The Total Antioxidant Capacity of Foods: a reappraisal.

Application to commercial orange juices.

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Abstract

Since a few years, more and more attention has been specifically given to dietary antioxidants as agents promoting health and preventing the incidence of ~~pathologies~~diseases. As part of these efforts, analytical methods and assays have been developed to measure the antioxidant content in food substances. In this paper, the antioxidant capacity of 17 orange juices is determined by various assays (DPPH, ORAC, heamolysis, xanthine/xanthine oxidase) as the content in ascorbic acid and total phenolics. The results evidence all the complexity to evaluate the *in vitro* antioxidant capacity of foods. In very general terms, in spite of the wide utilization in these tests (FRAP, TAC, ORAC TRAP and others), their significance remains obscure. The discrepancy of the results and the absence of good correlation between the assays clearly highlight all the importance of understanding the strengths and weakness of assays evaluating antioxidant potential of a food at the risk of giving erroneous information to the consumer. It is clear that the use of "total antioxidant capacity" assays for the *in vitro* assessment of antioxidant quality of food does not be employed by food industrials as a marketing argument or for the assessment of the "wholesomeness" of a food.

Introduction

Since a few years, more and more attention has been specifically given to dietary antioxidants as agents promoting health and preventing the incidence of pathologies[1]. As part of these efforts, analytical methods and assays have been developed to measure the antioxidant content in food substances. ~~Globally, t~~The methods generally used to determine the total antioxidant capacity in foods ~~can be divided into two major groups: (a)~~are: 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 radical scavenging capacity (DPPH) assay, based on single electron transfer reaction, ~~and (b)~~ the crocin bleaching assay, the Total peroxyl Radical-trapping Antioxidant Parameter (TRAP) assay and the Oxygen Radical Absorbance Capacity (ORAC) assay, based on hydrogen atom transfer reaction. Additional methods have also been developed like scavenging of superoxide anion generated by the xanthine/xanthine oxidase system[2], red blood cell resistance to oxidative stress (haemolysis)[3] or cellular antioxidant activity (CAA)[4].

Among all these tests, the most publicized assay was the ORAC assay (Oxygen Radical Antioxidant Assay) initially developed two decades ago by scientists belonging to the National Institutes of Health and the U.S. Department of Agriculture (USDA)[5,6]. Briefly, the method measures the ability of compounds to prevent the formation of dichlorofluorescein (DCF) produced by the interaction between dichlorofluorescin and peroxyl radicals generated by the thermal decomposition of 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH). Once the publication of the database for Oxygen Radical Absorbance Capacity (ORAC) of Selected Foods, Release 2 in 2010 by USDA (www.ars.usda.gov/nutrientdata/orac), food companies rapidly began citing ORAC values to market their products' antioxidant benefits. Food marketers so started competing to see who could rack up the highest ORAC score. However, a large number of experimenters rapidly highlighted several weakness of the ORAC assay. In

recent papers of us, we have, as an example, shown how analytical procedures (sample dilution, weight of analyzed sample, extraction conditions (nature of solvents mixture, time and temperature) could greatly influence the ORAC value[7,8].

With respect to these observations, the purpose of the present study was, therefore, to compare the ORAC value with the antioxidant content (vitamin C, total polyphenols) and the antioxidant capacity determined by other methods (DPPH, red blood cell haemolysis and xanthine/xanthine oxidase assay) for various commercial orange juices. A fundamental question was to know if correlation could exist between all these tests and if the determination of the antioxidant capacity of foods has a real biological signification.

Material and methods

The study material was 17 orange juices (pure juices obtained by pressing or concentrate juices diluted to their original volume, no nectar) commercialized in super markets in Belgium. These juices are conditioned in tetra packs, in glass or plastic bottles.

Extraction

For each sample, one ml juice was mixed with 20 ml of extraction solvent: acetone (70%), water (28%), acetic acid (2%) as described by Tabart et al[9] ~~[9]~~. ~~The mixture was shaken for 1 hour at 4°C and centrifuged at 17000 g for 15 min. The supernatant was used for the assays. Each sample was independently extracted in triplicate. This method was used for DPPH, ORAC, ESR and total phenolic measurements. For ESR, 1 ml of the obtained extracts was evaporated and then 1 ml H_2O was added before use.~~

Measurement of the antioxidant capacity

For each orange juice, four *in vitro* antioxidant assays DPPH, ORAC, ESR and hemolysis were performed[10] over two measurement of compounds: ascorbic acid and total phenolic compounds All assays (except ESR) were carried out on a Victor3 (Perkin Elmer) 96-well plate reader.

