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Rapid Tests to Assess the Antioxidant Activity of *Phaseolus vulgaris* L. Dry Beans

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The antiradical activity of dry beans was evaluated in order to assess the validity of this test and to correlate the results with those achieved with the Folin−Ciocalteu method and with a rapid spectrophotometric method for the analysis of total flavonoids. Four landraces (12 samples) of common beans (*Phaseolus vulgaris* L.), collected in two regions of Italy (Tuscany and Basilicata) in three different years, were analyzed. The EC_{50} values ranged from 39 to 2810 mg sample/mg 1,1-diphenyl-2-picrylhydrazil radical. The phenolic content of each sample was expressed as gallic acid equivalents; it changed from 1.17 to 4.40 mg/g. The flavonoid content, expressed as mg of (+)-catechin per g of dry seeds, ranged from 0.24 to 1.43 mg/g. The qualiquantitative composition of polyphenols has been also elucidated by means of high-performance liquid chromatography (HPLC)−diode array detection and HPLC/MS. These investigations showed that rapid tests can contribute to assessing the quality of functional food.

KEYWORDS: Antiradical activity; DPPH test; total phenolics; total flavonoids

INTRODUCTION

Beans are largely consumed in view of their high protein content, and recent studies have pointed out that they supply the diet with, in addition to complex carbohydrates, soluble fibers, essential vitamins, and metals, as well as polyphenols (*1*, *2*) such as flavonoids, isoflavones, and lignans (*3*).

In the past few years, the antioxidant properties of food have been studied since reactive oxygen species are widely believed to be involved in many diseases such as cancer, diabetes, autoimmune conditions, various respiratory diseases, eye diseases, and schizophrenia (*4*). Diets rich in fruits and vegetables are associated with a reduced risk for these pathologies (*5*). The action of different antioxidants continues to be discussed (*6*) since it seems that the antioxidant activity of a pool of compounds may be greater than that of the single compounds.

Natural polyphenols exert their beneficial health effects mainly thanks to their antioxidant activity (*7*); these compounds are capable of removing free radicals, chelate metal catalysts, activate antioxidant enzymes, reduce α-tocopherol radicals, and inhibit oxidases (*8*). The antioxidant properties of phenolic compounds (*9*) have been determined with different methods, which are based on the above-mentioned different actions.

It has been shown that different parts of dry beans (hull and different fraction extracts) exhibit antioxidant activities (*10*), and because these beneficial effects may improve the value of functional food, it would be beneficial to have rapid methods to assess the antioxidant activity of beans, especially with regard to their antiradical activity.

The objectives of this investigation were to set up and interpret the results of rapid methods, which are generally used for the determination of antioxidant activity and to compare the results achieved with those relative to total phenolics and total flavonoids. The tested beans were typical of two Italian regions (Tuscany and Basilicata).

MATERIALS AND METHODS

Plant Materials. Beans (*Phaseolus vulgaris* L.) were collected in three different fields in 3 years. The fields were located in Pratomagno (Tuscany), Valle d'Agri (Basilicata), and Senise (Basilicata).

The samples analyzed were as follows: landrace Poverella (two samples, PVPM00 and PVPM01 grown in Pratomagno in 2000 and 2001); landrace Verdolino (three samples, VDVA01, VDVA02, and VDSE01, grown in Valle d'Agri and Senise and collected in 2001 and 2002); landrace O'Marrozzo (three samples, OMPM01 grown in Pratomagno and collected in 2001, OMVA01 and OMVA02 grown in Valle d'Agri and collected in 2001 and 2002); landrace Zolfino (three samples, ZOPM00 grown in Pratomagno and collected in 2000, ZOVA01 and ZOVA02 grown in Valle d'Agri and collected in 2001 and 2002).

A 1 g sample of ground dry seeds was extracted with 90 mL of 70% ethanol and adjusted to pH 2.0 with formic acid; it was left for one night at room temperature. The extracts were defatted with 3 × 30 mL of petroleum ether. The defatted extracts were evaporated to

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dryness under vacuum at room temperature and then redissolved in EtOH/H_2O (70:30) adjusted to pH 2.0 with formic acid, to a final volume of 2 mL. All data are mean values of three determinations.

Standards. Authentic standards of (+)-catechin, gallic acid, quercetin, quercitrin, kaempferol, and ascorbic acid were purchased from Extrasynthèse S. A. (Lyon, France), and DPPH• (1,1-diphenyl-2-picrylhydrazil radical) and the Folin−Ciocalteu reagent were purchased from Fluka (Switzerland).

Antioxidant Activity. Free radical scavenging activity was evaluated with the DPPH• assay. The antiradical capacity of the sample extracts was estimated according to the procedure reported by Brand-Williams et al. (*11*) and slightly modified.

