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Crocin Bleaching Assay Step by Step: Observations and Suggestions for an Alternative Validated Protocol

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Examination of the crocin bleaching assay performance and in-house validation were focused on probe and test compound characteristics, conditions for peroxyl radical generation, reaction monitoring, and expression of results. HPLC and spectrometric examination showed that any authentic commercial saffron (origin, grade) can be used for probe preparation given that (a) interferences, such as tocopherols, are removed, (b) working solution concentration is adequately adjusted, and (c) stock probe solution changes during storage are not neglected. As suggested by log *P* values, calculated for a great number of radical scavengers (AHs), any AH more polar than Trolox (common reference compound) can be tested in the aqueous environment of the assay. AH activities order obeyed principles of structure−activity relationships. The assay was robust toward preheating of the azo-initiator (2,2′-azobis(2-aminopropane) dihydrochloride). Reaction monitoring through periodic UV−vis spectra recording was very informative. An alternative expression of results as "percent inhibition of crocin bleaching value", % Inh = $[(\Delta A_0 - \Delta A)/\Delta A_0)] \times 100$, is proposed for [AH]/[crocin] = 1, instead of the so far used k_{rel} values. The above findings also lead to analysis cost and time reduction.

KEYWORDS: Crocin bleaching assay; in-house validation; partition coefficients; peroxyl radical scavengers; relative rate constants; saffron

INTRODUCTION

In the course of research on food and biologically active radical scavengers, many in vitro assays developed during past decades for testing compounds or extracts are now subjected to reconsideration. As stated by Huang et al. (*1*), when it comes to the selection of a reliable method, "the biggest problem is the lack of a validated assay that can reliably measure the antioxidant capacity of foods and biological samples". Today, the need for standardization of test protocols has become an issue of high priority (*2*, *3*), and ideal requirements have been proposed (*4*). These provisions turn out to be critical when structure−activity relationships are sought.

The "crocin bleaching assay" (CBA) was suggested by Bors et al. (*5*) as suitable for screening radical scavenging activity. Originally, inhibition of crocin bleaching by a range of substances was monitored by competition kinetics in the presence of photolytically produced alkoxyl radicals. Relative rate constants obtained were found to correlate well with the known antioxidant activities of selected compounds (phenolic antioxidants, model phenols, polyhydric and heterocyclic radical scavengers, etc.). At that time the chemistry behind the method was considered to be the same as that of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical assay, which was regarded as H-atom abstraction. In CBA, abstraction of hydrogen atoms and/or addition of the radical to the polyene structure of crocin results in a disruption of the conjugated system accounting for crocin bleaching (*5*). The latter is recorded as reduction of absorbance in the presence or absence of radical scavengers. Later on, Bors and co-workers (*6*) found that the absolute rate of crocin bleaching depends heavily on the sort of radical attacking the polyene structure. Selectivity was evidenced toward alkoxyl radicals produced after photolysis of hydroperoxides and peroxyl radicals produced after thermolysis of azo-initiators. Hydroxyl and azide radicals were not found to be suitable for this test due to their high reactivity toward almost any organic substance. Superoxide and alkyl radicals were found to be nonreactive. Quantitative information expressed as relative rate constants is derived from the equation

$$\frac{V_0}{V} = 1 + \frac{k_{AH}}{k_C} \times \frac{[AH]}{[C]} \quad (1)$$

where [AH] and [C] are the concentrations of the tested antioxidant and crocin and k_C and k_{AH} are rate constants for the reaction of the radicals with crocin and with AH, respectively. Second-order rate constants are obtained from kinetic curves in the presence (V) or absence (V_0) of antioxidants. By determining the V_0/V value at a known ratio of [AH] to [C], k_{AH}/k_C could then be calculated. At that time, the assay was proposed for application without further validation. Later, the kinetic test was used by Tubaro et al. (*7*), who evaluated the antioxidant capacity of complex mixtures of compounds in a

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modified form. In the latter, peroxyl radical formation was achieved by using azo-initiators (hydrophilic or lipophilic). In this way, Ursini and co-workers (*7*) made an effort to average antioxidant and pro-oxidant effects of the constituents of complex natural mixtures. Results were expressed with reference to α-tocopherol (for lipophilic molecules) or Trolox (for hydrophilic ones). Two years later, the same group reported some validation data for the assay in the course of application to plasma (*8*). By using different reference antioxidants (Trolox, ascorbic acid, uric acid, bovine serum albumin), some useful observations about the plasma pretreatment or the effect of reaction environment (pH, ionic strength) were made.

Since then, CBA has gained interest among investigators for the evaluation of the antioxidant activity of individual compounds, plant extracts, or plasma (e.g., see refs *9*−*13*). In all applications the analytical protocols are based on modifications or adjustments of that proposed by Bors et al. (*5*) as adapted by Tubaro et al. (*7*). A literature survey concerning the standardization and validation of CBA showed that there is not so far a protocol meeting the current analytical requirements (*14*, *15*), which are only scarcely addressed (*13*, *16*). Diversity in interest may explain why a complete study of the analytical parameters that may influence the evaluation of radical scavenging activity does not exist.

In the present study a step-by-step examination of method performance was accomplished. Special attention was paid to probe and test compound characteristics, conditions for radical generation, reaction monitoring, and expression of results. Qualitative and quantitative data were obtained using spectrometric and chromatographic procedures.

MATERIALS AND METHODS

Samples and Standards. Saffron red stigmas were donated by Saffron Cooperative of Kozani (Greece) and Verdu-Canto Saffron S.L. (Alicante, Spain). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and 4-hydroxy-3-methoxycinnamic acid (ferulic acid) were obtained from Aldrich Chemie (Steinhem, Germany). *p*-Hydroxybenzoic, protocatechuic, vanillic, syringic, *p*-coumaric, and sinapic acids were purchased from Sigma Chemical Co. (St. Louis, MO). Caffeic acid was a product of Riedel de Haën (Seelze, Germany).

