Food Anal. Methods (2015) 8:1294–1302
DOI 10.1007/s12161-014-0005-6

Modified DPPH and ABTS Assays to Assess the Antioxidant Profile of Untreated Oils

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Received: 30 May 2014 / Accepted: 16 September 2014 / Published online: 5 October 2014

Abstract This research dwells on two widely used spectrophotometric methods, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assays, which assess the free radical scavenging activity (RSA) of natural samples and standard compounds. In particular, these assays were modified in order to simplify the evaluation of RSA of untreated edible oils, as well as to assess the antioxidant profile of oils' hydrophilic and lipophilic extracts with the same analytical procedure. A thorough study highlighted the effect of solvents on the DPPH and ABTS methods and resulted in selecting 2-propanol and an ethanol/1-butanol solvent mixture as the reaction solvent for the DPPH method and the ABTS method, respectively. The developed methods were used to evaluate the RSA of 12 antioxidant compounds and 8 edible oils. Then, the contribution of lipophilic and hydrophilic extracts to the total RSA of oils was estimated. The obtained results demonstrate the applicability of the method to routine edible oil analysis.

Keywords ABTS · DPPH · Reducing scavenging activity · Edible oils

Electronic supplementary material The online version of this article (doi:10.1007/s12161-014-0005-6) contains supplementary material, which is available to authorized users.

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Introduction

Antioxidant compounds are able to significantly suppress, delay, or prevent harmful oxidation processes, mainly caused by reactive oxygen species. They are characterized as highly health beneficial compounds, because they protect cells and macromolecules from oxidizing species, resulting in the prevention of diseases and the slowdown of aging (Gulcin 2012; Karadag et al. 2009; Tripoli et al. 2005; Covas et al. 2006).

Antioxidants also affect and determine the shelf-life, nutritional value, and quality of the food products, as they can slow down the deterioration of food products during storage caused by oxidation processes. The concentration of antioxidants in food samples can also be used as origin and freshness indicators (Carocho and Ferreira 2013; Camilo and Denicol 2013). Many methods have been developed for the estimation of the antioxidant properties of natural products which can be classified into two main categories: (i) those based on the evaluation of the radical scavenging activity (RSA) of samples (i.e., 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay, oxygen radical absorbance capacity (ORAC) assay, chemiluminescent assays) and (ii) those based on the evaluation of reducing activity of natural samples (i.e., ferric reducing/antioxidant power (FRAP) assay, cupric reducing antioxidant capacity (CUPRAC) assay) (Antolovich et al. 2002; Sanchez-Moreno 2002; Laguerre et al. 2007; Roginsky and Lissi 2005).

In general, the assays for the evaluation of antioxidant properties follow two prevalent mechanisms, the electron transfer (ET) and the hydrogen atom transfer (HAT) reaction mechanism. The former includes the CUPRAC assay, DPPH assay, ABTS assay, FRAP assay, ferrous oxidation-xylenol orange (FOX) assay, ferric thiocyanate (FTC) assay, and aldehyde/carboxylic acid (ACA) assay (Moon and

Shibamoto 2009). The latter comprises the crocin bleaching assay, the total peroxyl radical-trapping antioxidant parameter (TRAP) assay, and the ORAC assay (Gulcin 2012; Karadag et al. 2009; Huang et al. 2005; Prior et al. 2005).

In all assays based on either of the two reaction mechanisms, the results are affected by the solvent which is used. For example, the solvent influences the reaction kinetics in ET-based methods and the H-atom donor activities in HAT-based assays (Celik et al. 2010). Especially for the DPPH and ABTS assays, only few studies have provided information on the appropriate solvent (Zhou and Yu 2004; Pinelo et al. 2004; Perez-Jimenez and Saura-Calixto 2006; Prevc et al. 2013).

The DPPH method is an easy and simple method which estimates the ability of antioxidants to scavenge free radicals. It is based on the decrease of the absorbance at 515 nm of the $DPPH^{\bullet}$ solution (when methanol is used as the solvent), due to the inactivation of the $DPPH^{\bullet}$ radicals from the antioxidants present into the sample. The reaction time is between 20 and 60 min (Cheng et al. 2006). In the case of edible oils, the DPPH method is usually used only to the hydrophilic extracts of the oils which are easily diluted in methanol, the common reaction solvent (Espin et al. 2000; Valavanidis et al. 2004; Tuberoso et al. 2007). Ethyl acetate has been used in some cases for the determination of antioxidant activity of untreated oils (Espin et al. 2000; Minioti and Georgiou 2010), but its low viscosity which renders difficult the handling of solutions is a limiting factor that hinders the widespread application of this approach (Prevc et al. 2013). The contribution of the RSA of the hydrophilic and lipophilic oil extracts to the total RSA has been estimated with the DPPH method by utilizing different solvents (e.g., ethyl acetate for the untreated oils and the lipophilic part and methanol for the hydrophilic part) (Espin et al. 2000). This approach to use the nonpolar ethyl acetate and the polar methanol for different parts of oil may lead to erroneous results when considering the effect of the solvent on the DPPH reaction.

