JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Spectrophotometric and Chromatographic Assessment of Contributions of Carotenoids and Chlorophylls to the Total Antioxidant Capacities of Plant Foods

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ABSTRACT: Carotenoids and chlorophylls are photosynthetic compounds and also efficient antioxidants. This study aims to identify and quantify carotenoids and chlorophylls in some vegetables (carrot, tomato, spinach), to measure the total antioxidant capacity (TAC) of these samples with two spectrophotometric methods, to correlate TAC data with carotenoid structure, and to compare the TAC results with HPLC findings. Separation of the individual antioxidant pigments was achieved on a C_{30} column using a developed gradient elution program involving methanol−acetonitrile (50:50, v/v) with 0.1% (v/v) triethylamine (TEA) (A) and acetone (B) mobile phases. Total antioxidant capacities of the acetone extracts of studied samples, in trolox and β-carotene equivalents, were in the order: spinach > tomato > carrot by both CUPRAC and ABTS methods. CUPRAC responded favorably to both chlorophylls a and b. The TAC calculated with aid of combined HPLC−spectrophotometry was very close to the spectrophotometric value (93−108%) for real samples and synthetic mixtures.

KEYWORDS: *carotenoids, chlorophylls, antioxidant capacity, CUPRAC, ABTS, HPLC*

INTRODUCTION

Carotenoids are a very important group of organic pigments with antioxidant properties, especially through the scavenging of singlet oxygen and peroxyl radicals, thereby playing a major role in the protection of plants against photooxidative processes.[1] These compounds are largely responsible for the red, yellow, and orange color of fruits and vegetables, and are also found in many dark-green vegetables. So far, over 750 carotenoids have been discovered.[2] Most carotenoids can be derived from a 40-carbon basal structure, which includes a system of conjugated double bonds in the polyene backbone of their structure, determining their light-absorbing properties and influencing their antioxidant activity. Carotenoids are classified by their chemical structure as: (1) carotenes that are constituted by carbon and hydrogen; (2) oxycarotenoids or xanthophylls that have carbon, hydrogen, and additionally oxygen. Carotenoids have also been classified as primary or secondary; primary carotenoids (β-carotene, violaxanthin, and neoxanthin) are required by plants in photosynthesis, whereas secondary ones (α-carotene, β-cryptoxanthin, zeaxanthin, anthenaxanthin, capsanthin, capsorubin) are found in fruits and flowers.[3−6] The major dietary carotenoids are β-carotene and α-carotene (in carrot), lycopene (in tomato and watermelon), β-cryptoxanthin (in oranges), lutein (in dark-green vegetables and eggs) and zeaxanthin (in corn and yellow pepper). Daily carotenoid intakes in adults in five European countries (U.K., Republic of Ireland, Spain, France, and The Netherlands) are in the range of 9.54 (in Spain) to 16.06 mg (in France).[7] Carotenoids have also been found in human plasma and in some other tissues such as skin.[8] Carotenoids are efficient antioxidants,[2,6,9−21] and the consumption of carotenoid-rich products has been demonstrated to have beneficial health effects such as the reduction of degenerative diseases (such as cancer and cardio- and cerebrovascular diseases).[9−11]

The activities of carotenoids differ among the various carotenoids.[6,9,12,14,16,21−23] The most widely used methods for determination of carotenoid antioxidant activity, though with major drawbacks, are FRAP (ferric reducing antioxidant power),[24] ABTS (2,2′-azinobis[3-ethylbenzothiazoline-6-sulfonate])/TEAC (trolox equivalent antioxidant capacity)[17,25] and DPPH (2,2-diphenyl-1-picrylhydrazyl)[26] assays.[6,9,12−14,16] In addition, the luminol−chemiluminescence-based peroxyl radical scavenging capacity (LPSC) assay has been used for determination of carotenoid antioxidant activity by Müller et al.[12,27] Mueller and Boehm also investigated the antioxidant activities of both β-carotene isomers and some nonretinoic metabolites with various in vitro assays,[13] and detected ferric-reducing activity for β-carotene metabolites but not for the different β-carotene isomers. All analyzed isomers were shown to have 2.5−3.0 times higher activity in bleaching $ABTS^{\bullet+}$ than α-tocopherol.

Bright-green natural pigments having photosynthetic activity, i.e., chlorophylls which are not considered as dietary antioxidants, are widely distributed among green fruit and vegetables[28] with chlorophyll a and b derivatives predominating in higher plants. All natural chlorophyll derivatives can be described as substituted tetrapyrrols with a magnesium ion bound in the center.[29] Chlorophylls and pheophytins (metal-free chlorophyll derivatives) have been reported to possess antimutagenic and antioxidant activity by breaking radical chain reactions caused by autooxidation of vegetable edible oils (stored in the dark) via a hydrogen-donating mechanism. There is limited work in literature on the determination of antioxidant activity of chlorophylls. Ferruzzi et al.[28] assessed the in vitro antioxidant activity of dietary chlorophyll derivatives by the use of ABTS and DPPH methods. Generally, chlorophyll a is more abundant than chlorophyll b by a 3 to 1 ratio. The antioxidant

Received: July 30, 2013
Revised: October 23, 2013
Accepted: October 29, 2013
Published: October 29, 2013

dx.doi.org/10.1021/jf403356h | J. Agric. Food Chem. 2013, 61, 11371−11381

capacity of native chlorophyll a was found to be significantly greater than that of chlorophyll b.[29] In another study,[30] the antioxidant activities of chlorophylls and their derivatives were examined by using DPPH and β-carotene bleaching[31] assays, the latter method exhibiting a dose-dependent response for all chlorophyll derivatives. All natural chlorophyll derivatives tested by DPPH were shown to have low antioxidant activity, while Cu−chlorophyllin, tested by both methods, presented a higher antioxidant activity than that of natural chlorophylls, emphasizing the role of the chelated metal ion.[31]

Reversed-phase high performance liquid chromatography (HPLC) is widely used to analyze carotenoids,[5,8,32−39] and chlorophylls[40−42] or both of them simultaneously[43,44] in different samples. In these studies C_{18} or C_{30} columns were generally used. Some studies have indicated that the C_{30} column can provide a much better resolution than a C_{18} column in separation of carotenoids and their geometrical isomers.[35,36]

The CUPRAC (cupric reducing antioxidant capacity) assay developed in our laboratories[45] is a rapid and reliable TAC method capable of simultaneously analyzing lipophilic and hydrophilic antioxidants[46] using bis(neocuproine)copper(II) chelate as chromogenic redox reagent. The results of this study demonstrated that in 90% aqueous acetone, only CUPRAC and ABTS/horseradish peroxidase (HRP)-H_2O_2[17] assays were capable of measuring carotenoids together with hydrophilic antioxidants. Capanoğlu et al.[47] stressed that during the processing of tomato fruit to tomato paste, the CUPRAC assay among other assays showed the highest antioxidant capacities for lipophilic extracts, and correctly reflected the TAC changes of the lipophilic fraction, confirming the sensitivity of this assay in organic solvents.

Methods integrated with chromatographic and spectrophotometric TAC assays are useful both in the determination of individual antioxidant components and their TAC values in a successful chromatographic run. These assays are either online[48,49] or offline procedures.[50−54] Offline HPLC integrated to spectrophotometric TAC assays do not require additional equipment (i.e., a postcolumn system) and TAC reagents when most of the antioxidant components can be identified and quantified, provided that their TEAC (trolox-equivalent antioxidant capacity, defined as the millimolar trolox equivalent concentration of 1 mM solution of the tested antioxidant) values are known before the chromatographic run. An assay for evaluation of plant foods and beverages involving both the identification/quantification of individual antioxidant constituents (especially polyphenolics that are the principal antioxidants of these samples) and measurement of their TAC values was named as 'combined HPLC−CUPRAC assay' for the first time by our research group[50] and applied to various samples.[50−52]

The aim of this study is to develop a rapid and reliable HPLC method for the analysis of carotenoids accepted as important dietary antioxidants and chlorophylls known for their antioxidant properties but not accepted as dietary antioxidants, to calculate the theoretical total antioxidant capacities of various plant foods (spinach, carrot, and tomato) and food industry wastes (of tomato paste and orange juice industries) utilizing their constituents identified and quantified by HPLC, and to compare these theoretical TAC values with those found by spectrophotometric CUPRAC[45] and ABTS[25] assays. Additionally, the carotenoid contribution to the CUPRAC-TAC results[45] of plant foods as well as the CUPRAC-TEAC values of chlorophylls (a and b) not classified under the common name of 'antioxidants' have been aimed to be reported for the first time in this study.

MATERIALS AND METHODS

Chemicals and Standards. The following chemicals were supplied from the indicated sources: $CuCl_2 \cdot 2H_2O$ (copper(II) chloride dihydrate), hydrogen peroxide (Merck), neocuproine (2,9-dimethyl-1,10-phenanthroline), ammonium acetate, β-carotene, α-carotene, lycopene, astaxantin, lutein, zeaxsantin, triethylamine (TEA), potassium persulfate (Sigma-Aldrich), ABTS (2,2′-azinobis[3-ethylbenzothiazoline-6-sulfonate]), chlorophyll a, chlorophyll b (Sigma), HPLC grade solvents (acetone, methanol (MeOH), ethanol (EtOH), acetonitrile (ACN)) (Riedel-de Haën),

Instrumentation. Extractions were carried out using a Bransonic 221 (U.S.A., Shelton) ultrasonic bath. Vegetable samples were lyophilized using a Telstar Cryodos (Terrassa, Spain) freeze-dryer. Spectrophotometric measurements were done with a Varian Cary 1E (Sydney, Australia) UV−vis spectrophotometer, and chromatographic separation and quantitation of carotenoids and chlorophylls were performed using a Waters HPLC system (Milford, MA, U.S.A.) equipped with a 1525 binary pump, a column oven with thermostat, a 2998 PDA (photodiode array) detector (Chelmsford, MA, U.S.A.) and inline degasser. Bidistilled water used throughout the experiments was obtained from Millipore Simpak1 Synergy 185 (France) ultrapure water system. Centrifugal separations were performed with an Elektromag (M 4812 P) laboratory centrifuge apparatus (Istanbul, Turkey).

