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Effect of germination and fermentation on the antioxidant vitamin content and antioxidant capacity of *Lupinus albus* L. var. Multolupa

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Abstract

The present work studies the antioxidant capacity as well as the vitamin C and E contents of raw, fermented and germinated seeds of *Lupinus albus* L. var. Multolupa. Vitamin C was quantified by micellar electrokinetic capillary electrophoresis and vitamin E isomers by high performance liquid chromatography. The antioxidant capacity was determined by spectrophotometry and expressed as trolox equivalent antioxidant capacity (TEAC) and by liposome methods using phospholipids bilayers. Germination, in general, brought about an increase in the content of α-tocopherol, a decrease in the content of γ-tocopherol and did not affect the content of δ-tocopherol, which resulted in an increment in the vitamin E activity. Vitamin C increased sharply but gradually after germination. Fermentation caused a drastic reduction in the antioxidant vitamin content, vitamin C was not detected and tocopherol isomers decreased significantly. Germination processes caused a significant increase in antioxidant capacity (TEAC) of both hydrophilic and lipophilic extracts and fermentation produced slight changes or total reduction in TEAC, depending on process conditions. The peroxidation of egg yolk phosphatidyl-choline (PC) was inhibited by all lupin extracts in comparison with control assay. Germination was selected as a good treatment to increase antioxidant capacity and vitamin C and E contents.
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Keywords: Lupins; Germination; Fermentation; TEAC; Antioxidant capacity; Vitamin C; Vitamin E; Tocopherol; Phosphatidyl-choline peroxidation

1. Introduction

There is a considerable interest in the relationship between plant-based diets and the prevention of certain human diseases, in which increased levels of radicals are implicated. Plant components, mainly provided by vegetables, fruits and whole grains, seem to be responsible for improving health and can prevent chronic diseases. The consumption of whole legumes is very high in several parts of the world, mainly in under-developed countries of Asia, Africa and South America where chronic human diseases are hardly found. Heart disease and cancer researchers are looking at potential benefits of specific foods such as soybean and other plant-derived diets. Bioactive compounds, such as antioxidants, including tocopherols, carotenoids, vitamin C and phenolic compounds, seem to be associated with these health benefits (Cadenas & Packer, 2002).

Legume seeds are protein-valuable foods which have been present in the Mediterranean diet since ancient times. Among them, lupins are a high protein crop and breeding programmes, started in 1928, have successfully obtained very low-alkaloid types (Gladstones, 1990).

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Germination of legume seeds is one of the processing methods for increasing nutritive value (Augustin & Klein, 1989; Ghorpade & Kadam, 1989) and health qualities (Deshpande et al., 2000) as sprouts. This process is simple and inexpensive and different seeds have been germinated for human consumption such as legumes (soybean, lentils, beans, chickpeas, peas), cereals (rye, wheat, barley, oats) and, recently, seeds of some other vegetables (alfalfa, radish, mustards, brassica).

Fermentation also is a very interesting process used in plant foods to increase the nutritional quality and remove undesirable compounds. Fermentation involving lactic acid bacteria offers potential for widespread applications, particularly with respect to the preservation of cereals, legumes and root crops and the provision of safe, low-cost weaning foods for developing countries (Deshpande et al., 2000).

Nowadays, there is a growing interest in Western countries in natural, minimally processed foods, and additive-free, nutritional and healthy foods. Germinated and fermented legumes can be offered as natural nutritive products, which can be beneficial to health. The low content of antinutritional factors in lupins suggests a role as an alternative to the soybean, for producing, tofu, tempe, miso and traditional Oriental sauces, and also for beverages and protein products, opening the market to this nutritious crop. Furthermore, these processes could enhance the composition and content of bioactive antioxidant compounds such as vitamins E and C.

Reports of the contents of tocopherols and vitamin C in legumes are very scarce. It is strongly established that these vitamins are antioxidants and that they can act synergically in living tissues. Lupins have high lipid contents (5–10%) compared with other legumes used for human consumption and probably have considerable vitamin E contents. Legumes, however, have very low contents of vitamin C which could be changed after these biotechnological processes. No information has been found about the effects of germination and fermentation on the antioxidant capacity of lupins.

The aim of this work was to study how different conditions of germination and fermentation affect the content of vitamins E and C and the antioxidant capacity of *Lupinus albus* L. var. Multolupa in order to obtain new foods with enhanced antioxidant properties.

2. Materials and methods

2.1. Seeds

Lupin seeds (*L. albus* L. var. Multolupa) were harvested in 2002 and provided by the Consejería de Agricultura y Medio Ambiente de la Junta de Extremadura (Badajoz, Spain). Seeds were stored in darkness under vacuum conditions in a cool room at 4 °C prior to processing.

2.2. Germination with discontinuous watering

300 g of seeds was soaked for 30 min with 1500 ml of 0.07% sodium hypochlorite. Then, the seeds were washed with distilled water until they reached neutral pH. Afterwards, seeds were soaked with 1500 ml of distilled water for 5 h and 30 min, shaking every 30 min. The hydrated seeds were located in germination trays where a wet laboratory paper was extended and they were then covered with the same wet paper. The tray was introduced into a germination machine G-120 model (ASL Snijders International S.L., Holland) and they were kept in darkness at 20 °C for 5 days. The seeds were watered every 24 h with 1000 ml of distilled water. The germinated seeds were freeze-dried, milled and passed through a sieve of 0.5 mm. The germinated flours were stored in darkness under vacuum conditions in a desiccator at 4 °C.

2.3. Germination with continuous watering

200 g of seeds was soaked for 30 min with 1000 ml of 0.07% sodium hypochlorite. Then, the seeds were washed with distilled water until they reached neutral pH. Afterwards, seeds were soaked with 1000 ml of distilled water for 5 h and 30 min, shaking every 30 min. The hydrated seeds were located in germination trays where a wet laboratory paper was extended and they were then covered with the same wet paper, which was in contact with the circulating water of the germinator, and the seeds were always wet by capillary. The trays were introduced to the germination machine G-120 model (ASL Snijders International S.L., Holland) and they were kept in darkness at 20 °C for 9 days. Trays were removed at 2, 3, 4, 5, 6 and 9 days. The germinated seeds were freeze-dried, milled and passed through a sieve of 0.5 mm. The germinated flour was stored in darkness under vacuum conditions in a desiccator at 4 °C.