Reagents and Solvents. 2,2′-Azobis(2-aminopropane) dihydrochloride (AAPH, >98%) was purchased from Fluka Chemie (Buchs, Switzerland). NaCl, KH_2PO_4, Na_2HPO_4, and KCl used for the preparation of phosphate-buffered saline (PBS) were from Panreac Química S.A. (Barcelona, Spain). HPLC grade methanol, acetone, acetonitrile, 2-propanol, and *n*-hexane were obtained from Riedel de Haën. Diethyl ether, stabilized with ethanol, H_2SO_4 (95%), NaOH, and NH_4OH solution (25%, v/v) were purchased from Panreac Química. Ultrahigh-purity water was produced using a Millipore-Milli-Q system.

Apparatus. A Shimadzu UV-1601 spectrophotometer (Kyoto, Japan) was used for all UV−vis absorbance measurements. The system was equipped with quartz cells (1 × 1 × 4 cm) thermostated at 39.5 ± 0.5 °C (*7*) with the aid of an outer water-circulating bath. Microcells (1 × 1 × 0.1 cm) from Hellma (Jena, Germany) were used in saffron authenticity test. Adjustment of pH was achieved using a Consort model 5231 portable pH-meter (Turnhout, Belgium).

Examination of Probe Characteristics. *Saffron Authenticity Test.* The absence of artificial water-soluble colorants in raw saffron samples was verified using the procedure of Zalacain et al. (*17*).

Spectrophotometric Estimation of $E^{1\%}$ Values. Estimation of coloring strength values ($E^{1\%}_{440nm}$) for the classification of raw saffron samples to commercial grades was carried out according to specifications and test methods of the ISO trade standard 3632-1,2 (*18*).

Crocin Stock and Working Solution Preparation. The method of Friend and Mayer (*19*) was modified as follows. Raw saffron (0.5 g) was washed three times with diethyl ether (3 × 15 mL × 5 min), and the residual ether drops were evaporated under a nitrogen stream. Each ether extract was separately stored in glass vials at 0 °C and examined further for its chemical composition. Purified saffron was suspended in 25 mL of methanol, stirred manually for 5 min, and filtered through RC 55 (0.45 μm, 0.25 mm) filters. The filtrate (20 mg of saffron/mL) was stored at −18 °C for a maximum of 3 months and used as the *crocin stock solution*. Crocin working solutions were daily prepared in methanol so that after adjustment the A_{433} value was ∼3.0. All treatments were carried out away from direct exposure to light.

HPLC Characterization of Triacylglycerols, Tocopherols, and Carotenoids. Standard RP-HPLC conditions (acetone/acetonitrile 60:40, v/v, isocratic elution, 1.2 mL/min) were applied for the separation of triacylglycerol species of ether extracts dried under vacuum and redissolved in acetone (3 mL). The solvent delivery system consisted of an SSI liquid chromatography pump (model 300; Scientific Systems Inc., State College, PA). Separation of triacylglycerols was achieved on a Nucleosil C_{18}, 5 μm (250 × 4 mm i.d.), column (Macherey-Nagel, Düren, Germany) using refractive index detection (RID-6A, Shimadzu Co., Tokyo, Japan) (*system I*). Tocopherols present in ether extracts (redissolved in methanol) were separated according to the method of Psomiadou and Tsimidou (*20*) using fluorescence detection (*system II*). The presence of nonpolar carotenoids in the ether extracts was studied using system II and UV−vis detection (*system III*). Separation of individual crocins present in stock solution was carried out using the method of Tarantilis et al. (*21*) adapted to laboratory facilities (*system IV*).

GC-FID Study of Fatty Acid Methyl Ester Composition. Fatty acid methyl esters, prepared using a cold alkali transesterification procedure, were analyzed on an Agilent 6890 GC system (Hewlett-Packard Inc., Palo Alto, CA) coupled with a flame ionization detector (FID) under the following standard operation conditions: HP FFA column (15 m × 200 μm × 0.30 μm); carrier gas, helium (1.1 mL/min); oven temperature, 50 °C (3 min), raised at 15 °C/min to 200 °C (10 min) and at 20 °C/min to 220 °C (6 min); injector temperature, 230 °C; detector temperature, 230 °C. Identification was based on the retention times of external standards tested under the above conditions.

Calculation of Partition Coefficient (Log *P*). Calculation of the log *P* values, simulating partitioning of tested compounds in an *n*-octanol/water (1:1, v/v) system, was based on Broto's fragmentation method and was accomplished using the CS ChemDraw Ultra 5.0 software (*22*).

CBA Kinetic Study. Peroxyl radical scavenging activity was evaluated according to the protocol of Tubaro et al. (*7*) with some modifications. Estimation of crocin concentration to ∼10 μM was based on an extinction coefficient reported in the literature, $\epsilon^{MeOH}_{433} = 133000$ M^{-1} cm^{-1} (*23*). A certain volume of crocin working solution was diluted with methanol to 5 mL (total volume) so that the A_{433} value was ∼1.3. The same volume of crocin working solution was then transferred into a 5 mL volumetric flask, along with increasing amounts of AHs (0.05−0.5 mL from 0.5 mM solutions in methanol). Stock AAPH solution (0.25 M) was daily prepared in 0.01 M PBS (0.08% w/v NaCl) and stored at 4 °C during the different sets of experiments. The precision of the pH-meter measurements obtained for the same buffer solution was satisfactory (% CV = 0.11, $n = 7$). Repeatability of buffer preparation procedure was also quite satisfactory (7.44 ± 0.04, $n = 7$). The reaction started with the addition of AAPH (250 μL) ($t = 0$ min). After dilution to 5 mL (total volume) with PBS and ∼30 s of stirring, the test solution was transferred into a 3 mL quartz cell, and absorbance monitoring (440 nm) was started at exactly 1 min after the addition of initiator. Recordings were taken every 6 s for a period of 10 min. Five to six different [AH]/[C] ratios (0.5−5 mol of AH/mol of crocin) were used in each set of experiments. AH concentration ranged from 0 to 50 μM. Reaction spectra were recorded in the region of 200−600 nm within 10 min after AAPH addition. Respective second derivatives were calculated by smoothing of 17 points and $d\lambda = 10$.

