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Assessment of in vitro antioxidant capacity of the seed coat and the cotyledon of legumes in relation to their phenolic contents

M. Dueñas, T. Hernández, I. Estrella *

Instituto de Fermentaciones Industriales, CSIC, C/Juan de la Cierva 3, 28006-Madrid, Spain

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Abstract

Methanolic extracts of the seed coat and the cotyledon of two varieties of lentils (*Lens culinaris* L.) and two varieties of dark peas (*Pisum sativum* L.), were analysed for their antioxidant capacities (EC_{50}), in the form of free radical-scavenging activities. Huge differences have been observed in the antioxidant capacity in the seed coat and the cotyledon in both legumes. The seed coat, in which are located, principally, phenolic compounds with flavonoid structures, presents higher antioxidant activity than the cotyledon in lentils and peas, showing differences in both species but not very large differences between varieties. An analysis of principal components was applied to the results in order to relate the antioxidant capacity of these legumes with their phenolic composition previously established by the authors.

Keywords: Lentils; Peas; Antioxidant capacity; Phenolic compounds

1. Introduction

Several epidemiological studies suggest a correlation between the consumption of foods with a high content of phenolics (such as fruits, vegetables, legumes and wine) with decreasing incidence of diseases, e.g., cancer and cardiovascular disease (Kris-Etherton et al., 2002; Ramarathnam, Osawa, Ochi, & Hawakishi, 1995; Remesy, Manach, Demigne, Texier, & Regerat, 1998).

Legume seeds, consumed largely by populations in developing countries, are an important source of macronutrients such as proteins, carbohydrates and dietary fibre. Also, the legumes provide micronutrients, vitamins, carotenoids (Adsule & Kadam, 1989) and phenolic compounds, all of which are considered to be bioactive compounds (De Pascual Teresa, Santos-Buelga, & Rivas-Gonzalo, 2000; Dueñas, Hernández, & Estrella, 2002, 2004).

Three different parts are recognised in the legume seeds: cotyledon, seed coat and embryonic axe, which represent, on average, 89%, 10% and 1%, respectively, of the total seed weight. The cotyledon contains the main reserve substances, basically proteins and carbohydrates. The seed coat, which acts as a protective barrier for the cotyledon, has the highest concentration of phenolic compounds (Dueñas, 2003; Dueñas, Sun, Hernández, Estrella, & Spranger, 2003a; Dueñas, Hernández, & Estrella, 2004; Shahidi, Chavan, Naczk, & Amarowicz, 2001; Troszynska, Bednarska, Latosz, & Kozlowska, 1997).

The distribution of phenolic compounds differs in the cotyledon and the seed coat, with non-flavonoid phenolic compounds, such as free and combined hydroxybenzoic and hydroxycinnamic acids, being located mainly in the cotyledon of lentils (Dueñas et al., 2002). Flavonoids, such as glycosides of flavonols and flavones, were identified in the seed coat of lentils, together with *trans*-resveratrol-3-*O*-glucoside, and higher concentrations of proanthocyanidins (Dueñas et al.,

* Corresponding author. Tel.: +34 915622900; fax: +34 915644853.
E-mail address: iestrella@ifi.csic.es (I. Estrella).

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2002, 2003a), although the lentil seed coat represents only a small percentage of the entire lentil seed weight, ranging from 8% to 11%.

A similar distribution of phenolic compounds was reported in peas. Flavonols and flavone glycosides and some hydroxybenzoic and hydroxycinnamic compounds were described in the seed coat of dark peas (Troszynska, Estrella, López-Amorós, & Hernández, 2002), together with some proanthocyanidins, and compounds conjugated with malic acid, while *trans*-*p*-coumaroyl-malic acid and *p*-hydroybenzoyl-malic acid were identified in both parts of the seed, and the stilbene, *trans*-resveratrol-3-*O*-glucoside, only in the seed coat (Dueñas et al., 2004).

Lentils show antioxidant activity related to their total phenolic contents (Fernández-Orozco, Zielinski, & Piskula, 2003). In lentils and peas, the antioxidant capacity is related to the total flavonoid content (Amarowicz, Karamac, & Weidner, 2001; Amarowicz, Karamac, & Shahidi, 2003).

