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Critical assessment of antioxidant-related parameters of honey

M.T. Sancho[1*], A. Pascual-Maté[1], E.G. Rodríguez-Morales[1], S.M. Osés[1], I. Escriche[2], A. Periche[2] and M.A. Fernández-Muiño[1]

[1]Department of Biotechnology and Food Science, University of Burgos, Plaza Misael Bañuelos s/n, 09001 Burgos, Castilla-León, Spain.

[*]Corresponding author Email: mtsancho@ubu.es

[2]Institute of Food Engineering for Development (IUIAD), Food Technology Department (DTA), Universitat Politecnica de Valencia, P.O. Box 46022, Valencia, Spain.

Abstract

In this study several antioxidant-related parameters were researched on 56 Spanish honeys, setting up and optimizing some assays. Melissopalinology and colour (L*, a*, b*) were determined. Solid phase extraction (SPE) was used to obtain honeys' phenolic extracts. Total phenolics, total flavonoids and trolox equivalent antioxidant capacity (TEAC) were determined in both honeys and extracts. It was verified that total flavonoids determination in neutral media must be carried out on extracts instead of on honeys, because of sugars' interferences; likewise, extracts' colours must be corrected in this assay. The end-point for honeys' trolox equivalent antioxidant capacity (TEAC) was researched. Significant linear relationships were found between TEAC values of honeys and honeys' phenolic extracts, as well as between the results of TEAC measured at different times. Therefore, it would be possible to reliably calculate TEAC at 60 minutes (end-point), measuring the absorbance at 6 minutes, thus saving analysis time and reducing costs.

Key words. Honey; Colour; Phenols; Flavonoids; Antioxidant activity.

Introduction

Honey has a wide range of phenolic compounds and therefore, it has been reported to possess an antioxidant ability, which greatly depends on its composition that is, in turn, conditioned by the botanical source of this foodstuff. Studies about the antioxidant potential of various unifloral, multifloral and honeydew honeys are interesting in order to later check if some honeys have actually antioxidant effects when they are ingredients of other food products, and/or within the body after consumption (*in vivo* assays). The latter research is of particular interest since the European Food Safety Authority denied the health claims with regard to antioxidant-related properties of honey because this food *"...has not been sufficiently characterized in relation to the claimed effects"* (European Food Safety Authority, 2010 and 2011). Flavonoids and other phenolics are the main compounds responsible for honey antioxidant activity (Malenica-Staver *et al.*, 2014). Honey flavonoids, as a whole, are usually determined by aluminum chloride chelation methods that must be carried out after sugars' removal, because these substances hamper proper chelation (Denni & Mammen, 2012). However, in most published papers, authors determine total flavonoids in neutral media directly on honeys, and sometimes, with no sample's colour correction. Trolox equivalent antioxidant capacity (TEAC), is a simple and widely used procedure to determine antioxidant activity of foods. Nevertheless, before using it to measure the antioxidant capacity of a particular food, the endpoint of the assay should be previously researched (Van den Berg *et al.*, 1999; Prior *et al.*, 2005).

The aims of this work were: First, to study antioxidant-related features of honeys from different botanical origins analyzing such parameters as colour (L*, a*, b*), total phenolics, total flavonoids, and TEAC. Second, to go in depth in the method for honeys' total flavonoids analysis carried out in neutral media. Finally, to research the endpoint for honey's TEAC

determination, in order to set up a reliable honey's antioxidant activity analysis by TEAC method.

Materials and methods

Samples

This work was carried out on 56 representative artisanal and unpasteurized Spanish honeys, whose botanical origins had been determined by melissopalinology (Von der Ohe *et al.*, 2004), with the result of 21 multifloral, 16 honeydew, 10 heather (*Erica* sp. and *Calluna vulgaris*), 5 lavender (*Lavandula* sp.), 3 clover (Leguminosae Type *Trifolium* sp.), and 1 sainfoin (Leguminosae Type *Onobrychis* sp.) honeys. Sampling was carried out within the region of Castile-Leon, covering an area larger than 94,200 square kilometers. Samples were stored at 4ºC until analysis in dark conditions.

Apparatus

Colour parameters were determined with a Hunter Lab colorimeter (ColorFlex EZ System®, Reston, VA, USA). Total phenolics, total flavonoids and TEAC assays were carried out by visible spectrophotometry with a 400Bio UV-visible spectrometer (Varian®, Mulgrave Victoria, Australia).

