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Changes in the content of bioactive polyphenolic compounds of lentils by the action of exogenous enzymes. Effect on their antioxidant activity

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Abstract

The study of the effect of the enzymes tannase, α-galactosidase, phytase and viscozyme on the phenolic composition of lentil flours, in a semi pilot scale stirred fermentor, shows that important modifications occur. Among them, hydroxycinnamic compounds and proanthocyanidins are significantly decreased after the enzymatic treatments. However, quercetin 3-O rutinoside and luteolin increase and reach the highest concentration with tannase. The formation of *trans*-resveratrol was observed by the action of tannase and phytase, and gallic acid by the action of phytase, α-galactosidase and tannase. The antioxidant capacity of the methanolic extracts was determined by their free radical scavenging activity, using the DPPH test, to study the differences in the behaviour of polyphenolics compounds as antioxidants after the different enzymatic treatments. The treatments with viscozyme, α-galactosidase or tannase produce an increase in the antioxidant activity when compared to raw lentils. The results of the analysis of principal components to examine the relationship among antioxidant activity (EC_{50}) (DPPH test) and the concentrations of polyphenolics in all lentils samples, show that the quercetin 3-O rutinoside appears to be the compound with the greatest influence on the EC_{50} values.
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Keywords: Lentils; Polyphenolic compounds; Tannase; α-Galactosidase; Phytase; Viscozyme; Antioxidant activity

1. Introduction

Legumes contain antinutritional factors which must be minimized or eliminated before ingestion. The most common procedures used to reduce the amount of antinutritional factors and thus increase their nutritional value are soaking, cooking, germination, fermentation and addition of enzymes (Cowan, 1995; Frías, Vidal-Valverde, Sotomayor, Díaz-Pollán, & Urbano, 2000; Tabera, Frías, Estrella, Villa, & Vidal-Valverde, 1995; Vidal-Valverde et al., 1994). The biotechnological procedures of enzyme addition to legume flours have been recognised in the food industry, and the main objective is to improve the utilisation of nutrients in raw materials. In the food industry, enzymes are considered a nutritional supplement and have been used as technological coadjutant in increasing the biological value of certain foods.

Current enzymology and molecular biology techniques are selecting sources of enzymes on the basis of substrate selectivity and reaction conditions (James & Simpson, 1996; Estell, 1993). Usually substrates are complex and the commercial enzymes are mixtures, thus the use of pure enzymes, with specific catalytic properties and concrete activity on a concrete type of substrate is of great interest. The outcome of the enzymatic action depends on the biochemical reactions which occur during treatment, so the substrate must be perfectly identified and characterised and the reactions must occur under the right conditions of pH, temperature, time etc., for each enzyme.

The application of commercial enzymes in the treatment of legumes for human consumption can minimize or eliminate certain antinutritional factors, improving their nutritional value, as was observed in a study of ground soybeans (Classen, Balnave, & Bedford, 1993). Several authors have

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reported that the selective addition of enzymes to legume flour decreases phytic acid levels (Fredrikson, Biot, Alminger, Carlsson, & Sandberg, 2001; Frías, Doblado, Antezana, & Vidal-Valverde, 2003a; Wu, Ravindran, & Hendriks, 2004) or α-galactoside levels (Frías, Doblado, & Vidal-Valverde, 2003b; Mulinari & Devendra, 1997).

Some of the most used enzymes in the food industry are:

Phytases. These are the enzymes responsible for the dephosphorylation of phytates and are widely distributed in microorganisms, plants and animals (Konietzny, Greiner, & Jany, 1995). The addition of phytases to foods can decrease the antinutritional effects of phytic acid, which makes these enzymes important in biotechnology applications of foods and animal feeds (Fredrikson et al., 2001; Konietzny & Greiner, 2002; Wu et al., 2004).

α-*Galactosidases*. These are the enzymes responsible for the hydrolysis of α-galactosides since they hydrolyse the α-(1-6) bonds in the galactose molecules. The α-galactosides in legumes are the reason for flatulence since they are degraded by microorganisms producing carbon dioxide, hydrogen and methane (Price, Lewis, Wyatt, & Fenwick, 1988), and α-galactosidases can diminish or eliminate these compounds (Frías et al., 2003b). These enzymes occur in a large number of plants and microorganisms (Dey & Pridman, 1972; Mansour & Khalil, 1998).

