Original article

Use of cyclic voltammetry to determine the antioxidant capacity of berry fruits: correlation with spectrophotometric assays

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Summary

Background of the study – Among fruits, berry fruits contain a very large complex of chemical compounds that are responsible for their antioxidant properties. As far as we know there is no data available from cyclic voltammetry studies of berry fruits. **Objectives** – The first objective of the present work was to investigate the electrochemical properties of antioxidants present in four types of berry fruits: strawberries, blackberries, blueberries and red raspberries. The second objective was to establish the correlation between antioxidant capacities obtained from cyclic voltammograms with those obtained using spectrophotometric techniques. **Methods** – Evaluation of antioxidant activity of 15 berry samples (strawberry, blackberry, blueberry and raspberry) has been carried out using cyclic voltammetry (CV). CVs were taken in the potential range between 0 mV and 800 mV with a scan rate of 100 mV s⁻¹. **Results** – A pronounced anodic current peak observed between 0.524 V and 0.540 V in all analyzed berry samples can be ascribed to anthocyanins, which are the major contributors to the antioxidant capacity of investigated berries. Results obtained from CVs were compared with those obtained by using DPPH, ABTS, FRAP and RP assays. ACI parameters were determined for each berry sample by assigning all the assays an equal weight. According to obtained results, the highest ACI had blueberry samples, followed by blackberry, raspberry and strawberry samples. Also, high positive correlations were found between antioxidant capacities deduced from CVs with those obtained using FRAP ($R^2=0.8630$, $p=0.0006$), RP ($R^2=0.7426$, $p=0.0004$), DPPH ($R^2=0.7680$, $p=0.0001$) and ABTS ($R^2=0.8710$, $p=0.000002$). In addition, high positive correlations were also found between total phenols, flavonoids, anthocyanins and CV antioxidant capacity ($R^2=0.8091$, $p=0.0005$; $R^2=0.8341$, $p=0.0002$; $R^2=0.7163$, $p=0.0007$), respectively. **Conclusion** – Obtained results showed that the cyclic voltammetry is a suitable method for the determination of the reducing capacity based on the electrochemical properties of compounds under the test.

Significance of this study

What is already known on this subject?

- Berry fruits are one of the richest sources of polyphenols, but there is no data available from cyclic voltammetry studies of berry fruits.

What are the new findings?

- CV method is suitable for determination of the reducing capacity of polyphenols. The antioxidant power of blueberry is the highest, followed by blackberry, raspberry and strawberry.

What is the expected impact on horticulture?

- The capability of the method to rapidly evaluate the antioxidant activity of berries could be useful for horticulture industry.

Keywords

antioxidant composite index, blackberry, blueberry, raspberry, strawberry, total anthocyanins, total flavonoids, total polyphenols

Abbreviations

CV	cyclic voltammetry
ABTS	2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonate
DPPH	2-diphenyl-2-picrylhydrazyl hydrate
FRAP	ferric reducing-antioxidant power
RP	reduction power Fe(III) to Fe(II)
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
TPTZ	2,4,6-tri(2-pyridyl)-S-tirazine
AEAC	Ascorbate Equivalent Antioxidant Capacity
TEAC	Trolox Equivalent Antioxidant Capacity
ACI	Antioxidant Composite Index

Introduction

Fruits are well-known sources of health-promoting compounds. These compounds include vitamins, minerals and polyphenolic antioxidants. Among fruits, berry fruits are very popular and appreciated because of their beneficial

health effects. The recommendations based on epidemiological studies are such that berry fruits ensure good protection against the development of diseases caused by oxidative stress, such as cancer, coronary heart disease, diabetes and hypertension (Halvorsen et al., 2002; Seeram, 2008a, b; Szajdek and Borowska, 2008; Paredes-López et al., 2010). The World Health Organization (WHO, 2002) also emphasizes the importance of antioxidant activity of phenolic components, especially from small colorful fruits, for prevention of some diseases. Berry phenolics include flavonoids (anthocyanins, flavonols and flavanols), condensed tannins (proanthocyanidins), hydrolysable tannins (ellagitannins and gallotannins), stilbenoids and phenolic acids (Szajdek and Borowska, 2008; Paredes-López et al., 2010). Because of these natural antioxidants and due to their high concentration and quality diversity, people have a growing interest in the consumption of berries in diet as natural functional food. The total antioxidant activity of berry fruits has been evaluated by various assays and it is given in review works of Giampieri et al. (2012), Rao and Snyder (2010), Lee et al. (2012) and Nile and Park (2014), respectively, and in a study of Tulipani et al. (2008) and Wang and Millner (2009).

However, as far as we know there is no data available from cyclic voltammetry studies of berry fruits. Since the chemical activities of phenolics in terms of their reducing properties as hydrogen or electron-donating agents could predict their potential to act as antioxidants (Yang et al., 2001), studying of electrochemical properties could help to better understand structurally different phenolics. Cyclic voltammetry has numerous advantages over the other mentioned methods; it is simple, rapid and sensitive enough for determination of physiological concentrations of antioxidants.

For that purpose, the first objective of the present work was to investigate the electrochemical properties of antioxidants present in four types of berry fruits: blackberries (*Rubus* spp.), blueberries (*Vaccinium corymbosum*), red raspberries (*Rubus idaeus*) and strawberries (*Fragaria × ananassa*). The second objective was to establish the correlation between antioxidant capacities obtained from cyclic voltammograms with those obtained using spectrophotometric techniques, as well as to establish correlation between the results of antioxidant capacity and total phenol, flavonoid and anthocyanin contents. In addition, the total antioxidant capacities of berry fruits were measured using five *in vitro* methods: cyclic voltammetry (CV), 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH[•]) scavenging activity, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical cation scavenging activity, ferric reducing-antioxidant power (FRAP) and reduction power (RP) Fe(III) to Fe(II).

