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Analytical Methods

Antioxidant and radical-scavenging activities of Slovak honeys – An electron paramagnetic resonance study

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Abstract

The antioxidant properties of 15 honey samples from different floral sources and various Slovak regions were investigated by means of electron paramagnetic resonance spectroscopy. Cation radical of ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) diammonium salt), DPPH (1,1-diphenyl-2-picrylhydrazyl) and hydroxyl radicals generated by the photochemical decomposition of hydrogen peroxide were used as oxidants. The antioxidant activities found with $ABTS^{\cdot+}$, expressed as trolox equivalent antioxidant capacity (TEAC), ranged from 0.15 to 1.14 mmol kg^{-1}, and those determined with DPPH, from 0.04 to 0.32 mmol kg^{-1}. TEAC values correlated well with results found by elimination of DPPH, and both values revealed a linear relationship with the concentration of phenolics obtained with the Folin–Ciocalteu phenol test (expressed as gallic acid equivalents, GAE). The colour coordinates (CIE $L^*a^*b^*$), as well as reflectance spectra determined for original honeys using a white background, demonstrated that the colour difference (ΔE^*) and coordinate b^* interrelate with TEAC values. The radical-scavenging capacities (RSC) of the honey samples determined in the experiments with photochemically decomposed hydrogen peroxide, generating reactive $^{\cdot}OH$ radicals in the presence of spin trapping agent, differ from those found with $ABTS^{\cdot+}$ and DPPH. Here, probably, the reactive $^{\cdot}OH$ radicals, having higher redox potential, are scavenged by a variety of compounds not effective with $ABTS^{\cdot+}$ and DPPH (e.g., saccharides, proteins).

Keywords: Honey; Antioxidant; Phenolics; EPR; TEAC; DPPH; DMPO; Colour coordinates

1. Introduction

For the protection of human health, considerable attention is currently focused on the consumption of functional foods. In particular, the role of dietary antioxidants capable of scavenging the oxidants and free radicals responsible for initiating various diseases, has been intensively discussed (Kris-Etherton et al., 2004; Rice-Evans, Miller, & Paganga, 1996). Systematic investigations of the antioxidant properties of various foods, beverages, spices and herbs have been performed (Aruoma, 2003; Butkovic, Klasinc & Bors, 2004; Polovka, Brezová & Staško, 2003; Roginski & Lissi, 2005; Sanchez-Moreno, 2002; Staško, Polovka, Brezová, Biskupič & Malík, 2006) and the number of papers addressing the health-protective and antioxidant characteristics of honey is increasing. Honey has for a long time been used as a natural source of sugars, as well as an important

Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) diammonium salt; DMPO, 5,5-dimethyl-1-pyrroline N-oxide; DPPH, 1,1-diphenyl-2-picrylhydrazyl; EPR, electron paramagnetic resonance; F–C, Folin & Ciocalteu's phenol reagent; GAE, gallic acid equivalent; NHE, normal hydrogen electrode; ORAC, oxygen radical absorbance capacity; RSC, radical-scavenging capacity; SW, magnetic field sweep width; TEAC, trolox equivalent antioxidant capacity; TEMPOL, 4-hydroxy-2,2,6,6-tetramethylpiperidine N-oxyl; UV/vis, ultraviolet/visible.

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ingredient in traditional medicine, having antimicrobial and antiinflammatory properties (Küçük et al., 2007; McKibben & Engeseth, 2002). The health-protective and therapeutic impacts of honeys were formerly attributed to the presence of various antioxidant components, such as flavonoids, phenolic acids, organic acids, enzymes and vitamins (Gheldof & Engeseth, 2002; Gheldof, Wang, & Engeseth, 2002, 2003; Inoue et al., 2005; McKibben & Engeseth, 2002; Schramm et al., 2003; Wang, Gheldof & Engeseth, 2004). On the other hand, the main sugar constituents of honey, i.e., glucose and fructose, were reported to be either inhibitors or accelerators of mutagenic activity (Busserolles, Gueux, Rock, Mazur, & Rayssiguier, 2002). The hypertriglyceridemic and prooxidant effects observed in rats fed with a high fructose diet were eliminated by substituting honey for fructose (Busserolles et al., 2002). The antimutagenic effects of various honeys against the food mutagen Trp-p-1, depend on the honeys' origins and on their concentrations (Wang, Andrae, & Engeseth, 2002). A detailed identification and quantification of the antioxidants in honeys from various floral sources, showed the contribution of a variety of compounds (phenolics, peptides, organic acids, enzymes) to the demonstrated antioxidant capacities (Gheldof et al., 2002). The antioxidant capacities of honeys, evaluated by the oxygen radical absorbance capacity (ORAC) assay, showed a linear dependence on the total phenolics content (Gheldof & Engeseth, 2002). A combination of spectroscopic techniques with chemometrics was previously applied in the standardisation of the antioxidant characteristics of honey samples, and the results indicated the necessity of using different antioxidant tests and assays for the precise characterisation of honey antioxidants (Beretta, Granata, Ferrero, Orioli, & Facino, 2005).

The production of honey in Slovak Republic was approximately 2600 tons in the year 2002, and honey represents an important part of nutrition (http://www.vcely.sk/index.php?name=News&file=article&sid=152). However, no previous investigations of the antioxidant characteristics of Slovak honeys have been published. The aim of our work is to evaluate the antioxidant and radical-scavenging properties of 15 Slovak honey samples by electron paramagnetic resonance (EPR) spectroscopy using various radical oxidants, and to establish their relationships with the colour and the phenolics content of the honey.