The difficulties in elucidating the reaction mechanism of the $DPPH^{\bullet}$ radical with antioxidants have triggered the interest of many researchers, and a number of studies have been published to address this issue (Sharma and Bhat 2009; Huang et al. 2005). Initially, the $DPPH^{\bullet}$ radical can be converted to either $DPPH_2$ or $DPPH^{-}$ depending on whether the conversion is carried out with the addition of a hydrogen atom from the antioxidant (hydrogen atom transfer reaction mechanism) or with electron transfer (electron transfer reaction mechanism) from the antioxidant to the radical, respectively. However, in each case, the final product remains the same, since if the $DPPH^{-}$ is formulated, then it can be converted rapidly into $DPPH_2$ after reaction with a H^{+} (a mechanism called sequential proton-loss electron transfer (SPLET) (Litwinienko and Ingold 2007). At first, the mechanism followed was thought to be of a hydrogen atom transfer. Then, new scientific findings corroborate that both mechanisms occur simultaneously, while the solvent and the antioxidants of the reaction will probe to the prevalent mechanism (Karadag et al. 2009; Huang et al. 2005).

The ABTS assay is the other most common, easy, and simple method for estimating the scavenging ability of free radicals (free radical scavenging ability). It is based on the decrease of the absorbance of the solution of the radical $ABTS^{+\bullet}$, within the range 710–760 nm, depending on the solvent, due to its inactivation from antioxidants. The reaction time is usually within 5–30 min; thus, it is a more rapid method compared to the DPPH method. The method has been applied to various food items including both hydrophilic and lipophilic antioxidant systems (Floegel et al. 2011; Kim et al. 2002; Przygodzka et al. Przygodzka et al. 2014). The ABTS method has been applied mainly to hydrophilic oil extracts (Minioti and Georgiou 2008), while when implemented to the evaluation of total RSA of oils, ethanol was the solvent of choice (Pellegrini et al. 2001, 2003). However, oil solutions at dilutions higher than 1 % *v*/*v* are insoluble in ethanol and form turbid solutions rendering it a deficient solvent. Another study applied a new approach to ABTS method by using freeze-dried ABTS radical powder and a methanol/chloroform mixture (Durmaz 2012). The disadvantage of this approach is that freeze-dried ABTS radical powder is not commercially available so one extra step to the procedure is required.

The reaction mechanism of the radical $ABTS^{+\bullet}$ with the antioxidants within the sample has been extensively studied. The radical $ABTS^{+\bullet}$ is reduced by antioxidants in the sample either by electron transfer or by hydrogen atom transfer and is deactivated. It has also been proven that antioxidants can react with the radical and form various products of addition or degradation of $ABTS^{+\bullet}$ (Osman et al. 2006).

On these grounds, this research dwells on the modification of two widely used spectrophotometric methods, DPPH and ABTS, in order to simplify their application to untreated oils and study the contribution of lipophilic and hydrophilic part of the oil to the total radical scavenging activity (RSA).

Materials and Methods

Apparatus

Absorption measurements were performed on a JASCO V-500 spectrophotometer.

Reagents and Solutions

All chemicals were of analytical purity and were used without further purification. 1-Butanol, hexane, methanol, and ethanol were purchased from Panreac. Oleuropein was purchased

from Extrasynthese. Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), caffeic acid, ascorbic acid, catechin α-tocopherol, gallic acid, chlorogenic acid, pyrocatechol, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and 2,2-diphenyl-1-picrylhydrazyl (DPPH), were purchased from Sigma-Aldrich. All commercial edible oils (extra virgin olive oils, refined olive oils, sunflower oil, soybean oils, sesame oil, and corn oil) were purchased from local supermarkets. Antioxidant standard stock solutions were prepared by accurately weighing and dissolving with ethanol.

Sample Preparation

The radical scavenging activity (RSA) of edible oils, as well as their corresponding hydrophilic or lipophilic extracts, was measured by testing solutions of the oils diluted in 2-propanol (DPPH method) or 1-butanol (ABTS method).

