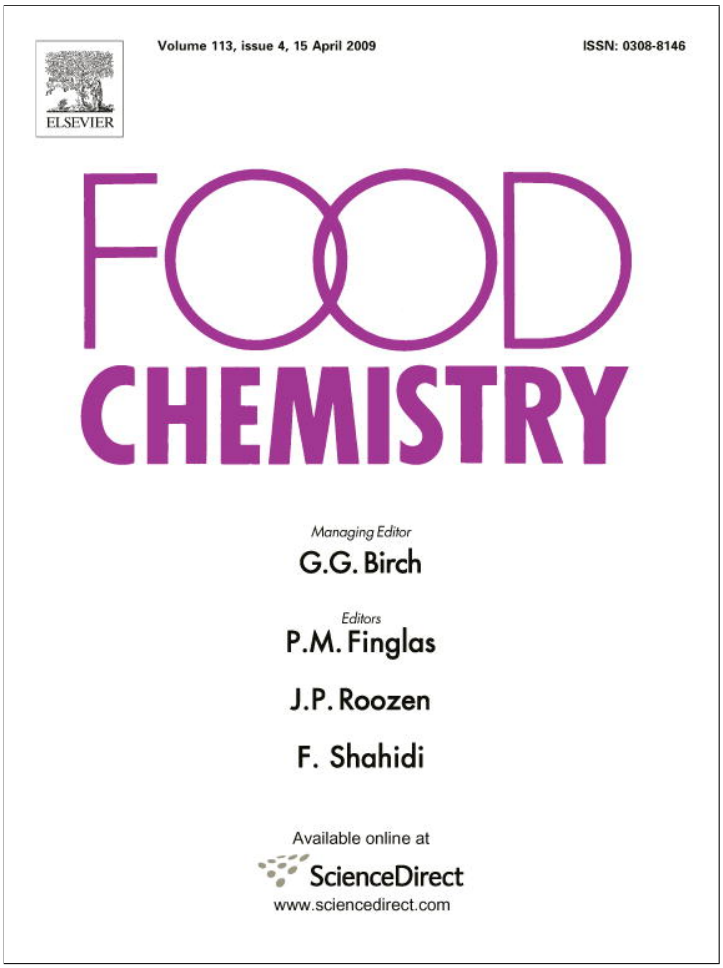

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Analytical Methods

Comparative antioxidant capacities of phenolic compounds measured by various tests

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ABSTRACT

The purpose of this study was to compare the antioxidant capacities of standard compounds (phenolic compounds, ascorbic acid, and glutathione) as measured by various assays. Five methods were selected so as to span a diversity of technical approaches: TEAC (radical 2,2′-azino-bis(3-ethylbenzothiazoline)-6 sulphonic acid), DPPH (radical 2,2-diphenyl-1-picryhydrazyl used to measure reducing capacity), ORAC (oxygen radical scavenging capacity), red blood cell haemolysis (protection of biological sample), and ESR (electron spin resonance for direct free radical evaluation). Most compounds showed significant differences in free radical scavenging activity according to the method used. Of the 25 tested compounds, only a few, such as myricetin and gallocatechin, gave comparable activities in the various tests. To standardise reporting on antioxidant capacity, it is proposed to use a weighted mean of the values obtained using the DPPH, ORAC, resistance to haemolysis, and ESR assays.

This strategy was used to test the antioxidant capacity of several beverages. The highest antioxidant capacity was observed for red wine, followed by green tea, orange juice, grape juice, vegetable juice, and apple juice.

1. Introduction

According to numerous epidemiological studies, the antioxidants from foods appear to be closely related to prevention of several pathologies, including different types of cancer, cardiovascular and neurological diseases, and aging-related disorders (Willet, 2001). Besides vitamins A, C, and E, foods of plant origin also provide our diet with other antioxidants in large amounts: carotenoids and phenolic compounds such as flavonoids (anthocyanins, flavonols, catechins, etc.). Some widely consumed beverages like tea, red wine, and cocoa are also rich in phenolic phytochemicals well known for their high antioxidant activities (Fernandez-Pachon, Villano, Troncoso, & Garcia-Parrilla, 2006; Lee, Kim, Lee, & Lee, 2003). Additionally, such compounds display antiviral and antimicrobial activity and can chelate iron, inhibit enzymes (matrix metalloproteinases), regulate gene expression, and significantly improve endothelial function (Lee et al., 2003).

It is therefore of great interest to assay properly the antioxidant capacity of the foods we consume. We thus need convenient methods for quick, simple quantification of the antioxidant capacity. The methods most commonly used to determine the total antioxidant capacity fall into two major groups: assays based on a single electron transfer reaction, monitored through a change in colour as the oxidant is reduced, and assays based on a hydrogen atom transfer reaction, where the antioxidant and the substrate (probe) compete for free radicals (Huang, Ou, & Prior, 2005).

Electron transfer reaction assays include the Trolox equivalent antioxidant capacity (TEAC) assay, the ferric reducing ability of plasma (FRAP) assay, the copper reduction (CUPRAC) assay, and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity assay. Hydrogen atom transfer reaction assays include the crocin bleaching assay, the total peroxyl radical-trapping antioxidant parameter (TRAP) assay, and the oxygen radical absorbance capacity (ORAC) assay. Other methods not included in these two groups have been developed, such as the total oxidant scavenging capacity (TOSC) assay, the chemiluminescence assay, and the electrochemiluminescence assay (Huang et al., 2005; Prior, Wu, & Schaich, 2005).

