ADVANCES IN THE ANALYTICAL METHODS FOR DETERMINING THE ANTIOXIDANT PROPERTIES OF HONEY: A REVIEW

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Abstract

Free radicals and reactive oxygen species (ROS) have been implicated in contributing to the processes of aging and disease. In an effort to combat free radical activity, scientists are studying the effects of increasing individuals' antioxidant levels through diet and dietary supplements. Honey appears to act as an antioxidant in more ways than one. In the body, honey can mop up free radicals and contribute to better health. Various antioxidant activity methods have been used to measure and compare the antioxidant activity of honey. In recent years, DPPH (Diphenyl-1-picrylhydrazyl), FRAP (Ferric Reducing Antioxidant Power), ORAC (The Oxygen Radical Absorbance Capacity), ABTS [2, 2-azinobis (3-ehtylbenzothiazoline-6-sulfonic acid) diamonium salt], TEAC [6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox)-equivalent antioxidant capacity] assays have been used to evaluate antioxidant activity of honey. The antioxidant activity of honey is also measured by ascorbic acid content and different enzyme assays like *Catalase (CAT), Glutathione Peroxidase (GPO), Superoxide Dismutase (SOD)*. Among the different methods available, methods that have been validated, standardized and widely reported are recommended.

Key words: Honey; antioxidant properties; DPPH; FRAP; ORAC; TEAC; ABTS

Introduction

Honey is a natural product made by honeybees (*Apis mellifera*). It has highly variable sensorial and physicochemical characteristics due to climatic, environmental conditions, as well as diverse origin of plants from which it is harvested. Therefore, different honey types have varying phenolic concentrations which contribute to different antioxidant activities. In addition, processing, handling and storage of honey may influence its composition (Gheldof et al., 2002, Khalil et al., 2010, Turkmen et al., 2006). Due to its potential and positive medicinal properties, honey is particularly recommended for children and sportsmen as it can help to support the overall well-being of the elderly as well as the invalids (Blasa et al., 2006). Due to this fact, it is crucial that the antioxidant potential of honey is determined which can also help in the authentication of different types of honey. Bertoncelj, Dobersek et al. (2007) in their research have investigated different honey types and reported significant differences in their antioxidant properties.

Scientific evidence suggests that antioxidants reduce the risk for chronic diseases including cancer and heart disease (Bjelakovic et al., 2004, Khalil and Sulaiman, 2010, Paolini et al., 2003, Vivekanathan et al., 2003). Besides honey, primary sources of naturally-occurring antioxidants are whole grains, fruits and vegetables (Beretta et al., 2005, Gheldof et al., 2002). The main characteristic of an antioxidant is its ability to mop up free radicals which are present in biological systems from a wide variety of sources. These free radicals may oxidize nucleic acids, proteins, lipids or DNA and have the potential to initiate degenerative disease. There are also preliminary reports pointing to the role of oxidative stress in ageing (Gilca et al., 2007, Muller et al., 2007). Therefore foods containing significant levels of antioxidants which can inhibit or delay oxidation of a substrate represent a healthy and logical diet choice. Antioxidants like phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydrogen peroxide or lipid peroxyl and thus inhibit the oxidative mechanisms that may lead to degenerative diseases. A number of clinical studies (Garc´ia and Castillo, 2008, Jendekova et al., 2006, Kandaswani and Middleton, 1994, Middleton et al., 2000) have suggested that the antioxidant contents in honey are the main factor for its observed therapeutic action in reducing the incidence of chronic diseases including heart disease and some cancers. The free radical scavenging activity of antioxidants in honey has been substantially investigated and reported in the literature (Blasa et al., 2007, Taormina et al., 2001).

Measuring the total antioxidant capacity of honey will provide an understanding of the functional properties of honey. The aim of our review is to evaluate the different assays used by various researchers for the determination of the antioxidant properties of honey and to highlight their advantages as well as disadvantages so that future researchers can make informed decisions when employing the various methods in their studies.

