A Fast and Simple Method for the Measurement of Total Antioxidant Potential and a Fingerprint of Antioxidants

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The goal of this paper is to demonstrate a new approach to the chromatographic total antioxidant potential (TAP) assay and chromatographic fingerprints of honeys. The analyte is analyzed using reversed-phase high-performance liquid chromatography with electrochemical (amperometric) detection. The TAP measure was the total surface area of all recorded chromatographic peaks (on the amperometric detector in the oxidation potential range) on the chromatogram. The proposed assay is superior to already described assays because TAP can easily be related to the different potentials of working electrodes, as well as different groups of compounds separated on the column. The fingerprinting of antioxidants (antiradicals) has been applied for the quality assessment of honeys.

Introduction

Free radicals are atoms, compounds or ions with odd numbers of electrons. They are usually more reactive than other chemical species and harmful to biological cells and living organisms, like human beings (1). They are implicated in the pathogenesis of many diseases including, cancer, Alzheimer's disease, rheumatoid arthritis and Parkinson's disease (2). Free radicals are inactivated by free radicals scavengers and/or antioxidants. Occasionally, both terms are used interchangeably. Usually, antioxidants are also identified by sample antiradical activity because oxygen free radicals or, more generally, reactive oxidant species (ROS) are the primary oxidants occurring in biological samples. Antioxidants can be analyzed using high-performance liquid chromatography (HPLC) (3). In this case, however, it is difficult to know whether all antioxidants are estimated. Additionally, interactions between particular antioxidants may influence the final results. Therefore, frequently the total value, called total antioxidant potential, or TAP (capacity, status and reactivity) is measured. TAP has been introduced for evaluating the antioxidant capacity of complex biological and food samples. The literature contains descriptions of many methods of their analysis (4). The classical tests exploit different free-radical generators (usually thermolabile diazo compounds generating peroxy radical) and oxidation of the analyzed samples (5). Because of competition, the sample inhibits interaction between the sensor compound and the radical. Results are calculated from the delay time during which antioxidants are consumed. The reaction products are analyzed using different analytical techniques (fluorometric, photometric or chemiluminescence) (6).

HPLC assays are based on the reaction of hydroxyl radicals, generated in a Fenton-like reaction with the sensor and sample. The products of the sensor reaction are analyzed using reversed-phase (RP)-HPLC with different detection systems, among them the electrochemical detectors. Decrease of the peak caused by the sample is the TAP measure (7). Alternative, electrochemical (potentiometric, voltammetric or electrogravimetric) methods of TAP estimation have been developed. The disadvantage of the voltammetric methods is their poor sensitivity.

This paper presents the preliminary results of the application of RP-HPLC followed by amperometric detection to TAP estimation. TAP has been measured by recording HPLC chromatograms at different potentials of the working electrode. The potentials were selected such that only antioxidants were obtained on the chromatogram. Glassy carbon was used as a working electrode. The TAP measure was the surface area of all chromatographic peaks. Different kinds of honeys from Podlachia region in Poland were used to test the elaborated assay.

Experimental

Instrumentation

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 and ClinLab Digital Amperometric Detector EC3000 (Recipe; Munich, Germany) with glassy carbon working electrode (reference electrode, Ag/AgCl; auxiliary electrode, Pt), Smartline Diode Array Detector 2800 and Autosampler Smartline 3900 (all from Knauer; Berlin, Germany) and a Smartline 4000 Column Thermostat (Industrial Electronics; Langenzersdorf, Austria). Samples were separated on a Eurospher RP-18 5 μm, 250 × 4 mm i.d. column (Knauer). The system was controlled and data acquisition was performed on an IBM PC computer with ClarityChrom V 2.6 2007 software.

pH was measured using a pH-meter OP-208/1 (Radelkis; Budapest, Hungary) with OSH 10-10 electrode (Metron, Switzerland).

