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Review

Electrochemistry as a Tool for Studying Antioxidant Properties

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Electrochemical methods provide high potential for investigation of antioxidant compounds, assessment of antioxidant capacity, and measurement of electrochemical index. Different types of electrodes can be used for the assay purposes. The devices can be stationary or flow through, and based on cyclic or differential pulse voltammetry as well as potentiostatic analysis. The methods are known for their suitability for food control and monitoring the levels of antioxidant capacity in other biological samples and matrices. In this review, the application of both stationary and flow electrochemical methods for analysis of plant and clinical samples with respect to study of their antioxidant properties is described. The advantages and disadvantages of the methods are discussed.

Keywords: Antioxidants; Voltammetry; Amperometry; Flow Injection Analysis; Electrochemical Index

Abbreviations: 3,4-DHBA – 3,4-dihydroxybenzoic acid; 4-HBA – 4-hydroxybenzoic acid; CV – cyclic voltammetry; DNA – deoxyribonucleic acid; DPV – differential pulse voltammetry; DPPH – diphenyl-1-picrylhydrazyl; EDTA – ethylenediaminetetraacetic acid; EI – electrochemical index; FCR – Folin-Ciocalteu reagent; FIA – flow injection analysis; FIA-ED – flow injection analysis with electrochemical detection; FRAP – ferric reducing antioxidant power; GSH – reduced glutathione; GSSG – oxidized glutathione; H_2O_2 – hydrogen peroxide; HIV – human immunodeficiency virus;

HMDE – hanging mercury drop electrode; HPLC – high-performance liquid chromatography; HPLC-ED – high-performance liquid chromatography with electrochemical detection; LMWA – low molecular weight antioxidant; LOD – limit of detection; $O_2^{\cdot -}$ – superoxide anion; $OH^{\cdot}$ – hydroxyl radical; PBS – phosphate buffered saline; RESAC – rapid electrochemical screening of antioxidant capacity; ROS – reactive oxygen species; RSD – relative standard deviation; SCE – saturated calomel electrode; SOD – superoxide dismutase; SW-AdCSV – square wave-adsorption cathodic stripping voltammetry; SWV – square wave voltammetry; TEAC – trolox equivalent antioxidant capacity; Trx ox – oxidized thioredoxin; Trx red – reduced thioredoxin; TrxPx – thioredoxin peroxidase; TrxR – thioredoxin reductase; UV – ultra violet

1. INTRODUCTION

An antioxidant capacity belongs to the most important antioxidant parameters. The capacity is defined as the ability of compound (or mixture of compounds) to inhibit the oxidative degradation of various compounds like preventing lipid peroxidation. These methods are usually based on the direct reaction between studied compounds and free radicals (quenching or scavenging) or on the reaction with transition metals [1-6]. Spectrometric methods are mainly used in the analysis of antioxidant properties. However, these methods are dependent on many parameters, such as temperature, time of the analysis, character of a compound or mixture of compounds (extracts), concentration of antioxidants and prooxidants and many other substances [7-15].

Electrochemical methods used for the determination of antioxidant capacity have been still developing. These provide rapid, simple and sensitive alternative method in the analysis of bioactive compounds associated with the scavenging of the radicals as well as the antioxidant capacity itself. They are low-cost and usually do not require time consuming sample preparation. They are used both in stationary systems, where differential pulse polarography and voltamperometric methods belong to the most used techniques, and dynamic, flow, eventually electromigration system, where the advantage of voltammetry and amperometry is considered. Dynamic systems are used and cover rapid and sensitive quantification of simple analytes; on the other hand, stationary systems are suitable for the quantification of limited amount of analytes and for studying formation of complexes of simple as well as more complicated biomolecules with target molecule(s). The development of the new instrumentation with the possibility of miniaturization in contrast to the conventional, routinely used systems is the main object of electrochemical techniques in the analysis of bioactive compounds [16-21].

Antioxidant compounds can act as reduction agents and, in solutions, they tend to be easily oxidized on the inert electrodes. Based on this fact, the relationship between electrochemical behavior of compounds with antioxidant activity and their resulting “antioxidant power” (capacity) is very interesting, because the “low oxidation potential” corresponds to the “high antioxidant power” [22]. It is a common knowledge that oxidative stress relates with imbalance between concentration of prooxidants and the antioxidants. Moreover, it appears that these changes are very important in the pathophysiology of critically diseased patients. Direct measurement of reactive oxygen species and oxidative stress markers is still difficult task in clinical medicine. The level of oxidative stress is measured as the total change of antioxidant capacity or, alternatively, as antioxidant status [23-29].

In the present review, there are summarized basic electrochemical techniques used for the determination of the characteristics of the antioxidants in **a)** plant samples and in **b)** clinical diagnostics, for which electrochemical methods have been proposed and developed. In the first part, the attention is focused on the using the carbon electrodes in stationary systems. The second part describes using the flow techniques. Advantages as well as disadvantages of the application of electrochemical methods for analysis of different types of samples are discussed.

2. STATIONARY SYSTEMS

A carbon working electrode is the most used sensor in the electrochemical detection. This electrode can be based on the fluid (carbon paste electrodes) [30] or solid (printed carbon electrode) [31-33] matrix (binder). Besides common graphite powders, both paraffin and silicone oils can be also mixed with other carbonaceous materials, forming more or less special carbon pastes [34,35]. The possibility of the modification of the carbon paste electrode as addition of inorganic or organic compounds, which increases the selectivity and specificity, is the most important advantage [36]. These electrodes are used in voltammetric as well as amperometric measurements, and cyclic voltammetry is the most used method. Voltammetric techniques are considered the most effective for studying antioxidant properties [37-42].

2.1 Cyclic voltammetry for determination of antioxidant capacity

Cyclic voltammetry (CV) is the most commonly used technique for the characterization of redox system. It can provide information about the number of redox states as well as qualitative information about the stability of these oxidation states and the electron transfer kinetics [43-46]. CV involves scanning the voltage of a working electrode while recording the anodic current produced by the low molecular mass antioxidants in the solution, which are oxidized on the surface of the working electrode [47]. The current produced is proportional to the combined concentration of the antioxidants [48,49]. CV on the carbon electrode seems to be suitable method for the determination of antioxidant capacity, especially due to its simplicity, rapidity and possibility to be used directly in biological and crude samples [50-54]. Australian scientists studied series of compounds based around combining the neuroprotective properties of non-competitive N-methyl D-aspartic acid receptor antagonists with antioxidant functionalities [55]. The redox chemistry of these compounds has been evaluated using the cyclic voltammetry, and the results have been compared with their radical-scavenging properties obtained from two standard biological assays, the inhibition of lipid peroxidation and the Sapphire colorimetric assay. The antioxidant activity of a set of pyrimidines has been shown to be dependent on the presence of three amino substituents [55].

