Pol. J. Food Nutr. Sci., 2015, Vol. 65, No. 2, pp. 87–91
DOI: 10.1515/pjfns-2015-0024
http://journal.pan.olsztyn.pl

Original article
Section: Food Chemistry

Antioxidant Properties of Honey from Different Altitudes of Nepal Himalayas

Bishnu Prasad Neupane[1], Komal Prasad Malla[1], Atis Kaundinnyayana[1], Prakash Poudel[1], Rashmi Thapa[1], Sabina Shrestha[2]*

[1]School of Health and Allied Sciences, Pokhara University, Lekhnath-12, Kaski, PO Box No. 427, Nepal
[2]Faculty of Biotechnology, College of Applied Life Science, Jeju National University, Jeju 690–756, Republic of Korea

Key words: Nepalese honey, antioxidant activity, phenolic content, physicochemical parameters

Twenty two multifloral honey samples representing central western parts of Nepal were examined spectrophotometrically for their antioxidant properties and total phenol content. The modified Folin-Ciocalteu method was used to determine total phenol content and 2,2-diphenyl-1-picrylhydrazyl radicals (DPPH•) assay for antiradical activity. In all samples, physicochemical parameters like moisture, reducing sugar, sucrose, ash, free acidity and water insoluble matter were also measured according to harmonized methods of the International Honey Commission (IHC). The results of physicochemical analysis showed that all the values, except for moisture of a small number of high altitude honey samples, are in good agreement with the current Nepalese standard. The total phenolic contents of honey, collected from high and low altitude, ranged from 154.87 to 41.90 mg gallic acid equivalent (GAE/100 g) respectively, at corresponding antiradical activity using DPPH• expressed as percent inhibition of 76.66% and 25.69%. The IC_{50} values of selected high altitude honey samples ranged from 56 to 72 mg/mL. The total antioxidant properties were correlated ($P<0.01$) between total phenol content and antiradical activity ($r=0.992$). The obtained results demonstrate that the Nepalese honey collected from high altitude region contained more antioxidants than honey of low altitude region.

INTRODUCTION

Honey is naturally sweet and viscous liquid made from the nectar of flowers collected by honey bees. It comes in numerous varieties with different colours, textures and flavours. The flavour, colour and sweetness of honey depend on climatic and environmental conditions and diverse botanical origins from which it is harvested [Gheldof & Engeseth, 2002; Küçük *et al*., 2007; Aljadi & Kamaruddin, 2004]. Man's use of honey goes back tens of thousands of years for its nutritional as well as curative purpose [Kaal, 1991]. The usage has continued into present-day folk medicine and is increasingly becoming a part of modern professional medicines. The role of honey in the treatment of various ailments has received a considerable attention recently, and its therapeutic value has been partly attributed to its antioxidant properties [Gheldof & Engeseth, 2002; Aljadi & Kamaruddin, 2004]. Antioxidant molecules prevent or inhibit reactive oxygen species (ROS) produced in metabolic and physiological process, and harmful oxidative reactions occurring in organisms [Young & Woodside, 2001]. Under certain conditions, the increase in oxidants and decrease in antioxidants cannot be prevented, and the oxidative or antioxidative balance shifts towards the oxidative status. Consequently, oxidative stress can be responsible for over 100 disorders [Halliwell *et al*., 2000]. Therefore, as food additive antioxidant potential of honey can play a positive role to overcome human health issues. Many methods for determining the antioxidative activity in honey have been used such as determination of active oxygen species, their radical scavenging ability [Gheldof & Engeseth, 2002], the 2,2-diphenyl-2-picrylhydrazil (DPPH) antioxidant content [Chen *et al*., 2000] and enzymatic and non-enzymatic measurements of lipid peroxidation inhibition [Chen *et al*., 2000; McKibben & Engeseth, 2002; Nagai *et al*., 2001]. The selection of honey samples with various altitude origins in this study was based on the assumption that varying total phenol content and antioxidant capacity is expected for honey produced from diverse floral sources having different geographic regions of Himalayan foothills to the plains of Terai region. The extensive exploitation of honey combs by honey hunters and traders has led to drastic decline of accessible honey combs of Himalayan rock bee (*Apis laboriosa*) in Himalayan region. In spite of its high medicinal values, virtually little research data are available on the phenolic antioxidants and radical scavenging activity in the context of Nepal.