Enzymes which act on non-starch polysaccharides. The non-starch polysaccharides are located in the cellular wall of vegetables and can limit the access of endogenous enzymes to nutrients reducing their use. The use of multienzymatic complexes, such as viscozyme, which contains cellulases, arabinases, hemicellulases, gluconases and xylanases, causes the rupture of the cell walls, favouring the extraction of useful compounds from the vegetable tissues, increasing their digestibility and absorption (Bedford, 2002; Peterson, Wiseman, & Bedford, 1999). Antezana et al. (2003) observed that the action of viscozyme 120 l on pea flour increases the soluble fiber content, improving the ratio of insoluble fiber/soluble fiber, causing an increase in the usefulness of these flours.

Tannase. This enzyme hydrolyses the depside and ester bond of hydrolysable tannins releasing gallic acid and glucose (Ramírez-Coronel, Viniegra-González, Darvill, & Augur, 2003). Tannase is an enzyme produced by various filamentous fungi, mainly *Aspergillus* and *Penicillium* in the presence of tannic acid (Ramírez-Coronel et al., 2003). This enzyme is used in several food procedures, such as the production of instant tea (Agbo & Spradlin, 1995; Boadi & Neufeld, 2001), or the production of coffee aroma additives for soft drinks (Chae & Yu, 1983; Pourrat, Regerat, Pourrat, & Jean, 1985). It has also potential use in the clarification of beers and fruit juices (Cantarelli, Brenna, Giovanelli, & Rossi, 1989). The release of gallic acid by the action of tannase on gallic tannins would be beneficial since this compound is supposed to have great antioxidant power (Netzel, Shahrzad, Winter, & Bitsch, 2000).

Lentils present flavonoid and non-flavonoid phenolic compounds which are distributed between the cotyledon and the seed coat in a qualitatively different way (Dueñas, Hernández, & Estrella, 2002). Proanthocyanidins are the most abundant polyphenolics, which are found mainly in the seed coat and which together with flavones and flavonols represent over 80% of the total phenolic compounds in lentils of Pardina variety (Dueñas, Sun, Hernández, Estrella, & Spranger, 2003). Phenolic compounds are considered bioactive substances, with antioxidant activity through their free radical scavenging activity; this activity is directly related to the chemical structure of polyphenolics, such as number of hydroxyl groups, degree of glycosylation, etc. (Baderscheneider & Winterhalter, 2001; Decker, 1997; Montoro, Braca, Pizza, & De Tommasi, 2005; Natella, Nardini, Di Felice, & Scaccini, 1999).

Some processes carried out on legumes, have been shown to increase in the antioxidant capacity in relation to their polyphenolic composition, for instance the fermentation of cowpeas with *Lactobacillus plantarum* produced an increase in their antioxidant capacity (Dueñas, Fernández, Hernández, Estrella, & Muñoz, 2005). The same improvement in the antioxidant capacity was observed by germination of lupines, peas and lentils (Hernández, Estrella, Dueñas, & Fernández, 2004; López-Amorós, Estrella, & Hernández, 2006).

Several authors (Antezana, 2002; Dueñas, 2003; Frías et al., 2003a; Frías et al., 2003b) have observed the effects of the treatment of Pardina lentil flour with the enzymes phytase, α-galactosidase, viscozyme or tannase with the purpose of reducing or eliminating antinutritional factors. To our knowledge, there are few reports on the effect of enzyme addition on the polyphenolic composition of legumes and the ones available frequently refer only to overall tannin evaluation and not to polyphenolic content in a detailed way. Therefore, the aim of this work was to study the effect of the addition of the enzymes phytase, α-galactosidase, viscozyme or tannase on the phenolic composition of lentil (*Lens culinaris*) flours in aqueous medium at pH 5.5 and 37 °C, at a semi pilot scale in a stirred fermentor. The antioxidant activity of the samples was evaluated by the free radical scavenging activity using the DPPH method. An analysis of principal components was carried out in order to correlate the antioxidant activity and the content of polyphenolic compounds in the different enzymatic treatments.