2.1.1 Determination of antioxidant activity of food and polyphenolics compounds

CV can be used in the food and plant products analysis. It is possible to determine total antioxidant capacity. On the other hand, experimental data must be related to standard, it means to compound with antioxidant properties and well-characterized chemical structure, which can be similar to structure of compounds present in evaluated sample [56]. Born et al. [57] determined compounds with low oxidation potential, which possessed only week antioxidant activity, by CV. The antioxidant properties of polyphenols chlorogenic acid, cordigol, cordigone, danthrone, 1,5-dihydroxy-3-methoxyxanthone, eriosematin, flemichin D, frutinone A, mangiferin, quercetin, 1,3,6,7-tetrahydroxyxanthone and verbascoside were investigated. The results revealed that compounds with a catechol group (*meta* arrangement) were oxidized below 0.4 V. Compounds having one or more isolated phenolic groups showed an oxidation potential between 0.45 and 0.8 V, excepting 1,5-dihydroxy-3-methoxyxanthone (0.45 V), danthrone (0.96 V) and eriosematin, which showed no or modest antioxidant activity. Simic et al. [58] monitored antioxidant properties of phenolics compounds, namely salicylic acid, *m*-hydroxybenzoic acid, *p*-hydroxybenzoic acid, protocatechuic acid, *o*-coumaric acid, *m*-coumaric acid, *p*-coumaric acid, caffeic acid, quercetin and rutin, by using the CV. Authors were aimed at the structure-antioxidant capacity relationship. Their data point at the importance of multiple hydroxyl substitution and conjugation in the resulting antioxidant activity and electrochemical properties of studied compounds. The compounds with the low oxidation potential (E_{pa} lower than 0.45) demonstrated significant antioxidant activity, whereas compounds with high oxidation potential (E_{pa} values >0.45) acted as prooxidants [58]. Using the cyclic voltammetry, phenolics and flavonoids were characterized by Yakovleva et al. [59]. Artega et al. [60] performed extend study focused on the antioxidant activity of low-molecular antioxidants present in the spice and some pharmaceuticals (gallic acid, sesamol, eugenol, thymol, carvacrol, vanillin, salicylaldehyde, limonene, geraniol, 4-hexylresorcinol). Zhang et al. [61] studied the electrochemical properties of 14 flavonoid standards using CV (area under anodic wave and oxidant peak potentials) and the structural parameters (bond dissociation enthalpy and ionization potential) were investigated. These results were compared with the results of four spectrophotometric assays, namely, diphenyl-1-picrylhydrazyl (DPPH) method, Folin-Ciocalteu reagent method, ferric reducing ability of plasma (FRAP) method, and trolox equivalent antioxidant capacity (TEAC), to analyze the chemical reasons for the varying antioxidant activities of flavonoids under different assays. They established that flavonoids showed inconsistent antioxidant activities depending on the assay used. Using CV, the antioxidant activity of the flavonoids in the DPPH, FCR, and FRAP assays were determined mainly by the ease of the charge transferring in the first oxidation step. The results of TEAC assays were primarily influenced by the amount of the charge transfer in the multiple oxidation steps of flavonoids. In the theoretical calculation, the bond dissociation enthalpy (BDE) values of the selected flavonoids had considerably higher correlations with the results of the DPPH assay ($r = 0.89$). Neither the ionization potential values nor BDE values had satisfactory correlation with the antioxidant activities of the flavonoids in the TEAC assay ($r = 0.57$, $r= 0.54$, respectively) [61].

On the base of determination of anodic peak potential according to pH, Rene et al. [62] suggested two possible mechanisms of the behaviour of phenolics on the electrode, for both one- and

two-electron oxidation. The different polyphenols versus superoxide anion radical ($O_2^{\bullet -}$) were investigated in an aprotic solvent dimethylformamide by CV. The results highlight that the proton-transfer and radical-transfer pathways take place for polyphenols, with the relative contributions of the two pathways depending on the polyphenol structure. Polyphenols containing an *o*-diphenol ring (as in flavonoids) were found to have the highest reactivity. The electrochemical behavior and the antioxidant effectiveness of ascorbic acid, caffeic acid, quercetin, catechin, hesperetin and their equimolar binary mixtures were evaluated by means of CV and luminol Co(II)-EDTA chemiluminescence, respectively, with a view to the prediction of a potential prooxidant or synergistic behavior of mixtures of antioxidants [63].

Caffeic acid is very interesting polyphenolics with the ability to induce the highest oxidation current. Thus, it serves as the model compound in the determination of phenolics and their antioxidant activity in the biological samples as showing Photinon et al. [64], who used thick-film screen printed working electrode modified with an iridium-carbon as an electrochemical sensor. Oxidation mechanism of caffeic acid was also studied by CV with a plastic formed carbon and/or a glassy carbon electrode in a microflow electrolytic cell in connection with on-line electrochemistry/electrospray ionization mass spectrometry [65].

CV can be utilized widely for identification of polyphenols and estimation of the relative reducing strength of flavonoids, phenolic acids and resveratrol in biological samples of red and white wine, tea, coffee, juices as well as in blood serum [66-68], for direct evaluation of antioxidant capacity in real samples of red wine and white wine [69,70] by ability to slower methyl linoleate oxidation. Kilmartin et al. [48] and Makhotkina et al. [71] used the CV for the quantification of antioxidants on a carbon electrode in wine samples. Anodic peak potentials in a model wine solution (pH 3.3) supplemented by standards within the concentration range from 0.1 to 0.2 mM were studied. In the study [72], the authors investigated the antioxidant potential of yellow dye synthesized by fermentation *Epicoccum nigrum* Link. strain. It was found that the fungal dye antioxidant activity in stable emulsions can be associated with its electrochemical behavior.

2.1.2 Determination of antioxidant activity of clinical samples

CV can be also widely applied in clinical studies. CV on a bare glassy carbon electrode was applied to determine human and horse plasma antioxidant activity [25]. The CV response of human plasma consisted of two broad voltammetric peaks observed within the potential range from 0.2 to 0.6 V and from 0.6 to 0.9 V. Horse plasma showed no voltammetric response on the non-activated bare glassy carbon electrode. Parameters that indicate the antioxidant capacity of the samples, i.e. the peak potential Ep, the peak current density i(p) and the charge Q below voltammetric waves were calculated for both waves. Based on the CV scans measured in human and horse plasma, which have indicated considerably different concentration of low-redox potential low molecular weight antioxidants, it can be concluded that the Total Antioxidant Status results include contribution of the high formal redox-potential low molecular weight antioxidants (LMWAs) comprising the second CV wave.

Interesting study was published by the scientific team from the university in Toulouse [73]. In this study, CV is proposed as a new method for the evaluating the antioxidant capacity of skin based on the reducing properties of low molecular weight antioxidants. Experiments were performed simply by recording the anodic current at 0.9 V of a platinum microelectrode placed directly on the epidermis surface without any gel or water. This method ensured a direct, rapid (less than 1 min), reliable (accuracy 12%) and non-invasive measurement of the global antioxidant capacity of the *stratum corneum* with a high spatiotemporal resolution.