The aim of this study was to determine the antioxidant properties of twenty two honey samples available in central western part of Nepal, with special focus on two different altitudinal ranges of honey collection, high altitude (1500––3500 m asl) and low altitude (800–1500 m asl). In addition, in all of the honey samples, physicochemical parameters were also measured.

* Corresponding Author: Tel.: +977–9841533885;
E-mail: bnsudesh8@gmail.com (Bishnu Prasad Neupane)

MATERIALS AND METHODS

Honey samples

Twenty two raw honey samples of varying geographical origins (11 from low altitude, 800–1500 m asl and 11 from high altitude, 1500–3500 m asl) were used for this study. The honey samples were obtained directly from beekeepers and honey hunters of Kaski district of Nepal during April-May 2013. All tested honey samples were multifloral honeys as derived from at least 55% pollen contribution from more than one floral source [Von der Ohe *et al*., 2004]. Five grams of each honey sample was distributed into test tubes and diluted to 50 mL with distilled water using a vortex mixture. The solution was then filtered through Whatman No. 1 filter paper and analysed for physicochemical parameters, total phenol content and antioxidants. The tests of all determination were performed in triplicate and expressed as mean±SD.

Chemicals

Folin-Ciocalteau reagent and 2,2-diphenyl-2-picrylhydrazine (DPPH$^{\bullet}$) free radical were purchased from Sigma-Aldrich, Germany. All other chemicals and reagents used in this study were of analytical grade.

Physicochemical parameters

In all samples, the physicochemical parameters such as moisture, reducing sugars, sucrose, ash, free acidity, water insoluble matter and pH were determined according to the methods recommended by International Honey Commission [Bogdanov, 2009]. pH was determined using pH meter (PH500 Benchtop) by dissolving 10 g honey sample in 75 mL carbon dioxide free water. The free acidity was quantified volumetrically, titrating a honey sample with a solution of 0.05 N NaOH, up to pH 8.3, and expressing the results in milliequivalent of acids at 1000 g of honey. Moisture was determined using the refractometric method of Chataway [1932]. All measurements were taken using an Abbe refractometer, and the moisture (g /100 g honey) was obtained from the refractive index of the honey sample by consulting a standard table (Chataway table). Sugar and sucrose were determined by Fehling solution method [Lane & Eynon, 1923]. For the determination of water insoluble matter, the gravimetric method was used. Twenty grams of honey were diluted with 200 mL water, filtered through crucible and washed carefully, until free from sugars. The presence of sugars was tested by the addition of 1% phloroglucinol in ethanol to some filtrate and few drops of concentrated sulphuric acid, because sugars produce colour at the interface. The crucible was dried at 135±1°C for an hour [Lord *et al*., 1988]. The gravimetric methodology was used for the determination of ash content. Ten grams of the sample were transferred to the crucible and two drops of olive oils were added. Afterwards, the sample was heated in a hot plate until carbonized. The sample was kept in the preheated furnace at 600±25°C for at least one hour. The crucible was cooled in desiccator and weighed. The ashing procedure was continued until constant weight has been reached.

Total phenolic content

The determination of the total phenol (TP) content of honey samples was performed according to the Folin-Ciocalteau method with slight modifications [Singleton *et al*., 1999]. A 0.5 mL of aliquot of the freshly prepared honey solution was added to 2.5 mL of 0.2 N Folin-Ciocaleau reagents and mixed for 5 min, followed by the addition of 2 mL of 75 gm/L sodium carbonate. After incubation at room temperature for 2 h, the absorbance of the reaction mixture was measured at 760 nm. The TP content was expressed as mg gallic acid equivalents GAE/100 g of honey, using the calibration curve of gallic acid (0–200 mg/L) standards [Meda *et al*., 2005].

Antiradical activity

Antiradical activity of honey samples were determined by using the 2, 2-diphenyl-1-picrylhydrazyl radicals (DPPH$^{\bullet}$) assay. It was measured according to the method previously described by Zhang & Hamauzu [2004]. Each honey sample was precisely diluted to 4° BX (Refractrometer, ERMA, Tokyo) with distilled water. A 1.5 mL aliquot of 0.1 mmol/L DPPH$^{\bullet}$ solution in methanol was mixed on a vortex and left to stand at 25°C in the dark for 60 min. Then, the decrease in absorbance was measured at 517 nm on spectrophotometer against a methanol blank. The radical scavenging activity (A %) was calculated from the following equation:

$$A\,\% = \frac{A_0 - A_A}{A_0} \times 100$$

where A_A - absorbance of the studied sample and A_0 - absorbance of the control sample. The parameter IC_{50} was also determined for those samples having high radical scavenging activity. IC_{50} parameter was calculated from linear fitting of the radical scavenging activity to the DPPH radical as a function of antioxidants concentration (20–100 mg/mL). Absorption measurements were performed using a UV-VIS Shimadzu 1601spectrophotometer.