Ten grams of the oil sample were diluted in 10 mL hexane, and the hydrophilic part was extracted three times with 20 mL methanol/water (60/40 *v*/*v*) by centrifugation for 10 min at 3000 cpm. The hydrophilic and the lipophilic extracts were collected, and the solvents were removed in a rotary evaporator under vacuum at temperatures lower than 100 °C. 12.74 mL of the appropriate solvent (2-propanol for DPPH and 1-butanol for ABTS) was added to the solid residue of the hydrophilic extract, and the mixture was stirred vigorously. Then, 0.40 mL of this solution was diluted to 5.00 mL with the appropriate solvent to obtain a final solution of the hydrophilic extract solution of the oil containing 4.0 % *v*/*v* in 1-butanol for ABTS method, and 2.0 % *v*/*v* in 2-propanol for DPPH method.

Radical Scavenging Activity Against DPPH$^{\bullet}$

In our endeavor to select an appropriate solvent which solubilizes sufficiently all the oil constituents, as well as the DPPH$^{\bullet}$ reagent, the following procedure ensued: DPPH$^{\bullet}$ stock solutions were prepared in concentrations ranging from 20 to 100 μM in all tested solvents (acetonitrile, dichloromethane, ethanol, diethyl ether, acetone, 1-butanol, tetrahydrofuran, toluene, chloroform, ethyl acetate, carbon tetrachloride, 1-propanol, 2-propanol). The response curves of the absorbance values at 515 nm vs. the concentration of DPPH$^{\bullet}$ were prepared. Then, the radical scavenging activity (%RSA) of an extra virgin olive oil (EVOO) solution 0.5 % *v*/*v* diluted in all the tested solvents was measured at 515 nm in a 50-μM DPPH$^{\bullet}$ solution after 1 h. The (%RSA) was calculated by Eq. 1:

$$\mathrm{RSA}(\%) = \frac{A_o - A}{A_o} \times 100 \quad (1)$$

where A and A_o are the absorbances of the tested sample and the blank sample, respectively. Three measurements were performed for each tested solvent. Finally, upon selecting the appropriate solvent, 2-propanol, the employed analytical procedure was the following: starting from a DPPH$^{\bullet}$ stock solution (1.00×10^{-3} M in methanol), a DPPH$^{\bullet}$ working solution of 8.75×10^{-5} M in 2-propanol was prepared. Then, 1.00 mL of the sample diluted in 2-propanol and 4.00 mL of DPPH$^{\bullet}$ solution were mixed so that the final DPPH$^{\bullet}$ concentration was equal to 7.00×10^{-5} M. The free radical scavenging activity against DPPH of oil solutions was determined by using Eq. 1 by measuring the reduction of the absorbance at 515 nm after 1 h.

Radical Scavenging Activity Against ABTS$^{\bullet+}$

The appropriate quantity of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) diammonium salt and potassium persulfate was transferred to a 10.00-mL volumetric flask and diluted so that the final concentrations were equal to 7.00 and 2.45 mM, respectively. The solution was left in the dark for 12–16 h for the formation of the ABTS$^{\bullet+}$ radical. Then, the solution of ABTS$^{\bullet+}$ radical was diluted with ethanol until the absorbance of the solution was equal to 1.0. The radical scavenging activity of oil against ABTS$^{\bullet+}$ was determined after mixing 0.500 mL of the examined sample diluted in 1-butanol with 2.00 mL of alcoholic solution ABTS$^{\bullet+}$ and measuring the reduction of the absorbance at 734 nm after 15 min. At least five measurements were performed for each tested sample, and the %RSA was also estimated by Eq. 1.

Statistical Analysis

All measurements were obtained in five replicates, and values were averaged and reported along with the standard deviation (SD). The Kolmogorov-Smirnov tests were applied to the results of the spectrophotometric assays to examine normality. A nonnormal distribution was detected, and the Spearman correlation was applied to extract the correlation coefficients. These calculations were performed with the SPSS (IBM SPSS Statistics. version 19.0. Chicago, IL, USA) statistical software for Windows.

Results and Discussion

Method Development

The previously proposed ABTS and DPPH assays (Camilo and Denicol 2013; Antolovich et al. 2002;

Sanchez-Moreno 2002) cannot be efficiently applied to edible oils due to the presence of water in the reaction mixture, which leads to the formation of turbid solutions. The appropriate solvent must solubilize well all the reagents, the hydrophilic and lipophilic antioxidants, and the untreated oils. In this context, the possible use of a range of solvents was examined to enable the assessment of the RSA of untreated oils and the corresponding hydrophilic and lipophilic extracts.

