JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 1455–1461 1455
DOI:10.1021/jf903467x

Novel Semiautomated Method for Assessing in Vitro Cellular Antioxidant Activity Using the Light-Scattering Properties of Human Erythrocytes

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The novel method developed for screening cellular antioxidant activity relies on differences in light-scattering properties (turbidity) between intact and lysed human erythrocytes. AAPH, a peroxyl radical generator, was used to enhance lipid peroxidation. The consequent hemolysis triggered a loss of the light-scattering ability in the lysed erythrocytes. When an antioxidant was added, the area under the absorbance decay curve (AUC) was linearly proportional to the concentration of antioxidant compound. This erythrocyte cellular antioxidant activity (ERYCA) method was found to be relatively fast, sensitive, accurate, and repeatable, even when using erythrocytes from different donors and for different storage times. The method was used to assess the antioxidant capacity of pure phenolic compounds, fruit juices, stimulant beverages, and blood plasma and compared with ORAC values. The values resulting from the two methods did not correlate as the mechanisms involved were different.

KEYWORDS: Erythrocytes; cellular antioxidant activity; ORAC; hemolysis; turbidity

INTRODUCTION

Reactive oxygen species (ROS) are natural byproducts of the human body's metabolism. When present in excess, they can degrade or transform molecules such as lipids, proteins, and enzymes and cause gene mutations, especially in DNA (*1*). Consequently, ROS and free radicals are implicated in many cell disorders and the development of several diseases, including cardiovascular diseases, atherosclerosis, chronic inflammation, and neurodegenerative diseases such as Alzheimer's and Parkinson's diseases (*1*, *2*). In healthy individuals, free radical production is continuously balanced by natural antioxidant defense systems. Nonetheless, this balance can be disrupted by, among other things, aging, disease, smoking, or bad diet, leading to oxidative stress (*1*−*3*). Although organisms have endogenous antioxidant defenses, further antioxidants are provided by the diet, mainly through fruits and vegetables (*2*−*4*).

From the nutritional viewpoint, therefore, the antioxidant potential of phytochemicals, food products, and even biological fluids to check the body's antioxidant status needs to be assessed. Most of the common methods that have emerged recently for assessing antioxidant capacity involve only chemical reactions, often performed under nonphysiological conditions and making their results questionable for predicting antioxidant capacity in vivo (*5*). Alternative biological methods are therefore needed.

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One group—in vitro cellular models—is expected to represent more closely the complexity of biological systems in vivo (*4*−*6*). However, models involving cells are often intricate and difficult to automate, making the development of screening assays difficult. To overcome this challenge, erythrocytes can be used, as they are robust cells and easy to collect, handle, and preserve under refrigeration without antibiotic or chemical preservatives. Hence, the complex and painstaking cell culture step can be eliminated. Additionally, erythrocytes are actively involved in the in vivo transportation and metabolism of antioxidants (*7*) and, so, present a good medium for studying the processes of cell damage induced by oxidizing agents.

The advantages of erythrocytes are that (a) they are living, energy-producing cells that represent the most abundant cell type in blood (*3*, *8*, *9*); (b) they can scavenge reactive oxygen and nitrogen species and play critical roles in antioxidant protection in the blood and body (*3*, *7*−*9*); (c) they do not have nuclei and so cannot perform cell signaling and, consequently, do not contribute to oxidative damage through the mitochondrial production of ROS (*7*); and (d) they carry the most common redox enzymes (*6*). For all of these reasons, human erythrocytes appear to form a convenient basis on which to construct a cellular antioxidant test.

Erythrocytes have been already used for the indirect evaluation of antioxidant capacity by determining the inhibition level of hemolysis caused by peroxyl free radicals (*1*, *2*, *8*−*11*). Erythrocytic membranes are rich in polyunsaturated fatty acids and

Published on Web 01/20/2010

proteins, which are both susceptible to free-radical-mediated peroxidation, leading to membrane damage and consequent release of hemoglobin (*12*−*14*). Consequently, hemolysis has traditionally been assayed by using spectrophotometric readings of the released hemoglobin at 540 nm after centrifugation to remove lysed cells and insoluble fragments. When antioxidant compounds are present, they scavenge peroxyl radicals, converting them to nonreactive species and, thus, inhibiting hemolysis and consequent release of hemoglobin (*8*).

However, because centrifugation is needed, these hemolytic assays, as currently performed, are tedious and time-consuming, thus precluding automation or kinetics studies for screening large numbers of samples. In addition, extra care is needed to ensure that the responses measured are specifically of hemolysis and do not include the subsequent oxidation of hemoglobin that is directly exposed to free (peroxyl) radicals (*15*).