Statistical analysis

The results of all experiments were expressed as mean ± SD values and are representative of three independent experiments. Statistical analysis was carried out by t-test (2 tailed) one using PRISM version 5.0 statistical analysis software (GraphPad Software, Inc., San Diego). Values of $P<0.01$ were considered significant.

RESULTS AND DISCUSSION

Physicochemical parameters

The seven different physicochemical parameters namely; moisture, reducing sugar, sucrose, ash, free acidity, pH and water insoluble matter were summarised in Table 1. The moisture of four high altitude honey samples (2, 5, 6 and 7) was found in between 29.1–25.0%. These values are higher than the maximum permissive content for honey described by Nepalese standard. The permissible standard value of moisture for the honey in Nepal is not more than 23% [FNCCI/AEC, 2006]. Moreover, the results of physicochemical studies showed that all the average values of different

TABLE 1. Physicochemical parameters of the Nepalese honey samples (n = 22).

Sample No.	Moisture (%) (Mean±SD)	Reducing sugars (%) (Mean±SD)	Sucrose (%) (Mean±SD)	Ash (%) (Mean±SD)	Free acidity (mmol/kg) (Mean±SD)	Water insoluble matter (%) (Mean±SD)	pH
High altitude sample							
1.	22.0±0.34	62.55±0.70	4.4±0.06	0.05±0.00	14.0±0.15	0.3±0.14	4.76
2.	25.3±0.70	64.03±2.33	2.55±0.99	0.04±0.00	13.0±0.13	0.25±0.13	4.80
3.	20.6±0.70	66.57±0.84	5.57±0.49	0.04±0.00	27.7±0.16	0.22±0.12	4.56
4.	21.3±0.30	64.77±0.80	5.84±0.85	0.44±0.07	31.3±0.14	0.12±0.11	4.38
5.	29.1±0.20	61.71±2.03	4.83±0.90	0.14±0.05	43.3±0.10	0.11±0.13	4.89
6.	25.5±0.50	62.51±1.05	2.87±0.81	0.45±0.06	44.7±0.11	0.15±0.10	5.09
7.	26.13±0.23	67.85±0.78	5.65±1.58	0.37±0.12	34.7±0.15	0.14±0.14	4.44
8.	20.13±0.23	63.15±1.81	6.51±0.42	0.34±0.92	33.7±0.17	0.16±0.16	4.51
9.	21.13±0.30	71.44±6.43	5.16±0.05	0.20±0.00	17.3±0.12	0.19±0.15	4.68
10.	22.60±0.20	64.87±2.62	3.16±0.58	0.32±0.04	17.3±2.54	0.10±0.14	4.70
11.	23.20±0.20	68.25±0.43	4.13±0.01	0.24±0.00	12.7±3.67	0.18±0.13	4.85
Low altitude sample							
12.	22.01±0.10	62.15±0.10	6.35±0.02	0.35±0.81	14.0±0.18	0.13±0.12	4.79
13.	20.12±2.01	65.13±1.00	3.39±0.01	0.25±0.25	13.3±0.11	0.15±0.11	4.71
14.	20.25±2.10	63.71±2.00	2.38±0.05	0.31±0.12	11.3±0.14	0.11±0.8	4.72
15.	27.05±1.05	62.12±1.50	8.55±0.20	0.45±0.12	52.3±0.13	0.12±0.6	4.84
16.	25.02±1.01	56.02±2.15	5.25±0.91	0.31±1.50	53.3±0.16	0.14±0.9	4.84
17.	22.66±1.50	59.13±1.25	7.58±0.30	0.48±1.60	15.0±0.18	0.11±0.10	4.68
18.	21.1±0.20	55.23±1.65	4.34±0.49	0.37±0.05	20.7±0.12	0.16±0.11	4.48
19.	22.46±0.15	57.21±0.056	3.01±0.45	0.47±0.01	22.0±0.13	0.14±0.13	4.52
20.	22.23±0.22	68.32±0.55	6.75±0.75	0.48±0.20	54.7±0.13	0.16±0.13	4.84
21.	22.01±0.24	62.12±0.34	4.02±0.15	0.35±0.15	51.3±1.50	0.10±0.16	4.88
22.	21.1±0.20	55.23±1.65	3.45±0.15	0.47±0.01	34.0±0.13	0.15±0.14	4.13

parameters were within the limits set by Nepalese standard indicating the use of good practices by bee farmers in central western part of Nepal. The noted higher moisture content in high altitude honey samples might be due tendency of bees to build hives near water source and dependence on water for cooling as well as thinning honey to be fed to larva and the immediate collection of raw honey from the natural habitat along with some waxes.