Materials and methods

Chemicals

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was purchased from Acros Organics (Morris Plains, New Jersey, USA). ABTS (2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonate), DPPH (2-diphenyl-2-picrylhydrazyl hydrate), TPTZ ((2,4,6-tri(2-pyridyl)-S-tirazine), kaempferol, malvidin-3-O-glucoside chloride, coumaric acid, ferrulic acid, caffeic acid, gallic acid, (+)-catechin, rutin and quercetin (HPLC grade), were purchased from Sigma Aldrich (Steinheim, Germany). Cyanidin-3-O-glucoside chloride and quercetin-3-O-β-glucoside (HPLC grade) were from ChromaDex (Irvine, CA, USA). Folin-Ciocalteu's phenol reagent, potassium peroxodisulfate, ammonium iron(II) sulfate hexahydrate,

iron(III) chloride, potassium hexacyanoferrate(III), sodium hydroxide, sodium acetate, sodium nitrite, sodium carbonate, sodium sulphate, aluminum chloride hexahydrate, sodium dihydrogen phosphate, sodium hydrogen phosphate, ascorbic acid, three chloro acetic acid, hydrochloric acid, acetic acid, and formic acid, were purchased from Merck® (Darmstadt, Germany). Ethanol (96% by vol.) and methanol (HPLC grade) were from J.T. Baker (Deventer, The Netherlands). Purified water (18 MΩcm), prepared by a MicroMed purification system (TKA Wasseraufbereitungssysteme GmbH, Niederelbert, Germany), was used to prepare all samples and standards.

Samples

Strawberry fruits of three cultivars (S1–S3), blueberry (Be1–Be3), blackberry (Bc1–Bc5), and raspberry (R1–R4) were collected from different commercial plantations in two regions of Serbia. Strawberry samples was selected from Southern Serbia (Leskovac), blueberry samples were originated from Ljig (Western Serbia), blackberry samples were from Valjevo (Western Serbia), whereas the raspberry samples originated from commercial plantations in Ivanjica and Arilje (Western Serbia). Samples were harvested during June–August 2016. The amount of each berry cultivar collected for the analysis was about 500 g.

Preparation of berry fruits

Ultrasound-assisted extraction has been recently reported as more efficient method for extraction of anthocyanins and flavonols from berry fruits than conventional solvent extraction (Chen et al., 2007; Oancea et al., 2013; Ivanovic et al., 2014). In this work, the ultrasound-assisted extraction with acidified 80% methanol (0.01%, v/v HCl) was used to prepare anthocyanin and flavonol-containing extracts from berry samples. Frozen berry fruits were milled in the blender to obtained puree which was subsequently used for extraction. The ultrasonic extractions were performed at room temperature (25°C) for 15 minutes. Briefly, 2.0000 g ± 0.0001 g of each berry sample was weighed and the flask with a sample was put into the ultrasonic bath and sonicated for 15 min at 25°C. After sonication, the solvent was removed by vacuum evaporation. The extracts were stored in the fridge at -18°C prior to analysis.

Instruments

An Agilent 8453 UV/Vis spectrophotometer (Agilent Technologies, Santa Clara, California, USA) was used for absorbance measurements and spectra recording, using optical cuvettes of 1 cm optical path. The pH measurements were made with Hanna instrument pH-meter (Hanna Instruments, Smithfield, Rhode Island, USA) equipped with glass electrode. Cyclic voltammograms were recorded on a CHI760B instrument (CH Instruments, Austin, Texas, USA). The cell was equipped with GC electrode, an accessory platinum electrode of larger area (Model CHI221, cell top including Pt wire counter electrode) and an Ag/AgCl reference electrode (Model CHI111). All measurements were taken at ambient temperature.

Total polyphenolic content (TP)

Total polyphenols were determined according to the Folin-Ciocalteu procedure (Slinkard and Singleton, 1977; Singleton et al., 1999; Stratil et al., 2006), using gallic acid as standard and expressing the results as gallic acid equivalents (GAE) per gram fresh weight of berry samples (mg GAE g⁻¹ fw). All measurements were performed in triplicate.

Total flavonoid content (TF)

The total flavonoid content was measured by the aluminum chloride spectrophotometric method described by Zhishen et al. (1999). Catechin was chosen as a standard and the results expressed as gram of catechin equivalents (CE) per gram fresh weight of berry samples (mg CE g⁻¹ fw). The levels of total flavonoid contents in berries were determined in triplicate.

Total anthocyanins (TA)

The monomeric anthocyanin contents of the acidified methanol extracts were determined using the pH-differential method (Guisti and Wrolstad, 2001). The total anthocyanin content was calculated as micrograms of cyanidin-3-glucoside equivalents/100 g fresh weight (mg cy-3-glu 100 g⁻¹ fw), using an extinction coefficient of 26.900 L mol⁻¹ cm⁻¹ and molecular weight of 449.2 g mol⁻¹.

Antioxidative assays

For DPPH method (Brand-Williams et al., 1995), which is slightly modified, a solution of DPPH (1×10^{-4} mol L⁻¹) was prepared in methanol. 5.0 mL of this solution and 100 µL of berry extract were mixed in 10-mL volumetric flask and filled with methanol to the mark. The discoloration of the DPPH radical was measured at 520 nm, 30 min after the reaction started. The Trolox calibration curve was plotted as a function of the decrease in absorbance of DPPH radical scavenging activity. The final results were expressed as millimoles of Trolox equivalents (TE) per gram of fresh weight of berry samples (mmol TE g⁻¹ fw).