A recent improvement of hemolysis inhibition assays, using erythrocytes, is the so-called "cell-based antioxidant protection in erythrocytes" (CAP-e) test (*6*). In this assay, erythrocytes are incubated with a fluorescent dye precursor and then exposed to peroxyl radicals. The dye precursor penetrates cells and becomes fluorescent when it is subjected to oxidative damage. Thus, the intensity of fluorescence reflects the amount of oxidative damage. This assay does not require centrifugation before the fluorescence readings are taken and can be semiautomated for screening cell antioxidant activity. However, the test is largely qualitative, although some semiquantitative comparisons with antioxidant standards have been performed (*6*). Also, the antioxidant capacity is essentially based on the ability to oxidize the dye precursor while erythrocytes only host the reaction.

This paper introduces a more direct and simpler method that takes advantage of differences in light-scattering properties (turbidity) between intact and lysed erythrocytes. The shape of erythrocytes is homogeneous, with the cells looking like biconcave disks (*16*). When the cells are suspended at low concentrations in physiological solution, they scatter light of wavelengths between 650 and 700 nm (*16*). As hemoglobin does not absorb these wavelengths, a clear signal, representing the concentration of intact cells suspended in the medium, is obtained. When free radicals are added to the medium, polyunsaturated lipids are oxidized, leading to the disorganization of erythrocytic membranes (*14*, *17*). The membranes become damaged, forming hemolytic holes (*18*) that lead to potassium leaking into the extracellular medium (*19*), cell disruption, and the release of hemoglobin (hemolysis). As a result, the light-scattering properties are lost (*20*, *21*).

Hemolysis of erythrocytes suspended at low concentrations in physiological solutions can therefore be followed directly by monitoring turbidity values. Under these conditions, various authors (*20*) have already found good correlation between hemolysis assessed by absorbance readings at 650 or 700 nm and the conventional assay, using absorbance at 540 nm after centrifugation. These findings, however, were used for other purposes.

In this paper, the light-scattering properties of diluted suspensions of erythrocytes in physiological medium are used to assess cellular antioxidant activity of some natural compounds, fruit juices, beverages, and human blood plasma.

MATERIALS AND METHODS

Reagents. The following reagents were obtained from Sigma-Aldrich Corp. (St. Louis, MO): quercetin ($C_{15}H_{10}O_7$, MW 302.24), catechin ($C_{15}H_{14}O_6$, MW 290.3), gallic acid ($C_7H_6O_5$, MW 170.1), tannic acid ($C_{76}H_{52}O_{46}$, MW 1701.2), chlorogenic acid ($C_{16}H_{18}O_9$, MW 354.31), ascorbic acid, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), and reagents for obtaining phosphate-buffered saline solution (PBS). Ellagic acid ($C_{14}H_6O_8$, MW 302.19) was purchased from Fluka Biochemika (Buchs, Switzerland) and 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) from Wako Chemicals USA Inc. (Richmond, VA).

Fruit Juices and Beverages. Juice from tropical highland blackberry (*Rubus adenotrichus* Schlech.) was microfiltered and antioxidant activity performed directly on the clarified juice. Commercial clear juices of apple and grape were also used directly. Pulpy orange juice was obtained by pressing fresh orange fruits and, then, extraction with acetone/water (70:30 v/v) was performed to obtain an extract that was directly used to assess antioxidant activity. Costa Rican coffee (4 g of toasted ground coffee beans) and yellow tea (2 g of dried leaves) were macerated with 400 and 200 mL, respectively, of boiling water for 10 min. An aliquot was then filtered through a syringe filter (0.45 μm) before being used directly for testing.

Blood Plasma. Fasting blood samples were collected from five healthy volunteers who had participated in a previous clinical study (not published yet). Their health was confirmed according to common criteria, using clinical laboratory tests and history questionnaires. Before their blood was collected, all subjects followed a 4-day washout, which consisted of a controlled diet with a low intake of dietary antioxidants. Ethical approval for the study was obtained from the Committee for Scientific Ethics of the University of Costa Rica. All subjects were informed and also signed a written consent before they took part in the study. Venous blood samples were obtained by antecubital venipuncture, with a heparin vacutainer. Blood samples were immediately centrifuged at 12000*g* for 3 min, and the supernatant was stored at −80 °C until analysis.

