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Cyclic Voltammetric Determination of Antioxidant Capacity of Cocoa Powder, Dark Chocolate and Milk Chocolate Samples: Correlation with Spectrophotometric Assays and Individual Phenolic Compounds

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Summary

Phenolic antioxidants in cocoa powder, dark chocolate and milk chocolate samples are quantified electrochemically using cyclic voltammetry with a glassy carbon electrode. Cyclic voltammograms were recorded from 0 to 800 mV at a scan rate of 100 mV/s. Phenolics with an *ortho*-diphenol group show the first oxidation peak in the potential range between 370 and 460 mV (*vs.* Ag/AgCl) in 0.1 mol/L of the acetate buffer, pH=4. The first and third anodic current peaks, close to 670 mV, can be ascribed to the first and second oxidation of the catechin-type flavonoids that are present at a high concentration in samples. The second peak at around 560 mV can be ascribed to phenolic acids. Procyanidins B1, B2, B3 and B4 did not have any visible waves. Strong positive correlation was established between antioxidant activities deduced from cyclic voltammograms with those determined using spectrophotometric assays. HPLC method was used for the quantification of individual phenolic compounds.

Key words: cocoa powder and chocolates, cyclic voltammetry, antioxidant capacity, spectrophotometric assays, catechins, procyanidins

Introduction

Chocolate drinks have become one of the most popular beverages after water, tea and wine throughout the world. Cocoa and chocolate are complex products with a high content of organic compounds as well as a rich source of many essential elements. Also, chocolate has always been favourite food among consumers. Many kinds of chocolate are produced today and they can be classified into three types: dark, milk and white chocolate. Dark chocolate is formulated with a higher percentage of cocoa liquor than milk chocolate. White chocolate does not contain chocolate liquor. Dark chocolate and cocoa have been identified as having high polyphenolic and flavonoid content, particularly catechins, epicatechins and procyanidins (*1–3*). In recent years, cocoa, dark choco-

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late, tea and wine have increasingly been viewed as sources of dietary components with potentially beneficial functional activities (*4–7*). Flavonoids from different cocoa and chocolate preparations have been identified as the major antioxidant components (*8–10*). Also, commercial chocolate products were found to decrease lipid oxidation when added to low-density lipoprotein (LDL) preparations *in vitro* (*11,12*). Cocoa and dark chocolate flavonoids have also been linked to reduced cardiovascular diseases (*13,14*). Several epidemiology studies speculate that the antioxidants present in the cocoa and chocolate provide health benefits (*15,16*).

Many chemical methods have been developed for assessing the antioxidant activity of flavonoids. The majority of methods are based on the scavenging capacity assays against specific oxidants such as 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, Fe(III), Cu(II), *etc.* (*17*). As far as we know, there is no electrochemical determination using cyclic voltammetry of antioxidant capacity of cocoa and chocolate. Cyclic voltammetry (CV) is a widely used electrochemical technique for the evaluation of total antioxidant capacity. The main advantage of CV is its capability to rapidly observe the total redox behaviour over a wide potential range without the necessity of measuring the specific antioxidant capacity of each component alone. Also, it could be an attractive alternative to spectrophotometric assays to determine total phenolics.

This study shows a possibility of applying cyclic voltammetry to characterize samples containing chocolate. The aim of this work is to investigate the electrochemical properties of antioxidants present in cocoa and chocolate and to establish the correlation between the antioxidant capacities deduced from cyclic voltammograms and those obtained by spectrophotometric assays.

Materials and Methods

Chemicals

The 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was purchased from Acros Organics (Morris Plains, NJ, USA), while 2,2-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diamonium salt (ABTS), 2,2-diphenyl-1-picrylhidrazyl (DPPH), 2,4,6-tri(2-pyridyl)-*s*-triazine (TPTZ), gallic acid, protocatechuic acid, (+)-catechin, (–)-epicatechin, and procyanidins B1, B2, B3 and B4 were purchased from Sigma-Aldrich (Steinheim, Germany). Folin-Ciocalteu's phenol reagent, potassium peroxydisulphate, ammonium iron(II) sulphate 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, trichloroacetic acid, hydrochloric acid, acetic acid, hexane and acetone were purchased from Merck (Darmstadt, Germany). Ethanol (96 %, by volume) and methanol (HPLC grade) were from J.T. Baker (Deventer, The Netherlands).

Instruments

Cyclic voltammograms were recorded on a CHI760B instrument (CH Instruments, Inc., Austin, TX USA). The cell was equipped with a glassy carbon electrode (CHI104, 2 mm), an accessory platinum electrode of a larger area (Model CHI221, cell top including Pt wire counter electrode, r=1 mm, A=3.14 mm^2) and an Ag/AgCl reference electrode (Model CHI111). An Agilent 8453 UV/Vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) was used for absorbance measurements and spectrum recording, using optical cuvettes of 1 cm optical path. For HPLC analysis, a model 1200 with analytical column C_{18} Zorbax Eclipse XDB-C18, 5 μm, 4.6×150 mm (Agilent Technologies) was used. The pH measurements were made with Hanna instrument pH-meter (Hanna Instruments, Smithfield, RI, USA) equipped with a glass electrode. All measurements were taken at ambient temperature.

Preparation of cocoa powder and chocolate samples

Nineteen commercially available cocoa powder and chocolate samples (5 cocoa powder products (C1–C5), 5 dark (DC1–DC5) and 9 milk chocolates (MC1–MC9)) were purchased from the local supermarkets. All samples were prepared for the analysis as described by Adamson *et al.* (*10*) with slight modifications. Chocolate samples (100 g) were ground to obtain a homogeneous material. A mass of 10 g of ground chocolate and cocoa powder was defatted 3–5 times with 10 mL of hexane, and the residue was dried under a gentle nitrogen stream. The defatted material (1 g) was then extracted three times with 10 mL of acetone/water/acetic acid (70:29.5:0.5, by volume) by sonication (37 °C for 10 min) and centrifugation (1431×g for 10 min). The supernatant was decanted to a clean 50-mL volumetric flask and diluted with water to the mark.