2.4. Natural fermentation of whole lupins

100 g of lupin seeds was washed with distilled water and suspended in 333 ml of sterile distilled water in a 500 ml Erlenmeyer flask protected from the daylight with aluminium foil, and left to naturally ferment – only with the microorganisms present on the seed – in an orbital shaker incubator (Unitron, Infors AG, Switzerland) at 220 rpm for 48 h at 37 °C. After fermentation, the seeds were freeze-dried, milled and passed through a sieve of 0.5 mm. The fermented flour was stored in darkness under vacuum conditions in a desiccator at 4 °C.

2.5. Natural fermentation of lupin flour

Lupin seeds were washed with distilled water, dried with a hand cleaning paper, milled and passed through a sieve of 0.5 mm. 100 g of lupin flour was suspended in 333 ml of distilled water in a 500 ml Erlenmeyer flask protected from daylight with aluminium foil. Flour suspension was left to ferment naturally – only with the microorganisms present on the seed – in an orbital shaker incubator (Unitron, Infors AG, Switzerland) at 220 rpm for 48 h at 37 °C. After fermentation, samples were freeze-dried and stored in darkness under vacuum conditions in a desiccator at 4 °C.

2.6. Inoculated fermentation of lupin flour

Lupin seeds were washed three times with sterile distilled water in aseptic conditions and dried at 55 °C for 24 h in order to reduce the competitive microflora of starter culture of the inoculated fermentations. After drying, lupins were milled and flour was passed through a sieve of 0.5 mm. 100 g of lupin flour was suspended in 333 ml of sterile distilled water in a sterile 500 ml Erlenmeyer flask protected from daylight with aluminium foil. Flour suspension was inoculated with a 10% inoculum (v/v) of *Lactobacillus plantarum* CECT 748, as by Doblado, Frias, Muñoz, and Vidal-Valverde (2003), and left to ferment in an orbital shaker incubator (Unitron, Infors AG, Switzerland) at 220 rpm for 48 h at 37 °C. After fermentation, samples were freeze-dried and stored in darkness under vacuum conditions in a desiccator at 4 °C.

2.7. Analysis of tocopherols

2.7.1. Extraction

The extraction of tocopherols was carried out according to Sierra, Prodanov, Calvo, and Vidal-Valverde (1996) with the following modifications: 2 g of sample was suspended in 50 ml of methanolic solution (Scharlau) with 0.5% ascorbic acid (Sigma) and 10 ml of 50% KOH (Panreak). The suspension was heated under reflux for 20 min at 80–90 °C without light exposure under nitrogen atmosphere. The lupin suspension was then centrifuged twice at 8000 rpm at 5 °C, removing the water-phase with 100 ml of distilled water. The water-phase was extracted three times with 100 ml of diethyl ethyl ether (SDS) containing 0.1% BHT (Fluka). Finally, the organic phases were collected and washed with distilled water to neutral pH and then concentrated under vacuum in a rotavaporator at 30 °C. The residue was dissolved in 5 ml of *n*-hexane:diisopropyl ether (90:10 v/v) (Scharlau, HPLC grade) and it was filtered through a 0.45 m membrane and stored at −20 °C under N_2 in darkness.

2.7.2. Standard solutions

Individual standard stock solutions of α-, δ- and γ-tocopherol (Sigma) were prepared by dissolving suitable amounts of each isomer in absolute ethanol and their concentration calculated using the appropriate extinction coefficients and absorbance measured using a Beckman spectrophotometer. The stock standards solutions were diluted in *n*-hexane (Scharlau, HPLC grade) as working standard solutions and stored at −20 °C under N_2 in darkness. The working standards were subjected to the same extraction procedure employed with the samples.

2.7.3. HPLC conditions

The analysis of tocopherols was carried out by using a modular chromatograph system (Water Associates, Mildford, CT, USA), equipped with a model 510 pump, a Rheodyne 7000 sample injector and a Waters 470 scanning fluorescence detector at λ_{ex} 295 and λ_{em} 330 nm. A 90:10 mixture of *n*-hexane:diisopropyl ether (Scharlau, HPLC grade) was used as mobile phase at a flow of 1.2 ml/min. A Lichrosorb Si 60 (250 × 46 mm i.d., 5 μm) column (Technokroma) connected to a guard column Phenyl/Corasil Bondapak (40 × 46 mm i.d.) and a 50 μl loop were used. Data were processed on a PC (NEC Corporation, Boxborough, MA) with a Maxima Database (Millipore Corporation, Waters Chromatography division, Melford, MA, USA).

The vitamin E activity of the samples was defined in terms of RRR-α-tocopherol calculated equivalents (α-TEs). One α-TE is the activity of 1 mg of RRR-α-tocopherol. The vitamin E activity was calculated using the factors for conversion of tocopherols to RRR-α-tocopherol equivalents (Eitenmiller & Landen, 1999). Vitamin E activity (α-TE/100 g) = [α-tocopherol (mg) × 1.0 + γ-tocopherol (mg) × 0.1 + δ-tocopherol (mg) × 0.03].

2.8. Analysis of vitamin C

The vitamin C quantification in lupin flours, by micellar electrokinetic capillary electrophoresis (MECC), was based on the procedure described by Thompson and Trenerry (1995) with the following modifications: for the extraction, 40 ml of 3% metaphosphoric acid (Sigma) was added to 2 g of lupin flour and the mixture blended in an Ultraturrax processor for 2 min. The volume was adjusted to 50 ml with 3% metaphosphoric acid. The resultant slurry was filtered through a Whatman No. 1 filter paper. 1.2 ml of the filtrate was added to 100 μl of isoascorbic acid (Sigma) as internal standard (600 μg/ml) in aqueous 0.2% D,L-dithiothreitol (Sigma), made up to 2 ml with aqueous 0.2% D,L-dithiothreitol, mixed thoroughly and filtered through a 0.45 μm membrane, discarding the first drops. D,L-dithiothreitol is added to prevent the

oxidation of the ascorbic acid to the corresponding dehydroascorbic acid.