Erythrocyte Collection. A blood bank provided healthy human blood samples after the shelf life had expired, 30 days after collection. The blood was maintained in storage at 4−6 °C. Only O Rh-positive blood was used, even though blood from other groups had been successfully tested. Whole CPDA-blood was washed five times with PBS solution (150 mM NaCl, 8.1 mM Na_2HPO_4, 1.9 mM NaH_2PO_4, pH 7.4) and centrifuged for 5 min at 1300*g* to remove all residual plasma and leukocytes. The residue was resuspended and centrifuged for 10 min at 1300*g*. The packed erythrocytes were redispersed in PBS solution to obtain a 2% (v/v) suspension, which was then used immediately for the antioxidant activity test.

Erythrocyte Cellular Antioxidant Assay (ERYCA). Aliquots (50 μL) of PBS solution containing suspended erythrocytes were added to the wells of a flat-bottom, transparent, 96-well microplate (Corning Costar 9018, Corning Inc., Corning, NY). The final concentration of erythrocytes in the wells was 0.4% (about $(1.8 \pm 0.2) \times 10^7$ cells/mL), corresponding to an optical density A_0 at 700 nm of around 1 ($A_0 = 1 \pm 0.2$). Only the 60 wells in the center of the plate were used. The outer wells were filled with water to prevent border effects.

All antioxidant standards, fruit juices, plant extracts, or plasma were diluted only in PBS to perform the ERYCA test. Each well received a final volume of 100 μL of PBS solution that contained one of the following: antioxidant standards, juices, extracts, or plasma.

The well plate was then loaded into the rack of an automatic plate spectrophotometer (Synergy HT, BioTek Instruments, Inc., Winooski, VT) and maintained at 37 ± 0.4 °C with gentle shaking 30s. The spectrophotometer was programmed to transfer, at time 0, 100 μL of the AAPH solution (to reach a final concentration of 100 μM) to each well. Hemolysis time was then monitored by recording, every 5 min, decreases in absorbance (A_t) at 700 nm. Before each reading, the equipment was programmed to shake the microplate gently for 15 s. A typical analysis lasted an average of 6 h until absorbance readings were < 10% of initial absorbance and no further changes were registered.

The protective effect of the antioxidant was measured by assessing the area under the absorbance decay curve (AUC) of the sample and comparing it with that of the PBS/AAPH blank, which had not received any antioxidant compound. Quercetin was used as a standard for the calibration curve, which was completed in triplicate. Samples were usually analyzed in sextuplicate. The AUC was calculated, using a Microsoft Excel spreadsheet (Microsoft Corp., Redmond, WA), following eq **1**

$$\mathrm{AUC} = 1 + \frac{A_1}{A_0} + \frac{A_2}{A_0} + \frac{A_3}{A_0} + \frac{A_4}{A_0} + \ldots + \frac{A_t}{A_0} + \ldots + \frac{A_f}{A_0} \qquad (1)$$

where A_0 is the initial absorbance at 0 min, A_t is the absorbance at time t, and A_f is the final absorbance. Free radical action was followed to

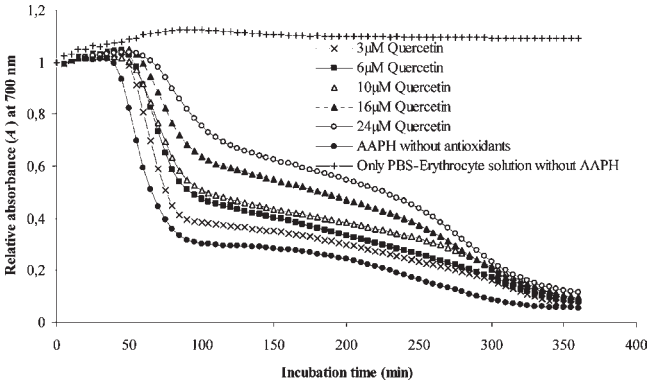

Figure 1. Example of absorbance decay curves of PBS−erythrocyte solution induced by AAPH in the presence of different concentrations of quercetin.

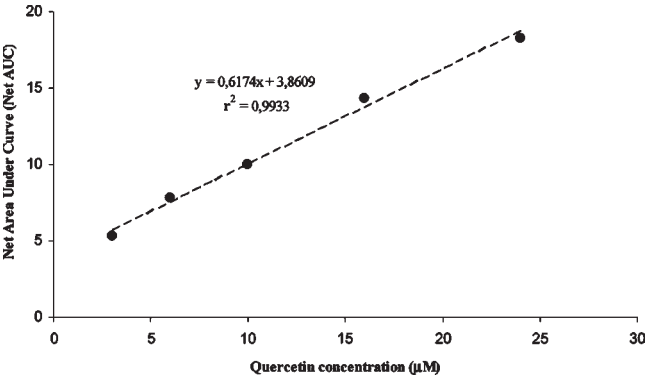

Figure 2. Example of linear relationship between net area under the absorbance decay curve and concentration of quercetin standard.

completion for all solutions, recording the decrease in absorbance from the initial average value of A_0, where $A_0 = 1 \pm 0.2$, until absorbance A_f, where A_f/A_0 was at least < 0.1.