Expression of Results. Loss in absorbance values within 10 min of reaction, in the absence (V_0) or in the presence of AH (V), was calculated, and relative rates (V_0/V) were plotted against the [AH]/[C] ratios. The linear regression slopes representing relative rate constants ($k_{rel} = k_{AH}/k_C$) were then divided by the respective value of Trolox, resulting in the index "Trolox equivalents". Within-day repeatability of the relative rate in the presence of Trolox was calculated at five

random days of analysis (% CV = 1.8−5.1, 4.1−7.5, and 3.9−10.6 for [AH]/[C] = 1, 2, and 3, respectively). Within-day repeatability, % CV, of the k_{rel} at five random days ranged between 2.1 and 7.9% (n = 5).

Statistical Analysis. Statistical comparisons of the mean values of each set of experiments were performed at a significance level of 95% by Student's t test and one-way ANOVA using SPSS 11.5 software.

RESULTS AND DISCUSSION

Performance characteristics of the CBA were tested step by step. Observations made on our results and critical appraisal of published ones were based on principles set by Prior et al. (*4*) for the selection of a method for further standardization.

Probe Characteristics. *Crocin*, used as the probe in the assay, is not a pure compound. Under this collective name, a mixture of structurally similar esters of crocetin (8,8′-diapocarotene-8,8′-dioic acid) with sugar and/or methyl moieties is expected, the major compound being crocin 1 [α-crocin, crocetin-di(β-D-gentiobiosyl) ester] (*24*). Raw material, saffron, is extracted according to a specific protocol adopted by all investigators so far to yield a stock solution, crocin (*19*). A dilute working solution is then used as the probe in the assay. Sources of uncertainty due to raw saffron characteristics, extraction method, and changes during storage were carefully explored because the literature was rather confined on this matter.

Raw Saffron Characteristics. In a recent paper (*13*) Indian saffron was proposed as a cheaper alternative crocin source to the product provided by a specific supplier. To justify under which conditions CBA performers can use commercial saffron, we examined the effect of origin and commercial grade of raw material on crocin preparation (*18*). Saffron is usually marketed in packets of different size that guarantee authenticity and origin. Uncertified material can be also found in the spice markets of the world. This product is frequently adulterated or misbranded. In our study, methanol extracts of representative authentic Iranian, Greek, and Spanish saffron samples were analyzed under HPLC conditions usually applied for the separation of crocins (*21*). As shown in **Figure 1**, all samples had a similar qualitative composition of crocins.

The origin of commercial saffron, used as a source of the assay probe, is, therefore, not expected to increase uncertainty if authenticity is guaranteed. In case the product is unlabeled, the absence of interfering adulterants, such as red or yellow synthetic colorants, can be checked using the method recently proposed by Zalacain et al. (*17*). According to the latter, crocins are removed by precipitation and preconcentration of remaining colorants is achieved on SPE-polyamide cartridges. Examination of the second-derivative spectra of aqueous extracts in the region above 480 nm (as illustrated in **Figure 2** for the Greek sample) must not indicate the presence of artificial colorants. Application of such a screening procedure prior to CBA is suggested as it provides the researcher with evidence of sample authenticity in case this was not guaranteed on purchase.

Neither origin nor commercial grade of raw saffron was found to differentiate method performance as exemplified in the case of Trolox (**Table 1**). To verify that the origin of the raw material does not affect the performance of the probe, we prepared three different crocin stock solutions using Iranian, Greek, and Spanish representative raw saffron. For each solution five sets of experiments were then carried out using Trolox as a test compound. The three respective relative rate constants shown in **Table 1** were found to be of the same size even though statistically significant differences were observed in some values.

