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Total antioxidant potential assay with cyclic voltammetry and/or differential pulse voltammetry measurements

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ABSTRACT

In the literature many different TAP assays are described. They use various analytical methods. However, it seems that *natural* methods of investigation of the redox reactions are electroanalytical techniques. Indeed, TAP has been already estimated potentiometrically and voltammetrically.

In the paper we propose new electrochemical assay of TAP measurements. In this case TAP measure is the integral of the product of current and exponent from the potential (related to the standard reduction potential of the hydroxyl radicals) along normal or differential pulse voltammetric peak. It was found that TAP values can be accurately and robustly measured using described assay. They are directly proportional to the sample concentration and dependent on its redox potential. The proposed assay has been tested on pure compounds and on the mixtures. It turned out that in the second case TAP is an additive value for non-interacted compounds. On the other side, it is non-additive for compounds interacting with each other. As the real samples to test elaborated assay the extracts of various herbs are selected. Melissa is characterized by the strongest antioxidative properties.

1. Introduction

Free radicals are atoms, compounds or ions, with the odd numbers of electrons, characterized by paramagnetic properties. They contribute to cellular degenerative changes, aging and are implicated in many diseases (cancer, Alzheimer's and Parkinson's diseases, myocardial infarction, rheumatoid arthritis, etc.) [1,2]. They are present in every living cell. In the pathological conditions their over-production and/or decreased capacity of antioxidant systems induce the redox homeostasis to be severed. This leads to cell degeneration and ultimately-death [3].

During the evolution living organisms elaborated the *defense* systems, antioxidants inactivated the oxidants and free radicals scavengers removing the free radicals [4]. Sometimes, both terms, as well as the terms reducers and antiradicals, are used interchangeably. Generally, antioxidant is defined as a substance (compound) that in small amount inhibits the oxidation (reaction with the free radicals) of other, usually biologically active, compounds. Antioxidants can be analyzed using many analytical techniques [5,6], including electroanalytical ones [7]. It turned out that frequently composition of real samples is unknown. It means that we do not have much confidence whether all antioxidants are estimated or not. Additionally, some of the antioxidants interact with each other inactivating one another or giving synergetic effect [8]. Therefore, instead of the estimation of concentrations of all particular antioxidants in the sample, sometimes more information is obtained from the cumulative potential (total antioxidant potential, TAP, although other names are also used like activity, capacity, reactivity, status etc.) of the sample components to scavenge the free radicals [9].

In the literature many TAP assays are described [10]. They are based on various analytical techniques (photometric, fluorymetric, luminescence measurements, MS, HPLC, GC, thermogravimetric etc.) [11]. The *classical* photometric or fluorymetric tests exploit different free-radical generators (usually thermolabile di-azo compounds generating peroxyl radicals) or stable, colorful radicals. Oxidation of the analyzed sample and what is known as *sensor* is measured [12]. The TAP measure is in this case the inhibition time, induced by the sample. HPLC assays are based on the hydroxyl radicals generation by the Fenton reaction. These radicals react with both, the sensor and the sample. This competition decreases the chromatographic peak of the product of sensor reaction with radicals. This decrease (height or area) is the TAP measure [5]. These procedures present some drawbacks. They require the use of specific reagents and/or time consuming sample preparation.

It seems that *natural* methods of examination of oxidation process should involve electroanalytical techniques [13]. As a matter of fact, in the literature many such methods are already described. Potentiometric one is based on the shift of the electrode potential

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by redox reaction between sample and sensor [14]. The authors used a potential shift of the redox system $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$. The righteous TAP measure is a standard redox potential, which is directly correlated with the half-wave potential in polarography and the voltammetric peak potential. However, this measure can be applied only to rather pure compounds than to the complex, real samples, like food products or blood serum.

Voltammetric (amperometric and coulometric) methods were applied to the electrogeneration of the free radicals or other reactive oxidants [15] or as analytical assays [16]. The electroanalytical techniques have been also used as detection systems in HPLC [17] or FIA [18,19].

Usually, voltammetric data provide two parameters (i) peak or half-wave potential of anodic oxidation and (ii) current limit or peak current, which reflect the concentration of antioxidant. These two parameters were used to evaluate the antioxidative activity [20]. The potentials mentioned are directly related to the potential of formal redox system, $E^{\circ\prime}$. They make it possible to assess whether the oxidation reaction occurs. The formal potential can thus be regarded as a measure of antioxidant power. Using this criterion, Blasco et al. [18] distinguished antioxidants with high ($E^{\circ\prime}$ < 0.3 V) and medium power ($E^{\circ\prime}$ = 0.5 V). Yang et al. [21] proposed the evaluation of the antioxidant power of flavonoids on the basis of the half-wave potential ($E_{1/2}$) of the first wave of anodic oxidation. Regardless of them, Kilmartin et al. used the potentials of first anodic peak of cyclic voltammetry to the characterization of polyphenols in wines, tea and coffee [22]. For the determination of antioxidant activity (in terms of concentration or content antioxidants in the sample) the peak current was used or, less commonly, the area (charge) under wave of the anodic peak [23–25]. A special case is the use of voltammetry in detection of free radicals, such as ABTS or DPPH [26,27] or reduction of oxygen on electrode and then indirect TAP estimation [28]. Another attempt is based on the electrochemical generation of free radicals or, more generally reactive oxygen species, and analysis of the products of their reaction with the what is called *sensors* using other analytical techniques.