The **DPPH** assays (absorbance decrease due to the reduction of the radical 2,2-diphenyl-1-picryhydrazyl) were carried out as in Kevers et al[11]. ~~The temperature of the incubator was set at 30°C. A fresh stock solution was prepared daily by stirring 75 mg DPPH in 1 L methanol for 30 min in the dark and warmed to 30°C. In the assay, 0.1 mL extract, standard (50 to 100 µM Trolox), or blank (methanol) and 0.2 mL DPPH solution were mixed. The absorbances at 520 nm of samples, standards, and blanks were determined after exactly 5 min. Ten microliters of 4 mM Trolox solution was added to each well of the microplates to correct for any sample absorbance at 520 nm.~~

For the **ORAC** assays, the procedures were based on the method of Wu et al[12]and modifications were described by Kevers et al[11]~~.~~. ~~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 535 nm. Briefly, daily fresh solutions of fluorescein (3 µM), Trolox (1 mM) and AAPH (221 mM) were incubated for 15 min at 37°C. A mixture containing 30 mL fluorescein and 5 mL AAPH was prepared just before injection of 175 µl into each well of the microplate. Twenty-five microliters of diluted sample, blank, or Trolox calibration solution (50 to 200 µM) was added.~~

For these two methods, Trolox was used as standard and the antioxidant capacity was expressed in mg Trolox equivalent (TE) per 100 ml of juice.

Superoxide anion (O_2^-)-scavenging capacities were measured by electron spin resonance (**ESR**) spectroscopy as previously described by Tabart et al[10]. ~~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,~~

To determine **the resistance of red blood cell to oxidative stress (hemolysis),** blood was centrifuged at 3000g 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 3000g 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 diluted juices (in PBS) or PBS (blank) were incubated for 3 h at 37°C[10]. Various concentrations of ascorbic acid (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. The results were expressed in mmole Ascorbic Acid (AA) equivalent per 100 ml juice. All results presented are means (±SEM) of at least three independent experiments.

Measurement of the content in ascorbic acid and total phenolic compounds

The concentration of **reduced ascorbic acid** was measured by the 2,6-dichloroindophenol (DCIP) method of the Association of Vitamin Chemists[11]. ~~Briefly, each molecule of vitamin C converted a molecule of DCIP into a molecule of $DCIPH_2$, and the conversion was monitored as a decrease in the absorbance at 520 nm. A standard curve was prepared using a series of known ascorbic acid concentrations. 1 mL of diluted juices (20 times in 5% metaphosphoric acid) or ascorbic acid calibration solutions was mixed with 500 µL 10% metaphosphoric acid. 300 µL citrate buffer (pH 4.15) and 300 µL DCIP (0.1 mg/mL) were added to 600 µL of this mixture. The optical density blanching was used. For each sample, the blank value was determined after addition of 60 µL ascorbic acid (1 mg/mL) in the aim to evaluate the interference due to the sample colour.~~ The results were expressed as µg of ascorbic acid (AA) per 100 ml juice.

Total phenolics were determined according to the Folin-Ciocalteu method described by Kevers et al[10]. ~~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~~ acid (CAE) per 100 ml juice.

All results presented are means (±SEM) of at least three independent experiments.

Results and discussion

Consumption of fruit juices has become a dietary concern worldwide. The physicochemical and organoleptical characteristics of fruits are retained in juices produced from them and their intake also should contribute to maintain health. Health benefits of fruit juices are attributed to a large number of compounds with biological activity; ascorbic acid, vitamin E, beta carotene, and phenolic compounds would be excellent antioxidants able to stabilize free radicals.

Oranges and orange juices are highly consumed, thus they may be highlighted as an important source of vitamin C and polyphenolic compounds, more generally of antioxidants in the diet. Orange juice contributes to essential micronutrient and antioxidant intakes in the United States Population[13].