In fact, the solvent effect was evident to the response lines of absorbance vs. DPPH concentration and to the evaluation of RSA of the tested sample as the results depended on the solvent that was used (Online Recourse 1). The toxicity of certain solvents (toluene, carbon tetrachloride, and chloroform) and the low viscosity and low boiling point of others (acetone, ethyl acetate, diethyl ether) constituted an additional criterion to dismiss these solvents from further evaluation. 2-Propanol was the solvent which facilitated the highest RSA and was selected for further studies. Furthermore, 2-propanol proved to be an appropriate solvent for both lipophilic and hydrophilic extracts of oils and also when the hydrophilic and lipophilic antioxidants were tested.

On the other hand, for the ABTS method, 1-propanol and 2-propanol, although solubilized fully the oil solutions, when mixed with the alcoholic solution of the $ABTS^{+\bullet}$ radical, produced turbid solutions. The turbidity of the solutions hindered the measurement of absorbance due to light scattering. Consequently, for the ABTS method, 1-butanol was chosen as sample's solvent, due to its complete solubilization of oil solutions, including both the hydrophilic and lipophilic antioxidants, as well as its compatibility with the alcoholic solution of the $ABTS^{+\bullet}$ radical.

Application to Hydrophilic and Lipophilic Compounds

The modified DPPH and ABTS assays evaluated the RSA of 12 standard compounds, both hydrophilic and lipophilic (Tables 1 and 2). The response curves of the RSA values vs. the concentration of each antioxidant compound were prepared, using five standard solutions that were each measured at least five times. It must be noted that for the first time, the same analytical procedure allows the assessment of RSA of hydrophilic and lipophilic compounds by the DPPH method. By comparing the slopes of the response lines and the limits of detection of the two methods, it becomes obvious that the sensitivity of the DPPH assay is higher than that of the ABTS assay.

To delineate the solvent effect in DPPH method, the response curves of the RSA values vs. the concentration of each antioxidant compound were also prepared when ethanol was used as the solvent (Online Recourse 2). The concentration of the standard compounds which reduce the initial absorbance of the DPPH radical by 50 % (IC_{50}) was estimated using the equations of the regression lines for each antioxidant. Moreover, due to the strong dependence of the IC_{50} to the experimental conditions, the RSA of each compound expressed as IC_{50} was compared to the IC_{50} values of gallic acid. Therefore, the RSA of each compound could be

Table 1 Regression lines (%RSA = $A(\pm S_A) \backslash B(\pm S_B) \times \log C_{analyte}$), LoD, LoQ, IC_{50}, and GEAC values in increasing order of 12 antioxidant compounds by the modified DPPH method

Compound	Linear range (μM)	$A(\pm S_A)$	$B \times 10^6$ ($\pm S_B$)	R (N)*	LoD (μM)	LoQ (μM)	IC_{50}±SD (μM)	GEAC
Caffeic acid	1.0–10.0	317.1 (±7.4)	53.5 (±1.2)	0.998 (5)	0.78	2.36	1.012±0.058	0.26
α-Tocopherol	1.0–20.0	437 (±25)	78.6 (±5.2)	0.995 (4)	0.57	1.74	1.19±0.45	0.30
Gallic acid	1.0–10.0	499 (±20)	83.1 (±4.0)	0.9995 (5)	0.46	1.38	3.95±0.23	1.00
Pyrocatechol	0.8–20.0	384 (±18)	63.6 (±3.4)	0.998 (4)	1.33	4.04	5.60±0.23	1.42
(±)-Catechin	0.8–20.0	312 (±10)	49.9 (±1.5)	0.9998 (5)	0.80	2.41	5.61±0.35	1.42
Chlorogenic acid	1.0–20.0	382 (±15)	63.9 (±2.9)	0.994 (4)	0.66	2.00	6.37±0.33	1.61
Trolox	1.0–20.0	346 (±10)	57.6 (±1.8)	0.9998 (4)	0.78	2.35	7.26±0.38	1.85
Ascorbic acid	2.0–10.0	478 (±20)	84.7 (±6.2)	0.998 (4)	0.54	1.65	8.84±0.41	2.23
Oleuropein	2.0–20.0	471 (±25)	83.9 (±3.8)	0.995 (5)	0.93	2.83	9.59±0.42	2.43
Butyl-hydroxy-toluene	50–2000	207.7 (±4.9)	41.7 (±1.5)	0.998 (4)	1.05	3.18	165.3±4.5	41.8
Resorcinol	50–4000	152.0 (±5.2)	31.6 (±1.4)	0.996 (6)	0.98	2.98	591±24	149
p-Coumaric acid	100–2000	78.81 (±7.3)	18.07 (±2.2)	0.98 (4)	0.39	1.18	2547±124	644

*Correlation coefficient (number of solutions used)

Table 2 Regression lines (%RSA=$A(\pm S_A)+B(\pm S_B)\times C_{analyte}$), IC_{50}, and GEAC values in increasing order of 12 antioxidant compounds by the modified ABTS method with 1-butanol as a solvent