2. Materials and methods

2.1. Materials

The original honey samples (forest H1, H4; sunflower H2, H10; colza H5–H8; mixed flower H3, H9, H11; acacia H12–H14; isosweet H15) produced in various Slovak regions were obtained from the Research Institute of Animal Production, Institute of Apiculture (Liptovský Hrádok, Slovak Republic). The samples were stored in the dark at a room temperature of 22 °C. The experiments were performed using freshly prepared 1.0% honey solutions in distilled water.

As sources of semi-stable free radicals we used 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) diammonium salt (ABTS; Sigma–Aldrich, St. Louis, MO) and 1,1-diphenyl-2-picrylhydrazyl (DPPH; Fluka, Buchs, Switzerland). Solutions of $ABTS^{\cdot+}$ cation radical were prepared by dissolving 17.2 mg of ABTS and 3.3 mg of $K_2S_2O_8$ (Sigma–Aldrich) in 5 ml of distilled water, and this mixture was left to stand for 16 h in the dark at room temperature according to recommendations published in Re et al. (1999). The stock solution of $ABTS^{\cdot+}$ was prepared by mixing 1 ml of this reaction mixture with 60 ml of distilled water, and the accurate concentration of $ABTS^{\cdot+}$ was determined by UV/vis spectroscopy, using the value of molar absorptivity at 735 nm of $1.5 \times 10^4\ mol^{-1}\ dm^3\ cm^{-1}$ (Arts, Haenen, Voss, & Bast, 2004). The prepared stock solution of $ABTS^{\cdot+}$ was diluted immediately before measurements in order to obtain an optimal concentration of the radical oxidant for EPR experiments (25 μM).

The spin trapping agent, 5,5-dimethyl-1-pyrroline N-oxide (DMPO; Sigma–Aldrich) was distilled before application and stored under argon at −18 °C. The concentration of photo-generated ·DMPO–OH adducts was determined using as standards aqueous solutions of 4-hydroxy-2,2,6,6-tetramethylpiperidine N-oxyl free radical (TEMPOL; Sigma–Aldrich). Ethanol (spectroscopy grade) and D-glucose monohydrate (analytical grade) were purchased from Mikrochem (Pezinok, Slovak Republic). Hydrogen peroxide (30%) and Na_2CO_3 were obtained from PLIVA-Lachema Diagnostika s.r.o. (Brno, Czech Republic). Gallic acid monohydrate and Folin & Ciocalteu's phenol reagent (F–C) from Sigma–Aldrich were used.

2.2. Methods and apparatus

2.2.1. $ABTS^{\cdot+}$ and DPPH techniques

The capacity of honey samples dissolved in distilled water (1.0 wt-%) to quench the semi-stable free radicals ($ABTS^{\cdot+}$ or DPPH) was monitored by EPR spectroscopy using a Bruker EMX EPR spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) working in the X-band. The aqueous $ABTS^{\cdot+}$ (25 μM) or ethanolic DPPH (5 μM) solution was placed in a syringe (volume 1 ml), and a second identical syringe was filled with 1 ml of the 1% honey solution. Both syringes were attached to a micro-mixing chamber and connected to a flat cell (WG-812, Wilmad-LabGlass, Buena, NJ) inserted into the TM-110 (ER 4103 TM) cylindrical cavity of the EPR spectrometer. After the simultaneous injection of both solutions into the flat cell, the time-course of EPR spectra was monitored for 630 s, obtaining a set of ten EPR spectra. (Each EPR spectrum represents the accumulation of 3 scans measured with a 21 s sweep time). Reference experiments were performed by replacing the honey solution with 1 ml of distilled water. From the monitored EPR spectra the integral intensities of $ABTS^{\cdot+}$ or DPPH were evaluated by the WIN EPR program (Bruker), and the resultant paramagnetic species concentrations were calculated using calibration curves. The

differences in $ABTS^{\cdot+}$ or DPPH concentrations evaluated after a reaction time of 630 s (the last spectrum in the individual time-course) for the reference or the honey solutions were used for the computation of the TEAC or the DPPH radicals terminated ($DPPH_{term}$) per 1 kg of honey.

2.2.2. Spin trapping technique using DMPO

The hydroxyl radicals were generated upon *in situ* irradiation of hydrogen peroxide solutions in the presence of DMPO as the spin trap, enabling the detection of hydroxyl radical as ·DMPO–OH adduct (Li, Cummings, Roethling, Buettner, & Chignell, 1988). The samples were prepared immediately before measurement by mixing 50 μl of H_2O_2 aqueous solution (100 μM), 50 μl DMPO (0.2 M in water) and 200 μl of honey solution (1.0%). Then the solutions were carefully saturated with argon and immediately transferred to a small quartz flat cell (WG 808-Q, Wilmad-LabGlass) optimised for the TE_{102} (ER 4102 ST) Bruker rectangular EPR cavity. In reference experiments honey solutions were replaced by water. The samples were irradiated at 293 K directly in the EPR spectrometer resonator, and the EPR spectra were recorded *in situ*. As an irradiation source an HPA 400/30S lamp (400 W, Philips) was used; the radiation source was focused to obtain the highest intensity in the active part of the cavity. A Pyrex glass filter (thickness: 1 mm) was applied to eliminate radiation wavelengths below 300 nm. The first spectrum in the time-course was measured without radiation, and subsequently we started the exposure, monitoring 10 spectra upon irradiation. (An individual EPR spectrum represents an accumulation of 3 scans measured with a 21 s sweep time). The concentration of ·DMPO–OH adduct was evaluated from double-integrated EPR spectra, using the calibration curve obtained by measuring the EPR spectra of TEMPOL aqueous solutions under strictly identical conditions. The value of the radical-scavenging capacity (RSC) of the honey sample was obtained after an exposure of 630 s, from the difference of the ·DMPO–OH concentration measured in the reference system (honey-free), and in the presence of a honey sample possessing antioxidant and radical-scavenging properties. The values obtained were finally recalculated per 1 kg of honey. The photoexcitation of honey components upon irradiation may be neglected due to the application of diluted honey solutions.