Reagents and Solutions. β-Carotene, α-carotene, lycopene, astaxantin, lutein, zeaxantin, chlorophyll a, and chlorophyll b solutions were prepared as stock solutions in HPLC grade acetone and were used fresh; if necessary, they were stored at −20 °C for less than one week and checked by absorbance at maximum absorption wavelength before use. For all experiments, working solutions were prepared by diluting the related stock solution with acetone before each experiment.

The reagents for the normal CUPRAC ($CUPRAC_N$) assay[45] were: 1.0×10^{-2} mol L^{-1} copper(II) chloride and 1.0 mol L^{-1} ammonium acetate at pH 7.0 in distilled water, 7.5×10^{-3} mol L^{-1} neocuproine in EtOH.

The reagents for the CUPRAC assay with EtOH ($CUPRAC_{EtOH}$) were: 2.0×10^{-2} mol L^{-1} copper(II) chloride in distilled water, 1.0 mol L^{-1} ammonium acetate at pH 7.0, and 7.5×10^{-3} mol L^{-1} neocuproine in EtOH.

The reagent for the ABTS/persulfate method[25] was produced by reacting 7.0×10^{-3} mol L^{-1} stock solution of ABTS with 2.45×10^{-3} mol L^{-1} potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12−16 h prior to use.

Sample Preparation. Fresh spinach (*Spinacia oleracea*), carrots (*Daucus carota*), and tomatoes (*Lycopersicon esculentum*) were supplied from the local market. The wastes of tomato paste and orange juice industries were obtained from Assan Foods (Balıkesir, Turkey) and Konfrut Gıda San. ve Tic. A.Ş. (Istanbul, Turkey), respectively. All samples were chopped into small pieces with hands (carrots only with ceramic knife) and freeze-dried at −40 °C for 16−24 h. They were crushed to fine powder in a porcelain mortar prior to analysis.

Several organic solvents such as acetone, *n*-hexane, *n*-hexane/diethylether (1:3, v/v), *n*-hexane/ethylacetate (9:1, v/v), and hexane/ethanol/acetone (2:1:1, v/v/v) were used to compare the extraction efficiency of carotenoids in carrots selected as representative of vegetables.

The extraction procedure for carotenoids and chlorophylls was applied as follows: (i) the lyophilized sample powder was weighed (0.5−1.0 g) in stoppered flasks and (ii) extracted for 10 min with 10 mL of solvent in an ultrasonic bath at room temperature protected from light; (iii) the upper phase was decanted, and (ii) and (iii) were repeated for five times. The extracts were combined and completed to 50 mL and were centrifuged at 4000 rpm for 5 min; the supernatants were filtered with GF/PET (glass fiber/polyethylene terephthalate) 1.0/0.45 μm microfilters.

The extracts were generally used freshly but, if necessary, were stored at −20 °C and checked for their absorbances at maximum absorption wavelength before use.

Preparation of Synthetic Mixtures. Five different synthetic ternary mixtures (Syn Mix 1−5) solutions in acetone were prepared.

The compositions of the synthetic mixtures were regulated to contain the pigments most probably existent in the analyzed sample extracts. They were prepared to contain pigments of the final concentrations declared below:

(i) 2.2×10^{-5}, 3.4×10^{-5}, and 1.0×10^{-5} M of β-carotene, α-carotene, and chlorophyll a, respectively.
(ii) 9.8×10^{-6}, 1.7×10^{-5}, and 2.7×10^{-5} M of β-carotene, α-carotene, and lycopene, respectively.
(iii) 1.1×10^{-5}, 1.1×10^{-5}, and 7.0×10^{-6} M of lutein, chlorophyll b, and β-carotene, respectively.
(iv) 1.8×10^{-5}, 1.0×10^{-5}, and 1.2×10^{-5} M of zeaxanthin, astaxanthin, and lutein, respectively.
(v) 8.6×10^{-5}, 4.3×10^{-6}, and 1.2×10^{-5} M of β-carotene, chlorophyll a, and lutein, respectively.

$CUPRAC_N$ Method. The normal CUPRAC method, as described by Apak et al.,[45] was applied as follows: A mixture comprising 1 mL of 1.0×10^{-2} M copper(II) chloride, 1 mL of 1 M ammonium acetate buffer at pH 7.0, and 1 mL of 7.5×10^{-3} M neocuproine solution was prepared, x mL sample solution and $(1 - x)$ mL of acetone were added, and well mixed (total volume: 4.0 mL). This final mixture in a stoppered test tube was allowed to stand at room temperature for 30 min. At the end of this period, the absorbance at 450 nm was measured against a reagent blank.

$CUPRAC_{EtOH}$ Method. In this method, all reagents were prepared in EtOH, except the copper(II) solution in distilled water. The method was applied as follows: A mixture comprising 0.5 mL of 2.0×10^{-2} M copper(II) chloride and 0.5 mL of EtOH, 1 mL of 1 M ammonium acetate buffer at pH 7.0, and 1 mL of 7.5×10^{-3} M neocuproine solution was prepared, x mL of sample solution and $(1 - x)$ mL of acetone were added and then well mixed (total volume: 4.0 mL). This final mixture in a stoppered test tube was allowed to stand at room temperature for 30 min. At the end of this period, the absorbance at 450 nm was measured against a reagent blank. The aim of this modification was to eliminate the turbidity occurring with the tomato paste waste extract.

Linear regression equations for both methods were determined using β-carotene and trolox, separately. Therefore, TAC results were expressed as both trolox and β-carotene equivalents.

Additivity experiments were also performed by adding known amounts of β-carotene into extracts and the error percentages in recoveries were calculated.

ABTS/Persulfate Method. In this method, the matured ABTS radical solution with blue-green color was diluted with EtOH at a ratio of 2:50 (v/v). To 3 mL of the radical cation solution was added 1 mL of acetone, and the absorbance at 734 nm was read at the end of the sixth minute (i.e., this optimal time was experimentally determined for β-carotene and lycopene, and also found to be adequate for routinely monitoring $ABTS^{+\bullet}$ radical quenching chemistry by Tian and Schaich[55]). The procedure was repeated for the antioxidant pigment by adding 3 mL of the radical cation solution to x mL of sample solution and $(1.0 - x)$ mL of acetone, and recording the absorbance readings at the end of the sixth minute. The absorbance difference (ΔA) was found by subtracting the absorbance of the antioxidant pigment from that of the reagent blank (radical solution).[25] Linear regression equations were determined using both trolox and β-carotene as ΔA versus molar concentration.

Chromatographic Analysis. In the developed method, the analyses were carried out using Waters YMC C_{30} column (5 μm, 250 mm × 4.6 mm) with 20 μL sample injection. For pigments analysis, two different solutions of the mobile phase, i.e., methanol/acetonitrile (50:50, v/v) with 0.1% (v/v) TEA (A) and acetone (B) were used in gradient elution. Detection was performed within the wavelength range 200−800 nm with a PDA detector. The elution rate was 1.5 mL min^{-1}, and column temperature was 35 °C.

Gradient elution program was: 15 min 100% A, 10 min from 100% to 30% A (slope, 4), 15 min from 30% to 0% A (slope, 4) (using Empower 2 Software, Waters Corporation).

Using the above working mode, the calibration curves and linear equations of peak area versus molar concentration were established for the carotenoids and chlorophylls tested with two injections.

Qualitative and quantitative analyses of the sample solutions were performed with at least two injections. The chromatographic column was washed for 10 min with acetone prior to injection, and equilibrated for 10 min with solvent A.

Theoretical TAC Determination. Theoretical TAC values as trolox equivalents were calculated by multiplying individual concentrations of constituents determined by HPLC by their corresponding TEAC coefficients (CUPRAC and ABTS) and adding the products.[50−52]

$$\text{theoretical TAC} = \sum_{i=1}^{n} C_i(\text{TEAC})_i \quad (1)$$

where c_i is the concentration of constituent i measured by HPLC and $(\text{TEAC})_i$ is the TEAC coefficient of constituent i measured by the corresponding spectrophotometric method.

A similar calculation was made using β-CEAC (β-carotene equivalent antioxidant capacity, defined as the mmol L^{-1} β-carotene equivalent concentration of 1 mmol L^{-1} solution of the tested carotenoid) coefficients. Both TEAC and β-CEAC coefficients were unitless, as they represented antioxidant power relative to a reference compound (i.e., trolox and β-carotene, respectively).

Since the calculated (theoretical) capacities involve the combined use of chromatographic and spectrophotometric methods, HPLC−$CUPRAC_N$ and HPLC−ABTS terms were used to express the summed products of HPLC-assayed individual antioxidant concentrations and their TEAC (or β-CEAC) coefficients found with $CUPRAC_N$ and ABTS/persulfate methods, respectively.