Total polyphenolic content

Total polyphenols (TP) were measured spectrophotometrically at 760 nm after the reaction with Folin-Ciocalteu's phenol reagent, according to the method described by Singleton *et al.* (*18*). Gallic acid was used as the standard and the results were expressed as milligrams of gallic acid equivalents (GAE) per gram of sample. All measurements were performed in triplicate.

Total flavonoid content

Total flavonoid (TF) content was measured by the aluminium chloride spectrophotometric methods described by Jia *et al.* (*19*) and Yang *et al.* (*20*) with minor modifications. Reaction mixture was prepared by mixing 0.25 mL of sample, 3 mL of deionised water and 0.3 mL of 5 % $NaNO_2$. After incubation at room temperature for 5 min, 1.5 mL of 2 % $AlCl_3{\cdot}6H_2O$ were added. Again, the flask was kept at room temperature for 5 min and then 2 mL of 1 mol/L of NaOH were added. The flask was filled with deionised water to the mark. Absorbance of the reaction mixture was measured against the prepared reagent blank at 510 nm. Catechin was chosen as a standard and the results were expressed as milligrams of catechin equivalents (CE) per gram of sample. Total flavonoid content was determined in triplicate.

Spectrophotometric analysis of antioxidant capacity

For DPPH assay (*21*), a solution of DPPH (10^{-4} mol/L) was prepared in methanol. The discolouration of the solution was measured at 520 nm, 30 min after the reaction

started. The Trolox calibration curve was plotted as a function of DPPH radical scavenging activity measured as the decrease in the absorbance:

$$\Delta A = A_{blank} - A \qquad /1/$$

The final results were expressed as micromoles of Trolox equivalents (TE) per gram of sample.

The ABTS radical scavenging activity was measured using the methods of Re *et al*. (*22*) and Arts *et al*. (*23*). The Trolox calibration curve was plotted as a function of the ABTS radical cation scavenging activity measured as the decrease in the absorbance (according to the Eq. 1) at 734 nm. The final results were expressed as micromoles of TE per gram of sample.

Ferric reducing ability of plasma (FRAP) assay was performed as previously described by Benzie and Strain (*24*). In the FRAP assay, antioxidants in the sample reduce Fe^{3+}-TPTZ complex to the ferrous form at low pH (pH=3.6), measured as an increase in the absorbance at 595 nm. FRAP values were expressed as micromoles of Fe^{2+} equivalents (FE) per gram of sample.

Reducing power (reduction of Fe(III) to Fe(II)) of samples was determined as described by Oyaizu (*25*) and expressed in ascorbic acid equivalents (AAE). Increased absorbance of the reaction mixture measured at 700 nm indicated increased reducing power.

Cyclic voltammetry

Cyclic voltammograms were recorded at ambient temperature. Prior to each run, the surface of the glassy carbon electrode was freshly abraded with alumina powder (particle size of 1.0, 0.3 and 0.05 μm), rinsed with redistilled water and degreased in ethanol in ultrasonic bath. The scan was done in the potential range between 0 and 800 mV, with a scan rate of 100 mV/s. Cyclic voltammograms were also recorded in the concentration range of 2–80 μmol/L to calculate catechin equivalents. A calibration curve, obtained by plotting anodic current *vs*. concentration of catechin, was used to calculate catechin equivalent antioxidant capacity of the studied samples.

Antioxidant composite index

Antioxidant composite index (ACI) was determined according to Seeram *et al.* (*26*) and calculated as:

$$\text{ACI} = (\text{sample score}/\text{best score}) \times 100 \qquad /2/$$

Analysis of individual phenolic compounds using HPLC

Individual phenolic compounds were determined using an Agilent chromatograph. Separation was performed with a C_{18} Zorbax Eclipse column kept at 25 °C, at a flow rate of 0.8 mL/min and an injection volume of 20 μL. Detection was performed by scanning from 260 to 400 nm (using UV/Vis and fluorescence detector). For the gradient elution, the following programme, slightly modified, was used (*27*): solvent A (acetonitrile) and solvent B (0.1 % formic acid in water) as follows: 10 % A for 0 min, then 10 % A for 15 min, followed by 35 min at 30 % A. Individual phenolic compounds were separated within 50 min. Identification was carried out by comparing the retention times and spectral data with those of standards. Quantitative determination of individual phenolic compounds in samples was done using calibration lines.

Statistical analysis

Data are presented as the mean values±standard deviations (S.D.) of triplicate determinations. Statistical analysis was performed by paired Student's *t*-test. A probability of $p<0.05$ was considered to be statistically significant (*28*). Principal component analysis (PCA) was used to make the results more easily interpretable. All statistical calculations were made using a statistical package running on a computer (STATISTICA v. 8.0, StatSoft, Tulsa, OK, USA).

Results and Discussion

Cyclic voltammograms of (+)-catechin and (–)-epicatechin were recorded in the buffer solutions in the pH range from 3 to 8. The highest current peak of the investigated compounds was around pH=4 and it decreased with an increase of pH. Cyclic voltammograms of (+)-catechin and (–)-epicatechin recorded at pH=4 are shown in Fig. 1. (+)-Catechin and (–)-epicatechin had similar anodic waves. Two oxidation peaks are associated with the oxidation centres present in the molecules (*29*,*30*). Martinez *et al*. (*29*) showed that spontaneous deprotonation does not occur below pH=6 for catechin-type flavonoids, which means that the neutral molecules of this type of flavonoids participate in an electrochemical oxidation and adsorption processes up to pH=6. Authors postulate that electron or hydrogen abstraction occurs at higher pH values (in the range of physiological pH).