The MECC buffer consisted of 1.08 g of sodium deoxycholate (Sigma) dissolved in 50 ml of a 1:1 mixture of 0.02 M sodium tetraborate (Panreak) and 0.02 M potassium dihydrogen orthophosphate (Panreak) at pH 8.6. The buffer was filtered through a 0.45 μm membrane before use. The standard solutions were prepared by dissolving L-ascorbic acid in aqueous 0.2% D,L-dithiothreitol. Isoascorbic acid was also dissolved in 0.2% D,L-dithiothreitol and used as the internal standard at a concentration of 30 μg/ml. Sample extraction and standard solutions were prepared daily.

The extracts were analyzed with a fused silica capillary TSP075375 (47 cm × 75 μm i.d.) purchased from Composite Metal Services LTD (The Chase, Hallow, Worcester, UK), using the buffer previously described. A P/ACE system 2050 (Beckman Instruments, Fullerton, CA, USA) at +18kV and at 28 °C was used for the analysis. Samples were loaded from the anodic end of the capillary by 4 s vacuum injection. Detection was at 40 cm from the injection end and was on-line UV detection at 254 nm. The capillary was flushed with 0.1 M NaOH, distilled water and running buffer for 2 min between runs. Data were processed with a Beckman Gold System Software Chromatopac (Beckman Instruments, Fullerton, CA, USA).

2.9. Total antioxidant capacity

2.9.1. Determination by ABTS

The antioxidant capacity of raw and processed lupins was determined with a method based on the fading of the ABTS radical cation on interaction with antioxidants where the radical is reduced. The inhibitory response of the radical cation is proportional to the antioxidant concentration and 10 min was selected to have the reaction completed.

2.9.2. Trolox standard solution

2.5 mM trolox (Sigma) was prepared in PBS or ethanol for hydrophilic and lipophilic antioxidant capacity measurement, respectively, and used as a stock standard solution (Miller, Rice-Evans, Davies, Gopinathan, & Milner, 1993). 150, 400, 500, 1000 and 1500 μl were taken to obtain the inhibition curve. Fresh working standards were prepared daily (Re et al., 1999).

2.9.3. ABTS solution

ABTS (Sigma) was dissolved in water to a 7 mM concentration. ABTS radical cation ($ABTS^{\cdot+}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (Aldrich) and allowing the mixture to stand in the dark at room temperature for 16 h before use, and it was stable in the dark at room temperature for two more days.

2.9.4. Hydrophilic extract

The hydrophilic antioxidant capacity of raw and processed lupins was determined following the procedure described by Re et al. (1999). The extraction of the compounds with hydrophilic antioxidant capacity was carried out by weighing 0.5 g of sample and adding 20 ml of 5 mM phosphate buffered saline, pH 7.4 (PBS). The mixture was blended in an Ultraturrax processor for 2 min. The volume was then adjusted to 25 ml with PBS. 150, 400, 500, 1000 and 1500 μl were taken to obtain the inhibition curve.

2.9.5. Lipophilic extract

The lipophilic antioxidant capacity of raw and processed lupins was determined as by Pelligrini, Re, Yang, and Rice-Evans (1999). The extraction of the compounds with lipophilic antioxidant capacity was carried out by weighing 2 g of lupin flour and adding 10 ml of dichloromethane (SDS) and 10 ml of distilled water. The mixture was blended in an Ultraturrax processor for 2 min and centrifuged at 15,000 rpm for 10 min to enhance the separation of the two phases. The extraction process was repeated twice more by adding a further 10 ml aliquot of dichloromethane to the water layer. The dichloromethane extracts were combined and subjected to rotary concentration at 30 °C to remove the organic solvent, and the extract was reconstituted in 5 ml of dichloromethane. 250, 500 and 750 μl of this solution were added to 1 ml of dichloromethane to obtain the inhibition curve.

2.10. Lipid peroxidation in phospholipid bilayers

Egg yolk phosphatidyl-choline (PC) (Sigma) was purified to remove contaminant peroxides by HPLC (Terao, Asano, & Matsushita, 1985; Terao, Piskula, & Yao, 1994), dissolved in chloroform and stored at −20 °C under N_2 in darkness prior to use. The solvent of the peroxide-free egg-yolk PC solution was removed in a stream of nitrogen followed by vacuum; the residue was dissolved in 1 ml of 0.01 M Tris–HCl buffer (Sigma) at pH 7.4 containing 0.5 mM diethylenetriaminepentaacetic acid (DTPA) (Sigma), vortexed for 30 s and exposed to ultrasonic waves for 30 min. Unilamellar liposomes were prepared by extruding the sample 21 times in a LipoFast^{TM}-Basic (Avestin Co, Ottawa, Canada) apparatus with a polycarbonate membrane (pore size 100 nm). The resulting liposome suspension (0.5 ml) was suspended in 0.5 ml Tris–HCl buffer and 50 μl of the PBS extract added. The mixture was placed in a light-protected shaker bath at 37 °C. After 5 min, 2,2′-azobis(2-amidinopropane)hydrochloride (AAPH) (Sigma), 100 mM in Tris–HCl buffer, was added to the mixture as radical initiator. The amount of phosphatidyl choline peroxides (PC-OOH) formed at 37 °C during 7 h of incubation was determined at 235 nm at 60 min

intervals by an HPLC method using a Beckman Gold System (Beckman Instruments, Fullerton, CA, USA), a C8 Nova Pak column (Waters), methanol (Scharlau, HPLC grade) and distilled water (94:6, v/v) as eluting solvent and 20 μl injection volume. A standard curve of peroxides was prepared with standard PC-OOH according to Terao et al. (1985).

