STUDIA UNIVERSITATIS BABEŞ-BOLYAI, CHEMIA, LIV, Sp.Iss. 1, 2009

THE ELECTROCHEMICAL BEHAVIOR OF SOME BERRIES EXTRACTS

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ABSTRACT. In this paper, cyclic voltammetry was used as method to estimate the antioxidant activity of anthocyanins extracts from frozen fruits of some berries. Extraction in methanol for four types of indigene berries, *Ribes nigrum* L., *Ribes rubrum* L., *Vaccinium myrtillus* L., *Prunus spinosa* L. and *Rubus fruticosus* L., have been made. The voltammograms were recorded at room temperature. Some information on the electrochemical properties of the extracts could be provided by comparing the oxidation potentials at different pH values. The antioxidant capacities of the extracts were also evaluated by DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging method and correlated with their oxidation potentials.

Keywords: anthocyanins, antioxidant activity, cyclic voltammetry, DPPH

INTRODUCTION

Anthocyanins are natural pigments widely distributed in flowers, fruits (especially in berries) and vegetables, being responsible for their bright colors. Interest for anthocyanins has intensified due to their biological activities [1,2,3], the most important being the strong antioxidant activity in metabolic reaction, and their possible health benefits [4,5,6].

The aglycon forms, anthocyanidins, are the basic structure of anthocyanins (Figure 1) [7]. The most common anthocyanidins are cyanidin, delphinidin, pelargonidin, peonidin, petunidin and malvidin. In plants, anthocyanidins occur in their glycosidic form (bonded to a sugar moiety), anthocyanins.

Anthocyanins may exist in a variety of protonated, deprotonated, hydrated, and isomeric forms. The relative proportion of these forms is strongly dependent on pH (Figure 2) [3,8]. At very low pH values (pH 1-3), the red flaviulium cation is the predominant species. At pH 4-5, a colorless carbinol pseudo-base is generated, which can further undergo to pale yellow chalcones (Figure 2, C_E and C_Z). At pH 6-7, the flavylium cation can alternatively be transformed to quinoidal-base isomers through deprotonation and proton-transfer reactions and can be further converted to the blue-purple quinonoid anions [3,8]. The possible pH effect on the antioxidant properties of anthocyanins is of great interest due to the presence of different pH values in different human body fluids.

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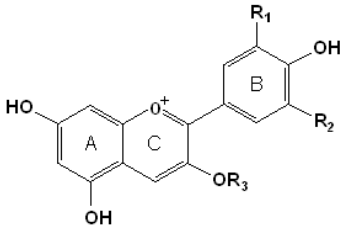

Anthocyanidin	R_1	R_2	R_3
Pelargonidin	H	H	H
Cyanidin	OH	H	H
Delphinidin	OH	OH	H
Peonidin	OCH_3	H	H
Petunidin	OCH_3	OH	H
Malvidin	OCH_3	OCH_3	H