Further, CV was used for the evaluation of the antioxidant status and the oxidative stress in healthy subjects and in chronic (*diabetes mellitus*) and acute patients (subjected to total body irradiation prior to bone marrow transplantation) [74]. CV could be widely employed for rapid evaluation of the status of subjects (in health and disease) for monitoring of their response to treatment and/or nutritional supplementation as well as for screening of specific populations [74]. In the human blood plasma of patients suffering from *diabetes mellitus*, antioxidant properties of thioctic acid were determined [75]. CV method was also used for the monitoring of the total oxidant-scavenging capacities of plasma from glycogen storage disease type Ia patients, where focus was devoted especially to the determination of uric acid as one of the most powerful antioxidants in organisms [76]. Barroso et al. published interesting study entitled "Electrocatalytic evaluation of DNA damage by superoxide radical for antioxidant capacity assessment" [77]. The integrity of DNA purine bases was herein used to evaluate the antioxidant capacity. Unlike other DNA-based antioxidant sensors reported so far, the radical enzymatically generated by the xanthine/xanthine oxidase system was the selected damaging agent. An adenine-rich oligonucleotide was adsorbed on carbon paste electrodes and subjected to radical damage in the presence/absence of several antioxidant compounds. As a result, partial damage on DNA was observed.

2.1.3. Study of the antioxidant activity in pathological processes and infectious diseases

Though generation of oxidative stress and depletion of LMWAs is not the main pathological process of infection diseases, the process follows the other pathologies presented in infected hosts. The pathological processes become the most evident during sepsis [78,79]. Suitability of exogenous LMWAs application is discussed in several studies [80,81]. Especially, mitochondria seem to be the most vulnerable by oxidative stress during sepsis and LMWAs application would be useful for their protection against oxidative damage during sepsis [82,83]. Uric acid and glutathione are the two most important LMWAs (Fig. 1). They show strong antioxidant capacity and, in addition, there are also markers of the "antioxidant protection" of organism [6,84-86]. Total antioxidant capacity and oxidation stress in organism are two important markers of immunity failure and eventually seriousness of a disease [87-90]. Alterations in antioxidant status are clearly evident in chronically developing diseases as well. Human immunodeficiency virus (HIV) positive patients are recommended to intake elevated doses of ascorbic acid and vitamin E. An ascorbic acid in the dose of 750 mg per a day is recommended as the lower plasmatic level of ascorbic acid is associated with faster progression of the disease [80,81,91].

In addition, we can example an effort to assess the role of oxidative stress in seriously infected laboratory animals. The hosts were infected with *Francisella tularensis* Dorofejev, a causative agent of tularemia disease, in a group of experiments. CV played a significant role in the assay. *F. tularensis* is able to survive stress conditions in macrophages where it proliferates [92]. Escape from the macrophages is caused by a pyroptotic process and reactive oxygen species are released, too. Use of CV for assay of LMWAs in tularemia infected hosts was chosen in some experiments as the method is gently to consumption of samples and fast enough to process high quantity of samples in a short time. The experiments were performed on screen printed sensor strips with platinum working and auxiliary electrodes. Reference electrodes were made as a composite of Ag/AgCl. In a total, 20 µl of sample was enough for one successful assay. The plasma sample was injected on sensor and antioxidants appeared as an anodic wave once antioxidants become electrochemically oxidized. The experiments proved different susceptibilities in murine and microtine hosts. Though CV proved depletion of antioxidants in plasma of mice [93], common voles and even European brown hares were able not only re-cover but also increase plasmatic level of the assayed antioxidants [93,94].

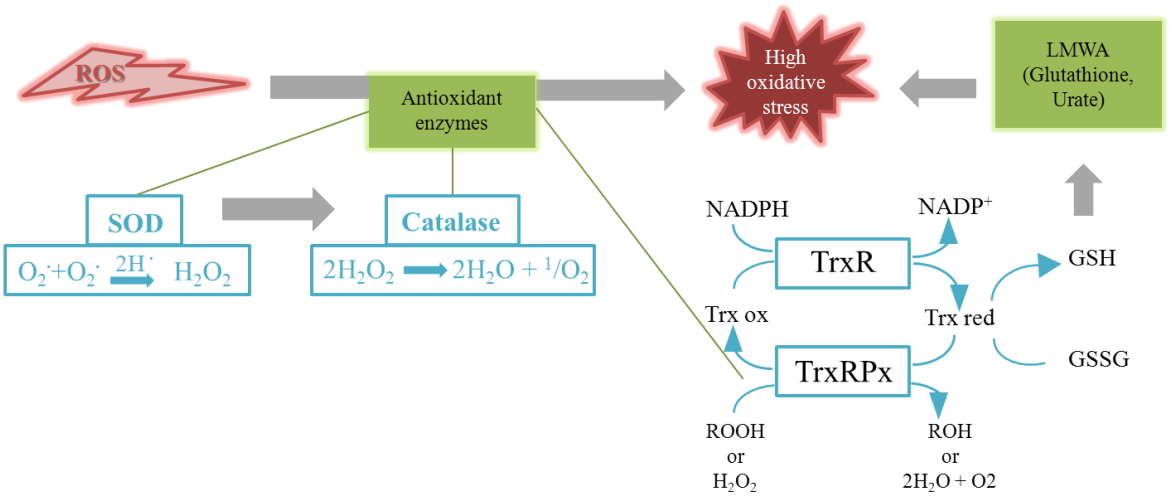

Figure 1. Scheme of the genesis of oxidative stress in the connection with reactive oxygen species (ROS) and protective systems provided by antioxidant enzymes superoxide dismutase (SOD, enzymes responsible for the conversion of the superoxide anion into hydrogen peroxide in the cytoplasm and in the mitochondrial matrix [95,96]), catalase (CAT, catalyzes the dismutation of hydrogen peroxide to oxygen and water [97]) and LMWAs, especially glutathione and uric acid (urate). Modified according to Graca-Souza et al. [98]. Abbreviations: ROS - reactive oxygen species; $O_2^{\cdot -}$ - superoxide anion; H_2O_2 - hydrogen peroxide; SOD - superoxide dismutase; TrxR - thioredoxin reductase; TrxPx - thioredoxin peroxidase; Trx red - reduced thioredoxin; Trx ox - oxidized thioredoxin; GSH - reduced glutathione; GSSG - oxidized glutathione; LMWA - low molecular weight antioxidant.