Statistical Analysis. Spectrophotometric assays were carried out in triplicate for each sample and standard. Descriptive statistical analyses were performed using Excel software (Microsoft Office 2010) for calculating the means and the standard error of mean. Results were expressed as mean values ± standard deviation (SD), except for the confidence limits of the slope and intercept of a regression line. The precision and accuracy of different methods applied to the same sample were compared by F-test and Student's t test, respectively.

RESULTS AND DISCUSSION

Choice of Solvent for Extraction. For selection of a suitable solvent, carrots as the representative vegetable were extracted with different solvents and solvent mixtures (acetone, n-hexane, n-hexane/diethyleter (1:3, v/v), n-hexane/ethylacetate (9:1, v/v), and n-hexane/ethanol/acetone (2:1:1, v/v/v)). All experiments were carried out under subdued light. For the comparison of carotenoid contents of extracts, the results were evaluated according to the study reported by Biehler et al.[3] The amount of total carotenoids was determined using an average molar absorption coefficient (1.353×10^5 L mol^{-1} cm^{-1} at 450 nm) and average molecular weight (548 g mol^{-1}). As can be seen from Table 1, there were no significant differences among the mean recoveries depending on solvents (at 99% confidence level for solvent mixtures involving acetone, and at 95% confidence level for the others), and the precisions of all extraction systems were alike (at 95% confidence level). Acetone was chosen as the

Table 1. Total Carotenoid Content of Carrot Extracts Determined by Spectrophotometry for Different Solvents and Solvent Mixtures

solvent	total carotenoid content[a] of extract (mg g^{-1} dw[b])
acetone	0.93 ± 0.01
hexane	0.98 ± 0.01
hexane/diethyl ether	1.01 ± 0.02
hexane/ethyl acetate	1.04 ± 0.01
hexane/ethanol/acetone	1.07 ± 0.02

[a]As mean ± SD, where n = 3. [b]dw: Dry weight.

dx.doi.org/10.1021/jf403356h | J. Agric. Food Chem. 2013, 61, 11371−11381

Table 2. Linear Regression Data Obtained for Some Carotenoids and Chlorophylls by the CUPRAC$_N$ Method (n = 3)

standards	regression equations[a]	(r)	linear range (mol L^{-1})
	Carotenoids		
β-carotene	$y = 0.52 \times 10^5 x - 0.01$	0.9992	2.50×10^{-6} – 2.00×10^{-5}
α-carotene	$y = 0.81 \times 10^5 x - 0.04$	0.9999	1.25×10^{-6} – 1.20×10^{-5}
lycopene	$y = 0.48 \times 10^5 x - 0.01$	0.995	2.25×10^{-6} – 2.25×10^{-5}
lutein	$y = 0.77 \times 10^5 x - 0.01$	0.9996	1.40×10^{-6} – 1.25×10^{-5}
astaxanthin	$y = 0.34 \times 10^5 x - 0.05$	0.997	3.00×10^{-6} – 3.00×10^{-5}
zeaxanthin	$y = 0.33 \times 10^5 x - 0.05$	0.997	3.20×10^{-6} – 3.00×10^{-5}
	Chlorophylls		
chlorophyll a	$y = 1.20 \times 10^5 x - 0.01$	0.9991	0.90×10^{-6} – 0.80×10^{-5}
chlorophyll b	$y = 1.04 \times 10^5 x - 0.01$	0.998	1.00×10^{-6} – 0.90×10^{-5}

[a] y stands for absorbance, x for molar concentration, and r for linear correlation coefficient.

Table 3. Linear Regression Data Obtained for Some Carotenoids and Chlorophylls by the ABTS/Persulfate Method (n = 3)

standards	regression equations[a]	(r)	linear range (mol L^{-1})
	Carotenoids		
β-carotene	$y = 0.42 \times 10^5 x - 0.06$	0.995	0.25×10^{-5} – 0.20×10^{-4}
α-carotene	$y = 0.91 \times 10^5 x - 0.04$	0.991	0.12×10^{-5} – 0.10×10^{-4}
lycopene	$y = 0.66 \times 10^5 x + 0.01$	0.992	0.20×10^{-5} – 0.15×10^{-4}
lutein	$y = 0.67 \times 10^5 x - 0.03$	0.9996	0.20×10^{-5} – 0.15×10^{-4}
astaxanthin	$y = 0.15 \times 10^5 x - 0.01$	0.9998	0.70×10^{-5} – 0.60×10^{-4}
zeaxanthin	$y = 0.30 \times 10^5 x + 0.01$	0.991	0.40×10^{-5} – 0.30×10^{-4}
	Chlorophylls		
chlorophyll a	$y = 0.32 \times 10^5 x + 0.11$	0.991	0.35×10^{-5} – 0.30×10^{-4}
chlorophyll b	$y = 0.08 \times 10^5 x - 0.01$	0.995	1.25×10^{-5} – 1.20×10^{-4}

[a] y stands for absorbance, x for molar concentration, and r for linear correlation coefficient.

Table 4. TEAC and β-CEAC Coefficients of the Carotenoids and Chlorophylls with Respect to the CUPRAC$_N$ and ABTS/Persulfate Methods

standards	TEAC$_{CUPRAC}$	TEAC$_{ABTS}$	β-CEAC$_{CUPRAC}$	β-CEAC$_{ABTS}$
		Carotenoids		
β-carotene	3.10	1.60	1.00	1.00
α-carotene	4.90	3.50	1.58	2.19
lycopene	2.90	2.50	0.93	1.56
lutein	4.60	2.60	1.48	1.63
astaxanthin	2.00	0.60	0.65	0.38
zeaxanthin	2.00	1.10	0.64	0.69
		Chlorophylls		
chlorophyll a	7.20	1.20	2.32	0.75
chlorophyll b	6.20	0.30	2.00	0.19

extraction solvent due to its miscibility with water, i.e., a distinct advantage for applications of spectrophotometric TAC assays. Rodriguez-Amaya[37] reported that the traditional carotenoid extraction with acetone has the following additional advantages: it penetrates the food matrix well and dissolves both carotenes and xanthophylls efficiently, and subsequent partitioning to a nonpolar solvent occurs more easily.

Application of CUPRAC$_N$ and ABTS Methods to Various Carotenoid and Chlorophyll Standards. Carotenoids and chlorophylls that are expected to be found in tested samples, namely β-carotene, α-carotene, astaxantin, zeaxantin, lutein, lycopene, and chlorophylls a and b, were assayed using the CUPRAC$_N$ and ABTS/persulfate methods, with the latter as reference. The linear regression equations of the tested pigments together with linear working ranges are given in Tables 2 and 3. In the regression equations given in Tables 2 and 3, y stands for absorbance, x for molar concentration, and r for linear correlation coefficient.

The linear regression equations of tested carotenoids and chlorophylls gave the molar absorption coefficient (ε) as the slope. This molar absorption coefficient divided by that of trolox (for CUPRAC method, $\varepsilon = 1.67 \times 10^4$ L mol^{-1} cm^{-1} and for ABTS/persulfate method, $\varepsilon = 2.60 \times 10^4$ L mol^{-1} cm^{-1})[50] under the same conditions gave the TEAC coefficients of those pigments. Also, the β-CEAC coefficients of these compounds were calculated in the same manner. These values are presented in Table 4.

Carotenoids are divided into two classes on the basis of their chemical structures: carotenes and xanthophylls.[3,21,38] Since the members of both groups exhibited antioxidant capacity[8,11,12,15] and β-carotene is the most abundant carotenoid, this compound was selected as the representative of all carotenoids to evaluate total antioxidant capacity of the concerned samples.

The studied six carotenoids gave different TEAC coefficients to CUPRAC$_N$ and ABTS methods. The differences in findings of the two mentioned methods can be explained by their different mechanisms and by the nature and position of substituent groups on the carotenoid molecules. Müller et al.[12] reported that the activity of the carotenes increased with increasing number of conjugated double bonds. In the present study, both methods rated α-carotene and lutein as the most effective antioxidant compounds with close TEAC coefficients, probably due to their structural similarity (with the exception of two additional −OH groups of lutein attached to the cyclohexene rings) and the length of the conjugated π-electron system.[16]

The TEAC coefficient of chlorophyll a was higher than chlorophyll b in both assays, as reported by Ferruzzi and co-workers.[29] Comparing β-CEAC coefficients, carotenoids and chlorophylls showed a similar tendency in both assays, with the exception of very low ABTS coefficient for chlorophyll b. Considering the mutual cyclic tetrapyrrole-like structure of chlorophylls and of the human plasma antioxidant, bilirubin, and the significant antioxidant capacity of bilirubin exerted in both CUPRAC and ABTS/persulfate methods,[56] this extremely low β-CEAC coefficient of ABTS for chlorophyll b (i.e., 0.19 in Table 4) is rather surprising, and reminiscent of problems of antioxidant accessibility by the hindered ABTS$^{\bullet+}$ radical.[55]

Application of Developed HPLC Method to Various Carotenoid and Chlorophyll Standards. To date, several HPLC methods were applied to carotenoid and chlorophyll analysis.[32−44] Polymeric C_{18} column widely utilized for carotenoid separation has recently been surpassed by the polymeric C_{30} column, introduced in 1994. As C_{18} columns

