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Research Article

Changes in Protein, Nonnutritional Factors, and Antioxidant Capacity during Germination of *L. campestris* Seeds

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The changes in SDS-PAGE proteins patterns, oligosaccharides and phenolic compounds of *L. campestris* seeds, were evaluated during nine germination days. SDS-PAGE pattern showed 12 bands in the original protein seeds, while in the samples after 1–9 germination days, the proteins located in the range of 28–49 and 49–80 kDa indicated an important reduction, and there was an increase in bands about 27 kDa. On the other hand, oligosaccharides showed more than 50% of decrease in its total concentration after 4 germination days; nevertheless after the fifth day, the oligosaccharides concentration increases and rises more than 30% of the original concentration. Phenolic compounds increased their concentration since the first germination day reaching until 450% more than the original seed level. The obtained results are related with liberation or increase of phenolic compounds with antioxidant properties, allowing us to suggest that the germination would be used to produce legume foods for human consumption with better nutraceutical properties.

1. Introduction

Legume seeds are important staple foods, particularly in developing countries, due to their relatively low cost, long conservation time, and high nutritional value; among these meals it is *Lupinus* seeds and their derivatives. This legume is one of the richest sources of vegetable protein, and although the protein content and amino acid profile vary between species, the intraspecies variability is low. In 2009, the FAOST reported that the area harvested was 662712 Ha, and *L. albus* and *L. angustifolius* were the most widely used. About 100 wild species have been reported throughout México [1]. These wild lupins have not been exploited at a commercial level. For this reason, in the present work we consider them as potential providers of vegetable proteins for human consumption. *Lupinus campestris* seed, like other *Lupinus* species, has high protein content (44%) [1, 2]. Lupin seeds offer some advantages in comparison with soy bean, since it contains only small amounts of trypsin inhibitors, tannins, phytates, saponins, α-galactosides, and so forth [3, 4]. However, a limitation for the wider use of lupins has been their high content of quinolizidine alkaloids [5, 6] as well as condensed tannins [7, 8]. Consequently, it is desirable to develop transformation processes which could improve the nutritional quality of legumes and also provide new derived products for the consumers. Germination is considered a potentially beneficial process for legume seed transformation which may decrease undesirable components such as alkaloids and phytates [9], and during germination, some grade of transformation of alkaloids to other more bioactive compounds, such as esters, occurs [7]. Cuadra et al. [3] and De Cortes-Sánchez et al. [7] found a slight increase in alkaloids during germination of *L. albus, L. angustifolius*, and *L. campestris*, and no α-pyridone alkaloids, such as the highly toxic anagyrine and cytosine, were detected in any of these species. Germination also increases nutrients such as vitamin C [10] and increase protein digestibility [11], consequently improving nutritional quality. Additional advantages of germination are reduction in cooking time and improvement of the product sensorial attributes [11]. Germination has been shown to decrease the level of α-galactosides of different legume seeds including soybean, black bean, and lupin seed, with the corresponding decrease in carbohydrates available for fermentation in the large human intestine. The content of trypsin inhibitors and phytates is also decreased, but considerable amounts of these factors are still present after germination [6]. On the other hand, it is widely accepted that antioxidant activity of food is related to high phenolic content. Phenolic compounds are capable of removing free radicals, chelating metal catalysts, activating antioxidant enzymes, and inhibiting oxidases [12]. Legume seeds are a rich source of many substances with antioxidant properties, including plant phenolics. *Lupinus* is a potential source of bioactive components with antioxidant activities. Although the interest in *Lupinus* species as a valuable component of functional food is increasing and has let to investigate on the determination of antioxidant activity in *Lupinus* seeds and its products, the information is scarce [13, 14]. The objective of this work was to evaluate the original content of proteins, oligosaccharides, and phenolic compounds, the antioxidant capacity in *Lupinus campestris* seed, and the changes of these parameters during the germination process.

2. Material and Methods

2.1. Samples and Germination Process. L. campestris seeds (wild type) were collected along 50 km of the Oaxtepec-Xochimilco highway in the Morelos State, México.

