed with the extraction with acetone (90.3 mg/kg). High phenolic activities were found in the extracts yielded by several two solvents in three-step extraction systems (amm, eea, eem, emm, and mma) with bioactive compound content above 120 mg/kg.

Antioxidant activity

Antioxidant activities of the sequentially extracted samples are presented in Table 3. It is apparent from the table that relevant values for the sweet cherry varied between 4372 and 5272 mg/kg depending on the solvents used. The single-solvent three-step extraction procedure with methanol provided an extract with the highest antioxidant activity (5272 mg/kg in ascorbic acid units). The efficiency of the extraction with ethanol and acetone might be characterised by slightly lower values (4978 and 4372 mg/kg, respectively). In the cases of binary systems, the application of several solvent combinations led to extracts with antioxidant activity above 5000 mg/kg (emm, mma, maa, mme, and mee). The involvement of methanol in the three-step extraction procedure enhanced the efficiency. Similar tendency were observed with the ternary solvent systems, with antioxidant activity val-

Table 3. The antioxidant activity of sweet cherry, blackberry, and elderberry obtained with the three step extraction procedure with three different solvents: acetone, ethanol, and methanol in different variations. The first three rows show the results with pure solvents, the following 12 raws contain results with two different solvents in different variations, the results with three different solvents in different sequences are indicated in the last 6 rows

Solvent	Sweet cherry				Solvent	Blackberry				Solvent	Elderberry			
	1st step	2nd step	3rd step	sum		1st step	2nd step	3rd step	sum		1st step	2nd step	3rd step	sum
aaa	3124.0	1125.0	123.0	4372.0	aaa	1372.0	432.0	82.0	1886.0	aaa	3255.0	1323.0	125.0	4703.0
eee	3462.0	1328.0	138.0	4928.0	eee	2564.0	1126.0	153.0	3843.0	eee	3505.0	1427.0	232.0	5164.0
mmm	3624.0	1436.0	212.0	5272.0	mmm	2689.0	1435.0	223.0	4347.0	mmm	3428.0	1356.0	186.0	4970.0
aae	3123.6	1124.9	122.4	4370.9	aae	1371.6	431.9	74.8	1878.3	aae	3254.6	1322.8	215.3	4792.8
aee	3123.8	1198.3	124.5	4446.6	aee	1371.8	2104.6	144.1	3620.5	aee	3254.8	1325.1	215.4	4795.4
aam	3123.8	1124.9	178.0	4426.7	aam	1371.8	431.9	97.5	1901.3	aam	3254.8	1322.9	194.4	4772.1
amm	3123.9	1665.9	224.0	5013.7	amm	1371.5	2813.5	219.7	4404.7	amm	3254.9	1428.1	190.0	4873.0
eea	3461.8	1327.9	109.1	4898.8	eea	2563.8	1125.9	40.1	3729.8	eea	3504.8	1426.9	116.0	5047.8
eaa	3461.9	1015.2	116.7	4593.8	eaa	2563.9	231.2	52.9	2848.0	eaa	3504.9	1228.7	120.9	4854.5
eem	3461.8	1327.9	145.8	4935.5	eem	2563.8	1125.9	249.2	3939.0	eem	3504.8	1426.9	121.3	5053.0
emm	3461.9	1503.2	216.1	5181.2	emm	2563.9	1505.0	226.0	4294.9	emm	3504.9	1326.2	184.2	5015.3
mma	3623.6	1435.8	146.5	5205.9	mma	2688.6	1434.8	187.4	4310.8	mma	3427.6	1355.8	130.6	4914.1
maa	3623.7	1304.9	142.7	5071.3	maa	2688.7	846.6	160.7	3696.0	maa	3427.7	1393.2	131.6	4952.5
mme	3623.8	1435.9	145.8	5205.5	mme	2688.8	1434.9	171.0	4294.7	mme	3427.8	1355.9	225.0	5008.7
mee	3623.9	1390.1	210.1	5224.1	mee	2688.9	1180.9	209.3	4079.0	mee	3427.9	1395.6	187.5	5011.0
aem	3123.8	1198.3	181.1	4503.2	aem	1371.2	847.2	120.0	2338.3	aem	3254.8	1325.1	222.1	4802.0
ame	3123.9	1665.9	138.0	4927.8	ame	1371.9	846.7	92.0	2310.6	ame	3254.9	1428.1	220.3	4903.3
eam	3461.8	1015.2	187.6	4664.6	eam	2563.8	807.3	182.3	3553.3	eam	3504.8	1228.7	234.5	4968.0
ema	3461.7	1503.3	143.7	5108.8	ema	2563.7	1505.1	184.9	4253.8	ema	3504.7	1326.3	131.9	4962.9
mae	3614.8	1301.7	141.6	5058.2	mae	2679.8	843.8	146.1	3669.7	mae	3418.8	1389.6	226.2	5034.6
mea	3623.9	1390.1	145.1	5159.1	mea	2688.9	1180.9	175.9	4045.6	mea	3427.9	1395.6	131.7	4955.2

a – 70% acetone with 0.01% HCl; e – 70% ethanol with 0.01% HCl; m – 70% methanol with 0.01% HCl

ues above 5000 mg/kg having been obtained in three cases (ema, mae, and mea). The antioxidant activity of the blackberry extracts ranged from 1886 mg/kg to 4347 mg/kg when one solvent was used only in the sequential extraction procedure. The application of the binary solvent compositions resulted in extracts with antioxidant capacity beyond 4000 mg/kg in several cases (emm, mma, mme, and mee). For ternary solvent combinations, extracts with antioxidant activity above 4000 mg/kg was gained in two cases (ema and mea). Higher antioxidant activities were found in elderberry extracts as a result of single-solvent three-step extraction procedure (4703–5164 mg/kg). The use of two solvent systems resulted in extracts with high (above 5000 mg/kg) antioxidant activities in the case of eea, eem, emm, mme, and mee. The application of only one specific ternary solvent combination (mea) led to the yield of extracts with antioxidant activity exceeding 5000 mg/kg.

CONCLUSION

The extraction efficiency of specific combinations of different solvents exhibited significant variability with diverse fruit species. Antioxidant activity, as well as anthocyanin and phenolic contents, was assessed by using both single-solvent extraction and combined, optimised three-step extraction procedures involving methanol, ethanol, and acetone in acidic media. By comparison of the efficiencies of the distinctive extraction agents, it may be stated that in most of cases ethanol and acetone can be regarded as the most suitable solvents for providing the highest yields of active compounds. Three-step sequential extractions proved to be more effective in terms of yielding bioactive substances than the single-solvent extraction procedures. The extraction yields were found to be twice that of the average obtained with conventional methods. In consequence

to the comparison of several sequential methods, the most suitable multiple extraction protocol may be suggested for possible food industrial applications. The novelty of the work is supported by the fact that such combinations of different extraction procedures have not been described previously, single-solvent extractions mainly prevailing in both the industrial and laboratory applications. The highest anthocyanin content was found for sweet cherry using the sequential extraction procedure with the application of ethanol (sequential, single-solvent ethanol extraction). The highest phenolic content, however, was assessed in the blackberry extracts obtained with methanolic extraction. DPPH antioxidant activity was found to be the highest in the case of sweet cherry using methanol as the extraction agent. The combined method has one more important advantage over the conventional ones: it does not require the use of elevated temperatures during the extraction process, which is beneficial for the heat sensitive natural compounds.

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