2.11. Statistical determination

Data were subjected to multifactor analysis of variance (ANOVA) using the least-squared difference test with the Statgraphic 5.0 Program (Statistical Graphic, Rockville, MD, USA) and multiple correlation using Statistica 5.1 Program (Statsoft, Tulsa, Okla, USA) for Windows using a PC-Pentium.

3. Results and discussion

Table 1 shows the contents of tocopherols, vitamin E activity and vitamin C in raw and germinated lupins (*L. albus* L. var. Multolupa). Raw lupins presented a content of 0.19 mg/100 g DM of α-tocopherol, 20.1 mg/100 g DM of γ-tocopherol and 0.25 DM mg/100 g of δ-tocopherol. The vitamin E activity of raw lupins was 2.21 α-TE mg/100 g. Germination brought about, in general, an increase in the content of α-tocopherol, a decrease in the content of γ-tocopherol and did not affect the content of δ-tocopherol, which was accompanied with an increment of the vitamin E activity. When germination was carried out for 5 days with discontinuous watering (GDW5), a large increment in α-tocopherol was obtained (1294%), γ- and δ-tocopherol did not significantly change ($P \leqslant 0.05$) and vitamin E activity increased twofold. When germination was carried out with continuous watering (GCW), α-tocopherol increased gradually, but significantly ($P \leqslant 0.05$), after 2 days of germination (GCW2) (153%), and a retention of 2058% was observed after 9 days (GCW9). The content of γ-tocopherol decreased by 31% after 2 days of germination (GCW2), 13% after 3 days (GCW3), 10% after 4 days (GCW4), 9% after 5 days (GCW5), 28% after 6 days (GCW6) and 66% after 9 days of germination (GCW9). The content of δ-tocopherol, however, only showed slight differences during germination. The vitamin E activity decreased significantly ($P \leqslant 0.05$) only after 2 days of germination (24%) but from that time followed a gradual rise to 9 days when a 238% increment was observed (Table 1).

The content of vitamin C in raw lupin seeds (*L. albus* L. var. Multolupa) was 6.5 mg/100 g DM (Table 1). Germination brought about a sharp rise in the content of this vitamin. Germination carried out for 5 days with discontinuous watering (GDW5) increased the vitamin C content five times (Table 1). When the kinetics of germination were studied with continuous watering for 9 days, an increase of 322% after 2 days (GCW2) was observed, and it continued rising gradually up to 9 days (GCW9) when an increment of 866% was found (Table 1).

Table 2 shows the effect of fermentation on the contents of tocopherols, vitamin E activity and vitamin C of *L. albus* L. var. Multolupa. Naturally fermented grains (NFWL) presented the highest α-tocopherol content and an increase of 32% was obtained whilst decreases in γ- and δ-tocopherol (6% and 44%, respectively) were found. Vitamin E activity only decreased by 3%. When fermentation was performed with lupin flour, either naturally (natural fermentation of lupin flour, NFLF) or with *L. plantarum* strain (inoculated fermentation of lupin flour, IFLF) a sharp decrease in

Table 1
Effect of germination on the antioxidant vitamin content of *L. albus* var. Multolupa[A]

Lupins	α-tocopherol (mg/100 g DM)	γ-tocopherol (mg/100 g DM)	δ-tocopherol (mg/100 g DM)	Vitamin E activity (α-TE units/100 g DM)[B]	Vitamin C (mg/100 g DM)
Raw	0.19 ± 0.01[a]	20.1 ± 0.86[d]	0.25 ± 0.02[b]	2.21 ± 0.11[b]	6.48 ± 0.09[a]
Watering discontinuous germination					
GDW5	2.46 ± 0.21[e]	19.3 ± 0.70[c,d]	0.24 ± 0.02[a,b]	4.40 ± 0.25[e]	30.3 ± 1.13[d]
Watering continuous germination					
GCW2	0.29 ± 0.01[a]	14.0 ± 0.13[a]	0.22 ± 0. 01[a]	1.69 ± 0.02[a]	20.9 ± 1.16[b]
GCW3	0.51 ± 0.02[b]	17.6 ± 0.81[b]	0.30 ± 0.02[c]	2.27 ± 0.12[b]	22.9 ± 1.21[c]
GCW4	1.09 ± 0.13[c]	18.2 ± 2.08[b,c]	0.26 ± 0.03[b]	2.92 ± 0.39[c]	43.9 ± 1.48[e]
GCW5	1.40 ± 0.08[d]	18.2 ± 1.11[b,c]	0.26 ± 0.02[b]	3.23 ± 0.22[c]	46.6 ± 0.64[f]
GCW6	2.33 ± 0.09[e]	14.3 ± 0.32[a]	0.23 ± 0.01[a,b]	3.80 ± 0.13[d]	52.8 ± 1.09[g]
GCW9	3.91 ± 0.13[f]	13.4 ± 0.42[a]	0.22 ± 0.02[a]	5.26 ± 0.21[f]	56.1 ± 0.78[h]

GCW2, GCW3, GCW4, GCW5, GCW6 and GCW9 = Germination with continuous watering for 2, 3, 4, 5, 6 and 9 days, respectively.
Different superscripts in the same column mean significant difference ($P \leqslant 0.05$).
GDW5 = Germination with discontinuous watering for 5 days.
[A] Mean value ± SD of 4 determinations of 2 batches of germinated lupins.
[B] Vitamin E activity = (mg α-tocopherol × 1) + (mg γ-tocopherol × 0.1) + (mg δ-tocopherol × 0.03).