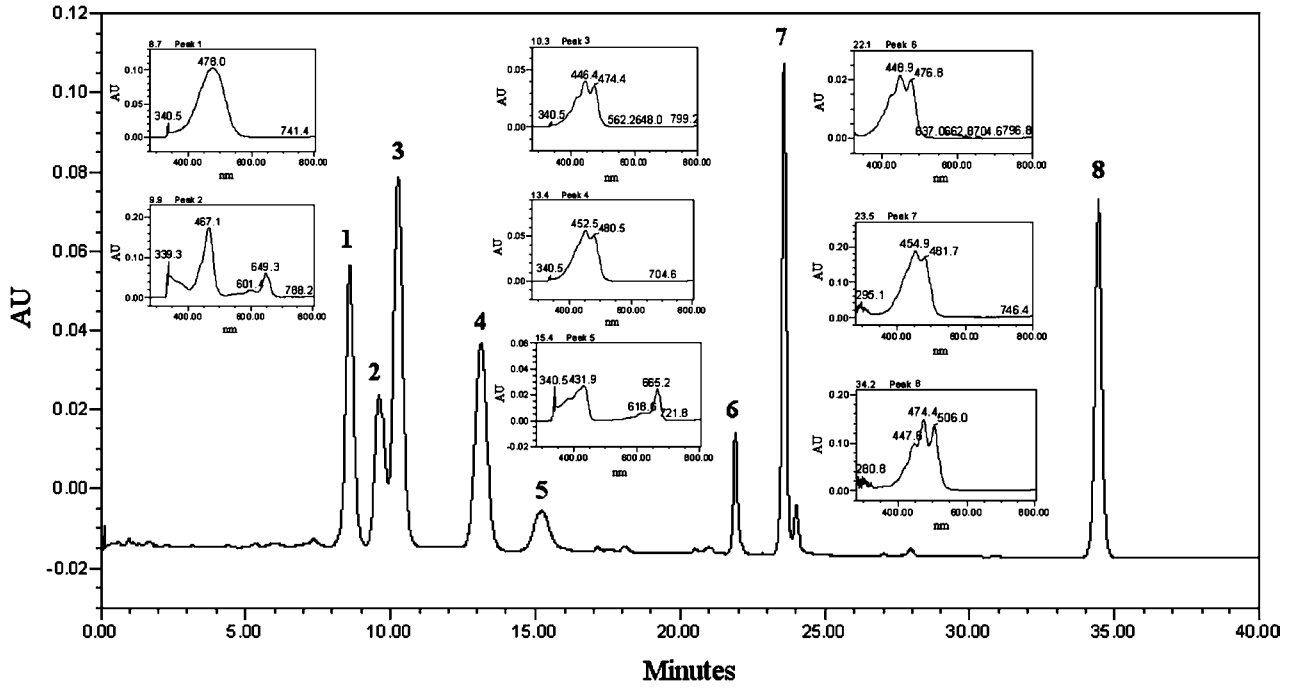

Figure 1. Chromatogram of a synthetic mixture of carotenoids and chlorophylls (detected at 450 nm), together with their PDA spectra (inset). (Peaks: 1, astaxanthin; 2, chlorophyll b; 3, lutein; 4, zeaxanthin; 5, chlorophyll a; 6, α-carotene; 7, β-carotene; 8, lycopene.)

Table 5. Linear Regression Data Obtained for Some Carotenoids and Chlorophylls by HPLC (n = 2)

standards	regression equations[b]	(r)	linear range (mol L^{-1})
	Carotenoids[a]		
β-carotene (455 nm)	$y = 0.76 \times 10^{11}x + 0.44 \times 10^{5}$	0.9998	0.25×10^{-6} – 0.12×10^{-5}
α-carotene (450 nm)	$y = 1.00 \times 10^{11}x - 0.28 \times 10^{5}$	0.998	0.18×10^{-6} – 0.12×10^{-5}
lycopene (475 nm)	$y = 0.68 \times 10^{11}x - 0.31 \times 10^{5}$	0.9998	5.30×10^{-6} – 2.65×10^{-5}
lutein (447 nm)	$y = 1.86 \times 10^{11}x + 1.20 \times 10^{5}$	0.998	5.02×10^{-6} – 2.50×10^{-5}
astaxanthin (478 nm)	$y = 1.13 \times 10^{11}x + 1.06 \times 10^{5}$	0.998	8.90×10^{-6} – 4.43×10^{-5}
zeaxanthin (453 nm)	$y = 0.73 \times 10^{11}x + 0.17 \times 10^{5}$	0.998	5.02×10^{-6} – 2.50×10^{-5}
	Chlorophylls[a]		
chlorophyll a (667 nm)	$y = 0.58 \times 10^{11}x - 0.03 \times 10^{5}$	0.997	2.50×10^{-6} – 1.00×10^{-4}
chlorophyll b (649 nm)	$y = 0.28 \times 10^{11}x - 0.45 \times 10^{5}$	0.998	2.50×10^{-6} – 1.00×10^{-4}
chlorophyll b (447 nm)	$y = 0.30 \times 10^{11}x - 0.45 \times 10^{5}$	0.997	2.50×10^{-6} – 1.00×10^{-4}

[a]The linear equations are calculated at the maximum absorption wavelengths (in the parentheses) of the pigments. [b]y stands for peak area, x for molar concentration, and r for linear correlation coefficient.

have often failed to provide adequate separation of food carotenoids, especially the geometric isomers,[37] the C_{30} column was preferred in this study.

Acetonitrile, methanol, or their mixtures are the major constituents of the mobile phases used in the analysis of carotenoids. Small amounts of other solvents (dichloromethane, hexane, chloroform, acetone, propanol, etc.) are added to optimize the separation of some carotenoids.[37,38] On the other hand, carotenoids may undergo losses or degradation on the column. Different studies have indicated that the addition of solvent modifiers, e.g., triethylamine (TEA) or ammonium acetate, to the mobile phase reduces losses or on-column degradation.[38,39] Another important factor that should be taken into account to achieve a satisfactory separation of carotenoids is the column temperature.[38] In recent years, several researchers have reported operating C_{18} and C_{30} columns at 30 °C and a slightly higher temperature for the analysis of carotenoids without any mention of degradation.[34]

A gradient elution program using mobile phases of methanol/acetonitrile (50:50, v/v) containing 0.1% (v/v) TEA (A) and acetone (B) was developed to separate carotenoids and chlorophylls within 40 min (Figure 1). A column operating temperature of 35 °C was suitable to satisfy efficiency criteria such as separation of major carotenoids and chlorophylls, and sharpness of peaks.

Figure 1 shows the chromatogram of a synthetic mixture of carotenoids and chlorophylls detected at 450 nm, together with their PDA spectra (inset).

Table 5 shows the linear regression equations of HPLC analysis of the tested pigments as chromatographic peak area versus molar concentration. In the regression equations, y stands for peak area, x stands for pigment molar concentration, and r stands for linear correlation coefficient.

Analysis of Real Samples and Synthetic Mixtures. Sample extracts and synthetic mixtures were spectrophotometrically assayed for TAC with CUPRAC and ABTS assays. The individual carotenoids and chlorophylls existing in these samples were identified and quantified with developed HPLC method (Figure 2).

The theoretical antioxidant capacities of real samples and synthetic mixtures were calculated using HPLC findings. The theoretical (calculated using eq 1) and experimental TAC (directly measured with a given spectrophotometric method) values for samples and synthetic mixtures were compared as μmol trolox and μmol β-carotene equivalent per g dw samples (Table 6).