Germination process was performed as described by De Cortes-Sánchez et al. [7]. Briefly, 800 *Lupinus campestris* seeds were used for the germination assay distributed in 10 trays, with 80 seeds each one. The seeds were spread on a moist sheet of filter paper (Albet 1516, 42–52 cm) and covered with another sheet of moist filter paper. They were put into a germination chamber under environmentally controlled conditions: 20°C, 8 h of light per day exposure, and watering of the seeds during germination keeps the paper always wet. Samples (80 seeds/tray) were taken at 0 (control), 1, 2, 3, 4, 5, 6, 7, 8, and 9 germination days. The germination process was repeated twice, and the germination capacity was evaluated by germination percentage and seed weights. Samples for analysis were constituted by germinated and moist seeds, discarding those that did not show any water absorption during the process. The germinated seeds were freeze-dried, milled, and passed through a sieve of 0.5 mm. The germinated flour was stored in darkness in a desiccator at 4°C until analysis.

2.2. Gel Electrophoresis. Denaturing gel electrophoresis (SDS-PAGE) was carried out according to the method of Schagger and von Jagow [15] using 10% polyacrylamide gels in the presence of 1% SDS; the proteins (1 μg) were loaded with or without β-mercaptoethanol. Standards used were phosphorylase B (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (20.1 kDa), and lysozyme (14.4 kDa).

2.3. Extraction and Quantification of Carbohydrates (CH). The method of Muzquiz et al. [16] was used for CH extraction. 0.1 g of grounded seeds was homogenized with aqueous ethanol solution (50% v/v, 5 mL) for 1 min at 4°C. Then the mixture was centrifuged for 5 min (2100 ×g) at 4°C, and the supernatant was recovered. The procedure was repeated twice, and the combined supernatants were concentrated under vacuum at 35°C. The concentrated supernatant was dissolved in deionized water (1 mL) and passed through a Waters minicolumn (Waters C-18 at 500 mg/cc) with Supelco vacuum system.

Samples (20 μL) were analyzed using a Beckman HPLC chromatograph f156 with refraction index detector. A Spherisob-5-NH_2 column (250 × 4.6 mm id) was used with acetonitrile: water (65 : 35, v/v) as the mobile phase at a flow rate of 1 mL min^{-1}. Individual sugars were quantified by comparison with standards of sucrose, raffinose, stachyose, and verbascose. Calibration curves were prepared for all these sugars, and a linear response was obtained for the range of 0–5 mg/mL with a determination coefficient (r^2) > 0.99.

2.4. Extraction and Quantification of Phenolic Compounds (PC). 1 g of sample was extracted with 10 mL methanol previous to phenolic determination. Total phenols content was estimated by using the Folin-Ciocalteu colorimetric method [17]. Briefly, the 0.02 mL of the extracts was oxidized with 0.1 mL of 0.5 N Folin-Ciocalteu reagent, and then the reaction was neutralized with 0.3 mL sodium carbonate solution (20%). The absorbance values were obtained by the resulting blue color measured at 760 nm with a Beckman spectrophotometer (California, USA) model DU-65 after incubation for 2 h at 25°C. Quantification was done on the basis of a standard curve of gallic acid. Results were expressed as mg of gallic acid equivalent per 1 g of dry weight.

2.5. TLC Analysis of Phenolic Compounds. TLC was performed on TLC sheets coated with 0.25 mm layers of silica gel 60 F254 (E. Merck, number 5554). Two mobile phases were used: ethyl acetate-formic acid-ethanol (65 : 15 : 20, v/v/v) and 1-butanol-acetic acid-water (7 : 0.5 : 2.5, v/v/v), upper phase. The chromatograms were evaluated in UV light at 360 nm before spraying them with 10% sulphuric acid [18].