Compound	Linear region (μM)	$A(\pm S_A)$	$B\times10^6$ ($\pm S_B$)	R (N)	LoD (μM)	LoQ (μM)	IC_{50}±SD (μM)	GEAC
(±)-Catechin	1.0–6.0	4.03 (±0.79)	13.8 (±2.1)	0.9998 (5)	0.57	0.19	3.33±0.1	0.85
Gallic acid	0.7–8.0	−3.2 (±1.5)	13.5 (±4.8)	0.9995 (5)	3.71	1.23	3.94±0.1	1.00
Caffeic acid	0.8–1.2	0.04 (±1.3)	7.6 (±2.6)	0.998 (5)	1.71	0.56	6.57±0.2	1.67
Trolox	1.0–20	1.15 (±0.88)	5.4 (±1.1)	0.9998 (5)	1.63	0.54	9.05±0.3	2.30
Pyrocatechol	2.0–17	−2.65 (±0.32)	5.64 (±0.67)	0.9998 (5)	0.57	0.19	9.34±0.3	2.37
Oleuropein	2.0–20	0.58 (±0.71)	4.55 (±0.81)	0.9995 (5)	1.56	0.51	10.9±0.4	2.76
Ascorbic acid	2.0–20	−2.09 (±0.35)	4.46 (±0.41)	0.9998 (5)	0.78	0.26	11.7±1.4	2.96
Chlorogenic acid	2.0–30	3.2 (±1.5)	3.09 (±0.92)	0.9994 (4)	4.85	1.60	15.1±0.5	3.84
α-Tocopherol	1.0–50	13.9 (±1.2)	1.6 (±0.13)	0.995(5)	7.50	2.48	22.6±0.8	5.73
Resorcinol	50–4000	29 (±3.6)	0.166 (±0.027)	0.996 (6)	216	71	127±4.3	32.1
Butyl-hydroxy-toluene	50–2000	26 (±4.9)	0.147 (±1.5)	0.998 (4)	333	110	163±5.5	41.4
p-Coumaric acid	100–2000	32 (±7.3)	0.060 (±2.2)	0.98 (4)	1216	401	300±10	76.1

expressed in equivalents of gallic acid (gallic acid equivalent antioxidant capacity (GEAC)) using Eq. 2:

$$\mathrm{GEAC} = \frac{\mathrm{IC}_{50}(\mathrm{compound})}{\mathrm{IC}_{50}(\mathrm{gallic\ acid})} \quad (2)$$

Interestingly, with the exception of resorcinol which provides almost identical IC_{50} values in both solvents, all the other standard compounds exhibit more potent RSA when 2-propanol is used as a solvent. This is in accordance with the results obtained during tests for the selection of the appropriate solvent for this method. Also, even variations in the ranking of the standard compounds according to their RSA values were noted, especially in α-tocopherol, caffeic acid, and Trolox. For instance, while Trolox in 2-propanol ranked seventh, it was ranked first when ethanol was used. Evidently, the solvent has a significant influence on the results and should be seriously considered before drawing conclusions on the RSA of a sample.

Application to Edible Oils

The RSA of edible oils was evaluated with the implementation of the DPPH and the ABTS methods. The total RSA as well as the contribution of the scavenging activity of the hydrophilic (RSA_{hydro}) and lipophilic (RSA_{lipo}) extract to the total scavenging activity (RSA) of oils was estimated by the two assays, and the results are shown in Table 3.

Gallic acid was chosen as the antioxidant compound to express RSA for edible oils using Eq. 3 for the DPPH assay

Table 3 RSA in GAE units of total (untreated) oil (RSA_{total}), lipophilic (RSA_{lipo}), and hydrophilic (RSA_{hydro}) extracts by the modified DPPH and ABTS methods

