J Am Oil Chem Soc (2017) 94:77–88
DOI 10.1007/s11746-016-2918-2

ORIGINAL PAPER

Quantification of Antioxidant Ability Against Lipid Peroxidation with an 'Area Under Curve' Approach

Temelkan Bakır[1] · İnci Sönmezoglu[2] · Reşat Apak[3]

Received: 6 June 2015 / Revised: 31 October 2016 / Accepted: 4 November 2016 / Published online: 18 November 2016
© AOCS 2016

Abstract When oxygen is passed through a linoleic acid (LA) emulsion containing copper(II), primary (hydroperoxides) and secondary oxidation products (aldehydes and ketones) are formed, monitored by ferric thiocyanate (Fe(III)-SCN) and thiobarbituric acid reactive substances (TBARS) colorimetry, respectively. As total antioxidant capacity (TAC) against lipid peroxidation was not quantified before, both methods were adapted to an 'area under curve (AUC)' approach. LA peroxidation followed pseudo-first order kinetics in aerated emulsions. Absorbance changes as a function of incubation time exhibited sigmoidal curves, enabling the calculation of 'area under curve' (AUC) and net AUC = $AUC_{blank}-AUC_{sample}$, standard calibration curve as net AUC *versus* concentration, and trolox-equivalent antioxidant capacity of the tested compounds. Garlic extract showed an antioxidative effect on hydroperoxide formation, but a prooxidative effect on TBARS. Although inhibition of lipid peroxidation was described qualitatively before, it was not evaluated quantitatively, e.g., the trolox-equivalent antioxidant capacities (TEAC values) of antioxidants with respect to their inhibitive effect against lipid peroxidation were not calculated and compared. Additionally, real-time monitoring of lipid oxidation products requires highly sophisticated but costly instrumental techniques, but no single oxidation product is a direct measure of lipid oxidation or its antioxidative prevention. The AUC approach is the first quantitative method measuring antioxidant protection against lipid oxidation, with a slightly different order of antioxidative effectiveness from reductive assays because of interfacial effects.

Keywords Lipid peroxidation · Antioxidant capacity · Colorimetry · Ferric thiocyanate · Thiobarbituric acid–reactive substances (TBARS) · Area under curve (AUC)

Abbreviations

LA	Linoleic acid
Fe(III)-SCN	Ferric thiocyanate
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid-reactive substances
MDA	Malondialdehyde
AUC	Area under curve
TEAC	Trolox-equivalent antioxidant capacity
k_1	Pseudo-first order rate constant with respect to hydroperoxides formation (measured by Fe(III)-SCN method in the presence of antioxidant)
k_1'	Pseudo-first order rate constant with respect to aldehydes and ketones formation (measured by TBARS method in the presence of antioxidant)
k_0	Pseudo-first order rate constant of the control reaction (in the absence of antioxidant)
I,%	Percentage inhibition
A	Absorbance, $A_{500\ nm}$ or $A_{532\ nm}$, proportional to the total concentration of hydroperoxides or secondary products (aldehydes and ketones)

✉ Reşat Apak
rapak@istanbul.edu.tr

[1] Department of Chemistry, Faculty of Science and Letters, Kastamonu University, Kuzeykent, 37150 Kastamonu, Turkey

[2] Department of Chemistry, Faculty of Science and Letters, Yildiz Technical University, Esenler, 34010 Istanbul, Turkey

[3] Division of Analytical Chemistry, Department of Chemistry, Faculty of Engineering, Istanbul University, Avcilar, 34320 Istanbul, Turkey

t	Incubation time
TocH	Tocopherol
TR	Trolox
AP	Ascorbyl palmitate
HQ	Hydroquinone
TBHQ	*tert*-Butyl hydroquinone
TBQ	*tert*-Butyl quinone
QR	Quercetin
MR	Morin
CT	Catechin
AA	Ascorbic acid
FOX	Ferrous oxidation xylenol orange
PV	Peroxide value
HAT	Hydrogen atom transfer
RG	Raw garlic
ESR	Electron spin resonance
CIS-MS	Coordination ion spray-mass spectrometry
ESI-MALDI-TOF	Electrospray ionization-matrix-assisted laser desorption/ionization-time of flight
TAC	Total antioxidant capacity
ROS	Reactive oxygen species

Introduction

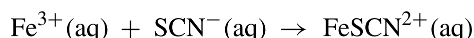
The combination of copper(II) salts and molecular oxygen in a linoleic acid (LA) emulsion causes the formation of primary and secondary oxidation products, namely hydroperoxides and aldehydes/ketones, respectively, and these products are known to give rise to rancidity of lipid foods. In order to measure the extent of lipid oxidation, one may use techniques targeting at oxygen absorption and free radical generation, along with the determination of initial substrate consumption or oxidation product formation. Free radical-induced lipid peroxidation has been hypothesized to cover the three stages of initiation, propagation and termination. Chain-breaking antioxidants can delay or inhibit lipid peroxidation by blocking one of the mentioned phases. For monitoring lipid peroxidation, spectrophotometric, chromatographic and immunochemical methods can be used [1]. To carry out kinetic analysis of the course of reaction, the accumulation with respect to time of primary products (such as conjugated dienes or lipid hydroperoxides) and/or of secondary products (aldehydes and ketones, symbolized with malondialdehyde) should be measured [2]. Lipid peroxidation in foods can be delayed or inhibited by antioxidant compounds acting as chain-breaking agents during various stages of oxidation.

In order to indicate the initial stages of oxidative damage (i.e. regarding the formation of primary oxidation products), a simple parameter is the iodometrically measured 'peroxide value'. Another reasonable choice is to measure the capacity of lipid peroxides to convert ferrous ions to ferric ions (i.e. Fe(II) → Fe(III)), determined by either the thiocyanate assay at 500–510 nm [3] widely applied to milk products, fats, oils and liposomes [4], or the FOX (xylenol orange) assay at 550–600 nm [5]. The colors of the ferric complexes formed with thiocyanate and xylenol orange are blood–red and blue–purple, respectively. The FOX method was applied for assessing the oxidative stability of meat [5], where AUC calculations were only performed for estimating the quantity of lipid hydroperoxides (as cumene hydroperoxide equivalents) accumulated in the system, but not for quantifying antioxidant ability.

LA peroxidation induced by copper(II) was reported to follow pseudo-first order kinetics with respect to the formation of hydroperoxides and aldehydes/ketones, subsequently determined by ferric thiocyanate and thiobarbituric acid-reactive substances (TBARS) methods, respectively [6]. The rate of production of ferric thiocyanate or TBARS chromophores as a function of time could be used to estimate the antioxidative or prooxidative effect of a test compound added to the LA emulsion (subjected to oxidation) by monitoring the weakening or strengthening of chromophore formation, respectively [7].

The antioxidant activity of protector compounds added to the medium is inversely proportional to the amount of Fe(III)–thiocyanate complex formed per unit time.

$$Fe^{2+} + LOOH \rightarrow Fe^{3+} + LO^{\cdot} + OH^{-}$$


$$Fe^{3+}(aq) + SCN^{-}(aq) \rightarrow FeSCN^{2+}(aq)$$

Chain-breaking antioxidants may interfere with various steps of lipid peroxidation, reduce lipid hydroperoxides or Fe^{3+}(aq) to produce less $FeSCN^{2+}$(aq), thereby decreasing the color intensity [3, 8].

The 'Area Under Curve' (AUC) concept is introduced here as a parameter to determine antioxidant capacity. For the function of an analytical property monitored along time $y = f(t)$, $\int f(t)dt$ integrated over a time interval between t_1 and t_2 indicates the cumulative amount of f(t), or the 'area under curve' remaining between the *f*(*t*) curve and the horizontal (time) axis. The benefit of the AUC approach is that it can be applied to antioxidants both having and not having a distinct lag phase. Furthermore, it takes into account the initial reaction rate and the total extent of inhibition (i.e. both kinetic and thermodynamic aspects of antioxidant action), covering slow-reacting or secondary antioxidant products formed [9].