Other authors have observed high antioxidant activity in lentils, faba beans and peas, mainly in the seed coat, due to the essential fact that large amounts of phenolic compounds, mainly flavonoids, are located in this part of the seed (Amarowicz, Naczk, Zadernowski, & Shahidi, 2000; Cardador-Martínez, Loarca-Piña, & Oomah, 2002; Nilsson, Stegmark, & Akesson, 2004; Shahidi et al., 2001; Takahata, Ohnishi-Kameyana, Furuta, Takahasi, & Suda, 2001; Troszynska & Ciska, 2002). Thus, the seed coat of legumes could be used as a natural source of antioxidants to replace the use of synthetic antioxidants in foods (Ronzio, Muanza, & Sparks, 1998).

The antioxidant activity of phenolic compounds is directly related to their structure. In the case of flavonoids, which are considered primary antioxidants (Decker, 1997), the position and degree of hydroxylation on the B ring are the most important factors associated with this activity (Rice-Evans, Miller, & Paganga, 1996). Chalcones, natural precursors of flavones and flavonols, have a potent antioxidant activity, and the 3,4-dihydroxychalcones are particularly effective antioxidants (Dziedzic & Hudson, 1983). Other phenolic compounds, such as hydroxycinnamic and hydroxybenzoic acids, also present antioxidant activity, which depends on the number and position of hydroxyl groups in the molecule (Dziedzic & Hudson, 1983). Hydroxycinnamic acids seem to be more effective than the corresponding hydroxybenzoics (Baderschneider & Winterhalter, 2001; Natella, Nardini, Di Felice, & Scaccini, 1999). Proanthocyanidins also have free radical-scavenging activity, influenced by the structure of the monomers and the degree of polymerisation (Hatano et al., 2002; Weisburger, 2001).

Individual antioxidant compounds do not act alone. They act in combination with other antioxidants, as interactions among them can affect total antioxidant capacity, producing synergistic or antagonistic effects (Niki & Noguchi, 2000).

In this study we evaluated the antioxidant capacity by free radical-scavenging activity of the seed coat and cotyledon of two lentil varieties and two pea varieties. This antioxidant activity is related to the content of phenolic compounds in the same samples, previously determined by the authors.

2. Materials and methods

2.1. Samples

Two varieties of lentils (*Lens culinaris* L.) Pardina and Castellana (Spain), and two samples of each variety, purchased from a local market, and two varieties of dark seed coat peas (*Pisum sativum* L.), Fidelia, supplied by Dr. Tronszynska (Institute of Animal Reproduction and Food Research of Poland) and ZP-840 from the Instituto Agrario of Valladolid (Spain), were analysed. The seed coat of lentils and peas was manually separated from the cotyledon, and both parts of the seed were ground in a small mill (Retsch MM 2000) through a 0.5 mm sieve screen. The flour obtained was put into plastic bags under vacuum and stored in the dark, at 4 °C, until used.

2.2. Sample preparation

The phenolic extracts were prepared according to the method described by Dueñas et al. (2002). 1.5 g of seed coat and 10 g of cotyledon were macerated separately with 3×80 ml of a solution of methanol–HCl (1‰)/water (80:20 v/v), in a bath at room temperature and stirred, separating the supernatants by centrifugation. The three combined supernatants were taken to a fixed volume (240 ml) of the methanol solution, yielding a methanol extract where the antioxidant activity was determined.

2.3. Antioxidant activity

The antioxidant activities (EC_{50}) of the seed coat and cotyledon of lentils and peas were determined by reaction with the 2,2′-diphenyl-1-picrylhydrazyl (DPPH) radical, according to the Brand-Williams method (Brand-Williams, Cuvelier, & Berset, 1995), modified by López-Amorós, Lomas, Estrella, and Hernández (1998), for legume samples. The reaction was carried out with 2 ml of a methanol solution of DPPH$^{\cdot}$ (0.025 g/l) and solutions of different concentrations from the different samples. The absorbance was measured at 1 min intervals at 515 nm, until the reaction reached a plateau (time at the steady state). The percentage of

remaining DPPH· (% $DPPH_{rem}$) was calculated as follows:

$$\%DPPH_{rem} = [(\text{Abs. 515 nm})\text{sample}/(\text{Abs. 515 nm})\text{control}] \times 100$$

This percentage was plotted against the sample concentration to obtain the EC_{50} defined as the amount of antioxidant (mg of seed coat or cotyledon flour) necessary to decrease absorbance by 50%. The lower the EC_{50} value, the higher was the antioxidant activity.