Chromatographic assay of reduced glutathione and antioxidants assayed by DPPH test confirmed the findings about antioxidants alterations assayed by CV. The level of reduced glutathione decreased to 25% of the original level, and the total level of low molecular weight antioxidants was less than 50% of the initial amount [99]. In the work entitled “Evaluation of the redox properties and

anti/pro-oxidant effects of selected flavonoids by means of a DNA-based electrochemical biosensor" of Lauda et al. [100], quercetin and rutin as well as catechin and epigallocatechin gallate were investigated, as widely distributed representatives of flavonols and flavanols, respectively, regarding their anti-/pro-oxidant properties. The flavonoids were irreversibly oxidized on a dsDNA-modified screen-printed electrode within 0.368 to 0.449 V vs. SHE without binding to DNA. Using the DNA biosensor the detection scheme of a DNA prevention/degradation exploits the $[Co(phen)(3)]^{3+}$ complex as an electrochemical DNA marker. Antioxidant activity of flavonoids was tested in a model cleavage mixture composed of 5×10^{-7} M $[Cu(phen)(2)]^{2+}$ as the catalyst, 1×10^{-3} M ascorbic acid as the chemical reducing agent and atmospheric oxygen as the natural oxidant, where reactive oxygen radicals are generated. The antioxidant activity increases with the concentration of flavonoids reaching a maximum where pro-oxidative behavior becomes of importance. The prooxidant potency of flavonoids depends on the presence of atmospheric oxygen and follows the order quercetin>rutin>epigallo-catechin gallate>catechin [100].

2.2 Differential pulse voltammetry for determination of antioxidant capacity

Differential pulse voltammetry (DPV) is a selective and sensitive technique, where the potential is changing linearly with the time (potential linear sweep) superimposed by the potential pulses of the amplitude between 10 and 100 mV for several milliseconds [30,101,102]. Voltage pulse is applied at the end of the drop time; the total time of the drop is directed electronically by a drop knocker. The difference between the currents applied immediately before the pulse application and at the end is registered. Dependence of the difference between these two currents on the applied potential goes through maximum, so it has a peak shape. The position of the peak on the potential axis is given by the quality of analyte, and its height depends on the concentration of the analyte [103,104].

2.2.1 Determination of antioxidant activity of food and polyphenolics compounds

Possibilities of the DPV application have been explored in the detection of natural polyphenolic antioxidants in both complex biological samples and matrixes containing several phenolics classes such phenolic acids and flavonoids, but also in the determination of antioxidant/oxidation damage of the clinical samples [105]. The analytical possibilities of this method can be used both, representative standards and real samples where the predominant flavonoid and phenolic acid classes are presented in different concentration. The DPV approach using graphite screen-printed electrodes could represent a quick screening method for the determination of polyphenols in natural extracts, derived from grape, olives, and green tea [106,107]. The pure compounds examined by this method include phenolic acids, flavones, flavonols, catechins, tannins, and oleuropein, a tyrosol esters of elenolic acid [108]. DPV has also helped establish the relationship between voltammetric behavior of samples and their *in vitro* antioxidant power [107]. Seruga et al. [109] analyzed samples of red wine in the view of total polyphenols content by DPV method. They determined strong correlations between the amount of total polyphenols determined by DPV, HPLC and spectrophotometrically using the Folin-Ciocalteu method.

In general term, the high antioxidant power was always related to predominant flavonoid and phenolic classes (acids and flavan-3-ols and flavonols) while the low antioxidant power was related to finger prints phenolic like dihydrochalcone phlorizin and glycosylated hydroquinone arbutin. In addition, a selective oxidation of flavonoids versus phenolic acids (at pH 7.5) was strategically proposed approaching the discrimination of different matrices and fruits (mainly at pH 2 the highest peak resolution was obtained Blasco et al. [110] RSDs < 9 %, recovery 100 %, LOD 0.1-4.2 μM) [107]. Antioxidant capacity of the wine samples was also investigated by Souza et al. [111]. In this study, the values obtained for gallic acid were used to estimate the antioxidant properties of the wine sample based on gallic acid oxidation. The suggested method is based on the gallic acid oxidation process at a modified carbon paste electrode (MCPE) containing 30% (*m*/*m*) of carbon nanotubes monitored at 0.35 V versus Ag/AgCl (KCl 3 M). Scientific team in Chile [112] used the DPV for the determination of electrochemical behavior of Chilean Cabernet Sauvignon red wine, *Vitis vinifera* L. grape and raspberry anthocyanin extracts in a model wine solution. Recently published study of Yardim [113] is focused on the determination of antioxidant capacity in the samples of coffee on the base of the oxidation peak of chlorogenic acid. Shapoval et al. [114] used DPV to study the antioxidant activity of thiamine (vitamin B 1) and its structural analogues at platinum and copper cathodes. The results indicate that the activity of thiamine relative to the hydroxyl radical is lower than the activity of 3-benzyl-4-methyl-5-(2-hydroxyethyl)thiazolium chloride. At the same time, thiamine can more efficiently react with hydrogen peroxide and bind divalent iron ions.

2.3 Square wave voltammetry for determination of antioxidant capacity

Another pulsed voltammetry technique called square wave voltammetry (SWV) uses a potential waveform. The advantage of SWV is that the entire scan can be performed on a single mercury drop in about 10 seconds. SWV saves time, reduces the amount of mercury used per scan by a factor of 100 [115-119]. SWV was found to be more sensitive in comparison with differential pulse voltammetry [106,120-122] and has more extend dynamic range and lower limit of detection in comparison with the linear sweep voltammetry [123,124] and the CV [125-128].

2.3.1 Determination of antioxidant activity of food and polyphenolics compounds

Adam et al. [129] suggested and optimized SWV method employing a carbon paste electrode for the determination of selected flavonoids and semisynthetic derivatives (quercetin, quercitrin, rutin, diosmin and chrysin) in the body fluids. They were primarily focused on the basic electrochemical behavior of the studied compounds. In the case of quercetin, quercitrin and diosmin, authors observed three peaks, where the first peak provided the highest signal. The first peak was observed at the potential of 0.25 V (quercetin), however, this first peak occurred at the potential of 0.35 V in quercitrin and at 0.51 V in diosmin. Malagutti et al. [130] used SWV with solid carbon polyurethane electrode (rigid carbon-polyurethane composite electrode) for comparing antioxidant capacity of green tea samples and determination of rutin. Detection limit was 7.1×10^{-9} M. Giannakopoulos et al. [131]

studied electrochemical interphase adsorption of a plenty of polyphenolics (i.e. polyhydroxybenzoic acids) on a hanging mercury drop electrode (HMDE) using the square wave-adsorption cathodic stripping voltammetry (SW-AdCSV) method at pH = 7.5. Polyhydroxybenzoic acids bearing one 4-hydroxybenzoic acid, two 3,4-dihydroxybenzoic acid (protocatechuic) or three 3,4,5-trihydroxybenzoic acid (gallic) hydroxyl groups at the positions C3, C4 and C5 on the benzene ring were studied. The complex interfacial electrochemical behavior of these molecules has been deconvoluted to (i) adsorption evens and (ii) redox formations at the HMDE interface. The approach presented was based on the comparative analysis of the SW-AdCSV signals in conjunction with the molecular structure of the molecules. Accordingly, theoretical calculations, involving nonlinear-fit, resulted in the identification of the interfacial reaction mechanism of adsorbed gallic acid on the HMDE. Moreover, a simple and sensitive electrochemical method for the determination of antioxidant capacity was investigated [20]. 4-hydroxybenzoic acid (4-HBA) was used as a trapping agent for photogenerated (center dot)OH radicals, leading to formation of 3,4-dihydroxybenzoic acid (3,4-DHBA). Addition of antioxidants induced the competition between 4-HBA and antioxidants toward (center dot)OH elimination, resulting in a decrease of the measured current. The suggested method was successfully compared to a fluorimetric one.