Oil	GAE ($\times10^{-7}$ M, ±SD, n=5) (%RSD)					
	DPPH			ABTS		
	RSA_{total}	RSA_{lipo}	RSA_{hydro}	RSA_{total}	RSA_{lipo}	RSA_{hydro}
EVOO 1	13.61 (±0.17) (1.3)	12.04 (±0.26) (2.2)	13.81 (±0.74) (5.4)	32.10 (±0.67) (2.1)	10.94 (±0.24) (2.2)	16.41 (±0.27) (1.7)
EVOO 2	14.05 (±0.26) (1.9)	11.19 (±0.45) (4.1)	10.03 (±0.54) (5.4)	19.50 (±0.37) (1.9)	11.14 (±0.38) (3.4)	12.95 (±0.22) (1.7)
EVOO 3	13.96 (±0.27) (2.0)	11.69 (±0.18) (1.6)	10.34 (±0.33) (3.2)	27.06 (±0.84) (3.1)	10.43 (±0.18) (1.7)	25.57 (±0.79) (3.1)
VOO	12.81 (±0.19) (1.5)	11.69 (±0.44) (3.8)	11.05 (±0.67) (6.1)	22.33 (±0.60) (2.7)	11.64 (±0.22) (1.9)	10.31 (±0.21) (2.1)
Corn oil	36.70 (±0.40) (1.1)	25.2 (±1.4) (5.7)	–	46.21 (±0.97) (2.1)	42.18 (±0.80) (1.9)	–
Sunflower oil	18.31 (±0.58) (3.2)	17.25 (±0.29) (1.7)	–	20.01 (±0.36) (1.8)	20.01 (±0.20) (1.0)	–
Soya oil	35.27 (±0.63) (1.8)	32.1 (±1.2) (3.6)	–	37.14 (±0.71) (1.9)	31.09 (±0.87) (2.8)	6.24 (±0.11) (1.9)
Sesame oil	13.15 (±0.68) (5.2)	15.68 (±0.65) (4.2)	–	19.00 (±0.46) (2.4)	14.46 (±0.25) (1.7)	7.76 (±0.20) (2.6)

EVVO extra virgin olive oil, *VOO* virgin olive oil

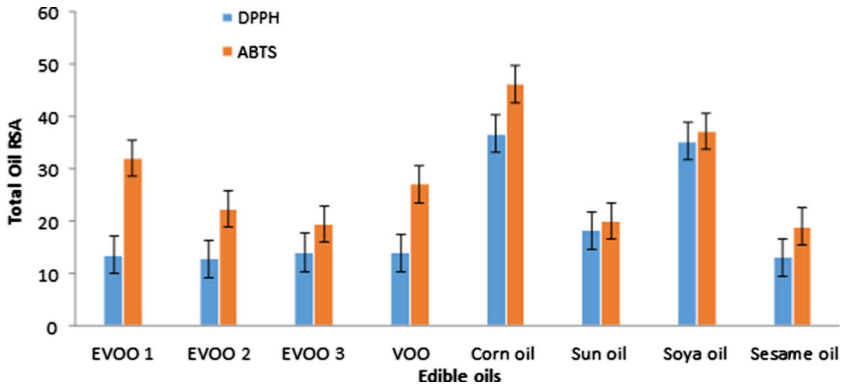

Fig. 1 Total RSA vs. type of edible oil in relation to the implemented methods

and Eq. 4 for the ABTS assay. The gallic acid equivalent (GAE) units were estimated for each sample.

$$RSA_{DPPH}(\%) = 499 + 83.1 \times C_{\text{gallic acid}} \quad (3)$$

$$RSA_{ABTS}(\%) = -3.20 + 13.5 \times 10^6 \times C_{\text{gallic acid}} \quad (4)$$

where C is the concentration in M.

The acquired results in DPPH method show that seed oils exhibit high RSA, particularly higher than those of olive oils. This is in accordance with results by other workers (Espin et al. 2000; Valavanidis et al. 2004). The hydrophilic extracts of seed oils do not display RSA while both the lipophilic and hydrophilic extracts of olive oils exhibit similar RSA. It has to be noted that the sum of RSA for the two extracts in most cases differs significantly from the total estimated RSA. This could be attributed to the alteration of the composition of the sample (e.g., higher moisture content) occurred during the separation stage of the two phases. Specifically, the DPPH method is especially sensitive to the conditions of the reaction (e.g., with residual moisture, minerals, etc.).

Regarding the ABTS method, the modifications proposed in this research highlight its scalability for the first time to assess antioxidants in both the lipophilic and hydrophilic extracts. Based on the results shown in Table 3, it is observed that olive and seed oils exhibit similar RSA. In fact, seed oils exhibited a low to none RSA in their hydrophilic extract, while olive oils portrayed similar RSA in both extracts. The difference of the sum of the RSA in hydrophilic and lipophilic extracts compared to the total estimated RSA is less than 6.2 % which is considered unimportant and demonstrates the additivity of the method.

When comparing the results of the total RSA estimated by DPPH and ABTS methods (Table 3 and Fig. 1), it becomes obvious that seed oils exhibited higher values in ABTS method in comparison to the DPPH assay. Interestingly, a converse trend was observed for the olive oils, as they exhibited higher values in the DPPH assay over the ABTS method.