When the accumulation of oxidized species (i.e. primary and secondary oxidation products of lipids, such as peroxides and aldehydes/ketones) are measured as a function of time, absorbance (*A*) as a measure of oxidized species integrated over the time interval $t_1 - t_2$ (i.e. $\int A(t)$

dt $_{blank}$) indicates the cumulative amount of oxidized species in the test system (blank, without antioxidant). In the presence of antioxidant, a lower amount of oxidized species will be accumulated in the sample (i.e. $\int A(t)\mathrm{d}t_{\ sample}$) within the same time interval t_1−t_2. The difference between these two integrals (ΔAUC) will represent the decrease in the build-up of oxidized species in the presence of antioxidant sample, thereby giving an indirect measure of the antioxidant capacity of the sample. The AUC method as a parameter of antioxidant assessment was widely applied in the past to hydrogen atom transfer (HAT)-based methods (such as the ORAC assay of antioxidant activity) where the fluorescence decay of a probe under free radical attack (inhibitable by antioxidants) was measured against time [10, 11]. To the best of our knowledge, this study is the first example of quantifying antioxidant ability against lipid peroxidation with an AUC concept. The advantage of the AUC approach for determining the relative order of antioxidant effectiveness is that it applies equally well to both antioxidants that exhibit distinct lag phases and those samples that have no lag phases. As the maximum absorbance of the oxidation products approached 1 [i.e. A_{max} ~ 1], the following parameters could be found: (i) area under the kinetic curve (AUC) and 'net AUC', [i.e. ΔAUC = (AUC_{blank}−AUC_{sample})]; (ii) standard calibration line by plotting ΔAUC against the concentration of the test compound (or trolox as reference compound); (iii) trolox-equivalent antioxidant capacity (TEAC) of the test compound by comparing the slopes of the relevant calibration lines [12, 13]. This approach unifies the lag time method and initial rate method, and is particularly useful for food samples which often have multiple ingredients and complex reaction kinetics. There is a direct linear correlation of AUC and a broad range of sample types, including raw fruit and vegetable extracts, plasma, and pure phytochemicals [13, 14].

In this study, the peroxidation of LA in the presence of Cu(II) ion alone and with antioxidants was investigated in aerated and incubated emulsions at 37 °C and pH 7. As TAC against lipid peroxidation was not quantified before, Fe(III)-SCN and TBARS methods were adapted to an 'area under curve (AUC)' approach so as to determine the TAC values of some antioxidants [e.g., quercetin (QR), morin (MR), catechin (CT), tocopherol (TocH), ascorbic acid (AA), ascorbyl palmitate (AP), hydroquinone (HQ), *tert*-butyl hydroquinone (TBHQ)] and one plant extract (raw garlic). Garlic (*Allium sativum*) was selected on the basis of its antioxidative ability to inhibit Cu(II)-induced low density lipoprotein oxidation [15] and to scavenge hydroxyl radicals (·OH) superoxide anion radicals ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) [16, 17]. Garlic aqueous extract reduces ·OH production and inhibits lipid hydroperoxide formation. In addition, garlic resists elevated temperature incubation, as shown by the preservation of the antioxidant potential of Polish, Ukrainian and Israeli garlic (lyophilized) samples subjected to heat treatment at 100 °C for 20 min [18].

Materials and Methods

Chemicals and Instruments

To determine the TAC against lipid peroxidation, certain antioxidant compounds (i.e. TR, QR, MR, CT, TocH, AA, AP, HQ, TBHQ) were used in the concentration range of (2.5–4.0) × 10^{-4} M, and raw garlic extract within 0.75–1.25 mg/mL. All chemical substances of analytical reagent grade were supplied by E. Merck AG (Darmstadt, Germany). Garlic cloves were purchased from a local market in Kastamonu (Taşköprü), a town well known for growing garlic. In determining the TAC of antioxidant compounds against LA peroxidation, the [Cu(II) + O_2] system was used as the primary oxidant [19]. Antioxidants were used in 80 mmol L^{-1} phosphate buffer at pH 7. Deionized distilled water was used throughout.

Spectral recording and absorption measurements were made with a SHIMADZU UVmini-1240 UV–Vis spectrophotometer (Schimadzu Corp., Kyoto, Japan manufactures) using a pair of matched quartz cuvettes with a 1-cm optical path. All experiments were carried out at 37 °C by means of a thermostatted system [19].

Experimental Design

The oxidation of linoleic acid emulsion with copper(II) and air oxygen (either in the presence or absence of antioxidants) was monitored for 3 h by spectrophotometric measurements (using Fe(III)-SCN and TBARS methods). In this study, change of absorbance due to the formation of ferric thiocyanate and thiobarbituric acid as a function of incubation time exhibited sigmoidal curves. Since Beer's law was generally not obeyed above an absorbance value of ≈1.0 for most spectrophotometric measurements, this value was not exceeded in subsequent determinations. The absorbance/time curves were evaluated as described in "AUC calculations and statistical analysis".

Kinetic Analysis and Determination of TAC of Antioxidants Against Lipid Peroxidation

The formation of primary and secondary oxidation products along time could be followed by absorbance values recorded periodically. During LA oxidation, the total concentrations of hydroperoxides and of aldehydes/ketones generated with pseudo-first order kinetics (having

Table 1 The effect of garlic extract on the rate constants of primary and secondary oxidation product formation in a linoleic acid–copper(II) system

Garlic conc. (mg/mL)	$k_1 \pm S_k$ (min^{-1}) Fe(III)-SCN	$k'_1 \pm S_k$ (min^{-1}) TBARS
0	$(2.47 \pm 0.17) \times 10^{-2}$	$(0.38 \pm 0.10) \times 10^{-2}$
0.75	$(1.66 \pm 0.12) \times 10^{-2}$	$(0.64 \pm 0.09) \times 10^{-2}$
1.00	$(1.43 \pm 0.25) \times 10^{-2}$	$(0.62 \pm 0.15) \times 10^{-2}$
1.25	$(1.36 \pm 0.39) \times 10^{-2}$	$(0.57 \pm 0.09) \times 10^{-2}$

k_1 pseudo-first order rate constant with respect to hydroperoxides formation (measured by Fe(III)-SCN method), k_1' pseudo-first order rate constant with respect to MDA formation (measured by TBARS method)

formation rate constants; k_1 and k_1') were monitored by ferric thiocyanate and TBARS methods, respectively [20] (Table 1). Then, the percentage inhibition (I,%) of lipid peroxidation by antioxidants was calculated by the following equation:

$$I, \% = 100\,[(k_0 - k_1)\,/\,k_0]$$

where k_0 and k_1 were the first-order rate constants of the concerned reaction in the absence and presence of antioxidant sample, respectively. As the maximum absorbance of the oxidation products approached 1 [i.e. $A_{max} \approx 1$], the net AUC, standard calibration curve, and the TEAC coefficient of the test compound could be found [12, 13].

Colorimetric Measurement of Primary and Secondary Oxidation Products

Peroxide production in the linoleic acid emulsion system was determined by the ferric thiocyanate method. The degree of oxidation was measured by sequentially adding ethanol (4.7 mL, 75% v/v), ammonium thiocyanate (0.1 mL, 30% w/v), sample solution (0.1 mL), and ferrous chloride (0.1 mL of 0.02 M in 3.5% v/v HCl). The peroxide concentration was spectrophotometrically determined by reading the absorbance of Fe(III)-SCN complex formed at 500 nm ($A_{500\ nm}$) against a reagent blank containing identical components without LA [19].

Secondary oxidation products (i.e. aldehydes/ketones, represented by malondialdehyde: MDA) in LA emulsion were determined by the TBARS method. The degree of oxidation was measured by sequentially adding water (2.65 mL), trichloroacetic acid (0.15 mL, 2.8% w/v), sample solution (0.1 mL) and thiobarbituric acid (TBA; 0.1 mL of 1% w/v solution in 50 mM NaOH) at pH 3.5. The absorbance ($A_{532\ nm}$) of the resulting pink-colored MDA-TBA adduct was measured at the absorption maximum wavelength of 532 nm against a reagent blank containing identical components without LA [20].