Orange juice is rich in ascorbic acid, vitamin B, fiber, potassium, iron, and antioxidant compounds[14], mainly hydroxycinnamic acids and flavonoids, among which flavanones are predominant. The vitamin C content in orange juices range from 150 to 450 mg/L; one glass of orange juice (200 mL) can deliver about 30-80% of recommended daily intake of vitamin C[15]. Hydroxycinnamic acids occur mainly as esters of ferulic, p-coumaric, sinapic and caffeic acids. Flavanones in oranges occur mainly as glycosides; glycosilation takes place at position 7 either by rutinose or neohesperidose[16]. Among flavanone aglycon, naringenin, hesperetin, eriodictyol and isokuranetin are the most common, but they are present in much smaller quantities than are glycosides[17]. Citrus flavonoids, especially hesperidin have shown a wide range of therapeutical properties such as anti-inflammatory, antihypertensive, diuretic, analgesic and hypolipidemic activities[18]. Papers on hydroxycinnamic acids and flavonone content in commercial orange

juices[16,19,20,21,22] are marginal and differentiated because of differences in varieties of oranges, their ripeness and the technological processes used to obtain commercial juice[17].

For the 17 commercial orange juices, we assayed *in vitro* DPPH, ORAC, red blood cells hemolysis and xanthine oxidase assay as well as total phenolic (TP) content and ascorbic acid (AA) levels (Fig.1). From a biological point of view, the xanthine/xanthine oxidase assay is the most important since it allowed to directly evidencing the scavenging effect of a food matrix on a physiological free radical that is superoxide anion. In the other assays, free radicals were synthetic or generated by pure chemical reactions. On second position, we highlighted the content in antioxidants (total polyphenolics and ascorbic acid) because this gave practical and precise information's about the nutritional quality of a food. A third group consisted of the two most popular assays described in the literature (ORAC and DPPH) and of the resistance of red blood cell to oxidative stress (hemolysis) for evaluating the global antioxidant capacity of a food.

As shown in Fig 1, data arising from the six investigated parameters did not allow adequately discriminating an orange juice from another. For instance, juice 13 exhibits a good antioxidant activity for DPPH while its antioxidant capacities measured by hemolysis or xanthine/xanthine assays are the lowest among all juices. Its ascorbic acid level and total phenolic compounds showed also the best value. Likewise, juice 9 has the highest ORAC value but its antioxidant capacity as assessed by the DPPH test or hemolysis is low. Juices 1 and 6 presented good value for DPPH and hemolysis assays and a good phenolic content; juices 9 and 13 showed a high content in ascorbic acid and phenolics and a good antioxidant capacity measured by ORAC and DPPH respectively. On the contrary, the antioxidant capacity measured by ORAC, xanthine/xanthine oxidase and hemolysis assays were low as the phenolic content for juice 10. Such variations between orange juices were previously observed especially by Stella et al[23]. In these conditions, it is difficult to compare the juices and to find the best juice.

From a general point of view, Table 1 clearly indicates large differences in the correlation coefficients between parameters. The correlation coefficients were used to measure the association between all pairs of variables. Moderate correlations were found between the antioxidant capacity estimated by ORAC or DPPH assays and total phenolics and between the antioxidant capacity estimated by DPPH assay and hemolysis. Surprisingly, the ORAC assay still considered as a reference for evaluating the antioxidant capacity of a food matrix poorly correlated with all the other tests, except total phenolics. Discrepancies may be explained by differences in the type of free radical generated in the test tube, conditions of oxidation, kinetics, temperature and the composition of the each investigated system[24]. At least, the selection of valid reference compounds for relative *in vitro* activity is also a controversial problem. In particular, the common use of Trolox as a reference to evaluate the relative antioxidant activity of compounds such as flavonoids is a matter of debate.

Therefore, evaluating the toal antioxidant capacity in a food matrix such as orange juices on the basis of ORAC value or another antioxidant test remains a challenge and can lead to misinterpretation. In 2005, Huang et al[25] wrote " any claims about the bioactivity of as sample based solely on assays such as ORAC, TEAC and FRAP would be exaggerated, unscientific, and out of context. Moreover, these assays do not measure bioavailability, *in vivo* stability, retention of antioxidants by tissues, and reactivity *in situ*". Face to this large number of criticisms, USDA finally decided to remove its ORAC database from its website in 2012 (http://www.ars.usda.gov/services/docs.htm?docid=15866). ~~explaining that *in vitro* measures of antioxidant capacity are "*routinely abused*" and "*have no relevance to the effects of specific bioactive compounds, including polyphenols on human health*". Consistently, o~~Official agencies (including EFSA) have taken a very strong position against the utilization of these tests to support health claims for food and nutritional supplements.