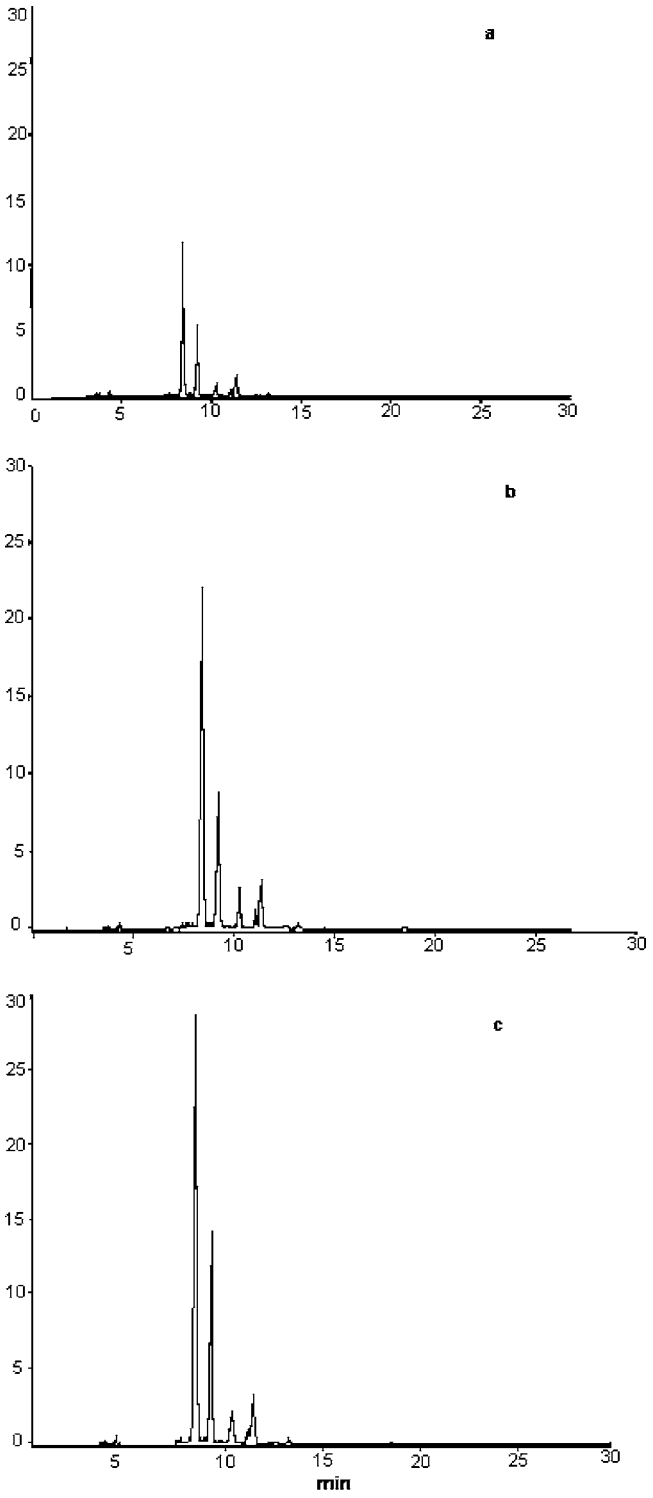

Figure 1. RP-HPLC profile of saffron methanol extracts at 440 nm: (**a**) Iranian, (**b**) Greek, and (**c**) Spanish origins.

This finding supports our suggestion that the origin of commercial authentic saffron is not expected to affect the reactivity of AHs. Commercial grades of saffron imply different levels of crocins in the raw material. Experimentation showed that appropriate adjustment of crocin concentration (to ∼10 μM) in working solutions obtained from raw saffron of different grades was enough to eliminate this source of uncertainty. The respective mean k_{rel} values were found to be of the same size (**Table 1**). The statistical difference observed was not considered

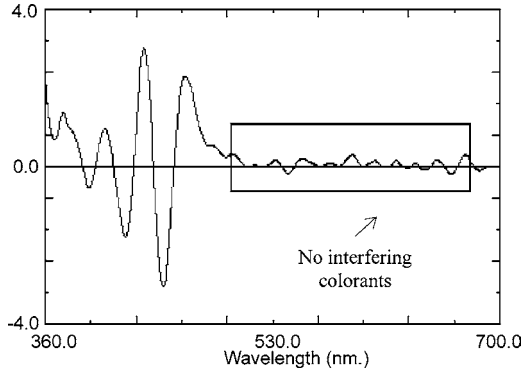

Figure 2. Typical second-derivative spectrum of aqueous saffron extract, according to Zalacain et al. (*17*). *Y* values from −1 to 3, in the region above 480 nm, are indicative of sample authenticity.

Table 1. Effect of Raw Saffron Characteristics on the Relative ROO• Scavenging Activity of Trolox

	$k_{rel} \pm$ SD[a] ($n = 5$)	intercept ± SD ($n = 5$)
origin		
Iranian	0.75 ± 0.06a[b]	0.82 ± 0.07a
Greek	0.68 ± 0.02a	0.91 ± 0.01b
Spanish	0.74 ± 0.07a	0.81 ± 0.06a
P	0.055	0.023
commercial grade		
I	0.78 ± 0.02a	0.91 ± 0.06b
III	0.68 ± 0.02b	0.91 ± 0.01b
t_{exptl}[c]	9.18	0.16
	t_{crit}[d] = 2.80 ($P < 0.05$)	

[a] Data from six-point linear regression curves. [b] Different letters indicate significantly different values within each category at $p < 0.05$. [c] t_{exptl} = Student *t* values calculated from experimental data. [d] t_{crit} = Student t value obtained from tables.

to be critical for the interpretation of results given that scavenging activity is expressed in terms of relative rate constants.

Stock and Working Crocin Solution Characteristics. The preparation procedure and storage of crocin solutions may be crucial steps in case reactive interferences remain or side products are formed. Storage conditions and the shelf life of crocin are other critical factors for the optimum CBA performance and reduction of overall uncertainty. A thorough investigation of the process of crocin preparation conducted on the basis of previous experience on raw saffron chemistry and analysis gave valuable evidence for the standardization of the protocol under examination.

(a) Removal of Interferences. As reported (*7*−*10*, *13*, *16*), "possible interfering substances are removed from saffron after repeated washes with diethyl ether". However, no information about the nature of "possible interference" is given. Our preliminary study on a petroleum ether saffron extract showed the presence of tocopherols, mainly α-tocopherol, which comprised ∼88% of the vitamin E fraction. The same extract also contained compounds, probably carotenoids that absorbed in the region 435−460 nm. Examination of the diethyl ether saffron fraction under various HPLC conditions (*systems I*−*III*) verified the presence of tocopherols, carotenoids, and triacylglycerols, whereas the presence of some mono- and diacylglycerol species could not be excluded. Fatty acid methyl ester composition by GC analysis showed that triacylglycerols contained mainly capric (10:0), palmitic (16:0), linoleic (18:2), and linolenic (18:3) acids. The major triacylglycerol species was that of ECN 44. To our knowledge this is the first time that some evidence is given for the lipid composition of saffron. Careful washing with diethyl ether proved to be more than adequate because no tocopherols were detected in the third washing. Tocopherols were absent from the methanol extract, reinforcing our previous statement. Some less polar carotenoids that would possibly cause overestimation of crocin concentration were not detected in the ether extract using the elution system III. Therefore, the yellow color in the lipid fraction was rather due to the presence of crocins, as indicated by analysis (system IV). Diethyl ether washing seemed to remove picrocrocin [4-(β-D-glucopyranosyloxy)-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde] to a great extent as evidenced by analysis of both ether extract and crocin stock solution. Residual picrocrocin in the stock solution is not expected to interfere with the reaction kinetics.

