Journal of Food Composition and Analysis 30 (2013) 13–18

Contents lists available at SciVerse ScienceDirect

Journal of Food Composition and Analysis

journal homepage: www.elsevier.com/locate/jfca

Original Research Article

Comparative analysis of antioxidant activity of honey of different floral sources using recently developed polarographic and various spectrophotometric assays

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ARTICLE INFO

Article history:
Received 27 January 2012
Received in revised form 26 November 2012
Accepted 28 December 2012

Keywords:
Analytical method
Food analysis
Honey
Honey constituents
Food composition
Phenolics
Reducing sugars
Antioxidant activity
Hydrogen peroxide scavenge
DC polarography
Anodic current
Honey adulteration
Honey authenticity

ABSTRACT

Hydrogen peroxide scavenging (HPS) activity of honey of different floral sources and its constituents such as predominant honey flavonoids, phenolic acids, amino and organic acids, and carbohydrates have been assessed by direct current (DC) polarographic assay. The assay was based on decrease of anodic current of hydrogen peroxide complex, formed in alkaline solution, at the potential of mercury dissolution. High correlations between honey HPS activity, its total phenolic content (FC-GAE), antioxidant activity measured by four standard methods (DPPH, TEAC, FRAP and ORAC), and also the relative antioxidant capacity index, were obtained. Statistical evaluation by ANOVA and *F*-test further confirmed the assay validity. The results for individual compounds showed that HPS activity of honey reflects an integrated action of a wide range of constituents, both phenolics and non-phenolics. The polarographic assay applied is a fast, reliable and low cost alternative to spectrophotometric antioxidant assays commonly applied in analysis of honey and can serve as an indicator of honey quality.

1. Introduction

Honey consists of a saturated solution of sugars, of which fructose (38%) and glucose (31%) are the main contributors, but also contains a wide range of minor constituents, including phenolic compounds (Alvarez-Suarez et al., 2010a). A growing number of evidence about honey antioxidant (AO) activity, a parameter useful to evaluate biological function and possible therapeutic potential, has been accumulated. Honey intake increases blood vitamin C, β-carotene and glutathione reductase, and improves AO activity in human plasma (Schramm et al., 2003).

Honey AO activity appeared to be a result of the combined effect of a range of compounds. Phenolic compounds (flavonoids and phenolic acids), as well as non-phenolics (ascorbic acid, carotenoid-like substances, organic and amino acids, and proteins including certain enzymes such as glucose oxidase and catalase) can contribute to honey AO activity (Gheldof et al., 2002). Various assays have been applied to determine honey AO activity (Alvarez-Suarez et al., 2009; Ferreira et al., 2009). The most common ones are colorimetric assays, DPPH, FRAP (ferric reducing antioxidant power) and TEAC (Trolox equivalent antioxidant capacity), based on electron transfer, and ORAC (oxygen radical absorbance capacity) assay, based on hydrogen atom transfer (Alvarez-Suarez et al., 2009). The electrochemically based AO activity assays have been considered an important tool for evaluating AO activity of food and beverages (Blasco et al., 2007). However, they have rarely been applied in honey analysis. The application of amperometric flow injection analysis (FIA) to determine AO activity of honey has been reported by Buratti et al. (2007), while an electrochemical procedure based on oxidation current obtained at the fixed potential was used to determine electrochemical antioxidant index in honey (Ávila et al., 2006).

Various voltammetric techniques were applied to investigate honeybee products. Heavy metals in honey were determined by differential pulse anodic stripping voltammetry at mercury micro-electrodes (Sanna et al., 2000). Characterization of various phenolic compounds isolated from propolis by cyclic voltammetry, as well as

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http://dx.doi.org/10.1016/j.jfca.2012.12.004

determination of AO ability of chrysin, a flavone present in high concentrations in propolis and honey, by linear sweep voltammetry on static mercury electrode (Zheng et al., 2005) was reported. Usage of polarographic techniques on dropping mercury electrode (DME) has a long history dating back several decades, when polarographic determination of fructose was applied to distinguish artificial from natural honeys (Heyrovsky and Zuman, 1968). Recently, the content of hydroxymethylfurfural (HMF) in honey has been determined by direct current (DC) polarography on the basis of polarographic wave of HMF in artificial honeys allowing their identification in natural honey (Reyes-Salas et al., 2006). In the present report, applicability of DC polarography in determination of hydrogen peroxide scavenging (HPS) activity of honey was investigated.

Assays based on HPS activity, as one of the key aspects of AO activity, have been developed and applied on complex food and biological samples. Limitations in the peroxidase-based system, such as the presence of interfering compounds, have been surpassed using enzyme free methodologies. Highly sensitive chemiluminescent assays represent a significant advancement over enzyme based HPS activity assays, owing to elimination of the effects caused by radical reaction (Arnous et al., 2002). Novel HPS activity assays have been developed recently based on (i) the formation of gold nano shells (Li et al., 2009), (ii) cupric reducing AO capacity (CUPRAC) methodology (Özyürek et al., 2010), (iii) chronoamperometry (Karyakina et al., 2009), and (iv) direct current (DC) polarography (Sužnjević et al., 2011).

Development and optimization of an enzyme-free polarographic assay has been reported recently (Sužnjević et al., 2011). Briefly, the assay relies on sensitivity of anodic current originating from hydrogen peroxide complex formation in alkaline solutions, at potentials of mercury dissolution, to antioxidants and reducing compounds. Decrease of the anodic current upon addition of individual compounds or complex samples has been followed using DC polarography on DME. In this way, HPS activity of various complex samples was determined (Sužnjević et al., 2011; Gorjanović et al., 2010a,b, 2011; Novaković et al., 2011). Application of the assay on propolis samples has been reported only recently (Potkonjak et al., 2012).