2.4. Statistical analysis

Analyses were performed in triplicate, and the data are presented as means ± SD. Analysis of variance and principal component analysis (LSD, 5% level) were performed using the statistical package Statgraphics Plus 5.0 v. (Statistical Graphics Corporation, Inc. Rockville, MD, USA). The principal components analysis was performed on the percentage of phenolic compounds and antioxidant capacity to find the influence of the different groups of phenolic compounds on the antioxidant capacity.

3. Results and discussion

3.1. Lentils

Large differences were found between the EC_{50} values corresponding to the seed coat and the cotyledon (Table 1) of both the Pardina and the Castellana varieties. The seed coat shows greater free radical-scavenging capacity, with EC_{50} values between 0.05 and 0.07 (mg of sample) while, in the cotyledon, the values were between 21 and 29 (mg of sample), thus the quantity of seed coat sample needed to reduce DPPH solution absorbance to 50% is of the order of 500 times less than is needed in the case of the cotyledon sample.

In the seed coat, significant differences ($p \leqslant 0.05$) between varieties can be observed, as Pardina demonstrates greater activity than Castellana, but, among samples of each variety, no appreciable differences were observed. In the cotyledon, there is little antioxidant activity, and significant differences ($p \leqslant 0.05$) are observable among all the samples.

Table 1
Antioxidant activity (EC_{50}) (mg of sample) of the seed coat and the cotyledon of lentils

Samples	Seed coat	Cotyledon
Pardina 1	0.058 ± 0.005^{a}	24.7 ± 0.86^{b}
Pardina 2	0.055 ± 0.003^{a}	27.9 ± 1.97^{c}
Castellana 1	0.079 ± 0.004^{b}	29.5 ± 0.11^{c}
Castellana 2	0.072 ± 0.002^{b}	21.5 ± 0.51^{a}

Means ($n = 3$) followed by the same letter in a column are not significantly different (LSD, 5%).

Previous work done by our group showed the phenolic composition of the seed coats and cotyledons of the same samples of lentils (Dueñas et al., 2002; Dueñas, 2003; Dueñas et al., 2003a), and the minimum and maximum levels for the different groups of compounds were presented. The seed coat of both varieties presented high concentrations of catechins (919–1633 μg/g), dimer procyanidins (619–1122 μg/g), trimer procyanidins (441–498 μg/g) and dimers plus trimers of prodelphinidins (369–725 μg/g), and relatively low concentrations of galloylated procyanidins (69.3–123 μg/g), tetramer procyanidins (18.5–59.5 μg/g), glycosides of flavones (33.1–186 μg/g) and flavonols (9.6–241 μg/g), hydroxybenzoic (28.4–4.5 μg/g) and hydroxycinnamic (11.7–29.5 μg/g) acids, and *trans*-resveratrol glucoside (5.5–9.3 μg/g). The cotyledon phenolic compounds content was much less, and consisted mostly of hydroxybenzoic acids (1.8–2.2 μg/g), free (3.2–5.7 μg/g) and combined hydroxycinnamic acids (1.4–13.5 μg/g) and (+)-catechin (0.2–2.9 μg/g) (Dueñas, 2003).

The lentil seed coat and cotyledon antioxidant activity studied can be principally attributed to its phenolic composition, due to these compounds' ability for free radical-scavenging. In order to elucidate this relationship in the lentil samples, the EC_{50} values were correlated with the relative percentages of quantified phenolic compounds, with respect to the total content of phenolics, in the seed coat and the cotyledon of the four samples, through the analysis of principal components.

In the seed coat, the variables were the percentages of dimer procyanidins, trimer procyanidins, tetramer procyanidins, catechins, prodelphinidins, galloylated procyanidins, *trans*-resveratrol glucoside, flavonols and flavone glycosides, and hydroxybenzoic and hydroxycinnamic compounds (Table 2). Three components have been obtained from this analysis, of which the first two represent 93.1% of the total variance. The graph of the two first components (Fig. 1) shows that the variables, flavones, flavonols and trimer procyanidins, contribute the most antioxidant capacity to the seed coat, taking into account the inverse form of expressing antioxidant activity (EC_{50}). The catechins and dimer and tetramer procyanidins, are less related, while an intermediate correlation is observed for the *trans*-resveratrol glucoside, prodelphinidins and galloylated procyanidins.