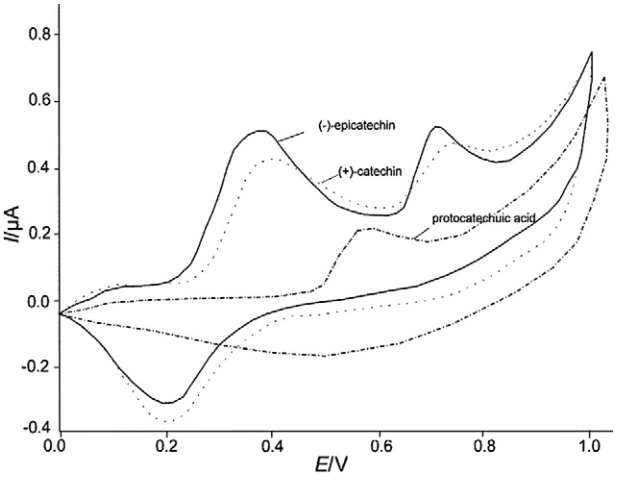

Fig. 1. Cyclic voltammograms of (+)-catechin, (–)-epicatechin and protocatechuic acid ($c=10^{-5}$ mol/L) in acetate buffer, pH=4. The scan rate was 100 mV/s

In order to cover all groups of antioxidant compounds, cyclic voltammograms of cocoa powder and chocolate samples in acetate buffer, pH=4, were recorded in the potential range of 0–800 mV. They were recorded in triplicate using a glassy carbon electrode at a scan rate of 100 mV/s. The cyclic voltammograms of cocoa powder and chocolate samples (Fig. 2) exhibit at least two oxidation peaks in the anodic wave. The peaks correspond to the sequent oxidation of the hydroxyl groups at different

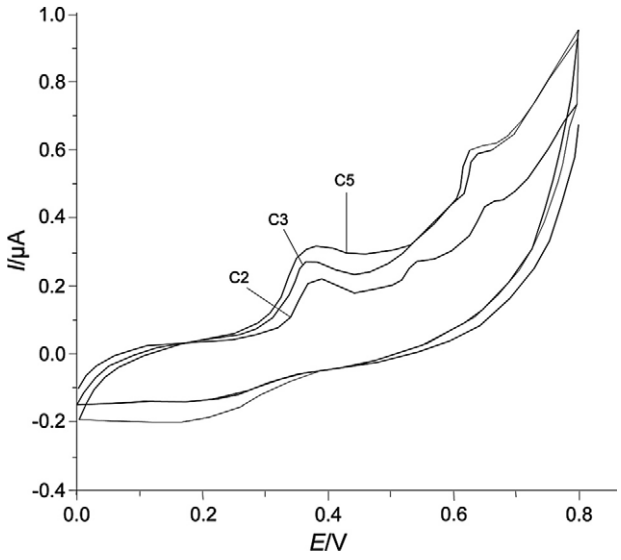

Fig. 2. Cyclic voltammograms of the tested cocoa powder samples C2, C3 and C5 in the range from 0 to 800 mV at a scan rate of 100 mV/s

positions. All the anodic peak potentials (E_{pa}) and anodic peak currents (I_{pa}) determined from the cyclic voltammograms of the tested samples are presented in Table 1. The first peak appears in the 370 to 460 mV potential range and corresponds to the oxidation of catechin-type flavonoids. These flavonoids contain an easily oxidizable *ortho*-diphenol group on the B-ring (*31–33*). The values given in Table 1 are consistent with previous literature studies, taking into account the influence of pH on the voltammetric response of the expected 59 mV per pH unit shift in the potential. With the increase of pH, the anodic peak potential shifts towards less positive values. Tests in pH intervals from 3 to 7 confirmed that the potential varied by 56 mV per pH unit for catechin (*33,34*). The second peak at around 560 mV can be ascribed to the oxidation of the monophenol group or the *meta*-diphenols on the A-ring of flavonoids, *i.e.* phenolic acids such as ferulic, vanillic, protochatechuic, *p*-coumaric and sinapic acids (*31,32,35*). The third anodic peak appears around 670 mV, which can be ascribed to the second oxidation of the catechins that are present at high concentrations in the tested samples. In contrast to catechins, procyanidins B1, B2, B3 and B4 did not produce any visible waves. This is probably due to the additional phenolic constituents present in a variety of orientations (*36*).

As reported by Kilmartin and Hsu (*37*), phenolic compounds may be ordered according to the reducing strength (better reducing agents have higher antioxidant activity) as follows: compounds containing triphenol group on the flavonoid B-ring>compounds containing catechol or gallate group>compounds containing monophenol or *meta*-dihydroxyphenol group. In the case of the tested chocolate and cocoa powder samples, it can be noted that their antioxidant activity may be attributed mostly to the compounds containing a catechol or a gallate group.

The determination of the area below the anodic current peak at 600 mV, Q_{600}, was used as a measure of the quantity of antioxidants present in the tested samples. This value is an alternative measure of concentration to

Table 1. Peak potential and currents from cyclic voltammograms of the tested chocolate and cocoa powder samples