Preparation of Standard and Sample Solutions and Kinetic Measurements

Linoleic acid emulsions and all other solutions were freshly prepared every day and stored at room temperature.

Standard {Linoleic Acid + Copper(II)} System Solution

The stock (0.02 mol L^{-1}) LA emulsion was prepared by mixing 0.2804 g of LA, 0.2804 g of Tween 20 (as emulsifier) and 50 mL of phosphate buffer (0.2 mol L^{-1}, pH 7), followed by homogenization of the mixture [19]. A stock solution of copper(II) nitrate was prepared at a Cu(II) concentration of 0.05 mol L^{-1}. The standard {LA + Cu(II)} solution was prepared as 25 mL (stock 0.02 mol L^{-1}) LA + 20 mL (stock 0.2 mol L^{-1}) phosphate buffer (pH 7) + x mL (stock 0.05 mol L^{-1}) Cu(II) + (5–x) mL absolute ethanol in a total reaction volume of 50 mL.

Preparation of Aqueous Extract of Raw Garlic (RG)

Garlic cloves were peeled, weighed (0.6 g), and crushed using a porcelain mortar and pestle by adding 6 mL of distilled water. This homogenate was centrifuged at 5,000 rpm for 10 min at 18 °C, and the supernatant was centrifuged at 14,000 rpm for 5 min at 4 °C. The soluble part of garlic extract was used in the experiments. The extract supernate was recovered and added to the working medium (i.e. linoleic acid + copper(II)) such that its final concentrations would be 0.75, 1.00 and 1.25 mg/mL [15].

Sample (Linoleic Acid + Copper(II) + Antioxidant) System Solution

This solution was prepared as 25 mL (stock 0.02 mol L^{-1}) LA + 20 mL (stock 0.2 mol L^{-1}) phosphate buffer (pH 7) + x mL (stock 0.05 mol L^{-1}) Cu(II) + y mL (stock 0.01 mol L^{-1}) antioxidant + [5–(x + y)] mL absolute ethanol in a total reaction mixture volume of 50 mL.

Sample {Linoleic Acid + Copper(II) + Garlic Extract} System Solution

This solution was prepared as 25 mL (stock 0.02 mol L^{-1}) LA + 20 mL (stock 0.2 mol L^{-1}) phosphate buffer (pH 7) + x mL (stock 0.05 mol L^{-1}) Cu(II) + y mL (stock 100 mg/mL) garlic extract + [5–(x + y)] mL absolute ethanol in a total reaction volume of 50 mL.

Kinetic Measurements

A 50-mL working solution containing 0.01 mol L^{-1} LA {emulsified by Tween 20} [19], 80 mmol L^{-1} phosphate

buffer, 1 $mmol^{-1}$ Cu(II), and aqueous extract of raw garlic at a series of concentrations 0.75, 1.00 and 1.25 mg/mL (or antioxidants between 250 and 400 µmol) was prepared in a 250-mL Erlenmeyer flask and placed in a water bath thermostatted at 37 °C. The pH of the sample solution was measured. During incubation, a stream of air (flow rate = 60 L/h) was passed through the flask so as to saturate the solution with oxygen [19]. Oxidation was followed during incubation for 3 h by monitoring absorbance *versus* time. Aliquots of 0.1 mL were periodically withdrawn at different intervals for spectrophotometric measurements (using Fe(III)-SCN and TBARS methods). Oxidation was monitored by recording absorbance *versus* time. Double experiments were conducted throughout, i.e. the oxidation of a standard {LA + Cu(II)} solution was followed in comparison with a similar solution additionally containing the antioxidants or garlic extract.

AUC Calculations and Statistical Analysis

All experiments were performed in triplicate, and the results for both methods (Fe(III)-SCN and TBARS) were expressed as {mean ± standard deviation}. The highest determination coefficient (R^2) was selected for each experiment, and the TEAC values were calculated. Descriptive statistical analyses were performed using MICROCAL ORIGIN 8.5.1 (Origin Lab Corp., Northampton, MA, USA) for calculating the relationship with the net area under curve (AUC) and concentration of antioxidant. The same program was used to fit experimental sigmoidal curves of absorbance *versus* time, complying with the nonlinear regression function, as described earlier [19]. The area under absorbance/time curves determined within fixed time intervals (where antioxidative effects were prevalent) was partitioned into a large number of trapezoid portions of equal width (using Microsoft Excel), the minute areas of which were summed to give the final AUC values. The accuracy of calculations was increased with the number of trapezoid portions (i.e. the height of the trapezoids on the *x*-axis should be kept as small as possible). Using SPSS software (SPSS Inc., Chicago, IL, USA) for Windows version 13, the data were evaluated by ANalysis Of VAriance (ANOVA) [21]. Standard calibration lines were produced by plotting net AUC against the concentration of tested compounds (or trolox as reference compound) (net AUC = $ax \pm b$, where b is the intercept, a is the slope, and x is the concentration of antioxidant). The trolox-equivalent antioxidant capacity (TEAC value) of the tested compound was found by comparing the slopes of the relevant calibration lines, calculated by comparative analysis of the 'area under kinetic curve' (AUC) and 'net AUC', the latter equal to ($AUC_{blank} - AUC_{sample}$).

Results and Discussion

The kinetic profile of lipid peroxidation is characterized by three major parameters, namely the lag time preceding rapid oxidation, the maximal rate of oxidation, and the maximal accumulation of oxidation products, whereas the addition of antioxidants alters this pattern, affecting the kinetic parameters of oxidation. In particular, antioxidants may prolong the lag phase or decrease the last two parameters [22]. In this study, oxidation was monitored by recording absorbance *versus* time, where the total concentrations of primary (hydroperoxides) and secondary (malondialdehyde) oxidation products were proportional to the formation of Fe(III)–thiocyanate (recorded as $A_{500\ nm}$) and TBARS (recorded as $A_{532\ nm}$), respectively. Changes of absorbance due to ferric thiocyanate and TBARS as a function of incubation time [6, 23] exhibited sigmoidal curves, as shown for some representative antioxidants (Fig. 1 for trolox, Fig. 2 for morin, Fig. 3 for AA, Fig. 4 for AP, Fig. 5 for HQ, and Fig. 6 for TBHQ). The intra-day and inter-day reproducibility of spectrophotometric measurements generally varied within (2–5) and (4–7)%, respectively, for both colorimetric assays, depending on the type of assay and the concentration of the tested antioxidant. From the absorbance/time curves, AUC and net AUC values of antioxidants as well as calibration curves expressing net AUC as a function of antioxidant concentration could be found, enabling the calculation of TEAC values of antioxidants by comparing the slopes of the corresponding calibration lines [12, 13].