Taking into account that all solvents used for the extraction are free of antioxidants, the removal of tocopherols in the lipid-soluble fraction of saffron increases the reliability of the probe.

(b) Stock Crocin Solution Preparation and Shelf Life. With regard to the extraction of crocins, methanol or mixtures with water are used by researchers for the preparation of the stock solution. To standardize this step, experimentation with water, methanol, and their mixture (1:1, v/v) was carried out. $E^{1\%}_{440nm}$ values of the extracts were quite similar in all cases (215, 220, and 270, respectively). Carotenoid profiles, checked using *system IV*, were qualitatively identical in all cases. Therefore, we propose the use of methanol in the standardized protocol because it ensures (a) satisfactory crocins yield, (b) easier solvent removal under reduced pressure, and (c) avoidance of mold growth.

The crocin bleaching assay cannot be considered a rapid test. For example, for each test compound or solution, calculation of k_{rel} values from six-point linear regression curves would require six different [AH]/[C] ratios. Considering that (a) construction of each curve requires at least 1 h, (b) three replications are needed for each curve, as suggested by analytical guidelines (*14*, *15*), and (c) the same procedure has to be followed for the reference compound, Trolox, the experiment becomes lengthy (∼6 h), and working solutions of crocins should be daily prepared. Consequently, preparation of a sufficient volume of stock crocin solution is a prerequisite. Some researchers have reported a period of 2 months as the maximum time during which a stock solution could be used for CBA studies (*13*, *16*) without further justification. In our study, the effect of storage within a three-month period at −18 °C was examined by periodic HPLC examination of the carotenoid profile, which did not deviate from that obtained just after extraction (see **Figure 1**). Use of the stored solution as a probe in the oxidation of Trolox resulted in ∼40% higher k_{rel} value (1.17 ± 0.11, $n = 5$) with regard to that estimated before storage of the solution (0.72 ± 0.06, $n = 3$). Such a deviation had not been experienced either in our daily experiments within the first month of use of the probe or when extraction was repeated using the same lot of raw saffron (0.78 ± 0.02, $n = 5$, see **Table 1**). These findings are better illustrated in the control chart constructed by plotting the k_{rel} values of Trolox over an extended period of time (**Figure 3**). Within a 2-month period all measurements laid within a narrow band. Higher k_{rel} values, out of the upper limit of +3*s*, were obtained for crocin stock solutions stored at −18 °C for a longer period.

Taking into account the sound effect of prolonged storage on the performance of the stock crocin solution, the researcher should predetermine the amount required for the analysis of a

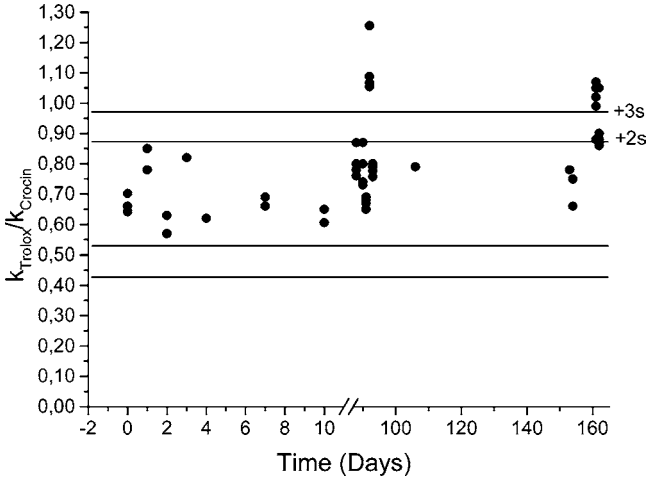

Figure 3. Ruggedness of CBA. Each point represents the k_{rel} value of Trolox calculated from each set of experiments within a working day.

series of test compounds/extracts. On the basis of the above findings and the simplicity of the extraction protocol, it is recommended that crocin stock solution be consumed within a month after extraction.

Test Compound Characteristics. CBA is considered to be applicable to "water-soluble" radical scavengers and related compounds. When "lipid-soluble" compounds are to be tested, a modification of the assay is proposed using either canthaxanthin as a probe (*5*) or a lipophilic initiator in organic solvent environment (*7*). However, a decisive criterion for the characterization of a test compound as water-soluble or not is missing. α-Tocopherol and Trolox, its polar analogue, are commonly used as reference compounds under nonaqueous or aqueous conditions, respectively. A physicochemical index of the water solubility of a substance is the partition coefficient (*P*) value in an *n*-octanol/water system (*25*). This parameter can be either determined experimentally or calculated with the aid of software widely used in chemical laboratories. Thus, we examined whether log *P* values of the test compounds could serve for the characterization of hydrophilicity/lipophilicity of test compounds under the conditions examined. Calculations were carried out using Broto's fragmentation method simulating partition of compounds in an *n*-octanol/water system (*22*). Working out log *P* values for a series of radical scavengers (**Table 2**) tested in the past using CBA in aqueous and nonaqueous environments (*5*), we observed that the values for the majority of them were lower than that of Trolox, the reference used under "aqueous conditions". Compounds exhibiting higher log *P* values (e.g., 3,5-di-*tert*-butyl-4-hydroxybenzoic acid, 2,4-di-*tert*-butylphenol, BHA, BHT) when tested in organic solvent reaction environment had a different performance. Therefore, it could be suggested that a log *P* value lower than that of Trolox is indicative of the suitability of a compound to be tested under the conditions of our study. A careful examination within applications of CBA (*7*−*9*, *13*, *16*, *26*, *27*) revealed that except for the case of some heterocyclic compounds (e.g., promethazine, chloropromazine, stobadine), most antioxidants tested so far under aqueous conditions were more polar than Trolox even though their hydro- or lipophilic nature was not clearly stated in the original studies. Taking into account that natural flavonoids, phenolic acids, and derivatives commonly studied for in vitro and in vivo antiradical activity are also consistent with the above requirement, it seems that the current CBA protocol might be applicable to a wide range of phenolic antioxidants. For this reason our further investigations were focused on CBA performance under aqueous conditions.

