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Correlation of tocopherol, tocotrienol, γ-oryzanol and total polyphenol content in rice bran with different antioxidant capacity assays

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Abstract

The relationship between antioxidant capacity and levels of various antioxidants in rice bran and brown rice powder was evaluated. Three different varieties of Venezuelan rice, namely, Cimarrón, Zeta 15 and FONAIAP-1, were studied using ferric reducing antioxidant power (FRAP), 2,2′-azinobis-3-ethylbenzotiazoline-6-sulphonic acid (ABTS), and oxygen radical absorbance capacity (ORAC) to measure antioxidant capacity. The results showed that rice varieties contained different levels and combinations of total polyphenols, γ-oryzanol, α- and γ-tocopherols and α-, γ- and δ-tocotrienols. Compared to brown rice powder, rice bran contained most of the antioxidants and had correspondingly higher values of antioxidant capacity. Principal components analysis and multiple regression on the data indicate that FRAP was sensitive to polyphenols and total tocotrienols, while ORAC was sensitive to polyphenols and total tocopherols. ABTS was the least sensitive of all assays tested. Thus, results from antioxidant capacity assays must be interpreted with caution particularly in complex systems and that further study is necessary to define more precisely the nature of the relevant chemical reactions.

Keywords: Rice bran; Brown rice; Antioxidants; Tocopherol; Tocotrienols; γ-Oryzanol; Polyphenols; ORAC; FRAP; ABTS

1. Introduction

There is a growing interest in natural antioxidants in food by virtue of their potential role in the prevention of oxidative stress-related diseases (Willcox, Ash, & Catignani, 2004; Young & Woodside, 2001). As such, there is a need for an antioxidant capacity assay for complex food systems that would also be indicative of biological efficacy. As a first step in achieving this goal, it is necessary to assess the coherence or inter-consistency of several existing antioxidant capacity assays currently in use with respect to their ability to measure the *in vitro* quenching of free radicals by well-known antioxidants. A major problem with these assays is that the free-radical reaction mechanisms upon which they operate may vary from one system to another (Huang, Ou, & Prior, 2005; Prior, Wu, & Schaich, 2005). Thus, two mechanistically different assays may give conflicting results when used to measure the activity of a given antioxidant in a food system. For example, the ferric reducing anti-oxidant power (FRAP) assay is based on a single-electron transfer mechanism while oxygen radical absorbance capacity (ORAC) assay is based on hydrogen atom transfer (Prior et al., 2005). As such, it is not unusual that the FRAP and ORAC assays may or may not correlate depending on the food system being tested. For example, one group reports that in berries, ORAC and FRAP

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results are correlated (Moyer, Hummer, Finn, Frei, & Wrolstad, 2002), while another group reports that these two assays are divergent when applied to common vegetables (Ou, Huang, Hampsch-Woodill, Flanagan, & Deemer, 2002).

In view of the above-mentioned complexities, it is premature for the food industry to choose any one of the currently available antioxidant assays and use it exclusively to project biological antioxidant efficacy of any particular food product. As a first step in resolving these difficulties, it is necessary to obtain more information on how various mixes and proportions of endogenous antioxidants present in food systems affect the performance of several well-known antioxidant assays. As a model system, we used rice bran which is known to contain high levels of natural antioxidants in different quantities and proportions depending on the variety (Nam, Choi, Kang, Kozukue, & Friedman, 2005; Shin & Godber, 1994; Xu, Hua, & Godber, 2001). The assays used were FRAP, ORAC and 2,2′-azinobis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) (Nilsson et al., 2005; Prior et al., 2003). The principal objective was to test whether these different antioxidant assays would report similar antioxidant capacities regardless of the nature and profile of the endogenous antioxidants. If the results for the different assays were found not to correlate, its second objective was to use statistical methods to identify which of the natural antioxidants are detected by assays employed. It is anticipated that results will be useful towards the eventual design of an antioxidant assay that would be generally applicable to a myriad of food systems.