Procedures

Phenolic extracts were obtained by solid phase extraction (SPE). 10 g honey was mixed with 15 ml acidified water and loaded onto Strata-X SPE cartridge (Phenomenex®, Torrance, CA, USA) previously conditioned with methanol and water. Sugars and other polar honey's constituents were completely removed with acidified and ultrapure water. After vacuum

drying, phenolic fractions were eluted from the cartridge with 3 ml 2:1 (v/v) methanol:acetonitrile (Bertoncelj *et al.*, 2011).

Colour parameters L* (lightness), a* and b* (chromaticity coordinates) were determined using illuminant D65 and 10° observer. Specimens were illuminated at 45º (Commission Internationale de L'eclairage, 2004).

Total phenolics (mg gallic acid/100 g) were determined by Folin-Ciocalteu method (Meda *et al.*, 2005). 0.5 ml of a filtered honey solution (100 mg/ml), or 0.5 ml of a diluted extract (40 µl/ml) were mixed with 2.5 ml of 0.2 N Folin Ciocalteu reagent. After 5 min, 2 ml of saturated sodium carbonate solution was added, and the mixtures were kept in the dark for 120 min. Then, the absorbance was read at 760 nm. Gallic acid was used to adjust the standard curve.

Total flavonoids (mg quercetin/100 g), were determined in the extracts by the Dowd aluminum chloride colorimetric assay in neutral media (Dowd, 1959; Isla et al., 2011; Meda *et al.*, 2005), and adapted for the analysed samples. 1 ml of a diluted honey extract (60 µl/ml) was mixed with the same volume of 2% aluminium trichloride in methanol. After 10 minutes, absorbance (A_1) was read at 415 nm against a blank constituted by 1 ml of 2% $AlCl_3$ in methanol and 1 ml of methanol instead of the diluted honey extract. Colour of extracts was corrected by determining the absorbance (A_2) of a solution containing 1 ml of a diluted honey extract mixed with the same volume of methanol against a blank of methanol. A_2 was substracted from A_1 before calculating. Quercetin was used to adjust the standard curve that was read against a blank of methanol. The same procedure was also applied to honey solutions (0.01 mg/ml).

TEAC antioxidant activity (µmol trolox equivalent/g), were determined by measuring the scavenging ability of antioxidants to the radical $ABTS^{\bullet+}$ (Re *et al.*, 1999). TEAC was

analyzed in both honeys and extracts, measuring the absorbance at 734 nm after 6, 30 and 60 minutes. The radical cation $ABTS^{\bullet+}$ was produced by the reaction of 7 mM ABTS stock solution with 2.45 mM potassium persulfate in the dark for 16 h. Then, the $ABTS^{\bullet+}$ solution was diluted to obtain an absorbance between 0.70 and 0.80 at 734 nm. For honey solutions (100 mg/ml), 10 µl of each honey solution was mixed with 990 µl of the diluted $ABTS^{\bullet+}$ solution. For honey extracts, first, 300 µl extract was diluted to 5 ml with methanol, and finally, 10 µl of each diluted extract was mixed with 990 µl of the diluted $ABTS^{\bullet+}$ solution. Blank was distilled water for honeys and methanol for honey extracts. Trolox (6-hydroxy-2,5,7,8-tetrametylchroman-2-carboxylic acid), was used to adjust the standard curve.

Statistical determinations were carried out with Statgraphics Centurion XVI (2010). All analytical procedures were carried out in triplicate.

Results

The results of colour (L*, a*, b*), total phenolics, total flavonoids and TEAC antioxidant activity (measuring the absorbance at different times) are summarized in Table 1. Total flavonoids results are those corresponding to honey extracts, after verifying the unfeasibility of the method when diluted honeys were used instead of their extracts, because for more than 95% honeys the absorbance of the colour correction (1 ml of honey solution plus 1 ml of methanol), was higher than the absorbance of the sample (1 ml of honey solution plus 1 ml of 2% $AlCl_3$ in methanol).