Reagents

Gallic acid, iron (II) sulfate, phosphate buffered saline (PBS) tablets, *p*-hydroxybenzoic (pHBA, 99%), 3,4-dihydroxybenzoic (DHBA, 97%) and HPLC-grade methanol were obtained from Sigma (St. Louis, MO). All other reagents (Fluka; Buchs, Switzerland; Alchem, Inform, and POCh, Poland) were of analytical-reagent grade and were used without further purification. Water was distilled three times from quartz. Mobile

phases were filtered through a 0.22-μm membrane filter (Millipore, Bedford, MA). The different commercial types of honeys, such as buckwheat, tilia, honeydew, multi-flower and robinia, were purchased at the local beekeeper in Siedlce.

Procedures

Chromatographic experiments were performed at 1.0 mL/min flow rate. The column was stabilized at 20°C by passage of mobile phase for 1 h before the chromatographic measurements. Phosphate buffer (100 mM, pH 6.6) + methanol (96 + 4)% v/v was used as a mobile phase. Twenty microliter samples were injected using an autosampler. The output signal from the electrochemical detector was continuously displayed on the computer. The measure of the TAP value was total surface area of all peaks recorded on the chromatogram at the given potential of working electrode.

Hydroxyl radicals were generated by Fenton reaction [Equation (1)] and both the sensor and the analyte scavenged these radicals:

$$Fe^{2+} + H_2O_2 \leftrightarrows Fe^{3+} + OH^- + HO^\bullet \qquad (1)$$

One mM Fe^{2+} and 0.03% H_2O_2 in 50 mM phosphate buffer (pH 7.4) in the presence of 1 mM pHBA acid were incubated at 37°C. The generation time was 7 min. The time was measured immediately after adding Fe^{2+} to the reaction mixture. The product of the reaction of pHBA with hydroxyl radicals, namely DHBA acid, was detected by HPLC with diode-array detection (DAD). The measurements were performed with and without the sample. The peak of the sample added to the reaction mixtures decreased because of competition reaction with the radicals. The results were recalculated to the gallic acid equivalence (GAE; 1 mg GA/1 g honey).

The honeys were prepared by dissolving them in water in the ratio 1:10 w/v, and filtering through a 0.22 μm Millipore filter.

Data analysis

The measurements were repeated three times for each sample and the results were averaged and expressed relative to the average result for the control solutes containing no sample (honey). Calculations were performed using Student's t-test for dependent variables. Significance was set at $P < 0.05$.

Results and Discussion

This paper presents a new attempt of TAP measurements. This new attempt is based on the measurement of antioxidant activity profile. Its measure is the surface area of all peaks recorded on the chromatogram. At the anodic (usually positive) potential of working electrode, the signal (reaction current) is obtained for each reductant (in other words, antioxidant) present in the sample. This means that the sum of surface areas of all peaks reflects the total concentration of all antioxidants in the sample. Contrary to other chromatographic TAP assays, the proposed method is superior because it allows TAPs to be obtained at different potentials of the working electrode, as

shown in Figure 1. The TAP values obtained at different working electrode potentials reflect those obtained by various assays, i.e., related to the different radicals. Additionally, the advantage of the application of chromatography, contrary to other analytical techniques (like photometric or fluorometric technizues), is that TAP can be estimated independently for different classes of compounds in the sample (Figure 2). Furthermore, the attempted assay is very fast and simple.

At low (0.0 and 0.2 V versus Ag/AgCl) values of oxidation potential, only very strong antioxidants are oxidized (peaks at 11.5 and 15.4 min in Figure 1). The increase of the potential means that weaker antioxidants can be also observed on the chromatograms. Therefore, increase of the potential increases TAP values because (i) more antioxidants are detected and (ii) the oxidation current increases (Randles-Ševčík equation) (8, 9).

The literature includes descriptions of different TAP assays (10). Generally, they are related to various radicals. The strongest, hydroxyl radicals, react with the most compounds, even including weak antioxidants like alcohols or sugars. On the other hand, weak radicals (peroxyl or DPPH•) react only with the strongest antioxidants (flavonoids). Therefore, different assays offer different information about the antioxidant properties of the investigated samples. The proposed assay allows results to be obtained that are related to various radicals.