2. Materials and methods

Samples. Lentils (*Lens culinaris* var. Pardina) were ground in a ball mill, sieved and the 0.050–0.250 mm fraction collected.

Enzymes. The following commercial enzymes were used: Phytase *EC 3.1.3.8* (Phytase Novo L, 5000 FYT/g; 1 FYT releases 1 μmol of phytic acid/min). α-Galactosidase *EC 3.2.1.22* (Novo Nordisk from *Aspergillus níger*, 1000 GALU/g; 1 GALU releases 1 μmol of galactose/min at pH 5.5 at 37 °C). Viscozyme [Novo Nordisk 120 l, 100 FBG (fungal β-glucanase)/g, including a mix of glucanase,

arabinase, hemicellulase and xylanase activities; 1 FBG is the amount of enzyme which under standard conditions liberates glucose or other reducing carbohydrates with a reduction power corresponding to 1 μmol glucose/min]. Tannase *EC 3.1.1.20* (Juelich Enzyme Products, from *Aspergillus ficuum*, 24.4 U/mg; 1 U releases 1 μmol of gallic acid in 1 min at 37 °C and a pH 5.5, from tannin substrate).

Treatment with addition of phytase, α-galactosidase, viscozyme or tannase. Lentil flour (300 g) was suspended in 300 mL of a buffer solution (acetic acid/sodium hydroxide, 0.1 N, pH 5.5) at 37 °C with the different commercial enzymes, in the optimal conditions of concentration and incubation time previously established for each of them (Table 1) (Antezana, 2002; Dueñas, 2003; Frías et al., 2003b; Frías et al., 2003a). The total volume was made up to 3000 mL with distilled water previously heated at 37 °C. The suspensions were incubated in a 5 l stirred fermentor (Microferm Fermentor, MF-100, New Brunswick Scientific, Edison N.J. USA). After the treatment with the enzymes, samples of lentil flours were centrifuged at 4 °C and 10,000 rpm for 10 min, and the supernatants were discarded. The residues were freeze-dried and stored for analysis.

Extraction of phenolic compounds. The raw and enzymatic treated lentil flours (10 g) were macerated with 3×80 ml of a solution of HCl–methanol ($1^{0}/_{000}$)/water (80:20 v/v). An aliquot of the macerate solution was extracted according to Dueñas et al. (2002). The obtained extracts were analysed by high-performance liquid chromatography (HPLC-PAD and HPLC-MS). The samples were prepared and extracted in triplicate.

Analysis by HPLC-PAD and HPLC-MS. The HPLC-PAD analyses of phenolic compounds were performed using a Waters chromatographic system 2690 (Milford, MA-USA) equipped with an autosampling system, a Waters 996 photodiode array detector and a Millenium 32 chromatography manager software, in the conditions reported by Dueñas et al. (2002).

Mass spectra were obtained using a Hewlett Packard 1100MS (Palo Alto, CA) chromatograph equipped with an API source, using a ESI interface by the method of Dueñas et al. (2002).

Identification of compounds was carried out by comparison of the retention times and UV spectra with those of standards and confirmed by HPLC-MS. Compounds for which no standards were available, such as hydroxycinnamic acid derivatives and proanthocyanidins were identified by their UV spectral parameters and by HPLC-MS (ESI) (Dueñas et al., 2002).

Quantification was made using the external standard method at 280 and 340 nm according to the maximum of absorption for each compound. The hydroxycinnamic acid derivatives were quantified using the corresponding free acid calibration curve and proanthocyanidins were quantified as (+) catechin.

Table 1
Optimal conditions of enzymes to act on lentil flours

Enzymes	*T* (°C)	pH	Incubation time (min)	Concentration (mg/g substrate)
Phytase[a]	37	5.5	60	1.60
α-Galactosidase[b]	37	5.5	90	1.16
Viscozyme[c]	37	5.5	60	1.50
Tannase[d]	37	5.5	120	0.50

[a] Frías et al. (2003a).
[b] Frías et al. (2003b).
[c] Antezana (2002).
[d] Dueñas (2003).