In respect of unifloral honeys, lavender samples showed the highest values for lightness (ranging from 43.16 to 71.01), and heather honeys the lowest ones (ranging from 29.8~~.16~~ to 57.36). Heather samples showed intense reddish tonalities (ranging from 7.19 to 15.64), whereas a* values widely ranged in the other honeys. b* values were in all cases positive, meaning that all samples were in the yellow area. Heather honeys showed the highest values

of total phenolics (ranging from 112.32 to 183.35 mg gallic acid/100 g), extracts' total phenolics (ranging from 15.62 to 60.30 mg gallic acid/100 g), extracts' total flavonoids (ranging from 3.41 to 6.98 mg quercetin/100 g) and TEAC (ranging from 5.0 to 10.0 μmol trolox equivalent/g, reading the absorbance at 60 minutes), whereas lavender samples showed the lowest values for total phenolics (ranging from 51.52 to 101.48 mg gallic acid/100 g), extracts' total phenolics (ranging from 8.41 to 27.34 mg gallic acid/100 g), extracts' total flavonoids (ranging from 1.10 to 4.49 mg quercetin/100 g) and TEAC (ranging from 1.83 to 6.37 μmol trolox equivalent/g, reading the absorbance at 60 minutes).

Results of most parameters fulfilled all assumptions necessary for one-way ANOVA test (90% confidence level). For a* and b* colour results, the non-parametric Kruskal-Wallis test were applied (90% confidence level). L* values grouped heather and honeydew honeys. a*, b* and total flavonoids grouped multifloral, honeydew, clover and lavender honeys. Total phenolics and TEAC grouped, on the one hand, sainfoin and lavender samples, and on the other hand, multifloral, honeydew, clover and heather samples.

Values of L* were significantly correlated with those of honeys' total phenolics (r= -0.7925), extracts' total phenolics (r= -0.7859), honeys' TEAC (r= -0.8882) and extracts' TEAC (r= -0.8389); values of honeys' total phenolics were significantly correlated with those of extracts' total phenolics (r= 0.6849), honeys' TEAC (r= 0.8373) and extracts' TEAC (r= 0.7029); values of extracts' total phenolics were significantly correlated with those of honeys' TEAC (r= 0.7684) and extracts' TEAC (r= 0.8647). Similar correlations among colour features, phenolics and antioxidant activities were described in the literature for both honeys from different botanical and geographical origins (Perna *et al.*, 2012; Kamboj *et al.*, 2013; Gambacorta *et al.*, 2014; Petretto *et al.*, 2015) and honey-based dairy products (Bansal *et al.*, 2014).

TEAC antioxidant activity (µmol trolox equivalent/g) of both honeys and extracts progressively increased with time up to 60 minutes. Significant linear relationships (90% confidence level), were found among all TEAC results (Table 2). Linear relationships were obtained between TEAC of honeys and TEAC of honeys' phenolic extracts, as well as between TEAC of honeys and extracts measuring absorbance at different times (Figure 1). With the results of honeys' TEAC at 6 minutes and the equations of Table 2, TEAC values at 60 minutes were calculated. Then, actual and calculated values of TEAC at 60 minutes were compared with t-test and One-way ANOVA (90% confidence level). Both procedures showed that there were no differences between actual TEAC values (µmol trolox equivalent/g) of both honeys (Table 3) and extracts, measuring the absorbance at 60 minutes, and calculated TEAC values (µmol trolox equivalent/g) at 60 minutes, by measuring absorbance at 6 minutes.

Discussion

The variability of colour among the samples was mainly related to honeys' botanical origin. According to the literature, the mean value for L* (< 50) obtained in this study, is typical of dark honeys, such as heather (González-Miret *et al.*, 2005) and honeydew (González-Miret *et al.*, 2005; Tuberoso *et al.*, 2014). These results are in concordance with the fact that the majority of the analyzed samples (55%), were heather unifloral, honeydew honeys or honeys rich in heather and honeydew. a* and b* data were particularly high in heather samples and honeys rich in heather, so that in addition to other parameters, a* and b* values could help characterize heather honeys.

As expected, honeys' total phenolics were higher than extracts' total phenolics because the method of analysis (Meda *et al.*, 2005) actually determines total reducing capacity and apart from phenolic compounds, honeys possess different reducing substances such as ascorbic acid, and reducing sugars, among others (Ferreira *et al.*, 2009). The relationships found

between values of total phenolics and those of L* and TEAC antioxidant activity agreed with literature (Wilczynska, 2014).