Sample	E_{pa}/V			I_{pa}/mA			Q_{600}/mC	ACI
	1	2	3	1	2	3		
MC1	0.38	–	–	2.27	–	–	0.001	0.2
MC2	0.39	0.58	–	1.09	1.76	–	0.001	0.2
MC3	0.37	0.54	–	1.00	2.42	–	0.042	6.5
MC4	0.38	0.56	0.68	1.26	3.30	4.05	0.047	7.3
MC5	0.37	0.56	0.67	1.31	3.65	5.04	0.035	18.4
MC6	0.40	–	0.64	1.57	–	4.80	0.052	8.1
MC7	0.40	–	0.64	1.37	–	3.73	0.088	13.7
MC8	0.40	–	–	1.78	–	–	0.021	3.3
MC9	0.38	–	0.65	1.59	–	4.14	0.090	14.0
DC1	0.37	–	0.68	2.01	–	–	0.075	11.7
DC2	0.39	–	0.69	2.94	–	8.80	0.124	19.3
DC3	0.38	–	–	2.76	–	–	0.381	59.3
DC4	0.38	–	0.66	1.59	–	4.31	0.080	12.4
DC5	0.38	0.55	0.68	2.05	5.05	7.00	0.207	32.2
C1	0.38	–	–	2.89	–	–	0.262	40.7
C2	0.37	–	0.68	1.19	–	8.70	0.281	43.7
C3	0.37	0.55	0.67	3.00	6.57	10.01	0.365	56.8
C4	0.36	–	0.67	3.00	–	11.26	0.558	86.8
C5	0.37	–	0.69	3.58	–	12.64	0.643	100.0

MC=milk chocolate, DC=dark chocolate, C=cocoa powder, E_{pa}=anodic peak potential, I_{pa}=anodic peak current, Q_{600}=charge passed to 600 mV, ACI=antioxidant composite index

I_a, and is more suitable for samples with several overlapping and broad peaks (*31,38*). Q_{600} values (in μC) of analyzed samples are given in Table 1. Cocoa powder samples exhibited the highest Q_{600} values in the range of 0.262 to 0.643 μC, followed by dark chocolate: 0.08–0.381 μC and milk chocolate samples: 0.001–0.090 μC. Cocoa powder samples C5 (0.643 μC), C4 (0.558 μC), C3 (0.365 μC) and dark chocolate DC3 (0.381 μC) had the highest Q_{600} values. All the remaining samples exhibited lower antioxidant capacity, reflected in the Q_{600} values of 0.001 μC, while the milk chocolate MC1 and MC2 had the lowest antioxidant capacity.

The results obtained for cyclic voltammetry are generally consistent with total phenolic and flavonoid content and with spectrophotometric antioxidant assays including DPPH, ABTS, FRAP and reducing power assays.

The total phenolic and flavonoid content of the tested cocoa powder and chocolate samples is shown in Table 2. As can be seen, the total phenolic content expressed as mg of GAE per g of milk chocolate, dark chocolate and cocoa powder samples varied from 2.84–5.59, 7.09–31.10 and 3.82–36.87, respectively. The total flavonoid content expressed as mg of CE per g of milk chocolate, dark chocolate and cocoa powder samples ranged from 1.73–3.70, 2.41–11.78 and 2.02–10.82, respectively. The highest total phenolic (expressed as mg of GAE per g) and flavonoid content (expressed as mg of CE per g) was found in cocoa powder samples C5, C4 and C3 (36.87 and 10.82, 18.62 and 5.07, and 10.95 and 4.71, respectively) and dark chocolate DC3 (31.10 and 11.78, respectively). Milk chocolate samples MC1 and MC2 had the lowest content of total phenols and flavonoids (2.84 and 1.73, and 2.94 and 2.15, respectively). These results are consistent with the ones obtained for antioxidant capacity derived from cyclic voltammograms.

Table 2. Total phenolic and flavonoid content of chocolate and cocoa powder samples

Sample	w(TP as GAE) / mg/g	w(TF as CE) / mg/g
MC1	(2.84±0.04)^a	(1.73±0.06)^a
MC2	(2.94±0.05)^a	(2.15±0.06)^b
MC3	(3.32±0.02)^b	(2.62±0.06)^c
MC4	(4.28±0.08)^d	(2.94±0.06)^d
MC5	(3.93±0.06)^c	(3.02±0.06)^d
MC6	(4.05±0.06)^c	(3.14±0.11)^e
MC7	(4.69±0.04)^e	(3.30±0.11)^e
MC8	(3.22±0.02)^b	(2.81±0.08)^e
MC9	(5.59±0.08)^f	(3.70±0.11)^e
DC1	(7.09±0.12)^g	(4.99±0.00)^g
DC2	(9.34±0.04)^i	(5.47±0.11)^h
DC3	(31.10±0.16)^l	(11.78±0.30)^i
DC4	(8.72±0.51)^h	(2.41±0.04)^c
DC5	(13.62±0.12)^j	(5.07±0.23)^f
C1	(3.82±0.06)^c	(2.02±0.04)^b
C2	(8.51±0.25)^h	(2.50±0.07)^c
C3	(10.95±0.16)^j	(4.71±0.17)^f
C4	(18.62±0.08)^k	(5.07±0.23)^h
C5	(36.87±0.33)^l	(10.82±0.17)^i

MC=milk chocolate, DC=dark chocolate, C=cocoa powder, TP=total phenols, GAE=gallic acid equivalent, TF=total flavonoids, CE=catechin equivalent

Values are the mean±S.D. (N=3). Values with different letters within columns are statistically different at $p<0.05$ according to paired Student's t-test

Different oxidants may oxidize flavonoids to different degrees, depending on their thermodynamic properties and the reactivity of the flavonoid hydroxyl groups (*39,40*). Therefore, the use of only one method is unsuitable for evaluating antioxidant capacity, because multiple oxidants are present in food.

The antioxidant capacity of the tested samples was evaluated using DPPH, ABTS, FRAP, and Fe(III)/Fe(II) assays. The results are shown in Table 3. In the DPPH assay, the antioxidant activity expressed as Trolox equivalents of cocoa powder samples was higher than the activity of dark and milk chocolate samples. Cocoa powder samples C5 (32.01 μmol/g), C4 (25.40 μmol/g) and C3 (22.43 μmol/g), and dark chocolate DC3 (24.82 μmol/g) and DC2 (23.31 μmol/g) had the highest, while milk chocolate MC2 (7.75 μmol/g) and MC1 (6.46 μmol/g) showed the lowest free radical scavenging capacity among the tested samples.