As with the ORAC method, the final ERYCA values were calculated by using a linear regression equation between quercetin concentrations and the net AUC under the absorbance decay curve (net $AUC_{sample} = AUC_{sample} - AUC_{AAPH}$).

$$\text{net AUC}_{\text{quercetin}} = a \times (\text{molarity}_{\text{quercetin}}) + b \quad (2)$$

Quercetin concentrations of 3, 6, 10, 16, and 24 μM were used as calibration standards. Regression coefficients (r^2) of > 0.98 were obtained and the concentration of quercetin equivalents (μmol of quer equiv) was calculated for each net AUC of samples as follows:

$$\mu\text{mol of quer equiv} = (\text{net AUC}_{\text{sample}} - b)/a \quad (3)$$

The ERYCA values were expressed in micromoles of quercetin equivalents per micromole of pure compounds or liters of fruit juice, beverage, or plasma.

ORAC Assay. ORAC assays were performed in 96-well polypropylene plates, as according to the method of Huang et al. (*22*) using the same microplate spectrofluorometer as previously described. The excitation and emission wavelengths were 485 ± 9 and 520 ± 20 nm, respectively. Solutions were prepared with 75 μM phosphate buffer (pH 7.4). Each well was filled with 160 μL of a 78.75 nM fluorescein (FL) solution (63 nM final in the well) and 20 μL of the buffer (blank).The plate was incubated at 37 °C for 15 min before the addition of 20 μL of a 178 μM AAPH solution (17.8 μM final). Fluorescence decay was measured every minute for 60 min. The final values were calculated by using a regression equation between the Trolox concentration and the net area under the FL decay curve. The AUC and the net AUC were calculated as

$$\text{AUC} = 0.5 + f_1/f_0 + f_2/f_0 + f_3/f_0 + \ldots + f_t/f_0 + 0.5(f_{60}/f_0) \quad (4)$$

where f_0 is the initial fluorescence reading at 0 min and f_t is the fluorescence reading at time t. The ORAC values were expressed as micromoles of Trolox equivalents, which were calculated as

$$\text{relative ORAC value} = [(\text{AUC}_{\text{sample}} - \text{AUC}_{\text{blank}})/(\text{AUC}_{\text{Trolox}} - \text{AUC}_{\text{blank}})](\text{molarity of Trolox/molarity of the sample})$$

Statistical Analysis. All statistical analyses were performed using *XL*STAT software (Microsoft Corp.).

RESULTS AND DISCUSSION

Figure 1 presents examples of curves corresponding to the decrease in relative absorbance at 700 nm registered for PBS solutions containing 0.4% (v/v) suspended erythrocytes. These solutions had been incubated with a 100 μM concentration of the peroxyl radical generator (AAPH), either without any antioxidant compounds or with increasing concentrations of quercetin. Absorbance for the PBS−erythrocyte solution without AAPH and antioxidants is also presented, showing stability of erythrocytes throughout the experiment with absorbance values remaining steady.

Table 1. Summary of Quercetin Calibration Curve for Blood of Four Different Donors (A−D) and for Different Days of Storage

blood donor/days of storage	r^2	slope (b)[a]	intercept (a)[a]
A/40	0.9986	0.5489	1.1861
B/32	0.9876	0.7550	5.1905
C/32	0.9186	0.9186	0.4954
C/38	0.9889	0.6329	3.5792
C/40	0.9905	0.6687	0.6322
C/48	0.9914	0.6538	0.5909
C/52	0.9873	0.6438	1.5011
C/58	0.9930	0.7820	2.1579
D/33	0.9909	0.4707	1.8065
av ± 2SD	0.98 ± 0.004	0.67 ± 0.24	2.08 ± 3.18

[a] Y (μM) = $a + bX$ (net AUC).