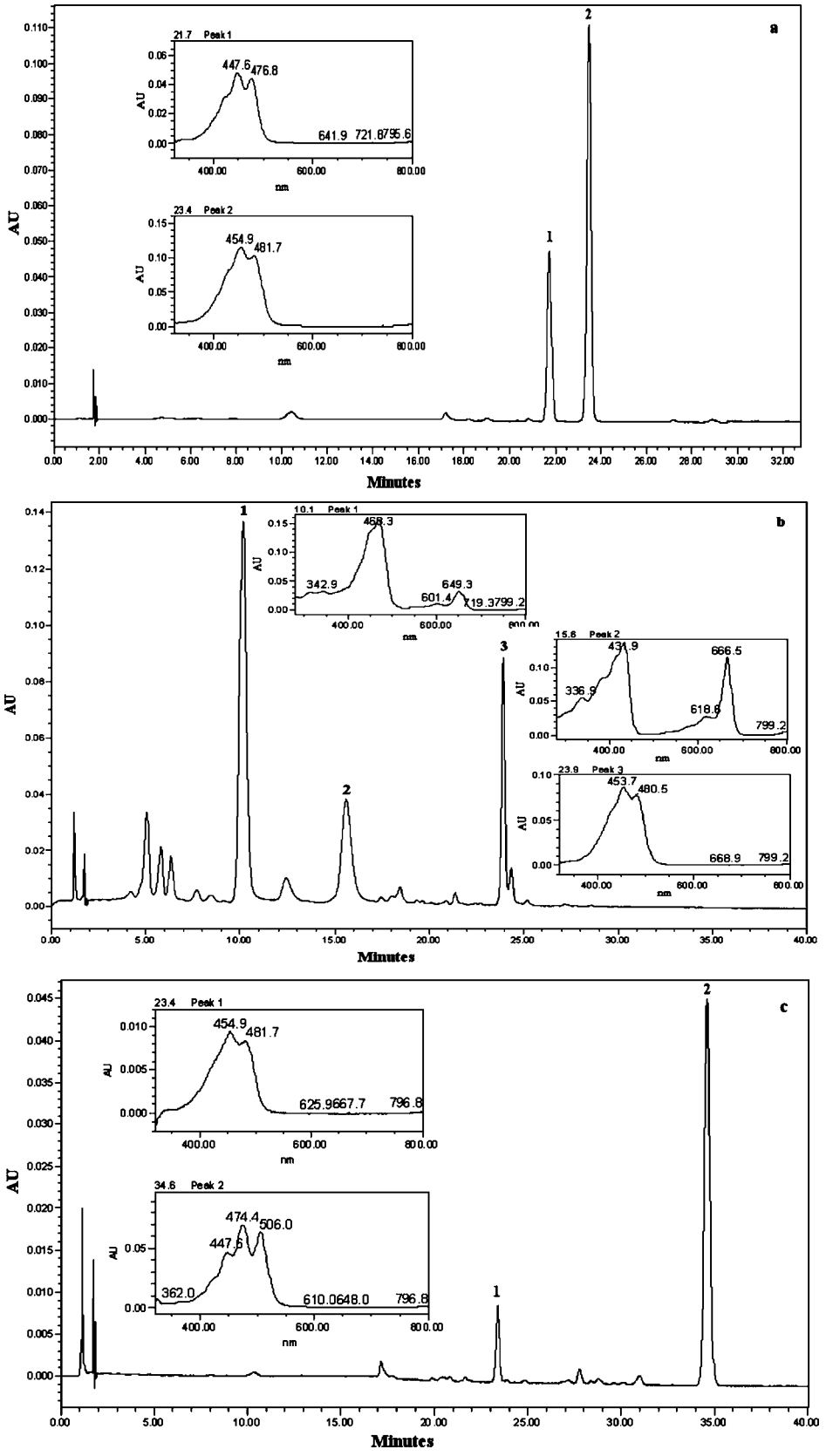

Figure 2. Chromatograms of samples (at 450 nm) and PDA spectra of identified peaks: (a) carrot (peaks: 1, α-carotene; 2, β-carotene); (b) spinach (peaks: 1, chlorophyll b; 2, chlorophyll a; 3, β-carotene); (c) tomato (peaks: 1, β-carotene; 2, lycopene).

As shown in Table 6, the TAC values (in trolox and β-carotene equivalents, as TE and β-CE, respectively) found with both spectrophotometric and combined HPLC−spectrophotometric (i.e., HPLC−CUPRAC and HPLC−ABTS) methods for real samples and synthetic mixtures were very close to each other except for spinach. For all synthetic mixtures, carrot and tomato,

dx.doi.org/10.1021/jf403356h | J. Agric. Food Chem. 2013, 61, 11371−11381

Table 6. Experimental and Theoretical TAC Values of Real Samples and Synthetic Mixtures Found by $CUPRAC_N$ and ABTS Methods; TAC units are given as μmol trolox g^{-1} dw and μmol β-carotene g^{-1} dw for real samples and as μmol (in 10 mL solution) for synthetic mixtures; combined HPLC−spectrophotometry enabled the theoretical calculation of experimental TAC values (with compensation percentage given in parentheses[a])

	$CUPRAC_N$[a]				ABTS[a]			
samples	TAC(TE)[b]	TAC(β-CE)[b]	HPLC-TAC(TE)[b,c]	HPLC-TAC(β-CE)[b,c]	TAC(TE)[b]	TAC(β-CE)[b]	HPLC-TAC(TE)[b,c]	HPLC-TAC(β-CE)[b,c]
carrot	4.90 ± 0.13	1.57 ± 0.05	4.70 ± 0.15(96)	1.50 ± 0.04(96)	2.74 ± 0.11	1.71 ± 0.06	2.68 ± 0.10(98)	1.67 ± 0.05(98)
spinach	49.50 ± 0.28	16.00 ± 0.22	45.10 ± 0.31(91)	14.50 ± 0.25(91)	11.70 ± 0.13	7.30 ± 0.21	9.00 ± 0.17(77)	5.49 ± 0.25(75)
tomato	9.70 ± 0.23	3.10 ± 0.18	9.30 ± 0.25(96)	2.98 ± 0.12(96)	8.24 ± 0.11	5.15 ± 0.13	7.83 ± 0.18(95)	4.95 ± 0.16(96)
Syn Mix 1	3.10 ± 0.06	1.01 ± 0.03	2.95 ± 0.03(95)	0.94 ± 0.03(93)	1.56 ± 0.03	1.00 ± 0.01	1.62 ± 0.28(104)	1.08 ± 0.01(108)
Syn Mix 2	1.65 ± 0.05	0.53 ± 0.04	1.60 ± 0.03(97)	0.51 ± 0.03(96)	1.10 ± 0.02	0.72 ± 0.02	1.07 ± 0.03(97)	0.71 ± 0.02(99)
Syn Mix 3	1.34 ± 0.02	0.43 ± 0.02	1.34 ± 0.03(100)	0.43 ± 0.02(100)	0.42 ± 0.03	0.26 ± 0.01	0.43 ± 0.03(102)	0.27 ± 0.01(104)
Syn Mix 4	1.16 ± 0.03	0.37 ± 0.01	1.13 ± 0.03(97)	0.36 ± 0.01(97)	0.61 ± 0.04	0.38 ± 0.02	0.61 ± 0.03(100)	0.36 ± 0.02(95)
Syn Mix 5	1.12 ± 0.01	0.37 ± 0.01	1.13 ± 0.01(101)	0.36 ± 0.01(97)	0.49 ± 0.02	0.29 ± 0.01	0.50 ± 0.02(102)	0.31 ± 0.02(107)

[a]As mean ± SD. [b]Symbols: TE: trolox equivalents, β-CE: β-carotene equivalents (n = 3 for spectrophotometric; n = 2 for HPLC analysis). [c]Values in parentheses of HPLC-TAC(TE) and HPLC-TAC(β-CE) columns represent the theoretically calculated percentages of the experimental TAC values.

Table 7. Comparison of the TAC Values (μmol β-CE g^{-1} dw) of Different Real Samples with Respect to the Two CUPRAC Methods (n = 3)

sample	$CUPRAC_{EtOH}$[a]	$CUPRAC_N$[a]
carrot	1.56 ± 0.02	1.64 ± 0.04
spinach	45.20 ± 0.92	44.70 ± 0.95
tomato waste	1.25 ± 0.01	−[b]
orange juice waste	4.30 ± 0.05	4.46 ± 0.06

[a]As mean ± SD. [b]Could not be measured due to the turbidity.

the calculated HPLC−spectrophotometric TAC values compensated for ≥95% of experimental TAC values in both methods, except for spinach where this compensation was 76% for HPLC−ABTS and 91% for HPLC−CUPRAC (Table 6). This means that experimental and theoretical antioxidant capacity values are quite compatible with one another.

Overall, the antioxidant/antiradical activity of carotenoids is based on a series of mechanisms including (i) electron transfer (ET), (ii) hydrogen atom abstraction, and (iii) radical adduct formation.[57] The different antioxidant behaviors of carotenoids is primarily caused by the different structure of their end groups

Table 8. Comparative Results (as β-CE) for All β-CAR Added Samples (at Concentrations of 1.49, 2.98, 4.47, 5.96, and 7.45 μM) (except tomato waste) and Their Error Percentages (n = 3)

	$CUPRAC_{EtOH}$			$CUPRAC_N$		
	β-CE (μM)			β-CE (μM)		
sample	expected	found	error (%)	expected	found	error (%)
			Carrot			
β-CAR	1.49			1.49		
carrot		2.69			2.94	
carrot + β-CAR	4.18	4.07	−2.63	4.43	4.38	−1.13
carrot + 2β-CAR[a]	5.67	5.54	−2.29	5.92	5.87	−0.84
carrot + 3β-CAR[a]	7.16	7.04	−1.68	7.41	7.49	1.07
carrot + 4β-CAR[a]	8.65	8.39	−3.00	8.90	9.05	1.68
carrot + 5β-CAR[a]	10.14	9.77	−3.65	10.39	10.65	2.50
			Spinach			
β-CAR	1.49			1.49		
spinach		3.37			3.22	
spinach + β-CAR	4.86	4.98	2.47	4.71	4.55	−3.40
spinach + 2β-CAR	6.35	6.21	−2.20	6.20	6.35	2.42
spinach + 3β-CAR	7.84	7.64	−2.55	7.69	7.50	−2.47
spinach + 4β-CAR	9.33	8.99	−3.64	9.18	9.34	1.74
spinach + 5β-CAR	10.82	10.35	−4.34	10.67	10.95	2.62
			Orange Waste			
β-CAR	1.49			1.49		
orange waste		6.39			6.65	
orange waste + β-CAR	7.88	7.85	−0.38	8.14	8.23	1.11
orange waste + 2β-CAR	9.37	9.32	−0.53	9.63	9.74	1.14
orange waste + 3β-CAR	10.86	10.57	−2.67	11.12	11.46	3.05
orange waste + 4β-CAR	12.35	11.96	−3.16	12.61	12.96	2.77
orange waste + 5β-CAR	13.84	13.39	−3.25	14.10	14.63	3.76

[a]2, 3, 4, 5β-CAR equivalents corresponding to 2.98, 4.47, 5.96, 7.45 μM β-CAR, respectively.