2. Materials and methods

2.1. Rice samples

Rice varieties Cimarrón, Zeta 15 and FONAIAP-1 were supplied by La Asociacion Venezolana de Productores de Arroz, (ASOPORTUGUESA, Acarigua, Estado Portuguesa, Venezuela). Rice was dehulled using a RIMAC dehuller MTH 35-A (Rice Machinery Supply Corp., Hialeah Gardens, FL). Brown rice powder was prepared by milling using a Fitz Mill Model D (Flitzpatrick Co., Chicago, IL). The powder was passed through an 80-mesh sieve and heat treated at 100 °C for 30 min to inactivate endogenous lipases (Juliano, 1985). Rice bran was obtained from brown rice by polishing for 90 min using a Grainman polisher model 60-115-60-2AT (Douglas International Corp, Coral Gables, FL). The rice bran was heat treated as above. Samples were lyophilised before analysis and were extracted in triplicate as described previously (Chen & Bergman, 2005). Briefly, 100 mg portions of the lyophilised samples were extracted with 6 ml then with 2 ml portions of methanol. The combined extracts were passed through 0.45 μm filters, evaporated to 4 ml, then made up to exactly 5.0 ml in a volumetric flask. The extracts were stored at −80 °C pending analysis.

2.2. Assays of polyphenols, tocopherols, tocotrienols and γ-oryzanol

Total polyphenols were assayed using the Folin–Ciocalteau method as described previously (Georgé, Brat, Alter, & Amiot, 2005). Briefly, Folin–Ciocalteu reagent (Sigma, Oakville, ON, Canada) was diluted with water 1:9 (v/v). To 1.25 ml of this reagent 50 μl sample were added. After 2 min incubation at room temperature, 1 ml sodium carbonate (75 g/L) was added. The mixture was incubated for 15 min at 50 °C and cooled quickly in an ice-water bath. The absorbance at 760 nm was read within 15 min. The readings were compared to a standard curve using gallic acid (Sigma, Oakville, ON, Canada).

Tocopherols, tocotrienols and γ-oryzanol were analyzed by HPLC using a Hewlett Packard (Avondale, PA) HPLC series 1050 equipped with fluorescence and absorbance detectors and a 250 × 4.6 mm Synergi Hydro-RP column (Phenomenex, Torrance, CA) kept at 25 °C. Detection was accomplished by fluorescence (285 nm excitation and 325 nm emission wavelengths) followed in series by absorbance at 330 nm. Mobile phase was CH_3OH/CH_3CN, isocratic at 15:85 (v/v) at a flow rate of 2 ml/min. The HPLC was calibrated using standards of α-, γ- and δ-tocopherols (Sigma, Oakville, ON, Canada) and γ-oryzanol (Gamma-Force, Granby, QC, Canada). Since tocotrienols by law cannot be imported to Canada even for use as scientific standards, the identity of putative tocotrienol peaks (Chen & Bergman, 2005; Cunha, Amaral, Fernandes, & Oliveira, 2006) was confirmed by measuring their fluorescence spectra under stopped-flow conditions during the HPLC analysis. The fluorescence standard curve generated for α-tocopherol was used to estimate the relative quantities of tocotrienols.

2.3. Antioxidant capacity assays

2.3.1. FRAP and ABTS

Both FRAP and ABTS methods for assaying antioxidant capacity were performed as described by Nilsson et al. (2005). The FRAP reagent was prepared fresh daily by mixing 0.1 mM sodium acetate buffer (pH 3.6), 10 mM 2,4,6-tri(2-pyridyl)-*S*-triazine (Sigma, Oakville, ON, Canada) and 20 mM ferric chloride in volume ratios of 10:1:1. Four concentrations of each of the triplicate extracts were added to 900 μl reagent. The absorbance at 593 nm was read after 10 min.

The ABTS radical cation reagent was prepared by stirring 7 mM ABTS (Sigma, Oakville, ON, Canada) in ethanol with potassium persulphate crystals to make a final concentration of 2.42 mM overnight and in the dark at room temperature. The reagent was passed through a 0.45 μm filter disk. Four concentrations of each of the triplicate extracts were added to 1.000 ml ABTS reagent. Absorbance at 734 nm was read after 6 min.

Antioxidant capacity was expressed as Trolox equivalents (μmol/g dry wt), extrapolated from standard curves

of Trolox (Sigma, Oakville, ON Canada) prepared daily for both FRAP and ABTS.

2.3.2. ORAC

ORAC assay was done as previously described (Prior et al., 2003) with the following modifications: Reactions were monitored using a Shimadzu Recording Spectrofluorophotometer Model RF-540 (Shimadzu Corp., Kyoto, Japan) equipped with Shimadzu Data Recorder DR-3 and a circulating water jacket to maintain cell temperature at 37 °C. Reaction mixtures contained 2.4 ml 18.75 μM fluorescein (Sigma, Oakville, ON, Canada) in 75 mM potassium phosphate buffer (pH 7.0), 0.75 ml 317 mM 2,2′-azobis(2-amidino-propane) dihydrochloride (Sigma, Oakville, ON, Canada) and 0.1 ml test solution. The decrease in fluorescence was followed for 40 min and ORAC values were calculated from areas under the curve corrected for blank. Four concentrations of each of the triplicate extracts were analyzed. Antioxidant capacity was expressed as Trolox equivalents (μmol/g dry wt), extrapolated from a standard curve prepared daily.