As was stated earlier, phenolic compounds with a flavonoid structure display greater antioxidant activity. Flavonols and flavones, present in relatively high concentrations in the seed coat, show high antioxidant activity, related to their structure (Fuhrman & Aviram, 2002; Jovanovic, Steenken, Simic, & Hara, 1998). Thus

Table 2
Percentage (%) of groups of phenolic compounds with respect to the total content of phenolics in the cotyledon and the seed coat of lentils

Groups	Cotyledon				Seed coat			
	P1	P2	C1	C2	P1	P2	C1	C2
Hydroxybenzoic acids	8.32	9.04	30.7	20.1	0.70	0.72	1.10	1.60
Free hydroxycinnamic acids	24.8	20.4	46.1	48.6	0.47	0.26	0.64	1.25
Combined hydroxycinnamic acids	54.4	66.1	19.8	20.1	–	–	–	–
Catechins	12.7	4.30	3.21	11.2	37.0	35.7	41.6	39.1
Glycosides of flavones	–	–	–	–	3.32	4.91	1.05	1.76
Glycosides of flavonols	–	–	–	–	2.86	7.07	0.73	0.41
Dimer procyanidins	–	–	–	–	23.5	24.5	31.9	26.3
Trimer procyanidins	–	–	–	–	10.9	11.6	7.21	8.33
Procyanidin tetramers	–	–	–	–	0.40	1.30	1.64	1.24
Galloylated procyanidins	–	–	–	–	2.98	2.20	2.28	2.95
Prodelphinidins	–	–	–	–	17.6	11.7	11.7	16.7
Glycoside of *trans*-resveratrol	–	–	–	–	0.23	0.14	0.18	0.23

P1:Pardina 1; P2: Pardina 2; C1: Castellana 1; C2: Castellana 2.
Data from Dueñas (2003).

luteolin, which is also present in the analysed samples, seems to be responsible for the antioxidant activity of some seeds (Shimoi et al., 1999).

On the other hand, the trimer procyanidins also seem to be more associated with antioxidant activity than catechins and dimer procyanidins, although these latter compounds are present in greater concentrations in the lentil seed coat. The high antioxidant activity of proanthocyanidins, which varies according to the degree of polymerization, with trimers presenting greater activity than dimer or monomer procyanidins, has been described (Dueñas, Hernández, Estrella, & Rabanal, 2003b; Lotito et al., 2000; Saint-Cricq de Gaulejac, Vivas, de Freitas, & Bourgeois, 1999). This could explain the existing relationship between the levels of trimer procyanidins in the lentil seed coat and the EC_{50} value. In addition a possible antagonism between these compounds in the studied samples may exist.

