A New Total Antioxidant Potential Measurements Using RP-HPLC Assay with Fluorescence Detection

Bronisław K. Główd[1,*], Paweł Piszcz[1], Katarzyna Czajka[1], and **Paweł K. Zarzycki[2]**

[1]Department of Analytical Chemistry, Institute of Chemistry, Faculty of Science, University of Podlasie, 3 Maja 54, 08-110 Siedlce, Poland and [2]Section of Toxicology and Bioanalytics, Koszalin University of Technology, Sniadeckich 2, 75-453 Koszalin, Poland

Abstract

In this paper, an improved total antioxidant potential (TAP) estimation using high-performance liquid chromatographic (HPLC) assay with fluorometric detection has been described. The principle of this method is based on the hydroxyl radicals generated in the Fenton-like reaction and subsequently detected using hydroxyterephthalic acid (HTPA), which is a reaction product of hydroxyl radicals and terephthalic acid (TPA), working as a sensing compound. HTPA quantity in the samples was measured by fluorescence detector working at excitation and emission wavelengths equal to 312 and 428 nm, respectively. A number of key experimental conditions including the influence of the reaction (incubation) time on the surface areas of HTPA peaks, concentration of Fe(II) ions as well as the influence of concentration of TPA on the surface area of the chromatographic peak of HTPA were optimized to the characteristic feature of TAP measurements. The elaborated assay has been used to evaluate TAP values of selected low-molecular mass compounds like pyrogallol, tryptamine, and *n*-alcohols (methanol, ethanol, and *n*-propanol) as well as chlorogenic and ascorbic acids and benzoic acid derivatives, which are commonly present in the food samples.

Introduction

Free radicals modify biologically active molecules as well as whole cells and are implicated in various degenerative diseases (carcinogenesis, mutagenesis, inflammation, and cardiovascular) and aging (1,2). Their mediated processes have been implicated in the pathogenesis of many diseases. It is widely believed that these modifications are preventable by exogenous antioxidants. Therefore, there is a need for a simple and robust method to assess and compare strength of particular antioxidants in order to select those of the highest potential for further development as dietary implementations or even as drugs (3). Many antioxidants can interact with each other, especially via red-ox reactions, increasing or decreasing synergetic effects. It is the main reason why more information can be obtained by measuring total antioxidant potential (TAP) of biological samples than concentration of particular antioxidants separately (4).

In the literature, there are few assays for the TAP measurements (2,4). Usually, they are based on the generation of free radicals and measurements of their competition reaction between sample and sensing compound (5,6). Under such conditions, the TAP measurement is based on the decrease (because of sample competition) of concentration of its product reaction with the generated radicals. Recently, it was shown that high-performance liquid chromatography (HPLC) can be used for TAP measurements (7,8). In this case, compounds used as sensors are the same as those applied for the measurements of hydroxyl radicals. The hydroxyl radicals are generated in the Fenton-like reaction, and they are detected using p-hydroxybenxoic acid (8). The product (3,4-dihydroxybenzoic acid) of its reaction with the hydroxyl radical has been subsequently analyzed using reversed-phase HPLC with the electrochemical (amperometric) detection. However, it should be noted that the use of electrochemical detector is inconvenient because of poisoning and instability of the electrodes. Therefore, some alternative detection methods involving different sensing compounds were developed. Particularly, it was shown that terephthalic (TPA) acid can be used as a spin trap of hydroxyl radicals (9,10). The product of its reaction with hydroxyl radicals, namely hydroxyterephthalic acid (HTPA), can be easily separated on the reversed-phase column and monitored fluorometrically. With such an analytical approach, the samples are monitored using excitation and emission wavelengths equal 312 nm and 428 nm, respectively. The aim of this paper is to optimize previously described protocol for the TAP estimation of selected low-molecular mass compounds commonly present in the food samples, particularly tryptamine and pyrogallol as well as chlorogenic, ascorbic, gallic, 3,4-dihydroxybenzoic, and 2,6-dimethoxybenzoic acids (11–13).

Experimental

Reagents and chemicals

Terephthalic, 3,4-dihydroxybenzoic, 2,6-dimethoxybenzoic, gallic and chlorogenic acids, and tryptamine were obtained from Sigma (St. Louis, MO). Acetonitrile and methanol, all HPLC-grade, were obtained from Labscan (Dublin, Ireland). All other reagents (Sigma, St. Louis, MO; Fluka, Buchs, Switzerland; and POCh, Gliwice, Poland) were of analytical-reagent grade and were used without further purification. Triplicate distilled from quartz water was used. Mobile phases were filtered through a 0.22-µm membrane filter (Millipore, Bedford, MA). The nettle (*Urtica dioica L)* leafs were obtained from the local supermarket.