Determination of the antioxidant capacity of food should take into account the overall concentrations and compositions of diverse antioxidants, because the total antioxidant capacity is due to the combined activities of diverse antioxidants, including phenolics.

The purpose of this study was to compare the antioxidant capacity values obtained by different methods (TEAC, DPPH, ORAC, red blood cell haemolysis, and ESR) for various standard

Abbreviations: AAPH, 2,2′-azobis-2-methyl-propanimidamide; DPPH, 2,2-diphenyl-1-picrylhydrazyl radical scavenging capacity; ESR, electron spin resonance; ORAC, oxygen radical absorbance capacity; SOD, superoxide dismutase; TEAC, Trolox equivalent antioxidant capacity.

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compounds (mainly phenolics) known as antioxidants. A second goal was to propose a way to standardise reporting on antioxidant capacity. The proposed approach was tested on several beverages (apple, orange, grape, and vegetable juices, ice tea, and red wine).

2. Materials and methods

2.1. Materials

Chemicals: Standards of flavonols and anthocyanins were obtained from Extrasynthese (Genay, France), flavanons, flavan-3-ols, phenolic acids, ascorbic acid, and glutathione were purchased from Sigma Chemical Co. (St. Louis, MO). Trolox [(±) 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid] was obtained from Fluka Chemie GmbH, Switzerland.

Beverages: Apple and orange juices (Minute Maid®, Coca-Cola Enterprises, Belgium), grape juice (N°1, Carrefour), vegetable juice V8 (Cambells Foods Belgium), ice tea green® (Lipton), red wine (Merlot 2004, Vallé des Rappel, Chile).

2.2. Sample preparation

Stock standard solutions (1 mmol/l) in methanol were prepared for each product and appropriate dilutions were done for each type of measurement with the specific solvent of each method.

The various beverages were centrifuged at 17,000*g* for 15 min. The resulting supernatants were used directly as final samples except in the case of wine. From the wine sample, alcohol was removed by evaporation at 38 °C to avoid ethanol interference and the volume was adjusted with distilled water as proposed by Fernandez-Pachon et al. (2006).

2.3. Evaluation of antioxidant capacity

One assay used to determine the antioxidant capacity was the TEAC assay (scavenging of the radical 2,2′-azino-bis(3-ethylbenzothiazoline)-6 sulphonic acid, ABTS) described by Re et al. (1999). The stock solution was prepared by stirring ABTS (7 mmol/l) and potassium persulfate (2.45 mol/l) in water overnight. Before use, this solution was diluted in ethanol to obtain an absorbance of 0.7 at 734 nm. In the assay, 5 µl extract, standard (0–0.1 mmol Trolox), or blank (ethanol) and 1 ml ABTS solution were mixed. The absorbance at 734 nm was determined after 4 min. For each extract, a blank with 1 ml ethanol; instead of the ABTS reagent, was included to correct for any sample absorbance at 734 nm.

Antioxidant capacity was also determined by scavenging of the radical 2,2-diphenyl-1-picryhydrazyl (DPPH) as described by Tadolini, Juliano, Piu, Franconi, and Cabrini (2000). The stock solution was prepared by stirring 75 mg DPPH in 1 l methanol overnight. In the assay, 0.75 ml extract, standard (0–0.1 mmol Trolox), or blank (methanol) and 1.5 ml DPPH solution were mixed. The absorbance of samples, standards, and blanks at 517 nm was determined after 5 min. For each extract, a blank with 1.5 ml methanol, instead of the DPPH reagent, was included to correct for any sample absorbance at 517 nm.

ORAC assays were carried out on a Fluoroskan Ascent FL Thermolabsystems (Finland) plate reader. The temperature of the incubator was set at 37 °C. Procedures were based on the method of Wu et al. (2004). Briefly, AAPH was used as a peroxyl radical generator, Trolox as a standard, and fluorescein as a fluorescent probe. Filters were used to select an excitation wavelength of 485 nm and an emission wavelength of 520 nm. Twenty-five microlitres of diluted sample, blank, or Trolox calibration solution (0–100 µmol) were mixed with 150 µl of 4 µmol fluorescein and incubated for 15 min at 37 °C before injection of 25 µl AAPH solution (173 mmol/l). The fluorescence was measured every 2 min for 4 h. All samples were analysed in duplicate at three different dilutions. The final ORAC values were calculated using the net area under the decay curves.

Red blood cell resistance to oxidative stress (haemolysis) was measured on a reader plate (Labsystems IEMS reader MF). Blood was centrifuged at 3000*g* and 4 °C for 5 min to separate the red cells from the plasma. The cells were washed twice with PBS (pH 7.4) and centrifuged at 3000*g* for 2 min. A 5% suspension of red blood cells was used for the test. In each well of a 96-well plate, 100 µl cell suspension, 160 µl AAPH, and 30 µl sample or PBS (blank) were incubated for 3 h at 37 °C. Various concentrations of Trolox (0.01–1 mmol) were used to obtain a calibration curve. Over the 3-h incubation period, the absorbance at 450 nm was determined every 2 min. Then the time corresponding to 50% haemolysis was determined and compared with the calibration curve (Girodon et al., 1997).

For these four methods, Trolox was used as a standard and the antioxidant capacity was expressed in µmol Trolox equivalent (TE) per mmol standard or per 100 ml beverage.