Table 2
Effect of fermentation on the antioxidant vitamin content of *L. albus* var. Multopupa[A]

Lupins	α-tocopherol (mg/100 g DM)	γ-tocopherol (mg/100 g DM)	δ-tocopherol (mg/100 g DM)	Vitamin E activity (α-TE units/100 g DM)[B]	Vitamin C (mg/100 g DM)
Raw	0.19 ± 0.01[b]	20.1 ± 0.86[b]	0.25 ± 0.02[d]	2.21 ± 0.11[b]	6.48 ± 0.09[a]
Whole lupin fermentation					
NFWL	0.25 ± 0.02[c]	18.9 ± 2.70[b]	0.14 ± 0.01[c]	2.14 ± 0.20[a]	ND
Flour lupin fermentation					
NFLF	0.10 ± 0.02[a]	1.93 ± 0.06[a]	0.01 ± 0.01[a]	0.29 ± 0.02[a]	ND
IFLF	0.08 ± 0.01[a]	2.35 ± 0.16[a]	0.04 ± 0.01[b]	0.31 ± 0.02[a]	ND

NFWL = Natural fermentation of whole lupins; NFLF = Natural fermentation of lupin flour; IFLF = Inoculated fermentation of lupin flour.
Different superscripts in the same column mean significant difference ($P \leqslant 0.05$).
[A] Mean values ± SD of 4 determinations of 2 batches of fermented lupins.
[B] Vitamin E activity = (mg α-tocopherol × 1) + (mg γ-tocopherol × 0.1) + (mg δ-tocopherol × 0.03).

all the tocopherol isomers was found (47% and 58% for α-tocopherol, 90% and 88% for γ-tocopherol, and 96% and 84% for δ-tocopherol, respectively), and the vitamin E activity dropped significantly (87% and 86%, respectively) (Table 2). Vitamin C disappeared completely after fermentation, irrespective of the conditions utilized (NFWL or NFLF).

The antioxidant capacity, determined as TEAC, of raw lupins was 71.4 μmol trolox/g DM for the hydrophilic extract and 1.21 μmol trolox/g DM for the lipophilic extract (Table 3). During a 5-day germination with continuous watering, TEAC of hydrophilic extracts increased sharply (35%), whilst TEAC of lipophilic extracts did not significantly change ($P \leqslant 0.05$). When germination was carried out with continuous watering (GCW), TEAC of hydrophilic extracts increased gradually from 8% after 2 days (GCW2) to 46% after 9 days (GCW9). TEAC of lipophilic extracts, however, modified slightly for the first days of germination and an increase of 53% was observed after 9 days of continuous watering germination (GCW9, Table 3).

Natural fermentation of whole lupins (NFWL) produced a reduction of 23% of hydrophilic TEAC which did not change significantly ($P \leqslant 0.05$) when it was carried out with lupin flours (NFLF). However, when *L. plantarum* was inoculated to lupin flour (IFLF) a slight, but significant ($P \leqslant 0.05$) rise (5%) of hydrophilic TEAC was observed (Table 4). TEAC, for lupin lipophilic extracts, did not significantly change ($P \leqslant 0.05$) for natural fermentation of whole seeds, whilst it was not detected when fermentation was carried out with lupin flours, irrespective of the type of fermentation conditions used (Table 4).

From the results obtained in this work, positive correlations have been obtained between antioxidant capacity of hydrophilic extracts and vitamin C content ($r = 0.80$, $P \leqslant 0.05$).

The antioxidative capacities of continuous watering germinated and fermented PBS extracts of lupin seeds

Table 3
Effect of germination in the antioxidant capacity of *L. albus* var. Multopupa[A]

Lupins	Hydrophilic extracts TEAC (μmol trolox/g DM)	Lipophilic extracts TEAC (μmol trolox/g DM)
Raw	71.4 ± 0.08[a]	1.21 ± 0.10[a,b]
Germination with discontinuous watering		
GDW	96.6 ± 1.24[e]	1.17 ± 0.08[a]
Germination with continuous watering		
GCW2	77.3 ± 1.28[b]	1.33 ± 0.16[a,b,c]
GCW3	79.1 ± 0.21[b]	1.52 ± 0.04[b,c]
GCW4	84.6 ± 1.35[c]	1.50 ± 0.21[b,c]
GCW5	91.1 ± 2.57[d]	1.56 ± 0.23[c,d]
GCW6	95.2 ± 0.64[e]	1.34 ± 0.14[a,b,c]
GCW9	104 ± 1.9[f]	1.86 ± 0.04[d]

Different superscripts in the same column mean significant difference ($P \leqslant 0.05$).
GDW5 = Germination with discontinuous watering for 5 days.
GCW2, GCW3, GCW4, GCW5, GCW6 and GCW9 = Germination with continuous watering for 2, 3, 4, 5, 6 and 9 days, respectively.
[A] Mean values ± SD of 4 determinations of 2 batches of germinated lupins.

Table 4
Effect of fermentation in the antioxidant capacity of *L. albus* var. Multopupa[A]

Lupins	Hydrophilic extracts TEAC (μmol trolox/g DM)	Lipophilic extracts TEAC (μmol trolox/g DM)
Raw	71.4 ± 0.1[b]	1.21 ± 0.10[a]
Fermentation of whole lupins		
NFWL	55.1 ± 1.2[a]	0.99 ± 0.12[a]
Fermentation of lupin flours		
NFLF	71.8 ± 1.8[b]	ND
IFLF	75.2 ± 0.6[c]	ND

Different superscripts in the same column mean significant difference ($P \leqslant 0.05$).
ND = Not detected; NFWL = Natural fermentation of whole lupins; NFLF = Natural fermentation of lupin flour; IFLF = Inoculated fermentation of lupin flour.
[A] Mean values ± SD of 4 determinations of 2 batches of fermented lupins.