In this paper our preliminary results of the application of cyclic voltammetry (CV) and differential pulse voltammetry (DPV) to the estimation of TAP are presented. Glassy carbon has been used as a working electrode. The surface area (where potential is expressed in the exponential form in relation to hydroxyl radical redox potential) of all peaks has been used as a TAP measure. Different kinds of herbs have been used to test the elaborated assay.

2. Material and methods

2.1. Instrumentation

Electrochemical measurements were performed using the Autolab PGSTAT20 potentiostat/galvanostat (Eco Chemie, Utrecht, Netherlands). A three-electrode system was used throughout the study. As a working electrode a glassy carbon (GC) disk, 2 mm in diameter, polished before each measurement, was used. A platinum wire served as an auxiliary electrode, and a saturated Ag/AgCl electrode was used as a reference electrode. System was controlled and data acquisition performed on the IBM PC type computer with GPES v 4.9 software.

Chromatographic measurements were performed by means of a chromatograph comprising an Interface Box, 4 channel Smartline Manager 5000 with Degasser K-5004, Solvent Organizer K-1500, Dynamic Mixing Chamber, HPLC Pump Smartline 1000, Autosampler Smartline 3900 (all from Knauer, Berlin, Germany), ClinLab Digital Amperometric Detector EC3000 (Recipe; Munich, Germany) with glassy carbon working electrode (reference electrode-Ag/AgCl, auxiliary electrode-cell body), and Smartline 4000 Column Thermostat (Industrial Electronics, Langenzersdorf, Austria). Samples were separated on a Eurospher RP-18 5 μm, 250 × 4 mm I.D. (Knauer) column. System was controlled and data acquisition performed on the IBM PC type computer with ClarityChrom V 2.6 2007 software.

Photometric measurements were performed on spectrophotometer Thermo-Spectronic, Helios Epsilon (USA). pH was measured using pH-meter OP-208/1 (Radelkis, Budapest, Hungary) with OSH 10-10 electrode (Metron-WCF, Czekanów, Poland).

2.2. Reagents

1R-(-)-10-camphorsulfonic (CSA), gallic (GA) and ascorbic acids (AA), iron(II) sulfate, phosphate buffered saline (PBS) tablets, 1,1-diphenyl-2-picrylhydrazyl (DPPH) and HPLC grade methanol were obtained from Sigma (St. Louis, MO, USA). All other reagents (Fluka, Buchs, Switzerland; Alchem, Poland; Inform, Poland and POCh, Gliwice, Poland) were of analytical reagent grade and were used without further purification. Water was three times distilled from quartz apparatus. Mobile phases were filtered through a 0.45 μm membrane filter (Millipore, Bedford, MA, USA).

The studies used dried herbs: melissa (*Melissa officinalis L.*), strawberry (*Fragaria L.*), marjoram (*Origanum majorana L.*), salvia (*Salvia officinalis L.*), equisetum (*Equisetum arvense L.*), coltsfoot (*Tussilago farfara L.*), hyssop (*Hyssopus L.*), nettle (*Urtica L.*), wild mint (*Mentha arvensis L.*), calendula (*Calendula L.*), common hollyhock (*Alcea rosea L.*), melilotus (*Melilotus officinalis L.*). They were gathered in June in Siedlce district (the south-west Podlachia region, Eastern Poland, 52°10′N; 22°04′E) which is located 150 m above sea level. Siedlce is a small-sized city with a population of roughly 80,000. It lies at the boundary of the continental climate zone, which is characterized by frosty winters and warm summers. The mean annual rainfall is about 500 mm, and the annual average temperature is 8 °C. The prevailing wind directions are westerly and southwesterly. All plants leafs were normal and had the natural green color. In the summer (June) the sky is sunny and partially cloudy with a rarely rain during the day. The weather was the usual condition for that time of year without any natural disasters. Green tea, cornflower (*Centaurea cyanus L.*) and ginkgo (*Ginkgo biloba L.*) were purchased from the local supermarket.

2.3. Procedures

Electrochemical measurements (10 mL) were performed in 100 mmol L^{-1} $NaClO_4$ used as a basic electrolyte, 1 mmol L^{-1} CSA (added to protect working electrode against irreversible adsorption of the analyzed samples) and 100 mmol L^{-1} PBS, pH 7.4 at a scan rate of 50 mV s^{-1}. All cyclic voltammograms measurements were performed at positive potentials, usually in the range 0.2–1.5 V. DPV measurements were performed at scan rate – 30 mV s^{-1}; pulse amplitude – 30 mV and sampling time – 3 ms. Each sample was analyzed three times. Prior to use, the GC working electrode was prepared by polishing with Micro Polish cloth (Buehler) in 1.0–0.3 μm alumina/water slurries and then rinsed with water. All solutions were degassed (deoxidized) prior to use by 5 min argon percolating.

Chromatographic experiments were performed at 1.0 mL min^{-1} flow rate. Column was stabilized at 20 °C by passing through mobile phase for 1 h prior to the chromatographic measurements. Phosphate buffer (pH 6.6) was used as the mobile phase, 20 μL samples were injected using autosampler. Output signal from the electrochemical detector was continuously displayed on a computer.

Total concentration of the polyphenols has been estimated using modified Folin–Ciocalteau (FC) assay [29]. Briefly, 50 μL of