Figure 1. Structures of common anthocyanidins

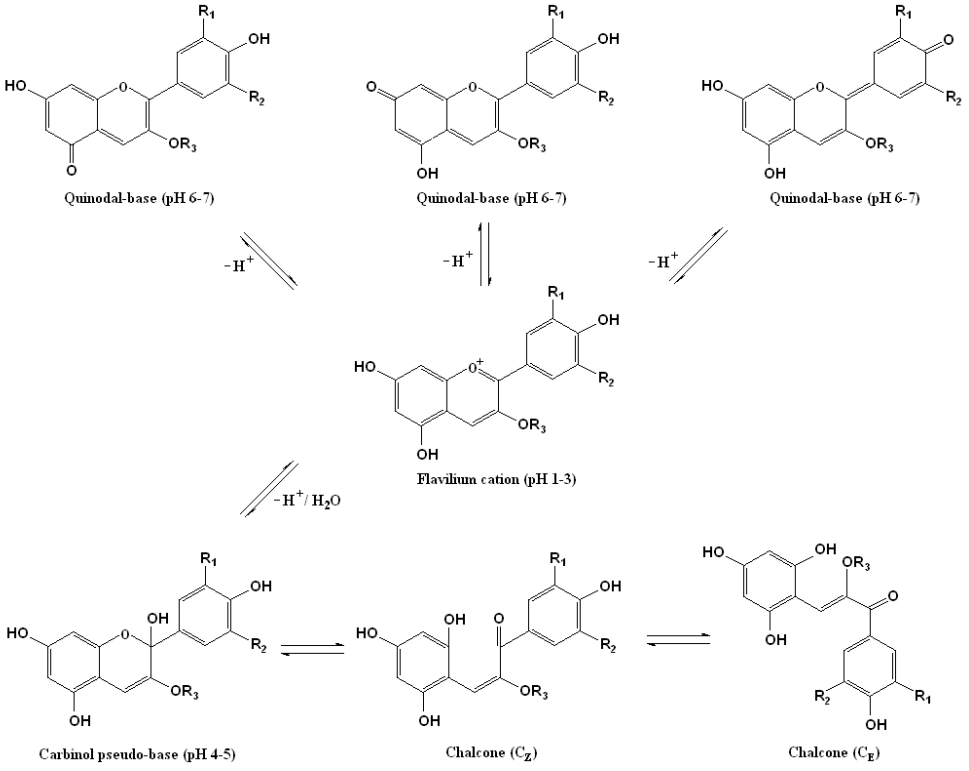

Figure 2. Structural changes of anthocyanins in aqueous solution at different pH

The antioxidant potential of anthocyanins occurs due to phenolic hydroxyl groups attached to their three-ring flavonoid structure and can change with the hydroxyl substituents and the sugar moieties bonded to the molecule [3,9].

There are several methods for in vitro determination of antioxidant capacity, mainly based on reaction between an antioxidant and a chromogen compound [10]. Among these methods, the electrochemical methods are simple, rapid and economic. Cyclic voltammetry is the most used electrochemical

technique for evaluation of the overall reducing capacity of low molecular weight antioxidants from biological samples (blood plasma, body fluids) and plant extracts [11,12,13,14].

In this work, cyclic voltammetry was used to evaluate antioxidant activity of the methanolic extracts of some indigene fruits rich in anthocyanins: black currants (*Ribes nigrum* L.), red currants (*Ribes rubrum* L.), bilberries (*Vaccinium myrtillus* L.), blackthorns (*Prunus spinosa* L.) and blackberry (*Rubus fruticosus* L.). For all the extracts, the oxidation profile in several background electrolytes at different pH values was investigated. The antioxidant capacity of the studied extracts was also determined using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging method [15]. In this assay, the purple chromogen radical DPPH is reduced by antioxidant compounds to the corresponding pale yellow hydrazine [16]. The reduction of DPPH was followed by a spectrophotometric method and the antioxidant capacities of the extracts evaluated by this assay were correlated with their oxidation potentials and anthocyanins content.

RESULTS AND DISCUSSION

Anthocyanins content of the extracts

The anthocyanins content in studied extract were determined by a pH differential method. Anthocyanins present a maximum absorbance at a wavelength of around 520 nm at pH 1.0. At pH 1.0, the flavylium cation is the predominant species and has a red color, in aqueous media at pH 4.5 hydration reaction generates the colorless carbinol pseudo-base (Figure 2).

The total monomeric anthocyanins are calculated based on the molecular weight and extinction coefficient of the predominant anthocyanin in the sample. If the ε value of the major pigment is not available or if the sample composition is unknown, anthocyanins content is calculate as cyinidin-3-glucoside [17] which is the most abundant in nature.

The composition of fruits extracts analyzed in this paper is not completely determined. We tried to establish the qualitative composition of the extracts through HPLC analysis. For all fruits extracts, chromatograms revealed the presence of a large number of anthocyanins: bilberries – 15 compounds, black currants – 18 compounds, red currants – 13 compounds, blackberries – 2 compounds and blackthorns – 7 compounds. We have succeeded to identify all anthocyanins only in bilberries extract [19]. We had no available commercial pure standards for all compounds. For this reason, the anthocyanins content is calculate as cyaniding-3-glucoside (MW = 449.2 and ε = 26900) and the obtained values are presented in Table 1.