ABTS assay is based on the ability of antioxidants to scavenge ABTS radicals. As seen in Table 3, the ABTS values expressed as Trolox equivalents varied from 0.063 to 0.175 μmol/g with a 3-fold difference. Cocoa powder samples C5 (0.175 μmol/g), C3 (0.136 μmol/g) and C4 (0.133 μmol/g) and dark chocolate DC2 (0.171 μmol/g), DC4 (0.164 μmol/g), and DC3 (0.163 μmol/g) had the highest, while milk chocolate MC2 (0.085 μmol/g) and MC1 (0.063 μmol/g) had the lowest free radical scavenging capacity among the tested samples.

To correlate the results obtained with different assays, as well as between assays and total phenols (TP), total flavonoids (TF), total catechins (TC) and total procyanidins (TPC), a regression analysis was performed and correlation coefficients (R^2) are given in Table 4. The correlation between the results of DPPH and ABTS assays was high (R^2=0.8152, $p<0.0001$).

The FRAP and Fe(III)/Fe(II) assays measure reducing capacity based on the reduction of Fe(III) to Fe(II) ions. In general, the analyzed samples had a high antioxidant capacity. As indicated in Table 3, The FRAP and Fe(III)/Fe(II) values (expressed as FE and AAE, respectively) varied from 9.73 to 137.51 μmol/g with 14-fold difference, and from 56.69 to 647.93 μmol/g with 11-fold difference, respectively. Cocoa powder samples C5 (137.51 μmol/g) and C4 (113.33 μmol/g) and dark chocolate DC4 (126.23 μmol/g) and DC3 (114.86 μmol/g) had the highest, and milk chocolate MC2 (13.38 μmol/g) and MC1 (9.73 μmol/g) the lowest FRAP values. The highest Fe(III)/Fe(II) values were obtained in cocoa powder samples C5 (647.93 μmol/g) and C4 (478.58 μmol/g) and dark chocolate DC3 (495.59 μmol/g) and DC4 (434.71 μmol/g).

Table 3. DPPH, ABTS, FRAP and reducing power assay of chocolate and cocoa powder samples

Sample	DPPH as TE	ABTS as TE	FRAP as FE	Fe(III)/Fe(II) as AAE
	μmol/g	μmol/g	μmol/g	μmol/g
MC1	(6.46±0.45)^{a}	(0.063±0.004)^{a}	(9.73±0.71)^{a}	(56.69±3.28)^{a}
MC2	(7.75±0.45)^{b}	(0.085±0.004)^{b}	(13.38±0.32)^{b}	(70.18±1.31)^{b}
MC3	(9.02±0.45)^{c}	(0.104±0.004)^{c}	(23.86±0.32)^{c}	(108.28±1.31)^{d}
MC4	(11.50±0.34)^{d}	(0.120±0.002)^{d}	(26.98±0.45)^{d}	(118.41±3.94)^{e}
MC5	(9.49±0.23)^{c}	(0.115±0.002)^{d}	(24.55±0.52)^{c}	(104.05±1.96)^{d}
MC6	(11.73±0.45)^{d}	(0.136±0.002)^{e}	(27.15±0.58)^{d}	(139.85±2.62)^{f}
MC7	(12.62±0.79)^{e}	(0.142±0.004)^{f}	(28.54±0.45)^{d}	(145.47±2.84)^{f}
MC8	(7.90±0.23)^{b}	(0.094±0.004)^{cb}	(22.04±0.58)^{c}	(81.29±3.93)^{c}
MC9	(13.01±0.61)^{d}	(0.145±0.004)^{f}	(30.35±0.45)^{d}	(146.69±3.28)^{f}
DC1	(21.37±0.34)^{g}	(0.155±0.002)^{f}	(73.78±0.32)^{e}	(291.82±3.28)^{h}
DC2	(23.31±0.34)^{g}	(0.171±0.002)^{g}	(93.88±0.26)^{e}	(326.39±2.63)^{h}
DC3	(24.82±0.23)^{g}	(0.163±0.003)^{g}	(114.86±0.45)^{f}	(495.59±1.31)^{k}
DC4	(22.98±0.34)^{g}	(0.164±0.002)^{g}	(126.23±0.19)^{f}	(434.71±1.97)^{j}
DC5	(23.78±0.11)^{g}	(0.151±0.002)^{f}	(76.79±0.26)^{e}	(195.48±1.31)^{g}
C1	(11.65±0.56)^{de}	(0.099±0.004)^{c}	(22.45±0.52)^{c}	(398.35±3.28)^{i}
C2	(17.00±0.23)^{f}	(0.131±0.002)^{e}	(85.43±0.45)^{e}	(361.80±3.94)^{i}
C3	(22.43±0.23)^{g}	(0.136±0.003)^{e}	(78.34±0.52)^{e}	(327.32±2.62)^{h}
C4	(25.40±0.56)^{g}	(0.133±0.002)^{e}	(113.33±0.32)^{f}	(478.58±1.97)^{k}
C5	(32.01±0.23)^{h}	(0.175±0.002)^{g}	(137.51±1.99)^{g}	(647.93±2.63)^{l}

MC=milk chocolate, DC=dark chocolate, C=cocoa powder, TE=Trolox equivalent, FE=μmol of Fe(II) equivalent, AAE=ascorbic acid equivalent
Values are the mean±S.D. (*N*=3)
Values with different letters within columns are statistically different at p<0.05 by paired Student's *t*-test

Table 4. Correlation coefficient (R^2) between cyclic voltammetry (CV), DPPH and ABTS radical scavenging activity, FRAP and reducing power assays, total phenols, total flavonoids, total catechins and total procyanidins