Superoxide anion ($O_2^{\cdot-}$)-scavenging capacities were measured by electron spin resonance (ESR) spectroscopy. Measurements were performed at room temperature on a JEOL – Jes-FR30 spectrophotometer. A standard superoxide-generating mixture of xanthine and xanthine oxidase (XOD) was prepared in PBS (pH 7.4). The reaction was initiated by addition of XOD and recorded after 2 min. In this system, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) dissolved in NaCl (9 g/l) was used at 44 mmol concentration as a spin-trap. Various final concentrations of SOD (0.25–1.5 U/ml) were added to the system to generate a calibration curve. The mixture finally contained 0.4 mmol xanthine, 2.5 U/ml XOD, 2.2 mmol DMPO, and 50 µl sample, PBS, or SOD in a final volume of 1 ml (Koide, Noda, Liu, Agata, & Kamada, 2000). The results were expressed in units of SOD equivalent (U SODE) per mmol standard or per 100 ml beverage.

The running conditions were as follows: 9.5 GHz frequency, 100 kHz modulation frequency, 4 mW microwave power, 335.6 mT centre field, 2500G Gauss modulation amplitude, 1 s time constant, and 4 min sweep time.

2.4. Total phenolics

Total phenolics were determined by the Folin–Ciocalteu method (Caboni et al., 1997). Although not very specific for phenolics, this protocol gives a good idea of the total phenolic content. Appropriately diluted extract (3.6 ml) was mixed with 0.2 ml Folin–Ciocalteu reagent and 3 min later, 0.8 ml sodium carbonate (20% w/v) was added. The mixture was heated at 100 °C for 1 min. After cooling, the absorbance at 750 nm was measured. Chlorogenic acid (CA) was used as standard, and the results were expressed in mg of CA equivalent (CAE) per mmol standard or per 100 ml beverage.

All samples were prepared in triplicate. Each sample analysis was performed in triplicate. All results presented are means (±SEM) of at least three independent experiments. Statistical analysis (ANOVA with a statistical significance level set at $P < 0.05$ and linear regression) was carried out with Microsoft Excel (Microsoft, Roselle, IL).

3. Results

3.1. Antioxidant capacity of standard compounds

The antioxidant capacities of flavonols, anthocyanins, flavanons, flavan-3-ols, several phenolic acids, ascorbic acid, Trolox, and reduced glutathione were evaluated by the TEAC, DPPH, ORAC, haemolysis, and ESR assays. The results clearly varied according to the assay used.

With the TEAC assay (Fig. 1), kaempferol and rutin showed the same activity as Trolox, whereas quercetin, myricetin, and especially myricetin-3-rhamnoside showed higher activities; kaempferol-3-*O*-glucoside showed lower activity. The tested anthocyanins showed rather similar antioxidant activities, at least twice as high as the Trolox activity. In the flavanon group, the activity of hesperidin was low and that of naringenin was almost zero. Amongst the flavan-3-ols, the activities of gallocatechin and epigallocatechin gallate were higher than that of Trolox (five and two times, respectively). Of the phenolic acids, only gallic acid differed significantly from Trolox, its activity being twice as high. The activity of ascorbic acid was lower than that of Trolox.

In the DPPH assay (Fig. 1), the antioxidant activity of flavonols was similar to that of Trolox used as the reference antioxidant, except for myricetin and myricetin-3-rhamnoside (twice as high) and kaempferol-3-*O*-glucide (no significant activity). The anthocyanins