Antioxidant properties

Since phenolic substances have been shown to be responsible for the honey antioxidant activity, total phenol content of the honey samples was investigated. The results of antioxidant activity and total phenolic content (TP) of the samples determined by DPPH assay and Folin-Ciocalteu method are presented in Table 2. Significant TP content differences were recorded among the honey types. The total phenolic contents were found to vary from 41.90 to 72.14 mg GAE/100 g in low altitude and 65.23 to 154.87 mg GAE/100 g in high altitude honey samples respectively. Similar phenolic contents (78.96–114.75 mg GAE/100 g) of several honeys from variuos floral sources were reported in literature, among which the highest TP contenet was found in strawberry tree (*Fragaria ananassa*) honey and honeydew honey samples [Gheldof & Engeseth, 2002; Beretta *et al*., 2005; Bertoncelj *et al*., 2007]. Several investigations have found a significant level of phenolic compounds in the honey samples of different floral origins [Meda *et al*., 2005; Bertoncelj *et al*., 2007; Ferreres *et al*., 1991; Gil *et al*., 1995; Martos *et al*., 2000; Blasa *et al*., 2006]. There is a significant difference ($P<0.01$) between the mean TP content of low altitude honeys 61.77 mg GAE/100 g and high altitude honeys 118.65 mg GAE/100 g.

The antiradical activity of tested honeys was evaluated and found significantly potential in the DPPH$^{\bullet}$ radical reaction system. The radical scavenging potential, expressed as % inhibition, of honey collected from high and low altitude with respect to DPPH radical was in between 76.66–38.23% and 37.27–25.59% respectively (Table 2). The average percent inhibition determined in the high altitude honey (59.53%) was found to be significantly higher than

TABLE 2. Total phenol (TP) content with respect to gallic acid equivalents (GAE) and antiradical activity by DPPH• assay of the analysed honey samples.

Sample no.	TP (mg GAE/100 g±SD)	DPPH assay (~% inhibition±SD)
High altitude samples		
1.	150.60±3.69	74.553±6.096
2.	154.87±3.41	76.667±2.814
3.	115.87±3.20	58.354±6.61
4.	114.52±2.65	59.167±8.04
5.	104.65±3.44	51.809±2.16
6.	98.45±3.34	50.224±2.70
7	139.18±4.23	68.902±0.38
8	135.04±3.66	66.850±1.07
9.	144.85±1.34	71.707±1.25
10.	65.23±3.45	38.232±3.22
11.	77.60±3.55	38.415±2.63
Mean	118.65	59.53
Low altitude samples		
12.	41.90±3.42	25.69±3.694
13.	61.91±5.35	30.65±8.676
14.	61.32±3.50	35.30±2.076
15.	59.45±625	29.43±2.928
16.	69.55±3.78	34.43±2.144
17.	65.22±7.49	37.24±1.890
18.	66.02±3.25	32.68±10.020
19.	55.41±5.25	34.86±2.542
20.	59.25±3.30	29.33±5.770
21.	67.41±1.28	36.34±1.582
22.	72.14±3.56	35.71±2.782
Mean	61.77	32.87

that of the low altitude honey (32.87%). It is difficult to compare the obtained values with data obtained by other authors that investigated Polish honeys due to different modes of presentation such as results expressed as mmol TEAC/kg [Rodríguez *et al*., 2012; Kuś *et al*., 2014]. However, the results are similar to those obtained by Socha *et al*. [2011]. A significant correlation was found between antioxidant activity determined by DPPH assay and phenolic content (r=0.992). Similar to our findings, some literature also reported strong correlation between the antioxidant capacity and total phenol content (r=0.873) [Beretta *et al*., 2005; Bertoncelj *et al*., 2007]. Among all honeys tested for their antioxidant capacity, the high altitude honey samples (Sample 1, 2 & 9) with the highest percentage inhibition in DPPH• assay were subjected for IC_{50} determination.

