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A Three-Step Approach to Estimation of Reduction Potentials of Natural Mixtures of Antioxidants Based on DPPH Test; Illustration for Catechins and Cocoa †

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Abstract: The aim of this study is to propose a methodology to assess electrochemical properties of complex mixtures of antioxidants, such as plant extracts, based on the results of simple and popular DPPH test. The first, most difficult step, involves determinations of standard reduction potentials (E^0) for the series of purified compounds (here catechins). The next step is the calculation of stoichiometric values (n_{10}) based on the results of DPPH test for the same compounds. Finally, a correlation equation is formulated, which is then employed to estimate "cumulative reduction potential" (E_c) for the mixture of interest (here cocoa) using DPPH test results.

Keywords: standard reduction potential; stoichiometry value; DPPH test; antioxidants

1. Introduction

The enhancement of endogenous antioxidant defense system through dietary supplementation with antioxidants, such as bioactive phenolic phytochemicals, has been widely accepted and advocated as a reasonable approach to reduce the level of oxidative stress and the risk of associated diseases. Unfortunately, despite the large number of publications about antioxidants, there is still no consensus which parameters should be used as a guidance enabling reliable predictions of the impact of redox active substances on cellular redox homeostasis. It may be presumed that electrochemical properties, i.e., standard reduction potential, of a given substance could be a good predictor of this substance's ability to modulate antioxidant status of the cell. Our previous research for a series of catechins [1] supported this hypothesis. However, the precise determination of this physicochemical parameter is difficult even for pure compounds, not to mention complex mixtures, such as antioxidant rich foods.

In this study, we propose the methodology that enables estimation of E^0-like value for complex mixtures based on results of DPPH test. Initially, electrochemical properties, i.e., standard reduction potentials (E^0), were determined which express the ability of a compound to accept electrons. In the next stage, antioxidant activity of the same set of compounds was evaluated using an easy to perform popular spectrophotometric assay employing DPPH radicals. Importantly, the results of DPPH test were calculated so as to include kinetic aspects of the redox reaction. The created relationship was then used to assess electrochemical properties of cocoa serving as a model of the actual food product rich in antioxidants.

2. Materials and Methods

2.1. Chemicals and Reagents

The following redox active compounds were used for the study: (+)-catechin (C), (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), (−)-epigallocatechin gallate (EGCG) propyl gallate (PG) from Extrasynthese (Lyon, France), *L*-ascorbic acid (AA), isoascorbic acid (iAA), sodium ascorbate (NaAA), calcium ascorbate (CaAA) as well as glutathione (GSH) from Sigma-Aldrich (Saint Louis, MO). Solution of potassium hexacyanoferrate (III) from Sigma-Aldrich (USA) was applied as a titrant in potentiometric titration. For spectrophotometric tests, 1-diphenyl-2-picrylhydrazyl (DPPH) from Sigma-Aldrich (Saint Louis, MO, USA), analytical grade ethanol and methanol from POCH (Gliwice, Poland) were used. Tablets of phosphate buffered saline (PBS) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). PBS solution was prepared by dissolving one tablet in 200 mL ultrapure water from a Millipore Milli-Q system.

2.2. Standard Reduction Potential by Potentiometric Titration

The determination of standard reduction potentials was performed by potentiometric titration as described previously [1]. Briefly, potassium hexacyanoferrate (III) was selected as a titrant. Analysed antioxidants (1 mg/mL) and the titrant were dissolved in PBS (pH = 7.4). Potentiometric titration was carried out vs. 3 M KCl Ag|AgCl reference electrode and a platinum measuring electrode at 25, 37 and 41 °C using JENCO 6230 N, ORP-146 C Micro Oxidation-Reduction equipment (San Diego, CA, USA). Temperature during measurement was maintained by Ultra Thermostat (PolyScience, Niles, IL, USA), while the temperature of the reaction was controlled in the range ±0.1 °C using JENCO 6230-AST thermometer. Mixing of the reactants was ensured by bubbling inert, high purity N_2 gas. The titrant was added to the antioxidant solution in increments of 0.5 mL and potential was read after stabilisation. Each potentiometric titration was performed in three independent repeats. Obtained titration curves (E [mV] vs. $V_{titrant}$ [mL]) were analysed by non-linear regression (Marquardt-Levenberg algorithm) using SigmaPlot (Systat Software Inc., London, UK) software. Obtained reduction potentials of tested compounds vs. standard hydrogen electrode (SHE) were calculated.