Conclusions

Our paper evidences all the complexity to evaluate the *in vitro* antioxidant capacity of foods. In very general terms, in spite of the wide utilization in these tests (FRAP, TAC, ORAC TRAP and others), their significance remains obscure. Our paper clearly highlights all the importance of understanding the strengths and weakness of assays evaluating antioxidant potential of a food at the risk of giving erroneous information to the consumer. It is clear that the use of "total antioxidant capacity" assays for the *in vitro* assessment of antioxidant quality of food does not be employed by food industrials as a marketing argument or for the assessment of the "wholesomeness" of a food[26].

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Table1 :

Correlation matrix between the various variables

	DPPH	ORAC	RBC Haemolysis	XO	AA	TP
DPPH	1	0.01	0.37	0.30	0.17	0.34
ORAC		1	-0.07	0.28	0.16	0.49
RBC Haemolysis			1	0.26	-0.44	0.05
XO				1	-0.34	0.12
AA					1	0.55
TP						1

Correlations between the various assays of antioxidant activity and the content in ascorbic acid (AA) and total phenolics (TP). XO : xanthine/xanthine oxidase assay.

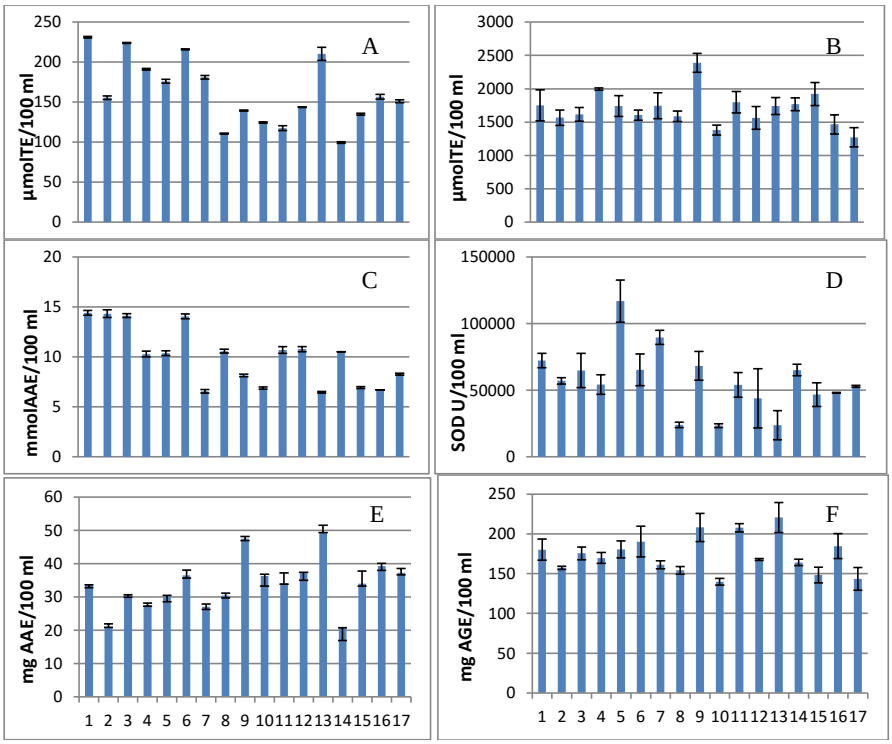

Figure 1: Antioxidant activity measured by various assays: DPPH (A), ORAC (B), red blood cell haemolysis (C), and xanthine/xanthine oxidase (D) and ascorbic acid (E) and total phenolic (F) contents in 17 orange juices. Mean and standard deviation (SD) calculated for each variable. TE=Trolox equivalent; AAE= ascorbic acid equivalent; GAE=gallic acid equivalent; SOD U=unities of superoxide dismutase