2.3.2 Determination of antioxidant activity of clinical samples

Study of Pohanka et al. [132] introduced analysis of LMWAs in blood plasma by the use of SWV. Plasma samples from five Cinereous Vultures (*Aegypius monachus* Linnaeus) accidentally intoxicated with lead were used to represent real biological matrices with different levels of LMWAs. Blood was collected prior to and one month after treatment with Ca-EDTA from the birds. SWV measurements resulted in two peaks. The first peak, with the potential value of 466 ± 15 mV, was recognized as ascorbic and uric acids, while the second one (743 ± 30 mV) represented glutathione, tocopherol, ascorbic acid and in a minor effect by uric acid, too. Contribution of individual antioxidants was recognized by separate assays of LMWA standards.

Scientific team from the Shandong Agricultural University [133] evaluated DNA damage and antioxidant capacity of protein sericin by a DNA electrochemical biosensor based on dendrimer - encapsulated Au-Pd/chitosan composite by SWV. The relationship between sericin concentration and scavenging percentage of ·OH was studied. With the increasing concentration of sericin, the scavenging percentage increased too. The antioxidant capacities of the sericin in comparison to that showed for the Trolox (89 %) were slightly differed.

Determination of a DNA damage by SWV, but also by CV and the electrochemical impedance spectroscopy (EIS) is described in the study of Ziyatdinova and Labuda [134]. They assembled DNA biosensor composed of dsDNA adsorptive layer on a carboxylated single-walled carbon nanotubes-chitosan composite deposited at a commercial carbon based screen-printed electrode. This sensor was prepared and applied for a complex investigation of damage to DNA by the Fenton type cleavage agent (hydroxyl radicals formed in the mixture of Cu^{2+}, H_2O_2 and ascorbic acid) and copper(II)-quercetin system in 0.1 M PBS pH 7.0 under aerobic conditions. Initial enhancement of the intrinsic

guanine and adenine moieties SWV response over that of original dsDNA one indicates opening of the helix structure in the first stage of damage. At the same time, decrease in the intercalated thioridazine response confirms damage of the helix structure in parallel to deep degradation of the DNA chain and its removal from the electrode surface [134]. Using SWV, oxidation stress induced by UV radiation was studied on DNA [135]. Techniques is based on the enzymatic conversion of the damaged DNA bases to single-strand breaks (ssb), single-stranded (ss)DNA regions, or both. Supercoiled DNA exposed to UV light was specifically cleaved by T4 endonuclease V, an enzyme recognizing pyrimidine dimers, the major products of photochemical DNA damage. This technique offers much better sensitivity and selectivity of DNA base damage detection than any other electrochemical method. It is not limited to DNA damage *in vitro*, but it can detect also DNA base damage induced in living bacterial cells [135].

We decided to use SWV method on graphite screen printed electrodes for the determination of antioxidant capacity of blood samples of patient suffering from malignant head disease in our internal study. This patient (6 years old child) has diagnosed neuroblastoma. Voltammogram showed two peaks marked as a_1 and a_2. Peaks a_1 and a_2 were positioned at 562 ± 20 mV and 839 ± 17 mV, respectively. Typical voltammogram of a plasma sample is shown in Fig. 2. Average square of the peaks was 6.88×10^{-3} μAV for the peak a_1 and 4.24×10^{-3} μAV for the peak a_2. Though the peak a_1 was present in all of the tested samples, the peak a_2 was nearly depleted in some samples. It could be appointed that significant pathology related to not all of the endogenous antioxidants but to only a specific group of them, but their effect is not fully understood.

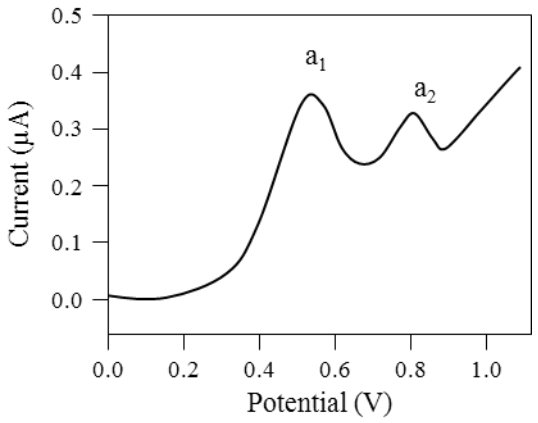

Figure 2. Typical voltammogram achieved by a plasma assay. Two peaks are measured: peak a_1 at the lower and a_2 at the higher potential.

2.4 Amperometric measurement

The amperometric method is based on measurement of electric current resulting from oxidation of the substance (or the mixture) being studied on the surface of a working electrode at a certain voltage potential. The nature of the working electrode as well as the voltage potential applied determines the sensitivity of the amperometric method. The antioxidant activity may be measured by using the value of oxidation of such compounds on the working electrode of the amperometric

detector. The signal is registered as differential dynamic curves [136]. In the summarizing review of Balsco et al. [106] there is clearly concluded that the amperometric method used for an evaluation of antioxidant capacity had the same advantages and disadvantages as the compared spectrophotometric methods.

2.4.1 Determination of antioxidant activity of food and polyphenolics compounds

Large study focused on the determination of compounds with antioxidant properties in the food by the help of amperometric detection was carried out by Yashin et al. [136], who analyzed 1140 food products and beverages and determined total antioxidant capacity of these products. Moreover, in the study of Ignatov et al. [137] a method for the electrochemical detection of antioxidants, which is based on a radical measurement with a cytochrome c modified electrode, was developed. A controlled enzymatic production system for superoxide radicals based on xanthine oxidase was used. The addition of antioxidants facilitated the decomposition of the radical in addition to the spontaneous dismutation. The steady-state of superoxide generation and decomposition was thus shifted to a new situation due to the higher decomposition rate after antioxidant addition. This resulted in a decreased current level at the electrode. Antioxidant activity could be quantified from the response of the sensor electrode by the percentage of the signal decrease. The applicability of this method was showed by analyzing the hierarchy of antioxidant capacity of flavonoids.

Korotkova et al. [37] introduced a highly attractive, convenient and especially sensitive amperometric approach for the study of antioxidant properties and determination of their activity in the work, where antioxidants are substances, which interrupt radical-chain oxidation processes in organic and inorganic molecules. The comparative analysis of the activity of well-known antioxidants such as ascorbic and citric acids, glucose, their compound solutions, some food products (green tea extract, apple vinegar) and pharmaceuticals (Haemodesum, Polyglucinum, Ringer solution) was carried out. The character of the antioxidant influence on the oxygen electrochemical reduction was investigated.