2.6. HPLC Analysis of Phenolic Compounds. HPLC analysis was performed on an Agilent Technologies 1200 series liquid chromatograph (G1311A quaternary pump, UV-VIS DAD G1315D detector, ALS G1329A injector, G1322A Deggaser, and TCC G1316A thermostat column), equipped with a Zorbax Eclipse XDB-C18 column (150 × 4.6 mm, 5 mm particle size) (Agilent Technologies, USA), and thermostated at 30°C. A gradient elution was used to separate the extracted phenolics. Solvent (A) was 5.0% formic acid in water, and solvent (B) was acetonitrile. Elution was performed at a solvent flow rate of 1.0 mL/min. The gradient profile of the system was 0% solvent B at the initial stage, 0% solvent B at 3 min, 30% solvent B at 5 min, 60% solvent B at 20 min, 100% solvent B at 25 min, and 0% solvent B at 30 to 35 min.

The eluted phenolic compounds were monitored at 280 nm. Quantitative levels were determined by comparing with a catechin standard curve. Phenolic concentration was expressed as mg catechin equivalent per gram of dry sample.

2.7. Free Radical DPPH Scavenging Capacity. 2,2-diphenyl-1-picrylhydrazyl (DPPH) is a free radical used for assessing antioxidant activity. Reduction of DPPH by an antioxidant or by a radical species results in a loss of absorbance at 515 nm. PC extracts were adjusted at a concentration of 0.24 mg gallic acid equivalent/mL prior to antioxidant capacity evaluation. Determination of antioxidant capacity, previously adapted for microplates [19], was performed as follows: 0.02 mL of extract (500 μM gallic acid equivalent) or standard (gallic acid, 500 μM) was added to a 96-well flat-bottom plates containing 0.2 mL of DPPH solution (125 μM DPPH in 80% methanol). Samples were prepared in triplicate. The plate was covered, left in the dark at room temperature, and read after 90 min in a visible-UV microplate reader (680 XR Microplate Reader, Bio-Rad Laboratories, Inc) using a 520 nm filter. Data are expressed as a percentage of DPPH· discoloration [20].

2.8. Statistical Analysis. All analyses were carried out in triplicate, and the report data are the average of the results and the standard error in each case.

3. Results

3.1. Germination. In Figure 1 it is shown the germination capacity expressed as percent of germinated seeds. This germination percentage increases from day 1 to day 4, and after that, no significant increase in germination is observed.

The gain in weight is observed in Figure 1, an increase in weight can be observed since the first day, this weight augmentation was due mainly to the water that has shrunk, and the germination percentage was 5% only. The total increase in weight was three times plus from the initial weight of the seed. By the second day, the germination and the weight have increased to 27% and 14 g/80 seeds (Figure 1), and additionally root has left the head. The greatest increment in the number of germinated seeds (from 27% to 82%) is observed between the second and third day. At the fourth day, 98% of the seeds showed development of the stem and root. A maximum germination percentage of 100% was obtained, which shows the good viability of *L. campestris*. These results agree with De Cuadra et al. [3] who reported a high degree of germination (up to 100%) for two *Lupinus* species.

3.2. Electrophoretic Analysis. Figure 2 shows the electrophoretic profile of *L. campestris* seeds subjected to different germination times. As it is observed the seed without any germination time showed greater amount of protein bands located between 20 and 75 kDa. As the germination time

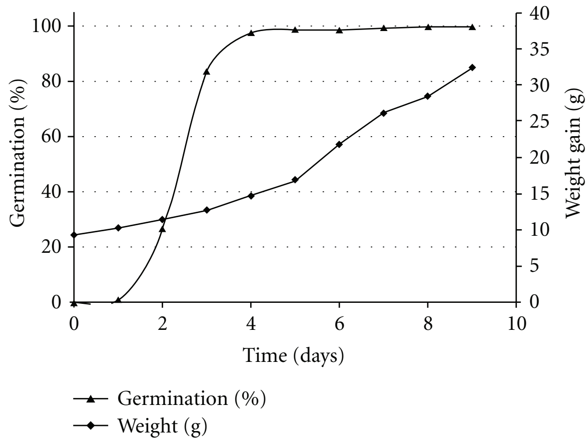


Figure 1: Germination (%) and weight gain (g) of the *L. campestris* seeds. The results represent the average of three independent experiments ± S. E.

advances, the proteins located in the range of 28–49 and 49–75 kDa almost disappeared after nine germination days of Lupinus seed, and there was an increase in bands about 27 kDa. These results confirm previous findings about storage proteins, which are hydrolysed and mobilised after germination [21, 22]. This behavior lets us to suggest that the principal storage protein molecules, the globulins 7 s, and 11 s constituted by three and six subunits, respectively, were hydrolyzed in lower molecular weight compounds which has a best digestibility and consequently a better biological value.