The ABTS radical scavenging activity was measured using the method of Re et al. (1999) and Arts et al. (2004). 100 µL of berry extract was mixed with 3.9 mL of diluted ABTS radical cation solution. After reaction at room temperature for 6 min, the reduction in absorbance at 734 nm was measured. The Trolox calibration curve was plotted as a function of the decrease in absorbance of ABTS radical cation scavenging activity. The final results were expressed as millimoles of (TE) per gram of fresh weight of berry samples (mmol TE g⁻¹ fw).

Ferric reducing-antioxidant power (FRAP) assay was performed as previously described by Benzie and Strain (1999). In the FRAP assay, antioxidants in the sample reduce Fe³⁺-TPTZ complex to the ferrous form at low pH (pH=3.6) with an increase in absorbance at 595 nm. FRAP values were expressed as millimoles of Fe²⁺ equivalents (FE) per gram of fresh weight of berry samples (mmol FE g⁻¹ fw).

Reducing power (RP) assay Fe(III) to Fe(II) was determined as described by Oyaizu (1986). Reducing power was expressed in relation to the reducing power of ascorbic acid as a positive control (AEAC, Ascorbate Equivalent Antioxidant Capacity). RP values were expressed as millimoles of ascorbic acid equivalents (AAE) per gram of fresh weight of berry samples (mmol AAE g⁻¹ fw).

Electrochemical determination of antioxidant capacity

Prior to each run, the surface of the glassy carbon electrode was freshly abraded with 1.0, 0.3 and 0.05 µm alumina powder, rinsed with redistilled water and degreased in ethanol in ultrasonic bath. Cyclic voltammetric experiments were performed with a solution of standard/extracts mixed with 0.1 mol L⁻¹ sodium acetate-acetic buffer (pH 4.5) at a ratio of 1:1 (v/v) in acidified 80% methanol. The sodium acetate-acetic buffer also acted as a supporting electrolyte for the CV measurements. The voltammetric experiments were performed at room temperature using an apparatus cell. The

scan was taken in the potential range between 0 mV and 800 mV with a scan rate 100 mV s⁻¹ at 2 mV intervals. Cyclic voltammograms were also recorded for Trolox, vitamin E equivalent, in the concentration range (2–80 µmol L⁻¹) (Piljac-Zegarac et al., 2010; Veljković et al., 2013). A calibration curve, the area below the major voltammetric anodic peak (Q₅₀₀) versus concentration (c) obtained for this standard, was used to calculate TEAC (Trolox Equivalent Antioxidant Capacity) of studied berries.

Antioxidant composite index

Antioxidant potency composite index was calculated for each sample as score = (sample score/best score) × 100 (Seeram et al., 2008). An index value of 100 was assigned for the best score for each test to the best score for each test, and then calculated an index score for all other samples within the test. The average of index scores obtained for all tests for antioxidant capacities was defined as its antioxidant composite index (ACI). All assays were given equal weight, and an overall mean index value was calculated on a normalized basis for each sample.

Statistical analysis

Data are presented as the mean ± standard deviation (SD) for triplicate determinations. Differences in the antioxidant activity and total polyphenols, flavonoids and anthocyanins content between cultivars and subsamples were tested by the Tukey's test. Statistical analysis was performed using a statistical package running on a computer (Statistica 8.0, StatSoft, Tulsa, Oklahoma, USA). A probability of $p < 0.05$ was considered to be statistically significant (Miller and Miller, 2008).

Results and discussion

Cyclic voltammograms of analyzed berry fruits were obtained in the potential range 0–800 mV in order to cover all groups of antioxidant compounds and presented in Figure 1. Oxidation potentials of some phenolic compounds are given in Table 1. The first anodic peak appearing between 0.310 V and 0.350 V in the cyclic voltammograms of blackberry and raspberry samples could be attributed to the oxidation of flavonols (quercetin and its glucoside) and caffeic acid. As can be seen from the Table 1, cyclic voltammograms of quercetin and quercetin-3-*O*-β-glucoside have shown first reversible oxidation peak at E_{pa} = 0.320 V and E_{pa} = 0.350 V, respectively, which corresponds to the oxidation of the 3'4'-dihydroxy substituent on the B-ring. The second oxidation peak at 0.537 V of quercetin is probably due to the formation of an intermediate radical produced during the oxidation process. Quercetin and quercetin-3-*O*-β-glucoside exhibit almost reversible oxidation steps. The reduction peak of the 3'4'-diquinone formed during quercetin or quercetin-3-*O*-β-glucoside oxidation process, appeared at E_{pc} = 0.273 V and E_{pc} = 0.318 V, respectively. Caffeic acid also undergoes an irreversible oxidation. The anodic oxidation of caffeic acid show irreversible anodic (E_{pa} = 0.344 V) and cathodic (E_{pa} = 0.180 V) waves. Several authors have studied the electrochemical oxidation mechanism of caffeic acid (Hotta et al., 2002; Trabelsi et al., 2004). It is generally accepted that oxidation of caffeic acid leads to *o*-quinones via semiquinone forms. Quercetin, quercetin-3-*O*-β-glucoside and caffeic acid with two hydroxyl groups at ortho positions with reference to each other showed distinct reduction peaks (Table 1). Thus, these compounds are electrochemically reversible at pH 4.5. Quercetin can donate an electron faster than any other compound tested, because it has the lowest anode peak voltage.

TABLE 1. Electrochemical data for phenolic compounds.