DPPH (Diphenyl-1-picrylhydrazyl) assay

DPPH is one of the most stable free radical and is frequently-used in the evaluation of radical scavengers in natural foods (Burda and Oleszek, 2001). DPPH assay method is very simple and is also quick for manual analysis of antioxidant contents. The DPPH method which can be used for solid or liquid samples is not only specific to any particular antioxidant, but also applies to the overall antioxidant capacity of the sample.

http://dx.doi.org/10.4314/ajtcam.v9i1.5

The DPPH test is based on the ability of the stable 2, 2-diphenyl-1-picrylhydrazyl free radical to react with hydrogen donors (Inoue et al., 2005, Fahey and Stephenson, 2002). The DPPH radical displays an intense ultra violet visible (UV-vis) absorption spectrum. In this test, a solution of radical is decolorized after reduction with an antioxidant (AH) or a radical (R) in accordance with the following equation (Parejo et al., 2000):

DPPH + AH → DPPH-H + A, DPPH + R→ DPPH-R.

The resulting decolorization is stoichiometric with respect to the number of electrons captured. The antioxidant activity of honey for DPPH has been assessed by several authors following the methodology described by other authors (Baltrušaitytė et al., 2007, Meda et al., 2005, Velazquez et al., 2003). Typically, 0.3 ml honey samples are dissolved in 2.7 ml methanol and each sample is then mixed with DPPH in methanol solution with the blank sample containing the same amount of methanol and DPPH.

Sangsrichan and Wanson (2008) however, prepared honey samples with deionized water followed by the addition of 0.2 ml of sample to 3.0 mL of 6 X 10^{-5} M solution of DPPH. The absorbance at 515 nm is measured using an UV-vis spectrophotometer after which the solution was allowed to stand in the dark for 30 min at room temperature. A lower absorbance reading of the reaction mixture indicates higher free radical scavenging activities expressed as the percentage of inhibition of free radical by the sample and is calculated using the equation shown below:

$$\% \text{ radical scavenging activity} = 100 \times (A_{control} - A_{sample})/A_{control}$$

A = Absorbance

The better way to make a comparison of antioxidant activity between honey samples are Inhibition Concentration (IC50) values defined as the concentration of sample required for 50% inhibition of free radicals. IC50 is determined from the plot between the remaining absorbance of free radical and concentration with each analysis in triplicates. In this test, quercetin, 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox), tocopherol and ascorbic acid are useful when used as positive controls (Isabel et al., 2009, Piljac-Žegarac et al., 2009).

The DPPH analysis is a fast and an uncomplicated test ensuring reliable result. Furthermore, it requires only a UV-*vis* spectrophotometer to perform, which explains its widespread use in screening antioxidant properties. However, the method may sometimes be complicated when test compounds have spectra that overlap with DPPH at 515 nm. The assay is not a competitive reaction because DPPH is both a radical probe and an oxidant. DPPH is stable nitrogen radical that bears no similarity to the highly reactive and transient peroxyl radicals involved in lipid peroxidation.

A disadvantage of DPPH method is the fact that many antioxidants that may react quickly with the radical peroxide are almost or entirely inert to DPPH. Despite having the above limitations, DPPH is stable, is commercially-available and does not have to be generated before carrying out assays like [2, 2-azinobis (3-ehtylbenzothiazoline-6-sulfonic acid) diamonium salt] (ABTS). For these reasons it is considered as an easy and useful spectrophotometric method with regards to screening or measuring the antioxidant activity of honey as recommended by Alvarez-Suarez et al. (2009).

FRAP (Ferric Reducing Antioxidant Power) assay

FRAP method is another method that can be employed for the determination of total antioxidant activities of honey. Even though it is primarily used for determining antioxidant activity of plasma, it has also been successfully applied to measure antioxidant activity of a number of biological samples and pure substances (Ghiselli et al., 1998; Ou et al., 2002(a); Ou et al., 2002). Since antioxidant and antiradical properties of honey extract have mainly been attributed to the presence of phenolic compounds (Beretta et al., 2005; Soares et al., 1997), it is expected that the effectiveness of a honey fraction is proportional to its phenolic concentrations. FRAP assay has so far been widely-used to directly test the total antioxidant potential of several foods and plant extracts based on the reduction of complexes of 2, 4, 6-tripyridyl-s-triazine (TPTZ) with ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), which are almost colorless. The solution will eventually turn slightly brownish forming blue ferrous complexes after complete reduction (Benzie and Strain, 1999).