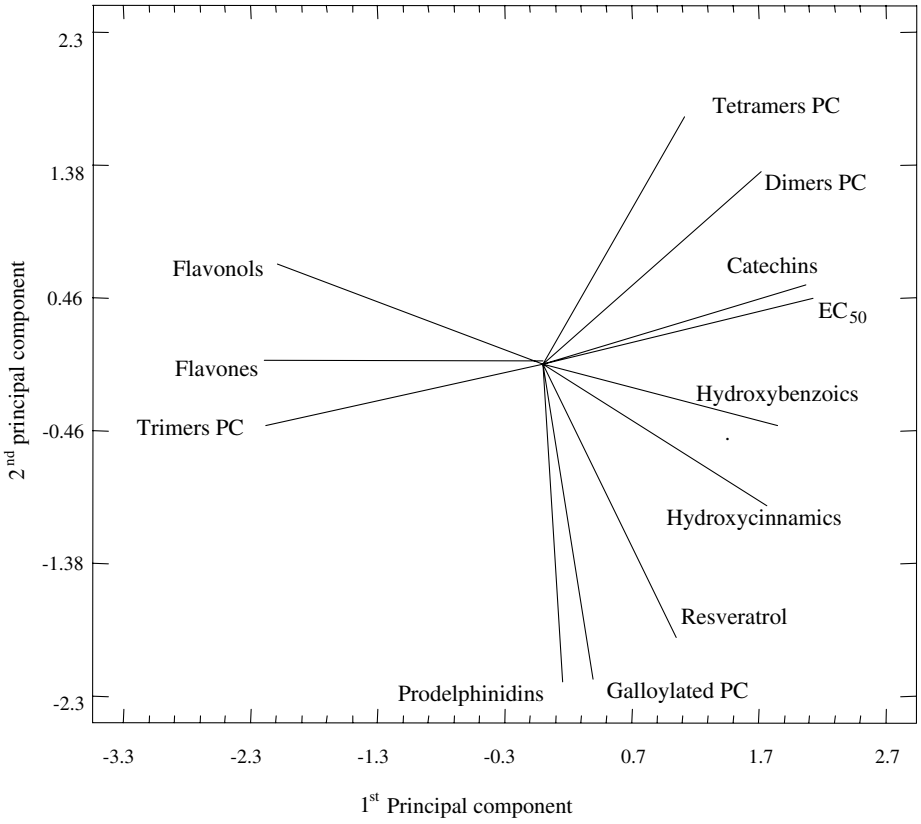

Fig. 1. Plot of the principal components of the percentage (%) of phenolic compounds and antioxidant activity (EC_{50}) of the seed coat of lentils PC: procyanidins.

Shahidi (2002), furthermore, found a high correlation between antioxidant capacity and the catechin content in the seed coats of the beach pea. Also, Amarowicz et al. (2000) related the total tannin content in the seed coat of some legumes to the scavenging effect for DPPH radicals.

In the cotyledon, the principal components analysis was also applied to the EC_{50} value (Table 1) and to the relative percentage of compounds (Table 2). The variables were free and combined hydroxycinnamic acids, hydroxybenzoic acids and (+)-catechin, considering the four samples of lentils. Three components have been obtained from this analysis, of which the first two represent 97.9% of the total variance. The graph of the two first components (Fig. 2) shows that the (+)-catechin, which is a flavonoid compound, is most highly associated with the antioxidant activity. Non flavonoid phenols have less antioxidant activity than flavonoids (Baderschneider & Winterhalter, 2001).

In view of these results, it can be concluded that the high concentrations of phenolic compounds, especially flavonoids, contained by the seed coat, are responsible for the greater antioxidant activity of this part of the seed compared to the cotyledon, with its much lower phenolic content.

3.2. Peas

In both pea varieties, ZP-849 and Fidelia, it was observed (Table 3) that the seed coat presents greater antioxidant activity, with EC_{50} values between 0.11 and 0.14 (mg of sample), than the cotyledon, with values between 8 and 28 (mg of sample). No significant differences ($p \leqslant 0.05$) exist between varieties in the seed coat but, in the cotyledon, there are noticeable differences, with the Fidelia variety showing a higher antioxidant activity than ZP-849.

In previous works, the authors (Dueñas, 2003; Dueñas et al., 2004) determined the phenolic composition of the seed coat and the cotyledon of the same samples of pea varieties, and the minimum and maximum levels

Table 3
Antioxidant activity (EC_{50}) (mg of sample) of the seed coat and the cotyledon of peas

Samples	Seed coat	Cotyledon
ZP-849	0.11 ± 0.02^{a}	7.98 ± 0.08^{a}
Fidelia	0.14 ± 0.03^{a}	28.2 ± 0.13^{b}

Means ($n = 3$) followed by the same letter in a column are not significantly different (LSD, 5%).