To determine the (unitless) TEAC coefficient of each compound, the ratio of the slope (m) of the linear regression curve of the tested compound to that of Trolox (Fig. 7) was used:

$$TEAC = m_{compound}/m_{Trolox}$$

The calculated trolox equivalents can then be used for comparative analysis of the antioxidant capacity of the various samples tested in the chosen LA oxidation system (Tables 2, 3). As a result of this new AUC approach, the TEAC coefficients of antioxidants in a LA emulsion with respect to the Fe(III)-SCN method were: hydroquinone (HQ) > *tert*-butyl hydroquinone (TBHQ) > trolox (TR) > morin (MR) > ascorbyl palmitate (AP) > tocopherol (TocH) > catechin (CT) ≥ ascorbic acid (AA) > quercetin (QR) (Table 2), with good correlation of Pearson's probability (P) ($P = 0.521 > 0.500$) and $p = 0.003 < \alpha = 0.05$ (confidence level), where α level was arbitrarily set at 0.05. In a previous work of the authors not involving AUC calculations, the inhibitive order of flavonoids in the protection of LA peroxidation was: morin > catechin ≥ quercetin, agreeing with that of formal reduction potentials *versus*

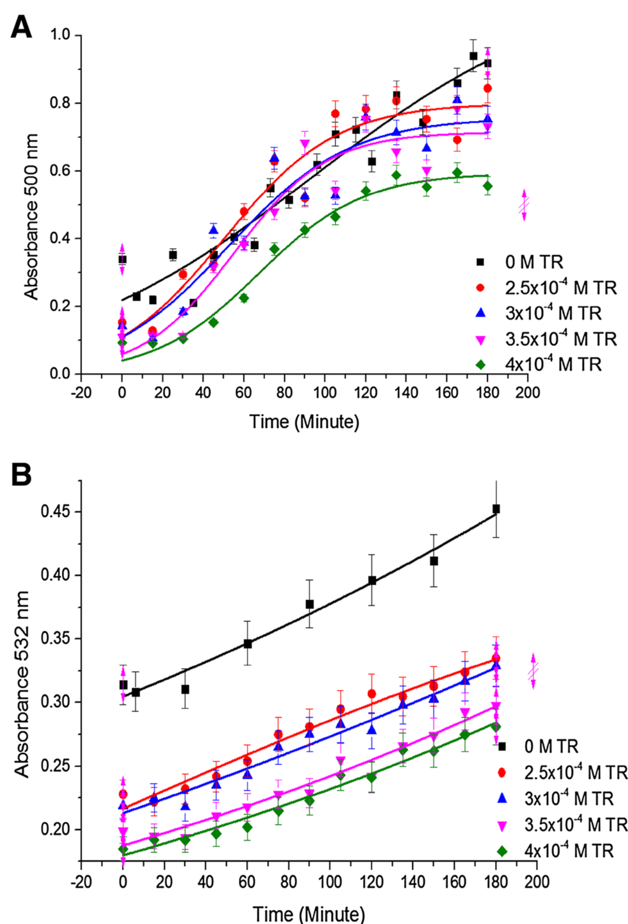


Fig. 1 Changes of absorbance in linoleic acid oxidation induced by a copper(II) system for trolox (TR) [measured by Fe(III)-SCN (**a**) and TBARS (**b**) assays]

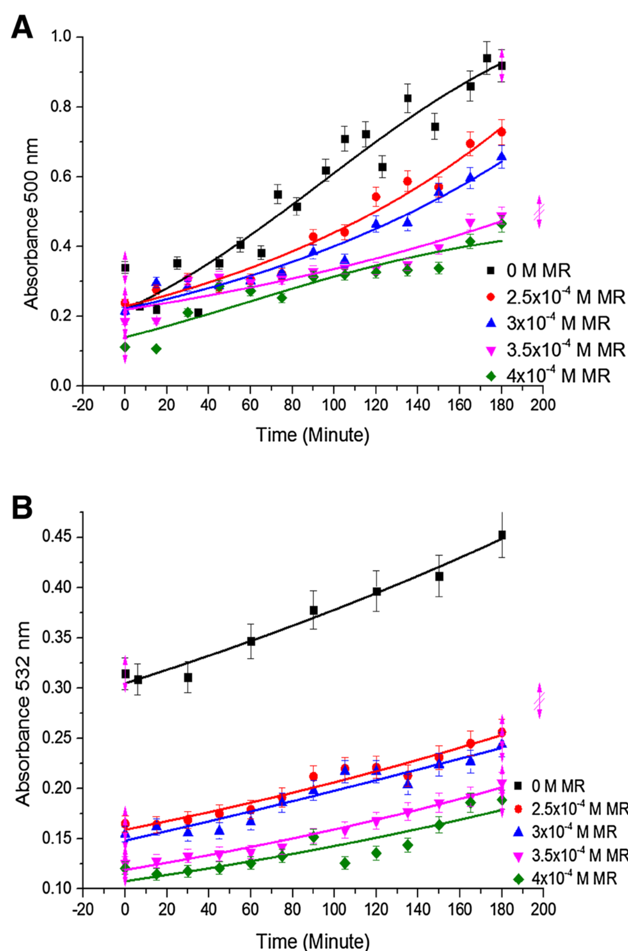


Fig. 2 Changes of absorbance in linoleic acid oxidation induced by a copper(II) system for morin (MR) [measured by Fe(III)-SCN (**a**) and TBARS (**b**) assays]

NHE at pH 7, i.e. 0.60, 0.57 and 0.33 V for MR, CT, and QR, respectively [19], also in accordance with the present findings. On the other hand, the TEAC order based on the AUC approach with respect to the TBARS method were: TBHQ > AP > TocH > MR > TR > AA > CT ≥ HQ > QR (Table 3), with good correlation of P values ($P = 0.555 > 0.500$) and $p = 0.003 < \alpha = 0.05$ (confidence level), where α level was arbitrarily set at 0.05. Naturally, the capability of antioxidants to concentrate at interfaces (in an aqueous LA emulsion) seemed to be important for TAC evaluation with the TBARS method, besides their reductive ability. In the analysis of both sets of data (with respect to the ferric thiocyanate and TBARS methods) among antioxidants, the found P value indicated the 'goodness of fit' with respect to the chosen regression model, and p value pointed out that the experimental effect was genuine at 95% certainty. On the other hand, the Fe(III)-SCN and TBARS methods did not correlate at 95% confidence level because they measured different properties (i.e. primary and secondary products of lipid peroxidation, respectively). However, it was noteworthy that three of the first four and three of the last four antioxidants agreed in the two orders (of ferric thiocyanate and TBARS sorting with respect to antioxidant power).

The observed TEAC sequences in the two colorimetric assays (Fe(III)-SCN and TBARS) can be interpreted in terms of the differences in structure and interfacial distribution of the tested antioxidants. As homologous pairs of antioxidants having similar structures but different hydrophilicities (i.e. TR and TocH, AA and AP, HQ and TBHQ) were comparatively used in the assay, it is noticeable that in the Fe(III)-SCN assay, TEAC differences between hydrophilic-lipophilic partners of the homologous pairs were not significant because of structural similarities. The ferric thiocyanate test is a relatively straightforward assay where the initially formed hydroperoxide products of LA oxidation can oxidize Fe(II) to Fe(III) which is in turn complexed by SCN^- to form a red color; therefore, antioxidants having similar structures and redox potentials can react with

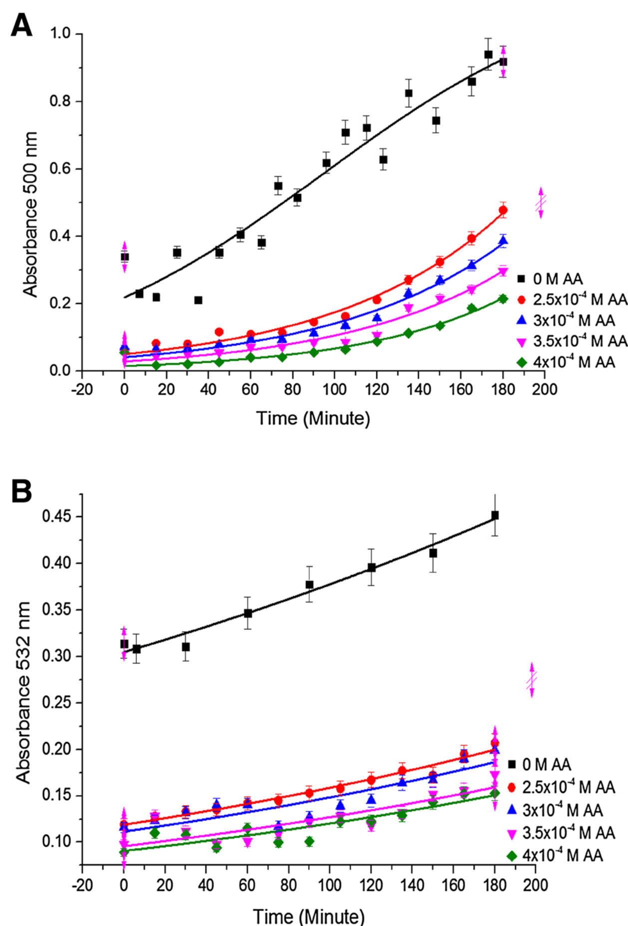


Fig. 3 Changes of absorbance in linoleic acid oxidation induced by a copper(II) system for ascorbic acid (AA) [measured by Fe(III)-SCN) (**a**) and TBARS (**b**) assays]