It was found that bilberries extract provided the highest content of anthocyanins pigments from analyzed species of fruits. Smaller amounts of anthocyanins were found in the other fruits extracts, in the following order: black currants > blackthorns > blackberries > red currants.

Cyclic voltammetry

The electrochemical behavior of the fruits extracts was investigated with two different working electrodes: a platinum electrode (Pt) and a glassy carbon electrode (GCE).

Platinum electrode had no relevant response in the three supporting electrolytes chosen in this study. As example, in figure 3, are presented the cyclic voltammograms obtained on Pt electrode for black currants extract.

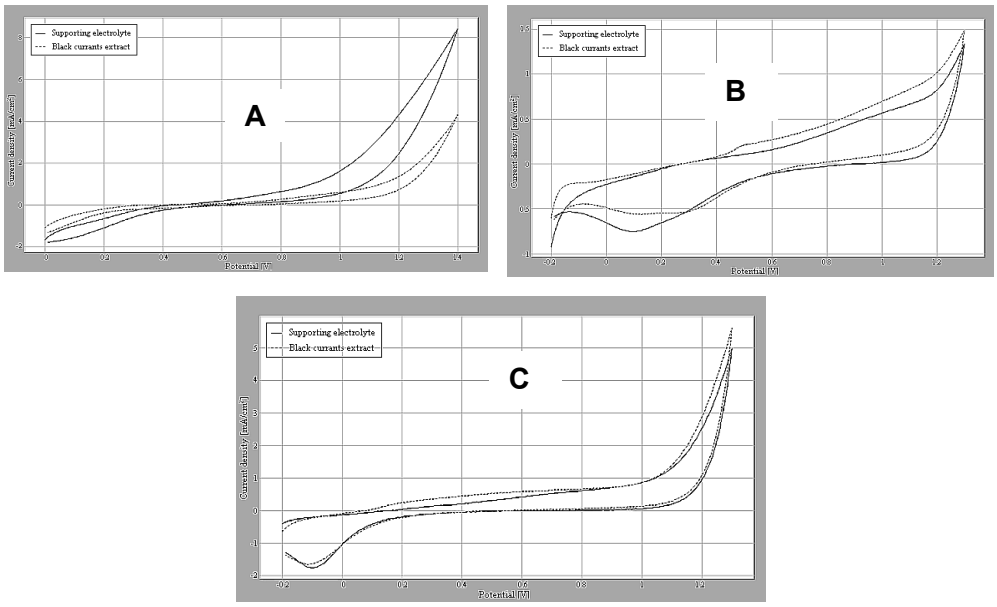

Figure 3. Cyclic voltammograms (Pt) of black currants extract at w=500 mV/s, supporting electrolyte: (**A**) NaClO4 0.1M in methanol; (**B**) acetate buffer (pH=3); (**C**) phosphate buffer (pH=7.6)

Unlike the results obtained using the platinum electrode, the voltammetric curves obtained with glassy carbon electrode present well defined oxidation peaks.

The electrochemical behavior of all the extracts on glassy carbon electrode was studied at different scan rates in the three support electrolytes.

Cyclic voltammograms were recorded from 0 to 1400 mV for NaClO4 0.1M in methanol and from -200 to 1300 mV for the other two supporting electrolytes at scan rates ranging from 100 to 500 mV/s. The best defined peaks were obtained at 500 mV/s and are exemplified for black currants extract using as supporting electrolyte NaClO4 0.1M in methanol (Figure 4). The following measurements were recorded at 500 mV/s.