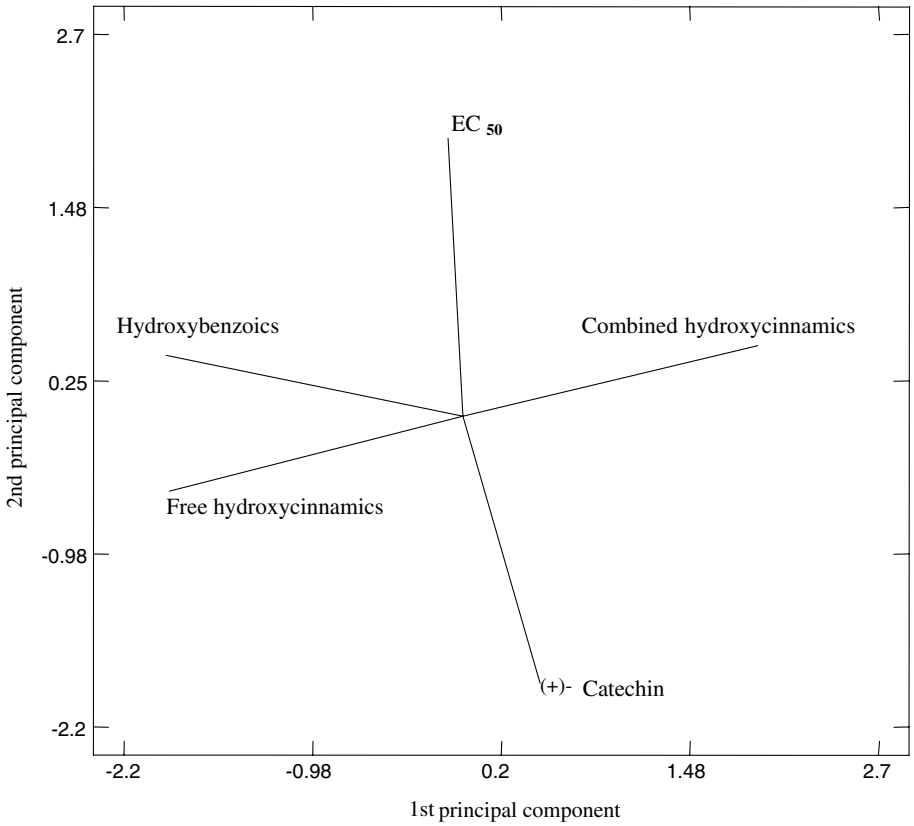

Fig. 2. Plot of the principal components of the percentage (%) of phenolic compounds and antioxidant activity (EC_{50}) of the cotyledon of lentils.

Table 4
Percentage (%) of groups of phenolic compounds with respect to the total content of phenolics in the cotyledon and the seed coat of peas

Groups	Cotyledon		Seed coat	
	ZP-849	Fidelia	ZP-849	Fidelia
Hydroxybenzoic acids	85.8	87.2	16.3	32.3
Free hydroxycinnamic acids	0.93	3.60	–	0.80
Combined hydroxycinnamic acids	1.22	1.14	2.15	6.45
Catechins	–	–	1.16	1.08
Gallocatechins	–	2.56	13.1	1.25
Dimer prodelphinidins	–	–	4.77	–
Glycosides of flavones	1.40	2.07	29.5	53.8
Glycosides of flavonols	2.26	1.43	1.89	0.03
Glyc. tetrahydroxydihydrochalcone	8.47	2.35	31.8	4.07
Glycoside of *trans*-resveratrol	–	–	nd	0.89

Data from Dueñas et al. (2004).

of the different groups of phenolic compounds were shown. In the cotyledon hydroxybenzoic acids (49.8–54.8 μg/g), free (0.6–2.0 μg/g) and combined (0.7–0.8 μg/g) hydroxycinnamic acids, glycosides of flavones (0.8–1.3 μg/g) and flavonols (0.9–1.3 μg/g), (−)-epigallocatechin (1.6 μg/g), only observed in Fidelia variety, and a glycoside of tetrahydroxydihydrochalcone (1.5–4.9 μg/g) were detected. The seed coat contains hydroxybenzoic acids (105–240 μg/g), free (6.0 μg/g), in Fidelia, and combined hydroxycinnamic acids (13.5–48.0 μg/g), glycosides of flavones (169.3–344.6 μg/g) and flavonols (12.1–0.2 μg/g), glycoside of tetrahydroxydihydrochalcone (30.3–208 μg/g), gallocatechins (9.4–85.2 μg/g), catechins (7.6–8.1 μg/g), dimer prodelphinidins (38.1 μg/g), only in ZP-849 and *trans*-resveratrol glucoside (6.7 μg/g) in Fidelia.

The principal components analysis, applied to the EC_{50} value (Table 3) and to the relative percentage of the different groups of phenolic compounds in relation to the total of the phenolics in the seed coat of the two samples (Table 4), bring out the relationship between these values. The variables were percentages of