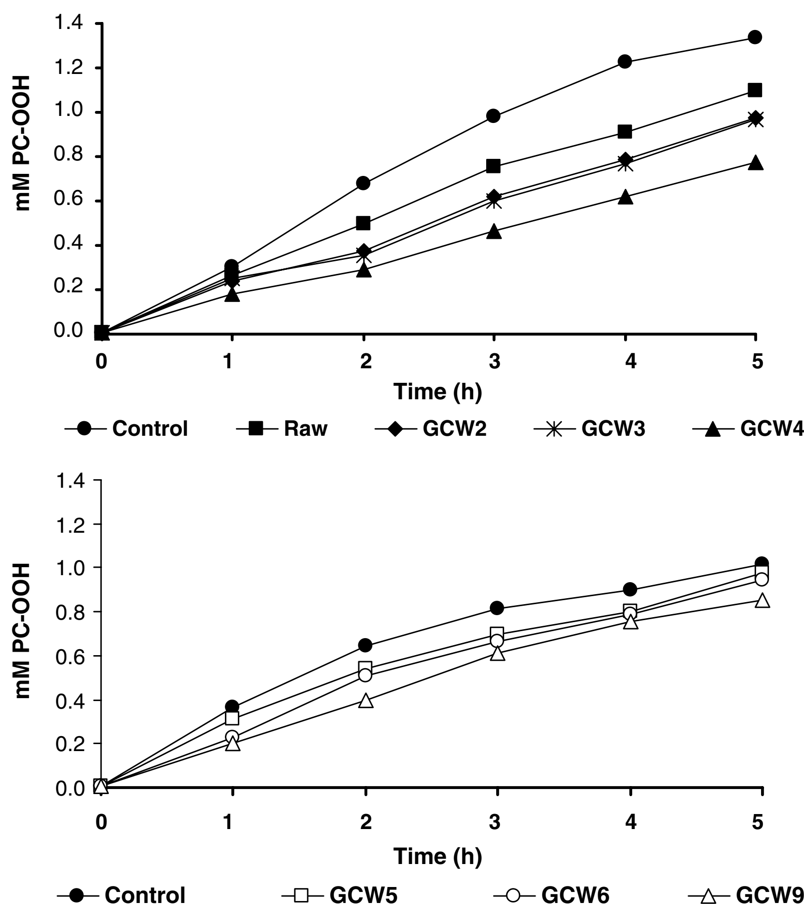

Fig. 1. Inhibition by germinated lupin extracts of AAPH-initiated peroxidation of PC liposomes.

in phospholipid bilayers, measured by the inhibition of lipid peroxidation in large unilamellar vesicles composed of egg yolk phosphatidylcholine (PC), are collected in Figs. 1 and 2. Peroxidation of PC in large unilamellar vesicles was studied by measuring PC-hydroperoxides (PC-OOH) formed by azo compound-initiated radical chain reaction according to Koga, Takahashi, Yamauchi, Piskula, and Terao (1997).

The PC peroxidation was inhibited by every lupin extract in comparison with the control assay (Figs. 1 and 2). The lupin extracts of 2-, 3- and 4 day-germinated seed (GCW2, GCW3 and GCW4) caused higher inhibition than the raw lupin extract. The inhibition of PC oxidation with 9 day-germinated seed (GCW9) extract was higher than those for 5 or 6 day-germinated seeds (GCW5 and GCW6) extracts (Fig. 1). Slight changes were observed in the inhibition of PC peroxidation between the raw and the naturally fermented whole seed (NFWL) extracts and also between the natural (NFLF) and *L. plantarum* fermentation (IFLF) of lupin flours (Fig. 2).

No information has been found about the content of tocopherols in lupin seeds. In other legumes, Wyatt, Pérez-Carballido, and Méndez (1998) reported vitamin E activities of 3.54 mg α-TE/100 g DM for lentils and chickpeas and, for kidney beans, 0.09 mg α-TE/100 g DM, γ-tocopherol being major isomer present in these seeds. Fordham, Wells, and Chen (1975) found 0.1–0.3 mg/100 g of α-tocopherol in peas and Plaza, De Ancos, and Cano (2003) found 0.09 mg/100 g of α-tocopherol in soybean. These authors did not give information about the contents of any other isomers.

There are few references about the effect of germination on the tocopherol content of legumes. In lentils germinated for 4 days, Frías et al. (2002) found an increase of α-tocopherol between 110% and 607%, depending on the variety studied, and the vitamin E activity also rose between 100% and 464%. Plaza et al. (2003) reported that the content of α-tocopherol in soybean sprouts (*Glycine max*) was multiplied by six compared with the content of the raw seed. Fordham et al. (1975) similarly observed an increment in tocopherol of 335% and 825% in two varieties of peas whilst, in kidney beans, they reported a decrease in tocopherol content as a consequence of germination. Marero, Payumo, Aguinaldo, Homma, and Igarashi (1991) showed an increase in α-tocopherol content during germination of mung-beans and cowpeas for 48 h,

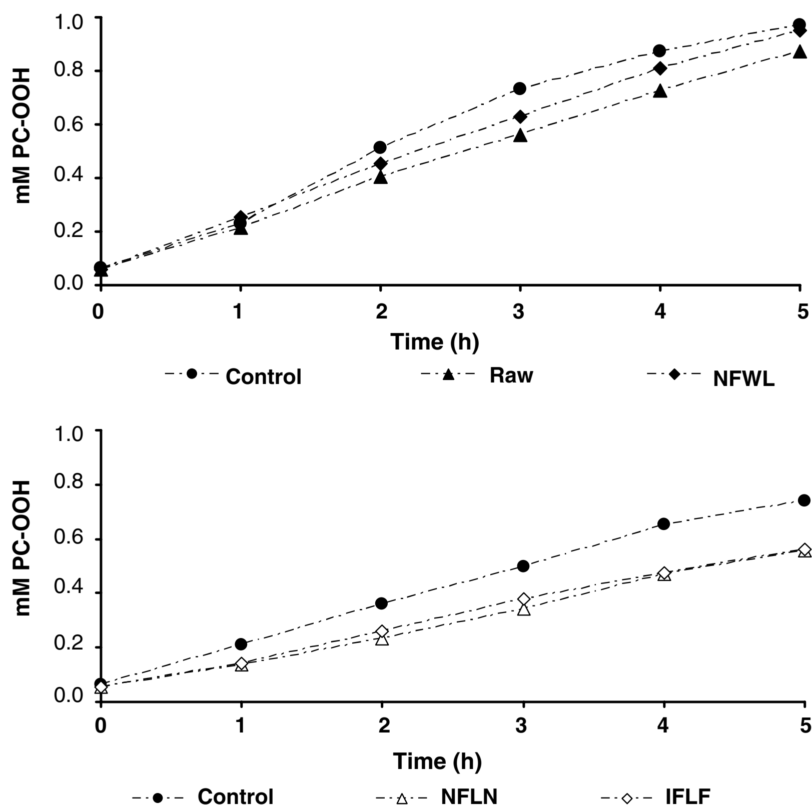

Fig. 2. Inhibition by fermented lupin extracts of AAPH-initiated peroxidation of PC liposomes.

whilst the content of γ-tocopherol decreased sharply. Similar results were reported by Lee and Chang (1993) during soybean germination.