Moreover, the DPPH method could not evaluate the antioxidant activity of hydrophilic extracts in seed oils, while the ABTS method partially assessed their antioxidant profile probably due to the very small amount of phenolic antioxidants in these extracts. Correlation analysis between the results obtained by the three methods (Table 4) shows that the results of the total RSA and RSA_{lipo} in all assays correlate significantly positively. The equivalence of their response indicates that these methods bear a similar analytical principle which renders their results comparable.

Table 4 Pearson correlation coefficients for GAE units of total (untreated) oil (RSA_{total}), lipophilic (RSA_{lipo}), and hydrophilic (RSA_{hydro}) extracts for results by the DPPH, ABTS, and ABTS-FIA methods

Pearson, correlation coefficient		DPPH			ABTS		
		RSA_{total}	RSA_{lipo}	RSA_{hydro}	RSA_{total}	RSA_{lipo}	RSA_{hydro}
DPPH	RSA_{total}	1					
	RSA_{lipo}	0.938[a]	1				
	RSA_{hydro}	−0.238	–	1			
ABTS	RSA_{total}	0.838[a]	0.682	0.822	1		
	RSA_{lipo}	0.964[a]	0.874[a]	–	0.802[b]	1	
	RSA_{hydro}	−0.451	–	−0.083	0.021		1

[a] Correlation is significant at the 0.01 level (two-tailed)

[b] Correlation is significant at the 0.05 level (two-tailed)

Table 5 RSA in GAE units of total (untreated) oil before and after spiking 2.0 % *v/v* oil with 1.00×10^{-6} M gallic acid by the proposed DPPH method

Oil	RSA GAE ($\times10^{-7}$ M, ±SD, $n=5$) (%RSD)			Recovery (%)
	Total oil	Spiked oil	Expected	
EVOO 1	16.33 (±0.70) (4.3)	26.36 (±0.71) (2.7)	26.33	100
EVOO 2	14.90 (±0.76) (5.1)	24.62 (±9.3) (3.8)	24.90	99
EVOO 3	14.05(±0.51) (3.6)	22.31 (±0.94) (4.2)	24.05	93
VOO	11.20 (±0.33) (3.0)	20.4 (±0.98) (4.8)	21.20	96
Corn oil	21.54 (±0.73) (3.4)	32.5 (±1.0) (3.1)	31.54	103
Sunflower oil	16.01 (±0.65) (4.1)	23.50 (±9.8) (4.2)	26.01	90
Soya oil	51.1 (±1.5) (3.1)	64.6 (±1.7) (2.6)	61.10	106
Sesame oil	9.82 (±0.41) (4.2)	18.72 (±0.67) (3.6)	19.82	94

EVVO extra virgin olive oil, *VOO* virgin olive oil

Method Validation

The quality characteristics of each method (linearity, precision, accuracy, limits of detection and quantification) were also investigated. *Limit of detection* (LoD) was calculated by using the equation $LoD=3.3\times s_a/b$ and the *limit of quantitation* (LoQ) by using the equation $LoQ=10\times s_a/b$, where s_a corresponds to the standard error of the intercept and b the slope of the calibration lines.

Linearity was established by measuring the absorbance of at least five solutions of each antioxidant compound at known concentrations. Each measurement was repeated five times, and the mean value was used for calculation of the regression lines. *Precision* was evaluated by estimating the repeatability and reproducibility (intraday and interday precision) of the method. The former was investigated by estimating the standard deviation of the five measurements of each antioxidant compound. The latter was calculated by measuring the reducing power of six edible oils and three gallic acid solutions at three different days.

Accuracy was estimated by using recovery assays. For the DPPH assay, the recovery experiments were carried out by measuring the RSA values of 4.0 % *v/v* oil solutions before and after spiking with 100 μM of gallic acid by using the following procedure: 1.00 mL of 2.50×10^{-5} M gallic acid is mixed with 0.100 mL of oil and diluted to 5.00 mL with 2-propanol. From this solution, 1.00 mL is mixed with 4.00 mL solution DPPH$^{\bullet}$ 8.75×10^{-5} M and, hence, the final solution contains 2.0 % *v/v* oil in gallic acid 1.00 μM and DPPH$^{\bullet}$ 7.00 μM. Mixed solutions without gallic acid were also prepared.

In the case of the ABTS assay, the recovery experiments were carried out by measuring the RSA of 2.0 % *v/v* oil solutions before and after spiking with 1 μM of gallic acid by using the following procedure: 50 μL of 1.0×10^{-3} M gallic acid solution is mixed with 0.250 mL of oil and diluted to 5.00 mL with 1-butanol. The final solution contains 5.0 % *v/v* oil and gallic acid 1.00×10^{-5} M. Mixed solutions without gallic acid were also prepared.