2.2.3. Folin–Ciocalteu's method for the determination of phenolics

The total phenolics content was determined according to a modified Folin–Ciocalteu procedure (Singleton, Orthofer, & Lamuela-Raventos, 1999), where the diluted honey solutions (1.0%) were added to the F–C reagent. The F–C reagent oxidises phenolic compounds in honey and the dark blue colour developed ($\lambda = 765$ nm) is followed by UV/vis spectroscopy. UV/vis spectra were recorded using a UV/vis spectrometer (PC 2000, Sentronic GmbH, Dresden, Germany) with a DH 2000 lamp. As a first step, a calibration curve, i.e., absorbance at 765 nm *versus* gallic acid concentration, was obtained by mixing the F–C reagent with variously concentrated gallic acid solutions. In this way the gallic acid equivalents (GAE) for the honey solutions were evaluated and finally recalculated per 1 kg of honey. Samples were analysed in triplicate.

2.2.4. Colour coordinates

The reflectance spectra ($\lambda = 380$–730 nm; $\Delta\lambda = 10$ nm) and colour coordinates (CIE $L^*a^*b^*$) of the honey samples were measured by a Spectrolino SpectroScan (X-Rite Incorporated, Grand Rapids, MI) using white backgrounds (illumination, D_{50}; observer angle, 2°; density standard, DIN; filter, no). The experiments were performed using the original honey samples (0.05 g) placed between two cover slips (2 × 2 cm, Menzel Glaser GmbH, Braunschweig, Germany) and heated to 45 °C, to dissolve the sugar crystals. The reference spectra were obtained using the two microscopic cover slips only. The measurements were repeated 5 times with different sample positions, and the average values of the colour coordinates or reflectance spectra were calculated.

2.2.5. Statistics

The statistical analysis was carried out using the Origin (OriginLab, Northampton, MA) program. The correlation matrix was calculated by linear regression and ANOVA analysis at the 95% confidence level.

3. Results and discussions

3.1. TEAC and $DPPH_{term}$

The characterisation of antioxidant status by TEAC values as found by the elimination of $ABTS^{\cdot+}$ cation radical, as well as by the capability to scavenge DPPH radical, is suitable for the investigation of the radical-scavenging activity of systems containing hydrogen/electron-donating compounds, especially phenolics (Aruoma, 2003; Klein & Lukeš, 2006; Roginsky & Lissi, 2005; Sanchez-Moreno, 2002). In our experiments the decrease of $ABTS^{\cdot+}$ or DPPH concentration was monitored using EPR spectroscopy. Fig. 1a illustrates the characteristic time-courses of EPR spectra of cation radical $ABTS^{\cdot+}$ detected within 630 s after mixing with distilled water, as well as with diluted honey samples possessing different antioxidant capacities (H15, H9, H3, H1). The EPR spectrum of $ABTS^{\cdot+}$ is characterised by a complex hyperfine structure (Landolt-Börnstein, 1989); however the higher value of the modulation amplitude used in the EPR spectrometer setting (0.2 mT) facilitates its monitoring as a single-line signal with $g = 2.0036$. No changes in $ABTS^{\cdot+}$ EPR signal intensity were observed in the honey-free reference solution. On the other hand, the mixing of $ABTS^{\cdot+}$ with diluted honey solutions (1.0%) resulted in a decrease of its EPR signal during measurements (Fig. 1a). The integral intensities of $ABTS^{\cdot+}$ were evaluated for time-courses of experimental EPR spectra, and the concentrations of $ABTS^{\cdot+}$ for the individual reaction times were calculated. The differences of $ABTS^{\cdot+}$ concentrations evaluated after a reaction time of 630 s (the last

spectrum in the individual time-course in Fig. 1a) for reference or for honey solutions were used for the computation of the TEAC per 1 kg of honey. A variety of antioxidants present in honey samples of different origins is manifested here in the varying amounts of $ABTS^{\cdot+}$ cation radical eliminated, resulting in the diversity of the TEAC values. Fig. 2 summarises the results for all investigated honey samples H1–H15, specifying their floral origin. The samples are ordered according to their decreasing TEAC values, and this order is retained throughout the manuscript. High TEAC values were found for dark forest honeys (0.94 and 1.14 mmol kg^{-1}), and the lowest, for honey obtained from corn hydrolysate isosweet (0.15 mmol kg^{-1}), where the content of plant flavonoids and phenolic acids is limited. The sunflower and mixed flower honeys demonstrated substantial differences in TEAC values (0.62–1.03 mmol kg^{-1}), most probably caused by the origin of the floral nectar collected and by the geographic region. On the other hand, the four colza honeys showed comparable TEAC values (0.68–0.77 mmol kg^{-1}), and relatively low values were found for the acacia honeys (0.38–0.52 mmol kg^{-1}), in accordance with data published by