In contrast, when 100 μM AAPH was added to the medium, absorbance at 700 nm remained almost stable for about 1 h and then decreased sharply, indicating cell disintegration and hemolysis. The free radical generator (AAPH) decomposed at a steady rate in aqueous solutions at physiological temperature (37 °C) for an extended period of time (half-life = 175 h) into alkyl radicals, which convert to peroxyl radicals in the presence of oxygen (*2*, *9*). The 1 h lag time observed, during which erythrocytic membranes resist the constant attack of free radicals, may be the result of action by endogenous antioxidants (*17*). Only after 1 h can alteration of cell membrane be observed. Lipid peroxidation and protein oxidation were indicated by the decrease in the suspended erythrocytes' light-scattering properties, which signified that the number of intact erythrocytes was declining and, hence, the hemolysis level was increasing. **Figure 1** illustrates that, as the concentration of antioxidant compound (in this case, quercetin) increases, the decrease of absorbance is hindered proportionally. The protective effect of the antioxidant compound can be assessed by measuring the AUC and comparing it with that of the blank carrying AAPH only. As for the ORAC method, the net AUC (net $AUC_{sample} = AUC_{sample} - AUC_{AAPH}$) enabled a complete assessment of antioxidant capacity in which the time and degree of inhibition are assessed as the reaction is completed.

The representation of the net AUC (**Figure 2**) versus quercetin concentration shows good linearity, with a high correlation

Table 2. Reproducibility and Accuracy of ERYCA Test for Different Blood Donors and Storage Days

	blood donor B (40 days)		blood donor C (32 days)		blood donor C (48 days)		interexperimental	
catechin	10 μM	18 μM	10 μM	18 μM	10 μM	18 μM	10 μM	18 μM
mean net AUC	5.80	9.48	15.10	23.13	11.62	20.91		
mean concn	10.00	17.98	9.98	18.04	9.35	15.03	9.78	17.02
SD	0.15	0.55	0.65	1.27	0.98	1.76	0.37	1.72
%RSD	1.50	3.07	6.60	7.10	11.80	11.70	0.04	0.10
%REC	100.00	99.89	99.80	100.22	93.50	83.50	97.77	94.54
n	3	3	3	3	6	6	3	3
ellagic acid	10 μM	18 μM	10 μM	18 μM	10 μM	18 μM	10 μM	18 μM
mean net AUC	11.23	17.89	15.04	20.40	12.74	19.13		
mean concn	10.39	17.29	10.76	16.83	10.84	16.48	10.66	16.87
SD	0.67	0.49	0.61	0.23	0.96	2.31	0.24	0.41
%RSD[a]	6.46	2.83	5.70	1.40	8.90	14.00	0.02	0.02
%REC[b]	103.90	96.06	107.60	93.50	108.40	91.56	106.63	93.70
n	3	3	3	3	6	6	3	3
Trolox	24 μM	40 μM	24 μM	40 μM	24 μM	40 μM	24 μM	40 μM
mean net AUC	4.36	6.14	9.44	13.94	11.94	15.58		
mean concn	25.78	37.00	24.46	39.43	26.23	34.42	25.49	36.95
SD	4.07	2.12	1.12	1.72	2.47	1.72	0.92	2.51
%RSD	15.82	5.73	2.20	4.40	9.40	4.99	0.04	0.07
%REC	107.42	92.50	101.92	98.58	109.29	86.05	106.21	92.38
n	3	3	3	3	6	4	3	3

[a] RSD, relative standard deviation (SD/mean concn). [b] REC, percent recuperation (mean concn obtained/initial concn).

Table 3. Ruggedness of ERYCA Values for Trolox Determined Using Blood Type O Rh-Positive from Various Donors (A−D) and Different Days of Blood Storage

blood donor	storage days	ERYCA (μM equivalent quercetin)
A	50	0.48 ± 0.02 (*n* = 6)
B	35	0.45 ± 0.03 (*n* = 6)
B	40	0.41 ± 0.04 (*n* = 3)
C	32	0.41 ± 0.06 (*n* = 3)
C	40	0.53 ± 0.05 (*n* = 6)
C	48	0.56 ± 0.03 (*n* = 6)
C	52	0.41 ± 0.06 (*n* = 6)
C	58	0.43 ± 0.04 (*n* = 6)
D	33	0.46 ± 0.04 (*n* = 6)
av ± SD		0.46 ± 0.06

coefficient (r^2 = 0.993). A good linear relationship (with $r^2 > 0.98$) between net AUC and standard antioxidant concentration (quercetin) was obtained for all of the blood samples collected from different donors over several storage times until 28 days after arrival at our laboratory (i.e., 58 days after blood collection) (**Table 1**). Good linear relationships between net AUC and concentration were also obtained for all other antioxidant standards tested such as catechin, gallic acid, ellagic acid, and Trolox (data not shown).

