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Determination of Iron (III) Reducing Antioxidant Capacity for Manuka Honey and Comparison with ABTS and Other Methods

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Authors' contributions

This work was carried out in collaboration between all authors. Authors ROA and PSN designed the study, wrote the protocol. Author HIMY conducted experimental work, managed the analysis. Authors HIMY and ROA wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Applying multiple assays with trolox as the sole reference compound is a recent AOAC proposal to improve the reliability of total antioxidant capacity determinations. The aim of this study was to evaluate, iron (III) reducing antioxidant capacity (*iRAC*) for Manuka honey samples and comparisons with ABTS and other well-known assays.

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Study Design: In-vitro, laboratory-based study.
Place and Duration of Study: School of Biomedical Sciences, Faculty of Life and Health Sciences, Ulster University, Cromore Road, Coleraine, BT52 1SA, UK; September 2015-May 2016.
Methodology: Manuka honey rated Unique Manuka Factor (UMF) 5+, 10+, 15+, 18+ and a non-rated (NR) sample were analysed using five assays for total antioxidant capacity namely, *iRAC*, ABTS, DPPH, FRAP, and Folin assays. Values for total antioxidant capacity were normalized as Trolox Equivalent Antioxidant capacity (TEAC) for comparison within and between assays.
Results: The TAC were correlated for all methods (R^2 = 0.83-0.99) and also correlated with the total phenols content. Actual TEAC value for a given honey ranged by 21-70-fold depending on the assay method with the following general order of increase; DPPH < FRAP (pH 3.6) < *iRAC* (pH 7.0) < ABTS (pH7) < Folin (pH ~11). The trends in TAC values are discussed alongside of TEAC values for 50 food items and some challenges for comparing different antioxidant methods are highlighted.
Conclusion: Total antioxidant capacity of Manuka honey changes in a regular manner probably affected by assay pH. The findings are important for attempts to standardize antioxidant methods as currently applied to foods, beverages and dietary supplements. Further research is recommended to examine the effect of normalizing antioxidant methods for solvent composition and pH.

Keywords: ABTS; antioxidants; honey; TEAC; total antioxidant capacity; food analysis.

ABBREVIATIONS

ABTS : 2,2'-azinobis-3-ethylbenzothiazoline 6-sulfonic acid,
DPPH : 2,2-diphenyl-1-picrylhydrazyl
FRAP : ferric reducing antioxidant power;
iRAC : iron (III) reducing antioxidant capacity
TEAC : trolox equivalent antioxidant capacity

1. INTRODUCTION

A high dietary antioxidant intake is associated with decreasing risk of chronic diseases including, atherosclerosis, cardiovascular disease, frailty in the elderly, colorectal cancer, and stroke [1-4]. Dietary antioxidant intake is inversely correlated with urinary 8-isoprostane biomarker for oxidative stress [5] and with C-reactive protein marker for chronic inflammation [6]. Large databases for total antioxidant capacity (TAC) of food items and food groups are being compiled for public health research [7,8].

Current guidelines support using multiple assays for TAC [9,10]. The AOAC recommends using trolox as the sole baseline antioxidant reference for foods, beverages and dietary supplements [11]. Some TAC assays were evaluated by professional organizations [11-13] and subjected to inter-laboratory testing with mixed success [14]. Currently, *in-vitro* methods do not reflect the entire antioxidant activity under physiological conditions [15]. Comparing results from different TAC assays remains challenging also [9-11,16]. Further research is needed to improve TAC assays for legislation, industry and health applications.

Manuka honey has significant commercial value linked with reports of antibacterial activity, the Unique Manuka factor (UMF) rating, methylglyoxal, leptosperin, total phenols content and other factors [17,18]. Honey is a good source of dietary antioxidants, with phenolic acids and flavonoids being major constituents [17,18]. The TAC of Manuka honey was reported from our laboratory [19-22] but analysis using multiple methods has not been published. There is no consensus regarding the antioxidant power of honey as a commodity. The aim of this paper is to evaluate the TAC for Manuka honey using a newly described method for iron (III) reducing antioxidant capacity (*iRAC*) and to compare the results with values determined using DPPH, ABTS, Folin and FRAP assays. Values for TAC of Manuka honey and nearly 50 food items are also discussed and some challenges for comparing different antioxidant methods are highlighted.