Compound*	E_{pa} vs Ag/AgCl, (V)**	E_{ca} vs Ag/AgCl, (V)**
Coumaric acid	0.759	–
Ferrulic acid	0.604	0.032
Caffeic acid	0.344	0.128
Quercetin	0.320; 0.537	0.273
Rutin	0.351; 0.549	–
Kaempferol	0.548	–
Quercetin-3-O- β -glucoside	0.350	0.318
Malvidin-3-O-glucoside	0.428; 0.543	–
Cyanidin-3-O-glucoside	0.529; 0.734	–

*c = 1 mmol L⁻¹.**Sodium acetate-acetic acid buffer (0.1 mol L⁻¹, pH = 4.5) in acidified 80% methanol; scan rate 100 mV s⁻¹.

The second, third and fourth anodic peak detected at potentials between 0.426 V and 0.444 V, 0.524 V and 0.540 V, and 0.638 V and 0.756 V, respectively, may be ascribed to anthocyanins and cinnamic acids (ferrulic and coumaric acid). As can be seen from the Table 1, the cyclic voltammogram of malvidin-3-O-glucoside presented two peaks at 0.428 V and 0.543 V. The first peak is attributed to the oxidation of -OH group of B-ring, and the second oxidation peak may be due to 5,7-dihydroxyl moiety of A-ring. This is in accordance with literature data for raspberry extracts given by Aguirre et al. (2010). Cyanidin-3-O-glucoside displays two oxidation processes at E_{pa} = 0.529 V and E_{pa} = 0.734 V, respectively. These

two peaks correspond to 3',4'-OH oxidations in B-ring. Ferrulic and coumaric acids showed two peaks at 0.604 V and 0.759 V, respectively. The anodic peak appears to correspond to an irreversible process, as no current is observed in the reverse scan. Considering kaempferol, -OH group presents on the B-ring is responsible for the oxidation peak at 0.548 V. All these results are in accordance with pioneering work that studied the structure-activity relationship of natural antioxidants (Rice-Evans et al., 1996).

The area underneath the anodic peak corresponds to the charge used up in the experiment up to potential of 500 mV (Q_{500}), and is used as a measure of antioxidants' content pres-

FIGURE 1. Cyclic voltammograms of strawberry (S1–S3), blackberry (Bc1–Bc5), blueberry (Be1–Be3) and raspberry (R1–R4) samples. Operative conditions: sodium acetate-acetic buffer (pH = 4.5) in acidified 80% methanol; potential up to 800 mV; scan rate 100 mV s⁻¹.

ent in the analyzed sample, i.e., antioxidant capacity. All the peak potentials (E_p) and peak currents (I_p) determined from the cyclic voltammograms as well as Q_{500} and TEAC values of analyzed berry samples are presented in Table 2. From the Table 2 it is evident that the blueberry samples have the highest TEAC, followed by blackberry, raspberry and strawberry samples, which is accordance with its high total anthocyanin and flavonoid contents (Table 3).

As the most natural antioxidants are multifunctional (Manach et al., 2005), the antioxidant capacities of samples cannot be fully described by one single method. In the present study, in addition to electrochemical method (cyclic voltammetry), spectrophotometric techniques such as DPPH radical scavenging activity, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) radical cation scavenging activity (ABTS), ferric reducing-antioxidant power (FRAP) and re-

duction power (RP) were also used to determine antioxidant activity of berry samples. The DPPH, ABTS, FRAP and RP values of berry samples are shown in Table 3. DPPH and ABTS assays are based on the ability of antioxidants to scavenge free radicals, while the FRAP and RP assays are based on the capacity of antioxidants to reduce ferric(III) ions to ferrous(II) ions. DPPH radical scavenging activity and ABTS activity is ranged from 5.05 mmol TE g⁻¹ to 13.1 mmol TE g⁻¹ and from 0.2108 mmol TE g⁻¹ to 0.5462 mmol TE g⁻¹, respectively. Most of tested berry samples had very close values for ABTS activities. In addition, the values obtained by the DPPH assay were higher than those obtained by the ABTS assay. The antioxidant activity of berry samples measured by the FRAP and RP assays is ranged from 21.2 mmol FE g⁻¹ to 52.6 mmol FE g⁻¹ and from 12.6 mmol AAE g⁻¹ to 31.6 mmol AAE g⁻¹, respectively. Obtained results for FRAP and RP as-

TABLE 2. Peak potential (E_p) and currents (I_p) from cyclic voltammograms of berry fruits.

Berries	E_{pa} (V)				I_{pa} (μA)				Q_{500} (μC)	TEAC (μmol Trolox g ⁻¹)
	1	2	3	4	1	2	3	4		
S1	–	0.439	0.545	0.756	–	13.0	16.6	23.0	2.70	4.596
S2	–	0.444	0.539	0.742	–	9.1	11.9	17.5	2.40	4.296
S3	–	0.442	0.540	0.750	–	11.2	13.8	22.4	2.10	4.079
Bc1	–	0.440	0.540	–	–	15.7	18.3	–	2.36	4.953
Bc2	–	0.437	0.539	0.650	–	15.7	16.8	17.9	2.36	4.998
Bc3	–	0.435	0.534	–	–	14.7	16.8	–	2.20	4.579
Bc4	–	0.438	0.535	–	–	16.3	19.1	–	3.36	5.702
Bc5	0.350	0.430	0.534	–	13.1	19.5	18.2	–	5.56	10.58
Be1	–	0.433	0.528	–	–	18.3	19.6	–	3.45	6.041
Be2	0.338	0.430	0.530	–	7.8	11.2	15.1	–	3.83	6.590
Be3	0.352	0.428	0.540	0.638	14.5	20.1	2.47	27.2	5.70	9.830
R1	0.340	0.432	0.528	–	7.7	14.4	1.63	–	3.60	6.116
R2	0.337	0.434	0.540	–	8.6	16.2	1.73	–	4.55	7.100
R3	–	0.432	0.526	–	–	14.7	1.74	–	3.05	5.043
R4	0.310	0.426	0.524	–	6.3	12.9	1.51	–	3.88	6.223

TABLE 3. Antioxidant activity, total phenol, flavonoid and anthocyanin contents* in berry fruits.