2.3. Antioxidant Activity by Spectrophotometic Method

The colorimetric determination of antioxidant activity was performed by standard method employing DPPH radical as described previously [1]. In short, stock solution of radical was diluted in methanol before measurement until absorbance reached 0.9 ± 0.05 at 515 nm. All reactions were carried out in 48-well plates at 25, 37 and 41 °C. Stock solutions of antioxidants were prepared in analytical grade ethanol at a concentration of 10 mM. Stock solutions of antioxidants were diluted appropriately with the same solvents to achieve concentrations falling within a linear range of the assay. The DPPH solution (1 mL) was mixed with solutions of antioxidants (30 µL) and the absorbance was measured at 515 nm after 10 min. The absorbance measurements were performed with the use of a TECAN Infinite M200 spectrophotometer (Tecan Group Ltd., Männedorf, Switzerland).

The results of antioxidant activity evaluations obtained by DPPH test were expressed as stoichiometry values (n) using modified method [2]. The stoichiometry value was determined as a regression coefficient, and defined as the slope of concentrations of a radical scavenged vs. concentrations of the tested antioxidant present in the mixture after 10 min of reaction (n_{10}). The concentration of radicals scavenged by the tested compound in reaction media was calculated with the use of the Beer–Lambert–Bouguer law (Beer's law) according to the equation:

$$S_R = \frac{(A_0 - A_f)}{\varepsilon \times l} \quad (1)$$

where S_R—the concentration of scavenged radicals [M]; A_0—the initial absorbance of the radical solution; A_f—the absorbance of the radical solution after the reaction time 10 min; ε—the molar extinction coefficient (11,240 M^{-1} cm^{-1} for DPPH radical at 515 nm [3]), l—optical path length (1 cm).

2.4. Statistical Analysis

All values are expressed as means ±SD of three independent experiments. Correlations between values of standard reduction potentials and antioxidant activity determined by DPPH test were examined using Pearson's coefficients. All statistical analyses were performed using Prism 4.0 software package (GraphPad Software, Inc., San Diego, CA, USA).

3. Results

The stoichiometry values n_{10} may be regarded as chemical indicators of antioxidant activity. Thus, the higher the n_{10} value is determined for a compound, the better antioxidant properties it exhibits. In turn, E^0 describes the ability of a compound to accept electrons. The lower the value of the standard reduction potential of a compound, the better an electron donor it is, which means that the compound exhibits stronger antioxidant properties. The results of antioxidant activity determinations for 9 redox active compounds expressed as either standard reduction potentials E^0 or stoichiometry values n_{10} were used to formulate the relationship described by the function $n_{10} = f(E^0)$ as shown in Figure 1 (Panel 1 and 2) using epicatechin as an example and measurement temperature of 37 °C. The two sets of values turned out to have strong inverse correlation for all temperatures tested (r = −0.894 for 25 °C, r = −0.889 for 37 °C, r = −0.858 for 41 °C). Based on experimental data determined at 37 °C, the following function equation was calculated: $n_{10} = -19.71x + 7.34$, where x denotes E^0 equivalents.