In addition, as shown in **Table 2**, the order of radical activity within each category of test compounds obeyed to a great extent classical principles of structure−antioxidant activity relationships (SAR) (*28*). It can be deduced that applicability of CBA in SAR studies seems to be promising. The latter is currently investigated in our laboratory.

Azo-initiator Performance. Despite reservation about their relevance to biological substrates, thermolabile azo-initiators (RN═NR) are widely used in quantitative studies of oxidation kinetics due to the advantage of generating localized peroxyl radicals at a constant rate (*29*). Reactions taking place under

Table 2. Partition Coefficient (log *P*) Values and Ranking Order of Radical Scavenging Activity of Selected Antioxidants Tested under CBA Conditions by Bors et al. (*5*)

		ranking order of AHs with CBA (k_{rel})				ranking order of AHs with CBA (k_{rel})	
	log *P*	method A[a]	method B		log *P*	method A	method B
phenols				standards			
2,4-di-*tert*-butylphenol	5.23	1	2	BHT	5.64	7	–[b]
isoeugenol	2.49	5	5	BHA	3.48	6	1
2,6-dimethoxyphenol	1.74	2	3	Trolox	3.18	3	2
guaiacol	1.61	5	4	ABTS	2.83	4	–
phenol	1.48	6	1	uric acid	–0.36	5	–
hydroquinone	1.09	4	–	ascorbic acid	–1.54	2	–
sesamol	0.96	3	–	isoascorbic acid	–1.54	1	–
phenolic acids				phenolic acid derivatives			
3,5-di-*tert*-butyl-4-hydroxybenzoic acid	4.58	6	2	4-hydroxybenzoic propyl ester	2.05	4	6
				4-hydroxybenzoic ethyl ester	1.59	3	5
sinapic acid	1.83	5	1	2,4,5-trihydroxybutyrophenone	1.52	1	2
ferulic acid	1.70	9	4	methyl gallate	1.27	2	–
caffeic acid	1.18	8	–	vanillin	1.09	9	4
syringic acid	1.10	3	3	2-hydroxy-4-methoxy-benzaldehyde	1.09	8	7
dihydrocaffeic acid	1.09	7	–				
vanillic acid	0.97	4	5	salicylic aldehyde	0.95	5	3
salicylic acid	0.83	10	–	3,4-dihydroxybenzaldehyde	0.45	7	-
protocatechuic acid	0.45	2	–	2,4,6-trihydroxyacetophenone	0.16	10	8
ellagic acid	0.06	1	–	acetovanillon	1.06	6	1
chlorogenic acid	–0.63	4	–				

[a] Method A, reaction with *t*-BuO• in aqueous solution; method B, reaction with *t*-BuO• in *n*-hexane. [b] No data available.

Table 3. Effect of AAPH Preheating (39.5 ± 0.5 °C) on the ROO• Scavenging Activity of Trolox

	k_{rel} ± SD (n = 5)	intercept ± SD (n = 5)
preheating	0.91± 0.08	0.84 ± 0.04
no preheating	1.00 ± 0.08	0.87 ± 0.05
t_{exptl}[a]	1.83	0.74
$t_{exptl} < t_{crit}$[b] = 2.80 ($P < 0.05$)		

[a] t_{exptl} = Student t values calculated from experimental data. [b] t_{crit} = Student t value obtained from tables.

CBA conditions can be described by the following scheme (*7*):

$$RN{=}NR \xrightarrow{heat} 2R^{\bullet} + N_2 \quad (R1)$$

$$R^{\bullet} + O_2 \leftrightarrow ROO^{\bullet} \quad (R2)$$

$$ROO^{\bullet} + \text{crocin} \rightarrow ROOH + \text{crocin}^{\bullet} \quad (R3)$$

$$ROO^{\bullet} + AH \rightarrow ROOH + A^{\bullet} \quad (R4)$$

$$A^{\bullet} + \text{crocin} \rightarrow AH + \text{crocin}^{\bullet} \quad (R5)$$

Peroxyl radicals, formed after a unimolecular thermal degradation of initiator (eq R1) in the presence of air (eq R2), cause crocin bleaching (eq R3) while a hydrogen atom is also abstracted from radical scavengers (eq R4) present in the solution. The antioxidant-derived radicals may also bleach crocin (eq R5) at a rate lower than that of eq R3. The exact mechanism of crocin reaction with peroxyl radicals (eq R3) has not yet been elucidated. Bleaching of crocin could be attributed to either H-atom abstraction and/or addition of radical to the polyene structure, as in the case of β-carotene (*30*).

Careful examination of the literature indicated that in most CBA applications, high molar ratios (>1000) of AAPH to probe are used similar to those found in applications of other probe-dependent assays such as the ORAC or TRAP (e.g., see refs *31* and *32*). This practice is not in line with ratios reported for AAPH-induced oxidation of micelles, methyl linoleate emulsions, low-density lipoprotein (LDL), or triglycerides. In the latter cases ratio values were <40 (*7*, *9*, *10*, *33*−*35*). Taking into account that all of the above tests are usually carried out under similar thermal conditions (37−40 °C), it is expected that molar ratios depend on the oxidation rate of the substrate (*36*, *37*).