*Author to whom correspondence should be addressed: email bkg@onet.eu.

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Apparatus

Measurements were performed by means of a chromatograph comprising a Interface Box, 4 channel Degasser K-5004, Solvent Organizer K-1500, Dynamic Mixing Chamber, HPLC Pump K-1001, Diode Array UV Detector (DAD) K-2600, Eurochrom 2000 chromatographic data acquisition and analysis software (all from Knauer GmbH, Berlin, Germany), Basic+ marathon Autosampler (Spark Holland B.V., Emmen, The Netherlands), Jet-Stream Plus Column Thermostat (Industrial Electronics, Langenzersdorf, Austria) and fluorometric detector RF-10AXL (Shimadzu, Tokyo, Japan). Samples were separated on a Hibar RP-18 5 µm, 250 × 4 mm i.d. (E. Merck, Darmstadt, Germany) column.

Analytical protocols

Chromatographic experiments were performed with a flow rate 1 mL/min. Column was stabilized at 20°C by passage of mobile phase for 1 h prior to the chromatographic measurements. A 100 mM phosphate buffer (pH 6.6) was used as mobile phase. 10 mM stock solutions of the analyzed compounds were prepared in the quaternary distilled from quartz water and diluted to the required concentration before use. Samples (20 µL) were injected using an autosampler. Output signal from the photometric detector working simultaneously at 210, 254, and 280 nm as well as fluorescence detector working at excitation and emission wavelengths equal 312 nm and 428 nm, respectively, were continuously displayed on the computer. Every sample was injected six times and the average was taken for further elaboration.

Hydroxyl radicals were generated by Fenton reaction and both the sensing compound and the analyte scavenge the radicals. They were generated through Fenton reaction by 7 min incubation of 1 mM Fe^{2+} and 10 mM H_2O_2 in 50 mM phosphate buffer (pH 7.4) in the presence of 1 mM terephthalic acid (TPA) and analyzed sample at 37°C. Product of reaction of TPA with hydroxyl radicals, namely hydroxyterephthalic acid (HTPA), was detected fluorometrically. When the sample is added to the reaction mixture it decreased its peak because of competition reaction with radicals. If the analyte reacts quicker than the sensing compound, generation of the HTPA is decreased. This assay enables the comparison of the OH radical scavenging performance of various substances, measured as a decrease of peak surface area of HTPA.

The nettle (*Urtica dioica L)* extracts were prepared by infusion of 1 g of sample in 10 mL of boiling water for 15 min. Then the solutes were percolated by a Millipore filter.

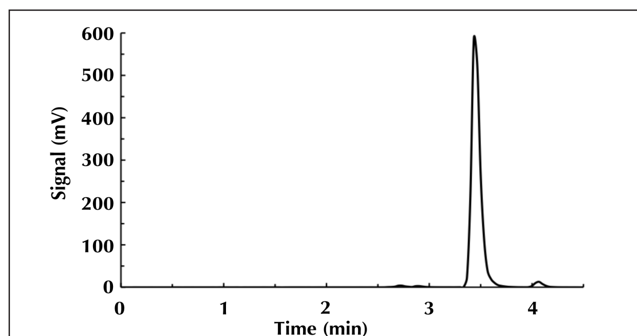

Figure 1. RP-HPLC chromatogram of the products of Fenton reaction with TPA. Reaction conditions: concentration of Fe^{2+}, 1; H_2O_2, 10; phosphate buffer (pH 7.4), 50 and TPA, 1 mM; temperature, 37°C. Chromatographic conditions: flow rate, 1 mL/min; temperature, 20°C; mobile phase, 100 mM phosphate buffer (pH 6.6); injection volume, 20 µL; fluorescence detector, 312/428 nm; column, Hibar RP-18 5 µm, 250 × 4 mm i.d.

Data analysis

The measurements (Fenton reaction and chromatographic analysis) of the antioxidant potential were repeated 3 times for each sample, and the results were averaged and expressed relative to the average result for the control samples containing no sample. This means that the TAP value is equal the difference of the surface area of HTPA obtained in the Fenton reaction with and without sample. Intra-subject comparisons were performed by using Student's *t*-test for dependent variables. Significance was set at $P < 0.05$.