FRAP assay uses antioxidants as reductants in a redox-linked colorimetric method, employing an easily reduced oxidant system present in stoichiometric excess. Usually, aliquots of the honey solution are mixed with a FRAP reagent (10 mM of TPTZ solution in 40 mM HCl, 20 mM $FeCl_3$ and 0.3 M acetate buffer at pH 3.6) followed by spectrophotometric measurement of the absorbance of the reaction mixture after incubation at 37ºC for 10 min at 593 nm against the blank. The final results can be expressed as the concentration of antioxidants having a ferric reducing ability equivalent to that of 1 mM $FeSO_4$ used as the standard solution. Trolox and ammonium ferrous sulfate [$Fe(NH4)_2(SO4)_2 \cdot 6H_2O$] can also be used for the calibration curve with the final results expressed as micromoles of Trolox Equivalent (TE) or ammonium ferrous sulfate per 100 gram of honey respectively.

Using the FRAP method; some authors have also attempted to correlate the antioxidant activity of honey with the amount of L-ascorbic acid (Ferreira et al., 2009; Gheldof et al., 2002; Kesic et al., 2009). It is concluded that the total antioxidant activity of analyzed samples of honey was higher in samples with higher content of L-ascorbic acid (Kesic et al., 2009).

FRAP method however, has its own limitations, especially for measurements below non-physiological pH values i.e. at pH 3.6. In addition, this method is unable to detect slowly-reacting polyphenolic compounds and thiols (Ou et al., 2002; Jerkovic and Marijanovic, 2010). Furthermore, any compounds (even without antioxidant properties) with redox potential lower than that of the redox pair $Fe(3^+)/Fe(2^+)$, can theoretically reduce $Fe(3^+)$ to $Fe(2^+)$ contributing to an increase in the FRAP value and thus inducing false positive results (Alvarez-Suarez et al., 2009). On the other hand, not all antioxidants reduce $Fe(3^+)$ at a rate fast enough to allow its measurement within the observation time (typically 4 min). Indeed, many polyphenols react more slowly and require longer reaction times (30 min) for total quantification. Moreover, some polyphenolic compounds such as quercetin, caffeic, ferulic and tannic acids may have slower reactions, requiring longer time (approximately 30 min) until the complex reduction process was completed. When used to determine the

antioxidant potential of polyphenols in water and methanol (the solvent typically used for extraction of antioxidant from honey), the change in absorbance continued after 4 min (Blasa et al., 2006). Therefore, the FRAP values for these compounds cannot be accurately determined in 4 min. Hence for this reason, the ideal reaction time should be at least 10 min.

ORAC Assay (The Oxygen Radical Absorbance Capacity)

ORAC assay is a method for quantifying the antioxidant strength of substances. It involves combining the sample to be tested (i.e. the antioxidant) with a fluorescent compound as well as a compound that generates free radicals at a known rate. As free radicals are being generated, the fluorescent compound (compounds that exhibit fluorescence, such as fluorescein) is damaged and subsequently loses its fluorescence.

When antioxidants are present, it mops up the free radicals being produced and therefore inhibits the loss of fluorescence as described. The stronger the antioxidant property of a substance, the higher is the degree of inhibition on the loss of fluorescence. The measurement is standardized Trolox which has a known ORAC value and is reported in terms of Trolox equivalents (µM TE). This method serves as an excellent way to quantify the ability of various compounds to quench free radicals. ORAC assay in honey can be carried out by using a modified procedure described by Cao et al. (1993); Ou et al. (2001); Ou et al.(2002) and Rasmussen et al.(2008).

Gheldof et al. (2002) measured the *in vitro* antioxidant activity of honey polyphenols by comparing ORAC with the total phenolics concentrations. They showed that out of 14 honey samples, Buckwheat Illinois honey have the highest ORAC values with total phenolic content of 16.95 µmol trolox equivalent/g and 796 gallic acid equivalent/kg respectively.