	CV	DPPH	ABTS	FRAP	Fe(III)/Fe(II)	TP	TF	TC	TPC
CV	1.0000	0.7791	0.4053	0.7359	0.8743	0.8481	0.6959	0.3609	0.6357
DPPH		1.0000	0.8152	0.9525	0.8617	0.8238	0.7557	0.5502	0.8008
ABTS			1.0000	0.7797	0.6230	0.5539	0.5947	0.5891	0.8051
FRAP				1.0000	0.8866	0.8008	0.6913	0.5041	0.7818
Fe(III)/Fe(II)					1.0000	0.8119	0.6822	0.4001	0.6665
TP						1.0000	0.9377	0.6830	0.8342
TF							1.0000	0.7586	0.8499
TC								1.0000	0.8076
TPC									1.0000

CV=cyclic voltammetry, TP=total phenols, TF=total flavonoids, TC=total catechins, TPC=total procyanidins

Milk chocolate MC1 (56.69 μmol/g) and MC2 (70.18 μmol/g) had the lowest Fe(III)/Fe(II) values among the tested samples. The results showed that the Fe(III)-reducing activities of selected flavonoids obtained by two assays are well correlated (R^2=0.8866, p<0.0001), which is identical to the results of a previous report in which the data of the two assays are strongly correlated (*41*).

Moreover, FRAP values were highly correlated with the results of the DPPH assay (R^2=0.9525, p<0.0001) and moderately correlated with those of the ABTS (R^2=0.7797, p<0.0001). The antioxidant activities measured by Fe(III)/Fe(II) assay correlated well with the results of the DPPH assay (R^2=0.8617, p<0.0001), but the correlation with antioxidant values in the ABTS assay (R^2=0.6230, p<0.005) was weak.

Significant correlation was also found between the CV and Fe(III)/Fe(II) assay (R^2=0.8743, p<0.0001), CV and FRAP assay (R^2=0.7359, p<0.0001), and CV and DPPH assay (R^2=0.7791, p<0.0001). The lowest correlation was found between the CV and ABTS assay (R^2=0.4053).

High degree of positive correlation was also observed between the CV (R^2=0.8481, p<0.0001), DPPH (R^2=0.8238, p<0.0001), FRAP (R^2=0.8008, p<0.0001) or Fe(III)/Fe(II) (R^2=0.8119, p<0.0001) assays and total phenolic content determined by the Folin-Ciocalteu's method. These results confirm a relationship between the concentration of phenolic compounds in cocoa powder and chocolate samples and their free radical scavenging and ferric reducing capacities. Therefore, the presence of phenolic compounds in the tested samples contributes significantly to their antioxidant potential. Moreover, antioxidant properties of phenolic compounds are directly linked to their structure (*42*,*43*).

Weak correlations between total catechins and the antioxidant capacity estimations (Table 4) suggest that catechins are not primary contributors to cocoa antioxidant capacity. On the other hand, better correlations were obtained between total procyanidins and other assays (Table 4), suggesting that procyanidins account for a major portion of the antioxidants in chocolate and cocoa powder samples. These results are in accordance with those obtained from the literature (*10*,*27*,*44*). Another reason for weak correlations between catechins and antioxidant assays is the effect of fermentation and drying. This means that whenever cocoa beans are processed into chocolate, all polyphenols are not affected to the same degree. For example, alkalinization of cocoa powder does not equally reduce the amount of catechins as compared to procyanidins. During fermentation, a 6-fold variation in epicatechin concentration was observed, while procyanidin levels were shown to decrease 3-fold (*45*).

Because particular antioxidants have various contributions to the total antioxidant capacity, no single assay will accurately reflect all of the radical sources or all antioxidants in a complex system. Seeram *et al*. (*26*) and Piljac-Žegarac *et al*. (*46*) calculated antioxidant composite index (ACI) of polyphenol-rich beverages (red wine, fruit juices and fruit tea). Therefore, in this work the ACI indices were calculated from the results of five antioxidant capacity assays (scaled to relative percentages), which are shown in Table 5. Cocoa powder sample C5 has the highest ACI, followed by sample C4 and DC3, which is in accordance with their high Q_{600} value and total phenolic and flavonoid content. Milk chocolates MC1 and MC2 had the lowest ACI values, which is in accordance with their lowest Q_{600} values and total phenolic and flavonoid content.

The results of HPLC analysis are given in Table 6. Representative chromatograms of catechins and procyanidins from chocolate and cocoa powder samples are presented in Fig. 3. The HPLC results show that catechin and procyanidin content varied significantly in milk chocolate, dark chocolate and cocoa powder, as well as in the chocolate and cocoa powder samples of the same type. Catechin and procyanidin content in milk chocolate, dark chocolate and cocoa powder samples ranged from 0.081 to 0.23 and from 0 to 0.178, 0.088–0.48 and 0.123–0.39, 0.095–0.33 and 0–0.30 mg/g, respectively. Milk chocolate samples had the lowest catechin and procyanidin content. The content of (–)-epicatechin was higher than of (+)-catechin in most tested samples. The most abundant procyanidin was B2, followed by B1, B3 and B4. The highest content of catechins and procyanidins was found in cocoa powder samples C5 (0.33 and 0.30 mg/g) and C4 (0.165 and 0.214 mg/g), and dark chocolate DC3 (0.65 and 0.39 mg/g) and DC5 (0.48 and 0.218 mg/g).