The moderately significant relationship found between total phenolics of honeys and extracts is interesting, because it shows that the proportion of total reducing substances in honeys and their corresponding extracts appears to be somehow constant, even for very different honey samples from various botanical origins.

With regard to flavonoids' contents, the values of this work were in general lower than those described for different honeys by other authors. After a thorough literature revision, it must be explained that in most published papers, the spectrophotometric assay based on aluminum complex formation conducted in neutral media was carried out directly on a honey solution with no sugars' removal and, in some cases, with no sample's colour correction. The reason is likely due to the fact that those papers followed previous references that were in turn, based on procedures (Arvouet-Grand *et al.*, 1994; Popova *et al.*, 2005) published for propolis extracts in which there were no sugars' interferences. Thus, if the assay is carried out on a honey solution, the results depend on the specific flavonoid composition of the sample because, on the one hand, flavonoids do not react uniformly and, on the other hand, glycosylation prevent chelation of Al(III) with some flavonoids, but not with all flavonoids (Pekal & Pyrzynska, 2014).

In addition, in the literature there is no agreement about the blank when total flavonoids are analyzed directly on a honey solution in neutral media. As it has been commented above, the assays of all manuscripts were based on others, being the common principle for all of them the initial spectrophotometric Dowd's procedure with some modifications. The original method (Dowd, 1959), used an aluminum chloride reagent blank. Nevertheless, for honey solutions, in some articles the blank employed was methanol, thereby neither the reagents nor the samples colour were corrected, so that the final flavonoids' values could be overestimated.

Despite the fact that a blank of reagents (as in Dowd, 1959), is usual in spectrophotometric measurements, very few authors employed such blank for the analysis of honeys' flavonoids in neutral media, and their manuscripts cited Isla *et al.* (2011) as a reference, which was in turn based on Popova *et al.* (2005) procedure that had been set up and applied to 6 poplar Turkish propolis, in which the colour of the extracts could have not interfered. Values of honey solutions' total flavonoids of those papers (based on the Popova *et al.*, 2005 manuscript), might also be overestimated because matrix interferences were not substracted. When a honey solution is used, a sample's colour correction is compulsory, since the results are based on the absorbance measurement at 415 nm (or 425 nm), and at this(these) wavelength(s), there is a colour interference of the honey itself, which is particularly important for dark samples. In most published papers about total flavonoids analysis on honey solutions, authors claimed that they followed Meda *et al.* (2005) procedure, based in turn on Arvouet-Grand *et al.* (1994) assay for propolis extracts, which used as blank a solution of the sample and the solvent, thus only correcting the colour of the samples, but not the interference in the absorbance recording due to the aluminum chloride. Therefore, data of honey solutions' total flavonoids of those manuscripts (based on Arvouet-Grand *et al.* 1994 paper), could be overestimated, as well.

Both for honeys' extracts and for honeys' solutions, we followed the procedure described in this manuscript, in which a blank of reagents was used, and then absorbance of the colour of the samples was substracted, in a similar way to that described in the official method of AOAC (2005) for the analysis of proline in honey.

We verified that, when using honey solutions (instead of honey extracts), for the vast majority of samples, the absorbance values at 415 nm (and also at 425 nm), were considerably higher for colour correction than for the sample with flavonoid-aluminium complex, showing the unreliability of the procedure if sugars and other interferences were not removed. Therefore,

spectrophotometric analysis of honeys' flavonoids in neutral media must be always carried out after getting rid of sugars; otherwise the results could be specious.

Our TEAC results were similar to the values described in the literature for Brazilian honeys (Sant'Ana *et al.*, 2012), and slightly lower than the antioxidant activities described for South African samples (Serem & Bester, 2012). Our TEAC data were also similar to those described in the literature for other honeys from different botanical and geographical origins that were analyzed by another method combined to a flow injection analysis (Álvarez-Suárez *et al.*, 2010a,b; Gorjanovic *et al.*, 2013).