The free radicals in ORAC method are produced by 2,2'-azobis (2-amidino-propane) dihydrochloride (AAPH) followed by the oxidation of the fluorescent indicator protein phycoerthrin (β-PE) (Gheldof, 2002). This loss of fluorescence can be inhibited by antioxidants and was monitored using a microplate fluorescence reader. All reagents are prepared in phosphate buffer (75 mM), pH 7.0 with Trolox (0-4 µM), which is used as a standard. Before use, the honey samples are suitably diluted in the phosphate buffer. Quercetin dehydrate (1 µM) (positive control) is dissolved in methanol followed by a dilution with buffer (1:249, v/v). Methanol is used in the control sample, blank and standard without having an effect on the 1:1 relationship between Trolox and ORAC value. The reaction mixtures consisted of 1 mL of β-PE (0.92 nM) which has been pre-incubated for 15 min at 37°C), 60 μL of test compound, 40 µL of 75 mM phosphate buffer (pH 7.0) and 100 μL of AAPH (500 mM). Once the AAPH is added, the plate is automatically shaken for 3 sec and the fluorescence is measured every 2 min for 70 min with emission and excitation wavelengths at 565 and 540 nm respectively using a micro plate fluorescence reader FL600 (BioTek, Inc., VT) which was maintained at 37°C. The ORAC values are calculated according to (Cao et al., 1993) and expressed as μM Trolox equivalent (TE)/g. Analysis of variance with post-hoc comparisons using Tukey's test is performed to compare the ORAC readings shown by the different honey samples using SAS Software (NC, version 8, SAS Institute Inc, Cary, 1999).

Utilization of a β-PE method provides an additional advantage as the substrate "self-prevents" free radical generations due to its oxidation. Therefore, it is good to determine the capacity of hydrophilic and hydrophobic samples simply by changing the generating source of radicals and the solvent. One limitation of this method however, is that the protein photo bleached under plate-reader conditions has large inter-batch differences and may interact with polyphenols due to non-specific protein binding and therefore may loss fluorescence even without the addition of a free radical generator (Alvarez-Suarez et al., 2009)

ABTS [2, 2-azinobis (3-ehtylbenzothiazoline-6-sulfonic acid) diamonium salt] assay

ABTS is a measure of antioxidant activity in contrast to antioxidant concentration which might include a proportion of biologically-inactive antioxidants (Rice-Evans and Miller, 1995, Salah et al., 1995). ABTS permits the measurement of antioxidant activity of mixtures of substances, hence helping to distinguish between additive and synergistic effects (Miller et al., 1996, Rice-Evans et al., 1995, Rice-Evans et al., 1996). The original assay is based on the activation of metmyoglobin with hydrogen peroxide in the presence of ABTS to produce the radical cation either in the presence or absence of antioxidants. This has been criticized on the basis that the faster reacting antioxidants might also contribute to the reduction of the ferryl myoglobin radical (Re *et al.*, 1999). A more appropriate assay method is using a decolorization technique because the directly generated radical is stable prior to reacting with the putative antioxidants. This improved technique for generation of ABTS involves the direct production of the blue/green ABTS chromophore via the reaction between ABTS and potassium persulfate (Re *et al.*, 1999)which has absorption maxima at wavelengths 645 nm, 734 nm and 815 nm (Miller, 1994; Miller, 1993;Re et al., 1999) with the more commonly used maximum absorbance reported to be at 415 nm (Huang et al., 2005, Re et al., 1999).

The addition of antioxidants to the pre-formed radical cation reduces ABTS on a time-scale to a certain extent, depending on the antioxidant activity of the samples analyzed, the concentration of the antioxidant and the duration of each reaction. Thus, the extent of decolorization as percentage inhibition of the ABTS radical cation is determined as a function of both concentration and time and is calculated relative to the reactivity of Trolox standard under similar conditions. A modification of the method utilised to determine the antioxidant capacity of honey was also developed by (Piljac-Žegarac et al., 2009). For the evaluation of antioxidant activity, ABTS solution is diluted with ethanol (96%) to obtain an absorbance of 0.700 (± 0.020) at 734 nm. Two mL of ABTS solution are mixed with 100 µL of honey solution in a cuvette and the decrease in the absorbance is measured after 6 min. The reagent blank is prepared by adding 100 mL of ethanol instead of the sample. Ascorbic acid was then used as the standard at different concentrations (0-100 mg/L) prepared in 96% ethanol and assayed under a similar procedure as that conducted on the samples with the means of the three values expressed as mg (Ascorbic Acid Equivalents) AAE/100 g of honey.

ABTS assay is beneficial as it reduces labor time, material cost and sample volume. Some of the assays have also been adapted for more convenient mass screening using quantitative spectrophotometer assays as well as being applied in agriculture and food industries. Although this method has been reported and commercialized by CAYMAN (Gupta et al., 2009) the method does not incorporate any blank samples which could result in further inaccuracies in the measurements.