Table 5. Antioxidant potency composite index of the tested samples calculated from five antioxidant capacity measurements scaled to relative percentages

Sample	DPPH index	ABTS index	FRAP index	Fe(III)/Fe(II) index	Q_{600} index	ACI
MC1	20.2	36.0	7.1	8.7	0.2	14.4
MC2	24.2	48.5	9.7	10.8	0.2	18.7
MC3	28.2	59.4	17.4	16.7	6.5	25.6
MC4	35.9	68.6	19.6	18.3	7.3	29.9
MC5	29.6	65.7	17.9	16.1	18.4	29.5
MC6	36.6	77.7	19.7	21.6	8.1	32.7
MC7	39.4	81.1	20.8	22.5	13.7	35.5
MC8	24.7	53.7	16.0	12.5	3.3	22.0
MC9	40.6	82.9	22.1	22.6	14.0	36.4
DC1	66.8	88.6	53.7	45.0	11.7	53.2
DC2	72.8	97.7	68.3	50.4	19.3	61.7
DC3	77.5	93.1	83.5	76.5	59.3	77.9
DC4	71.8	93.7	91.2	67.1	12.4	67.2
DC5	74.3	86.3	55.8	30.2	32.2	55.8
C1	36.4	56.6	16.3	61.5	40.7	42.3
C2	53.1	74.9	62.1	55.8	43.7	57.9
C3	70.1	77.7	57.0	50.5	56.7	62.4
C4	79.4	76.0	82.4	73.9	86.8	79.7
C5	100.0	100.0	100.0	100.0	100.0	100.0

MC=milk chocolate, DC=dark chocolate, C=cocoa powder, Q_{600}=charge passed to 600 mV, ACI=antioxidant potency composite index

Table 6. Individual phenolic content of chocolate and cocoa powder samples

Sample	w/(mg/g)								
	Protocatechuic acid	(+)-Catechin	(–)-Epicatechin	Catechins	Procyanidin B1	Procyanidin B2	Procyanidin B3	Procyanidin B4	Σ(B1–B4)
MC1	0.014±0.0005	0.021±0.001	0.060±0.003	0.081±0.004	n.d.	n.d.	n.d.	n.d.	0
MC2	0.011±0.0004	0.024±0.001	0.062±0.003	0.086±0.004	n.d.	n.d.	n.d.	n.d.	0
MC3	0.017±0.0006	0.033±0.001	0.102±0.005	0.135±0.006	0.0097±0.0005	0.036±0.001	0.024±0.001	0.0098±0.0005	0.079±0.003
MC4	0.011±0.0004	0.055±0.002	0.148±0.007	0.203±0.009	0.0207±0.0009	0.049±0.001	0.029±0.002	0.0164±0.0007	0.115±0.004
MC5	0.009±0.0003	0.038±0.001	0.106±0.005	0.144±0.006	0.011±0.001	0.052±0.002	0.034±0.002	0.0137±0.0004	0.111±0.009
MC6	n.d.	0.044±0.001	0.162±0.008	0.206±0.009	0.0192±0.0006	0.056±0.002	0.035±0.002	0.0171±0.0008	0.127±0.005
MC7	0.038±0.001	0.052±0.002	0.168±0.008	0.22±0.01	0.0229±0.0009	0.065±0.001	0.041±0.001	0.0167±0.0005	0.146±0.003
MC8	n.d.	0.026±0.001	0.102±0.005	0.128±0.006	n.d.	n.d.	n.d.	n.d.	0
MC9	n.d.	0.049±0.001	0.181±0.009	0.23±0.01	0.035±0.001	0.078±0.003	0.046±0.002	0.0193±0.0007	0.178±0.007
DC1	0.075±0.002	0.043±0.001	0.045±0.002	0.088±0.003	0.0082±0.0004	0.067±0.002	0.043±0.002	0.0051±0.0003	0.123±0.005
DC2	0.064±0.002	0.089±0.003	0.187±0.009	0.28±0.01	0.038±0.002	0.075±0.002	0.063±0.003	0.027±0.002	0.203±0.009
DC3	0.061±0.002	0.182±0.005	0.47±0.02	0.65±0.03	0.041±0.001	0.194±0.006	0.116±0.004	0.039±0.001	0.39±0.01
DC4	0.064±0.002	0.081±0.002	0.163±0.008	0.24±0.01	0.0207±0.0006	0.073±0.004	0.047±0.002	0.0158±0.0006	0.156±0.007
DC5	0.073±0.002	0.167±0.005	0.313±0.01	0.48±0.02	0.0199±0.0006	0.112±0.003	0.069 ±0.003	0.0169±0.0008	0.218±0.007
C1	n.d.	0.033±0.001	0.091±0.004	0.124±0.005	n.d.	n.d.	n.d.	n.d.	0
C2	0.102±0.003	0.049±0.001	0.046±0.002	0.095±0.003	0.0251±0.0009	0.084±0.004	0.052±0.003	0.0145±0.0007	0.176±0.009
C3	0.252±0.008	0.056±0.002	0.057±0.003	0.113±0.005	0.0163±0.0008	0.054±0.002	0.034±0.001	0.0122±0.0005	0.116±0.004
C4	0.175±0.006	0.073±0.002	0.092±0.004	0.165±0.006	0.0287±0.0009	0.097±0.004	0.067±0.003	0.0215±0.0009	0.214±0.009
C5	0.114±0.004	0.184±0.006	0.142±0.007	0.33±0.01	0.0365±0.0008	0.148±0.004	0.093±0.005	0.0247±0.0009	0.30±0.01

MC=milk chocolate, DC=dark chocolate, C=cocoa powder
The results are expressed as mean values±S.D. (N=3)
n.d.=not detected