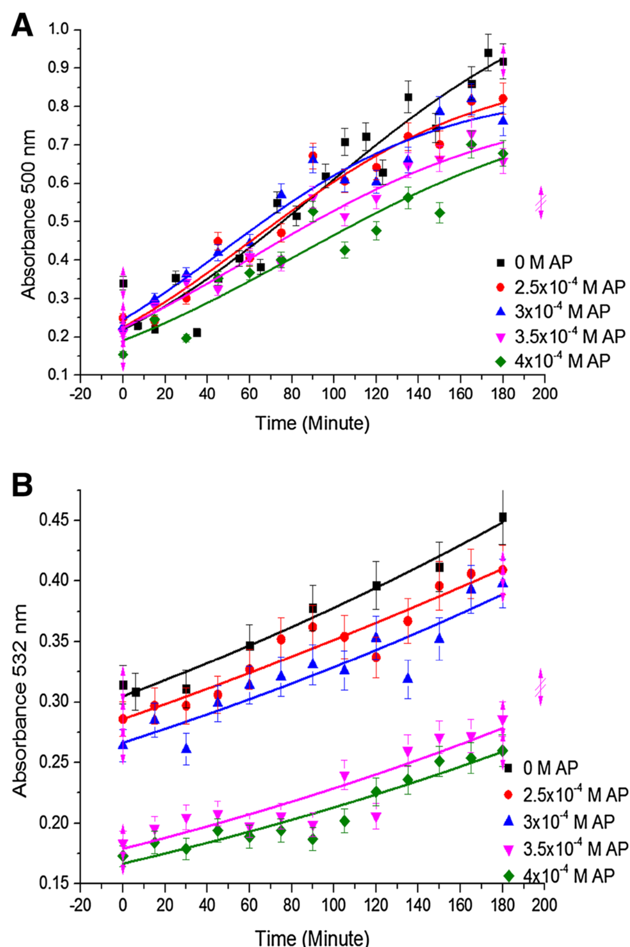


Fig. 4 Changes of absorbance in linoleic acid oxidation induced by a copper(II) system for ascorbyl palmitate (AP) [measured by Fe(III)-SCN (**a**) and TBARS (**b**) assays]

hydroperoxides (responsible for ferrous-ferric oxidation) in a similar manner, regardless of their hydrophilicities. However, in the TBARS test, the lipophilic antioxidants showed higher TEAC values than their hydrophilic partners in the homologous pairs (i.e. TBHQ > HQ; AP > AA; and TocH > TR). The TBARS test is a rather indirect assay where the initially formed alkoxyl radicals produced by the breakdown of lipid hydroperoxides can undergo further reactions to yield various secondary products, i.e. aldehydes and ketones [24], and this is closely related to the distribution of antioxidants at the interfaces which may be more important than the redox potential. This behavior is in agreement with the 'polar paradox' hypothesis, stating that hydrophobic antioxidants that can concentrate at the interfaces of lipid emulsions may function as stronger antioxidants, whereas polar antioxidants remaining in the aqueous phase are more diluted and less effective [25]. For example, Koleva *et al*. reported that trolox (TR) was a less effective antioxidant than α-tocopherol (TocH) in emulsified linoleic acid because of the interfacial distribution effect [24].

In accordance with theoretical expectations [15], the garlic extract showed a distinct dose-dependent antioxidant effect on inhibition of LA peroxidation with respect to hydroperoxide formation (Table 1), as measured by Fe(III)-SCN colorimetry. Surprisingly, TBARS results showing faster oxidation of LA in the presence of garlic with higher pseudo-first order rate constants (Table 1) were contradictory to this finding, showing the prooxidative effect of garlic extract on secondary product formation during peroxidation. This unexpected result may stem from the generation of reactive sulfur species from garlic extract under the Cu(II)-induced oxidizing conditions of the TBARS assay, enhancing (rather than retarding) sulfhydryl and LA oxidation in model systems of lipid oxidation which is better expressed in secondary product formation characteristically measured by the TBARS test [26].

The antioxidative/prooxidative behavior of the homologous pairs of antioxidants are collectively shown in Fig. 8a–c. It is noteworthy that the distinct antioxidative (i.e. lipid

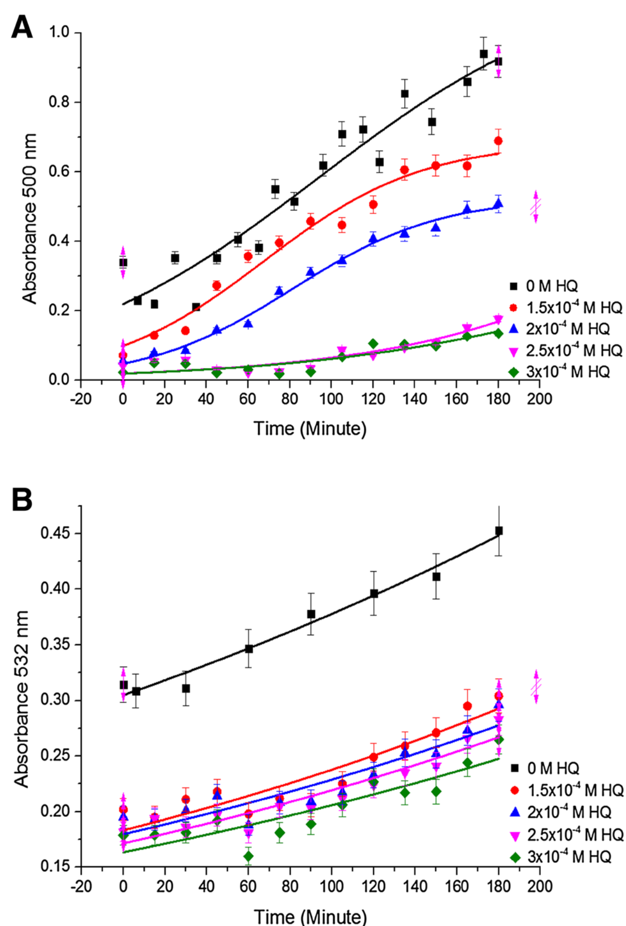


Fig. 5 Changes of absorbance in linoleic acid oxidation induced by a copper(II) system for hydroquinone (HQ) [measured by Fe(III)-SCN (**a**) and TBARS (**b**) assays]