Two milliliters of the sample solution, suitably diluted with ethanol, was added to 2 mL of an ethanolic solution of DPPH• (0.025 g/100 mL), and the mixture was allowed to stand. After 20 min, the absorption was measured at 517 nm (LAMBDA 25, Perkin-Elmer spectrophotometer) vs ethanol, as a blank. Each day, a calibration curve of DPPH• was carried out. The antioxidant activity is expressed as EC_{50}, the antioxidant dose required to cause a 50% inhibition (*12*). EC_{50} was calculated plotting the ratio:

$$[\text{DPPH}^\bullet \text{ concentration at } t = 20']/[\text{ DPPH}^\bullet \text{ concentration at } t = 0]$$

against the concentration of the antioxidant. EC_{50} is expressed as mg antioxidant/mg DPPH•.

Total Phenolic Content. The total phenolic content was determined using the Folin−Ciocalteu method, described by Singleton et al. (*13*) and slightly modified according to Dewanto et al. (*14*). To 125 μL of the suitably diluted sample extract, 0.5 mL of deionized water and 125 μL of the Folin−Ciocalteu reagent were added. The mixture was allowed to stand for 6 min, and then, 1.25 mL of a 7% aqueous Na_2CO_3 solution was added. The final volume was adjusted to 3 mL. The mixture was allowed to stand for 90 min, and the absorption was measured at 760 nm against water as a blank. The amount of total phenolics is expressed as gallic acid equivalents (GAE, mg gallic acid/g sample) through the calibration curve of gallic acid. The calibration curve range was 20−500 μg/mL ($R^2 = 0.9969$).

Total Flavonoid Content. The total flavonoid content was determined using a colorimetric method described by Dewanto et al. (*14*) and slightly modified in our laboratory. To 0.25 mL of the suitably diluted sample, 75 μL of a 5% $NaNO_2$ solution, 0.150 mL of a freshly prepared 10% $AlCl_3$ solution, and 0.5 mL of 1 M NaOH solution were added. The final volume was adjusted to 2.5 mL with deionized water. The mixture was allowed to stand for 5 min, and the absorption was measured at 510 nm against the same mixture, without the sample, as a blank. The amount of total flavonoids is expressed as (+)-catechin equivalents [CE, mg (+)-catechin/g sample] through the calibration curve of (+)-catechin. The calibration curve range was 10−500 μg/mL ($R^2 = 0.9946$).

Total Condensed Tannins. The analysis of condensed tannins (procyanidins) was carried out according to the method of Broadhurst and Jones (*15*) and slightly modified in our laboratory. To 50 μL of the suitably diluted sample, 3 mL of a 4% methanol vanillin solution and 1.5 mL of concentrated hydrochloric acid were added. The mixture was allowed to stand for 15 min, and the absorption was measured at 500 nm against methanol as a blank. The amount of total condensed tannins is expressed as CE [mg (+)-catechin/g sample]. The calibration curve range was 50−600 μg/mL ($R^2 = 0.9978$).

High-Performance Liquid Chromatography (HPLC)−Diode Array Detection (DAD) and HPLC/MS. Analyses of flavonols and phenolic acids were carried out using a HP 1100L liquid chromatograph equipped with a DAD detector and managed by a HP 9000 workstation (Agilent Technologies, Palo Alto, CA). Flavonols and phenolic acids were separated by using a 150 mm × 3.9 mm i.d. 4 mm Nova-Pak C18 column (Waters) operating at 26 °C, according to the method of Romani et al. (*15*). UV/vis spectra were recorded in the 190−600 nm range, and the chromatograms were acquired at 260, 280, 305, 330, and 350 nm.

HPLC/MS analyses were performed using a HP 1100L liquid chromatograph linked to a HP 1100 MSD mass spectrometer with an API/electrospray interface (Agilent Technologies). The mass spectrometer operating conditions were as follows: gas temperature, 350 °C; nitrogen flow rate, 10.0 L/min; nebulizer pressure, 40 psi; quadrupole temperature, 40 °C; and capillary voltage, 3500 V. The mass spectrometer was operated in positive and negative modes at 80−120 eV.

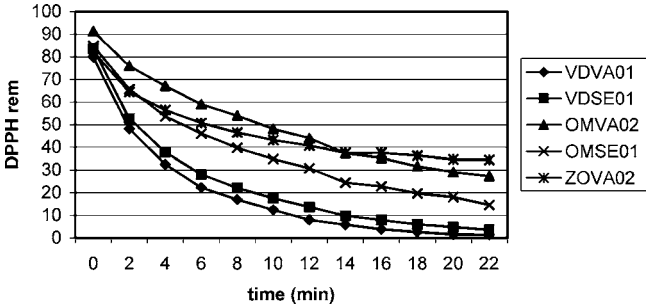

Figure 1. Kinetic behavior of three landraces. The concentration of the antioxidant = 10 mg.