3.3. Changes of Oligosaccharides in L. campestris Seeds during the Germination. The *L. campestris* germinated seeds were also evaluated as for the variation of present oligosaccharides. The obtained results are showed in Table 1. The concentration of total oligosaccharides in the seed without germinating was of 90.26 mg/g; this concentration was diminished near 15% in the first day, and then 25, 46, and 58% in the period were comprised since the second to the fourth germination day. Then oligosaccharide concentration increased its value from the fifth to the ninth day, reaching 30% above than the original content. The composition of oligosaccharides varies during the germination process. In the seed without treatment, the sucrose was present with an initial content of 21.45 mg/g increasing to 55.36 mg/g at five days of germination. Since the sixth day, sucrose diminished until reaching 14.84 mg/g of seed at the nine day of germination. This increase in sucrose concentration can be due to hydrolysis of oligosaccharides by the α-galactosidase enzyme, which selectively acts on the galactosides such as raffinose, stachyose, and verbascose releasing sucrose [23]. Muzquiz et al. [16] has reported a similar behavior in other species of *Lupinus*. After the fourth day of germination, the oligosaccharides proportion has increased substantially, mainly in the stachyose percentage, which is almost twice of the originally presented. Even though there is a substantial reduction of these carbohydrates, they are not totally eliminated, since it has been informed for other species of *Lupinus* whose diminution is bigger than 80–100% after four days

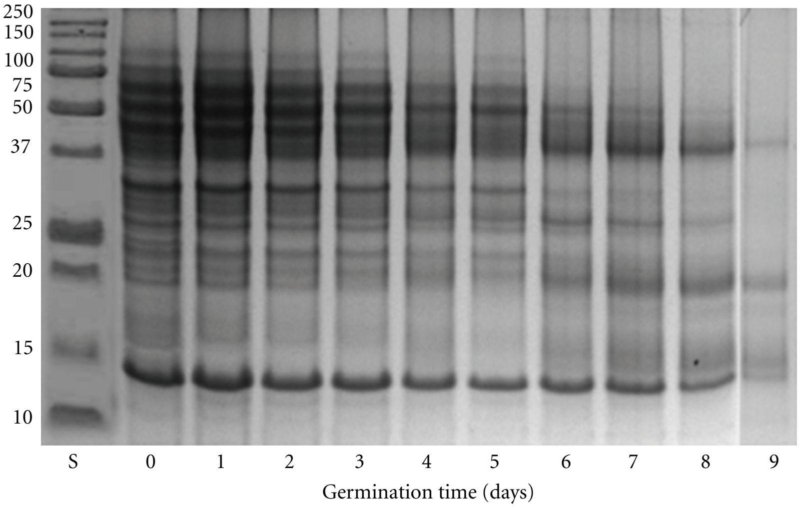


Figure 2: Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of present proteins in the *L. campestris* germinated seed. S = standards (kDa).

Table 1: Behavior of carbohydrates of the *L. campestris* germinated seed (mg/g of seed).