Results and Discussion

Total antioxidant potential (TAP) can be measured chromatographically by quantification of the peak, which corresponds to the reaction product of the sensing compound with the free radicals followed by an electrochemical detection (2,8). In this case, hydroxyl radicals were generated in the Fenton-like reaction. They were analyzed using a method adopted from the that originally elaborated to the analysis of hydroxyl radicals (14). Namely, *p*-hydroxybenzoic acid has been used as a sensing compound. A product of its reaction with hydroxyl radicals, 3,4-dihydroxybenzoic acid (3,4DHBA), has been monitored, using reversed-phase HPLC with electrochemical (amperometric) detection. In this method, decrease of the chromatographic peak surface area of 3,4DHBA, before and after adding the sample to reaction mixture, has been used as TAP measure. Despite of the fact that the method has high selectivity and sensitivity, the application of an electrochemical detector is usually inconvenient because of poising of the working electrode surface. Therefore, the possible use a fluorometric detector instead to the amperometric one was investigated. The literature described the application of fluorometric detection to the analysis of hydroxyl radicals (9). In this case as a sensing substance, terephthalic acid (TPA), was used. With hydroxyl radicals, it gives hydroxyterephthalic acid (HTPA), which can be detected by fluorescence detector working at excitation and emission wavelengths equal to 312 and 428 nm, respectively.

We have applied the same wavelengths of the fluorescence detector as it was used for the analysis of hydroxyl radicals involving HTPA reaction product (9). However, other experimental conditions should be optimized for the characteristic features of TAP measurements. The product of the Fenton reaction with TPA is presented in Figure 1. It turned out that one main product of the reaction, HTPA, has been obtained. However, other reaction products (like dihydroxyterephthalic acid) are visible (surface area of their peaks is around 10% of the surface area of HTPA). Therefore, the HPLC driven protocol is superior to the simple fluorimetric analysis (9,10). The problem of interfering signals are a real concern when using multicomponent samples, including herbs, food, blood serum, etc. As an example, chro-

matograms of the nettle extract and the nettle extracts after reaction with the hydroxyl radicals are presented on Figures 2 and 3, respectively. It was found that although additional peaks (components of the sample and/or products of their reaction with the hydroxyl radicals) were obtained on the chromatograms, they did not overlap the HTPA peak. Again, the advantage of using HPLC with the fluorescence detection contrary to the fluorescence analysis alone is clearly seen. The time of the chromatographic analysis of HTPA is shorter: ~ 5 min (Figure 1). In real samples it may be necessary to increase it, to remove all interference (Figure 3). The influence of the reaction (incubation) time on the surface areas of HTPA peaks is presented in Figure 4A. It turned out that, at the constant, other conditions, asymptotic curve (increase of the peak surface area) is observed. This asymptote is reached after, ~ 10 min. Therefore, this time has been selected for further measurements.

In a similar way, optimal and final concentration of Fe(II) at a level of 0.8 mM has been selected, as is presented in Figure 4B. It is worth to note that this dependence is more complicated (sigmoid) because iron on both steps of oxidation can react with hydrogen peroxide, hydroxyl radicals as well as terephthalic acid (15). Finally, the influence of concentration of terephthalic acid on the surface area of the chromatographic peak of HTPA have been tested (Figure 4C). A nearly linear dependence was found at the reasonable TPA range of concentration. Particularly, 0.6 mM TPA was selected for further experiments.

The elaborated assay has been firstly tested using a number of low molecular mass compounds that can be present in the food samples (tryptamine and pyrogallol as well as chlorogenic, ascorbic, gallic, 3,4-dihydroxybenzoic and 2,6-dimethoxybenzoic acids). The calibration curves obtained for chlorogenic acid are presented in Figure 4D. Similar curves have been obtained for all other investigated compounds. According to the expectation, increase of the sample (in this case chlorogenic acid) concentration decreased HTPA peak area, which is a TAP measure. Obviously, the limitation of this process is the area of HTPA peak (the peak can be completely decreased but not to give negative value). Therefore, asymptotic dependence is obtained, as it is presented in Figure 4A. Nevertheless, within a low concentration range, the linear dependencies were observed (Figure 4D). Therefore, all experiments were performed using low antioxidants (samples) concentrations, corresponding to the linear range of the calibration curves. It enables easy comparisons of TAP values of different samples (antioxidants). TAP can be expressed as (*i*) decrease of the chromatographic peak of HTPA (at constant sample volume and concentration), (*ii*) slope of the calibration curve, or (*iii*) the sample concentration at which HTPA peak decreased two times. In the linear calibration ranges, the first two attempts are directly proportional and equivalent. The first one has been selected by us because of its simplicity.