2.3.3. Miscellaneous chemicals

Solvents were OPTIMA quality from Fisher Scientific (Nepean, Ont., Canada). All other chemicals used were reagent grade, or the highest purity available.

2.4. Statistical analysis

Statistical analyses were performed using routines available in SPSS Release 13.0 (Chicago, IL). Number of replicates are indicated in the presentation of results. In general, extractions were done in triplicate and the determinations on each extract were done in quadruplicate.

3. Results and discussion

Table 1 shows that bran from the three rice varieties have different concentrations of each of the antioxidants measured. Zeta 15 bran has the most γ-oryzanol, whereas FONAIAP-1 bran has the most tocopherols. α-Tocopherol and α-tocotrienol were undetectable in Cimarrón bran, but this variety has significantly ($p < 0.05$, Tukey b) higher γ-tocopherol and δ-tocotrienol than all the other samples. Polyphenol content in all bran samples was not significantly different ($p = 0.07$). The levels of these antioxidants were in the same magnitude as those published previously for other varieties of rice (Chen & Bergman, 2005; Luh, 1980).

Table 1 also shows the antioxidant content of brown rice powder. Brown rice is made up of a starchy endosperm and embryo coated with the layer of bran. As expected, the values of rice brown powder were consistently lower compared with the values for rice bran, a confirmation that micronutrients are located in the bran rather than in the endosperm (Luh, 1980). However, there seems to be some exceptions. In Cimarrón, α-tocopherol and α-tocotrienol were found in brown rice powder but were undetectable in the bran suggesting that in this variety, these antioxidants are associated with layers closer to the endosperm. Furthermore, Table 2 suggests that certain antioxidants are distributed asymmetrically across the bran layers in that polishing results in enrichment of one relative to the other in the bran versus the endosperm fractions. For example, in FONAIAP-1, polishing results in the enrichment in γ- over δ-tocotrienol in the bran relative to brown rice (Table 2). The opposite was true for polyphenol versus γ-oryzanol and total tocotrienols versus total tocopherols in the same variety.

Fig. 1 shows that total antioxidant capacity expressed as Trolox equivalents (μmol/g dry wt) has a complex relationship with antioxidant content. First, it is clear that bran exhibits more antioxidant capacity than brown rice powder as expected from the fact that bran contains more antioxidants per unit weight (Table 1). However, ORAC values are around 4-fold higher than either the corresponding FRAP or ABTS values in both bran and brown rice powder. It has previously been shown that FRAP and ORAC

Table 1
Antioxidant content of rice bran and brown rice powder

Antioxidants	Rice varieties – rice bran			Rice varieties – brown rice powder		
	Cimarrón	Zeta 15	FONAIAP-1	Cimarrón	Zeta 15	FONAIAP-1
Polyphenols (μmol gallic acid eq/g dry wt)	6.11 ± 0.40[a]	6.84 ± 0.23	6.04 ± 0.44	1.16 ± 0.30[a]	1.01 ± 0.04	1.16 ± 0.30
γ-Oryzanol (mg/g dry wt)	1.55 ± 0.10[*]	2.72 ± 0.12[*]	2.12 ± 0.26[*]	0.328 ± 0.013	0.388 ± 0.045	0.201 ± 0.018[*]
Total tocols (μg/g dry wt)	196 ± 8	210 ± 8	219 ± 8	50.8 ± 6.7	65.0 ± 5.3[a]	46.4 ± 4.1
Total tocopherols	41.0 ± 1.9	47.3 ± 2.6	61.3 ± 9.6[*]	13.3 ± 3.0	12.6 ± 2.6	6.70 ± 1.4[*]
α-Tocopherol	nd[b]	26.4 ± 2.7	35.0 ± 8.9	7.83 ± 1.8	8.51 ± 1.5	nd[b]
γ-Tocopherol	41.0 ± 1.9[*]	20.9 ± 0.6[*]	26.3 ± 2.2[*]	5.49 ± 1.5	4.04 ± 1.4	6.70 ± 1.4
Total tocotrienols	155 ± 6	163 ± 10	158 ± 9	37.5 ± 3.8	52.5 ± 3.4[*]	39.7 ± 4.0
α-Tocotrienol	nd[b]	15.9 ± 0.8[*]	24.3 ± 4.2[*]	7.08 ± 7.8	7.18 ± 1.2	3.45 ± 2.0[*]
δ-Tocotrienol	17.0 ± 0.6[*]	7.21 ± 0.36	6.50 ± 0.45	2.08 ± 0.54	2.58 ± 0.42	4.06 ± 0.40[*]
γ-Tocotrienol	138 ± 6	140 ± 9.7	127 ± 5	28.3 ± 0.86[*]	42.7 ± 3.9[*]	36.2 ± 2.0[*]