Time (days)	Sucrose	Raffinose	Stachyose	Verbascose	Total oligosaccharides
0	21.45 ± 0.76	13.65 ± 0.89	57.16 ± 0.95	19.45 ± 0.59	90.26 ± 0.73
1	26.49 ± 3.86	8.51 ± 0.51	54.35 ± 3.38	11.48 ± 2.14	74.34 ± 1.17
2	32.21 ± 1.80	7.92 ± 0.51	49.10 ± 0.51	10.94 ± 0.55	67.96 ± 0.02
3	34.38 ± 1.37	6.49 ± 0.68	36.75 ± 1.06	5.53 ± 1.06	48.77 ± 0.03
4	46.14 ± 0.34	4.03 ± 1.03	29.99 ± 0.13	6.78 ± 0.12	40.80 ± 0.07
5	55.36 ± 5.15	7.08 ± 1.85	47.95 ± 0.69	8.44 ± 0.69	63.47 ± 0.65
6	35.09 ± 1.65	8.18 ± 2.23	65.17 ± 0.12	9.30 ± 0.12	82.65 ± 0.32
7	27.85 ± 1.45	10.19 ± 1.01	84.75 ± 0.57	9.64 ± 0.58	104.58 ± 0.13
8	22.95 ± 1.57	11.38 ± 0.10	93.09 ± 0.24	10.82 ± 0.24	115.29 ± 0.02
9	14.84 ± 3.76	11.34 ± 4.23	98.17 ± 2.53	11.93 ± 0.23	121.44 ± 0.30

*The values represent the average of two separated germinations with extractions made by triplicate ± S.E.

of germination [3, 24]. This difference can be due to the germination conditions in which the seeds were carried out. With the obtained results it is observed that the germination diminishes the concentration of oligosaccharides, being the lower value in the fourth day.

3.4. Phenolic Compounds in Seed of L. campestris during the Germination. Total phenolic compounds concentrations in the germinated *L. campestris* seed during nine germination days are presented in Figure 3. Control seed, without germination, presented 5.27 mg gallic acid equivalent per g of seed. This value remained almost constant during the days one and two. After that, the concentration of total phenolics increased gradually reaching twice the original value. However, phenolic content was in the range reported for other legumes such as yellow pea, green pea, lentils, common beans, and soybean [25] and similar to the content in other *Lupinus* species [26]. Contrary to the behavior of the oligosaccharides, the phenolic compounds increase as the time of germination occurs. The behavior shown for *Lupinus* germinated seeds differs from the observed by Muzquiz et al. [27] who indicated a reduction of 76% in phenolics from lentil but is similar to *Cajanus* seed [28] which showed a fivefold increment in total phenolic content during a period of five germination days.

3.5. Phenolic Compounds by HPLC. Although individual phenolics remain unidentified, they were quantified on the basis of a catechin calibration curve. In Table 2, the changes in composition and quantity of phenolics as determined by HPLC are shown.

There are two main groups of peaks. The first one which is presented since cero day increases at seven and eight days and decrease a little in the ninth day. This group is formed by peaks marked as peak 1 to 6 and peak number 10. The other group of peaks appears between the third and fourth day of germination, increases its concentration at the same days the other group does, and also diminished at nine day. This group is formed by peaks number 7–9 and 11–15 (Table 2). As the time of germination occurs, the complexity and quantity of total phenolics increase, being the germinated seeds on seven to nine day more complex in composition than no germinated seeds or those on the first germination days. Also, seeds in the seventh day are the richest in total phenolic composition.

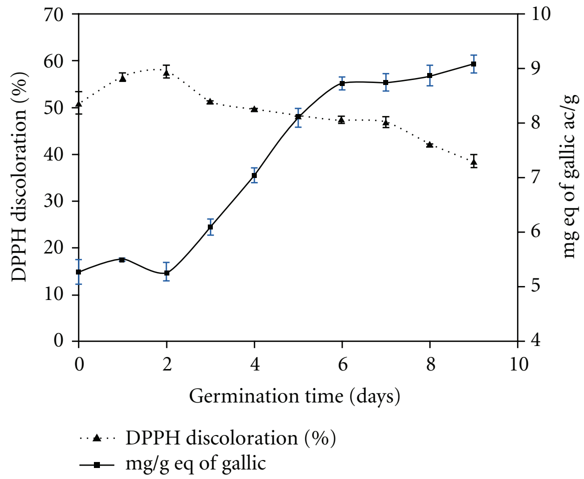


Figure 3: Phenolic compound variation and antioxidant capacity in *L. campestris* germinated seed.