Berries	DPPH (mmol TE g ⁻¹)	ABTS (mmol TE g ⁻¹)	FRAP (mmol FE g ⁻¹)	RP (mmol AAE g ⁻¹)	Total phenols (mg GAE g ⁻¹)	Total flavonoids (mg CE g ⁻¹)	Total anthocyanins (mg cy-3-glu 100 g ⁻¹)
S1	5.7 ± 0.0 ^a	0.238 ± 0.001 ^p	24.6 ± 0.2 ^b	14.7 ± 0.3 ^p	3.23 ± 0.03 ^s	0.67 ± 0.02 ^a	28.19 ± 0.03 ^c
S2	5.3 ± 0.1 ^a	0.221 ± 0.001 ^o	23.0 ± 0.1 ^a	13.7 ± 0.0 ^o	3.37 ± 0.09 ^s	0.53 ± 0.00 ^a	24.34 ± 0.01 ^b
S3	5.05 ± 0.05 ^a	0.2108 ± 0.0006 ^a	21.2 ± 0.1 ^a	12.6 ± 0.3 ^a	2.32 ± 0.08 ^a	0.40 ± 0.02 ^a	21.42 ± 0.01 ^a
Bc1	7.79 ± 0.08 ⁱ	0.2588 ± 0.0001 ^t	22.6 ± 0.2 ^a	19.5 ± 3 ⁱ	3.38 ± 0.00 ^u	0.64 ± 0.00 ^a	98.96 ± 0.01 ⁱ
Bc2	7.6 ± 0.1 ^v	0.2541 ± 0.0006 ⁱ	33.2 ± 0.2 ^z	19.9 ± 0.6 ⁱ	3.6 ± 0.1 ^v	0.52 ± 0.00 ^a	99.19 ± 0.04 ⁱ
Bc3	7.78 ± 0.05 ⁱ	0.2542 ± 0.0005 ⁱ	27.9 ± 0.2 ^r	22.4 ± 0.1 ^e	3.5 ± 0.1 ^w	0.56 ± 0.02 ^a	90.49 ± 0.02 ^h
Bc4	7.98 ± 0.05 ⁱ	0.2544 ± 0.0006 ⁱ	39.5 ± 0.3 ⁱ	22.9 ± 0.2 ^e	3.76 ± 0.02 ^v	0.59 ± 0.02 ^a	132.99 ± 0.01 ^j
Bc5	13.1 ± 0.1 ^q	0.5462 ± 0.0000 ^e	52.6 ± 0.3 ^h	31.6 ± 0.1 ^q	5.96 ± 0.07 ^f	1.59 ± 0.03 ^c	365.13 ± 0.04 ^m
Be1	7.5 ± 0.1 ^v	0.3127 ± 0.0006 ⁱ	32.0 ± 0.1 ^y	18.9 ± 0.1 ^d	3.72 ± 0.06 ^y	0.63 ± 0.02 ^a	242.54 ± 0.04 ^k
Be2	8.17 ± 0.08 ^e	0.3411 ± 0.0006 ⁱ	35.3 ± 0.1 ^g	20.7 ± 0.2 ^e	4.37 ± 0.1 ^x	0.92 ± 0.05 ^a	272.5 ± 0.2 ^l
Be3	12.19 ± 0.08 ^f	0.5089 ± 0.0005 ⁱ	49.0 ± 0.3 ^h	29.4 ± 0.2 ^f	5.93 ± 0.06 ^f	1.57 ± 0.03 ^b	382.5 ± 0.1 ⁿ
R1	7.16 ± 0.08 ^u	0.253 ± 0.000 ^t	30.3 ± 0.1 ^t	17.8 ± 0.2 ^r	2.82 ± 0.03 ^o	0.52 ± 0.02 ^a	58.05 ± 0.04 ^f
R2	6.1 ± 0.1 ^o	0.2494 ± 0.0005 ^r	34.5 ± 0.1 ^g	19.2 ± 0.2 ^d	4.72 ± 0.06 ^y	0.64 ± 0.02 ^a	70.15 ± 0.02 ^g
R3	6.25 ± 0.1 ^q	0.2610 ± 0.0006 ⁱ	27.0 ± 0.2 ^p	16.1 ± 0.1 ^r	3.0 ± 0.1 ^q	0.59 ± 0.00 ^a	45.88 ± 0.05 ^d
R4	7.2 ± 0.1 ^u	0.3220 ± 0.0006 ⁱ	31.2 ± 0.1 ^y	18.4 ± 0.1 ^d	2.96 ± 0.08 ^o	0.53 ± 0.02 ^a	53.53 ± 0.02 ^e

*Values are the mean ± SD (n=3). Values with different letters within columns are statistically different at p<0.05 using Tukey's test.

FIGURE 2. Statistical analysis – correlation between the cyclic voltammetry results and different spectrophotometric results: a) total phenols, b) total flavonoids, c) total anthocyanins, d) FRAP, e) RP, f) DPPH, g) ABTS.

TABLE 4. Antioxidant potency composite index of berry samples calculated from five antioxidant capacity measures scaled to relative percentages.