The identity of polyphenols was ascertained using data from HPLC/DAD and HPLC/MS analyses by comparison and combination of their retention times, UV/vis, and mass spectra with those of authentic standards. Quantification of individual polyphenolic compounds was directly performed by HPLC/DAD using a five-point regression curve ($R^2 \geq 0.998$) in the range of 0−30 mg on the basis of authentic standards; kaempferol and quercetin derivatives were determined using kaempferol 3-*O*-glucoside and quercetin 3-*O*-rutinoside as reference compounds; quantification of hydroxycinnamic derivatives was effected on the basis of caffeic acid as a reference standard.

RESULTS AND DISCUSSION

The data obtained from the DPPH• test can be considered from two possible points of view: one being correlated to the kinetics of the reaction between the antioxidant and the stable DPPH• radical and the other to the antioxidant efficiency of the sample. With regards the first point, **Figure 1** reports some curves for three different cultivars accounting for the time used to measure the absorption of the remaining DPPH• solution (20 min).

The kinetic behavior is described by a general equation (*11*) of the type

$$\ln [\text{DPPH}^\bullet \text{ rem}] = b\,t + a$$

The data of all samples fit this equation ($R^2 \geq 0.95$), and the slopes are correlated to the antioxidant activity (the greater the slope, the higher the antioxidant activity at a given concentration). However, this method cannot be used easily since the samples exhibit different antioxidant properties and it is difficult to evaluate the antioxidant activity of all compounds at the same concentration. For this reason, we compared the samples through the EC_{50} parameter, which is independent of sample concentration.

Table 1 lists the EC_{50} values, total phenolics according to the Folin−Ciocalteu method expressed as GAE, and total flavonoids expressed as CE. EC_{50} indicates the amount of beans, expressed in mg, necessary to reduce the activity of 1 mg of DPPH• by one-half; the lower the EC_{50} value, the higher the antioxidant activity of the sample. With this method, the EC_{50} values (mg of pure standard/mg DPPH) for kaempferol, quercetin, and quercitrin are 0.44 ($R^2 = 0.9999$), 0.20 ($R^2 = 0.9971$), and 0.49 ($R^2 = 0.9898$), respectively; for ascorbic acid, the EC_{50} value is 0.21 ($R^2 = 0.9945$).

EC_{50} data range from 39 to 2810. This very wide range, with respect to the variation of total phenolics and total flavonoids, indicates that this parameter is more greatly affected by the landrace and environmental conditions than the other two

Table 1. EC_{50} Expressed as mg Sample/mg DPPH•, R^2 Values within Brackets; GAE Expressed as mg Gallic Acid/g Sample, Standard Deviation within Brackets; CE Expressed as mg (+)-Catechin/g Sample, Standard Deviation within Brackets

sample	EC_{50}	GAE	CE	GAE/CE
PVPM00	2810 (0.9474)	1.17 (0.035)	0.30 (0.015)	3.9
PVPM01	2365 (0.9863)	1,29 (0.0084)	0.22 (0.003)	5.8
VDVA01	39 (0.9806)	3,90 (0.24)	0.64 (0.046)	6.09
VDVA02	82 (0.9684)	4.00 (0.39)	0.58 (0.036)	6.89
VDSE01	50 (0.9568)	3.71 (0.089)	0.60 (0.034)	6.18
OMPM01	96 (0.9517)	4.40 (0.15)	1.31 (0.082)	3.36
OMVA01	112 (0.9978)	4.08 (0.014)	1.43 (0.104)	2.85
OMVA02	135 (0.9899)	4.27 (0.18)	1.03 (0.056)	4.14
OMSE01	153 (0.9899)	4.20 (0.19)	1.28 (0.077)	3.28
ZOPM00	448 (0.9804)	3.32 (0.115)	0.327 (0.023)	10.15
ZOVA01	244 (0.9868)	3.09 (0.084)	0.24 (0.014)	12.87
ZOVA02	346 (0.975)	3.13 (0.132)	0.24 (0.022)	13.04

parameters. The phenolic content of the dry beans under study is of the same magnitude order as that previously found in one bean cultivar used in central Mexico (*10*). **Table 1** also reports the ratio of GAE to EC, which aids discrimination among the landraces.

The characterization and quantification of flavonols and phenolic acids by HPLC were also carried out for all samples: Kaempherol, quercetin, and caffeic acids derivatives were identified.

If we consider the antiradical activity, two mathematical models can be taken into account:

$$\ln[\mathrm{DPPH}^{\bullet}] = b[\text{antioxidant}] + a \quad (1)$$

$$\ln[\mathrm{DPPH}^{\bullet}] = b \ln [\text{antioxidant}] + \ln a \quad (2)$$

indicating exponential and multiplicative models, respectively.