The effect of increasing concentration of AAPH was tested up to 400 μM, but it was found to have a relatively small effect, slightly diminishing run time and increasing the slope of net AUC versus the standard's concentration. Indeed, higher concentrations of AAPH increased reaction rate and thus reduced accuracy. We found that 100 μM AAPH represented a good equilibrium between run time and accuracy. Average run time was 6 h (360 min) to reach an absorbance of < 10% of the initial value. Under these conditions, the values obtained for three antioxidant standards (catechin, ellagic acid, and Trolox; **Table 2**) were found to be reproducible and accurate, even when using blood from different donors and storing for 18 days in our laboratory (i.e., 48 days after collection). Precision, expressed as the relative standard deviation (%RSD) for all compounds tested, ranged from 1.4 to 16%, even though this value tended to increase with the number of days of blood storage.

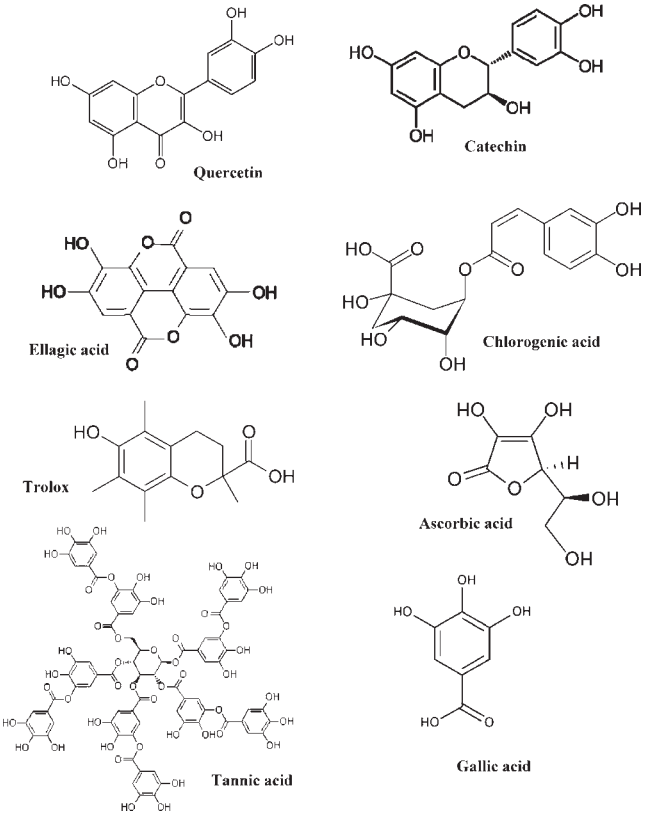

Figure 3. Chemical structures of phenolic compounds studied in this paper.

These values are nonetheless acceptable if they are compared with the ORAC method for which similar errors may result and when the fact that this test is based on a cellular model is taken into account. These findings show not only the stability of erythrocytes in blood medium during storage but also the homogeneous

behavior among red blood cells from different O Rh-positive donors. On the basis of previous results (**Table 2**), the method's accuracy varies from 92.5 to 107% within the individual run and between 94 and 106% between experiments.

To allow comparison with ORAC results, the test's rigor was assessed for Trolox, a hydrosoluble derivative of vitamin E. Blood samples from at least five donors were used after various storage times in our laboratory to a maximum of 28 days (i.e., 58 days after blood collection) (**Table 3**). The reproducibility of the antioxidant value expressed as micromolar quercetin equivalents was achieved with a maximum relative standard deviation (%RSD) of 15% for individual runs; about 15% for values obtained using the same blood sample, but various storage times; and about 12% for all experiments, using blood samples from different donors and different storage times.

Table 4. ERYCA of Pure Antioxidant Natural Compounds (Mean ± SD, $n = 6$)

compound	ORAC[a] (μM TE)	ERYCA[a] (μM TE)	ERYCA[b] (μM Qu)
tannic acid	8.70 ± 0.8	14.20 ± 3.46	6.53 ± 0.58
ellagic acid	1.29 ± 0.10	3.83 ± 0.37	1.76 ± 0.05
quercetin	5.59 ± 0.50	2.17 ± 0.20	1
chlorogenic acid	3.80 ± 0.30	2.13 ± 0.29	0.98 ± 0.10
catechin	7.86 ± 0.20	1.87 ± 0.17	0.84 ± 0.02
Trolox	1	1.00 ± 0.13	0.46 ± 0.06
gallic acid	1.00 ± 0.10	0.84 ± 0.08	0.38 ± 0.02
ascorbic acid	0.85 ± 0.01	0.53 ± 0.06	0.24 ± 0.03

[a] TE, μM Trolox equivalent (for ERYCA value, conversion was based on 1 μM Trolox = 0.46 ± 0.06 μM quercetin equivalent). [b] Micromolar quercetin equivalent.