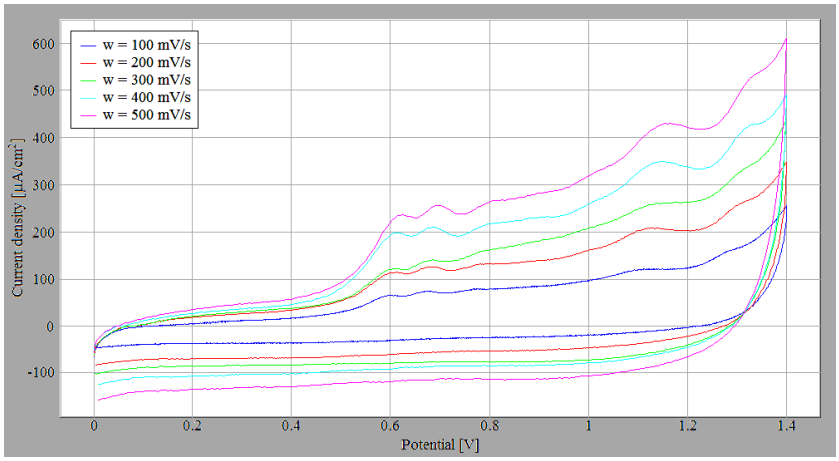

Figure 4. Cyclic voltammograms (GCE) of black currants extract at different scan rates, supporting electrolyte: NaClO4 0.1M in methanol

Also, it can be observed that the oxidation peak potentials shift to more positive values and the anodic peak currents become more accentuated with the increase of scan rate. These scan rate-dependent processes indicates that EC reaction mechanisms take place [20].

The electrochemical behavior in time of the anthocyanins extracts it has been also studied (Figure 5). In this type of experiments, the electrode remained immersed in the solution on entire duration of an experiment.

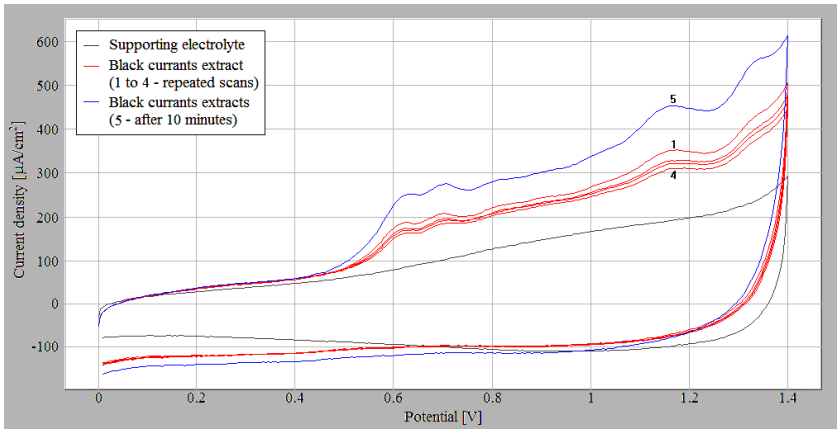

Figure 5. Cyclic voltammograms (GCE) of black currants extract at w=500 mV/s, supporting electrolyte: NaClO4 0.1M in methanol

After several repeated scans (Figure 5, the curves from 1 to 4), it has been observed the decrease of the current density for all oxidation peaks,

due to a decrease of species concentration at electrolyte-electrode interface. After 10 minutes, we expected that the electrode surface will be coated by the anthocyanins or their oxidation products which may be adhering to the electrode surface, lowering the efficiency with which a new quantity of anthocyanins are oxidized and significantly altering the current response of the electrode [12]. However, after 10 minutes, the anodic peaks increased (Figure 5, curve 5), probably due to the concentration restoration by diffusion of anthocyanins molecules at electrolyte-electrode interface.

The influence of supporting electrolyte is illustrated in Figures 6, 7, and 8. For testing were chosen three supporting electrolytes: $NaClO_4$ 0.1M in methanol (pH=2.35) because the most phenolic antioxidants are soluble in methanolic medium, and two buffer solution, one at acidic pH (acetate buffer pH=3) and the other one at alkaline pH (phosphate buffer pH=7.6).