(i.e., the π-electron density of the polyene chain is unevenly distributed and is greater toward the end groups[58]), their chain length (i.e., of minor importance), and the number and position of their methyl groups.[59] In a previous study, the antioxidant activities of carotenes and xanthophylls were evaluated with ABTS/MnO_2 method by Miller et al.,[16] the results of which showed that of the carotenes studied, lycopene scavenged the $ABTS^{\bullet+}$ radical cation more extensively than β-carotene, and that carotenes with 11 conjugated double bonds were generally more active radical scavengers than the corresponding xanthophylls (i.e., oxygenated carotenoids). In the mentioned study, the sequence for relative radical scavenging abilities of carotenes and xanthophylls were determined as follows: lycopene > β-cryptoxanthin ≈ β-carotene > lutein ≥ zeaxanthin ≥ α-carotene > echineone > canthaxanthin = astaxanthin. Both Miller et al.[16] and Jiménez-Escrig et al.[60] utilizing ABTS/MnO_2 and DPPH radical scavenging methods, respectively, marked lycopene as the most antioxidative compound among the tested carotenoids, possibly due to the number of conjugated double bonds ($n = 11$) aside from the two isolated double bonds of this molecule, and to its coplanar structure enabling complete overlap of C═C sp^2 orbitals for easy electron transfer. In our study, the antioxidant capacity order obtained by the CUPRAC method was: α-carotene > lutein > β-carotene ≥ lycopene > zeaxanthin ≈ astaxanthin, whereas the $ABTS^{\bullet+}$ radical cation (generated by persulfate) scavenging order was established as α-carotene > lutein ≥ lycopene > β-carotene > zeaxanthin > astaxanthin. Obviously, in the CUPRAC method, α/β-carotene and lycopene changed places in the order of antioxidant activities, compared to radical scavenging assays. Considering that CUPRAC is a pure ET-based assay, either conjugated C═C chain length (i.e., $n = 10$ or 11, as in α-carotene and lycopene, respectively) or resonance-stabilization of radical adducts may not be as important as in the ABTS and DPPH scavenging methods. Instead, the *cis*-configuration of the β-ionone double bond in α-carotene[60] may be important for stabilization of the carotenoid radical cation ($CAR^{\bullet+}$) after electron-transfer to the CUPRAC reagent (i.e., cupric−neocuproine chelate), or higher reactivity of α/β-carotenes than of lycopene may be important. For example, β-carotene, being a bicyclic olefin, autooxidized faster than lycopene (an open-chain olefin) at an oxygen partial pressure of $p_{O2} = 150$ Torr;[59] α-carotene in turn reacted faster than β-carotene in the protection of lipid peroxidation against azo-initiated peroxyl radicals, and both carotenes reacted faster than zeaxanthin (a xanthophyll) in the same system,[61] in accordance with the CUPRAC order of antioxidant capacities of carotenoids. It is not surprising that both ABTS and CUPRAC methods marked astaxanthin, a 4,4′-diketone having electron-withdrawing properties at the keto-sites, as the least antioxidant-active compound. As H-atom abstraction (i.e., CAR-H + $R^{\bullet}$→ $CAR^{\bullet}$ + RH) is one of the mechanisms of the radical ($R^{\bullet}$) scavenging action of carotenoids, the differences in antioxidant reactivity of carotenes and xanthophylls may be attributed to the presence of the hydroxy or keto substituents in the allylic C-4 and C-4′ positions, preventing hydrogen abstraction from these positions to give a resonance-stabilized neutral radical.[62] By similar reasoning, zeaxanthin may show considerable reluctance compared to α-carotene to form radical cations ($CAR^{\bullet+}$) through electron-transfer (ET) reactions because of its more positive redox potential, arising from its −OH functionality attached to the β-ionone rings.[59] In both TAC assays (i.e., CUPRAC and ABTS) used in this work, lycopene proved to be a stronger reducing agent than both zeaxanthin and astaxanthin, in accordance with the relative one-electron reduction potentials of carotenoid radical cations reported in literature.[63]

Aside from the structure of carotenoids, the differences in the findings with ABTS/persulfate and CUPRAC methods can naturally be explained by their different mechanisms, i.e., ABTS is a mixed mode (hydrogen atom transfer and electron transfer: HAT and ET, respectively) mechanism assay while CUPRAC is a pure ET method. The reactive part of carotenes is usually the conjugated polyene chain in the center of the molecule, making it difficult for steric demanding oxidants like $ABTS^{\bullet+}$ to interact with the carotenoid, especially with the bicyclic structures of β-carotene.[13] Reactivity with $ABTS^{\bullet+}$ appears to be controlled first and foremost by steric accessibility of test antioxidants to the $ABTS^{\bullet+}$ radical site rather than by chemical properties.[64] The recent experimental results obtained by Tian and Schaich[55] raised questions regarding the ability of reactions with the hindered $ABTS^{\bullet+}$ to rank actual radical quenching by compounds with different structures. In addition, it was previously established that the TEAC coefficients for various antioxidants are quite dependent on the methodology of the colored $ABTS^{\bullet+}$ radical production, giving rise to different TEAC coefficients for the same antioxidant compound.[25,51] In our study, the radical cation was prepared by preliminary oxidation with persulfate, whereas Miller and co-workers[16] had used manganese dioxide. On the other hand, the preference of the CUPRAC method for antioxidant capacity measurement of carotenoids and chlorophylls can be justified due to its low cost, reagent stability and accessibility, and response to both hydrophilic and lipophilic antioxidants. Moreover, the CUPRAC assay results were precise and reproducible, and the TAC of complex mixtures perfectly showed the property of additivity.

Although chlorophylls are widely distributed among green fruits and vegetables, only a small number of publications have appeared to date concerning the assessment of their antioxidant activity. Chlorophylls were reported to possibly prevent the autooxidation of vegetable oils (stored in the dark) by a H-atom donating mechanism breaking the radical chain reactions.[65] Ferruzzi et al.[29] reported that antioxidant activity of natural chlorophylls was significantly lower than that of commercial grade chlorophyllins. The antioxidant capacity of native chlorophyll a was found to be significantly higher than that of chlorophyll b,[29] as in our findings.

Comparison of $CUPRAC_{EtOH}$ and $CUPRAC_N$ Methods. The linear regression equations of β-carotene found by the two CUPRAC applications are given below:

$$A = (5.20 \pm 0.27) \times 10^4 c - 0.01 \pm 0.02$$
$$r = 0.999 \text{ (for } CUPRAC_N\text{)}$$

$$A = (4.50 \pm 0.34) \times 10^4 c - 0.02 \pm 0.03$$
$$r = 0.997 \text{ (for } CUPRAC_{EtOH}\text{)}$$

In particular, the $CUPRAC_{EtOH}$ method can be used to solve the turbidity problem occurring in the $CUPRAC_N$ determination of TAC values of tomato paste industry waste and similar samples.

The experimental TAC values of different samples, found by the two methods, i.e., normal CUPRAC and its ethanol modification, were very close (i.e., not different at 95% confidence level for spinach, and not different at 98% confidence level for carrot and orange juice waste) when expressed as β-carotene equivalents (Table 7).

Additivity assessments were made by adding different amounts of β-CAR to the sample extracts. The comparative results for all β-CAR added (1.49, 2.98, 4.47, 5.96, and 7.45 μM) samples (except tomato waste) and their error percentages are summarized in Table 8. Unreacted carotenoid was shown not to remain at the end of 30 min incubation by extraction with 4 mL hexane, because the 450-nm absorbance of the hexane phase was between 0.0051 and 0.0200.

Overall Evaluation of the Proposed Assay for Carotenoids and Chlorophylls. The present study showed that the CUPRAC assay can be used to determine antioxidant capacity of carotenoids and chlorophylls. The results of the CUPRAC assay demonstrated that the important carotenes found in human diet (α-carotene, β-carotene and lycopene) were efficient antioxidants and α-carotene had the highest TEAC coefficient in the sequence: α-carotene > β-carotene > lycopene. The TEAC coefficients of xanthophylls determined by the CUPRAC assay were found higher than those by the ABTS/persulfate assay. The differences in the findings of the two mentioned methods can be explained by their different reaction mechanisms (i.e., CUPRAC is a pure ET assay, while ABTS is a mixed mode (ET/HAT) assay) and by the different structural properties of the tested carotenoids (i.e., conjugated C═C chain length, the nature and position of substituent groups on the carotenoid molecules, especially those attached to the bicyclic ends).

Theoretical TAC calculations by the HPLC−CUPRAC method are shown for the first time in this work for synthetic mixtures and real samples of carotenoids, and the calculated TAC values proved to be close to the experimentally found ones. This means that if all the carotenoids in a sample could be identified and quantified with HPLC, it would be possible to estimate the actual (experimental) TAC of the sample with the proposed procedure thanks to the validity of the additivity of TAC in the CUPRAC assay. On the other hand, experimental and theoretical ABTS-TAC results were found compatible among each other for real samples of carrot and tomato but not for spinach. This may possibly be explained by the lack of additivity of TAC values of the spinach sample components (e.g., chlorophylls) in the ABTS method under the employed conditions or by the presence of unidentified components.

Additionally, it can be expressed that there were no significant differences (though at different confidence levels) between the TAC results of $CUPRAC_{ETOH}$ and $CUPRAC_N$ methods for carrot, spinach, and orange juice waste samples and also for the samples spiked with various amounts of β-carotene, enabling the safe use of $CUPRAC_{ETOH}$ method for turbidity-producing samples (*e.g.*, tomato paste industrial waste).