2. MATERIALS AND METHODS

2.1 Samples

Manuka honey rated Unique Manuka Factor (UMF) 5+, 10+, 15+ and 18+ were purchased from Comvita Ltd. (Maidenhead UK). Rowse honey selected as a non-rated (NR) honey with a presumed zero-UMF value was from Rowse Honey Ltd. (Oxfordshire, UK). All other reagents were purchased from Sigma-Aldrich, UK

(Gillingham Dorset, UK) unless otherwise stated. Spectrophotometric measurements were performed with a VersaMax, microplate reader (Molecular Devices, Sunnyvale, California, USA) and standard 96-well flat-bottomed microplates (Nunc, Sigma-Aldrich, UK).

2.2 Antioxidant Assays

The Folin-Ciocalteu method, FRAP, ABTS, and DPPH assays were adapted to a microplate format as described recently [19-22]. The reagents for *iRAC* comprised iron citrate (8 mM in deionized water, 1ml) as the soluble Fe (III) salt mixed with 9 ml of ferrozine (2.2 mM in 0.1 M Tris-HCl buffer, pH 7) immediately before use. Honey samples were diluted 1/10 with distilled water before analysis. For all assays, 20 µl of trolox (0-1000 µmol/l) or diluted honey was added to 96-well microplates followed by 280 µl of assay reagent using a multichannel pipette. Microplates were incubated for 30 minutes at 37 ºC, and absorbance values were recorded at 592 nm (FRAP & *iRAC*), 760 nm (Folin), 734 nm (ABTS) or 515 nm (DPPH) using a microplate reader.

Antioxidant methods were all calibrated using trolox. Calibration parameters were determined by plotting graphs of absorbance (Y-axis) versus concentration (mol/l) of trolox inside microplates (x-axis). Data were fitted by linear regression and the gradient (*m*) and squared regression coefficient (R^2) were recorded. The precision of analysis was determined from the average coefficient of variation (CV, %) where CV = (SD / mean) x 100. The minimum detectable concentration (MDC) was determined from the relation: MDC = (3 x SD_0 of "blank" solution) / *m*). Colorimetric readings for honey were expressed as trolox equivalent antioxidant capacity (TEAC) as described in Section 2.4. For comparison, gallic acid was used a second calibration compound and results were cited as gallic acid equivalents antioxidant capacity (GEAC). All experiments were repeated on two or more separate occasions with (n=) 8-16 replicates per data point.

2.3 Statistical Analysis

Statistical analyses were using IBM SPSS v. 22. One-way ANOVA was conducted to determine significant differences for mean values ($p<0.05$) with post-hoc analysis for the separation of means using Tukey or Dunnetts T3 test. Pearson 2-tailed test was used to examine correlations with significant results noted for $p<0.01$.

2.4 Additional Data Analysis

2.4.1 Calibration parameters for total antioxidant methods

Colorimetric analyses for antioxidants was modelled by Beer's equation (Fig. 1; Eq. 1), where ΔA_{TX} is absorbance for trolox corrected for a reagent blank, ε_{TX} (l/mol. cm) is molar absorptivity for trolox, C_{TX} is the concentration of trolox in the assay vessel (mol/l), and d (cm) is the optical pathlength for a microplate reader [21].

$$\Delta A_{TX} = \varepsilon_{TX}\, d\, C_{TX} = m.\, C_{TX} \quad (1)$$

Plotting a graph of ΔA_{TX} versus C_{TX} produced straight-lines (y = *m*x) confirmed by linear regression.

2.4.2 Total antioxidant capacity of honey

Colorimetric readings for honey (ΔA_H) conformed to Beer's equation (Eq. 2) where, C_H (g/l) is the concentration of honey; TAC refers to the *equivalent* concentration of trolox or TEAC (mol-trolox per gram of honey)

$$\Delta A_H = \varepsilon_{H.}\, d\, C_H = m'.\, C_H \quad (2)$$

The values of ΔA_H were converted to TAC [23] according to Eq. (3) and plotted as Fig. 2.

$$TAC = m'/\, m = \Delta A_H / (m\, .C_H) \quad (3)$$

It is noteworthy that replacing m (= ΔA_{TX} / C_{TX}) from Eq 3 produces the more familiar expression for TEAC [23] shown in Eq. (4). Also Eq (4) shows TEAC is a ratio quantity but that this parameter is not dimensionless;

$$TEAC = \Delta A_{H.}\, C_{TX} / (\Delta A_{TX}\, .C_H) \quad (4)$$

The units for TEAC (µmol trolox/100g) recommended by the AOAC for solids is obtained by multiplying Eq. 3 by 10^8 [11].