In addition, it allows the TAPs of different classes (groups) of compounds (Figure 2) to be estimated, in addition to individual compounds (Figure 3), even if they are not known. The results showed (Figures 2 and 3) that for small values of working electrode potentials, the TAP depends only on the strong antioxidants. TAP is a function of both concentration and power (redox potential) of antioxidants. Therefore, the percentage (contribution) of weak antioxidants (their concentration is often much higher than strong antioxidants) in TAP value increased with the increase of the potential. Such relationship provides additional information about contribution of all constituents of the sample on the total antioxidant potential. At the given potential, TAP depends primarily on the total concentration of antioxidants in the sample.

Retention in reversed-phase depends on many factors. In general, it is proportional to the hydrophobicity of sample and its molecular surface area (11). Therefore, less polar and bigger compounds are eluted later. The results showed (Figure 2) that in honey, the strong antioxidants (E ≤ 0.6 V) are mostly big, non-polar compounds ($t_R \geq 10$ min). Compounds characterized by short retention are weak antioxidants, shown on the chromatogram only at higher (approximately 1.0 V) potentials of the working electrode (Figures 2 and 3). Their concentrations are usually much higher than strong antioxidants (12).

As mentioned previously, with the increase of potential, more peaks are observed on the chromatogram. However, no more peaks were observed at the potential above 0.8 V. Therefore, this potential was selected as optimal for further studies.

The developed method was practically tested in the determination of TAP of various honeys, as shown in Figure 4. Buckwheat honey was found to characterize the strongest antioxidant potential. These results are consistent with changes in the concentrations of phenols in different honeys (13).
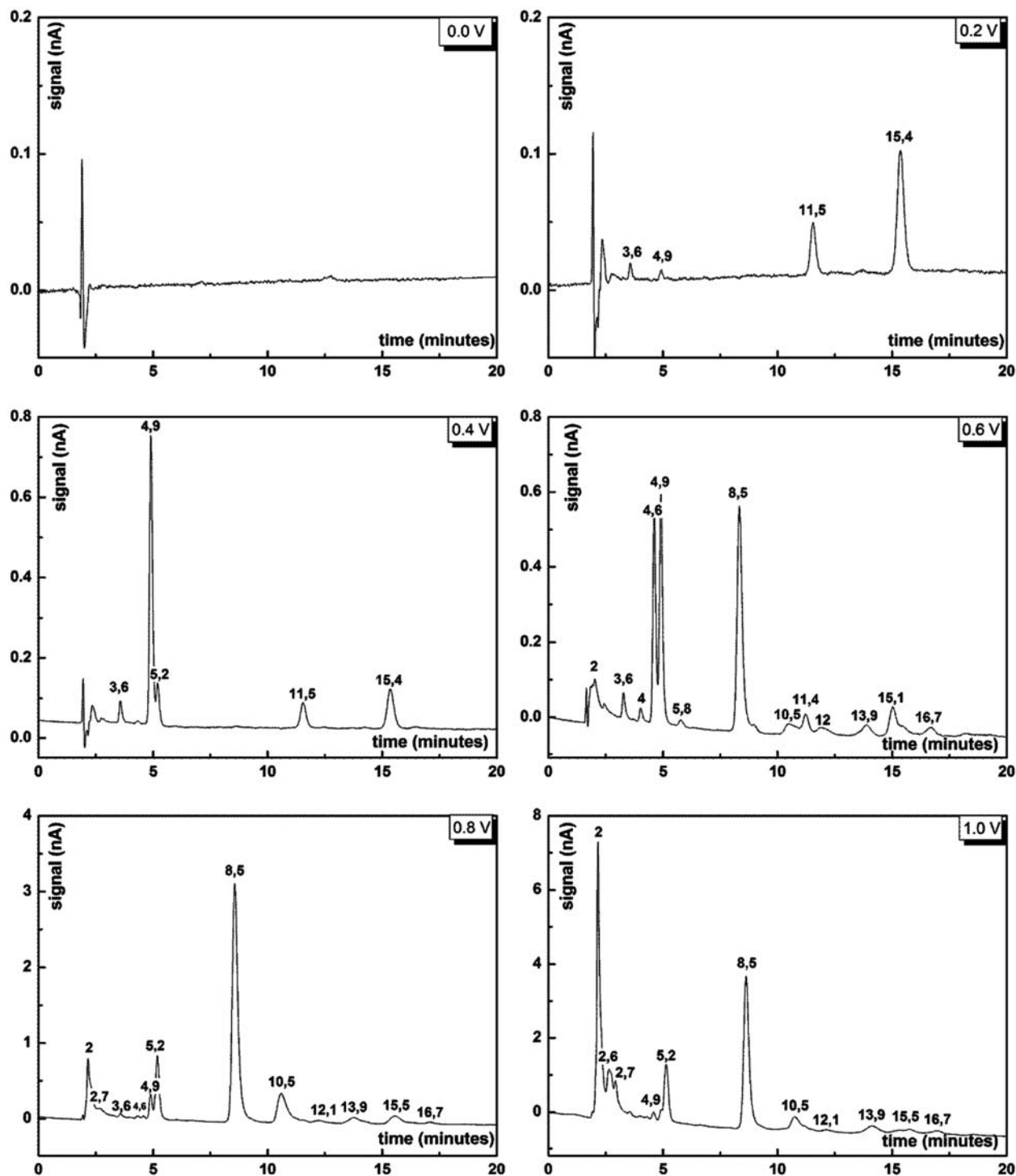
Figure 1. HPLC chromatograms of 1 g/L tilia honey. Chromatographic conditions: column, Eurospher C18 5 μm, 250 × 4 mm i.d. (Knauer); temperature, 20°C; flow rate, 1.0 mL/min; mobile phase, 100 mM phosphate buffer (pH 6.6) + methanol (96 + 4)% v/v; injection volume, 20 μL; electrochemical detector with the following working electrode potentials versus Ag/AgCl: 0 V (A); 0.2 V (B); 0.4 V (C); 0.6 V (D); 0.8 V (E); 1.0 V (F).