In respect of TEAC antioxidant activity, in the literature no agreement was found regarding the proper end-point for the absorbance measurement. Some researchers determined the absorbance at 1, 4, 6 and 10 minutes (Baltrusaityté *et al.*, 2007; Escriche *et al.*, 2014), whereas other scientists considered the end-point at 1 minute (Tuberoso *et al.*, 2013), at 6 minutes (Vit *et al.*, 2009; Sant'Ana *et al.*, 2012), at 7 minutes (Habib *et al.*, 2014), at 10 minutes (Silva *et al.*, 2013), at 15 minutes (Socha *et al.*, 2009; Kowalski, 2013; Wilczynska, 2014), at 20 minutes (Lachman *et al.*, 2010), and a 30 minutes (Serem & Bester, 2012). Our work shows that absorbance values at different times change proportionally for both honeys from different botanical origins and their corresponding extracts, so that it would be possible to calculate the TEAC antioxidant activity at 60 minutes, measuring the absorbance at 6 minutes, thus saving analysis time and reducing costs. However, it would be necessary to study if similar relationships occur in other honeys from different origins and harvested in different years, in order to propose an appropriate analytical procedure for the determination of honey's TEAC antioxidant activity.

Conclusions

This research has shown that honeys from different botanical origins share common antioxidant-related features, being the most important of which total phenolics and TEAC. Total flavonoids analysis with aluminium trichloride in neutral media must be carried out on honey extracts, because honey sugars interfere; furthermore, in this determination, extracts' colours must be corrected. In respect of TEAC assay, if similar equations are obtained for honeys from different botanical and geographical origins, it would be possible to save analysis time and money, calculating the values corresponding to the end-point from the data of absorbance at 6 minutes.

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Table 1

	Colour						Honeys' TEAC (µmol trolox/g)			Extracts' TEAC (µmol trolox/g)		
	L*	a*	b*	Honeys' total phenolics (mg gallic acid/100 g)	Extracts' total phenolics (mg gallic acid/100 g)	Extracts' total flavonoids (mg quercetin/100 g)	6 min	30 min	60 min	6 min	30 min	60 min
Mean	48.24	9.30	33.82	119.24	26.29	3.44	4.35	5.90	6.92	1.92	2.34	2.58
Median	45.45	9.67	33.71	130.05	27.51	3.41	4.55	6.23	7.52	1.99	2.45	2.68
Standard deviation	10.78	3.75	3.54	39.42	8.95	1.15	1.88	2.35	2.57	0.68	0.79	0.85
Minimum	26.07	0.17	23.80	29.10	7.98	0.93	0.97	1.39	1.64	0.49	0.62	0.76
Maximum	71.01	20.74	43.46	183.35	60.30	6.98	7.46	9.49	10.65	3.16	3.85	4.43

a*

Mean: 8.94

Median: 9.55

Standard deviation: 3.59

Minimum: 0.17

Maximum: 15.64

1 Table 2

2

Relationships	Correlation coefficient (r)
TEAC-Extracts= 0.59+0.30* TEAC-Honeys	0.9043
TEAC-Extracts-60= 0.22 + 1.23*TEAC-Extracts-6	0.9879
TEAC-Extracts-60 = 0.08 + 1.07*TEAC-Extracts-30	0.9978
TEAC-Extracts-30 = 0.12 + 1.16*TEAC-Extracts-6	0.9937
TEAC-Honeys-60= 1.00 + 1.35*TEAC-Honeys-6	0.9818
TEAC-Honeys-60 = 0.50 + 1.09*TEAC-Honeys-30	0.9959
TEAC-Honeys-30 = 0.49 + 1.24*TEAC-Honeys-6	0.9945
TEAC-Honeys&Extracts-60= -0.03 + 1.52*TEAC-Honeys&Extracts-6	0.9817
TEAC-Honeys&Extracts-60 = 0.50 + 1,09*TEAC-Honeys&Extracts-30	0.9959
TEAC-Honeys&Extracts-30 = 0.49 + 1.24*TEAC-Honeys&Extracts-6	0.9945

3

Table 3

	Actual TEAC-Honeys-60	Calculated TEAC-Honeys-60
Sample size	56	56
Average	6.91875	6.86411
Standard deviation	2.56686	2.52967
Coefficient of variation	37.10%	36.85%
Minimum	1.64	2.31
Maximum	10.65	11.06
Range	9.01	8.75
Stnd. Skewness	-1.44897	-0.543526
Stnd. Kurtosis	-1.36357	-1.59705
Variance	6,58875	6,39924
Degrees of freedom	55	55

Variance Check		
	Test	*P-Value*
Levene's	0.010297	0.91936

Comparison	*Sigma1*	*Sigma2*	*F-Ratio*	*P-Value*
ActualTEAC-Honeys-60 / CalculatedTEAC-Honeys-60	2.56686	2.52967	1.02961	0.9142