[a] The data are presented as mean ± SD for 3 samples.
[b] nd = Below the limit of detection at 0.5 μg/g.
[*] Significant differences between means for a given antioxidant either within the rice bran group or within the rice brown powder group ($p < 0.05$ ANOVA, Tukey b).

Table 2
Relative ratios of some antioxidants in rice bran and brown rice powder

	Cimarrón	Zeta 15	FONAIAP-1
Polyphenol/γ-oryzanol			
Rice bran	3.95 ± 0.51[a]	2.52 ± 0.08	2.85 ± 0.19
Brown rice powder	3.51 ± 0.79	2.64 ± 0.35	5.72 ± 1.0[*]
Tocotrienols/tocopherols			
Rice bran	3.78 ± 0.03	3.46 ± 0.41	2.60 ± 0.25
Brown rice powder	2.87 ± 0.41[*]	4.25 ± 0.62	6.11 ± 1.4[*]
γ-Tocotrienol/δ-tocotrienol			
Rice bran	8.11 ± 0.26	19.3 ± 0.71	19.6 ± 1.2
Brown rice powder	14.3 ± 4.2	17.0 ± 4.4	8.95 ± 0.44[*]

[a] Values are mean ± SD ($n = 3$).
[*] Difference between the values for rice bran and brown rice powder ($p < 0.05$, ANOVA, Tukey b).

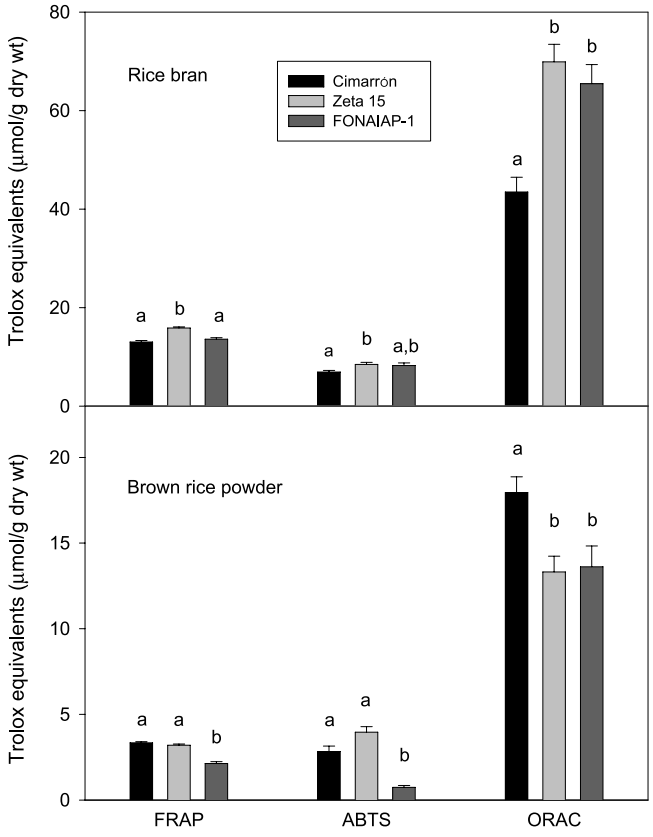

Fig. 1. Antioxidant capacity of rice bran and brown rice powder. Error bars represent SEM ($n = 12$ for rice bran and $n = 8$ for brown rice powder). Different superscripts indicate significant differences between the rice varieties within a specific assay group.

are similar or dissimilar depending on the food system tested (Ou et al., 2002). Consistent with the results here, FRAP and ABTS are more concordant although not necessarily identical (Nilsson et al., 2005). Second, by inspection of the data (Table 1 and Fig. 1), there is no apparent one to one relationship between antioxidant content and antioxidant capacity. For example, in rice bran but not in brown rice powder, FRAP, ABTS and ORAC values rise and fall in parallel with the corresponding γ-oryzanol content. Furthermore, while the high ORAC values in Zeta-15 and FONAIAP-1 bran are consistent with the presence of α-tocopherol and α-tocotrienol in these varieties, they also have paradoxically lower γ-tocopherol and δ-tocotrienol levels. It is interesting that the trends in association between antioxidant levels and antioxidant capacity in brown rice powder are different from those observed in the bran.