As previously explained, at the potential above 0.8 V, all (even weak) antioxidants contained in the tested honeys were oxidized. Similarly, TAP measured using hydroxyl radicals also contains information on all antioxidants in the sample (7). Therefore, one would expect a linear relationship between both values, as shown in Figure 5 ($R^2 = 0.872$). However, both oxidation mechanisms are different. Therefore, the relationship is not perfectly linear. In particular, the intercept is higher than zero for $TAP_{ED} = 0$ nAs/μg (sample does not contain antioxidants) $TAP_{OH} = 3.9$ GAE. This is because certain compounds
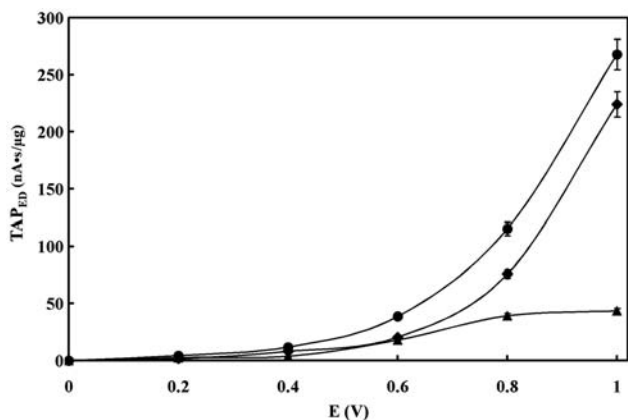
Figure 2. Dependence of the TAP values of tilia honey obtained using HPLC−ED assay for small and/or polar compounds (diamonds) ($t_R \leq 10$ min), large and/or non-polar compounds (triangles) ($t_R \geq 10$ min) and the sum of the compounds (circles). Other conditions are the same as those described in Figure 1. Data are presented as means from three independent experiments $\pm$ SD.
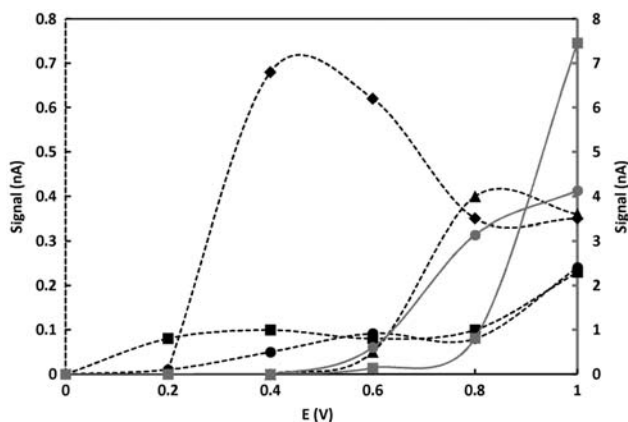