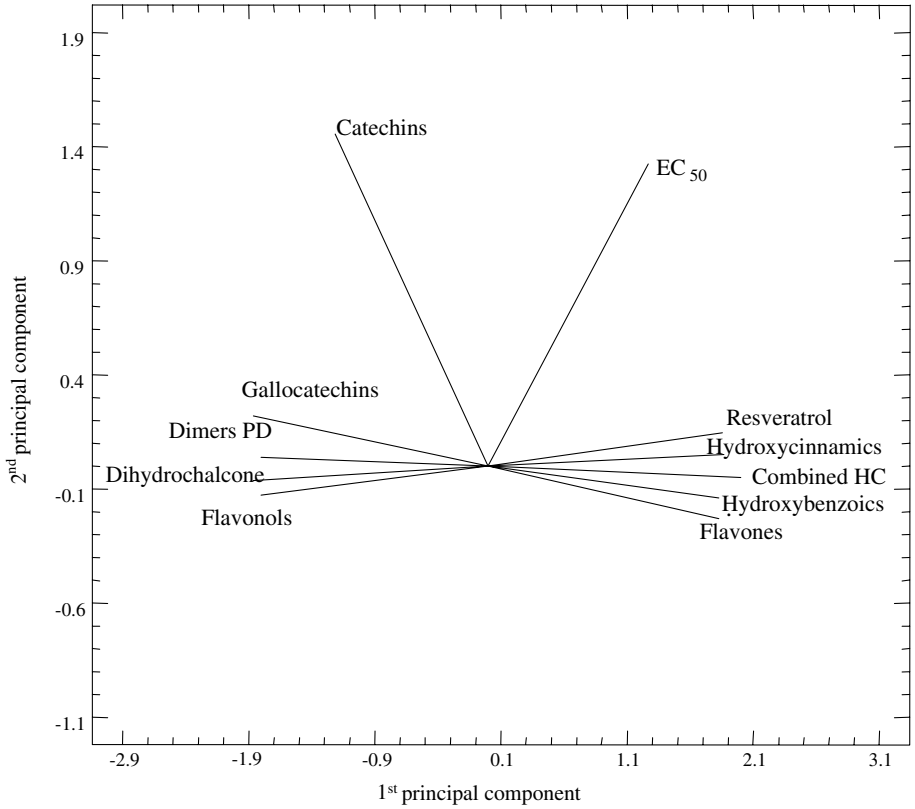

Fig. 3. Plot of the principal components of the percentage (%) of phenolic compounds and antioxidant activity (EC_{50}) of the seed coat of peas PD: prodelphinidins; HC: hydroxycinnamics.

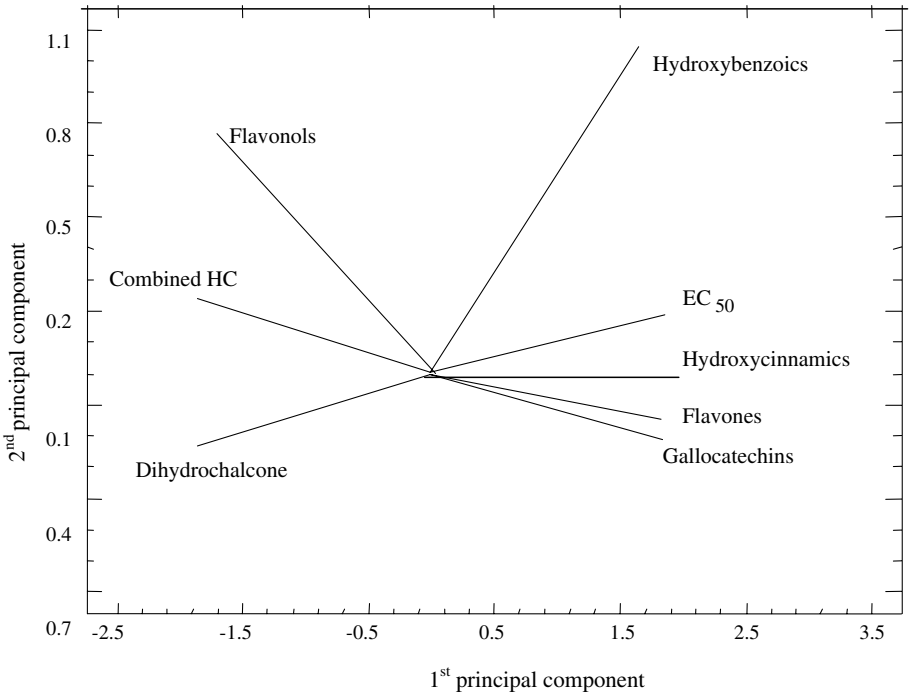

Fig. 4. Plot of the principal components of the percentage (%) of phenolic compounds and antioxidant activity (EC_{50}) of the cotyledon of peas HC: hydroxycinnamics.

flavonols and flavone glycosides, glycoside of tetrahydroxydihydrochalcone, dimer prodelphinidins, catechins, gallocatechins, *trans*-resveratrol glucoside, hydroxybenzoic acids and free and combined hydroxycinnamic acids. Three components were obtained from the analysis, of which the first two represent 95.1% of the total variance.