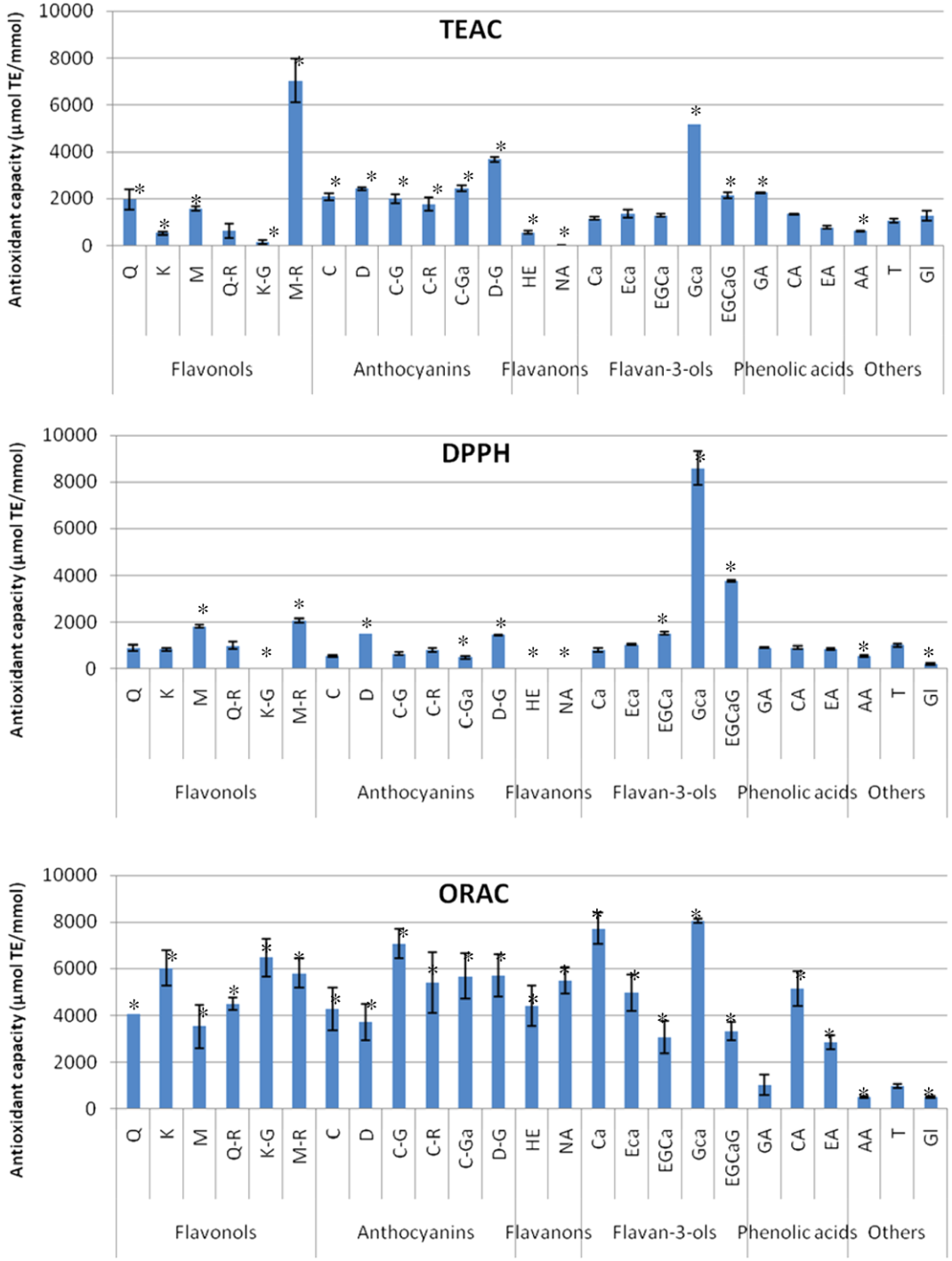

Fig. 1. Antioxidant activities (µmol TE/mmol) of standard compounds (1 mmol) determined by the TEAC, DPPH, and ORAC assays. *indicates a significant difference with respect to Trolox, determined by AVONA ($p < 0.05$). AA, ascorbic acid; C, cyanidin; C-G, cyanidin-3-*O*-glucoside; C-Ga, cyanidin-3-*O*-galactoside; C-R, cyanidin-3-*O*-rutinoside; Ca, catechin; CA, chlorogenic acid; D, delphinidin; D-G, delphinidin-3-*O*-glucoside; EA, ellagic acid; ECa, epicatechin; EGCa, epigallocatechin; EGCaG, epigallocatechin gallate; GA, gallic acid; GCa, gallocatechin; Gl, reduced glutathione; HE, hesperidin; K, kaempferol; K-G, kaempferol-3-*O*-glucoside; M, myricetin; M-R, myricetin-3-rhamnoside; NA, naringenin; Q, quercetin; Q-R, rutin; T, Trolox.

showed a similar range of activities, with delphinidin and delphinidin-3-*O*-glucoside exhibiting higher activity and cyanidin and cyanidin-3-*O*-galactoside lower activity than Trolox. With this assay the variability amongst anthocyanins was greater than with the TEAC assay. The antioxidant activity of the flavanons was near zero. As with the TEAC assay, the activities of some flavan-3-ols (gallocatechin and epigallocatechin gallate) were very high (respectively, four and eight times as high as Trolox). The phenolic acids tested had the same activity as Trolox, whereas ascorbic acid and reduced glutathione displayed lower activity.

In general, the antioxidant activities measured by the ORAC assay (Fig. 1) were considerably higher than that of Trolox (4–6 times for all flavonols, anthocyanins, and flavanons, 3–8 times for the flavan-3-ols, 3–5 times for the phenolic acids). The only exceptions were gallic acid, with similar activity, and ascorbic acid and reduced glutathione, with lower activity. The antioxidant capacities of most tested compounds ranged between 3000 and 8000 μmol TE/mmol, far higher than most of the TEAC and DPPH values. It is noteworthy that kaempferol-*O*-glucoside (flavonol), hesperidin, and naringenin (flavanons), displaying low to no antioxidant activity in the TEAC and DPPH assays, behaved as strong antioxidants in the ORAC assay.

Ten of the compounds (four flavonols, three anthocyanins, one flavanon, and two flavan-3-ols) delayed AAPH-induced haemolysis of red blood cells significantly longer than Trolox (Fig. 2). Of the remaining compounds, kaempferol, ellagic acid, ascorbic acid,