The TEAC coefficient of chlorophyll a was higher than of chlorophyll b, when tested by the CUPRAC assay. Chlorophylls a and b (especially the latter) had low antioxidant capacities when compared to that of trolox using the ABTS/persulfate assay, probably due to reagent accessibility problems.

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Funding

The authors express their gratitude to the Scientific Research Projects Coordination Unit (BAP) of Istanbul University for the funding of Project 2724. N.Ö. also extends his gratitude to the Scientific Research Projects Coordination Unit of Istanbul University for the support given to his M.Sc. Thesis Project 23192.

Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

TAC, total antioxidant capacity; CUPRAC, cupric reducing antioxidant capacity; ABTS, 2,2′-azinobis[3-ethylbenzothiazoline-6-sulfonate]; HPLC, high performance liquid chromatography; TEAC, trolox equivalent antioxidant capacity; FRAP, ferric reducing antioxidant power; DPPH, 2,2-diphenyl-1-picrylhydrazyl; LPSC, luminol−chemiluminescence-based peroxyl radical scavenging capacity; HRP, horseradish peroxidase; TEA, triethylamine; MeOH, methanol; EtOH, ethanol; ACN, acetonitrile; PDA, photodiode array; $CUPRAC_N$, normal CUPRAC; $CUPRAC_{EtOH}$, CUPRAC assay with EtOH; β-CEAC, β-carotene equivalent antioxidant capacity; TE, trolox equivalents; β-CE, β-carotene equivalents; ET, electron transfer; HAT, hydrogen atom transfer; CAR, carotenoid; SD, standard deviation; dw, dry weight

REFERENCES

(1) Stahl, W.; Sies, H. Antioxidant activity of carotenoids. *Mol. Aspects Med.* **2003**, *24*, 345−351.

(2) Han, R.-M.; Zhang, J.-P.; Skibsted, L. H. Reaction dynamics of flavonoids and carotenoids as antioxidants. *Molecules* **2012**, *17*, 2140−2160.

(3) Biehler, E.; Mayer, F.; Hoffman, L.; Krause, E.; Bohn, T. Comparison of 3 spectrophotometric methods for carotenoid determination in frequently consumed fruits and vegetables. *J. Food Sci.* **2010**, *75*, C55−C61.

(4) Delgado-Vargas, F.; Jiménez, A. R.; Paredes-López, O. Natural pigments: Carotenoids, anthocyanins, and betalains — Characteristics, biosynthesis, processing, and stability. *Crit. Rev. Food Sci. Nutr.* **2000**, *40*, 173−289.

(5) Kao, T. H.; Loh, C. H.; Inbaraj, B. S.; Chen, B. H. Determination of carotenoids in *Taraxacum formosanum* by HPLC−DAD−APCI-MS and preparation by column chromatography. *J. Pharmaceut. Biomed. Anal.* **2012**, *66*, 144−153.

(6) Fu, H.; Xie, B.; Ma, S.; Zhu, X.; Fan, G.; Pan, S. Evaluation of antioxidant activities of principal carotenoids available in water spinach (*Ipomoea aquatica*). *J. Food Compos. Anal.* **2011**, *24*, 288−297.

(7) O'Neill, M. E.; Carroll, Y.; Corridan, B.; Olmedilla, B.; Granado, F.; Blanco, I.; Van den Berg, H.; Hininger, I.; Rousell, A.-M.; Chopra, M.; Southon, S.; Thurnham, D. I. A European carotenoid database to assess carotenoid intakes and its use in a five-country comparative study. *Br. J. Nutr.* **2001**, *85*, 499−507.

(8) Simonovska, B.; Vovk, I.; Glavnik, V.; Černelič, K. Effects of extraction and high-performance liquid chromatographic conditions on the determination of lutein in spinach. *J. Chromatogr. A* **2013**, *1276*, 95−101.

(9) Sánchez-Moreno, C.; Plaza, L.; de Ancos, B.; Cano, M. P. Quantitative bioactive compounds assessment and their relative contribution to the antioxidant capacity of commercial orange juices. *J. Sci Food Agric.* **2003**, *83*, 430−439.

(10) Tibble, D. L. Further evidence of the cardiovascular benefits of diets enriched in carotenoids. *Am. J. Clin. Nutr.* **1998**, *68*, 521−522.

(11) Slattery, M. L.; Benson, J.; Curtin, K.; Ma, K.-N.; Schaeffer, D.; Potter, J. D. Carotenoids and colon cancer. *Am. J. Clin. Nutr.* **2000**, *71*, 575−582.

(12) Müller, L.; Fröhlich, K.; Böhm, V. Comparative antioxidant activities of carotenoids measured by ferric reducing antioxidant power (FRAP), ABTS bleaching assay (αTEAC), DPPH assay and peroxyl radical scavenging assay. *Food Chem.* **2011**, *129*, 139−148.

(13) Mueller, L.; Boehm, V. Antioxidant activity of β-carotene compounds in different in vitro assays. *Molecules* **2011**, *16*, 1055−1069.