Significant results were obtained with $NaClO_4$ 0.1M in MeOH (Figure 6). Cyclic voltammograms of the all studied extracts display more than one oxidation peaks in the range 400-1400 mV and practically no reverse reduction peak, indicating the irreversibility of the electrode processes, except for the blackthorns extract. The position of anodic peaks in the voltammograms suggests the antioxidant ability; peaks at lower potential mean a higher antioxidant capacity [11]. The lowest potential for the first distinct peak in this supporting electrolyte belongs to blackthorns extract. On the basis of electrochemical data, the order of antioxidant activity of analyzed fruits extracts is: blackthorns > black currants > bilberries > blackberries > red currants.

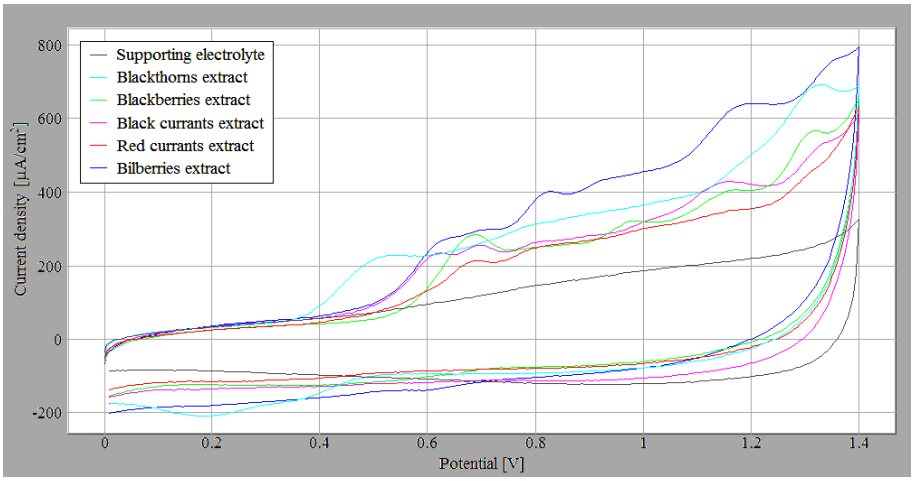

Figure 6. Cyclic voltammograms of of the extracts at w=500 mV/s, supporting electrolyte: NaClO4 0.1M in methanol

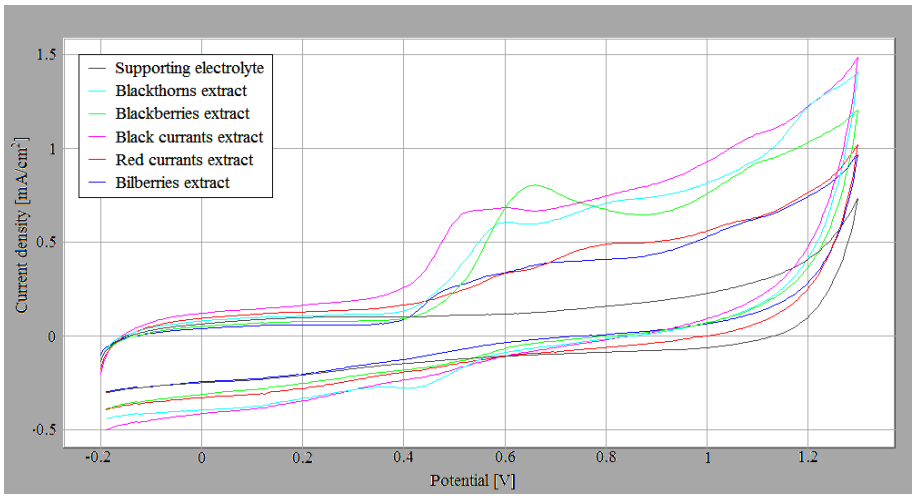

Figure 7. Cyclic voltammograms of the extracts at w=500 mV/s, supporting electrolyte: acetate buffer pH=3

In buffer solutions, the electrochemical behavior of the extracts is changed. The cyclic voltammograms shapes for the fruits extracts, in the two buffer solution, is quite similar, but different from those obtained with $NaClO_4$ 0.1M in MeOH, even if the acetate buffer solution and the methanolic solution have close values of pH. In the two buffer solution (Figure 7 and Figure 8), the less positive potential peak corresponds to black currants extract.