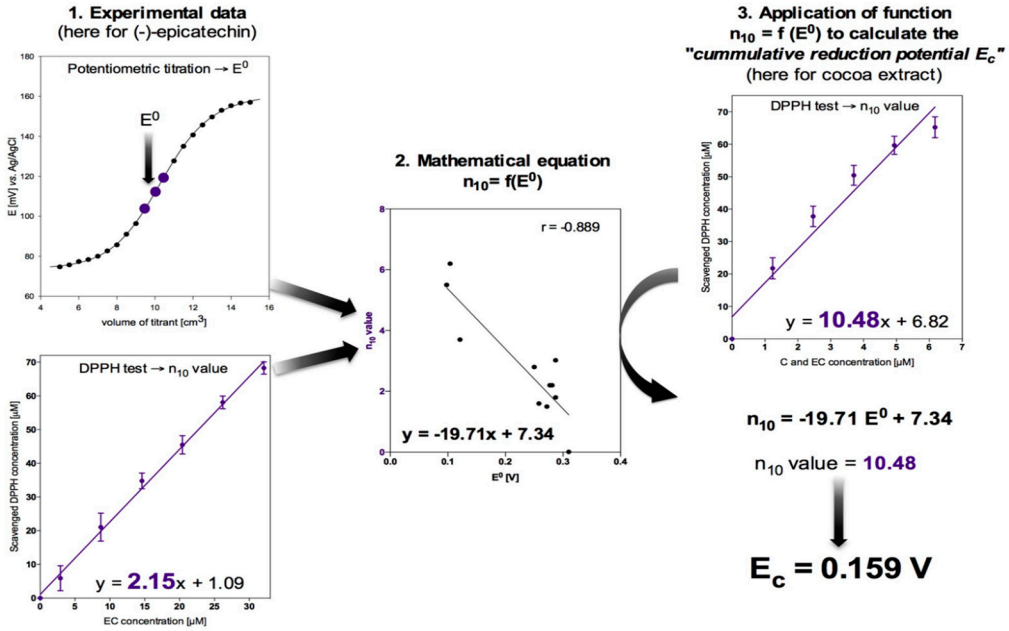

Figure 1. The three-step approach to estimate "cumulative reduction potential" E_c of complex mixtures of antioxidants illustrated for cocoa extract.

In a separate experiment carried out under the same, corresponding to physiological, conditions (37 °C, pH = 7.4), the n_{10} value was determined for cocoa extract by DPPH test (Figure 1, Panel 3). This value was then substituted into $n_{10} = f(E^0)$ function and the "cumulative reduction potential" E_c was calculated for this cocoa extract sample. The calculated E_c value of 0.159 V, falls between E^0 values determined for EGCG (0.104 V) and EC (0.277 V), two catechins that are present in cacao. The cellular antioxidant activity measured by CAA assay for cacao extract also exhibited

intermediate potency when compared with these two catechins, which confirmed the expected results of the proposed approach.

4. Discussion

Standard reduction potential of redox active compounds has been shown to be a helpful, chemically defined, unambiguous predictor of their impact on a number of biological activities in a cellular model, and thus probably also in vivo [1]. However, E^0 values cannot be easily determined for mixtures of redox active chemicals such as food products and components containing, e.g., antioxidant phytochemicals. Thus, the growing interest in dietary antioxidants requires the fast, cheap, reliable and accessible in every laboratory parameter to assess redox properties of foodstuffs. Stoichiometry value n_{10} determined by DPPH test meets these requirements to some extent. This parameter seems to be a reliable predictor of antioxidant activity being also in line with electrochemical properties evaluated using potentiometric titration. Its determination is less related to the sample concentration used. Moreover, when the measurements are made after fixed time, also kinetic aspects of redox reaction(s) in which the studied sample is implicated are taken into account. Still, standard redox potential E^0 appears even better parameter, because it directly characterizes the redox active compound. Therefore, we have made an attempt to estimate the latter for natural complex mixtures based on simple measurements performed by DPPH test. Although, theoretically any assay measuring antioxidant activity of samples of interest may be applied, our previous investigations performed for the sets of catechins demonstrated the best correlation between E^0 and n_{10} values determined with the aid of DPPH radical [1].

We propose three-step approach explained in detail in Figure 1, in which pure redox active compounds are used to provide experimental data (step 1) needed to generate the mathematical formula describing the relationship between E^0 and n_{10} (step 2). In this paper, the linear approximation was applied, however once more data for a larger number of compounds is available, the function $n_{10} = f(E^0)$ may become more complicated. This function equation, when published may be generally used by anybody interested. Finally, in the step 3, the n_{10} value is determined for any sample of interest with the use of strictly defined uniform DPPH test protocol and based on this determination, cumulative reduction potential E_c calculated from the $n_{10} = f(E^0)$ function equation. We named this parameter "cumulative", because it reflects all the redox interactions taking place in natural plant sample containing a mixture of redox active ingredients characterized by divergent electrochemical properties and present in various concentrations.

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