When considering the four standard solutions (quercetin, quercitrin, kaempferol, and ascorbic acid), we obtained linear trends ($R^2 \geq 0.95$) applying both models in the case of kaempferol and ascorbic acid, while the data fit the first model better ($R^2 \geq 0.95$) than the second ($R^2 \leq 0.88$) in the case of quercitrin and quercetin.

With regard to our samples, the data from most compounds fit both models showing that the reaction of the radical is performed with different classes of antioxidant compounds, which may account for the wider variation of EC_{50} with respect to that of GAE and EC.

The same conclusions may be drawn considering the GAE/EC ratios. In fact, a small ratio (higher flavonoid content) does not correspond to a higher antiradical activity.

In **Table 2**, the flavonols (quercetin and kaempferol glycosides) and phenolic acid (caffeic acids derivatives) contents obtained by HPLC analysis are reported. Comparing the data obtained with the rapid method (CE equivalent) and those from HPLC analysis on a molar basis, no great differences are found between the two methods in the case of Verdolino and Zolfino landraces, while for Poverella and O'Marrozzo the values (on a molar basis) of CE equivalents are 10−100 times higher than those from the HPLC analysis. The use of (+)-catechin as a standard in the spectrophotometric method demonstrates that positive absorptions are given, other than by flavonols, also by polymers (like procyanidins and condensed tannins), which are not detected with our HPLC method. Condensed tannins were evaluated according to a spectrophotometric method. The results showed a low amount of condensed tannins in the Zolfino and Verdolino landraces [0.36 and 0.25 mg (+)-catechin/g sample, respectively] and a high amount in the O'Marrozzo landrace [1.68 mg (+)-catechin/g sample]. Poverella exhibits a relatively high amount of condensed tannins [0.56 mg (+)-catechin/g sample], which accounts for the large discrepancy between the two sets of data.

Table 2. Flavonoids and Phenolic Acid Contents (mg/g) as Obtained from HPLC Measurements[a]

sample	flavonoids	phenolic acids
PVPM00	traces	0.051
PVPM01	0.0307	0.022
VDVA01	1.15106	0.070
VDVA02	1.274	0.084
VDSE01	0.807	0.059
OMPM01	0.119	0.071
OMVA01	0.174	0.060
OMVA02	0.138	0.063
OMSE01	0.16	0.089
ZOPM00	0.615	0.042
ZOVA01	0.5515	0.028
ZOVA02	0.555	0.038

[a] Coefficient of variation ≤ 5%.

Table 3. Mean Values (See **Table 1**) and Standard Errors (SE)[a]

landrace	EC_{50}	SE	GAE	SE	CE	SE	GAE/CE	SE
Verdolino	57.00^{Bc}	12.897	3.870^{A}	0.085	0.607^{B}	0.017	6.387^{B}	0.254
O'Marrozzo	124.00^{B}	12.550	4.238^{A}	0.067	1.263^{A}	0.084	3.408^{C}	0.269
Zolfino	346.00^{A}	58.891	3.180^{B}	0.071	0.269^{Bc}	0.029	12.020^{A}	0.936

[a] Tukey multiple comparison; capital letters, $p < 0.01$; small letters, $p < 0.05$.

There is a low correlation ($R^2 = 0.494$ at $p = 0.0234$) between the EC_{50} and GAE values if PVPM00 and PVPM01 data point are not considered. Better correlations were obtained in the case of Japanese edible plant (*17*) and raspberries (*18*).

No correlation has been found between EC_{50} and CE values. In the case of beans, the flavonols mixture includes quercetin and kaempferol derivatives for all four landraces (data not shown), but the relative abundance of the derivatives does not account for the differences, even if the antioxidant activity of quercetin is higher than that of kaempferol. Also, condensed tannins (*19*) and phenolic acids contribute to the antioxidant activity (*20*); for instance, it has been shown that the antioxidant activity of caffeic acid is similar to that of quercetin (*21*). In our case, however, there is no correlation even if the phenolic acids contribution is considered; such behavior has previously been pointed out (*22*).

With regards to the differences among the landraces, **Table 3** indicates that only the ratio between the Folin−Ciocalteu assay and the total flavonols can discriminate ($p \leq 0.01$) among the three landraces (the fourth landrace, Poverella, being very different from the other ones).

In the literature, it is very difficult to compare the antioxidant activity when complex extracts are analyzed due to the different methods, which used to measure the antioxidant activity (*23*) and, in the case of a given method, to the mathematical forms used to express the result.

We have compared beans of four landraces grown in different geographic areas and different years and have found similarities within the varieties and differences, which, although not significant from a statistical point of view, indicate that the EC_{50} parameter is greatly affected by the composition of the extract. Furthermore, when beans are considered, a 70-fold difference can be pointed out in their antiradical activity, showing how the EC_{50} value can help in assessing the functional characteristics of food.

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