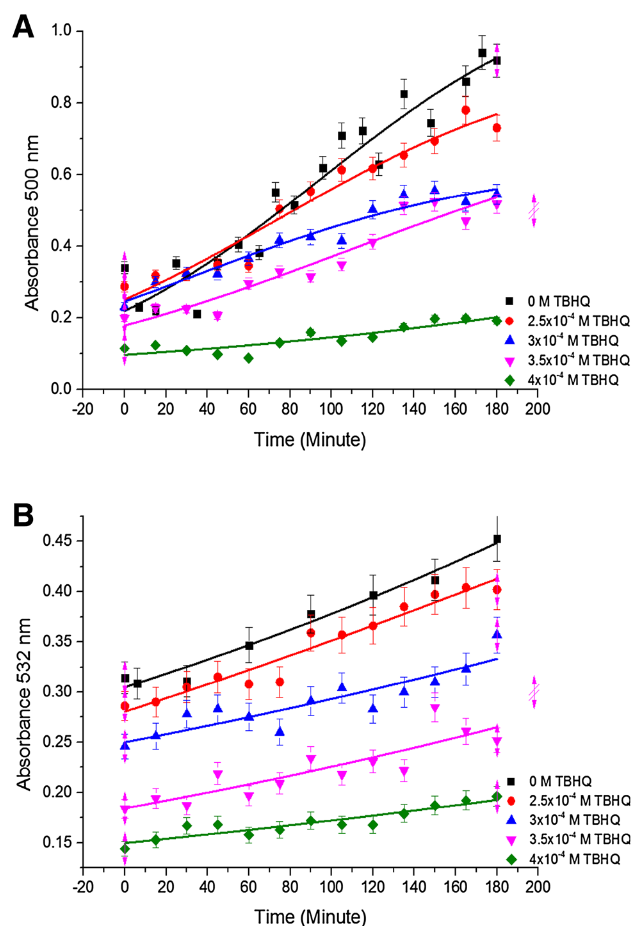


Fig. 6 Changes of absorbance in linoleic acid oxidation induced by a copper(II) system for *tert*-butyl hydroquinone (TBHQ) [measured by Fe(III)-SCN (**a**) and TBARS (**b**) assays]

peroxidation inhibitive) behavior of the tested antioxidants at lower concentrations [6] gradually decreased with increasing concentration (especially for TocH, for which prooxidative behavior was seen at intermediate concentrations in the TBARS test, Fig. 8a), possibly because of the Cu(II)-reductive ability of antioxidants at elevated concentrations giving rise to reactive species and enhanced secondary product formation in the TBARS test (Fig. 8a, b).

Obviously, a concentration-dependent inhibition (reflecting definite antioxidative behavior) was observed for TR and HQ in the FeSCN test (Fig. 8a, c) for the first six concentration values between 0.05 and 1.6 μM, as there was a positive correlation at $p < 0.05$ significance level for both antioxidants within the indicated concentration range (TR: $r = 0.865$ for $p = 0.026$, and HQ: $r = 0.878$ for $p = 0.022$). It was reported earlier that both TR and HQ behave as reducing agents toward oxidative species. Naturally, this observation is valid up to a critical concentration limit, above which the antioxidants could behave in a prooxidative manner (e.g.,

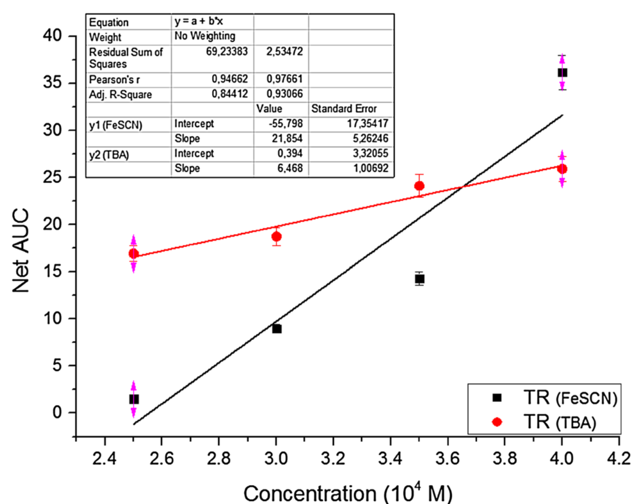


Fig. 7 Net AUC *versus* concentration of trolox (TR) in linoleic acid oxidation induced by copper(II) and oxygen [measured by Fe(III)-SCN (**a**) and TBARS (**b**) assays]

Table 2 Calculated TEAC (trolox equivalent antioxidant capacity) coefficients of antioxidants in the linoleic acid-copper(II) system (measured by Fe(III)-SCN method)

Compound (AOX)	Calibration equation Net AUC: $y = ax \pm b$	R^2	TEAC(unitless) $c = (2.5–4.0) \times 10^{-4}$ M
TR	$y = 21.8 \times 10^4 c - 55.8$	0.844	–
QR	$y = 8.37 \times 10^4 c + 62.0$	0.839	0.38
MR	$y = 18.2 \times 10^4 c - 22.1$	0.984	0.83
CT	$y = 12.9 \times 10^4 c + 41.0$	0.543	0.59
TocH	$y = 14.2 \times 10^4 c - 36.3$	0.979	0.65
AA	$y = 12.6 \times 10^4 c + 38.1$	0.893	0.58
AP	$y = 15.1 \times 10^4 c - 38.0$	0.771	0.69
HQ	$y = 45.5 \times 10^4 c - 37.4$	0.861	2.09
TBHQ	$y = 43.3 \times 10^4 c - 103.$	0.913	1.99
Garlic extract	$y = 17.9 \times c^* - 2.17$	0.466	0.82**

Garlic extract concentrations (c^*): 0.75; 1.00; 1.25 mg/mL (g/L)

TEAC of garlic (**): µmol of Trolox equiv per gram garlic extract (µM of Trolox equiv./g)

The correlation between antioxidants for the Fe(III)-SCN test; $P = 0.521 > 0.500$ (Good correlation, showing 'goodness of fit' for a sample size of $N = 30$). $p = 0.003 < \alpha = 0.05$ (The confidence level is arbitrarily set at $\alpha = 0.05$, and the given inequality shows that the observed experimental effect is genuine at 95% certainty)

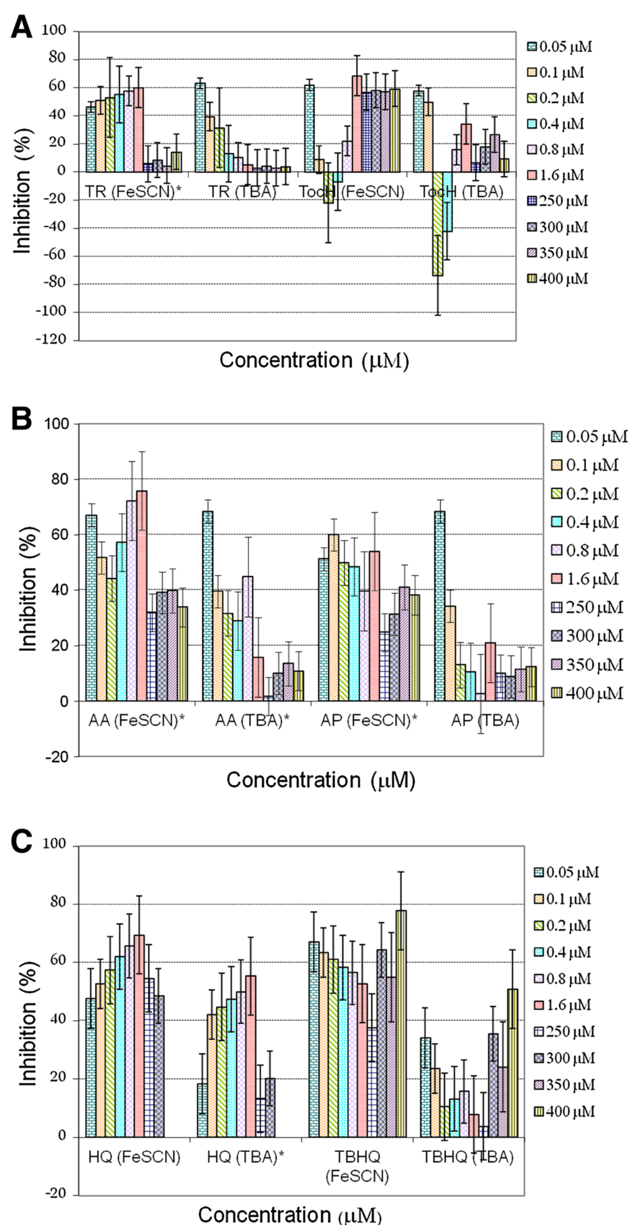
Table 3 Calculated TEAC (trolox equivalent antioxidant capacity) coefficients of antioxidants in the linoleic acid-copper(II) system (measured by TBARS method)