Moreover, a new approach for antioxidant capacity determination was suggested in order to reduce the risk of electrochemical interferences [138]. New method takes advantage of using a H_2O_2 amperometric biosensor (based on horseradish peroxidase wired by Os-redox polymer), measured at low applied potential (-0.100 V vs. Ag/AgCl). This biosensor was coupled with the xanthine-xanthine oxidase system which, as a result of the spontaneous dismutation of superoxide radicals ($O_2^{\cdot}$), generates H_2O_2. The addition of an antioxidant, by its scavenging ability for $O_2^{\cdot}$ radicals as well as by consumption of H_2O_2 decreased the H_2O_2 concentration. Finally, the antioxidant capacity for real samples of citrus juices were assessed by using the new approach, and were found in good correlation with those provided by the method based on electrochemical detection of $DPPH^{\cdot}$, as well as with their polyphenolics content, estimated using the Folin-Ciocalteu method, and their antioxidant activity content, assessed using a titrimetric method [138].

2.4.2 Determination of antioxidant activity of clinical samples

A novel version of a chronoamperometric method of antioxidant determination is described [139]. It is based on the use of the oxidation current of ferrocyanide formed in the reaction of antioxidants to be determined with potassium ferricyanide as an analytical signal. Thus, the total concentration of all antioxidants present in the analyzed solution, i.e. total antioxidant capacity, is measured. The results of blood plasma analysis by the newly presented and certified potentiometry method are in good agreement with each other, R = 0.9980. Study of Brainan et al. [140] describes the monitoring of antioxidant and oxidative activity of blood and blood fractions by the new potentiometric method. The source of information is the electrode potential shift observed when the analyzed sample is introduced into the medium containing a mediator system. Mediator system, measuring electrode, method of blood hemolysis, anticoagulant are selected.

3. FLOW SYSTEMS

The flow injection techniques are main techniques competitive to discrete systems, developed mainly for clinical analysis and environmental field measurements. Their present new trend of development is focused on the miniaturization of fluidics and detectors, design of multianalyte detection systems, and also hyphenation with high-performance separation instruments [141-144]. Their great advantage compared to stationary systems is based on the possibility of the automation of these systems [145-148].

3.1 Flow injection analysis with the electrochemical detection

Flow injection analysis with the electrochemical detection (FIA-ED) has a predominant role in the evaluation of antioxidant capacity because it allows to work at selective target potential giving an easy and direct way for both, evaluation of antioxidant capacity, and the quantification of the total antioxidants involved [106,149]. It has similar characteristic as high performance liquid chromatography with electrochemical detection (HPLC-ED), however, the HPLC technique enables to separate individual analytes compared to FIA-ED. In the light of the above-mentioned fact, FIA-ED is more rapid and needs only simple instrumentation. On the other hand, these electrochemical systems that work at the electrochemically constant potential are not able to “distinguish” analytes that are electroactive and electrochemical process on the applied potential [150-152].

3.1.1 Determination of antioxidant activity of food and polyphenolics compounds

FIA with amperometric detection is the commonly used method for the evaluation of antioxidant capacity of phenolic compounds. In addition, this method is adequate for measuring the total phenolics content in food samples, but values are usually lower than those found using the spectrometric method. This fact indicates higher selectivity of electrochemical approach [153].

Mannino et al. [154] developed simple method for determination of antioxidant capacity of wine samples based on the FIA system with amperometric detection with glassy carbon electrode as a working electrode at the potential of +0.4 V. The same research team used the suggested method for determination of antioxidant capacity of olive oil [155] and plant extracts [156]. New method was further tested for the determination of antioxidant capacity of red wine samples, and satisfactory correlation with the Folin-Ciocalteu method was obtained [157]. FIA with amperometric detection was also used for determination of antioxidant activity of total polyphenols and *o*-dihydroxyphenols in extra virgin olive oil samples [158,159]. Blasco et al. [153] used the FIA method with amperometric detection for determination of antioxidant capacity of various food matrices as apple and pear juices, fresh apples, pears and beans, processed beans and wine samples. The same method was used for determination of antioxidant capacity of total polyphenols in honey, propolis and royal jelly [149,160].

3.2 High-performance liquid chromatography with electrochemical detection

Recently, the method for robust and rapid electrochemical screening of antioxidant capacity (RESAC) which was correlated with common used photometric assays, was developed [19]. It has been shown that RESAC and classical HPLC-ED of antioxidant activity has many advantages as easy to use, high speed and a low price of analysis as well. These systems are especially valuable for detection of electroactive molecules, which can be also separated by reverse phase column i.e. that nonpolar character of the determined substance, is required. Majority of flow electrochemical detectors used in clinical or nutrition diagnostic applications uses the potentiostatic principles [70,161-163]. In HPLC-ED systems, the detector is consisting from counter electrode, reference electrode and working electrode. The working electrode can be made from different materials and based on its total area. We can easily distinguish between planar electrodes for amperometric detection and spherical electrodes as flow-through electrodes for coulometric detection. The planar electrodes represented by gold, platinum, silver, glassy carbon or boron doped diamond electrode have different upper potential limitation for oxidation of the substances but they allows to detect mostly 1 - to 5 % of the analyte, which is passing over the surface of the cell based on the redox change. In the case of the flow-through electrodes represented by porous graphite more than 90 % of analyte passing through the electrode can be changed and therefore total electric charge of the passing analyte can be detected in Coulombs [164].

3.2.1 Determination of antioxidant activity of food and polyphenolics compounds

Array techniques are frequently applied for determination of polyphenolics profile and antioxidant activity of plant matrices, which include vegetables and fruits. Aaby et al. [165] analyzed phenolic compounds in strawberry (*Fragaria* x *ananassa* (Weston) Duchesne ex Rozier) fruits. Electrochemical profiles obtained from the coulometric array detector contributed to the structural elucidation of 40 compounds. Quercetin-3-malonylhexoside and a deoxyhexoside of the ellagic acid were reported for the first time as a result of this method. Antioxidative properties of individual