2.1. Antioxidant activity

In the methanol solution the antioxidant activity (EC_{50}) was determined by the Brand-Williams method (Brand-Williams, Cuvelier, & Berset, 1995), with 2,2′diphenyl-1-picrylhydrazyl (DPPH) radical, adapted to the legume samples by López-Amorós, Lomas, Estrella, and Hernández (1998). The reaction was carried out with 2 ml of a methanol solution of $DPPH^{\bullet}$ (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 remained DPPH against the sample concentration was plotted to obtain the amount of antioxidant (mg of legume flours) necessary to decrease by 50%. A smaller EC_{50}value corresponds to a higher antioxidant activity. The samples were prepared and measured separately in triplicate.

2.2. Statistical analyses

Data were subjected to multifactor ANOVA using the least-square differences test (LSD, 5% level) and multiple correlation with the Statgraphics Plus 5.0 Program (Statistical Graphics Software System, Rockville, MD, USA).

3. Results

3.1. Modification of the phenolic composition by the action of exogenous enzymes

Hydroxybenzoic and hydroxycinnamic compounds, flavonols and flavones, stilbenes and flavanols, are identified and quantified in the raw and enzymatic treated lentil flours. These compounds had been previously identified in the seed coat and the cotyledon of Pardina lentils (Dueñas et al., 2002; Dueñas et al., 2003). In Tables 2 and 3, are shown the changes observed in these compounds by the action of the exogenous enzymes.

Hydroxybenzoic compounds. After the treatment with the enzymes phytase, α-galactosidase or tannase the formation of gallic acid was observed, a compound not detected in the raw lentils. This acid is produced in similar concentrations by treatment with tannase or phytase. Gallic aldehyde is observed after all of the enzymatic treatments, reaching the maximum concentration with the addition of phytase

Table 2
Effect of addition of commercial enzymes on the phenolic composition (μg/g dry matter) of lentil flours

Compounds	Raw lentils	Phytase	α-Galactosidase	Viscozyme	Tannase
Hydroxybenzoic and Hydroxycinnamic compounds					
Gallic acid	nd[a]	0.80 ± 0.03^{d}	0.66 ± 0.02^{bc}	nd[a]	0.83 ± 0.07^{d}
Gallic aldehyde	nd[a]	2.36 ± 0.17^{e}	1.61 ± 0.12^{bc}	1.44 ± 0.08^{b}	1.86 ± 0.05^{d}
Protocatechuic acid	1.45 ± 0.03^{c}	1.36 ± 0.06^{c}	1.18 ± 0.09^{ab}	1.29 ± 0.04^{bc}	2.72 ± 0.11^{e}
p-Hydroxybenzoic acid	3.25 ± 0.18^{d}	2.48 ± 0.16^{c}	1.57 ± 0.10^{a}	1.49 ± 0.12^{a}	2.00 ± 0.09^{b}
trans-p-Coumaroyl-malic acid	10.02 ± 1.07^{c}	2.69 ± 0.18^{ab}	2.53 ± 0.09^{ab}	1.97 ± 0.14^{a}	2.64 ± 0.62^{ab}
trans-p-Coumaroyl-glycolic acid	2.88 ± 0.15^{d}	0.93 ± 0.06^{a}	1.26 ± 0.08^{bc}	0.97 ± 0.04^{a}	1.31 ± 0.15^{c}
trans-p-Coumaric acid	5.74 ± 0.45^{d}	4.20 ± 0.23^{c}	2.74 ± 0.13^{b}	1.92 ± 0.12^{a}	3.87 ± 0.28^{c}
cis-p-Coumaric acid	0.73 ± 0.09^{c}	0.35 ± 0.03^{ab}	0.46 ± 0.06^{b}	0.66 ± 0.04^{c}	0.45 ± 0.06^{ab}
trans-Ferulic acid	0.74 ± 0.07^{cd}	0.62 ± 0.02^{b}	0.91 ± 0.06^{e}	0.34 ± 0.04^{a}	0.84 ± 0.05^{de}
Flavonols and flavones					
Myricetin 3-O rhamnoside	5.79 ± 0.44^{d}	0.40 ± 0.01^{b}	0.69 ± 0.03^{bc}	t[a]	0.87 ± 0.06^{c}
Luteolin 7-O glucoside	1.29 ± 0.07^{e}	1.04 ± 0.09^{d}	0.70 ± 0.03^{b}	0.39 ± 0.02^{a}	0.77 ± 0.04^{bc}
Apigenin 7-O apiofuranosyl glucoside	6.20 ± 0.45^{c}	3.25 ± 0.40^{ab}	3.73 ± 0.19^{b}	2.25 ± 0.17^{a}	5.23 ± 1.00^{c}
Apigenin 7-O glucoside	1.87 ± 0.07^{c}	t[a]	0.43 ± 0.03^{b}	nd[a]	1.79 ± 0.09^{c}
Luteolin glycoside	1.35 ± 0.19^{f}	0.97 ± 0.05^{de}	1.06 ± 0.09^{e}	0.76 ± 0.07^{bcd}	0.53 ± 0.05^{a}
Quercetin 3-O-rutinoside	5.24 ± 0.56^{a}	11.68 ± 1.55^{c}	8.28 ± 0.63^{b}	6.73 ± 0.36^{ab}	12.27 ± 2.03^{c}
Luteolin	0.33 ± 0.02^{b}	1.24 ± 0.12^{d}	0.19 ± 0.02^{ab}	0.81 ± 0.04^{c}	1.49 ± 0.23^{d}
Stilbenes					
trans-Resveratrol 3-O-glucoside	0.93 ± 0.09^{d}	0.42 ± 0.03^{b}	0.39 ± 0.06^{b}	0.23 ± 0.02^{a}	0.59 ± 0.04^{c}
trans-Resveratrol	nd[a]	0.34 ± 0.05^{bc}	nd[a]	nd[a]	0.41 ± 0.07^{c}