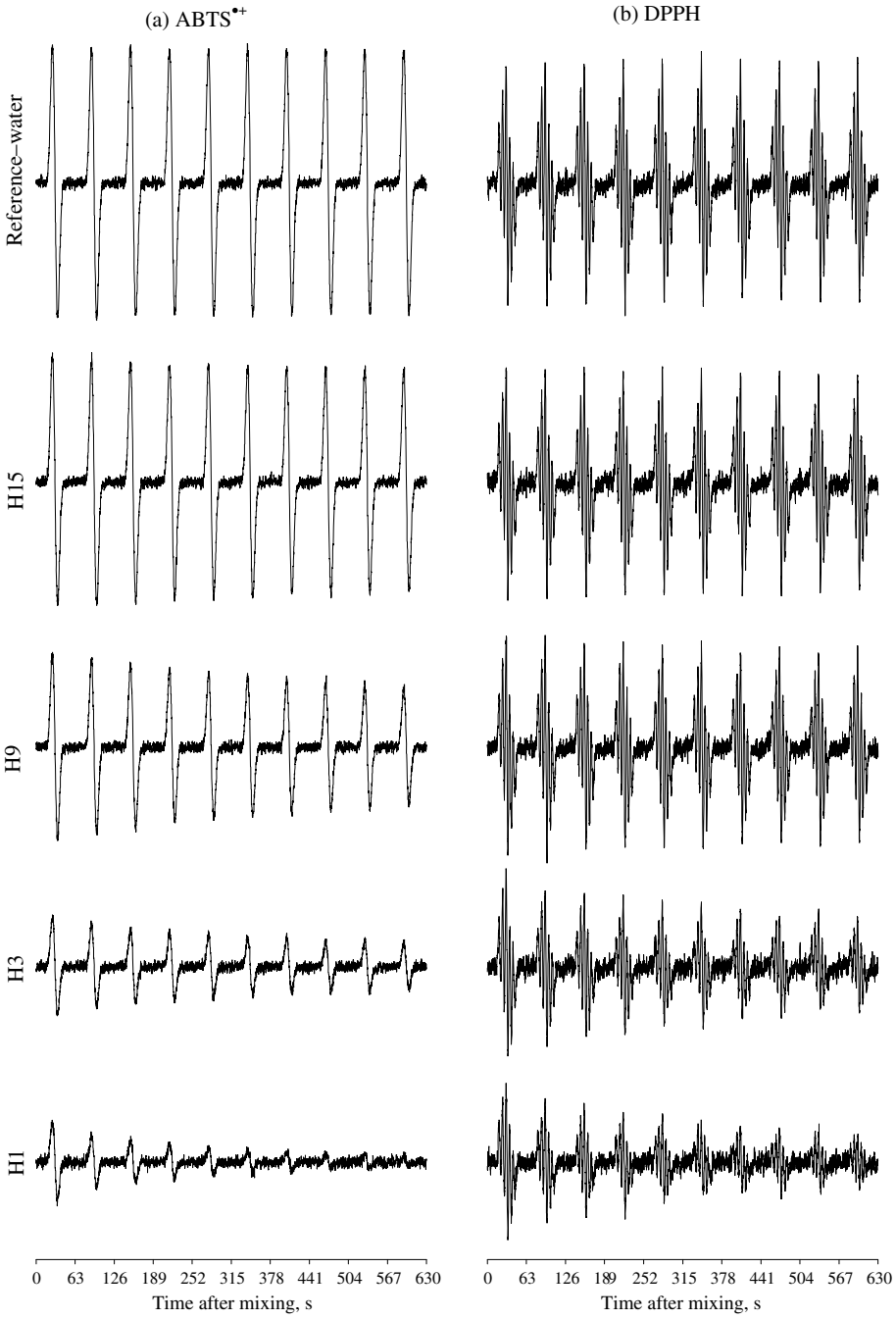

Fig. 1. The sets of 10 individual EPR spectra monitored after mixing the solution of radical oxidant (a) $ABTS^{\cdot+}$ (initial concentration 25 μM); (b) DPPH (initial concentration 5 μM) with honey-free water solution (reference), and with diluted (1.0%) honey samples H15, H9, H3 and H1.

other authors (Gheldof & Engeseth, 2002; Gheldof et al., 2002). Honey is a very complex system and its resulting antioxidant activity reflects predominantly the properties of the original floral sources (Baltrušaitytė, Venskutonis, & Čeksterytė, 2007; Beretta et al., 2005; Gheldof & Engeseth, 2002; Gheldof et al., 2002; McKibben & Engeseth, 2002). The results found in our study confirm a substantial influence of the floral sources on the TEAC values, as the honey prepared from the corn hydrolysate (H15) showed the lowest antioxidant capacity (Fig. 2).

An analogous set of experiments was performed with DPPH free radical. The time-courses obtained in experiments with diluted honey samples (H15, H9, H3, H1) are presented in Fig. 1b. As before, the EPR measurements were begun with the reference experiments, mixing ethanolic DPPH solutions with an identical volume of distilled water (Fig. 1b, reference-water) and monitoring 10 EPR spectra over 630 s. Under the given experimental conditions (1:1 ethanol:water) DPPH radical shows a quintet EPR spectrum well known from the literature ($a_{N1} = 0.927$ mT, $a_{N2} = 0.846$ mT; $g = 2.0036$; Yordanov & Christova, 1997). No changes in the EPR intensity of DPPH were observed in the reference experiments, but replacing distilled water with diluted honey solutions (1.0%) led to a decrease of EPR spectra intensity, which is dependent on the properties of the individual honey samples (Fig. 1b). As before, the integral intensities of DPPH were evaluated for time-courses of experimental EPR spectra, and the concentrations of DPPH were calculated. The experimental data were used to evaluate a molar amount of DPPH terminated per 1 kg of honey 630 s after the mixing of reagents ($DPPH_{term}$). The values of $DPPH_{term}$ for individual honey samples are summarised in Fig. 3a.