2.4.3 Comparison by interconversion of antioxidant values for foods

In accord with AOAC guidelines to use trolox as reference antioxidant [11], we converted antioxidant results e.g. vitamin C equivalent

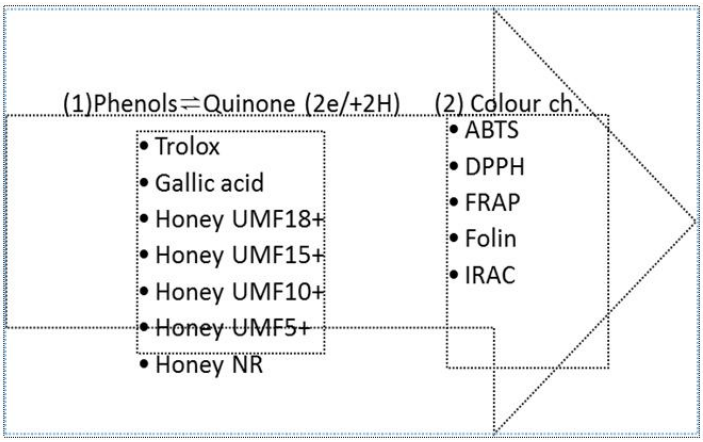

Fig. 1. Diagram for colorimetric antioxidant assays systems studied
Consecutive reactions occur between antioxidant/redox reaction (1) coupled to a fast colour changing processes (2)

antioxidant capacity (VCEAC) to units of TEAC, where TEAC (µmol/100 g) = VCEAC (µmol/100g) * F. The conversion factor (F) is the assay calibration slope for vitamin C divided by the calibration slope using trolox. For the ABTS method, F = 1.06 whilst F=1.14 for the DPPH method (unpublished data).

3. RESULTS

3.1 Calibration Parameters for Differing Assays and Pure Compounds

The line-gradient (*m)*, correlation coefficient (R^2), and other calibration parameters for different antioxidant methods are reported in Table 1. The optical pathlength for the microplate reader system was 0.7 cm for a total assay volume of 300 µl, determined as described previously [21].

3.2 Total Antioxidant Capacity of Honey

For Manuka honey rated UMF18+ values for TAC increased in the order, DPPH < FRAP < *iRAC* < ABTS < Folin, with a ratio of 1:3:8:9:21 TEAC (Fig. 2). However, the corresponding GEAC values for UMF18+ honey were ranked in a slightly different order, DPPH < FRAP < *iRAC* < Folin < ABTS with a ratio 1: 3: 11: 19:22. A Pearson's test showed that TEAC values using *iRAC*, DPPH, ABTS, FRAP and Folin assays were highly correlated (Table 2). The numerical values for TEAC were not identical, ranging by 70-fold for NR honey analyzed with DPPH versus the Folin assay. By comparison, the TEAC values assessed by ABTS and DPPH methods differed by, 31-fold (NR honey), 16-fold (UMF5+), 14-fold (UMF10+), 11-fold (UMF15+) or 9-fold (UMF18+).

3.3 Comparison by Interconversion of Antioxidant Values for Foods

Interconverting antioxidant values from VCEAC to TEAC for nearly 50 foods yielded a range of 27-2888 (µmol TEAC /100 g) for ABTS or 44-2502 (µmol TEAC /100 g) for DPPH analysis [10]. A Person's test confirmed that ABTS, DPPH

Table 1. Calibration parameters for microplate based antioxidant assays

Assays	Trolox				Gallic acid			
	m	MDC	R^2	CV%	m	MDC	R^2	CV%
ABTS	10590	8.00	0.9995	8.7	114170	3.60	0.9960	3.2
FRAP	23240	0.41	0.9981	1.0	82224	0.75	0.9987	3.0
DPPH	14449	3.51	0.9947	2.2	48780	1.04	0.9970	2.5
Folin	2976	15.6	0.9809	7.5	10889	4.26	0.9868	6.8
iRAC	878	65.0	0.9945	2.8	2070	17.0	0.9988	2.5