Compound (AOX)	Calibration equation Net AUC: $y = ax \pm b$	R^2	TEAC(unitless) $c = (2.5–4.0) \times 10^{-4}$ M
TR	$y = 6.47 \times 10^4 c + 0.39$	0.931	–
QR	$y = 2.01 \times 10^4 c + 45.7$	0.920	0.31
MR	$y = 8.05 \times 10^4 c + 9.61$	0.929	1.24
CT	$y = 3.76 \times 10^4 c + 29.6$	0.795	0.58
TocH	$y = 9.22 \times 10^4 c - 16.2$	0.983	1.42
AA	$y = 4.83 \times 10^4 c + 26.8$	0.945	0.75
AP	$y = 18.2 \times 10^4 c - 42.0$	0.862	2.81
HQ	$y = 3.70 \times 10^4 c + 19.2$	0.985	0.57
TBHQ	$y = 21.3 \times 10^4 c - 48.3$	0.931	3.29

The correlation between antioxidants for the TBARS test; $P = 0.555 > 0.500$ (Good correlation, showing 'goodness of fit' for a sample size of $N = 27$). $p = 0.003 < \alpha = 0.05$ (The confidence level is arbitrarily set at $\alpha = 0.05$, and the given inequality shows that the observed experimental effect is genuine at 95% certainty)

The Fe(III)-SCN and TBARS methods did not correlate significantly (results of statistical analysis to find differences among treatments for the two methods not shown) because they measured different properties (i.e. primary and secondary products of lipid peroxidation, respectively)

for the ten concentration values within the range of 0.05 and 400 µM, TR showed a negative correlation in the FeSCN test, with $r = -0.945$ for $p < 0.01$). However, a similar concentration-dependent response could not be obtained with TBA for most antioxidants tested (Fig. 8a–c) because of the complicated nature of the TBARS test generating secondary products, as Beer's law is generally valid for single chromogenic species from which a single chromophore is produced whose absorbance at a specified wavelength is proportional to its concentration. Except for AA (giving $r = -0.700$ with $p = 0.024$ for the ten concentration values up to 400 µM) and HQ (giving $r = -0.722$ with $p = 0.043$ for the first 8 concentration values up to 300 µM) yielding negative correlations, none of the tested antioxidants gave correlations at $p < 0.05$ significance level for the concentration-inhibition relationship in the TBA test (Fig. 8a–c). Only certain lipid peroxidation products may generate (though with low efficiency) malondialdehyde (MDA) emerging as the effective chromogen of the TBARS method, and it should be remembered that MDA is neither the sole end product of fatty peroxide formation/decomposition nor a substance generated exclusively through lipid peroxidation [27]. Covering all ten concentrations within 0.05–400 µM, ascorbic acid (AA) and ascorbyl palmitate (AP) gave negative correlations in the FeSCN test (Fig. 8b) (with $r = -0.782$ and $p = 0.008$ for AA and $r = -0.702$ and $p = 0.024$ for AP) due to the possible prooxidative behavior of AA and its derivatives in transition metal ion-containing solutions [28], because AA may reduce transition ions to their lower oxidation states to generate reactive species. Likewise, the distinctive prooxidative behavior of TocH was reflected in the negative inhibition (i.e. enhancement) values of this compound at certain concentrations in both FeSCN and TBA tests (Fig. 8a). A plausible explanation for this observation is that increasing concentrations of α-tocopherol under conditions of oxidative stress may result in increased levels of TocH radicals which may not be effectively detoxified by co-antioxidants existing in the system [28]. TBHQ, like AA, did not exhibit a concentration-dependent inhibitive effect within the full concentration range tested at $p < 0.05$ significance level in either FeSCN



◀**Fig. 8** **a** Concentration-dependent antioxidant/prooxidant activity of Trolox(TR)/α-tocopherol (TocH) pair in linoleic acid (LA) peroxidation induced by copper(II) system (measured by TBA and Fe(III)-SCN assays). A positive inhibition shows an antioxidant, while a negative inhibition prooxidant effect. The values for concentrations between 0.05 and 1.6 µM were taken from Ref. [6]. The calculated results (expressed as *vertical bars*) are given as mean ± SEM (standard error of the mean). *Asterisks* indicate that the statistical significance was accepted at $p < 0.05$. **b** Concentration-dependent antioxidant activity of ascorbic acid (AA)/ascorbyl palmitate (AP) pair in linoleic acid (LA) peroxidation induced by copper(II) system (measured by TBA and Fe(III)-SCN assays). The values for concentrations between 0.05 and 1.6 µM were taken from Ref. [6]. The calculated results (expressed as *vertical bars*) are given as mean ± SEM (standard error of the mean). *Asterisks* indicate that the statistical significance was accepted at $p < 0.05$. **c** Concentration-dependent antioxidant activity of hydroquinone (HQ)/*tert*-butyl hydroquinone (TBHQ) pair in linoleic acid (LA) peroxidation induced by copper(II) system (measured by TBA and Fe(III)-SCN assays). The values for concentrations between 0.05 and 1.6 µM were taken from Ref. [6]. The calculated results (expressed as *vertical bars*) are given as mean ± SEM (standard error of the mean). *Asterisks* indicate that the statistical significance was accepted at $p < 0.05$

or TBA tests (Fig. 8c) possibly because it was previously demonstrated that TBHQ is not a simple antioxidant capable of scavenging free radicals, but may also act as a prooxidant under certain conditions, e.g., toward a DNA probe [29]. However, TBHQ gave a negative correlation in the FeSCN test for the first six concentrations tested between 0.05 and 1.6 µM, with $r = -0.904$ for $p = 0.013$). The activation of TBHQ by Cu(II) was reported to result in the formation of TBQ, semiquinone anion radical and reactive oxygen species (ROS) [30].

Since the proposed method is a measure of the area under conversion/time curves, it has two dimensions of kinetic rate and thermodynamic efficiency, and therefore should be considered as superior to both kinetic ‘lag time’ assays and thermodynamic ‘fixed-time’ assays, each measuring a single parameter of antioxidant action. Integration of the curves to find the corresponding AUC values were made within concentration levels where antioxidants showed a distinct protective effect against lipid peroxidation. However, in cases when antioxidants showed a prooxidative effect below or above a certain critical concentration, this approach would still be valid, as negative areas would compensate for positive parts, yielding a smaller area (than for antioxidants exhibiting a uniform protective action throughout) as the algebraic sum of the corresponding AUC values. This offers future prospects of quantifying prooxidant ability along with antioxidant activity, though necessitating new experiments. As this is a mechanistically different approach to quantitatively express TAC in lipid peroxidation, antioxidant effectiveness followed a slightly different order from those seen in chemical reduction–based assays. It may be useful to mention that no previous assay in literature reported comparative TEAC values for antioxidants in protection against lipid peroxidation, and therefore the presented order of antioxidant effectiveness in this work could not be compared to those found by other reference methods. However the obtained results were reproducible and robust, giving ΔAUC values linearly varying with antioxidant concentration, thereby enabling the calculation of reliable TEAC coefficients for a selected set of hydrophilic and lipophilic antioxidants.

Conclusions

To the best of our knowledge, the proposed AUC-based approach of determining total antioxidant capacity is the first

application in antioxidative protection against lipid oxidation (which was qualitatively described previously). Although rate constants for lipid peroxidation in the absence and presence of antioxidants were reported in the literature, quantification of antioxidant capacities was not performed and the corresponding TEAC values of antioxidant compounds were not calculated before. Real-time monitoring of lipid oxidation products requires highly sophisticated but costly techniques such as ESR, CIS-MS or ESI-MALDI-TOF, however no single oxidation product is an exact measure of either lipid oxidation or its prevention by antioxidants. This study has confirmed that the ability of antioxidants to concentrate at interfaces in aqueous lipid emulsions may be equally important as their reductive power, and that an antioxidant sample such as garlic extract, definitely known to have antioxidative properties, can behave as a prooxidant under certain conditions of protection against secondary product formation during lipid oxidation. This work has also supplied additional proof to the generally known rule that antioxidants may behave as prooxidants above certain critical concentrations in lipid peroxidation catalyzed by transition metal ions. It is believed that quantitative estimation of lipid oxidation (in terms of both primary and secondary products formation) and of its inhibition by antioxidants with a simple and low-cost method are of utmost importance for food and lipid research. Additionally, the presented approach may open up new ways of quantifying food preservation together with antioxidant or prooxidant action, because conventional TAC assays utilizing AUC calculations can only simulate but not in reality reflect lipid oxidation.