In order to analyze further the complex relationship between the levels of endogenous antioxidants and antioxidant capacity, bran data (Table 1) were subjected to principal components analysis and multiple linear regression. Principal components analysis organizes the data into groups that vary independently from each other. Each group is composed of predictor variables that are statistically related to each other. The predictor variables yielded three principal components or groups that accounted for 96% of the total variability (Table 3). Table 3 shows, for example, that polyphenols and γ-oryzanol are colinear

Table 3
Principal components analysis – groupings of predictor Variables[a]

Component 1
Total tocopherols[b]
α-Tocotrienol
α-Tocopherol
Total tocols
Component 2
Polyphenols
γ-Oryzanol
Component 3
Total tocotrienols
γ-Tocotrienol

[a] γ-Tocopherol and δ-tocotrienol span components 1 and 2.
[b] The first predictor variable in each grouping is the most representative of the variation in that respective component.

Table 4
Multiple regression on the principal components of Table 3

	Standardized β coefficient[a]	Significance
FRAP		
Component 1	0.201	0.076
Component 2	0.644	0.001[*]
Component 3	0.402	0.001[*]
ABTS		
Component 1	0.325	0.051
Component 2	0.269	0.103
Component 3	0.008	0.963
ORAC		
Component 1	0.518	0.001[*]
Component 2	0.512	0.001[*]
Component 3	−0.024	0.848

[a] The higher the standardized β coefficient, the greater the influence of the component on the dependent variable.
[*] Statistical significance.

meaning that they vary in parallel and together act as an independent variable. The three components were used as independent variables in multiple regression analysis against FRAP, ABTS and ORAC as dependent variables. The results (Table 4) show that both FRAP and ORAC responded to variations in polyphenols and γ-oryzanol combined. FRAP, however, also responded to variations in total tocotrienols. γ-Tocotrienol was colinear with total tocotrienols (Table 3) most likely because it is the major component in this group of antioxidants (Table 1). In contrast, ORAC did not respond to component 3, but was sensitive to variations in component 1 most represented by total tocopherols (Tables 3 and 4). ABTS did not respond significantly to any of the components, although component 1 came close to statistical significance.

Principal component analysis and multiple regression on the principal components reveal the complexity of the relationships between antioxidants and antioxidant capacity measured by three different methods. The complexity arises from several fronts. First, FRAP, ORAC and ABTS do not necessarily measure the same antioxidant phenomenon (Huang et al., 2005). For example, FRAP is sensitive to single electron transfers while ORAC, to hydrogen atom transfer. ABTS is thought to be sensitive to both reactions. In effect, the three methods operate on different reaction mechanisms, accounting for the observation that they are not mutually equivalent. Second, bran contains several types of antioxidants and in varying proportions. The effects of the antioxidants are not necessarily additive, given the fact that they most likely use different reaction mechanisms with respect to the three assay methods used (Prior et al., 2005). The results of principal component analysis (Table 3) support this hypothesis in that while it is possible to group the predictors into three independent variables, there are some antioxidants that span the components, i.e., they contribute to the variability of at least two principal components. This is true for γ-tocopherol and δ-tocotrienol. This is also true for component 3 (Table 3), where total tocotrienols also interact with components 1 and 2 by virtue of the presence of α- and δ-tocotrienol in those groupings. Thus, further experiments must be done to explore synergistic effects and interactions between the various antioxidants that give rise to a net antioxidant capacity in any given food substance.

In conclusion, it was demonstrated that the FRAP, ABTS and ORAC methods for measuring antioxidant capacity are not equivalent by virtue of their varying sensitivities to different antioxidants found in rice bran and rice bran powder. The effects of different antioxidants did not appear to be additive, but were most likely governed by interactions that need to be explored further. The complexity of the relationships between combinations of antioxidants and various methods of antioxidant capacity assays in a food system such as rice bran bring into question the interpretation of data originating from more complex conditions such as antioxidant activity in intact living organisms.

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