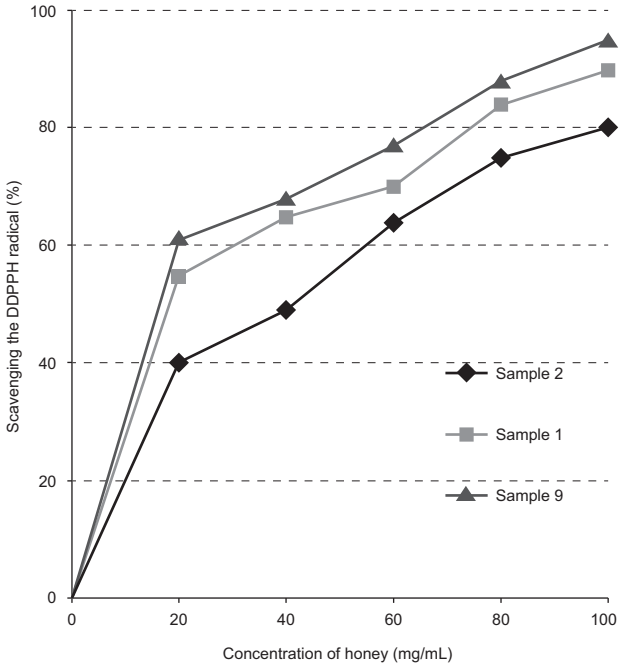

FIGURE 1. The capability of selected high altitude honey samples to scavenge the DPPH radical as a function of honey concentration for the determination of IC_{50} values.

From the results obtained it follows that sample 2 had big capability to scavenge the DPPH radicals with IC_{50} equal to 56 mg/mL. Samples 1 and 9 also had considerable capacity to scavenge DPPH radicals than low altitude honey samples and their IC_{50} equalled 68 mg/mL and 72 mg/mL, respectively. One can conclude that the values of IC_{50} parameter determined in this paper are comparable to those determined by other authors [Meda *et al*., 2005; Krpan *et al*., 2009; Kuś *et al*., 2014]. Figure 1 shows concentration dependence of the DPPH reduction for selected antioxidant high altitude honey samples.

CONCLUSION

The results of antiradical activity with respect to DPPH• radical and total phenol content revealed that the high altitude honey contained a higher level of antioxidants than low altitude honey, justifying that there would be the chance of synthesis of highly potent antioxidative secondary metabolites by the plants grown at high altitude Himalayan regions to cope with the harsh and extreme climatic conditions. The findings of present research demonstrated that the honey made from high altitude nectar by honeybees from plants grown at high altitude regions possessed high antioxidant capacity.

ACKNOWLEDGEMENTS

This research was funded by Research Division, University Grants Commission, Nepal, under the title Faculty Reserch Grants-2013. We are grateful to all local bee farmers and honey hunters of Kaski district, Nepal, for their coopertions during the field visits.