Table 6 RSA in GAE units of total (untreated) oil before and after spiking 5.0 % *v/v* oil with 1.00×10^{-6} M gallic acid by the proposed ABTS method

Oil	RSA GEAC ($\times10^{-6}$ M, ±SD, $n=5$) (%RSD)			Recovery (%)
	Total oil	Spiked oil	Expected	
EVOO 1	20.15 (±0.42) (2.1)	29.15 (±0.90) (3.1)	30.15	97
EVOO 2	10.55 (±0.18) (1.7)	20.67 (±0.37) (1.8)	20.55	100
EVOO 3	12.82 (±0.19) (1.5)	24.82 (±0.62) (2.5)	22.82	109
VOO	13.16 (±0.41) (3.1)	22.01 (±0.42) (1.9)	23.16	95
Corn oil	50.69 (±0.91) (1.8)	58.4 (±1.6) (2.8)	60.69	96
Sunflower oil	28.51 (±0.31) (1.1)	39.47 (±0.67) (1.7)	38.51	102
Soya oil	47.08 (±0.85) (1.8)	56.0 (±1.7) (3.1)	57.08	98
Sesame oil	21.50 (±0.49) (2.3)	33.22 (±0.93) (2.8)	31.50	105

EVVO extra virgin olive oil, *VOO* virgin olive oil

Recovery results for the DPPH and ABTS assays were calculated by using Eq. 5 and are summarized in Tables 5 and 6, respectively.

$$\text{Recovery}\ (\%) = \frac{\text{RSA of spiked samples}}{\text{expected RSA}} \times 100 \qquad (5)$$

From the validation results, it becomes apparent that in the DPPH assay, all antioxidant compounds examined exhibit antioxidant properties and can be measured at the sub-millimolar concentration with correlation coefficients higher than 0.99 (Table 1). Gallic acid, which was selected as the reference compound to express the RSA of edible oils, exhibited correlation coefficient equal to 0.9995 which was deemed satisfactory. For all compounds, the linear range was at least one order of magnitude and the limits of detection and quantification are shown in Table 1. The interday and intraday precisions were found equal to 2.1 and 4.7 %, respectively, and were deemed satisfactory. The mean recovery was estimated to be equal to 98±5 % (n=7) which was satisfactory as verified by Student test at a confidence level of 95 % (t_{theor}= 2.365>t_{exp}=0.43) postulating that the method does not exhibit systematic error.

Similarly, in the ABTS assay, all compounds examined can be measured at the sub-millimolar concentration with correlation coefficients higher than 0.999. Gallic acid, which was the selected reference compound for the expression of the RSA of edible oils, displayed a satisfactory correlation coefficient equal to 0.9995. For all tested compounds, the linear range was at least one order of magnitude (Table 2). The interday and intraday precisions were found equal to 1.5 and 3.2 %, respectively, and were deemed satisfactory. The mean recovery was estimated to be equal to 100.5±4.8 % (n=7) which was satisfactory as verified by Student test at a confidence level of 95 % (t_{theor}=2.365>t_{exp}=0.1) postulating that the method does not exhibit systematic error.

Conclusions

This research accomplished to modify two widely applied methods, ABTS and DPPH, in order to simplify the assessment of the antioxidant profile of untreated oils. The determination of total antioxidant profile of edible oils is important because it can be used as a quality index for the shelf-life and the stability of edible oils. The enhanced stability and self-life of edible oils with increasing antioxidant content are attributed to the ability of antioxidants to inhibit lipid oxidation, the main process that leads to the quality deterioration, degradation, and off-flavor formation in edible oils.

The developed methods were successfully used to evaluate the RSA of 12 antioxidant compounds and 8 edible oils. The contribution of lipophilic and hydrophilic extracts to the total antioxidant capacity of oils was also estimated. The modified DPPH and ABTS methods have all the quality factors required for the routine analysis of commercial edible oils in industrial or in-field settings as they are simple, quick, accurate, precise, do not require expensive instrumentation or experienced personnel, and could be easily incorporated in kits for in-field measurements.

Acknowledgments This research has been cofinanced by the European Union (European Social Fund (ESF)) and Greek national funds through the Operational Program "Education and Lifelong Learning" of the National Strategic Reference Framework (NSRF)-Research Funding Program: THALES. Investing in knowledge society through the European Social Fund.

Conflict of Interest Dionysios C. Christodouleas declares that he has no conflict of interest. Charalambos Fotakis declares that he has no conflict of interest. Aspasia Nikokavoura declares that she has no conflict of interest. Kyriakos Papadopoulos declares that he has no conflict of interest. Antony C. Calokerinos declares that he has no conflict of interest. This article does not contain any studies with human or animal subjects.

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