components in strawberries were estimated by their electrochemical responses. Fedina et al. [166] determined the content of total antioxidants in the food of plant origin by the HPLC with amperometric detection. They investigated herb extracts, tea, coffee, wine, brandy, balsams, beer, vegetables, fruit, and berries. Hoai et al. showed possibility of using HPLC fingerprinting methodology [167]. In that paper, HPLC fingerprints of different lengths (time of analysis) were developed for a number of *Mallotus* Lour. species. Further, a multivariate regression model was constructed to model the antioxidant activity of the *Mallotus* samples from the HPLC fingerprints with the aim to indicate peaks possibly responsible for this activity. For this purpose, after data pre-treatment, the calibration technique partial least squares (PLS) was applied [167]. In addition, determination of antioxidant capacity of less common fruits, such as honeysuckles, Saskatoon berry and Chinese hawthorn was performed by Gazdík et al. [168]. The antioxidant content evaluation was based on the total flavonoid amount, determined by HPLC-ED. Sochor et al. [11] monitored the antioxidant capacity of apricot fruits (20 cultivars in total). The results of study confirmed correlation with photometric methods. Described method is suitable for the evaluation of the biological value of fruit using the value of antioxidant capacity. Comparative analysis of radical scavenging and antioxidant activity of phenolic compounds occurring in everyday use spice plants was carried out by team of Stankevicius et al. [169]. Studies using HPLC system with electrochemical detector showed that bioactive phytochemicals can be separated and antioxidant activities of individual compounds evaluated without the need of a complex HPLC system with reaction detector. The results obtained using electrochemical detection correlate with the radical scavenging activity assayed using spectrophotometric method ($r = 0.893$). Moreover to these results, electrochemical analysis with the use of the HPLC technique of the total content of phenolic acids with antioxidant activity and polyhydroxylated derivatives of flavan was the technique selected for the determination of the total antioxidant capacity in the extracts of the berries of less common fruit species. Peaks showing detected antioxidant complex were summarized and their areas were then integrated to obtain the relative values. Obtained data was interpreted as a relative antioxidant capacity [170]. Polish team of scientists [171] determined total antioxidant potential of biogenic amines in alcoholic and non-alcoholic beverages. The results of this study revealed that suggested assay gives additional information about the $OH^{\cdot}$ scavenging performance and antioxidant properties of pure chemicals and complex food samples, in contrary to the described assays based on weaker radicals (peroxyl radical, DPPH radical, NO radicals, ABTS cation radical or superoxide anion radical).

3.2.2 Determination of antioxidant activity of clinical samples

The application of electrochemical detectors is useful in nutrition dependent clinical studies prior to cancer based on antioxidants because of the noise is reduced and this system enabled identification of the target substance in one retention time due to detection cell which can contain two-channel electrode [172,173]. Tea polyphenols, such as epicatechine and epigallocatechine, in urine and plasma samples using identification based on retention time and electrochemical profile is very useful method in studies concerning chemically induced tumourigenesis [173]. Determination of

polyphenolics level in blood, which shows on its antioxidant status, urine and rectal tissue after consummation of green tee by subjects suffering from colorectal carcinoma, and correlation with prostaglandin levels was determined by enzyme-linked immunosorbent assay (ELISA). Decreased levels of prostaglandin in rectal mucosa were observed at 4 and 8 h after consumption of green tea. Ten of 14 subjects demonstrated a response to green tea, as evidenced by at least a 50% inhibition of prostaglandin levels at 4 h. The conclusion of this study is that green tea constituents have biological activity in inhibiting prostaglandin synthesis [172]. The method for easy extraction of catechins or theaflavines from plasma, urine or tissue samples was suggested [174]. In addition, more sophisticated extraction method based on column switching and thus on the completely automated online extraction was designed and successfully tested for determination of vitamin E and Coenzyme Q10 in reduced and oxidized form in serum [175]. Next to the nutrition antioxidant questions the diagnosis the vascular diseases based on the determination of total homocysteine in plasma was done using the optimized isocratic elution and amperometric detection using glassy carbon working electrode [176].

There is a number of studies, which are using multi-channel coulometric detector for determination mainly the nutrient dependent studies of antioxidant levels as polyphenols in body fluids because it enables higher sensitivity and lower noise compared to amperometric detectors [177]. The catecholamines quantification in body fluids belongs to the most frequent application for the Coularray detector (coulometric detector manufactured by ESA Inc., USA) in antioxidation research area. Stability, sensitivity and reproducibility of the detector were estimated [178]. The complex study focused on the bioavalability of gallated and non-gallated forms of catechins were done through electrochemical determination of catechins in the samples of plasma, urine and faces of the tested subject after consumption of black tea [179] and in the plasma of subjects after the consummation of green tea [180]. Observation of the formation of conjugates of quercetin in blood by the determination of various forms of conjugates in the plasma was performed [181]. Determination of hydroquinone in human urine supported by the computer assisted verification was designed [182]. For the detection of some specific metabolites like homocysteine, the clinical marker of cardiovascular diseases, which normally must be derivatized because of fluorescent detection, the coulometric detector does not require any of these steps and thus avoids the complications with stability of derivative and the possibility of sample storing can be prolonged [183]. The homocysteine also was precisely detected in neonates [184]. The similar conclusions were achieved during determination of cysteamine, cystamine, thialysine and other sulfur containing metabolites in plasma and in the brain of the rats [185]. Even for metabolomics studies is the usage of coulometric detector for determination of the compounds of interest in the human plasma very beneficial [186]. In contrast to the increasing of numbers of applications for coulometric detection the issues, which can critically influence this type of detector, must be properly clarified and tested [170]. Glod et al. [187] determined antioxidant potential of blood plasma by the use of an improved of reversed-phase HPLC with electrochemical detection. Method was used for the estimation of antioxidant profile in blood plasma of patients treated with various drugs. In the study “Application of RP-HPLC-ED assay to analysis of the blood of Parkinson's disease patients” [188], there was determined total antioxidant potential in blood serum of patients treated with anti-Parkinson drugs. It was found that it increases in the concentration of hydroxyl radicals in blood serum, and of the total antioxidant potential related to the hydroxyl radical was inversely correlated

with the concentration of catecholamines in the serum. This suggests that catecholamines are pro-oxidants of hydroxyl radicals.

3.2.3. Electrochemical index

Content of compounds with antioxidant activity may be expressed as the electrochemical index. This term was originally established by Blasco et al. [153]. The most important aspects involved in it are: **a)** All phenolics react chemically with Folin-Ciocalteu reagent under specific experimental conditions (presence of Folin-Ciocalteu reagent, Na_2CO_3, room temperature). In the electrochemical protocol, other experimental conditions at which all polyphenolics should give a suitable electrochemical signal have been optimized. **b)** The spectrophotometric protocol is based on the chemical reaction of phenolic compounds (chemical reaction: complex formation), and this reaction being the basis of the method. Once the chemical reaction produces a "total signal" (absorbance at 750 nm). The electroanalytical protocol is based on the oxidation of polyphenolic compounds (electrochemical reaction: oxidation of alcohol(hydroxyl) group/s (OH) to ketone groups (C=O), and this reaction being the basis of the method. **c)** The total signal obtained in the spectrophotometric protocol is transferred to concentration units using a suitable standard. The previously established total signal in the electrochemical protocol is also transferred to concentration units using a representative standard. The choice of the standard is very difficult, because several phenolics classes occur in the extract. The most appropriate standard is selected considering the nature of the food medium studied. The "total signal" could be defined as (i.e.) the total amperometric current obtained at controlled potential (0.8 V) and neutral media (pH 7.5) from the oxidation of all polyphenolics. We suggest that the corresponding concentration obtained ("total phenolics") from this "total signal" could be defined as the "Electrochemical Index". In this way, the "Electrochemical Index" could be also understood as an approach for quantification of "total polyphenols" in samples without ascorbic acid (or alpha-tocopherol) and an approach for quantification of the "total natural antioxidants" index in those samples where ascorbic acid (or alpha-tocopherol) could be present [153]. All these data are summarized in Fig. 3.