In our study it was examined whether preheating of AAPH solution could lead to the use of lower amounts of initiator, which consequently would reduce the cost of analysis. Preheating of either the assay mixture or the AAPH solution is reported in some of the CBA applications without clear indication of the incubation period or its effect on reaction kinetics. Preheating of the initiator has been also found to reduce variability of results in ORAC applications (*38*). However, a 10-min preheating of AAPH solution (39.5 ± 0.5 °C) was not found to exert statistically significant changes in k_{rel} values of Trolox or even to reduce the variability of analytical results (**Table 3**). CBA was found to be robust toward this parameter. Therefore, standardization of the protocol to include initiator preheating is not necessary.

Reaction Monitoring and Expression of Results. In most CBA applications monitoring of the reaction kinetics is carried out at a single wavelength, that of crocin (440−450 nm). A series of experiments is then necessary to evaluate the radical scavenging activity in terms of k_{rel} values. Construction of linear regression curves for the calculation of k_{rel} values and evaluation of the activity of a series of compounds is, therefore, a considerably laborious and time-consuming procedure, as already argued. To overcome this shortcoming, we sought an alternative way for the evaluation of the ROO• scavenging activity. Spectra of all combinations of reaction components (**Figure 4**) obtained at 2-min intervals over a 10-min incubation period in the region of 200−600 nm were very informative toward this objective. Trolox was used as the standard AH.

As shown in **Figure 4a**−**c**, none of crocin (~10 μM), Trolox (10 μM), or AAPH (12.5 mM) solutions was unstable under the conditions of the assay (pH 7.4, 40 °C, 10 min). No evidence of interactions between crocin and Trolox was found (**Figure 4d**). Upon addition of AAPH to crocin or Trolox solution, noticeable changes were brought about in the spectra of the latter two (**Figure 4e**,**f**). The spectrum of the reaction mixture indicated clearly the competitive character of the reaction as protection of crocins was evidenced (**Figure 4g**). This protection was dependent on the concentration of Trolox as pointed out by tests on different molar ratios of [Trolox]/[C]. Parallel investigation of all respective derivative spectra indicated that a crossing point at 370 nm could not be considered as an isosbestic one (**Figure 4eD**,**fD**,**gD**). Thus, the only source of information for the oxidation of Trolox (or any other AH) is the stepwise reduction of absorbance in the region of 415−470 nm. Investigation of the spectra taken for a series of phenolic AHs at an equimolar ratio to crocin (data not shown) indicated that this stepwise reduction could be used not only for assigning the relative order of radical scavenging activity but also for quantitative purposes. This observation gave us the idea to adopt the term "percent inhibition of crocin bleaching value (% Inh)" used by Lussignoli et al. (*16*) but to introduce another way method of activity evaluation. Thus

$$\%\ \text{Inh} = [(\Delta A_0 - \Delta A)/\Delta A_0) \times 100 \quad (2)$$

where ΔA_0 and ΔA as in eq 1 (see Introduction) can be calculated from only one [AH]/[C] ratio instead of at least five ratios required for the calculation of k_{rel} or IC_{50} values (*7*, *16*). Trolox equivalent values can then be easily produced if $\%\ Inh_{AH}$ is divided by the respective $\%\ Inh_{Trolox}$. The validity of our suggestion was checked for a series of *p*-hydroxy phenolic acids for which k_{rel} values and, consequently, relative order of reactivity under similar experimental conditions had been reported (*9*). Results are presented in **Table 4**. The same trend was found using either k_{rel} values or the % Inh value with one exception. Within the benzoic acid series the order observed for syringic and protocatechuic acids, verified by a series of experiments for the calculation of the respective k_{rel} values, was different from that reported by Natella et al. (*9*). Our finding was considered to be the acceptable one as it was in line with trends based on BDE values (*39*) and radical scavenging activity experimental data for the two acids (e.g., see refs *40*−*42*). It should be pointed out that under the pH conditions of CBA (pH 7.4), AAPH-derived radicals probably carry ionizable $=NH_2^+Cl^-$ groups (*43*). Therefore, reaction with phenolic acids (see eq R4) might not be ascribed to only H-atom abstraction. Obviously, further substantiation is needed upon this hypothesis.

Concerning the magnitude of activity, our proposal seems to have the serious advantage of deducing information from the competitive reaction of equal numbers of moles of crocins and AH. In this way, the order and the magnitude of activity should be neither over- nor underestimated. Taking into account the different meaning of the terms "activity" and "capacity" of a test compound to scavenge peroxyl radicals (*3*), the parameters