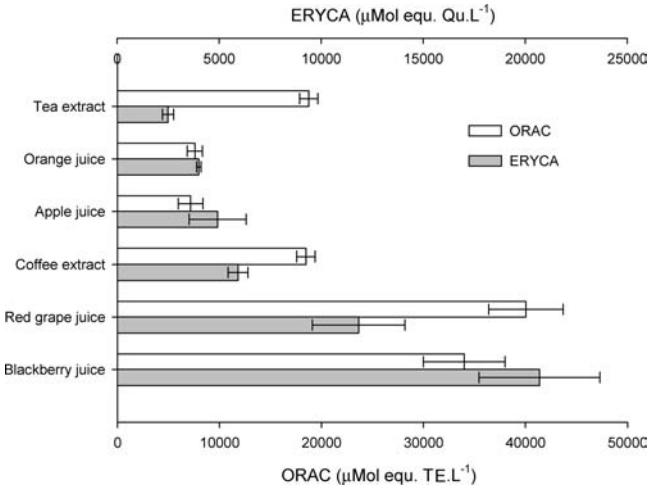

Figure 4. Comparison of ERYCA and ORAC values for different fruit juices and beverages. (Values expressed as micromoles of Trolox (TE) or quercetin (Qu) equivalent per liter ($n = 6$).)

Because it is a non-natural compound, Trolox was not used as a standard in this test. Instead, the standard chosen was the more natural quercetin, which is widespread among fruit and vegetable products and has been detected as occurring naturally in the human bloodstream (*23*). For quercetin, the method's limits of quantification and detection were estimated to be, respectively, 3 and 2 μM quercetin equivalents. The ERYCA method therefore appears to be slightly more sensitive than the ORAC (12.5 and 5 μM Trolox equivalents, respectively (*24*)). Hence, antioxidants can be tested at lower concentrations, which are closer to the physiological concentrations expected for blood plasma (*23*).

The ERYCA assay has therefore been used to assess the antioxidant capacity of pure and natural antioxidant compounds (**Figure 3**). Results were compared with ORAC values (**Table 4**), which were, for standard compounds, similar to those reported previously by other authors (*24*, *25*). Although no correlation was found between the values of the two methods ($r^2 = 0.405$), the ranking was similar for compounds of lower antioxidant capacity (ascorbic acid and gallic acid) and those of higher antioxidant activity (tannic acid).

When the same standard Trolox equivalents were used to compare antioxidant capacity by both methods, values for ellagic acid appeared to be much higher than for Trolox or gallic acid. In contrast, for the ORAC method, the antioxidant capacity of this compound was similar to that of Trolox or gallic acid. Catechin, however, had relatively low ERYCA antioxidant activity, but relatively high antioxidant activity with the ORAC method, being almost comparable to tannic acid.

Nevertheless, comparisons between the two methods are perilous, as different antioxidant mechanisms are addressed, even though the ability of compounds to turn free radicals into nonreactive species within the extracellular solution is assessed by both methods. The ERYCA method probably differs by