Berries	DPPH index	ABTS index	FRAP index	RP index	Q ₅₀₀ index	ACI
S1	43.5	43.6	46.8	46.5	43.4	44.8
S2	40.5	40.5	43.7	43.4	40.6	41.7
S3	38.5	38.6	40.3	39.9	38.6	39.2
Bc1	59.5	47.4	43.0	61.7	46.8	51.7
Bc2	58.0	46.5	63.1	63.0	47.2	55.6
Bc3	59.4	46.5	53.0	70.9	43.3	54.6
Bc4	60.9	46.6	75.1	72.5	53.9	61.8
Bc5	100	100	100	100	100	100
Be1	57.3	57.3	60.8	59.8	57.1	58.5
Be2	62.4	62.4	67.1	65.5	62.3	63.9
Be3	93.1	93.2	93.2	93.0	92.9	93.1
R1	54.7	46.3	57.6	56.3	57.8	54.5
R2	46.6	45.7	65.6	60.8	67.1	57.2
R3	47.7	47.8	51.3	50.9	47.7	49.1
R4	55.0	59.0	59.3	58.2	58.8	58.1

says showed that the antioxidant power of blueberry samples was the highest, followed by the blackberry, raspberry and strawberry samples. This is in accordance with the results of antioxidant obtained from cyclic voltammetry.

Generally, it is known that total polyphenols are highly correlated with antioxidant activity, and the bioavailability of polyphenols has been reported (Manach et al., 2005). The results for total phenolic (TP), total flavonoid (TF) and total anthocyanin (TA) contents in the studied berry samples are presented in Table 3. Total phenolic, flavonoid and anthocyanin contents varied from 2.32 mg GAE g⁻¹ to 5.96 mg GAE g⁻¹, from 0.40 mg CE g⁻¹ to 1.59 mg CE g⁻¹ and from 21.4 mg cy-3-glu 100 g⁻¹ to 365 mg cy-3-glu 100 g⁻¹ respectively, among the tested berry samples. Strawberry samples had the lowest content, while blueberry samples had the highest content of total phenols, flavonoids and anthocyanins.

The ACI indices, along with the results from all five antioxidant capacity measures (scaled to relative percentages) are shown in Table 4. The highest ACI have blueberry samples followed by the blackberry, raspberry and strawberry samples which is in accordance with their high total phenols, flavonoids and anthocyanins content and with antioxidant capacity.

Furthermore, strong positive correlations were observed between total phenols, flavonoids, anthocyanins and CV antioxidant capacity ($R^2=0.8091$, $p=0.0005$; $R^2=0.8341$, $p=0.0002$; $R^2=0.7163$, $p=0.0007$), respectively (Figure 2a–c). In addition, high positive correlations were also found between antioxidant capacities deduced from CVs with those obtained using FRAP ($R^2=0.8630$, $p=0.0005$), RP ($R^2=0.7426$, $p=0.0004$), DPPH ($R^2=0.7680$, $p=0.0001$) and ABTS ($R^2=0.8710$, $p=0.000002$) (Figure 2d–g, respectively) indicating that polyphenolic antioxidants with -OH groups of B-ring are major contributors to the overall antioxidant capacity. This is in accordance with literature data given in review work of Heinonen (2007) that contribution of anthocyanins to the antioxidant effect of berries is significant. The highest degree of positive correlation was observed between total phenols and FRAP, which may be attributed to an electron transfer based mechanism governing these two assays. Also, Firuzi et al. (2005) found that the *o*-dihydroxy structure in the B-ring and the 3-hydroxy group and 2,3-double bond in the C-ring give the highest contribution to the FRAP antioxidant activity.

Differences in the TP, TF and TA as well as antioxidant activity observed among cultivars and subsamples were tested by the Tukey's test, respectively. Similarly to the polyphenols, flavonoids and anthocyanins content, the antioxidant activity of berry samples showed significant differences depending on cultivars. According to the literature data (Connor et al., 2002; Anttonen and Karjalainen, 2005; Anttonen et al., 2006; Milošević et al., 2012), cultivar and different growing environments as well as agricultural and climatic factors contribute to the phenolic status in plants.

Conclusion

The results obtained in this work by electrochemical method indicate that cyclic voltammetry is a good technique for the characterization of the antioxidant properties of berry samples. Furthermore, CV is based only on the chemical/physical properties of the molecules and does not require the use of reactive species. The capability of the method to rapidly evaluate the antioxidant activity of berries could be useful for food industry. The high degree of positive correlation with the spectrophotometry antioxidant capacity measures

indicates that the results derived from CV are reliable and comparable to the results of other spectrophotometric methods (FRAP, RP, DPPH and ABTS). This finding clearly indicates that the CV method as a suitable method for the determination of the reducing capacity based on the electrochemical properties of compounds under the test. A pronounced anodic current peak observed between 0.524 V and 0.540 V in all analyzed berry samples can be ascribed to anthocyanins, which are the major contributors to the antioxidant capacity of investigated berries. Obtained values for ACI parameter provides easy comparison of antioxidant capacity among tested berries. The highest ACI had blueberry samples, followed by blackberry, raspberry and strawberry samples.