Concentration of total phenolics by HPLC is lower than the obtained by the Folin-Ciocalteu method. Considering the heterogeneity of natural phenols and the possibility of interference from other readily oxidized substances such as ascorbic acid and mono- and disaccharides, this disagreement between methods is comprehensible [17, 29].

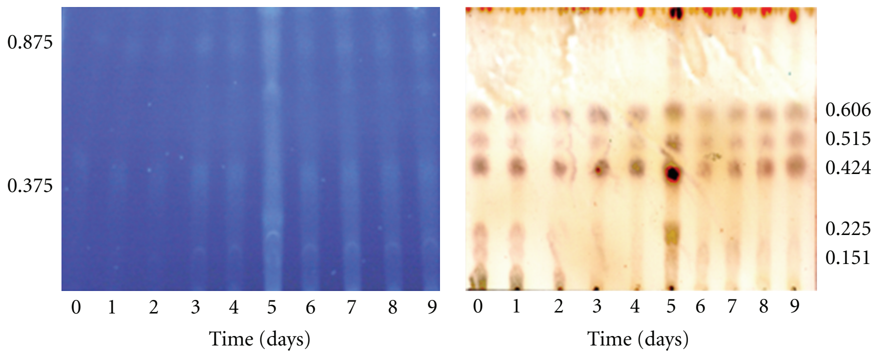
3.6. Antioxidant Capacity. All the extracts showed antioxidant capacity against DPPH-free radical, as measured by the decrease in absorbance at 520 nm. During the seeds germination, it was observed a light increase in antioxidant capacity nongeminated seeds until the second germination day (51–58%), followed by a continuing depression in antioxidant capacity until the ninth germination day (38%) (Figure 3). The initial antioxidant capacity (51%) is similar to that reported for other legumes [30]. An enhancement in antioxidant capacity by germination has been reported for *Lupinus albus* [14] and *Lupinus angustifolius* seeds [13] as measured in aqueous extracts. The *L. campestris* methanolic extracts showed a different behavior, which could be attributed to the kind of compounds that could be solubilized by methanol, and since water could solubilize other antioxidants such as vitamins. Fernandez-Orozco et al. [13] suggested that polyphenols extractability is better in buffer phosphate than in methanol. Correlations between antioxidant capacity toward DPPH-free radical and total polyphenols have been observed in beans [30] and in *L. angustifolius* germinated seeds [13]. In this study, polyphenol concentration did not correlate with antioxidant activity; while polyphenols increase as germination progress, antioxidant capacity decreases. It is interesting to note that polyphenol concentration was adjusted to 0.24 mg/mL in all samples, previously to antioxidant capacity determination. These results suggest again that it is composition but not concentration of polyphenols in the extracts, and possibly the presence of other antioxidants, which makes a difference in antioxidant capacity behavior. In order to confirm that composition affects antioxidant activity, the extracts were analyzed by TLC. The best profile was obtained with ethyl acetate-formic acid-ethanol (65 : 15 : 20, v/v/v), which is shown in Figures 4(a) and 4(b). 360 nm UV light shows that there is a spot with an Rf value of 0.375; although this yellowish fluorescent spot is in all samples, its relative intensity is bigger at the last germination days. There is another spot (Rf 0.875) that is present in all germination days (Figure 4(a)). On the other hand, the extracts would contain flavonoids and phenolic acids due to the yellow and the blue fluorescent bands under 360 nm UV light [18]. The Figure 4(b) shows the TLC plate revealed with sulphuric acid. There is a group of three spots at the medium of the plate in all extracts (Rf values = 0.424, 0.515, and 0.606); however it has higher intensity around five–seven days, this intensity suggests higher concentration of phenolics, as all the samples were applied in the same volume (Figure 4(b)). Another group of phenolics is observed in 0.031, 0.156, and 0.219 Rf values. The behavior of this second group differs from the previous one; the spots can be visualized in the seed without germination, and at one and two days, later the group is disappeared in the next two days, increased its intensity in fifth day, and once again, decreased in the last germination days. Changes in composition of the phenolic extract were confirmed by HPLC analysis, as described previously (Table 2) germination process increase, the complexity of the phenolic extract (Figure 4). There is a group of compounds around 25 min that should appear as a consequence of germination. According to the HPLC analysis these compounds must be lower polar, suggesting that their antioxidant activity could be less than more polar compounds.