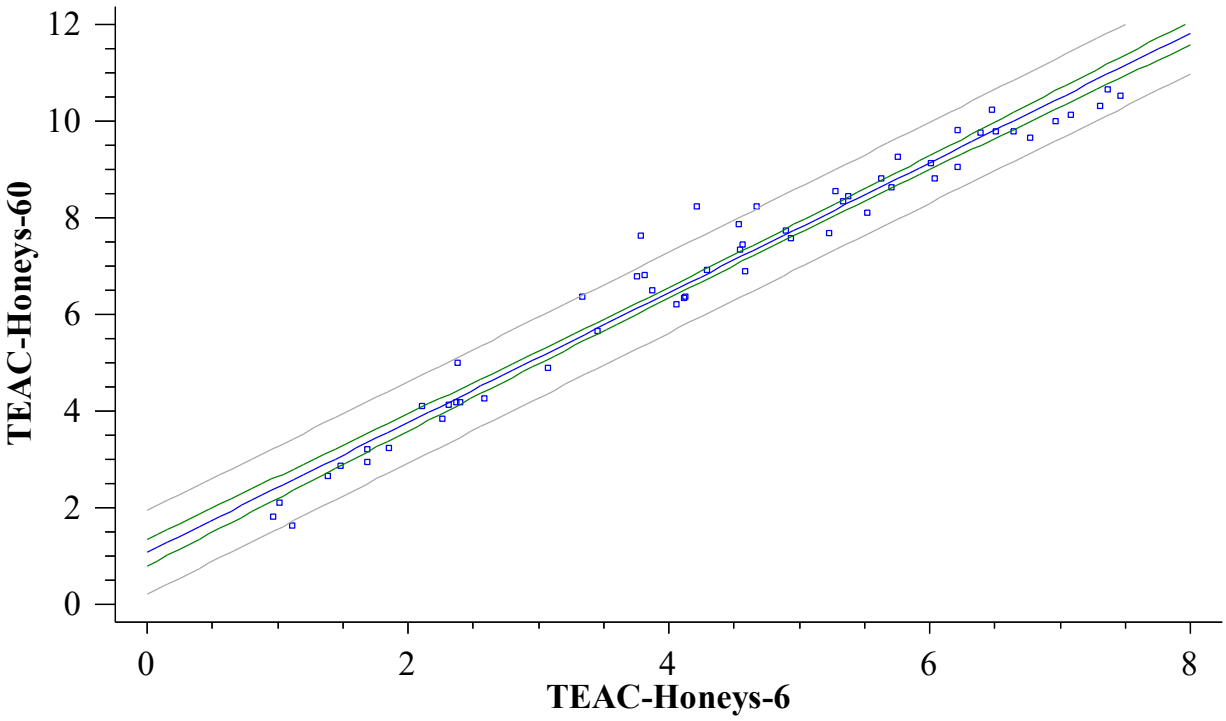

Figure 1

Table captions

Table 1: Mean, median, standard deviation, minimum and maximum values of L*, a*, b*, total phenolics of honeys and extracts, total flavonoids of extracts and TEAC antioxidant activity of honeys and extracts.

Table 2: Linear relationships among TEAC results of honeys and honeys' phenolic extracts.

Table 3: Summary of the results of t-test (90% confidence level) and variance check of one-way ANOVA (90% confidence level) applied to the results of honeys' TEAC actual values (μmol trolox equivalent/g) measuring the absorbance at 60 minutes (TEAC-Honeys-60), and the results of honeys' TEAC calculated values at 60 minutes (μmol trolox equivalent/g), measuring the absorbance at 6 minutes (TEAC-Honeys-6). **t = 0.113464. P-value = 0.90987**.

Figure legend

Figure 1: Relationships between TEAC values (µmol trolox equivalent/g) of honeys at 6 and 60 minutes. “TEAC-Honeys-60” is TEAC antioxidant activity measuring the absorbance at 60 minutes; “TEAC-Honeys-6” is TEAC antioxidant activity measuring the absorbance at 6 minutes). Equation: **TEAC-Honeys-60= 1.00 + 1.35*TEAC-Honeys-6. Correlation coefficient = 0.9818.**