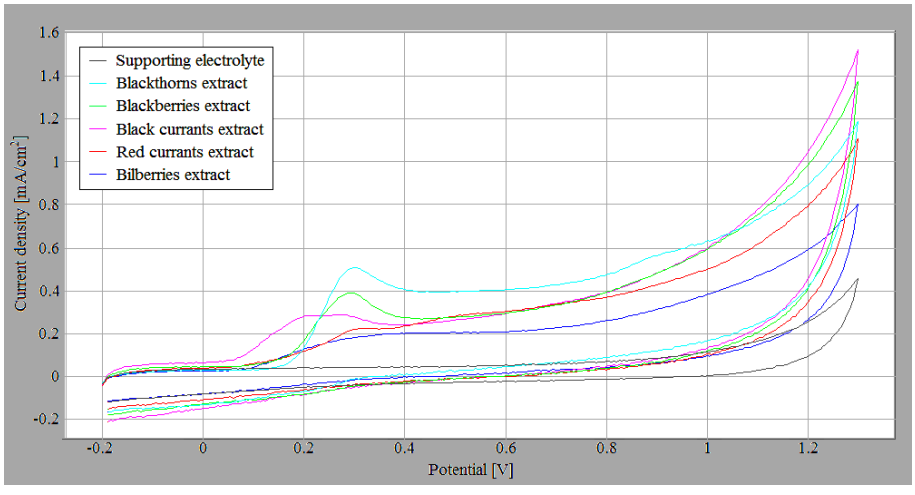

Figure 8. Cyclic voltammograms of of the extracts at w=500 mV/s, supporting electrolyte: phosphate buffer pH=7.6

Some information about the mechanism of anthocyanins oxidation could be provided by comparing oxidation potential at different pH values. In this case, is difficult to present an oxidation mechanism because the studied extracts are mixtures of anthocyanins with one or more hydroxyl groups attached in various positions to their three-ring flavonoid structure. However, it can be observed that, for all studies extracts, oxidation potentials shift to lower values at higher pH, indicating that the compounds are easier oxidized, so their antioxidant activity is increasing. These results are in agreement with data obtained by other authors for pure compounds [21].

Free radical scavenging activity

The antioxidant activity of the extracts was estimated by the ability to scavenging the DPPH radical. The DPPH concentration in the reaction medium was calculated from the calibration curve with the following equation determined by linear regression:

$$A_{515} = 11048 \cdot c_{DPPH} + 3.6828 \cdot 10^{-3} \quad (R^2 = 0.99987) \qquad (3)$$

For each extract, the amount of the remaining DPPH expressed as a percentage was calculated as:

$$\text{Remaining DPPH [\%]} = (c_{DDPH})_t / (c_{DPPH})_{t=0} \cdot 100 \qquad (4)$$

where $(c_{DDPH})_t$ is the value of DPPH concentration in the presence of extracts at time *t*.

The percentage of remaining DPPH concentration against reaction time for the five extracts is illustrated in Figure 9. In Table 1 is presented the percentage of remaining DPPH concentration after 4 hours. The lower this value, the higher is antiradical efficiency.