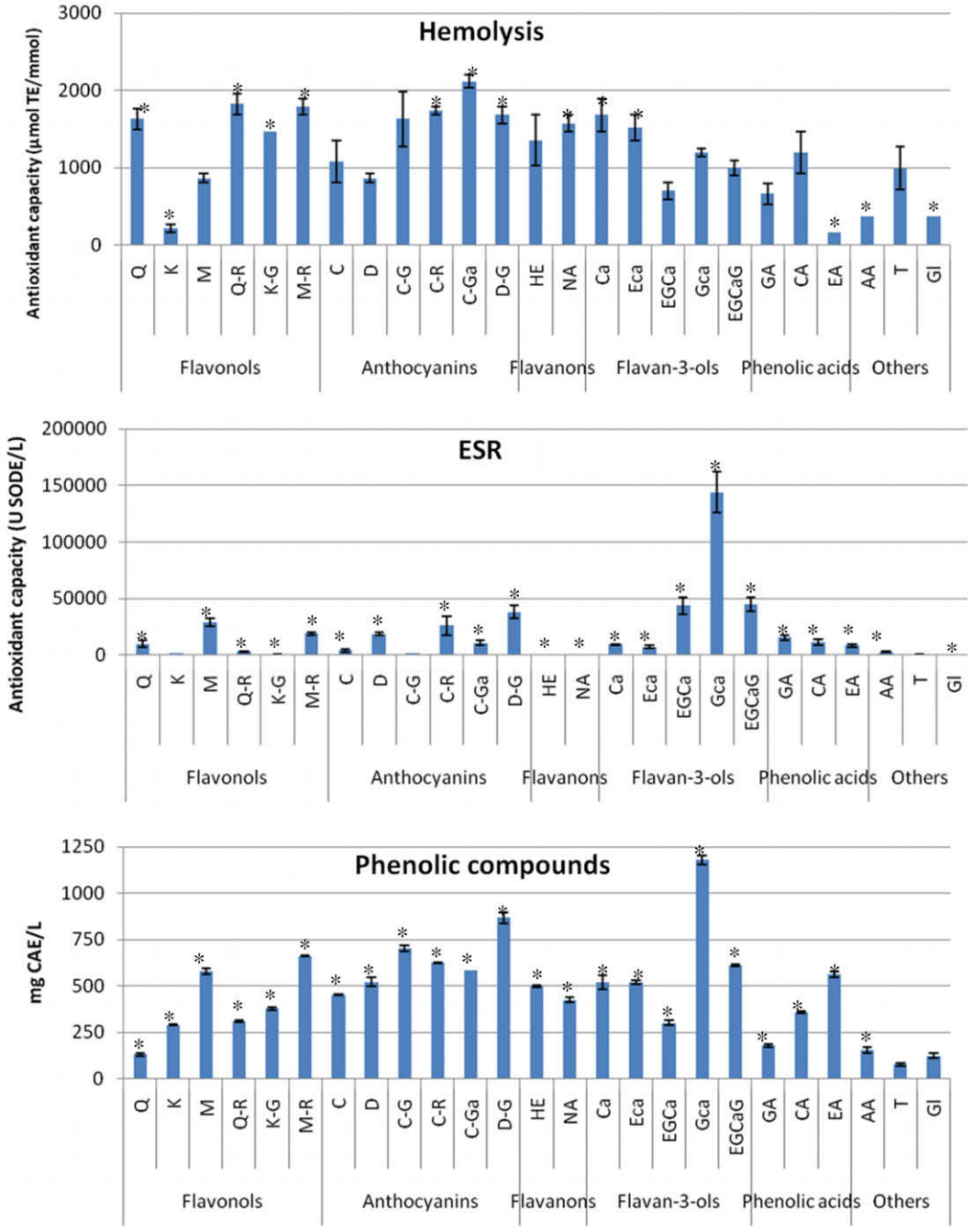

Fig. 2. Antioxidant activities of standard compounds (1 mmol) determined by the haemolysis (μmol TE/mmol) and ESR (U SODE/l) assays and Folin–Ciocalteu measurement of the phenolic content (mg CAE/l). *indicates a significant difference with respect to Trolox, as determined by AVONA ($p < 0.05$). *Abbreviations:* see legend of Fig. 1.

and reduced glutathione showed significantly lower antioxidant activity than Trolox, with myricetin, cyanidin, delphinidin, cyanidin-3-*O*-glucoside, hesperidin, epigallocatechin, gallocatechin, epigallocatechin gallate, and gallic acid, and chlorogenic acid displaying no significant difference with respect to the reference compound.

With the ESR method, Trolox exhibited very low antioxidant activity (Fig. 2), so the data were expressed using SOD (superoxide anion scavenger) as the reference antioxidant. Compounds showing a significantly higher antioxidant capacity than Trolox included the flavanols quercetin, myricetin, and myricetin-3-rhamnoside, and (just barely) rutin, the anthocyanins, the flavan-3-ols, the phenolic acids, and (just barely) ascorbic acid. The highest activities were recorded for the flavan-3-ols gallocatechin, epigallocatechin, and epigallocatechin gallate. Although this assay is quite different from the DPPH assay, it is noteworthy that these two assays yielded quite similar profiles.

All the phenolic compounds tested reacted with Folin–Ciocalteu reagent (Fig. 2). Amongst the flavonols, the intensity of the reaction varied by a factor of 5 from quercetin (which gave the lowest result of all phenolics tested) to myricetin-3-rhamnoside (myricetin also gave a strong reaction). The various anthocyanins also reacted differently but the differences were less pronounced. The flavanons reacted similarly; the results being near the average for all tested compounds. Amongst the flavan-3-ols, gallocatechin reacted about twice as strongly as the other tested compounds. The various phenolic acids also reacted quite differently. Although the non-phenolic compounds also reacted with the Folin–Ciocalteu reagent, the results were lower than those obtained with most phenolics.

3.2. Relative antioxidant capacities of various beverages

Apple, orange, grape, and vegetable juice, ice green tea, and red wine were tested for their antioxidant capacity by the five different methods (Fig. 3). Of all the assays, DPPH clearly emerged as the least sensitive. The TEAC, ORAC, and haemolysis assays generally yielded quite similar results for each beverage, with the exception of grape juice, in which case the ORAC assay gave a much higher result than the TEAC and haemolysis assays. The ESR method compared variably with the other methods according to the beverage studied: with red wine, grape juice, and ice tea the antioxidant activity determined by ESR was very high, but with vegetable, apple, and orange juice it was low.

The measured phenolic compound content was highest in wine and orange juice and lowest in apple and vegetable juice (Fig. 4).