There are very scarce data on the effect of fermentation on the tocopherol content of legumes. In okara koji, a food product based on fermented soy with *Aspergillus oryzae*, the presence of α-tocopherol was not detected, whilst γ- and δ-tocopherols were found (Matsou, 1997).

The content of vitamin C in legumes ranges from no detection in lentils and beans to 8 mg/100 g DM in peas (Souci, Fachmann, & Kraut, 1986) and 10 mg/100 g DM in soybean seeds (Plaza et al., 2003). We have not found references about the effect of germination on the content of vitamin C in lupins. In other legumes, such as soybean, phaseolus and cowpeas, different authors observed that germination caused an increment of vitamin C content (Chen, Wells, & Fordham, 1975; Fordham et al., 1975). (Abdullah & Baldwin, 1984; Plaza et al., 2003; Sattar, Durrani, Mahmood, Ahman, & Khan, 1989). No information has been found about the modifications of vitamin C during legume fermentation.

The hydrophilic antioxidant activity observed in lupin seeds (*L. albus* L. var. Multolupa) (71.4 μmoles trolox/g DM) was sharply superior to values found in the literature for other foods, while lipophilic TEAC was slightly lower. Thus, different authors found hydrophilic TEAC for wheat, barley, tomato and vegetable soup values of 2.3, 3.1, 19, 13–30 μmoles trolox/g DM, respectively, and, for tomato lipophilic TEAC, 5.72 μmoles trolox/g DM (Arnao, Cano, & Acosta, 1998; Yu, Perret, Wilson, & Melby, 2002; Yu et al., 2001).

The results obtained in the present paper show that germination increases TEAC of lupin (*L. albus* L. var. Multolupa) by 46% (Table 3), results which are in agreement with those found in the literature for soybeans (Doval, Romero, Sturla, & Judis, 2001). No information has been reported about the effect of fermentation on the antioxidant capacity of lupins. In *Phaseolus radiatus* var. Aurea fermented with *Bacillus subtilis* IMR-NK1, a superoxide dismutase (SOD)-producing bacterium, Chung, Chang, Chao, Lin, and Chou (2002) reported an increase of the antioxidant activity determined as scavenging effect on DPPH.

The liposome method is often used as a model for studying in vitro antioxidant activity because liposomes can be related to the lamellar structures of biological membranes found in vivo (Chatterjee & Agarwal, 1988). Azuma, Ippoushi, Ito, Higashio, and Terao (1999) observed a good correlation between the linoleic acid emulsion system and liposome phospholipid suspension systems in the evaluation of antioxidative effects of different vegetable extracts.

In liposome membranes, vitamin E is consumed by scavenging, not only by the aqueous radicals, but also by the phospholipid peroxyl radicals to interrupt the

chain propagation. Biomembranes seem to be attacked by aqueous oxygen radicals such as hydroxyl radicals or hydroperoxyl radicals, resulting in the accumulation of lipid hydroperoxides by radical chain oxidation via phospholipid peroxyl radicals within membranes (Koga & Terao, 1996). This fact could be related to the increase of vitamin E activity and the inhibition of PC peroxidation in large unilamellar vesicles observed in the germinated seeds. According to Azuma et al. (1999), the antioxidative activities in the liposome phospholipids suspension system correlated with total contents of ascorbic acid and polyphenols in vegetable extracts.

4. Conclusions

Germination of *L. albus* L. var. Multolupa is an adequate technology to increase the antioxidant vitamins E and C, to enhance the antioxidant activity measured as TEAC and to increase the inhibition of lipid peroxidation in large unilamellar vesicles.

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References

Abdullah, A., & Baldwin, R. E. (1984). Mineral and vitamin contents of seeds and sprouts of newly available small-seeded soybeans and market samples of mung beans. *Journal of Food Science, 49*, 656–657.

Arnao, M., Cano, A., & Acosta, M. (1998). Total antioxidant activity in plant material and its interest in food technology. *Recent Research Developments in Agricultural and Food Chemistry, 2*, 893–904.

Augustin, J., & Klein, B. P. (1989). Nutrient composition of raw, cooked, canned and sprouted legumes. In R. H. Metthews (Ed.), *Legumes: Chemistry, technology and human nutrition* (pp. 187–217). New York: Marcel Deckker.

Azuma, K., Ippoushi, K., Ito, H., Higashio, H., & Terao, J. (1999). Evaluation of antioxidative activity of vegetable extracts in linoleic acid emulsion and phospholipids bilayers. *Journal Science and Food Agriculture, 79*, 2010–2016.

Cadenas, E., & Packer, L. (2002). *Handbook of antioxidants*. New York: Marcel Dekker.

Chatterjee, S. N., & Agarwal, S. (1988). Liposomes as membrane model for study of lipid peroxidation. *Free Radical Biology and Medicine, 4*, 51–72.

Chen, L. H., Wells, C. E., & Fordham, J. R. (1975). Germinated seeds for human nutrition. *Journal of Food Science, 40*, 1290–1294.

Chung, Y. C., Chang, C. T., Chao, W. W., Lin, C.-F., & Chou, S. T. (2002). Antioxidative activity and safety of the 50% ethanolic extract from red bean fermented by Bacillus subtilis IMR-NK1. *Journal of Agricultural and Food Chemistry, 50*, 2454–2458.