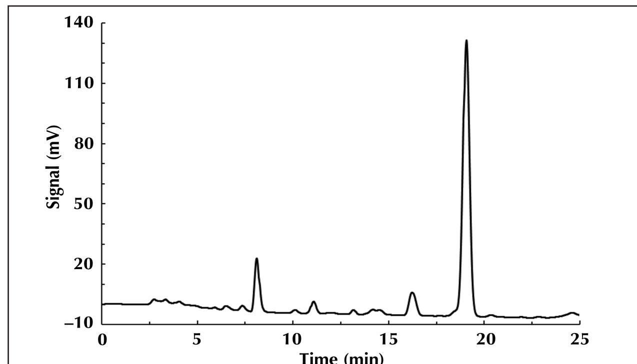

Figure 2. RP-HPLC chromatogram of the extract of nettle (*Urtica dioica* L). Chromatographic conditions are the same as in Figure 1.

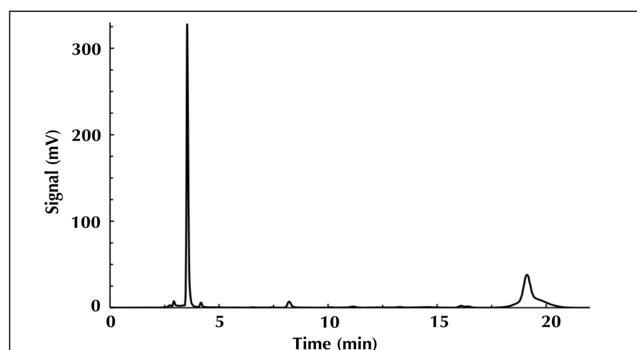

Figure 3. RP-HPLC chromatogram of the products of Fenton reaction with TPA in the presence of the extract of nettle (*Urtica dioica* L). Experimental conditions are the same as in Figure 1.

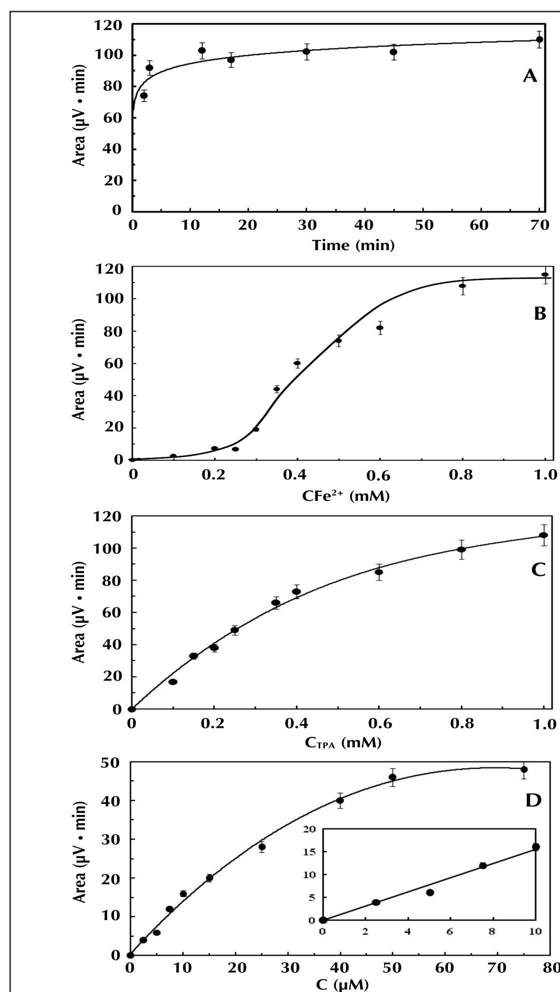

Figure 4. Influence of the reaction time (A), concentration of Fe(II) (B) and concentration of TPA (C) on the surface area of the chromatographic peak of HTPA and the calibration curves of chlorogenic acid (D). Reaction and chromatographic conditions are the same as in Figure 1.

The elaborated assay enabled us to obtain the antioxidant potential values for given samples, as can be seen in Figure 5. It turned out that, from the investigated compounds, the strongest antioxidant is chlorogenic acid and all strong antioxidants comprise the catechol group. It is worth to note very small TAP value of the ascorbic acid, which is widely assumed to be strong antioxidant (16). However, it is also a strong reducer. For example, it can reduce Fe^{3+} to Fe^{2+}, increasing production of hydroxyl radicals (17). This means that in the systems containing transient metals and hydrogen peroxide, the ascorbic acid is pro- and antioxidant at the same time.