Semi-stable radical DPPH can react with antioxidants along different pathways; the predominant reaction represents hydrogen/electron transfer from the antioxidant (Roginsky & Lissi, 2005; Staško, Brezová, Biskupič, & Mišík, 2007). The values of $DPPH_{term}$ obtained using diluted honey solutions (1.0%) showed trends analogous to those found by TEAC (Fig. 2). The highest value of $DPPH_{term}$ was gained for forest honey and the lowest for honey produced from corn hydrolysate (Fig. 3a). The decreased capability of honey antioxidants to scavenge DPPH (Fig. 3a), as compared to $ABTS^{\bullet+}$ (Fig. 2), correlates well with the values of the redox potentials of the radical oxidants, i.e., $DPPH/DPPH^{-} = 0.43$ V (Zhuang, Scholz, & Pragst, 1999) and $ABTS^{\bullet+}/ABTS = 0.68$ V *vs.* NHE (Scott, Chen, Bakac, & Espenson, 1993), respectively.

3.2. Phenolic compounds

As a biological product, honey is a complex system containing sugars, organic acids, minerals, vitamins, flavonoids and phenolic acids. According to data in the literature we had expected that $ABTS^{\bullet+}$ and DPPH paramagnetic species would be eliminated predominantly by hydrogen/electron-donating phenolic and polyphenolic compounds (Aruoma, 2003; Roginsky & Lissi, 2005; Sanchez-Moreno, 2002). The modified Folin–Ciocalteu phenol test (Singleton et al., 1999) was therefore used for the determination of total phenolics in the diluted honey samples (1.0%) and the results are expressed in GAE. Fig. 3b summarises the GAE values for individual honey samples, confirming that the content of phenolics is significantly dependent on the floral sources, in agreement with the data in the literature (Gheldof & Engeseth, 2002; Gheldof et al., 2002; McKibben & Engeseth, 2002). The honey samples with the highest GAE values also showed the highest capability to quench $ABTS^{\bullet+}$ (Fig. 2) and DPPH (Fig. 3a).

3.3. Quenching of hydroxyl radicals

The spin trapping technique enables us to monitor the generation of hydroxyl radicals during hydrogen peroxide

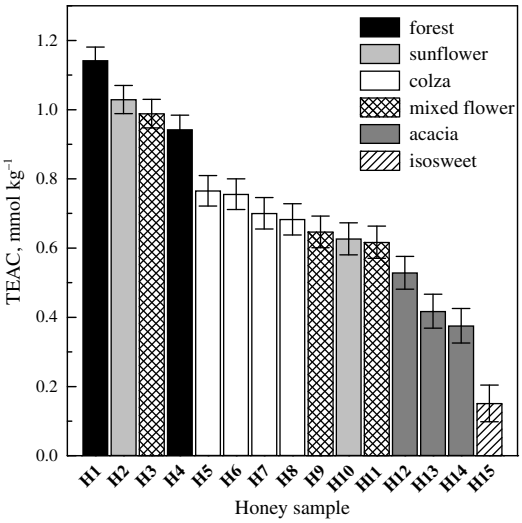

Fig. 2. The scavenging of $ABTS^{\bullet+}$ cation radical (expressed in TEAC) found for 15 investigated honey samples from different floral sources.