Notes: m = calibration graph slope or (ε_R) molar absorptivity (l/mol) for microplate analysis, MDC (µmol/l), minimum detectable concentration; Folin, Folin-Ciocalteu; FRAP, ferric reducing antioxidant power; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ABTS, 2,2'-azinobis-3-ethylbenzothiazoline 6-sulfonic acid, iRAC = iron (III) reducing antioxidant capacity

and ORAC results [10] were correlated (Fig. 3). The average value for TEAC for ABTS (620±621 µmol TEAC /100 g; n=49 foods) and DPPH analysis (673±557 µmol/100 g, n-49 foods) were not significantly different (p = 0.960). However, the ABTS and DPPH results were both lower (p ≤ 0.004) than the ORAC average (1944±2052 µmol TEAC /100g; n=43 foods). Comparing the preceding TEAC data suggests also that the TAC values for honey rank highly amongst the listed foods in terms of ABTS but not DPPH results (Table 3).

Table 2. Correlation matrix different antioxidant methods

	DPPH	FRAP	ABTS	IRAC	Folin	UMF
DPPH	1	0.969**	0.935*	0.966**	0.992**	0.994**
FRAP	0.969**	1	0.987**	0.874	0.972**	0.962**
ABTS	0.935*	0.987**	1	0.828	0.957*	0.926*
iRAC	0.966**	0.874	0.828	1	0.951*	0.963**
Folin	0.992**	0.972**	0.957*	0.951*	1	0.978**
UMF	0.994**	0.962**	0.926*	0.963**	0.978**	1

*Notes: **. Correlation is significant at the 0.01 level (2-tailed); *. Correlation is significant at the 0.05 level (2-tailed). Folin, Folin-Ciocalteu; FRAP, ferric reducing antioxidant power; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ABTS, 2,2'-azinobis-3-ethylbenzothiazoline 6-sulfonic acid, iRAC = iron (III) reducing antioxidant capacity, UMF = Unique Manuka Factor rating value (range 5+ to 18+)*

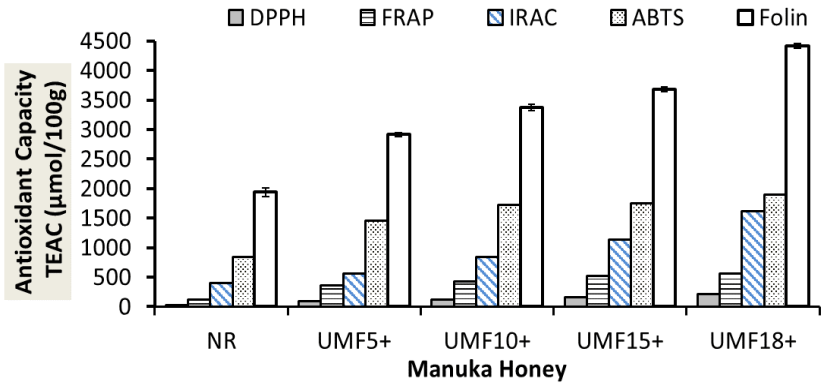

Fig. 2. Antioxidant capacity of Manuka honey samples determined by five methods

Antioxidant capacity was measured using five different assays. DPPH = DPPH radical quenching assay, FRAP = ferric reducing antioxidant power, ABTS = ABTS assay, Folin = total phenolic assay. & IRAC = iron (III) reducing antioxidant capacity. UMF = Unique Manuka Factor rating for Manuka honey, NR is standard honey