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References

- Aguirre, M.J., Chen, Y.Y., Isaacs, M., Matsushiro, B., Mendoza, L., and Torres, S. (2010). Electrochemical behavior and antioxidant capacity of anthocyanins from Chilean red wine, grape and raspberry. *Food Chem.* 121, 44–48. <https://doi.org/10.1016/j.foodchem.2009.11.088>.
- Anttonen, M.J., and Karjalainen, R.O. (2005). Environmental and genetic variation of phenolic compounds in red raspberry. *J. Food Comp. Anal.* 18, 759–769. <https://doi.org/10.1016/j.jfca.2004.11.003>.
- Anttonen, M.J., Hoppula, K.I., Nestby, R., Verheul, M.J., and Karjalainen, R.O. (2006). Influence of fertilization, mulch color, early forcing, fruit order, planting date, shading, growing environment, and genotype on the contents of selected phenolics in strawberry (*Fragaria × ananassa* Duch.) fruits. *J. Agric. Food Chem.* 54, 2614–2620. <https://doi.org/10.1021/jf052947w>.
- Arts, M.J.T.J., Haenen, G.R.M.M., Voss, H.P., and Bast, A. (2004). Antioxidant capacity of reaction products limits the applicability of the trolox equivalent antioxidant capacity (TEAC) assay. *Food Chem. Toxicol.* 42, 45–49. <https://doi.org/10.1016/j.fct.2003.08.004>.
- Benzie, I.F.F., and Strain, J.J. (1999). Ferric reducing/antioxidant power assay: Direct measure of total antioxidant activity of biological and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. *Methods Enzymol.* 299, 15–27. [https://doi.org/10.1016/S0076-6879\(99\)99005-5](https://doi.org/10.1016/S0076-6879(99)99005-5).
- Brand-Williams, W., Cuvelier, M.E., and Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *Lebensm. Wiss. Technol.* 28, 25–30. [https://doi.org/10.1016/S0023-6438\(95\)80008-5](https://doi.org/10.1016/S0023-6438(95)80008-5).
- Chen, F., Sun, Y., Zhao, G., Liao, X., Hu, X., Wu, J., and Wang, Z. (2007). Optimization of ultrasound-assisted extraction of anthocyanins in red raspberries and identification of anthocyanins in extract using high-performance liquid chromatography-mass spectrometry. *Ultrason. Sonochem.* 14, 767–778. <https://doi.org/10.1016/j.ultrasonch.2006.12.011>.
- Connor, A.M., Luby, J.J., and Tong, C.B.S. (2002). Genotypic and environmental variation in antioxidant activity, total phenolic content, and anthocyanin content among blueberry cultivars. *J. Am. Soc. Hortic. Sci.* 127, 89–97. <https://doi.org/10.21273/JASHS.127.1.89>.
- Firuzi, O., Lacanna, A., Petrucci, R., Marrosu, G., and Saso, L. (2005). Evaluation of the antioxidant activity of flavonoids by “ferric reducing antioxidant power” assay and cyclic voltammetry. *Biochim. Biophys. Acta* 1721, 174–184. <https://doi.org/10.1016/j.bbagen.2004.11.001>.