In Fig. 3 a graphic representation of the first two components can be seen. It can be observed that, in the seed coat, the variables, flavonols, glycoside of tetrahydroxydihydrochalcone, dimer prodelphinidins and gallocatechins, are concentrated; in the same way the variables flavones, *trans*-resveratrol glucoside, hydroxybenzoics and free and combined hydroxycinnamic acids are grouped. Whereas neither of the groups of compounds appears to have a strong relationship with antioxidant activity (EC_{50}), it can be deduced that the first group, which corresponds overall to flavonoid compounds, has a most positive correlation with the activity.

On the other hand, the highest antioxidant activity of the ZP-849 variety (Table 3) could be attributed to the more abundant concentration of gallocatechins and dimer prodelphinidins (Dueñas et al., 2004), due to the fact that these compounds present higher activity than the corresponding procyanidins (Rice-Evans et al., 1996; Saito et al., 2004).

In the principal components analysis, applied to the compounds of the cotyledon and the EC_{50} values, the variables were the percentage of glycosides of flavonols and flavones, hydroxybenzoic acids, free and combined hydroxycinnamic acids, gallocatechins, and glycoside of tetrahydroxydihydrochalcone (Table 4). Three principal components were obtained in the analysis, the first two accounting for 99.3% of the variance. The graph of the two first components (Fig. 4) shows that the glycoside of tetrahydroxydihydrochalcone is the compound that most highly and positively contributed to the EC_{50} values.

The antioxidant activity of the cotyledon of ZP-849 is higher than that of Fidelia, which could be explained by the higher content of the glycoside of tetrahydroxydihydrochalcone in ZP-849 (5 μg/g) than in Fidelia (1.5 μg/g) (Dueñas et al., 2004). It has been reported that the dihydrochalcones exhibit higher antioxidant activity than the corresponding flavones against the stable free radical, 1,1-diphenyl-2-picrylhydrazyl, and lipid peroxidation in the erythrocyte membrane (Nakamura, Watanabe, Miyake, Kohno, & Osawa, 2003).

4. Discussion

The analysed lentil varieties show greater antioxidant activity than the peas and, in both types of seeds, the seed coat demonstrates greater activity than the cotyledon.

The differences found in the EC_{50} values of lentils and peas may be related to the large qualitative and quantitative differences in the phenolics content, as can be found in the analysis of phenolic compounds done on these samples in previous studies (Dueñas et al., 2002,

2004; Dueñas, 2003). These differences were found not only between the seed coat and cotyledon of both kinds of legumes, but also among varieties.

In both lentils and peas, the cotyledon has a lower concentration of phenolic compounds than the seed coat, and the distribution of these compounds is different in the two seed parts as well. In both types of legumes, the cotyledon contains mainly non-flavonoid compounds, hydroxybenzoics and hydroxycinnamics, to which are attributed less antioxidant activity than flavonoids (Baderschneider & Winterhalter, 2001).

The scavenging effect on the DPPH radical by an antioxidant, e.g., phenolic compounds, depends on the chemical structure and the concentration of the antioxidant (Adom & Liu, 2002; Rice-Evans et al., 1996; Sanchez-Moreno, Larrauri, & Saura-Calixto, 1998). The free and combined hydroxycinnamic compounds show a free radical-scavenging activity greater than that of the corresponding hydroxybenzoics (Natella et al., 1999). The esterification of *p*-coumaric and ferulic acids has also been observed to increase antioxidant potential in in vivo tests (Meyer & Andreasen, 1999).

The lentil seed coat has a higher antioxidant activity than that of peas, which is because the lentil has larger amounts of flavonoid compounds, especially of proanthocyanidins, which contribute to the increasing of antioxidant activity. In peas, on the other hand, the seed coat has similar concentrations of flavonoid and non-flavonoid compounds, and in lower quantities.

From the obtained results it can be deduced that, even though the seed coat represents the lower part of the total weight of the seed (10–11%) in grain legumes, the phenolic compounds of this part should contribute more to the antioxidant capacity of the entire seed, although there may be antagonistic or synergistic effects between them.

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