Acknowledgements This work was supported by Yildiz Technical University, Research Fund, YTÜ-BAPK Project No: 29-01-02-03. One of the authors (Dr. Apak) would like to thank Istanbul University–Application & Research Center for the Measurement of Food Antioxidants (Istanbul Universitesi Gida Antioksidanlari Olcumu Uygulama ve Arastirma Merkezi) for the development of antioxidant/prooxidant test methods.

Compliance with ethical standards

Conflict of interest The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

1. Rop O, Mlcek J, Jurikova T, Valsikova M, Sochor J, Reznicek V, Kramarova D (2010) Phenolic content, antioxidant capacity, radical oxygen species scavenging and lipid peroxidation inhibiting activities of extracts of five black chokeberry (*Aronia melanocarpa* (Michx.) Elliot) cultivars. J Med Plants Res 4:2431–2437
2. Draper HH, Squires EJ, Mahmoodi H, Wu J, Agarwal S, Hadley M (1993) A comparative-evaluation of thiobarbituric acid methods for the determination of malondialdehyde in biological-materials. Free Radic Biol Med 15:353–363
3. Mihaljevic B, Katusin-Razem B, Razem D (1996) The reevaluation of the ferric thiocyanate assay for lipid hydroperoxides with special considerations of the mechanistic aspects of the response. Free Radic Biol Med 21:53–63
4. Cramer GL, Miller JF, Pendleton RB, Lands WEM (1991) Iodometric measurement of lipid hydroperoxides in human plasma. Anal Biochem 193:204–211
5. Tres A, Nuchi CD, Bou R, Codony R, Guardiola F (2009) Assessing rabbit and chicken tissue susceptibility to oxidation through the ferrous oxidation-xylenol orange method. Eur J Lipid Technol 111:563–573
6. Bakır T, Sönmezoğlu İ, İmer F, Apak R (2013) Polar paradox revisited: analogous pairs of hydrophilic and lipophilic antioxidants in linoleic acid emulsion containing Cu(II). J Sci Food Agric 93:2478–2485
7. Fukumoto LR, Mazza G (2000) Assessing antioxidant and prooxidant activities of phenolic compounds. J Agric Food Chem 48:3597–3604
8. Lea CH (1952) Methods for determining peroxide in lipids. J Sci Food Agric 12(3):586–594
9. Magalhaes LM, Segundo MA, Reis S, Lima JLFC (2008) Methodological aspects about *in vitro* evaluation of antioxidant properties. Anal Chim Acta 613:1–19
10. Ching SY, Hall J, Croft K, Beilby J, Rossi E, Ghisalberti E (2006) Antioxidant inhibition of oxygen radicals for measurement of total antioxidant capacity in biological samples. Anal Biochem 353:257–265
11. Apak R, Özyürek M, Güçlü K, Çapanoğlu E (2016) Antioxidant activity/capacity measurement. Hydrogen atom transfer (HAT)-based, mixed mode (electron transfer (ET)/(HAT), and lipid peroxidation assays. J Agric Food Chem 64:1028–1045
12. Apak R, Gorinstein S, Böhm V, Schaich KM, Özyürek M, Güçlü K (2013) Methods of measurement and evaluation of natural antioxidant capacity/activity (IUPAC Technical Report). Pure Appl Chem 85:957–998
13. Huang D, Ou B, Prior RL (2005) The chemistry behind antioxidant capacity assays. J Agric Food Chem 53:1841–1856
14. Cao G, Prior RL (1998) Comparison of different analytical methods for assessing total antioxidant capacity of human serum. Clin Chem 44:1309–1315
15. Pedraza-Chaverri J, Gil-Ortiz M, Albarran G, Barbachano-Esparza L, Menjıvar M, Medina-Campos ON (2004) Garlic's ability to prevent *in vitro* Cu^{2+}-induced lipoprotein oxidation in human serum is preserved in heated garlic: effect unrelated to Cu^{2+}-chelation. Nutr J 3:10. doi:10.1186/1475-2891-3-10
16. Torok B, Belagyi J, Rietz B, Jacob R (1994) Effectiveness of garlic on the radical activity in radical generating systems. Arzneimittelforschung 44:608–611
17. Pedraza-Chaverri J, Medina-Campos ON, Avila-Lombardo R, Zúñiga-Bustos AB, Orozco-Ibarra M (2006) Reactive oxygen species scavenging capacity of different cooked garlic preparations. Life Sci 78:761–770
18. Gorinstein S, Drzewiecki J, Leontowicz J, Leontowicz M, Najman K, Jastrzebski Z, Zachwieja Z, Barton H, Shtabsky B, Katrich E, Trakhtenberg S (2005) Comparison of the bioactive compounds and antioxidant potentials of fresh and cooked Polish, Ukrainian, and Israeli garlic. J Agric Food Chem 53:2726–2732
19. Yıldoğan-Beker B, Bakır T, Sönmezoğlu I, Imer F, Apak R (2011) Antioxidant protective effect of flavonoids on linoleic acid peroxidation induced by copper(II)/ascorbic acid system. Chem Phys Lipid 164:732–739
20. Bakır T, Sönmezoğlu İ, İmer F, Apak R (2014) Antioxidant/prooxidant effects of a-tocopherol, quercetin and isorhamnetin on linoleic acid peroxidation induced by Cu(II) and H_2O_2. Int J Food Sci Nutr 65:226–234

21. Miller JC, Miller JN (1993) Statistics for analytical chemistry, 3rd edn. Ellis Horwood, Prentice Hall, London
22. Pinchuk I, Lichtenberg D (2002) The mechanism of action of antioxidants against lipoprotein peroxidation, evaluation based on kinetic experiments. Prog Lipid Res 41:279–314
23. Watanabe Y, Ishido E, Xu Fang Adachi S, Matsuno R (2005) Oxidation kinetics of linoleic acid in the presence of saturated acyl l-ascorbate. J Am Oil Chem Soc 82:389–392
24. Koleva II, van Beek TA, Linssen JPH, de Groot A, Evstatieva LN (2002) Screening of plant extracts for antioxidant activity: a comparative study on three testing methods. Phytochem Anal 13:8–17
25. Porter WL (1993) Paradoxical behaviour of antioxidants in food and biological systems. In: By Williams GM (ed) Antioxidants, chemical, physiological, nutritional and toxicological aspects. Princeton Scientific, Princeton, pp 93–121
26. Brannan RG (2010) Reactive sulfur species act as prooxidants in liposomal and skeletal muscle model systems. J Agric Food Chem 58:3767–3771
27. Janero DR (1990) Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. Free Radic Biol Med 9:515–540
28. Rietjens IM, Boersma MG, Haan Ld, Spenkelink B, Awad HM, Cnubben NH, van Zanden JJ, Hv Woude, Alink GM, Koeman JH (2002) The pro-oxidant chemistry of the natural antioxidants vitamin C, vitamin E, carotenoids and flavonoids. Environ Toxicol Pharmacol 11:321–333
29. Badary OA, Taha RA, Gamal El-Din AM, Abdel-Wahab MH (2003) Thymoquinone is a potent superoxide anion scavenger. Drug Chem Toxicol 26:87–98
30. Li Y, Seacat A, Kuppusamy P, Zweier JL, Yager JD, Trush MA (2002) Copper redox-dependent activation of 2-*tert*-butyl(1,4) hydroquinone: formation of reactive oxygen species and induction of oxidative DNA damage in isolated DNA and cultured rat hepatocytes. Mutat Res Genet Toxicol Environ Mutagen 518:123–133