Honeys from different floral sources, individual phenolics present in honey, as well as non-phenolic compounds such as the most prevalent amino and organic acids, and the main carbohydrates, have been subjected to DC polarographic measurement of AO activity for the first time. Total HPS activity of honey samples has been correlated with AO activity determined by AO assays widely used in analysis of honey (FC, DPPH, ORAC, TEAC and FRAP), as well as with relative antioxidant capacity index (RACI) calculated by assigning equal weight to each applied assay. Reliability of DC polarographic assay has been estimated through correlations between obtained results and RACI. Validity of DC polarographic assay was confirmed based on correlations obtained, analysis of variance (ANOVA) and *F*-test. Post ANOVA's Tukey HSD test at the $p < 0.05$ confidence level was performed in order to access the statistically significant differences within each AO assay. In order to obtain better insight in the contribution of particular honey compound to its total HPS activity the most relevant representatives of carbohydrates, organic acids and amino acids, phenolic acids and flavonoids were included into the study. Based on the obtained results the combined contribution of both phenolics and non-phenolics has been discussed.

2. Materials and methods

2.1. Chemicals

All chemicals and solvents used were of analytical grade. 2,2′-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS diammonium salt), fluorescein and 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), pinocembrin and syringic acid were purchased from Fluka Chemie (Buchs, Switzerland). 2,2′-Azobis (2-methylpropionamidine) dihydrochloride (AAPH), ferrous sulfate ($FeSO_4$), sucrose, D-(+)-maltose monohydrate, D-(−)-fructose, D-(+)-glucose, sodium acetate trihydrate, ferrous ammonium sulfate, ferric chloride hexahydrate, 2,4,6-tripyridyl-triazine (TPTZ), chlorogenic acid, (+)-catechin hydrate, kaempferol, myricetin and quercetin were purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). Hydrogen peroxide, Folin–Ciocalteu reagent, D-gluconic acid potassium salt and amino acids (L-lysine monohydrate, L-arginine, L-phenylalanine, L-tyrosine free base and L-proline) were purchased from Merck (Darmstadt, Germany). 2,2-Diphenyl-1-picrylhydrazyl (DPPH), vanillic acid and trans-ferulic acid, was purchased from Aldrich (Milwaukee, WI, USA).

2.2. Honey samples

Honey samples ($n = 7$) considered in this study were of the following floral sources: pine (*Pine* spp.) honeydew, polyfloral forest honey, dead nettle (*Urtica dioica*), linden (*Tilia platyphyllos*), polyfloral meadow and acacia (*Robinia pseudoacacia*) honey (1 and 2). Three of them (*Urtica dioica*, *Tilia platyphyllos* and acacia 2), provided by local beekeepers living in different regions of Serbia, were artisanally produced, unpasteurized and collected during the 2009 year flowering. Three commercial honeys, Langenese brand, German (acacia 1, polyfloral forest and meadow) were purchased from the supermarket, while pine honeydew was obtained from a local market in the Greek island of Thasos. Honey color was determined using a C 221K Honey Color Analyzer Refractometer and results were expressed in millimeters (mm) Pfund grades (AOAC, 1990), compared to an analytical grade glycerol reference standard.

An artificial honey that reflected the main components of honey was prepared by dissolving sucrose (0.15 g), maltose (0.75 g), fructose (4.05 g) and glucose (3.35 g) in 1.7 mL of deionized water (Gheldof et al., 2002; Copper et al., 2002). This solution was used in all antioxidant assays to determine the contribution of sugars in each specific assay. Honey samples and artificial honey (500 mg) were diluted in distilled water (1 mL).

2.3. Determination of HPS activity using DC polarographic assay

Anodic current (polarograms) of H_2O_2 obtained by dropping mercury electrode (DME) in alkaline solutions was investigated (Sužnjević et al., 2011). In Clark-Lubs (CL) borate buffer (pH 9.8) at H_2O_2 concentrations higher than 1×10^{-3} M, a peak of anodic current was developed instead of a wave. Initial i_l value (i_{l0}) of anodic current of 5.0 mM H_2O_2 solution in buffer decreased upon addition of investigated samples. Honey samples, as well as artificial honey, were gradually added (in three equal aliquots of 100 μL) into the electrolytic cell with buffered H_2O_2 solution. Individual compounds were added in various amounts depending on their activity. Carbohydrates and glucuronic acid (0.5 g/mL H_2O) were added in aliquots of 100 μL, aminoacids (10^{-2} M) in aliquots of 25 μL, phenolic acids (2×10^{-3} M) in aliquots of 100 μL while flavonoids (2×10^{-3} M) were added in aliquots of 50 μL. Phenolic acids were dissolved in ethanol except vanillic acid dissolved in CL buffer pH 9.8. Flavonoids were dissolved in ethanol except kaempferol dissolved in 0.2 M NaOH. Height of initial peroxide limiting current was compared with residual peroxide limiting current (i_{lr}) obtained upon gradual addition of tested samples. Percentage of i_l decrease was calculated upon each addition of tested honey samples according to the equation:

$$\%\,\text{scavenged}\ [H_2O_2] = \left(1 - \frac{i_{lr}}{i_{l0}}\right) \times 100 \quad (1)$$