Gazdík et al. described methodology for the determination of relative antioxidant capacity of samples of less common fruit [161,168]. This methodology directly corresponds to determination of electrochemical index. The antioxidant content evaluation was based on the total flavonoid content determined by HPLC-ED. Peaks showing detected antioxidant complex(es) were summarized and their areas were then integrated (derived) to obtain the relative values. Thanks to ED it is possible to observe reactive kinetics of antioxidants in applied potentials, evaluate the structure of antioxidant complexes and their concern in total antioxidant capacity, whose different functional basis was determined in examined samples [168]. We used this published methodology for the determination of antioxidant capacity of three samples of Canadian blueberry (*Vaccinium myrtilloides* Michx.). Typical obtained chromatograms are shown in Fig. 4.

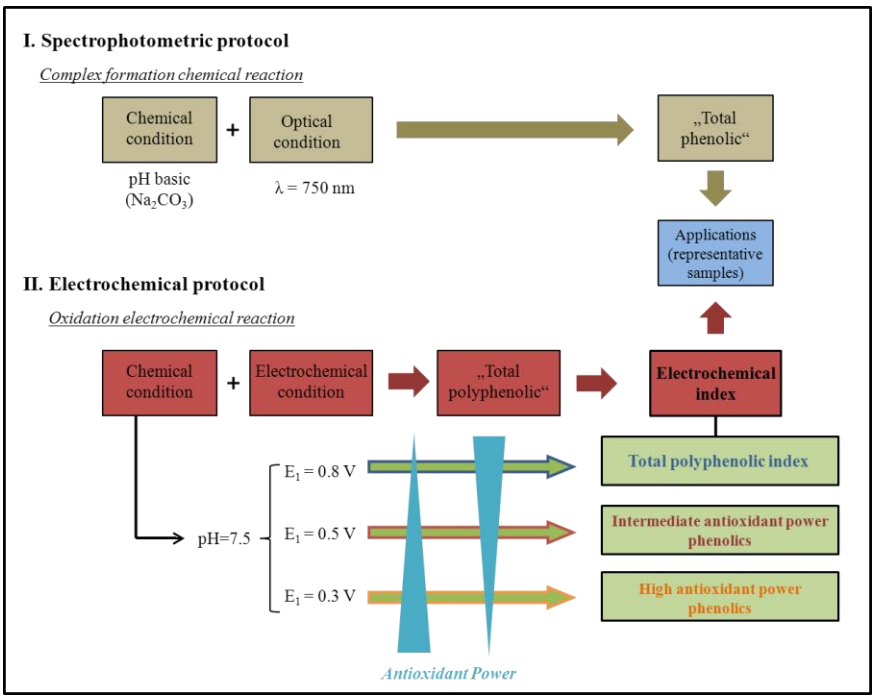

Figure 3. Definition/introduction of the "Electrochemical Index" concept. Scheme shows the keys to introduce the Electrochemical Index concept by comparison with the spectrophotometric protocol. Modified according to Blasco et al. [153].

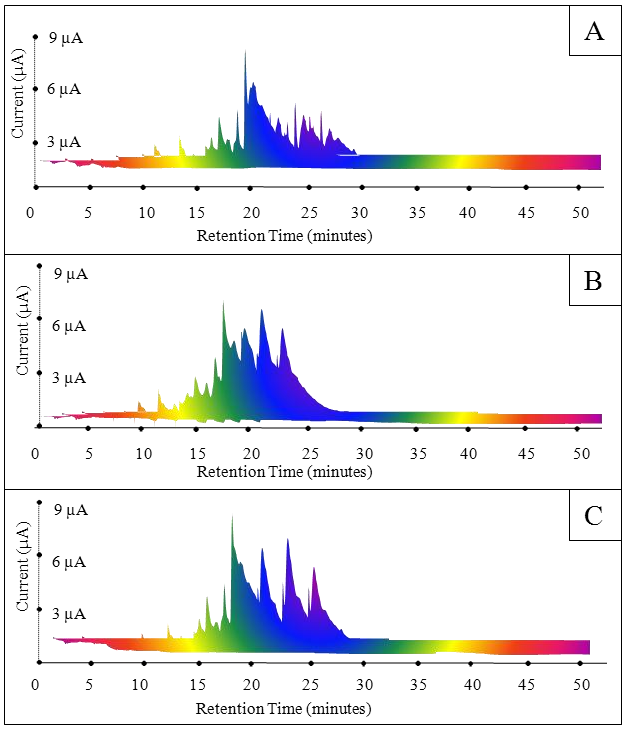

Figure 4. HPLC-ED chromatograms of the samples of Canadian blueberry, respectively its cultivars: **a)** Sinoglaska, **b)** Gerda and **c)** Kamcadalka. Coul-array detector was used. The potentials applied on the electrode were -80, 0, 80, 160, 240, 320, 400, 480, 560, 640, 720 and 800 mV. Summary of peaks of all potentials (summary of all electroactive molecules) is evaluated as the total antioxidant activity.

Electrochemical index is commonly used in the determination of antioxidants in the agricultural products, such as vegetables and fruits, or for the determination of antioxidants in herbs and herb extracts [189]. Polyphenols, ascorbic acid and alpha-tocopherol are the most important antioxidants in food. Electrochemical determination of "total" polyphenols, ascorbic acid and alpha-tocopherol may correspond to the oxidation measurement of all compounds with naturally antioxidant properties, which are common in food. In this view, we can understand electrochemical index as an expression of "total natural antioxidants". On the other hand, it can be interpreted also as a content of total polyphenols, especially in samples without ascorbic acid and alpha-tocopherol, it means as a "polyphenol index" [107,153,189,190]. Electrochemical index well correlated with the content of total phenolics determined spectrophotometrically in wine samples (r=0.99), in soya products samples (r=0.95) and in samples of honey. All these results indicate the possibility and suitability of the test for the determination of polyphenolics as an alternative method to spectrophotometric measurement. In addition, electrochemical index highly correlated with the antioxidant capacity (r=0.90). This fact indicates that the electrochemical protocol is becoming the new tool for the determination of antioxidant capacity [149,191]. Due to this fact, electrochemical index can be also called "antioxidant index".

4. CONCLUSION

This contribution accentuates the role of electrochemical techniques in the determination of antioxidant activity/capacity in the biological samples of plant origin and in clinical samples. Electrochemical techniques represent due to selectivity and sensitivity suitable tool for the determination of antioxidant capacity in biological samples, where these method are sensitive under the low concentrations of antioxidants. In addition, obtained peaks and their position enable to estimate possible antioxidant present in a sample. There are high demands on the rapid, accurate and specific analysis in the field of clinical diagnostics. Possibility of continuous monitoring and multiplexing is also very important.

Because total and direct antioxidant determination versus individual ones is highly desired, the main electrochemical approaches have been used the potential-controlled techniques: cyclic voltammetry, differential pulse voltammetry, other voltammetric approaches as well as a flow injection with amperometric detection. All these techniques must be appreciated in the light of the correlation with commonly used spectrophotometric methods [192].

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