The antioxidant activity of phenolic compounds is affected by their chemical structure. Structure-activity relationships have been used as a theoretical method for predicting antioxidant activity. Polymeric polyphenols are more potent antioxidants than simple monomeric phenolics: Hagerman et al. [31] demonstrated the higher antioxidant ability of condensed and hydrolyzable tannins at quenching peroxyl radicals over simple phenols; Yamaguchi et al. [32] observed that the higher the polymerization degree of flavanols, the stronger the superoxide-scavenging activity. A similar effect was reported for the capacity to inhibit the O_2^- radical, which increased with the degree of procyanidin polymerization [33].

The antioxidant activity also depends on the type and polarity of the extracting solvent, the isolation procedures, purity of active compounds, the test system, and substrate to be protected by the antioxidant [34].

4. Conclusion

The germination is a simple technological process of easy application and low cost. This process allows to the protein modification, obtaining peptides of low molecular weight and improving the nutritional quality. The oligosaccharides ones show diminution in the third germination day, nevertheless tend to increase as of the fourth day of this one process. On the contrary, the phenolic compounds concentration increases from the first day. With this, we can



(a) (b)

Figure 4: TLC analysis of phenolic compounds of *L. campestris* germinated seed eluted with a mixture of ethyl acetate-formic acid-ethanol (65 : 15 : 20, v/v/v) and revealed (a) UV light at 360 nm and (b) 10% sulphuric acid.

Table 2: Phenolic compounds in *L. campestris* germinated seeds by HPLC.

Peak number	Retention time (min)	Phenolic concentration, days of germination[1]									
		0	1	2	3	4	5	6	7	8	9
1	1.590	145.8	47.3	105.8	100.4	325.0	235.1	444.7	568.9	573.6	452.1
2	1.654	124.4	ND	76.4	118.4	238.9	252.1	347.0	499.2	498.0	408.9
3	1.764	362.8	ND	ND	ND	233.2	ND	231.0	306.8	310.7	272.5
4	7.454	524.7	133.6	149.7	108.3	321.6	137.2	357.7	474.2	482.8	431.3
5	8.941	665.0	ND	243.7	209.4	427.0	332.9	551.5	982.4	837.3	763.1
6	9.169	593.5	215.1	165.0	119.4	380.6	273.0	511.8	931.7	829.9	746.9
7	11.277	ND	ND	ND	ND	ND	111.4	ND	454.8	393.5	ND
8	12.038	ND	ND	ND	ND	ND	272.1	440.4	1270.5	1159.9	853.0
9	12.178	ND	ND	ND	ND	ND	162.2	257.3	696.4	785.6	561.1
10	12.626	395.7	64.5	98.8	ND	ND	134.7	288.5	451.1	400.7	341.3
11	14.455	ND	ND	ND	ND	ND	ND	214.6	354.0	308.4	265.1
12	15.953	ND	ND	ND	99.5	451.2	231.0	701.8	1157.9	668.6	452.8
13	25.195	ND	ND	ND	ND	212.4	87.0	318.9	262.2	253.2	209.4
14	25.361	ND	ND	ND	ND	85.4	ND	62.6	55.9	98.5	90.3
15	25.522	ND	ND	ND	ND	137.8	54.4	255.1	199.0	164.1	128.5
Total concentration		2811.9	460.5	839.5	755.4	2812.9	2282.9	4983.0	8665.1	7764.6	5976.3

[1] Phenolic concentration expressed as μg catechin equivalent per g of dry sample.
ND: not determined, under the detection limit.

conclude that it is necessary to control the time of germination to obtain an optimal concentration of nonnutritional factors to the third day.

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