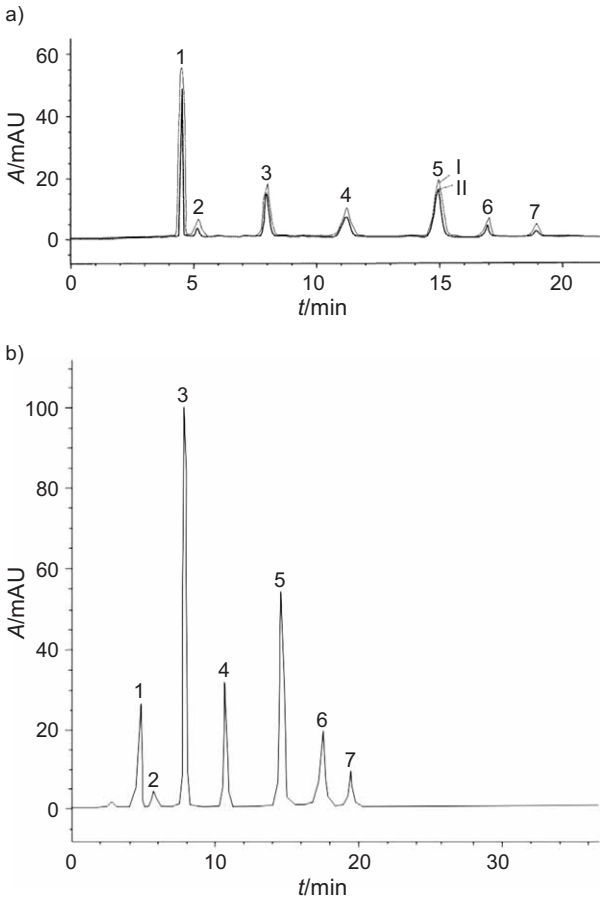

Fig. 3. Chromatograms of: a) cocoa powder (I) and dark chocolate (II) samples; b) standards. Recorded at 260 nm: 1=protocatechuic acid; recorded using fluorescence detector (λ_{E_x}=275 nm, λ_{E_m}=322 nm): 2=procyanidin B1, 3=(+)-catechin, 4=procyanidin B2, 5=(–)-epicatechin, 6=procyanidin B3, 7=procyanidin B4

Milk chocolate samples MC1 (0.081 mg/g) and MC2 (0.086 mg/g) had the lowest content of catechins and procyanidins among the tested samples. It can be expected that milk chocolate contains the lowest levels of procyanidins, since it contains the lowest amount of liquor (*45*). The lower levels of procyanidins found in dark chocolate could be explained by the fact that the liquor used to prepare chocolate was partially alkalized, which is known to cause chemical alterations in polyphenols. The obtained results suggested that the antioxidant capacity was related to catechin and procyanidin content. The content of protocatechuic acid varied in milk chocolate, dark chocolate and cocoa powder samples from undetectable level to 0.038, 0.061–0.075, and from undetectable level to 0.252 mg/g, respectively. The highest content was found in cocoa powder samples, followed by dark and milk chocolate samples.

Principal component analysis (PCA) was performed to classify samples on the basis of the similarity of their chemical properties. Starting point for the PCA calculations was the data matrix with 29 rows corresponding to milk chocolate, dark chocolate and cocoa powder samples, and 7 columns corresponding to the contents of individual phenolic compounds. One of the main objectives of PCA is to identify factors that are substantially meaningful. The principal components which have eigenvalues higher than 1 were extracted (Kaiser criterion) (*47*). This led to the formation of two principal components, because subsequent eigenvalues were all less than one. The first component accounted for 65.5 % (eigenvalue of 5.3) and the second for 25.7 % (eigenvalue of 1.1) of the total variation of the data. These two components accounted for 91.2 % of variance of all the data. The first component represents the maximum variation of the data set. The results of PCA calculations are illustrated in Fig. 4. They confirm that milk chocolate samples contain the lowest levels of individual phenolic compounds. These samples are grouped on the left side of the plot. On the other hand, cocoa powder and dark chocolate samples, with higher contents of individual phenolic compounds, can be found on the opposite side.

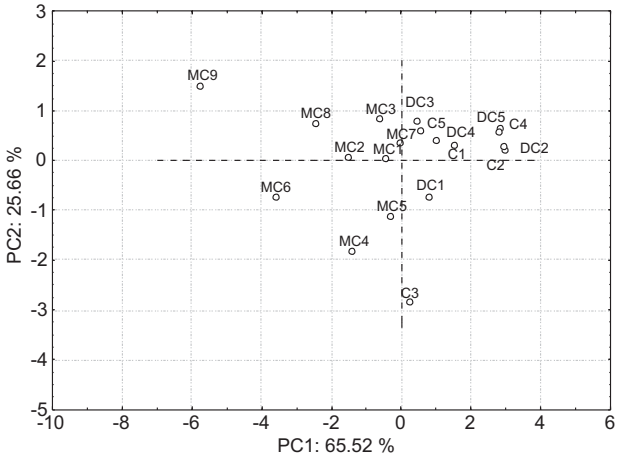

Fig. 4. Scatter plots of the first two principal component vectors (PC1 *vs.* PC2) for milk chocolate (MC), dark chocolate (DC) and cocoa powder (C) samples. Classification of 19 samples according to individual phenolic data

Conclusions

Cocoa powder, dark and milk chocolate samples were tested for their antioxidant capacity using cyclic voltammetry. Taking into account all the results obtained in this work, the proposed electrochemical technique can be employed for the direct and rapid determination of antioxidant capacity of tested samples. The high positive correlation between the antioxidant capacity values obtained from the CV and the results obtained from other spectrophotometrical assays indicates that the results derived from CV are reliable and comparable to the results of other commonly employed methods. The ACI parameter provides easy comparison of the antioxidant capacity of the tested samples obtained by different methods. Using HPLC method, individual catechins and procyanidins were determined. A significant relationship between the antioxidant capacity and total procyanidin content was found, indicating that procyanidins are the major contributors to the antioxidant properties of cocoa powder and chocolate samples. The obtained results also provide evidence for the applicability of PCA to group together samples according to their chemical similarity. The samples were classified into three groups. The first group (cocoa powder samples) and the second group (dark choco-

late) are close to each other, with higher mass fractions of individual phenolic compounds. The third group contains milk chocolate samples, with lower individual phenolic mass fractions.

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