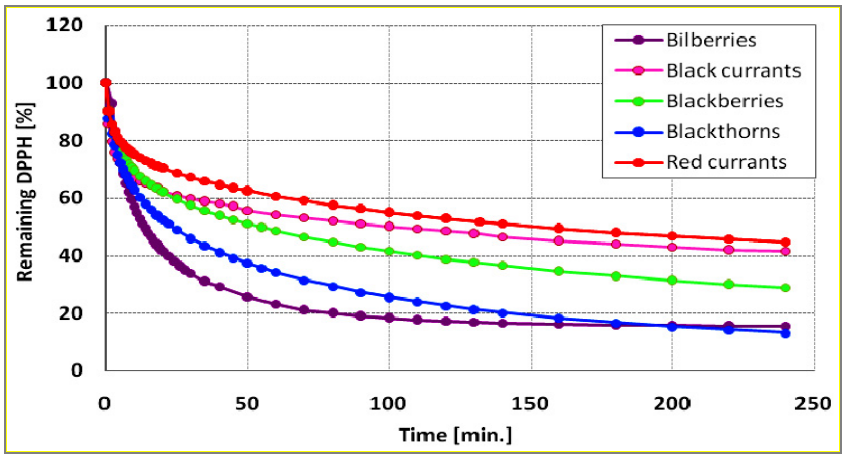

Figure 9. Kinetic behavior of DPPH scavenging

Table 1. Comparison between anthocyanins content, voltammetric behavior and DPPH radical scavenging activity of analysed extracts

Fruits extract	Anthocyanins content [mg/l]	Oxidation potentials [mV]						Remaining DPPH at the time t = 4h [%]
		E_{pa1}	E_{pa2}	E_{pa3}	E_{pa4}	E_{pa5}	E_{pa6}	
Bilberries	3888	637	708	830	927	1205	1357	15.38
Black currants	860	624	695	799	1160	1334	-	41.53
Blackberries	458	685	981	1172	1317	-	-	28.88
Blackthorns	709	529	unread	1328	-	-	-	13.16
Red currants	127	693	805	unread	unread	-	-	44.73

The scavenging of the free radical by the studied extract show similar pattern curves of remaining DPPH versus time. The reaction between samples and DPPH show a biphasic kinetic behaviour, the reaction occurs rapidly in the first minutes and then slowed. This behaviour type has been reported also by some authors [22,23]. The lower step can be due to the antioxidant properties of the slow reacting components originally present in the sample and/or due to the reaction products formed during rapid phase [23].

The DPPH radical scavenging activities reveal the following order for antioxidant activities of the extracts: blackthorns > bilberries > blackberries > blackcurrants > red currants.

Comparing the experimental results obtained by the two methods, it is observed that the order of antioxidant activities for studied fruits extracts established on the basis of oxidation potentials is confirmed by the DPPH assay with only one exception, the black currants extracts, which show a weak radical scavenging effect.

Considering the anthocyanins content, we expected a higher antioxidant activity for bilberries extract which has the highest concentration in anthocyanins. It is an interesting fact that the blackthorns extract presents the strongest antioxidant activity even if the anthocyanins content is much smaller than for bilberries. This phenomenon can be explained by the possible presence of other antioxidant compounds in methanolic extract which can amplify the antioxidant effect.

CONCLUSIONS

Cyclic voltammetry can be considered a rapid and simple technique to evaluate the antioxidant properties of natural extracts. Between the two tested electrodes, glassy carbon is an excellent electrode material to study the electrochemistry of natural antioxidants.

The obtained results show that the electrochemical behavior of studied berries extracts is dependent on supporting electrolyte and solutions pH. The blackthorns extracts present the less positive potential peak, so the higher antioxidant activity in $NaClO_4$ 0.1M in MeOH and black currants extract present the less positive potential peak in the two buffer solution. Depending on pH all extracts have the same behavior, oxidation potentials shift to lower values at higher pH.

The data obtained by the cyclic voltammetry were substantially confirmed by the DPPH method. The antioxidant activities of analyzed extracts decrease in the following order: blackthorns > bilberries > blackberries > red currants. Black currants extracts presents a different behavior. No correlation between antioxidant activity and anthocyanins content has been observed for unpurified studied indigene extracts.

The further objectives regard: separation, identification and quantitative determination of anthocyanins and anthocyanidins from different fruits like bilberry, black currant, red currant, raspberry, strawberry, mulberry, grapes, blackberry etc., and determination of their “in vitro” antioxidant activity and “in vivo” testing of extracts on rats with experimentally induced diabetic disease.