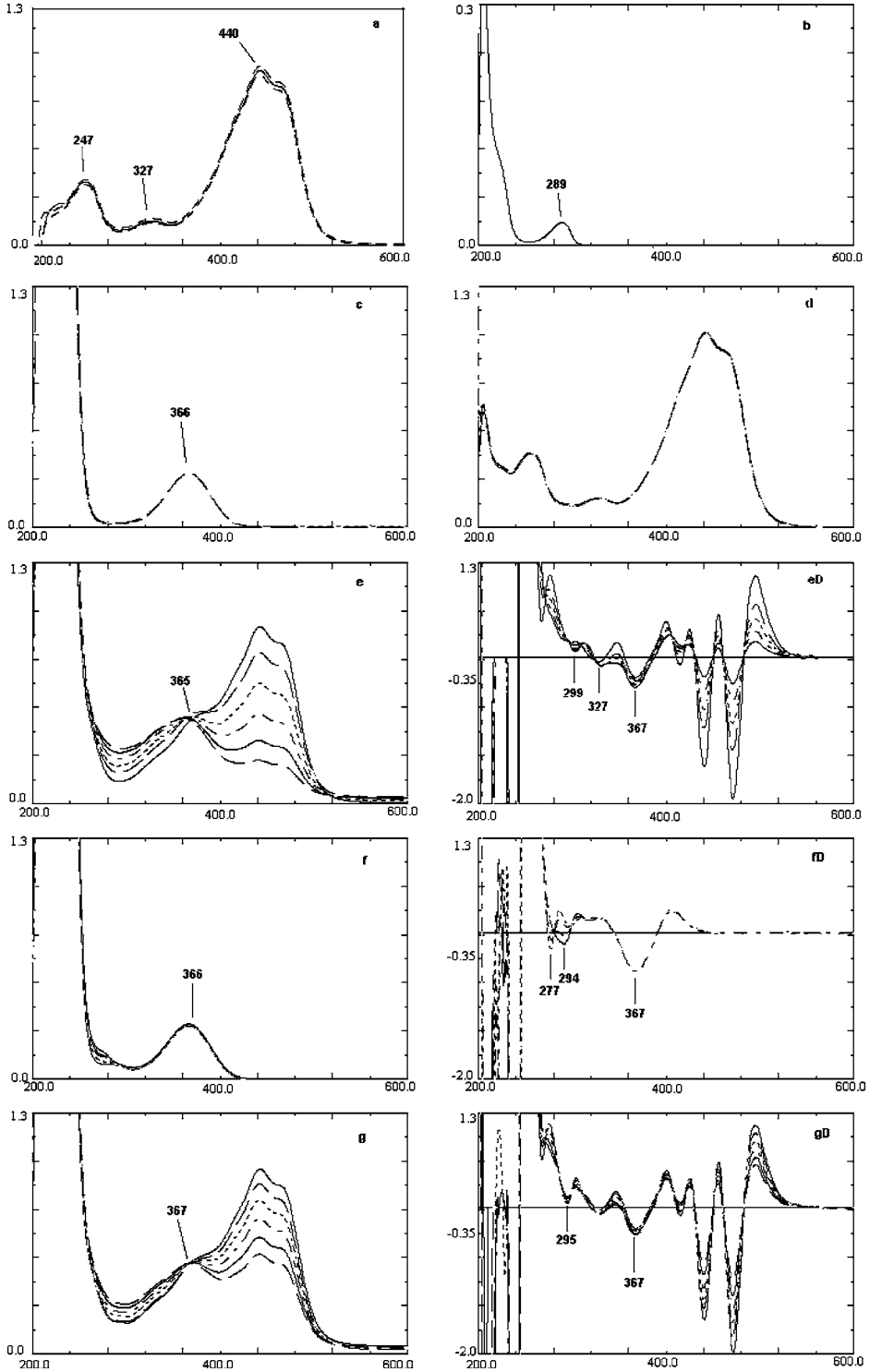

Figure 4. UV–vis spectra of the reaction components (pH 7.4) within 10 min of incubation at 40 °C: (**a**) crocin (10 μM); (**b**) Trolox (10 μM); (**c**) AAPH (12.5 mM); (**d**) crocin and Trolox (1:1 mol/mol); (**e**) crocin and AAPH; (**f**) Trolox and AAPH; (**g**) crocin, Trolox, and AAPH reaction mixture; (**eD**, **fD**, and **gD**) second derivatives of the respective spectra.

Table 4. Trolox Equivalents of *p*-Hydroxybenzoic and Cinnamic Acids, Calculated Using either % Inh or k_{rel} Values

benzoic acid derivatives					cinnamic acid derivatives				
	exptl			lit. data[a]		exptl			lit. data
AH	% Inh ($n = 3$)	Trolox equiv	f[b]	Trolox equiv	AH	% Inh ($n = 3$)	Trolox equiv	f	Trolox equiv
p-hydroxybenzoic	3.4 ± 1.4	0.10 ± 0.05	0.2	0.02	*p*-coumaric	9.2 ± 1.7	0.27 ± 0.03	0.6	0.04
vanillic	15.1 ± 0.6	0.45 ± 0.04	0.9	0.15	ferulic	40.3 ± 3.9	1.23 ± 0.05	2.4	0.90
protocatechuic	40.3 ± 1.2	1.19 ± 0.10	2.4	0.79	caffeic	74.6 ± 2.1	2.20 ± 0.14	4.4	3.97
syringic	22.0 ± 1.1	0.65 ± 0.03	1.3	1.30	sinapic	80.6 ± 0.7	2.38 ± 0.17	4.8	6.03

[a] Based on k_{rel} values published by Natella et al. (*9*). [b] Stoichiometric factor *f*: 2 mol of ROO•/mol of Trolox × Trolox equiv.

% Inh or k_{rel} are more likely to provide information about the reactivity of AHs. Due to the competitive reaction, absolute stoichiometry (*f*), that is, moles of ROO$^{\bullet}$ per mole of AH, is difficult to calculate. Accepting that each molecule of Trolox scavenges 2 mol of ROO$^{\bullet}$ (*44*), *f* values (**Table 4**) were found to be reasonably in line with the number of hydroxyl groups or other active sites of benzoic and cinnamic acid derivatives.

In summary, examination of the CBA performance and in-house validation included probe and test compound characteristics, conditions for peroxyl radical generation, reaction monitoring, and expression of results. HPLC and spectrometric examination showed that any authentic commercial saffron (origin, grade) can be used for probe preparation given that (a) interferences, such as tocopherols, are removed, (b) working solution concentration is adequately adjusted, and (c) stock probe solution changes during storage are not neglected. These findings lead to reduction of analysis cost. Considering the hydrophilic reaction environment, a wide range of antioxidants with lower partition coefficient values than that of Trolox may be tested. To shorten assay time, an alternative monitoring of the reaction involving UV−vis spectra recording was introduced. Calculation of the % Inh parameter allowed a reliable, straightforward estimation of peroxyl radical scavenging activity within a sixth of the time needed so far.

ABBREVIATIONS USED

DPPH, 2,2-diphenyl-1-picrylhydrazyl; ECN, equivalent carbon number; BHA, 2,3-di-*tert*-butyl-4-hydroxyanisole; BHT, 3,5-di-*tert*-butyl-4-hydroxytoluene; ORAC, oxygen radical absorbance capacity; TRAP, total radical-trapping antioxidant parameter; BDE, bond dissociation enthalpy.

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