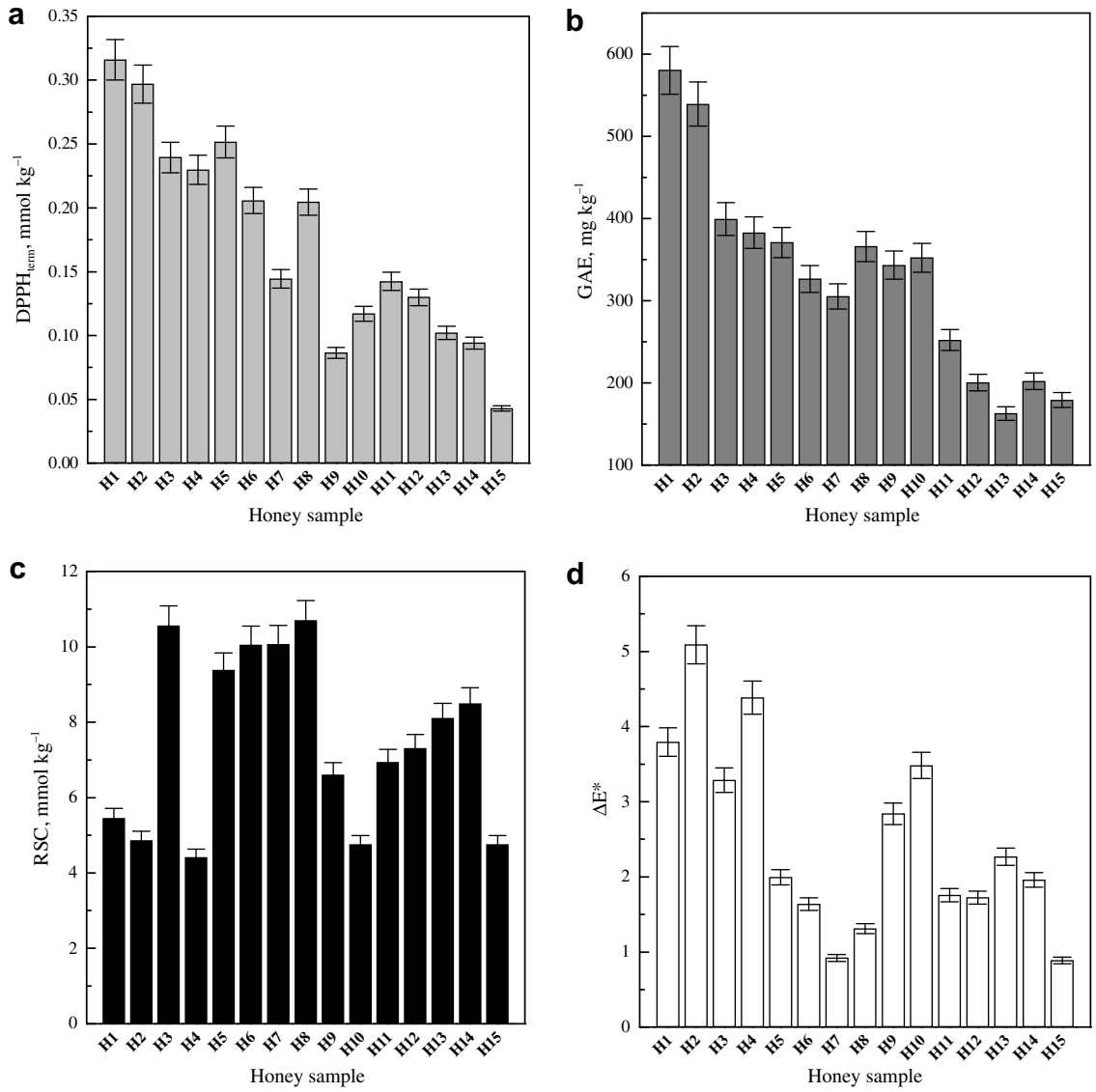

Fig. 3. An overview of experimental data: (a) termination of DPPH radical, $DPPH_{term}$; (b) phenolics content in gallic acid equivalent, GAE; (c) radical-scavenging capacity, RSC, monitored by EPR using spin trapping agent DMPO; (d) colour difference, ΔE^*; obtained by investigation of 15 honey samples from different floral sources.

photolysis. The photo-produced hydroxyl radicals react with DMPO spin trap and form the paramagnetic ·DMPO–OH adduct with a four-line EPR spectrum (Fig. 4b), well characterised by the spin Hamiltonian parameters ($a_N = 1.495$ mT, $a_H^{\beta} = 1.472$ mT; g-value = 2.0057; Li et al., 1988). Additionally, we observed a minor six-line EPR signal of very low intensity (* in Fig. 4b), attributable to the spin adducts of carbon-centred radicals produced by the degradation of the spin trap ($a_N = 1.570$ mT, $a_H = 2.225$ mT; $g = 2.0056$) (Li et al., 1988). Fig. 4a illustrates a set of EPR spectra monitored upon irradiation of H_2O_2 in the presence of DMPO, in the reference experiment with a honey-free system, as well as in experiments with the diluted solutions of honey samples. Obviously, the formation of paramagnetic ·DMPO-adducts is most pronounced in the reference system; the presence of honeys containing sugars, flavonoids, phenolic acids, enzymes and other compounds, which compete with the spin trap for the photo-generated hydroxyl radicals, hinders the formation of the above-mentioned ·DMPO-adducts (Fig. 4a and c). Consequently, a substantial decrease in EPR spectra intensity was observed for reaction mixtures containing the studied honeys. The concentrations of ·DMPO-adducts were evaluated from double-integrated experimental EPR spectra using precise solutions of TEMPOL as the standards. The values of radical-scavenging capacity (RSC) were determined from the difference between the ·DMPO-adduct concentration measured in the reference system (honey-free), and in the presence of honey, after a 630 s exposure to UV light. The procedure is illustrated in Fig. 4c for samples H15 and H3, and the RSC values found for individual honey samples are depicted in Fig. 3. It should be noted here that all diluted honey solutions showed higher capability to terminate