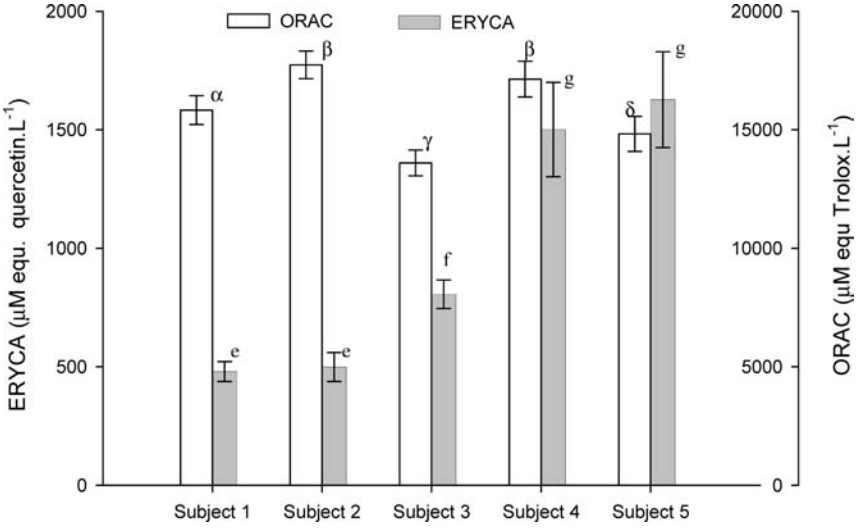

Figure 5. Comparison of ERYCA and ORAC values for fasting blood plasma of five healthy volunteers after controlled washout period low in dietary antioxidant ($n = 6$). (Statistically significant differences using Student's t test between values are marked with different letters, Greek letters for ORAC, Roman letters for ERYCA ($p < 0.05$).)

assessing not only the ability of compounds to quench peroxyl radicals in solution, but also the capacity to protect erythrocytic membranes, whether by specific association with or binding to the membrane or by penetrating the cells and acting from the intracellular space. The observed differences in activities between both methods can be also related to structural dissimilarities of standard compounds. For instance, it has been shown that O-methylated flavonoids such as quercetin exhibit much higher antioxidant capacity than catechin in microsomal lipid peroxidation assays (*26*), whereas antioxidant values of both compounds are similar in ORAC assays (*24*). Therefore, within a cellular-base test, physiologically relevant parameters can influence the antioxidant capacity of compounds, such as lipophilicity and membrane partitioning ability, both afforded by specific chemical structures (*26*).

The ERYCA and ORAC methods were also used to assess the antioxidant capacity of fruit juice and two stimulant beverages (coffee and tea) (**Figure 4**). Statistically, no good correlation between the methods was obtained ($r^2 = 0.576$). Even so, the ranking of antioxidant capacity is similar for both methods, except for tea extract and tropical highland blackberry juice. The juice ranked first in ERYCA and second in ORAC. It is also rich in antioxidant ellagitanins, up to 3.8 g of ellagic acid equiv/kg of fresh fruits (*27*), which can explain its higher ERYCA value, as ellagic acid presents also a higher ERYCA value (table **4**) .

The ERYCA method appears to be more interesting in its potential for the assessment of the antioxidant capacity of biological fluids, especially blood plasma. The method can test the antioxidant status within the same environment as encountered in vivo, without interference from chemical compounds or antibiotics. Hence, the test is biologically more relevant than ORAC because, instead of using intermediary fluorescent compounds, the light-scattering properties of erythrocytes are used directly, as a probe. **Figure 5** shows the ERYCA and ORAC values of blood samples from five healthy volunteers who fasted. Results differed sharply between methods, and no statistical correlation between methods was found ($r^2 = 0.018$).

The ERYCA method appears to discriminate more significantly between blood samples than the ORAC method. The antioxidant value assessed by ERYCA distinguishes three groups: low, intermediate, and high antioxidant capacity. Differences between groups are significant, unlike for ORAC values. Additionally, the average value of antioxidant capacity of blood plasma for the five volunteers assessed by ERYCA is low at 983 ± 582 μmol of quer equiv/L. This is about 2137 μmol of Trolox equiv/L, assuming a conversion value of 0.46 (**Table 3**), that is, about 7 times less than the average ORAC value (15827 ± 1682 μmol of Trolox equiv/L) obtained for the same blood sample.

The ERYCA method is an original attempt to use a cellular model for the automated determination of antioxidant activity. The method is relatively fast (6 h), compared with other cellular models. Because it can be automated, one run can assess at least seven samples with six replicates and calibrate three standard curves. The method was reproducible, whether using erythrocytes from different blood donors or the same blood for up to 58 days of storage under refrigeration. It was more sensitive and accurate than the well-known ORAC method. Additionally, for a cellular model, the ERYCA method is cost-effective, as the cell-culture step is eliminated.

In this first developmental stage of the method, only the global antioxidant activity is addressed. No distinction was made between molecules able to cross or interact with the erythrocytic membranes. Further research on adding a washing step after incubation of the erythrocytes with antioxidant compounds is under way. Such research may allow the specific measurement of the capacity of natural compounds to provide membrane protection by specific surface interaction or by acting from the intracellular space.

ACKNOWLEDGMENT

We thank Paula Valverde Camacho for impressive technical help with the ORAC determinations.

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Received for review October 1, 2009. Revised manuscript received December 15, 2009. Accepted December 22, 2009. This research was partially funded by the PAVUC Project (Producing Added Value from Under-Utilised Tropical Fruit Crops with High Commercial Potential), FP6-INCO project DEV-2, contract 015279, from the European Union.