nd, not detected; t, traces.
The same letters in the same row are not significantly different ($p \leqslant 0.05$).
[a] Values are the means of two determinations ± standard deviation ($n = 2$).

Table 3
Effect of addition of commercial enzymes on the proanthocyanidins (μg/g dry matter) of lentil flours

Compounds	Raw lentil	Phytase	α-Galactosidase	Viscozyme	Tannase
Prodelphinidin trimer (2GC-C)	31.05 ± 1.56^{c}	5.69 ± 0.26^{b}	5.80 ± 0.35^{b}	3.74 ± 0.10^{a}	5.10 ± 0.44^{ab}
Prodelphinidin dimer (GC-C) (1)	8.03 ± 0.98^{d}	2.98 ± 0.36^{b}	4.86 ± 0.18^{c}	0.37 ± 0.03^{a}	2.27 ± 0.10^{b}
Procyanidin digallate dimer	1.26 ± 0.15^{c}	4.49 ± 0.24^{d}	0.58 ± 0.01^{a}	0.51 ± 0.03^{a}	0.96 ± 0.07^{b}
Prodelphinidin dimer (GC-C) (2)	6.02 ± 1.00^{e}	2.08 ± 0.24^{c}	2.91 ± 0.12^{d}	nd[a]	0.93 ± 0.05^{b}
Dimer B_3	10.61 ± 1.07^{d}	2.48 ± 0.20^{a}	5.31 ± 0.73^{b}	2.06 ± 0.09^{a}	7.33 ± 0.55^{c}
Procyanidin trimer (PT1)	13.16 ± 1.12^{c}	2.03 ± 0.13^{b}	1.82 ± 0.31^{b}	0.45 ± 0.01^{a}	1.91 ± 0.28^{b}
(+)-Catechin 3-O glucose	31.50 ± 1.98^{c}	16.33 ± 1.56^{a}	17.18 ± 1.24^{a}	17.09 ± 1.96^{a}	25.15 ± 1.69^{b}
(+)-Catechin	0.77 ± 0.05^{d}	0.54 ± 0.04^{c}	t[a]	0.15 ± 0.01^{b}	0.19 ± 0.03^{b}
Dimer B_2	13.80 ± 0.96^{d}	6.64 ± 0.21^{c}	6.27 ± 0.59^{c}	2.82 ± 0.11^{a}	3.92 ± 0.27^{b}
Procyanidin trimer (PT2)	0.29 ± 0.05^{b}	0.17 ± 0.02^{a}	1.82 ± 0.02^{d}	0.16 ± 0.00^{a}	0.69 ± 0.06^{c}
Procyanidin trimer (PT3)	1.61 ± 0.23^{e}	0.51 ± 0.04^{b}	1.38 ± 0.19^{de}	0.26 ± 0.08^{a}	0.70 ± 0.06^{c}
(−)-Epicatechin	4.17 ± 0.69^{e}	0.85 ± 0.05^{ab}	2.41 ± 0.26^{d}	0.64 ± 0.03^{a}	2.01 ± 0.20^{cd}
Procyanidin trimer (PT4)	1.55 ± 0.06^{c}	2.43 ± 0.16^{d}	1.29 ± 0.19^{b}	0.63 ± 0.05^{a}	1.30 ± 0.13^{b}