Contrary to the assays related to weaker radicals (for example peroxyl or DPPH$^•$) elaborated assay enabled to estimate TAP's also for weak antioxidants. TAP values of the volatile alcohols (methanol, ethanol, and *n*-propanol) are presented in Figure 6A. It turned out that their antioxidant potentials increased with the increase of their aliphatic chain, although they were much smaller than TAP values of strong antioxidants (Figure 5). TAP values are proportional to their rate constants with hydroxyl radicals (Figure 6B), which are equal to 9.7×10^8; 1.9×10^9, and 2.3×10^9 mol L^{-1} s^{-1} for methanol, ethanol, and propanol, respectively (18).

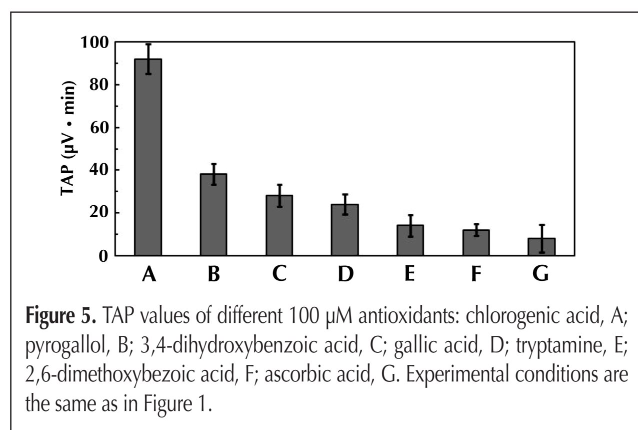

Figure 5. TAP values of different 100 µM antioxidants: chlorogenic acid, A; pyrogallol, B; 3,4-dihydroxybenzoic acid, C; gallic acid, D; tryptamine, E; 2,6-dimethoxybezoic acid, F; ascorbic acid, G. Experimental conditions are the same as in Figure 1.

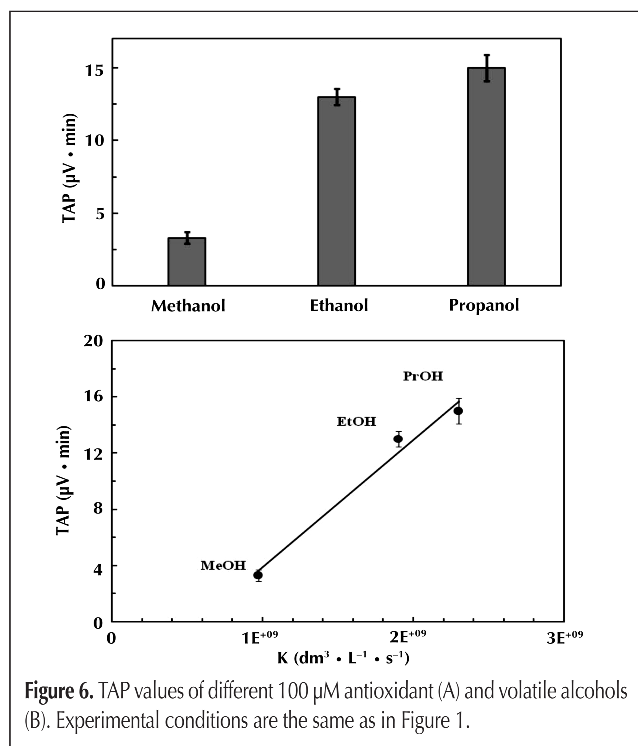

Figure 6. TAP values of different 100 µM antioxidant (A) and volatile alcohols (B). Experimental conditions are the same as in Figure 1.

Conclusions

It was shown that TAP values can be accurately and robustly measured using an assay based on RP-HPLC with fluorescence detection. The advantage of this assay is that TAP is related to hydroxyl radicals, the strongest oxidant responsible for damaging living organisms. However, it is limited to the water-soluble samples. It has been found that TAP values can be additionally affected by all interaction of components of interest (e.g., ascorbic acid) with iron ions, reduction of Fe(III) to Fe(II). Therefore, the proposed assay gives additional information about antioxidant properties of chemicals in contrary to the described in the literature assays based on weaker radicals.

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