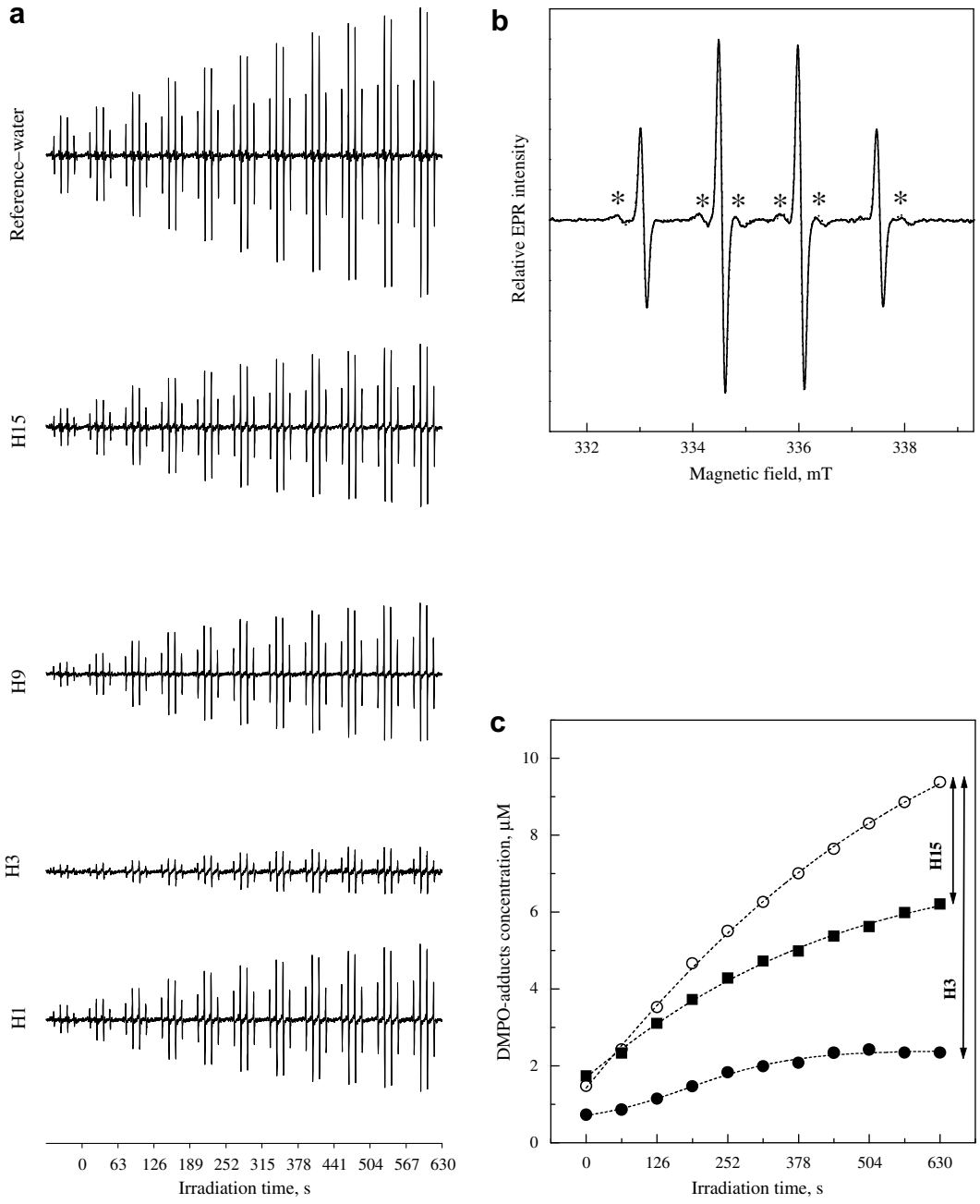

Fig. 4. Scavenging of the photo-generated hydroxyl radicals in the presence of honey samples monitored by EPR using DMPO spin trapping technique: (a) The sets of 11 individual EPR spectra (SW = 10 mT) monitored in the reference system (honey-free), and in the presence of honey samples H15, H9, H3 and H1 during continuous irradiation ($\lambda > 300$ nm); (b) experimental (solid line) and simulated (dotted line) EPR spectrum of ·DMPO–OH adduct (*) indicates the six-line spectrum of ·DMPO-carbon adducts); (c) scheme employed in the evaluation of RSC; the concentrations of ·DMPO-adducts after a 630 s irradiation monitored in the presence of honey samples H15 (■) and H3 (●) were subtracted from the reference value (○).

hydroxyl radicals than the analogous glucose solutions. Since hydroxyl radicals, with high redox potential (2.310 V *vs.* NHE at pH 7 (Buettner, 1993)), are able to react with a majority of the honey's components (sugars, flavonoids, phenolic acids, enzymes, etc. (Madden, 2007)) along different reaction pathways, the evaluated RSC values are inconsistent with TEAC, $DPPH_{term}$ and GAE, as presented in Fig. 5.

3.4. Colour measurements

Colour represents a very important characteristic of honey (Terrab, González-Miret, & Heredia, 2004). The differences in honey origin and composition are significantly exhibited in their colour, and the colour measurements can be used in the identification of the floral origin of honey (Terrab et al., 2004). As the highest values of TEAC,

$DPPH_{term}$ and GAE were found for the darker honey samples, we performed additional measurements of reflectance spectra and colour coordinates (CIE $L^*a^*b^*$).

The values of the colour differences: $\Delta E* = \sqrt{(L^*_{honey} - L^*_{ref})^2 + (a^*_{honey} - a^*_{ref})^2 + (b^*_{honey} - b^*_{ref})^2}$ found for individual original honey samples are summarised in Fig. 3d.

The experimental techniques and assays applied in the investigations of the antioxidant capacities of the honey samples brought diverse results, as depicted in Figs. 2 and 3. We therefore tested the relationships between pairs of data obtained with different methods (TEAC, $DPPH_{term}$, GAE, RSC, ΔE^*, b^*) by correlation analysis, with results presented in Fig. 5 and Table 1.

The significant linear correlation between TEAC and $DPPH_{term}$ ($r_{TEAC/DPPH_{term}} = 0.9175$; Fig. 5a and Table 1), as well as between TEAC or $DPPH_{term}$ and phenolics content expressed in GAE ($r_{TEAC/GAE} = 0.9110$; and $r_{DPPH_{term}/GAE} = 0.8669$) gives evidence that both semi-stable radicals are terminated predominantly by the phenolic and polyphenolic compounds possessing hydrogen/electron-donating activity (Table 1). On the other hand, no correlation was found between the RSC values determined by the DMPO spin trapping method with hydroxyl radicals and the experimental data from the other described assays (Table 1 and Fig. 5c). Most probably, in the DMPO spin trapping experiments, the competitive reaction kinetics of hydroxyl radicals represent diffusion-controlled reactions with sugars, which are the major components of honey (Madden, 2007).