GC (epi)galocatechin, C (epi)catechin, nd, not detected; t, traces.
The same letters in the same row are not significantly different ($p \leqslant 0.05$).
(1) and (2) are two different prodelphinidin dimers; PT1,PT2, PT3 and PT4 are different procyanidin trimers.
[a] Values are the means of two determinations ± standard deviations ($n = 2$).

(2.36 μg/g). Protocatechuic and *p*-hydroxybenzoic acids decrease in each of the treatments (Table 2), except in the treatment with tannase, in which an increase of 47% in protocatechuic acid is observed. In spite of the general decrease of these compounds, with respect to the raw lentil, the action of phytase seems to bring out the higher concentrations of gallic aldehyde and *p*-hydroxybenzoic acid.

Hydroxycinnamic compounds. These compounds, in general, decrease with all the enzymes, particularly their derivatives (Table 2). The *trans p*-coumaroylmalic acid suffers the most pronounced decrease (69–80%), and the greatest reduction is observed after viscozyme treatment. *Trans p*-coumaroylglycolic acid also decreases but in a smaller percentage (54–67%) and the smallest decrease is observed after treatment with tannase. The decrease of the hydroxycinnamic derivatives, *trans p*-coumaroylmalic and *trans p*-coumaroylglycolic acids, is not correlated with an increase of the corresponding free acid, *trans p*-coumaric acid, since it also decreases in all treatments, albeit at a smaller rate (26.8–66.5%), and the smallest concentration is observed after viscozyme treatment. The behaviour of *trans*-ferulic acid is different, it decreases after treatment with phytase and viscozyme and increases with treatment with tannase and α-galactosidase (14 and 23%, respectively).

Table 4
Antioxidant activity[a] (EC_{50}) of raw lentil flours and after the treatment with enzymes

Samples	Raw	Phytase	Viscozyme	α-Galactosidase	Tannase
Lentils	3.16 ± 0.10[c]	3.41 ± 0.07[d]	2.16 ± 0.04[a]	2.29 ± 0.05[a]	2.50 ± 0.08[b]

Means ± SD ($n = 3$); means followed by the same letter in row are not significantly different (LSD, 5%).
[a] A smaller value of EC_{50} correspond with a higher antioxidant activity.

Flavonols and flavones. The flavonol glycosides, myricetin 3-O rhamnoside and quercetin 3-O rutinoside, have a similar concentration in the raw lentil (5.2–5.7 μg/g) but the changes after enzymatic treatment depend on the enzyme (Table 2). The concentration of myricetin 3-O-rhamnoside decreases with every treatment, but the concentration of quercetin 3-O rutinoside shows great increases in all the enzymatic assays although with differences depending on the enzymes; the greatest increase (134%) is after treatment with tannase.

The flavone glycosides, luteolin 7-O glucoside, apigenin 7-O apiofuranosyl glucoside, apigenin 7-O glucoside and an unidentified luteolin glycoside generally decrease in all of the treatments when compared to the raw lentil. However, we need to point out the increase experienced by the flavone luteolin with all the enzymes except with α-galactosidase; the greatest increase is with tannase (77%). Luteolin increase might come from hydrolysis of its glycosides by enzymatic action.