Figure 3. Hydrodynamic voltammograms of the different compounds present in the tilia honey recorded at the following retention times: 2 min (gray squares); 3.6 min (black circles/dotted line); 4.9 min (black diamonds/dotted line); 8.5 min (gray circles); 10.5 min (black triangles/dotted line); 15.4 min (black squares/dotted line). Other conditions are the same as those described in Figure 1.
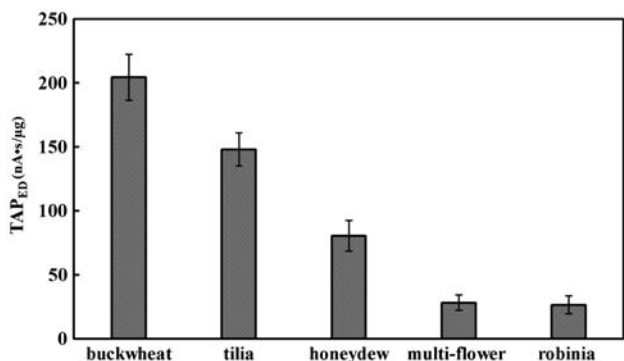

Figure 4. TAP values of the different honeys (final concentration 1 g/L). Chromatographic conditions are the same as those described in Figure 1. Data are presented as means from three independent experiments $\pm$ SD. Working electrode potential: 0.8 V versus Ag/AgCl.
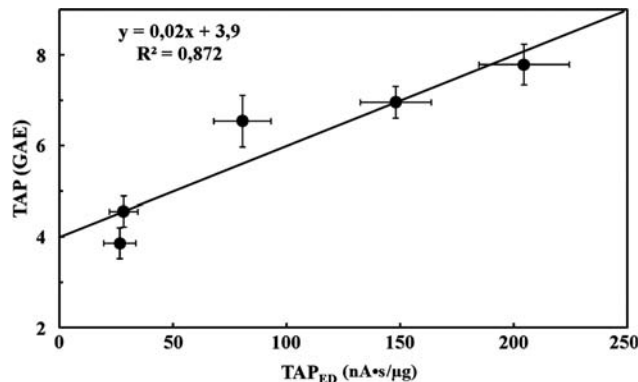

Figure 5. Correlation between TAP values obtained using the assay described in this paper, TAP_{ED}, and TAP_{OH} related to the hydroxyl radicals.

that are not antioxidants, such as alcohols or sugars, react (are oxidized) with hydroxyl radicals, but are barely oxidized electrochemically.

The investigated honeys could be ordered according to the increase in value of the TAP: robinia, multi-flower, honeydew, tilia and buckwheat.

Conclusions

This study showed that the total area of all peaks recorded (using an electrochemical detector) on an RP-HPLC chromatogram can be a measure of the TAP. It was found that the proposed method is much more sensitive than the voltammetric method because it lacks the capacitive current and a large convection current. The proposed assay is superior to the methods described in the literature, because the TAP can be easily related to the different values of the potential of working electrodes and various compounds (or their groups) separated chromatographically. It was found that TAPs of honeys are directly proportional to the results obtained using a test based on the Fenton reaction. An assay for the measurement of antioxidant (antiradical) activity profiles (fingerprinting) of free radical scavengers in honeys was elaborated. The elaborated assay is very fast and simple, and it needs no additional steps, like reactions producing the radicals.

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