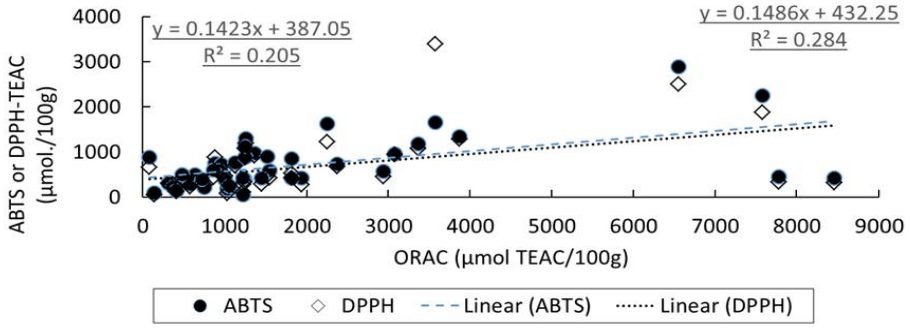

Fig. 3. Total antioxidant capacity values for 50 food items

Values were determined by ABTS, DPPH and ORAC methods. All values were converted from VCEAC to TEAC, (µmol/100g food). ORAC correlated with ABTS (p<0.0001) and DPPH (p =0.002) methods. Data replotted from Floegel et al. [10]

Table 3. Total antioxidant capacity for some foods compared with honey

Food	Total antioxidant capacity, TEAC (µmol/100g)		
	ORAC	ABTS	DPPH
NR Honey*	-	836.0	27.2
Spinach	1515	895.1	467.1
Apple	3082	961.8	937.4
Broccoli	1362	972.1	912.0
Tea, green	1253	1119.3	1081.6
Cherry, sweet	3365	1176.9	1077.0
Grape, red	1260	1299.3	1193.1
Wine, table, red	3873	1351.4	1281.2
Manuka honey UMF5+*	-	1455.0	89.2
Cabbage, red	2252	1627.2	1222.5
Strawberry	3577	1657.5	3396.7
Manuka honey UMF10+*	-	1722.0	121.6
Manuka honey UMF15+*	-	1753.0	166.3
Manuka honey UMF18+*	-	1900.0	207.7
Plum, black	7581	2254.4	1876.1
Blueberry	6552	2888.3	2501.7
Guava fruit extract⊥	2130	3112.0	2520.0

*Values are on a fresh weight basis. *This study- honey samples are, Rowse honey (NR), Manuka honey rated Unique Manuka Factor UMF5+, UMF10+, UMF15+ or UMF18+. All other values converted from [10]. ⊥ Average for 5 guava fruit varieties [24]*

4. DISCUSSION

Using many antioxidant assays should increase the reliability of TAC determinations for honey [11]. The ABTS and DPPH methods monitor free radical quenching or chain breaking, antioxidants [9,11,22,23] whilst *iRAC*, FRAP or Folin methods determine metal-ion reduction albeit with different solvent conditions and reactants. The five TAC assays used in this study [9,10] apply different antioxidant principles. We adopted AOAC guidelines for using trolox as a baseline compound in order to compare different assays effectively [11].

4.1 Regarding Calibration Parameters for Pure Compounds

Colorimetric assays for TAC involve a number of consecutive reactions (Fig. 1). For example, many phenols will undergo oxidation forming a semi-quinone, then a quinone and (2e $+H^+$) two reducing equivalents [25]. Reducing equivalents from phenol oxidation interact with a redox indicator to produce a colour change (Fig. 1). Since redox indicators e.g. ABTS are used "in-excess", the colorimetric response and molar absorptivity serve as a proxy for TAC [24]. Pure compounds produce colorimetric response in direct proportion to their TAC.

For a given antioxidant method (Table 1) we found the molar absorptivity for trolox and gallic acid differ by about 3-fold, reflecting the 1:3 ratio of hydroxyl groups in the two molecules (Table 1). Comparing other polyphenols to trolox can produce unexpected results due to secondary redox reactions [26]. For the FRAP assay, the molar absorptivity for iron (III) reduction to iron (II) was 22600 (l/mol cm) [27]. Consequently, data from Table 1 indicates 1.5 mol of iron (II) were formed per mol trolox oxidized (23240 /22600*0.7 = 1.5) or 5.2 mol of iron (II) were formed per mol gallic acid (82224 / (22600*0.7) = 5.2). Other investigations showed that structure-activity relations could be gained by comparing molar absorptivity values for many compounds analyzed using the *same* antioxidant method [28].