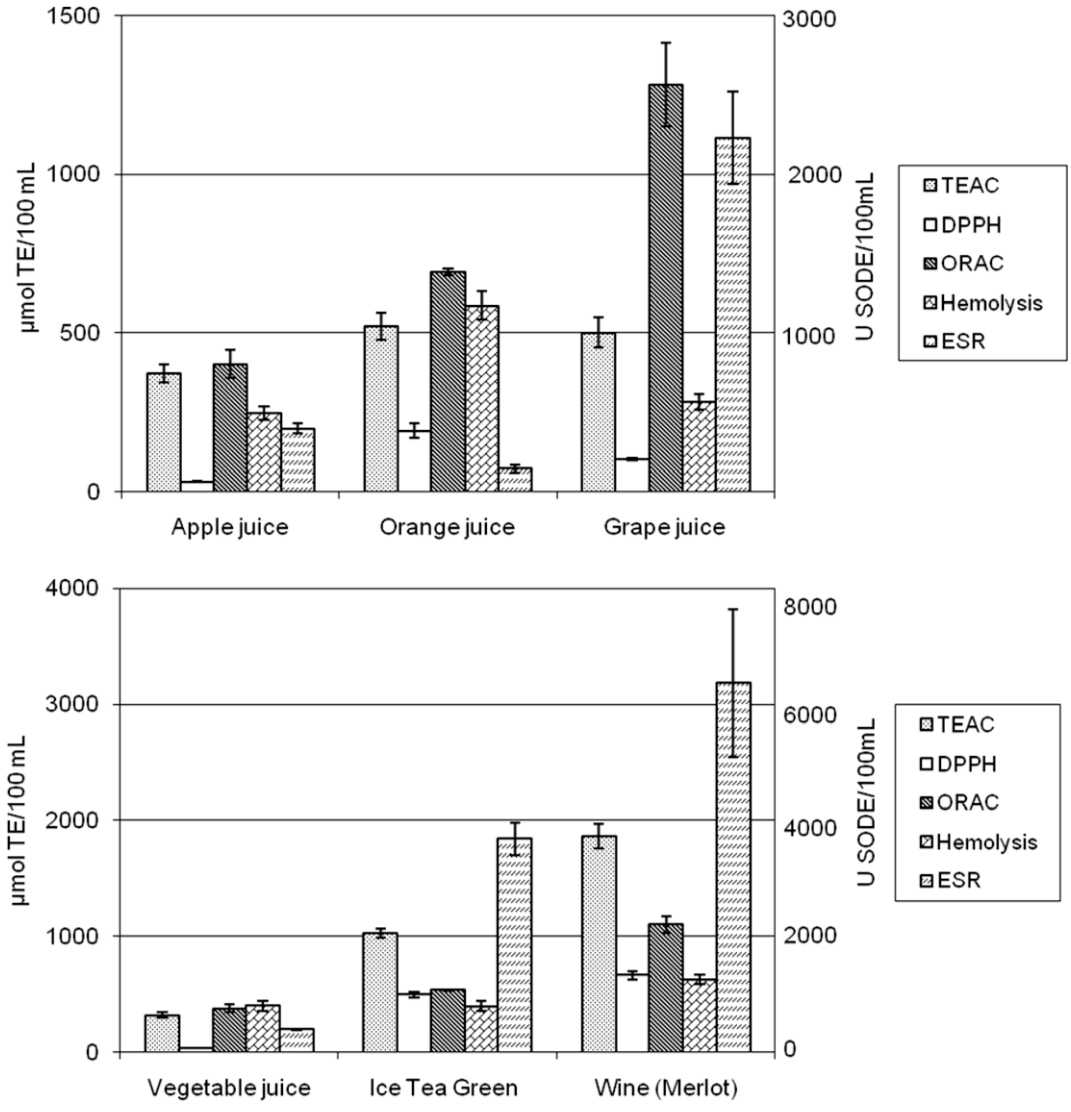

Fig. 3. Evaluation of the antioxidant capacities of various beverages by five methods: TEAC, DPPH, ORAC, haemolysis (µmol TE/100 ml), and ESR (U SODE/100 ml).

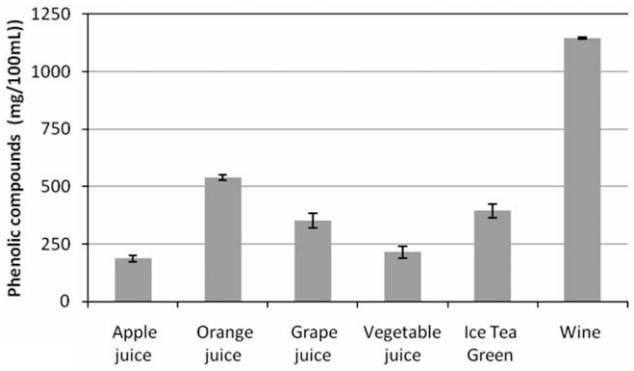

Fig. 4. Phenolic contents of various beverages (mg CAE/100 ml).

4. Discussion

4.1. Antioxidant capacities of standard compounds with the different methods

The antioxidant activities of some phenolic compounds have been determined previously by the TEAC assay under various experimental conditions, with results varying greatly from author to author. Our results additionally highlight the variability of results obtained, whatever the flavonoid family considered, according to the assay method used (see Table 1 for values normalised with respect to Trolox). As generally observed (Kevers et al., 2007), we noted a slight correlation ($r^2 = 0.356$) between the TEAC and DPPH values obtained for the various phenolic compounds, but the DPPH assay generally yielded a value closer to the Trolox activity (lower average, Table 1). TEAC and DPPH are commonly used to assess antioxidant activity *in vitro*. Reduction of the radicals used by a hydrogen-donating antioxidant is monitored through a decrease in optical density. The fact that both reactions have same mechanism explains the correlation between their results (Prior et al., 2005).