TEAC Assay (Trolox-equivalent antioxidant capacity)

TEAC measures the antioxidant activity of a given substance, as compared to the standard, Trolox (Huang et al., 2005). The three TEAC tests developed at different periods, namely TEAC assay I (ABTS) enzymatically-generated with met-myoglobin and hydrogen peroxide, TEAC II (radical generation with filtration over the manganese dioxide (MnO_2) oxidant and TEAC III [with potassium persulphate ($K_2S_2O_8$) oxidant] even though were totally different from one another, are interchangeably-used even when using different solvent media. This may create significant variability in the measurements of the antioxidants (Apak et al., 2007;Schleiser et al., 2002).

The 'pre-addition technique' (employed by adding antioxidants before radical generation) for TEAC I could result in an overestimation of antioxidant capacity because many substances interfere with the formation of the free radical; TEAC I measured the ability of delaying radical formation as well as scavenging of the radical (Schleiser *et al.*, 2002). Since the reagent is soluble in both aqueous and organic solvent media, the advantages of ABTS/TEAC are reported to be operational simplicity, reproducibility, diversity and the most important of all, flexible usage in multiple media to determine both hydrophilic and lipophilic antioxidant capacity of food extracts and physiological fluids (Awika et al., 2003).

Ascorbic acid content Assay

Besides phenolic compounds, honey may contain other compounds known to act as antioxidants, such as ascorbic acid (Alvarez-Suarez *et al.*, 2010). Determination of ascorbate by HPLC is based on the methods developed by (Lee and Coates, 1999). Triplicate extracts are prepared by diluting 5 g of honey to 10 mL with dithiothreitol solution (4.2 mM in 0.1 M K_2HPO_4, pH 7.0) followed by a through mixing. For this test, 1 mL of extract and 1 ml of 4.5% *m*-phosphoric acid are mixed followed by a 20 μL injection into the HPLC system (Gheldof et al., 2002). The stationary phase of the HPLC is a 150 mm; 3.9 mm i.d., 5-μM XTerra RP18 (Waters, MA) column. A linear gradient is generated using 50 mM KH_2PO_4 (pH 4.5) (solvent A) and methanol (solvent B) starting at 100% A and decreasing to 70% A in 8 min. The selected flow rate was 0.8 mL/min with detection done at 263 nm.

Low levels (<5 mg/100 g) of ascorbic acid have been reported in honey (White, 1975), although some authors (Ferreira *et al.*, 2009) detected values between 140 and 145 mg/kg in Portuguese honey samples using spectrophotometric method. In another study, ascorbic acid was not detected in any of the five monofloral Cuban honeys analyzed, using HPLC coupled to photodiode array detection (Alvarez- Suarez *et al.*, 2010). A similar result was obtained by (Gheldof et al., 2002) which was attributed to a loss of the antioxidant during sample processing and storage.

Enzyme Assays
i) Catalase (CAT) Assay

CAT activity is usually determined according to the method published by Gott (1991). Briefly, this assay involves the incubation of a test tube containing 0.5 mL of hydrogen peroxide and 0.1 mL of pancreatic homogenate. After incubation in a water bath at 37°C for 60 sec, the reaction is terminated by adding 0.5 mL of ammonium molybdate solution. A yellow complex of ammonium molybdate and hydrogen peroxide will be formed. The absorbance of this yellow color is measured at 405 nm using spectrophotometer. One unit of catalase is defined as the amount of enzyme that catalyzes the decomposition of 1 µmol of hydrogen peroxide per min.

A study conducted by Erejuwa et al. (2010) investigated the hypothesis that common antidiabetic drugs such as glibenclamide and metformin in combination with tualang honey offer additional protections against oxidative stress and damage for the pancreas of streptozotocin (STZ)-induced diabetic rats using CAT assays. The researchers found that CAT activity in the pancreas is significantly reduced among the diabetic control groups when compared with the rats in the control group. On the other hand, administration of honey or a combination of glibenclamide, metformin and honey significantly up-regulated catalase activity towards the normal control group, indicating that increased catalase activity is enhanced by honey.

ii) Glutathione Reductase (GR) Assay

GR activity is by and large measured according to the procedure described by Goldberg and Spooner (1983) using oxidized glutathione (GSSG) as a substrate . Briefly, 1 mL of 2.728 mM of GSSG solutions and 40 µL of pancreatic homogenate is incubated in a water bath at 37°C. After incubation for 5 min, the reaction is initiated by the addition of 200 µL of 1.054 mM NADPH solution and the decrease in absorbance is measured at 340 nm using spectrophotometer and recorded every 30 sec over a period of 5 min. GR activity is expressed as unit per mg protein based on the molar extinction coefficient of 6.22×10^3 L/mol/cm. One unit of GR is defined as the amount of enzyme that catalyzes the oxidation of 1 nmol of NADPH per min.