4.2 Challenges for Comparing Total Antioxidant Capacity of Honey by Different Methods

Adopting trolox as a sole calibration compound is *critical for effective* comparisons between different antioxidant methods [9,10,11]. Alterations in the value for TEAC can be expected because of well-known differences between antioxidant methods; (i) different redox indicators or chromophore are used, (ii) the wavelength for maximum absorption, molar absorptivity and other spectrophotometric characteristics are different, or (iii) the choice of solvent is different in many cases. Aqueous

solvents were used for the FRAP, ABTS, and *iRAC* methods whilst the DPPH assay was performed with 93% methanol as solvent [9, 10]. A newly modified DPPH method using buffered-methanol as solvent led to increased TAC [29]. Oxidation of polyphenols by free radicals species involved several non-exclusive mechanisms depending on the choice of solvent. Polar or H-bonding acceptor solvents promoted radical quenching via sequential proton loss electron transfer (SET). In contrast, non-polar and aprotic solvents favour a proton-coupled electron transfer or hydrogen atom transfer (PC/HAT) mechanism [30]. Finally, (iv) the pH for different assays is massively different leading to possible consequences for antioxidant activity [22].

In the present study, TEAC determined by *iRAC*, Folin, or FRAP methods were significantly different *(P=0.05*). Also the free radical quenching activity for honey was higher using the ABTS method compared with the DPPH method (Fig. 2). Overall, TEAC values for honey (Fig. 2) decreased along with the pH used for different antioxidant methods: Folin (pH 11.8) > ABTS (pH 7.0) ≈ *iRAC* (pH 7) > FRAP (pH 3.6) > DPPH assay. The pH of a methanolic DPPH system is indeterminate, but adding 50% buffer increased the values for TAC [29]. Hydroxy-benzoic acid and hydroxy-cinnamic acids associated with Manuka honey [17,18] will ionize with rising pH (pKa_1 = 4-5, pKa_2 ≈ 8.5-9.0, pKa = 11) leading to expected rises of TAC [22].

4.3 Comparing and Interconversion of Antioxidant Values for Foods

Formerly, ferric ammonium sulphate was the preferred calibration standard for the FRAP method. Gallic acid was used for calibrating the Folin assay. The ABTS and ORAC assays introduced trolox as a reference compound [9, 10]. Therefore, values for TAC were expressed in terms of ferric, gallic acid or trolox "Equivalent Antioxidant Capacity/Power". Trolox was selected for the ABTS assay originally because it is an analogue for α-tocopherol with enhanced water solubility [23]. The antioxidant character of trolox is also stable over a wide range of pH values [22]. Moreover, trolox has desirable kinetic attributes for TAC determination since it reacts rapidly with many redox indicators [25] compared to other phenols. Referencing TAC on the basis trolox may be advantageous, also because TEAC is a ratio-quantity (Eq.4) which is less affected by differences between assays. Finally, when using trolox as the sole reference compound all results are expressed as TEAC, which is important for inter-assay comparisons [11].

Converting values for VCEAC to TEAC units (Fig. 3) for 50 foods had no effect on the underlying correlations between ORAC, ABTS and DPPH methods [10]. By contrast, adopting TEAC units throughout allowed direct comparison of results, *beyond establishment of correlations*. ORAC values were significantly greater than ABTS or DPPH results [10]. By contrast, another study showed that TEAC values for guava juice extract were significantly lower with the ORAC method compared with ABTS (-30%), DPPH (-19%), or FRAP (-18%) methods [24]. Clearly, the relative sizes of TEAC values using different antioxidant methods is affected by the type(s) of food being analyzed.

5. CONCLUSION

Current recommendations are for using several antioxidant methods [9,10] alongside of trolox as the sole reference compound [11] in order to compare between different assays. In this study, the TAC of Manuka honey determined by *iRAC*, DPPH, FRAP, ABTS and Folin methods were highly correlated. By contrast, actual values for TEAC differed by 20-70 depending on the antioxidant method used for analysis. We speculated that the trends for TEAC could be related to solvent pH for different antioxidant methods [22]. Identifying if any specific antioxidant method overestimates or underestimates TAC remains a problem. The TAC determined by ABTS and *iRAC* methods indicated that Manuka honey has high TAC compared to some common foods (Table 3). The findings of this study are relevant for future efforts to standardize antioxidant methods [11-13,15]. Further research is recommended to examine the effect of standardizing antioxidant methods with respect to changes of solvent composition and pH.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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