Table 1
Antioxidant activities normalised with respect to the Trolox measurement

		ABTS	DPPH	ORAC	Haemolysis	ESR	Weighted average
Flavonols	Q	1.8	0.9	4.2	1.6	16.0	0.9
	K	0.5	0.8	6.2	0.2	2.7	0.5
	M	1.5	1.8	3.6	0.9	47.1	1.1
	Q-R	0.6	1.0	4.6	1.8	5.1	0.9
	K-G	0.2	0.0	6.6	1.5	0.6	0.7
	M-R	6.6	2.0	6.0	1.8	30.9	1.3
Anthocyanins	C	2.0	0.5	4.4	1.1	6.4	0.6
	D	2.3	1.5	3.8	0.9	30.5	0.9
	C-G	1.9	0.6	7.3	1.6	2.6	0.9
	C-R	1.7	0.8	5.5	1.7	42.0	1.1
	C-Ga	2.3	0.5	5.8	2.1	17.4	1.0
	D-G	3.5	1.4	5.9	1.7	61.4	1.4
Flavanons	HE	0.5	0.0	4.5	1.4	0.4	0.5
	NA	0.0	0.0	5.6	1.6	0.2	0.6
Flavan-3-ols	Ca	1.1	0.8	7.9	1.7	15.3	1.1
	ECa	1.3	1.0	5.1	1.5	11.5	0.9
	EGCa	1.2	1.5	3.1	0.7	70.7	1.2
	GCa	4.9	8.5	8.3	1.2	232.7	4.2
	EGCaG	2.0	3.7	3.4	1.0	72.5	1.7
Phenolic acids	GA	2.1	0.9	1.0	0.7	24.9	0.6
	CA	1.3	0.9	5.3	1.2	18.4	0.9
	EA	0.7	0.8	2.9	0.2	13.5	0.5
Others	AA	0.6	0.5	0.5	0.4	4.6	0.2
	GI	1.2	0.2	0.5	0.4	0.3	0.2
Average		1.7	1.3	4.7	1.2	30.3	

The right-hand column shows the weighted average (mmol TE/mmol) obtained by (1) dividing the antioxidant capacity of each compound, as determined by the specified method, by the average capacity determined for the whole set of compounds by the same method (bottom row), (2) summing the four (DPPH, ORAC, haemolysis, and ESR) results of this calculation, and (3) dividing the sum by four. *Abbreviations:* see legend of Fig. 1.

There was no correlation between either the TEAC or the DPPH data and the ORAC data, whatever the class of phenolics considered ($r^2 < 0.1$, Table 1). ORAC data have a different meaning, the results reflecting more than just radical scavenging. In this assay, free radicals from the thermal decomposition of AAPH are generated at a constant rate throughout the test. The ORAC assay is the only method combining both an inhibition time and a degree of inhibition, and it also has the particularity of resulting in a complete reaction. With this test, all the phenolic compounds tested displayed an antioxidant activity more than three times as high as the Trolox activity, whilst ascorbic acid and reduced glutathione showed lower values (as with the TEAC and DPPH assays). For each of these methods, differences between phenolic compounds can be explained largely by the specific structure of each compound (number of OH groups, side chain on benzoic acid, etc.) (Cao, Sofic, & Prior, 1997; Rice-Evans, Miller, & Paganga, 1996; Wang, Cao, & Prior, 1997), rather than by the phenolic family to which it belongs (Paquay, Haenen, Korthouwer, & Bast, 1997).

Electron spin resonance (ESR) spectroscopy determines the presence of unpaired electrons of oxygen, and is commonly used for free radical evaluation. It has been applied to some foods to measure free radical production and to establish their antioxidant capacities (Noda et al., 1997). Phenolic compounds are the most prevalent antioxidant phytochemicals in the plant kingdom and reportedly possess both singlet oxygen quenching activity and radical scavenging activity (Guo, Zhao, Shen, Hou, & Xin, 1999). Their reactivity in this assay was high, far higher than that of Trolox (on the average, 30 times as high), with the exception of flavanons. The ESR and DPPH profiles of this set of compounds were quite similar, a very high correlation ($r^2 = 0.917$) being found between the results obtained by these two methods.

In vitro oxidative haemolysis of human red blood cells is used as a model to study free radical-induced damage of biological membranes and the protective effect of phenolic compounds and known antioxidants. Here, most of the phenolic compounds inhibited oxidative damage as efficiently as, or more efficiently than Trolox (Table 1). Dai, Miao, Zhou, Yang, and Liu (2006) have previously reported that flavonols and their glycosides are effective antioxidants protecting human red blood cells from free radical-induced oxidative haemolysis. In our study, kaempferol was not effective, but as mentioned by these authors, this may be due to a dose-dependency effect (Dai et al., 2006). The protective effect of flavonoids can be linked to their binding to the plasma membrane (Blasa, Candiracci, Accorsi, Piacentini, & Piatti, 2007) and their ability to penetrate lipid bilayers (Lopez-Revuelta, Sanchez-Gallego, Hernandez-Hernandez, Sanchez-Yague, & Llanillo, 2006). The results obtained by this method were different from those obtained with the TEAC, DPPH, and ESR assays ($r^2 < 0.08$). A slight correlation ($r^2 = 0.396$) was found with the ORAC assay. The antioxidant effects of ascorbic acid and reduced glutathione were always very low.