REFERENCES

1. Aljadi A.M., Kamaruddin M.Y., Evaluation of the phenolic contents and antioxidant capacities of two Malaysian floral honeys. Food Chem., 2004, 85, 513–518.
2. Beretta G., Granata P., Ferrero M., Orioli M., Maffei F.R., Standardization of antioxidant properties of honey by a combination of spectrophotometric/fluorimetric assays and chemometrics. Anal. Chim. Acta., 2005, 533,185–191.
3. Bertoncelj J., Dobersek U., Jamnik M., Golob, T., Evaluation of phenolic content, antioxidant activity and colour of Solvenian honey. Food Chem., 2007, 105, 822–828.
4. Blasa M., Candiracci M., Accorsi A., Piacentini M.P., Albertini M.C., Piatti E., Raw Millefiori honey is packed full of antioxidants. Food Chem., 2006, 97, 217–222.
5. Bogdanov S., Harmonized methods of the international honey commission. International Honey Commission., 2009. Retrieved from [http://www.ihc-platform.net/ihcmethods2009.pdf].
6. Chataway H.D., Determination of moisture in honey. Can. J. Res., 1932, 6, 532–547.
7. Chen L., Mehta A., Berenbaum M., Zangerl A.R., Engeseth N.J., Honeys from different floral sources as inhibitors of enzymatic browning in fruit and vegetable homogenates. J. Agri. Food Chem., 2000, 48, 4997–5000.
8. Ferreres F., Tomas-Barberan F.A., Gil M.I., Tomas- Lorente F., An HPLC technique for flavonoid analysis in honey. J. Sci. Food Agri., 1991, 56, 49–56.
9. FNCCI/AEC., Mandatory quality standard of honey in Nepal. 2006, *in:* The study report on trade competitiveness of Nepalese honey. Federation of Nepalese Chambers of Commerce and Industry /Agro Enterprise Center, p. 3.
10. Gheldof N., Engeseth N.J., Antioxidant capacity of honeys from various floral sources based on the determination of oxygen radical absorbance capacity and inhibition of *in vitro* lipoprotein oxidation in human serum samples. J. Agri. Food Chem., 2002, 50, 3050–3055.
11. Gil M.I., Ferreres F., Ortiz A., Subra E., Tomas-Barberan F.A., Plant phenolic metabolites and floral origin of rosemary honey. J. Agri. Food Chem., 1995, 43, 2833–2838.
12. Halliwell B., Gutteridge J.M.C., (eds.) Free Radicals in Biology and Medicine. 2000, Oxford Science Publications, pp. 617 – 624.
13. Kaal J., Natural medicine from honey bees.1991, *in:* Apitherapy. Kaal's Printing House, pp. 7–8.
14. Krpan M., Markovic K., Saric G., Skoko B., Haruskar M., Vahcic N., Antioxidant activities and total phenolics of acacia honey. Czech J. Food Sci., 2009, 27, 245–247.
15. Küçük M., Kolayli S., Karaoğlu Ş., Ulusoy E., Baltacı, C., Candan F., Biological activities and chemical composition of three honeys of different types from Anatolia. Food Chem., 2007, 100, 526–534.
16. Lane J.H., Eynon L., Determination of reducing sugars by means of Fehling's solution with methylene blue as internal indicator. J. Soc. Chem. Ind. Trans., 1923, 42, 32–36.
17. Lord D.W., Scotter M.J., Whittaker A.D., Wood R., The determination of acidity, apparent reducing sugar and sucrose, hydroxymethylfurfural, mineral, moisture, water-insoluble solids contents in honey; collaborative study. J. Assoc. Publ. Anal., 1988, 26, 51–76.
18. Martos I., Ferreres F., Yao L.D., Arcy B., Caffin N., Tomas-Barberan F.A., Flavonoids in monospecific *Eucalyptus* honeys from Australia. J. Agri. Food Chem., 2000, 48, 4744–4748.
19. McKibben J., Engeseth N.J., Honey as a protective agent against lipid oxidation in ground turkey. J. Agri. Food Chem., 2002, 50, 592–595.
20. Meda A., Lamien C.E., Romito M., Millogo J., Nacoulma O.G., Determination of the total phenolic, flavonoid and proline contents in Burkina Fasan honey, as well as their radical scavenging activity. Food Chem., 2005, 91, 571–577.
21. Nagai T., Sakai M., Inoue R., Inoue H., Suzuki N., Antioxidative activities of some commercially honeys, royal jelly, and propolis. Food Chem., 2001, 75, 237–240.
22. Kuś P.M., Congiu F., Teper D., Sroka Z., Jerković I., Tuberoso C.I.G., Antioxidant activity, color characteristics, total phenol content and general HPLC fingerprints of six Polish unifloral honey types. LWT-Food Sci Technol., 2014, 55, 124–130.
23. Rodríguez B.A., Mendoza S., Iturriga M.H., Castaño-Tostado E., Quality parameters and antioxidant and antibacterial properties of some Mexican honeys. J. Food Sci., 2012 71, 121–127.
24. Singleton V.L., Orthofer R., Lamuela-Raventos R.M., Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin–Ciocalteu reagent. Meth. Enzymol., 1999, 299, 152–178.
25. Socha R., Juszczak L., Pietrzyk S., Gałkowska D., Fortuna T., Witczak T., Phenolic profile and antioxidant properties of polish honeys. Int. J. Food Sci. Technol., 2011, 46, 528–534.
26. Von Der Ohe W., Persano Oddo L., Piana M.L., Morlot M., Martin P., Harmonized methods of melissopalynology. Apidologie, 2004, 35, 18–23
27. Young, I.S., Woodside J.V., Antioxidants in health and disease. J. Clin. Pathol., 2001, 54, 176 – 86.
28. Zhang D., Hamauzu Y., Phenolics, ascorbic acids, carotenoids and antioxidant activity of broccoli and their changes during conventional and microwave cooking. Food Chem., 2004, 88, 503–509.

Submitted: 29 September 2014. Revised: 18 February 2015. Accepted: 23 February 2015. Published on-line: 15 April 2015.