(14) Prasad, K. N.; Chew, L. Y.; Khoo, H. E.; Yang, B.; Azlan, A.; Ismail, A. Carotenoids and antioxidant capacities from *Canarium odontophyllum* Miq. Fruit. *Food. Chem.* **2011**, *124*, 1549−1555.
(15) Donado-Pestana, C. M.; Salgado, J. M.; de Oliveira Rios, A.; dos Santos, P. R.; Jablonski, A. Stability of carotenoids, total phenolics and *in vitro* antioxidant capacity in the thermal processing of orange-fleshed sweet potato (*Ipomoea batatas* Lam.) cultivars grown in Brazil. *Plant Foods Hum. Nutr.* **2012**, *67*, 262−270.
(16) Miller, N. J.; Sampson, J.; Candeias, L. P.; Bramley, P. M.; Rice-Evans, C. A. Antioxidant activities of carotenes and xanthophylls. *FEBS Lett.* **1996**, *384*, 240−242.
(17) Arnao, M. B.; Cano, A.; Acosta, M. The hydrophilic and lipophilic contribution to total antioxidant activity. *Food Chem.* **2001**, *73*, 239−244.
(18) Dewanto, V.; Wu, X.; Adom, K. K.; Liu, R. H. Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. *J. Agric. Food Chem.* **2002**, *50*, 3010−3014.
(19) Di Vaio, C.; Graziani, G.; Marra, L.; Cascone, A.; Ritieni, A. Antioxidant capacities, carotenoids and polyphenols evaluation of fresh and refrigerated peach and nectarine cultivars from Italy. *Eur. Food Res. Technol.* **2008**, *227*, 1225−1231.
(20) Grassmann, J.; Schnitzler, W. H.; Habegger, R. Evaluation of different coloured carrot cultivars on antioxidative capacity based on their carotenoid and phenolic contents. *Int. J. Food Sci. Nutr.* **2007**, *58*, 603−611.
(21) Zanfini, A.; Corbini, G.; La Rosa, C.; Dreassi, E. Antioxidant activity of tomato lipophilic extracts and interactions between carotenoids and α-tocopherol in synthetic mixtures. *LWT-Food Sci. Technol.* **2010**, *43*, 67−72.
(22) Paiva, S. A. R.; Russell, R. M. β-Carotene and other carotenoids as antioxidants. *J. Am. Coll. Nutr.* **1999**, *18*, 426−433.
(23) Böhm, V.; Puspitasari-Nienaber, N. L.; Ferruzzi, M. G.; Schwartz, S. J. Trolox equivalent antioxidant capacity of different geometrical isomers of α-carotene, β-carotene, lycopene, and zeaxanthin. *J. Agric. Food Chem.* **2002**, *50*, 221−226.
(24) Benzie, I. F.; Strain, J. J. The Ferric Reducing Ability of Plasma (FRAP) as a measure of "Antioxidant Power": The FRAP Assay. *Anal. Biochem.* **1996**, *239*, 70−76.
(25) Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Bio. Med.* **1999**, *26*, 1231−1237.
(26) Brand-Williams, W.; Cuvelier, M. E.; Berset, C. Use of a free radical method to evaluate antioxidant activity. *LWT-Food Sci. Technol.* **1995**, *28*, 25−30.
(27) Müller, L.; Goupy, P.; Fröhlich, K.; Dangles, O.; Caris-Veynat, C.; Böhm, V. Comparative study on antioxidant activity of lycopene (Z)-isomers in different assays. *J. Agric. Food Chem.* **2011**, *59*, 4504−4511.
(28) Ferruzzi, M. G.; Schwartz, S. J. Overview of chlorophylls in Foods. In: *Current protocols in food analytical chemistry*, John Wiley & Sons, Inc. 2001, F4.1.1−F4.1.9.
(29) Ferruzzi, M. G.; Böhm, V.; Courtney, P. D.; Schwartz, S. J. Antioxidant and antimutagenic activity of dietary chlorophyll derivatives determined by radical scavenging and bacterial reverse mutagenesis assays. *J. Food Sci.* **2002**, *67*, 2589−2595.
(30) Lanfer-Marquez, U. M.; Barros, R. M. C.; Sinnecker, P. Antioxidant activity of chlorophylls and their derivatives. *Food Res. Int.* **2005**, *38*, 885−891.
(31) Miller, H. E. A simplified method for the evaluation of antioxidants. *J. Am. Oil Chem. Soc.* **1971**, *48*, 91−97.
(32) Lin, C. H.; Chen, B. H. Determination of carotenoids in tomato juice by liquid chromatography. *J. Chromatogr. A* **2003**, *1012*, 103−109.
(33) Meléndez-Martínez, A. J.; Vicario, I. M.; Heredia, F. J. A routine high performance liquid chromatography method for carotenoid determination in ultrafrozen orange juices. *J. Agric. Food Chem.* **2003**, *51*, 4219−4224.
(34) Akhtar, M. H.; Bryan, M. Extraction and quantification of major carotenoids in processed foods and supplements by liquid chromatography. *Food Chem.* **2008**, *111*, 255−261.
(35) Inbaraj, B. S.; Lu, H.; Hung, C. F.; Wu, W. B.; Lin, C. L.; Chen, B. H. Determination of carotenoids and their esters in fruits of *Lycium barbarum* Linnaeus by HPLC−DAD−APCI−MSJ. *Pharmaceut. Biomed. Anal.* **2008**, *47*, 812−818.
(36) Inbaraj, B. S.; Chien, J. T.; Chen, B. H. Improved high performance liquid chromatographic method for determination of carotenoids in the microalga *Chlorella pyrenoidosa*. *J. Chromatogr. A* **2006**, *1102*, 193−199.
(37) Rodriguez-Amaya, D. B. Quantitative analysis, in vitro assessment of bioavailability and antioxidant activity of food carotenoids-A review. *J. Food Compos. Anal.* **2010**, *23*, 726−740.
(38) Rodríguez-Bernaldo de Quirós, A.; Costa, H. S. Analysis of carotenoids in vegetable and plasma samples: A review. *J. Food Compos. Anal.* **2006**, *19*, 97−111.
(39) Hart, D. J.; Scott, K. J. Development and evaluation of an HPLC method for the analysis of carotenoids in foods, and the measurement of the carotenoid content of vegetables and fruits commonly consumed in the UK. *Food Chem.* **1995**, *54*, 101−111.
(40) Bohn, T.; Walczyk, T. Determination of chlorophyll in plant samples by liquid chromatography using zinc-phthalocyanine as an internal standard. *J. Chromatogr. A* **2004**, *1024*, 123−128.
(41) Kao, T. H.; Chen, C. H.; Chen, B. H. An improved high performance liquid chromatography−photodiode array detection−atmospheric pressure chemical ionization−mass spectrometry method for determination of chlorophylls and their derivatives in freeze-dried and hot-air-dried Rhinacanthus nasutus (L.) Kurz. *Talanta* **2011**, *86*, 349−355.
(42) Loh, C. H.; Inbaraj, B. S.; Liu, M. H.; Chen, B. H. Determination of chlorophylls in *Taraxacum formosanum* by High-Performance Liquid Chromatography−Diode Array Detection−Mass Spectrometry and preparation by column chromatography. *J. Agric. Food Chem.* **2012**, *60*, 6108−6115.
(43) Gökmen, V.; Bahçeci, S.; Acar, J. Liquid chromatographic method for the determination of chlorophylls, carotenoids, and their derivatives in fresh and processed vegetables. *J. Liq. Chromatogr. Relat. Technol.* **2005**, *25*, 1201−1213.
(44) Puspitasari-Nienaber, N. L.; Ferruzzi, M. G.; Schwartz, S. J. Simultaneous detection of tocopherols, carotenoids, and chlorophylls in vegetable oils by direct injection C30 RP-HPLC with coulometric electrochemical array detection. *J. Am. Oil Chem. Soc.* **2002**, *79*, 633−640.
(45) Apak, R.; Güçlü, K.; Özyürek, M.; Karademir, S. E. Novel total antioxidant capacity index for dietary polyphenols and vitamins C and E, using their cupric ion reducing capability in the presence of neocuproine: CUPRAC method. *J. Agric. Food Chem.* **2004**, *52*, 7970−7981.
(46) Özyürek, M.; Bektaşoğlu, B.; Güçlü, K.; Güngör, N.; Apak, R. Simultaneous total antioxidant capacity assay of lipophilic and hydrophilic antioxidants in the same acetone-water solution containing 2% methyl-β-cyclodextrin using the cupric reducing antioxidant capacity (CUPRAC) method. *Anal. Chim. Acta* **2008**, *630*, 28−39.
(47) Capanoglu, E.; Beekwilder, J.; Boyacioglu, D.; Hall, R.; De Vos, R. Changes in antioxidant and metabolite profiles during production of tomato paste. *J. Agric. Food Chem.* **2008**, *56*, 964−973.
(48) Shi, S.-Y.; Zhou, H.-H.; Zhang, Y.-P.; Jiang, X.-Y.; Chen, X.-Q.; Huang, K.-L. Coupling HPLC to on-line, post-column (bio)chemical assays for high-resolution screening of bioactive compounds from complex mixtures. *TrAC Trends Anal. Chem.* **2009**, *28*, 865−877.
(49) Çelik, S. E.; Özyürek, M.; Güçlü, K.; Apak, R. Determination of antioxidants by a novel on-line HPLC-cupric reducing antioxidant capacity (CUPRAC) assay with post-column detection. *Anal. Chim. Acta* **2010**, *674*, 79−88.
(50) Yıldız, L.; Sözgen Başkan, K.; Tütem, E.; Apak, R. Combined HPLC-CUPRAC (cupric ion reducing antioxidant capacity) assay of parsley, celery leaves, and nettle. *Talanta* **2008**, *77*, 304−313.
(51) Karaman, Ş.; Tütem, E.; Sözgen Başkan, K.; Apak, R. Comparison of total antioxidant capacity and phenolic composition of some apple juices with combined HPLC−CUPRAC assay. *Food Chem.* **2010**, *120*, 1201−1209.

(52) Karaman, Ş.; Tütem, E.; Sözgen Başkan, K.; Apak, R. Comparison of antioxidant capacity and phenolic composition of peel and flesh of some apple varieties. *J. Sci. Food Agric.* **2013**, *93*, 867−875.
(53) Van der Sluis, A. A.; Dekker, M.; Skrede, G.; Jongen, W. M. F. Activity and concentration of polyphenolic antioxidants in apple juice. 1. Effect of existing production methods. *J. Agric. Food Chem.* **2002**, *50*, 7211−7219.
(54) De Beer, D.; Joubert, E.; Marais, J.; Manley, M. Unravelling the total antioxidant capacity of pinotage wines: Contribution of phenolic compounds. *J. Agric. Food Chem.* **2006**, *54*, 2897−2905.
(55) Tian, X.; Schaich, K. M. Effects of molecular structure on kinetics and dynamics of the trolox equivalent antioxidant capacity assay with $ABTS^{+\bullet}$. *J. Agric. Food Chem.* **2013**, *61*, 5511−5519.
(56) Apak, R.; Güçlü, K.; Ozyurek, M.; Karademir, S. E.; Altun, M. Total antioxidant capacity assay of human serum using copper(II)-neocuproine as chromogenic oxidant: The CUPRAC method. *Free Radical Res.* **2005**, *39*, 949−961.
(57) Krinsky, N. I.; Yeum, K.-J. Carotenoid-radical interactions. *Biochem. Biophys. Res. Commun.* **2003**, *305*, 754−760.
(58) Britton, G. Structure and properties of carotenoids in relation to function. *FASEB J.* **1995**, *9*, 1551−1558.
(59) Martin, H. D.; Ruck, C.; Schmidt, M.; Sell, S.; Beutner, S.; Mayer, B.; Walsh, R. Chemistry of carotenoid oxidation and free radical reactions. *Pure Appl. Chem.* **1999**, *71*, 2253−2262.
(60) Jiménez-Escrig, A.; Jiménez-Jiménez, I.; Sánchez-Moreno, C.; Saura-Calixto, F. Evaluation of free radical scavenging of dietary carotenoids by the stable radical 2,2-diphenyl-1-picrylhydrazyl. *J. Sci. Food Agric.* **2000**, *80*, 1686−1690.
(61) Olatunde-Forombi, E.; Britton, G. Antioxidant activity of palm oil carotenes in organic solution: effects of structure and chemical reactivity. *Food Chem.* **1999**, *64*, 315−321.
(62) Woodall, A. A.; Britton, G.; Jackson, M. J. Carotenoids and protection of phospholipids in solution or in liposomes against oxidation by peroxyl radicals: Relationship between carotenoid structure and protective ability. *Biochim. Biophys. Acta* **1997**, *1336*, 575−586.
(63) Edge, R.; Land, E. J.; McGarvey, D.; Mulroy, L.; Truscott, T. G. Relative one-electron reduction potentials of carotenoid radical cations and the interactions of carotenoids with the vitamin E radical cation. *J. Am. Chem. Soc.* **1998**, *120*, 4087−4090.
(64) Apak, R.; Gorinstein, S.; Boehm, V.; Schaich, K. M.; Özyürek, M.; Güçlü, K. Methods of measurement and evaluation of natural antioxidant capacity/activity. *Pure Appl. Chem.* **2013**, *85*, 957−998.
(65) Endo, Y.; Usuki, R.; Kaneda, T. Antioxidant effects of chlorophyll and pheophytin on the autoxidation of oils in the dark. II. The mechanism of antioxidative action of chlorophyll. *J. Am. Oil Chem. Soc.* **1985**, *62*, 1387−1390.