The Folin–Ciocalteu method, generally used to assay the total phenolic compound content also measures the total reducing capacity of a sample. Total phenolics generally correlate with redox and antioxidant capacities measured by the TEAC or DPPH method (Tabart, Kevers, Pincemail, Defraigne, & Dommes, 2006; Tabart et al., 2007) or with ORAC values (Andre et al., 2007). For the standard compounds used here, the correlation with the other methods used was never very good ($r^2 < 0.5$). Dissociation of the phenolic proton leads to a phenolate anion, which is capable of reducing the Folin–Ciocalteu reagent. Yet this method is not specific to phenolic com-

pounds. Many non-phenolic compounds, such as ascorbic acid (Fig. 2) and saccharides present in fruits and vegetables, can reduce the Folin–Ciocalteu reagent (Stratil, Klejdus, & Kuban, 2007). Ethanol, furthermore, can interfere with antioxidant capacity measurement by the ORAC assay (Fernandez-Pachon et al., 2006).

4.2. A standardised antioxidant capacity: weighted average based on four methods

As stressed by Frankel and Meyer (2000) and Huang et al. (2005), no single method is adequate for evaluating the antioxidant capacity of foods, since different methods can yield widely diverging results. Various methods, based on different mechanisms, must be used. Here we have used assays based on four different principles: DPPH or TEAC (reducing capacity), ORAC (peroxyl radical scavenging capacity), haemolysis (protection of a biological sample), and ESR (free radical evaluation). To standardise reports on antioxidant capacity, we propose using an average of the results obtained by the DPPH, ORAC, haemolysis, and ESR methods with Trolox as reference. A simple mathematical mean is not adequate, because two of the four methods (ORAC and ESR) gave much higher values because of the poor performance of Trolox in these assays. This would give those assays undue preponderance in the mean. We therefore propose calculating a global antioxidant capacity as a weighted mean of the results obtained by the DPPH, ORAC, resistance to haemolysis, and ESR assays.

As the antioxidants analysed in this paper are the major antioxidants found in plants, we propose calculating, for each compound, a weighted average where the weighting factor applied to the antioxidant capacity determined by a given method is equal to 1 divided by the average capacity determined for this whole set of compounds by the same method. Results of this calculation are shown in Table 1 (last column). On this basis, gallocatechin appears as the best antioxidant, with a weighted average of 4.2. Some other flavan-3-ols (epigallocatechin and epigallocatechin gallate), anthocyanins (delphinidin-3-*O*-glucoside), and flavonols (myricetin and myricetin-3-rhamnoside) also show a high antioxidant capacity when it is calculated in this way.

4.3. Antioxidant capacity of beverages

As discussed above, assessment of the antioxidant capacity of food matrices requires the parallel use of several methods. Here, we have applied the TEAC, DPPH, ORAC, resistance to haemolysis, and ESP assays to various beverages. Although the DPPH results were almost always lower than the others, the results of the TEAC, ORAC, and haemolysis assays were quite similar in the case of apple, orange, and vegetable juice (Fig. 3). ESR gave very high results (grape juice, ice tea green, and especially wine). Of all the beverages tested, wine showed the best antioxidant capacity regardless of the method used. Phenolic compounds can explain this high antioxidant capacity (Fernandez-Pachon, Villano, Garcia-Parrilla, & Troncoso, 2004; Fernandez-Pachon et al., 2006; Kevers et al., 2007; Mullen, Marks, & Crozier, 2007). By calculating a weighted average of the results obtained in the four assays as described above it was possible to rank the beverages as follows: apple (84 μmol TE/100 ml) < vegetable juice (117 μmol TE/100 ml) < grape juice (176 μmol TE/(100 ml) < orange juice (198 μmol TE/100 ml) < ice green tea (256 μmol TE/100 ml) < wine (402 μmol TE/100 ml). Similar results have been reported for apple, orange, and grape juice on the basis of TRAP and TEAC assays (Pellegrini et al., 2003), but not when the FRAP assay was used. It would be interesting to know the type of compounds responsible for the antioxidant capacity. After specific analysis of different compounds present in red wine, Fernandez-Pachon et al. (2004) showed that 50% of the total radical scavenging activity (TEAC, DPPH assays) was attributable to polymeric phenolic compounds. The remaining activity was mainly attributed to anthocyanins and flavan-3-ols, followed by phenolic acids and flavonols.

Given the complex composition of foods, separating each antioxidant compound and studying it individually is costly and inefficient, notwithstanding the possible synergistic or antagonistic interactions amongst the antioxidant compounds in a food mixture. For instance, gallic acid and epigallocatechin gallate, the major antioxidants in tea, show strong antioxidant activity but may also act as pro-oxidants (Johnson & Loo, 2000).

5. Conclusion

Various methods, based on different mechanisms, must be used in parallel to evaluate the antioxidant capacity of compounds or beverages, since different methods can give very different results. We confirm here that different methods can give widely divergent results. We propose a means of standardising reporting on plant antioxidant capacities by using a weighted mean of the results of four methods based on different principles: DPPH (reducing capacity), ORAC (peroxyl radical scavenging capacity), haemolysis (protection of a biological sample), and ESR (estimation of free radicals). This approach highlighted considerable differences amongst the phenolic compounds, which all showed a greater antioxidant capacity than ascorbic acid. The same approach enabled us to rank various beverages according to their antioxidant activity.

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