- Giampieri, F., Tulipani, S., Alvarez-Suarez, J.M., Quiles, J.L., Mezzetti, B., and Battino, M. (2012). The strawberry: Composition, nutritional quality, and impact on human health. *Nutrition* 28, 9–19. <https://doi.org/10.1016/j.nut.2011.08.009>.
- Guisti, M.M., and Wrolstad, R.E. (2001). Characterization and measurement of anthocyanins by UV-visible spectroscopy. *Curr. Prot. Food Anal. Chem.* F1.2.1–F1.2.13. <https://doi.org/10.1002/0471142913.faf0102s00>.
- Halvorsen, B.L., Holte, K., Myhrstad, M.C.W., Barikmo, I., Hvattum, E., Remberg, S.F., Wold, A.B., Haffner, K., Baugerod, H., Andersen, L.F., Moskaug, O., Jacobs Jr., D.R., and Blomhoff, R. (2002). A systematic screening of total antioxidants in dietary plants. *J. Nutr.* 132, 461–471. <https://doi.org/10.1093/jn/132.3.461>.
- Heinonen, M. (2007). Antioxidant activity and antimicrobial effect of berry phenolics – A Finnish perspective. *Mol. Nutr. Food Res.* 51, 684–691. <https://doi.org/10.1002/mnfr.200700006>.
- Hotta, H., Ueda, M., Nagano, S., Tsvjino, Y., and Koyama, J. (2002). Mechanistic study of the oxidation of caffeic acids by digital simulation of cyclic voltammograms. *Anal. Biochem.* 303, 66–72. <https://doi.org/10.1006/abio.2002.5577>.
- Ivanovic, J., Tadic, V., Dimitrijevic, S., Stamenic, M., Petrovic, S., and Zizovic, I. (2014). Antioxidant properties of the anthocyanin-containing ultrasonic extracts from blackberry cultivar 'Čačanska Bestrna'. *Ind. Crop Prod.* 53, 274–281. <https://doi.org/10.1016/j.indcrop.2013.12.048>.
- Lee, J., Dossett, M., and Finn, C.E. (2012). *Rubus* fruit phenolic research: The good, the bad, and the confusing. *Food Chem.* 130, 785–796. <https://doi.org/10.1016/j.foodchem.2011.08.022>.
- Manach, C., Williamson, G., Morand, C., Scalbert, A., and Remesy, C. (2005). Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am. J. Clin. Nutr.* 81, 230S–242S. <https://doi.org/10.1093/ajcn/81.1.230S>.
- Miller, J.N., and Miller, J.C. (2005). *Statistics and Chemometrics for Analytical Chemistry* (London, England: Pearson Education).
- Milošević, T., Milošević, N., Glišić, I., and Mladenović, J. (2012). Fruit quality attributes of blackberry grown under limited environmental conditions. *Plant Soil Environ.* 58, 322–327. <https://doi.org/10.17221/33/2012-PSE>.
- Nile, S.H., and Park, S.W. (2014). Edible berries: Bioactive components and their effect on human health. *Nutrition* 30, 134–144. <https://doi.org/10.1016/j.nut.2013.04.007>.
- Oancea, S., Grosu, C., Ketney, O., and Stoia, M. (2013). Conventional and ultrasound assisted extraction of anthocyanins from blackberry and sweet cherry cultivars. *Acta Chim. Slov.* 60, 383–389.
- Oyaizu, M. (1986). Studies on products of browning reaction: antioxidative activity of products of browning reaction prepared from glucosamine. *Jpn. J. Nutr.* 44, 307–315. <https://doi.org/10.5264/eiyogakuzashi.44.307>.
- Paredes-López, O., Cervantes-Ceja, M.L., Vigna-Pérez, M., and Hernández-Pérez, T. (2010). Berries: Improving human health and healthy aging, and promoting quality life – A review. *Plant Food Hum. Nutr.* 65, 299–308. <https://doi.org/10.1007/s11130-010-0177-1>.
- Piljac-Zegarac, J., Valek, L., Stipcevic, T., and Martinez, S. (2010). Electrochemical determination of antioxidant capacity of fruit tea infusions. *Food Chem.* 121, 820–825. <https://doi.org/10.1016/j.foodchem.2009.12.090>.
- Rao, A.V., and Snyder, D.M. (2010). Raspberries in human health: A review. *J. Agric. Food Chem.* 58, 3871–3883. <https://doi.org/10.1021/jf903484g>.
- Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., and Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolonization assay. *Free Radic. Biol. Med.* 26, 1231–1237. [https://doi.org/10.1016/S0891-5849\(98\)00315-3](https://doi.org/10.1016/S0891-5849(98)00315-3).
- Rice-Evans, C.A., Miller, N.J., and Paganga, G. (1996). Structure antioxidant activity relationship of flavonoids and phenolic acids. *Free Rad. Bio. Med.* 20, 933–956. [https://doi.org/10.1016/0891-5849\(95\)02227-9](https://doi.org/10.1016/0891-5849(95)02227-9).
- Seeram, N.P. (2008a). Berry Fruits: Compositional elements, biochemical activities, and the impact of their intake on human health, performance, and disease. *J. Agric. Food Chem.* 56, 627–629. <https://doi.org/10.1021/jf071988k>.
- Seeram, N.P. (2008b). Berry fruits for cancer prevention: Current status and future prospects. *J. Agric. Food Chem.* 56, 630–635. <https://doi.org/10.1021/jf072504n>.
- Seeram, N.P., Aviram, M., Zhang, Y., Henning, S.M., Feng, L., Dreher, M., and Heber, D. (2008). Comparison of antioxidant potency of commonly consumed polyphenol-rich beverages in the United States. *J. Agric. Food Chem.* 56, 1415–1422. <https://doi.org/10.1021/jf073035s>.
- Singleton, V.L., Orthofer, R., and Lamuela-Raventos, R.M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Meth. Enzymol.* 299, 152–178. [https://doi.org/10.1016/S0076-6879\(99\)99017-1](https://doi.org/10.1016/S0076-6879(99)99017-1).
- Slinkard, K., and Singleton, V.L. (1977). Total phenol analysis: automation and comparison with manual methods. *Am. J. Encl. Vitic.* 28, 49–55.
- Stratil, P., Klejdus, B., and Kuban, V. (2006). Determination of total content of phenolic compounds and their antioxidant activity in vegetables-evaluation of spectrophotometric methods. *J. Agric. Food Chem.* 54, 607–616. <https://doi.org/10.1021/jf052334j>.
- Szajdek, A., and Borowska, E.J. (2008). Bioactive compounds and health-promoting properties of berry fruits: A review. *Plant Food Hum. Nutr.* 63, 147–156. <https://doi.org/10.1007/s11130-008-0097-5>.
- World Health Organization (2002). *The World Health Report Reducing Risk and Promoting Healthy Life* (Geneva: World Health Organization).
- Trabelsi, S.K., Tahar, N.B., and Abdelhedi, R. (2004). Electrochemical behavior of caffeic acid. *Electrochim. Acta* 49, 1647–1654. [https://doi.org/10.1016/S0013-4686\(03\)00995-2](https://doi.org/10.1016/S0013-4686(03)00995-2).
- Tulipani, S., Mezzetti, B., Capocasa, F., Bompadre, S., Beekwilder, J., Ric de Vos, C.H., Capanoglu, E., Bovy, A., and Battino, M. (2008). Antioxidants, phenolic compounds, and nutritional quality of different strawberry genotypes. *J. Agric. Food Chem.* 56, 696–704. <https://doi.org/10.1021/jf0719959>.
- Veljković, J., Pavlović, A., Mitić, S., Tošić, S., Stojanović, G., Kaličanin, B., Stanković, D., Stojković, M., Mitić, M., and Brcanović, J. (2013). Evaluation of individual phenolic compounds and antioxidant properties of black, green, herbal and fruit tea infusions consumed in Serbia: spectrophotometrical and electrochemical approaches. *J. Food Nutr. Res.* 52, 12–24.
- Wang, S.Y., and Millner, P. (2009). Effect of different cultural system on antioxidant capacity, phenolic content, and fruit quality of strawberries (*Fragaria × ananassa* Duch.). *J. Agric. Food Chem.* 57, 9651–9657. <https://doi.org/10.1021/jf902057s>.
- Yang, B., Kotani, A., Rarai, K., and Kusu, F. (2001). Estimation of the antioxidant activities of flavonoids from their oxidation potentials. *Anal. Sci.* 17, 599–604. <https://doi.org/10.2116/analsci.17.599>.
- Zhishen, J., Mengcheng, T., and Wu Jianming, W. (1999). The determination of flavonoids content in mulberry and scavenging effect on superoxide radicals. *Food Chem.* 64, 555–559. [https://doi.org/10.1016/S0308-8146\(98\)00102-2](https://doi.org/10.1016/S0308-8146(98)00102-2).

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