The colour of the honey samples was characterised by the colour difference ΔE^* and the b^* coordinate. Their linear correlation ($r_{\Delta E^*/b^*} = 0.9445$) confirms that the colour

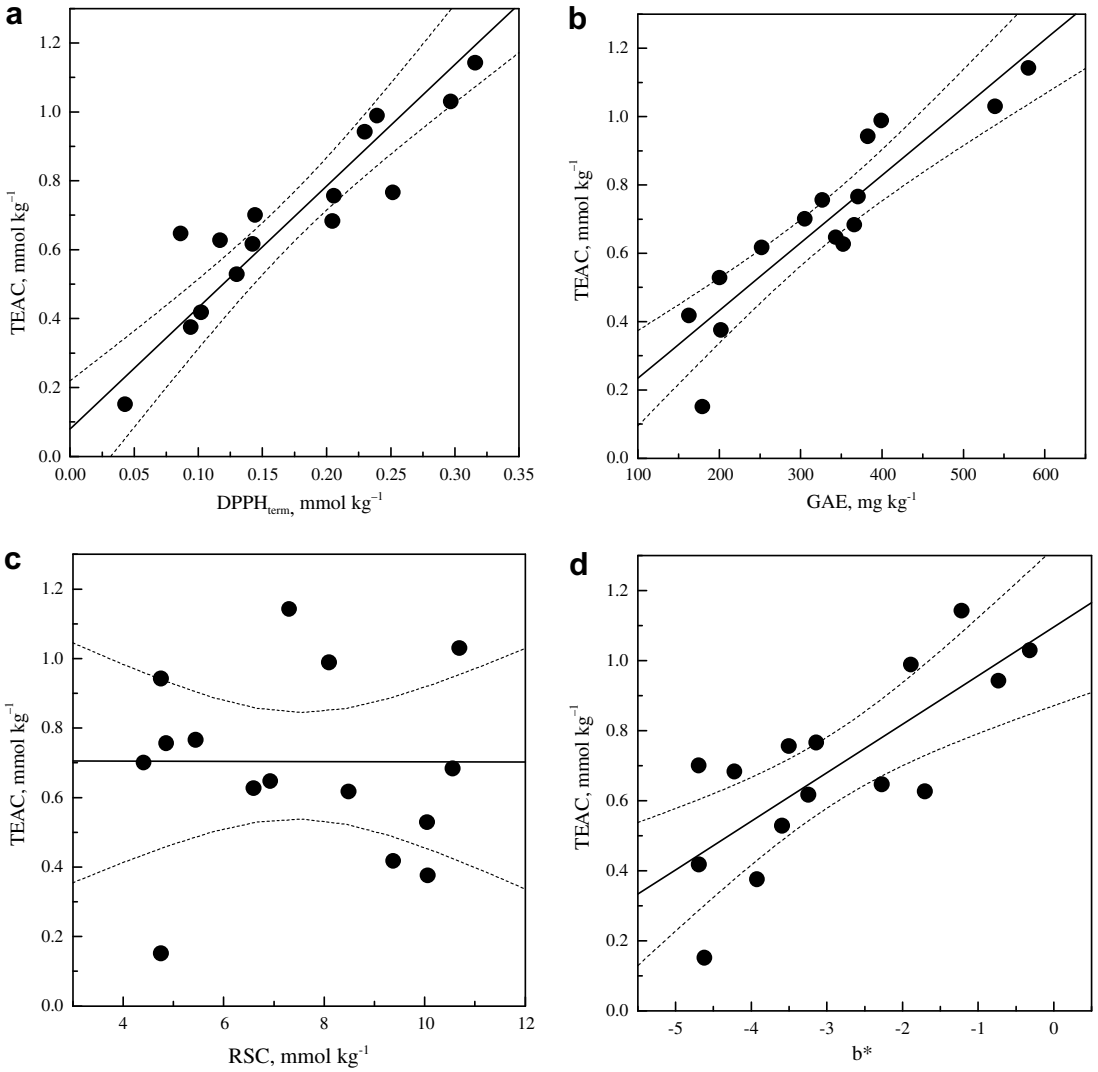

Fig. 5. The linear relationship at a 95% confidence level (confidence bands marked with dotted lines) between the capacity to scavenge cation radical $ABTS^{\cdot+}$ expressed in TEAC values and (a) ability to scavenge DPPH radical in $DPPH_{term}$ ($r_{TEAC/DPPH_{term}} = 0.9175$); (b) phenolics content in GAE ($r_{TEAC/GAE} = 0.9110$); (c) radical-scavenging capacity, RSC, monitored by EPR spin trapping technique with DMPO ($r_{TEAC/RSC} = -0.0101$); (d) colour coordinate b^* ($r_{TEAC/b^*} = 0.7683$).

Table 1
Correlation matrix among experimental data found for the 15 investigated honey samples from different floral sources

	TEAC	$DPPH_{term}$	RSA	GAE	b^*	ΔE^*
TEAC	1	0.9175	−0.0101	0.9110	0.7683	0.6944
$DPPH_{term}$	0.9175	1	0.0602	0.8669	0.6356	0.5793
RSA	−0.0101	0.0602	1	−0.1817	−0.5390	−0.5498
GAE	0.9110	0.8669	−0.1817	1	0.7877	0.7068
b^*	0.7683	0.6356	−0.5390	0.7877	1	0.9445
ΔE^*	0.6944	0.5793	−0.5498	0.7068	0.9445	1

difference of honey samples is mainly caused by changes in b^* values. Additionally, satisfying linear correlations were found between ΔE^* or b^* and GAE (Table 1), as well as between b^* and TEAC ($r_{TEAC/b^*} = 0.7683$ in Fig. 5d).

4. Conclusions

Comprehensive characterisation of antioxidant capabilities in biological and food systems, of which honey is one example, requires the application of several experimental methods and assays. The results presented in our study demonstrate the capability of honey samples to eliminate model radical oxidants $ABTS^{\bullet+}$ and DPPH, and this capacity correlates well with their phenolics content. However, no correlation was found between the monitored ability to scavenge hydroxyl radicals (responsible for most of the free-radical-related damage in living organisms) and either phenolics content or other experimental data.

Honey, representing a complex system containing a variety of organic compounds with antioxidant and radical-scavenging activity, has the potential to serve as a significant source of natural antioxidants in human nutrition. The correlation between the colour of honeys and their antioxidant properties, found in our study, may provide a good guide for consumers, when looking for products with the highest antioxidant power.

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