iii) Superoxide Dismutase (SOD) Assay

SOD activity is generally measured using an assay kit (Cayman, MI, USA) according to the manufacturer's recommendations. This kit utilizes a tetrazolium salt for the detection of superoxide radicals generated by xanthine oxidase

and hypoxanthine. One unit of SOD is defined as the amount of enzyme needed to produce 50% dismutation of superoxide radical. The SOD assay measures all the three types of SOD [Copper/Zinc (Cu/Zn), Manganese (Mn) and Iron (Fe) SOD].
In a research by Erejuwa et al. (2010), honey-treated diabetic rats showed significantly reduced SOD activity, but neither glibenclamide and metformin nor their combinations reduced SOD activity in diabetic rats indicating that super oxide dismutase activity is reduced by honey.

iv) Glutathione Peroxidase (GPO) Assay

GPO activity is measured using an assay kit (Cayman, MI, USA) according to the manufacturer's instructions. The measurement of GPx activity is based on the principle of a coupled reaction with glutathione reductase (GR). The oxidized glutathione (GSSG) formed after reduction of hydroperoxide by GPO is recycled to its reduced state by GR in the presence of NADPH. The oxidation of NADPH is accompanied by a decrease in absorbance at 340 nm. One unit of GPO is defined as the amount of enzyme that catalyzes the oxidation of 1 nmol of NADPH per min at 25^0C (Erejuwa, et al. 2010). .

v) Glutathione-S-Transferase (GST) Assay

GST activity is usually determined according to the method published by Habig et al. (1974). This procedure is based on the conjugation of glutathione (GSH) to 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate. Briefly, 2 mL of 0.3 M potassium phosphate buffer (pH 6.35), 75 µL of 30 mM CDNB solution, 725 µL of distilled water and 0.1 mL of pancreatic homogenate were pipetted into a test tube. The test tube was vortexed and incubated at 37°C for 10 min. After incubation, the reaction is initiated by the addition of 100 µL of 30 mM reduced glutathione solution and the decrease in the the absorbance is spectrophotometrically measured at 340 nm and recorded every 30 sec for 4 min. GST activity is calculated as unit per mg protein based on a molar extinction coefficient of 9.6×10^3 L/mol/cm. One unit of GST was defined as the amount of enzyme that catalyzes the conjugation of 1 nmol of GSH-CDNB per min.

vi) Lipid Peroxidation (LPO) Assay

The extent of lipid peroxidation is usually determined by the concentration of thiobarbituric acid reactive substances (TBARS). Briefly, 100 µL of pancreatic homogenates or malondialdehyde (MDA) standards are pipetted into test tubes containing 1.5 mL of 20% (w/v) glacial acetic acid (pH 3.5), 200 µL of 8.1% (w/v) Sodium dodecyl sulfate SDS, 1.5 mL of 0.8% (w/v) Thiobarbituric acid TBA and 700 µL of distilled water according to the method described by Ohkawa et al. (1979). The test tubes were incubated at 95°C for 60 min with a marble on top of each test tube. After incubation, the test tubes were cooled and then centrifuged at 3000 X g for 10 min and the amount of MDA formed was spectrophotometrically measured at 532 nm.

Conclusion

The antioxidant properties of honey are contributed by the presence of polyphenolics, flavonoids, vitamin C and monophenolics making the identification and quantification of these compounds important.
A range of analytical methods for the determination of antioxidant properties of honey is available. Our review clearly demonstrates that different assay methods differ from one another in terms of reaction mechanisms, oxidant species, reaction conditions and the way the final results were expressed. It is hoped that the summarised information on the various methods available for antioxidant determination of honey can provide the scientific community with reliable information to confirm the benefits of antioxidant effects of honey and help provide some basic information before a more expensive and time-consuming effort of identification and characterization of the antioxidant components of honey is embarked. It is crucial that only methods that have been validated and standardized were selected.

As a conclusion, the use of DPPH assay coupled with various other useful methods such as FRAP and ORAC are preferred because they are able to reflect the antioxidant properties of honey more accurately.

Acknowledgement

The first author is financially supported by Universiti Sains Malaysia RU grant (grant no. 1001/PPSP/815058).

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