Stilbenes. Trans-resveratrol 3-O glucoside, which is initially found in raw lentils, decreases upon enzymatic treatment. The corresponding simple form, *trans*-resveratrol is only detected in flours treated with phytase and tannase, but not in raw lentils, reaching the highest concentrations with tannase (Table 2). No relationship has been observed between the decrease of the glucoside and the concentration of the free form.

Catechins and proanthocyanidins. Catechins, procyanidins and prodelphinidins, generally experience a decrease in relation to the raw lentil in all the enzymatic treatments (Table 3), although the decrease is different depending on the enzyme. (+)-Catechin 3-O glucose, a flavanol monomer which is the major compound in the raw lentil, greatly decrease after all the enzymatic treatments, between 20% and 60%, but it remains in all cases as the major flavanol. In the case of proanthocyanidins, an increase in the concentration is only observed in the procyanidin digallate dimer after the phytase treatment and in two of the procyanidin trimers, PT2 after the acting of α-galactosidase and phytase and PT4 only with phytase.

3.2. Antioxidant capacity of lentil flours after enzymatic treatment

Evaluation of the antioxidant capacity was carried out after noting the changes in the polyphenolic composition of the lentil flours after the enzymatic treatments, in order to find out the changes as a function of the enzyme used, considering the relationship of these compounds with the free radical scavenging activity.

An increase of the antioxidant activity is observed in flours treated with viscozyme, α-galactosidase and tannase, but no significant differences ($p \leqslant 0.05$) are found between the treatment with viscozyme or α-galactosidase (Table 4). Treatment with phytase causes a decrease of the antioxidant activity.

The free radicals scavenging capacity (EC_{50}) of the processed lentils seems to be influenced not only by the changes observed in their phenolic composition, but also by other antioxidant components present in the seeds, such as vitamins and carotenoids.

The antioxidant capacity of polyphenolic compounds is related to their chemical structure, so in order to relate antioxidant activity to the polyphenolic compounds identified in the lentil flours, an analysis of principal components was carried out. Previously, a correlation analysis was carried out among all the identified compounds, in order to find out which had the highest correlation with the treatments with phytase, viscozyme, α-galactosidase or tannase.

Principal component analysis was done on the values of the antioxidant activity (EC_{50}) and the concentrations of the compounds which showed the highest correlation with the four treatments: *p*-hydroxybenzoic acid, *trans p*-coumaroylmalic acid, myricetin 3-O rhamonoside, quercetin 3-O rutinoside, luteolin 7-O glucoside, luteolin glycoside (+) catechin, prodelphinidin trimer (2GC-C), prodelphinidin dimer (2) and procyanidin trimer (PT1).

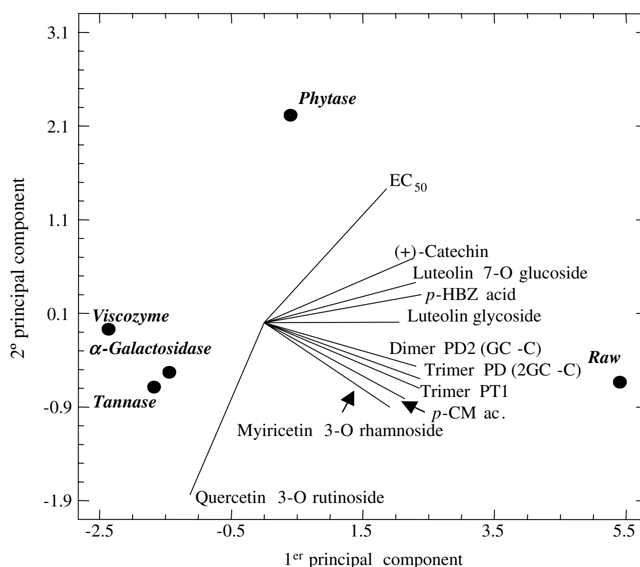

Fig. 1. Plot of the principal components of the polyphenolic compounds and antioxidant activity (EC_{50}) of the lentil flours before and after the treatment with the enzymes, phytase, tannase, α-galactosidase or viscozyme. HBZ, hydroxybenzoic; PD, prodelphinidin; PT, procyanidin timer; CM, coumaroylmalic; GC, gallocatechin; C, catechin.