EXPERIMENTAL SECTION

Extraction of Anthocyanins

The ultrasonication method in methanol acidified with 0.1% HCl, at 25°C, 59 kHz, 60 min. (ultrasonic bath FALC Instruments - Italy) has been used. 25 g of black currants, red currants, bilberries, blackthorns and blackberries frozen fruits (without seed) were treated with 100 ml extracting material for each experiment (solid to solvent ratio 1:4 w/v). After filtration through a Whatman no. 1 filter paper, the methanolic extracts were concentrated in a rotary evaporator at 40°C under vacuum (40-45 mbar) until complete solvent evaporation. For an accurate comparative analysis of extracts, they were brought to the same volume (25 ml) with methanol. The samples were kept in the freezer until analysis.

Anthocyanins content of the extracts

Anthocyanins content were quantified using the pH differential method described by Giusti and Wrolstad [17]. This method, based on reversible structural transformations of anthocyanin pigments in different pH solutions [18], permits a rapid and accurate measurement of the total anthocyanins even in the presence of polymerized degraded pigments and other interfering compounds.

A Jasco V 530 UV-Vis spectrophotometer was used for measurements. Samples were diluted in buffer solutions of pH 1.0 (0.025 M potassium chloride buffer) and pH 4.5 (0.4 M sodium acetate buffer) and then it has been made the measurements of absorbance for each solution at maximum wavelength (λ_{max}) and 700 nm (to correct for haze). The absorbance (A) was calculated using the following relation:

$$A = (A_{\lambda_{\max}} - A_{700})_{pH1.0} - (A_{\lambda_{\max}} - A_{700})_{pH4.5} \quad (1)$$

The monomeric anthocyanin pigment concentration was calculated using the following formula:

$$C[mg/l] = \frac{A \times MW \times DF \times 1000}{\varepsilon \times l} \quad (2)$$

where *MW* is the molecular weight, *DF* is the dilution factor, *ε* is the molar absorptivity and *l* is the pathlength (1 cm). Each sample was analyzed in duplicate and the results were expressed as the averages of the two measurements.

Cyclic voltammetry

Electrochemical experiments were carried out with a Voltalab 80 PGZ 402 apparatus from Radiometer Copenhagen, equipped with VoltaMaster 4 software, version 7.0 using a three-electrode electrochemical cell equipped with a working electrode, a platinum wire auxiliary electrode and Ag/AgCl, KCl sat. reference electrode. The potentials were recorded at room temperature.

Prior to each run, the electrode surface was cleaned by polishing with alumina powder and ultrasonified 10 minutes in HCl 5% solution. In order to minimize the adsorption of the compounds and their oxidation products onto the electrode surface, the voltammograms were recorded immediately after the immersing of the working electrode in the solution.

The following support electrolytes were tested: $NaClO_4$ 0.1M in methanol (pH=2.35), acetate buffer CH_3COONa/HCl (pH=3) and phosphate buffer Na_2HPO_4/NaH_2PO_4 (pH=7.6). 300 µl of extracts was diluted in 25 ml background electrolyte for electrochemical tests.

Free radical scavenging activity

The free radical scavenging activity of the studied fruits extracts was perform by using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay according to the procedure described by Brand-Williams et al. [15] with some modifications. This assay is based on the spectrophotometric measurements of the loss of DPPH colour caused by consumption of DPPH radical by antioxidant species present in the sample.

In order to evaluate antioxidant activities, each extract has been diluted 1:25 v/v with methanol. Antioxidant solution in methanol (0,1 ml) was added to 2,9 ml of a solution $6 \cdot 10^{-5}$ mol/l DPPH in methanol. The reduction of DPPH was followed by monitoring the decrease of absorbance at 515 nm during 4 hours. A Jasco V 530 UV-Visible spectrophotometer was used for measurements.

ACKNOWLEDGMENTS

This work is part of the project no. 52145 / 2008 - "Hypoglycemic and antioxidant dietary supplements with anthocyanidinic structure - SAHASA", carried out under the 4th Programme-Partnerships in priority areas of The National Plan for Research-Development and Innovation 2007-2013 (Romania).

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