Deshpande, S. S., Salunkhe, D. K., Oyewole, O. B., Azam-Ali, S., Battcock, M., & Bressani, R. (2000). *Fermented grain legumes, seeds and nuts. A global perspective*. Rome, Italy: FAO.

Doblado, R., Frias, J., Muñoz, R., & Vidal-Valverde, C. (2003). Fermentation of Vigna sinensis var. carilla flours by natural microflora and *Lactobacillus* species. *Journal of Food Protection, 66*(12), 2313–2320.

Doval, M. M., Romero, A. M., Sturla, M. A., & Judis, M. L. (2001). Antioxidant effect of soya bud in model systems. *European Food Research and Technology, 213*, 297–300.

Eitenmiller, R. R., & Landen, W. O. (1999). *Vitamin analysis for the health and food sciences*. New York: CRC Press.

Fordham, J. R., Wells, C. E., & Chen, L. H. (1975). Sprouting of seeds and nutrient composition of seeds and sprouts. *Journal of Food Science, 40*, 552–556.

Frías, J., Fernández-Orozco, R., Zielinski, H., Piskula, M., Kozlowska, H., & Vidal-Valverde, C. (2002). Effect of germination on the content of vitamins C and E of lentils. *Polish Journal of Food Nutrition and Science, 11/52*, 76–78.

Ghorpade, V. M., & Kadam, S. S. (1989). Germination. In D. K. Salunkhe & S. S. Kadam (Eds.). *CRC Handbook of world food legumes: Nutritional chemistry, processing technology, and utilization* (3, pp. 165–176). Boca Ratón, FL: CRC Press.

Gladstones, J. S. (1990). Development of lupins as new crop legume. *Food Australia, 42*, 270–276.

Koga, T., & Terao, J. (1996). Antioxidant behaviors of vitamin E analogues in unilamellar vesicles. *Bioscience Biotechnology and Biochemistry, 60*, 1043–1045.

Koga, T., Takahashi, I., Yamauchi, R., Piskula, M., & Terao, J. (1997). Kinetics studies on the formation of phosphatidylcholine hydroperoxides in large unilamellar vesicles by azo compounds. *Chemistry and Physics of Lipids, 86*, 85–93.

Lee, I. B., & Chang, K. W. (1993). Changes in concentration of tocopherols and fatty acids during germination and maturation of soybean (Glycine max). *Journal of Korean Agricultural Chemical Society, 36*, 127–133.

Marero, L. M., Payumo, E. M., Aguinaldo, A. R., Homma, S., & Igarashi, O. (1991). Vitamin E constituents of weaning foods from germinated cereals and legumes. *Journal of Food Science, 56*, 270–271.

Matsou, M. (1997). In vivo antioxidant activity of okara koji, a fermented okara, by *Aspergillus oryzae*. *Bioscience Biotechnology and Biochemistry, 61*, 1968–1972.

Miller, N. J., Rice-Evans, C., Davies, M. J., Gopinathan, V., & Milner, A. (1993). A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clinical Science, 84*, 407–412.

Pelligrini, N., Re, R., Yang, M., & Rice-Evans, C. (1999). Screening of dietary carotenoids and carotenoid-rich fruit extracts for antioxidant activities applying 2,2′-azinobis (3-etilenbenzotiazoline-6-sulfonic acid) radical cation decolorization assay. *Methods in Enzymology, 299*, 379–389.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine, 26*, 1231–1237.

Plaza, L., De Ancos, B., & Cano, P. (2003). Nutritional and health-related compounds in sprouts and seeds of soybean (Glycine max), wheat (*Triticum aestivum* L.) and alfalfa (*Medicago sativa*) treated by a new drying method. *European Food Research and Technology, 216*, 138–144.

Sattar, A., Durrani, S. K., Mahmood, F., Ahman, A., & Khan, I. (1989). Effect of soaking and germination temperatures on selected nutrients and antinutrients of mung bean. *Food Chemistry, 34*, 111–120.

Sierra, I., Prodanov, M., Calvo, M., & Vidal-Valverde, C. (1996). Vitamin stability and growth of psychotrophic bacteria in refrigerated raw milk acidified with carbon dioxide. *Journal of Food Protection, 59*, 1305–1310.

Souci, S. W., Fachmann, W., & Kraut, H. (1986). *Food composition and nutritional tables 1986/1987* (3rd ed.). Stuttgart, Germany: Wissenschaftliche Verlagsgesekkschaft GmbH.

Terao, J., Asano, I., & Matsushita, S. (1985). Preparation of hydroperoxy and hydroxy derivates of rat liver phosphatidylcholine and phosphatidylethanolamine. *Lipids, 20*, 312–317.

Terao, J., Piskula, M., & Yao, Q. (1994). Protective effect of epicatechin, epicatechin gallate and quercitin on lipid peroxidation in phospholipid bilayers. *Archives of Biochemistry and Biophysics, 308*, 278–284.

Thompson, C. O., & Trenerry, V. C. (1995). A rapid method for the determination of total L-ascorbic acid in fruits and vegetables by micellar electrokinetic capillary chromatography. *Food Chemistry, 53*, 43–50.

Wyatt, C. J., Pérez-Carballido, S., & Méndez, R. O. (1998). α- and γ-tocopherol content of selected foods in the mexican diet: Effect of cooking losses. *Journal of Agricultural and Food Chemistry, 46*, 4657–4661.

Yu, L., Perret, J., Wilson, J., & Melby, C. (2002). Antioxidant properties of cereal products. *Food Chemistry and Toxicology, 67*, 2600–2603.

Yu, W., Zhao, Y., Xue, Z., Jin, H., Wang, D., Liang, X., & Hu, T. (2001). Antioxidant activity of natural vitamin E extracted from soybean sludge as assessed by chemiluminescence. *Journal of the Science of Food and Agriculture, 82*, 230–233.