Four components were obtained of which the first two accounting for 91.1% of the total variance. Fig. 1 shows the graphic representation of the first two components.

The treatments with viscozyme, α-galactosidase and tannase are the ones which appear to be more positively related to the antioxidant activity, which means that these treatments produce an increase of such activity when compared to raw lentils, taking into account the inverse expression of EC_{50}. There are not either any great differences among these three enzymes, but there are some with phytase treatment. The treatments with viscozyme, α-galactosidase and tannase are close to quercetin 3-O rutinoside, which appears to be the compound with the greatest influence on the EC_{50} values and which is also the compound that shows the greatest increase versus the raw lentils.

4. Discussion

The addition of enzymes to lentil flours in a semi-pilot scale produces substantial changes in the concentration of phenolic compounds. These changes are different for each enzyme and depend on the type of compound. In these processes the endogenous enzymes of the flours must also be considered since they regulate different metabolic reactions which might involve the phenolic compounds. Dueñas (2003) observed this effect in lentil flours treated with tannase, by comparing samples without tannase addition, under the same conditions.

Among the hydroxybenzoic compounds gallic acid and gallic aldehyde must be remarked on, they are a product of the esterase action of the enzymes used on the galloylated compounds present in lentils, and also of oxidative non-enzymatic reactions of existing amino acids, such as phenylalanine, under existing conditions of pH, temperature and aqueous medium (Dewick & Haslam, 1969; Haslam, 1998).

The enzyme tannase causes a great increase in the amount of protocatechuic acid which might be produced from other phenolic structures, since this acid is an intermediate in the metabolism of other phenolic acids, such as vanillic, ferulic etc., by some enzymes (Cartwright & Smith, 1967). On the other hand some authors (Alberto, Gómez-Cordovés, & Manca de Nadra, 2004; Hopper & Mahadevan, 1997) have observed that the protocatechuic acid is an intermediate in the (+) catechin degradation, which could also influence the observed increase of this acid.

Among all the identified compounds the hydroxycinnamic ones are those most decreased, being the greatest after viscozyme treatment. In the case of other processes studied with Pardina lentil flours, such as fermentation (Bartolomé, Hernández, & Estrella, 1997) and germination (López-Amorós et al., 2006), a great decrease of this type of compounds was also observed, which might be due to the fact that they can react with hydroxyacids forming derivatives.

The treatment of lentil flours with phytase, α-galactosidase, viscozyme and tannase causes changes in the antioxidant activity. Treatment with viscozyme produces flours with greater antioxidant activity than those with tannase and α-galactosidase. Viscozyme, an enzymatic complex capable of acting on a great number of components, fundamentally acts on the soluble and insoluble fiber fraction (Antezana et al., 2003), and this causes modifications of several components and can release antioxidant compounds, different from the phenolic compounds, such as vitamins and carotenoids, which have an effect on the evaluation of the antioxidant activity.

The fact that the treatment with phytase produces flours with lower antioxidant activity might be due to the phytase acting on phytic acid, decreasing its concentration and liberating into the medium the phosphate groups and cations which such phosphates contain (Beal & Mehta, 1985; Frías et al., 2003b). Phytic acid is considered to be an antioxidant compound because it is a potent inhibitor of iron-catalysed hydroxyl radical formation by chelating free iron (Empson, Labuza, & Graf, 1991). On the other hand, the liberated cations might favour the oxidation of different components of the medium. These two facts could be the cause of lower antioxidant activity.

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). Studies carried out by other authors on these same lentil flours have shown that phytase treatment produced flours with low content of IP6 and IP5, and remarkable contents of IP4 and IP3 (Frías et al., 2003a) and the α-galactosidase treatment caused a decrease in the α-galactosides (Frías et al., 2003b). The previous results and the ones obtained in this work appear to indicate that the incubation of Pardina lentil flours with tannase, α-galactosidase or phytase would be appropriate for improving their functionality and obtaining flours with a greater added value.

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