

CHEMICAL AND PHYSICAL PROPERTIES OF GLOBULINS AND Γ -CONGLUTINS ISOLATED AT DIFFERENT pH VALUES FROM *LUPINALBUS*

P. Garzón-de la Mora¹, G. Avalos-Alcantara¹, J.R. Villafán-Bernal¹, E.A. Maciel Hernández¹,
C. Gurrola-Díaz², J. López³, D.C. Brune³, J.S. García-López⁴, P.M. García-López⁴ and
M. Ruiz-López⁴

¹Laboratorio de Investigación en Bioquímica

²Instituto de Enfermedades Crónico-Degenerativas, Departamento de Biología Molecular y Genómica, CUCS

³Proteomics Core Laboratory, Department of Chemistry and Biochemistry, Arizona State University, USA

⁴Laboratorio de Biotecnología, Departamento de Botánica y Zoología, CUCBA, Universidad de Guadalajara

Corresponding author's email: pgarzonm@yahoo.com

ABSTRACT

The nutraceutical properties of *Lupinus* proteins and the necessity of attaining pure conglutin- γ have prompted us to analyse an accepted conglutin fractionation procedure. Dehulled *L. albus* seeds were finely ground, defatted with hexane, air dried and stored in a drying chamber at 4°C until use. Following solubilisation of the defatted flour in 0.1 M NaOH, pH was adjusted to 9.0. After centrifugation at 10,000 rpm at 4°C twice, pellets were resuspended twice in 0.1 M NaOH. After pooling supernatants pH was adjusted to 6.5, 6.0, 5.5, 5.0, 4.5 and 4.0 respectively, and the recovered pellets were vacuum dried. Portions of each sample were amino acid analysed; SDS-PAGE separated using 17 to 250 kDa reference proteins.

On the other hand flowing proteins were separated through a 300SB-C3, 9.4 mm x 25 cm Zorbax column for a reverse phase HPLC while performing TFA water and acetonitrile gradient. The resulting fractions were recorded to achieve each fraction molecular weight under Matrix-assisted laser-desorption ionisation (MALDITOF-MS). Isolated proteins at different pH values displayed equal Met, more Gly and Arg and less Lys and Ala content than BSA. Also at increasing pH values Gln, Glu and Gly concentration also increased, reaching a maximum of 23%. SDS-PAGE revealed wider bands of 30 and 17 KDa at pH of 6.0 and 6.5 compared to other remaining pH values; however the 45 KDa band appeared more intense at pH 5.0 and 5.5 values. A 45.6 Kda peak corresponding to conglutins- γ in RPHPLC was higher at pH 5.5 and 6.0. MALDITOF-MS confirmed the 45.6 Kda conglutin- γ peaks. Thus, it seems that pH 5.5 to 6.0 is a better range than the recommended pH 4.5 for *L. albus* seed proteins to isolate conglutin- γ . We also recommend solubilising conglutin- γ in 0.25 M Na₂HPO₄/NaH₂PO₄ buffer at pH 8.5. Loading onto an HPLC column require of a very careful implementation.

KEY WORDS

Lupinus albus, isoelectric points, protein isolates, mass spectrometry, Conglutin- γ

INTRODUCTION

Lupins are predominantly cultivated as a ruminant feed and only secondarily for human nutrition, soil structure improvement, or for ornamental use in the flowering period (Faluyi *et al.* 2000; Huyghe, 1997). However, the recognition of the nutraceutical properties of conglutin- γ has recently augmented the interest in this crop. Along with soybean, domesticated lupins are one of the richest crops in terms of protein content and although it is still a minor crop, the interest in its production is growing due to the expected developments related to its use as a valuable protein source. *Lupinus albus* seed proteins are predominantly storage proteins, and depending on their genotype and location, they have a protein content ranging from 33% to 47% and low alkaloid content from 0.008% to 0.012% (Knight, 2000). Because lupin storage seed proteins are characterised by extreme polypeptide heterogeneity, a multigenic origin and very pronounced post-translational processing of the main protein components (Cerletti *et al.* 1978), their properties are variable and require proper handling to isolate high yields of conglutin- γ . Therefore, the aim of this study was to define better conditions to isolate globulins and conglutin- γ that are more rapid and that give higher yields.

MATERIALS AND METHODS

REAGENTS AND CHEMICALS

N-hexane, HPLC grade acetonitrile, sodium acetate and potassium acetate (p.a. grade), and other analytical grade chemicals were obtained from (Sigma-Aldrich St Louis MI). Reagents for derivatisation, OPA-3-MPA, FMOC, borate buffer and standards (10, 25, 100, 250 pmol), containing sixteen amino acids representative of

a protein hydrolysate, were supplied by Hewlett-Packard (AA reagent kit high sensitivity No. 5061-3347). A second amino acid standard, representative of a physiological sample containing 36 amino acids, was prepared from the commercial Pierce standards (20086+20087) and diluted 1:10 with 0.1 M hydrochloric acid. THF (silylation grade) was obtained from Pierce (Rodgau, F.R.G.). All reagents and samples were kept at 4°C in the cooled auto-sampler and all other reagents used were of analytical grade.

PLANT MATERIAL

Lupinus albus seeds were donated by E. van Santen (Auburn University, USA). After dehulling, *L. albus* seeds were ground into a fine flour using a homemade grinder (JNF_2006*) such that the flour passed through a 0.5 mm mesh sieve. The fat was removed from the flour for 9 h in hexane using a Soxhlet distillation equipment at 40°C until less than 5% of the fat remained. This flour was then stored within a drying chamber at 4°C until use.

PROXIMAL CHEMICAL ANALYSIS

De-oiled *L. albus* flour was analysed to determine the crude protein, moisture, ash, oil, titratable acidity, pH and crude fibre. The nitrogen content was determined by using the Kjeldahl method and multiplied by a factor of 6.25 to determine the crude protein content (No. 985.28, 975.44E and 988.15 31 AOAC, 1975; Faluyi *et al.* 2000). The moisture content was determined by drying the samples at 105°C to a constant weight, and the ash content was determined by maintaining the sample in a muffle furnace at 925°C until a constant weight was reached. The oil content was determined using the Soxhlet method and pH values were measured after 4 volumes of water were added to the sample. The titratable acidity was determined by titrating with 0.1 N NaOH up to pH 8.1 and expressed as sulfuric acid (AOAC, 1984; Skoog *et al.* 1996). The crude fibre content of the defatted samples was determined by decomposing starches with acids, and proteins with a base, followed by filtering and drying. All the measurements obtained were expressed as dry weights.

PREPARATION OF PROTEIN ISOLATES

Aliquots of 100 g of defatted flour were dissolved in double distilled and demineralised water (10:1 water:flour ratio) for 12 h at 4°C in order to extract the albumins, which were then freeze dried. After centrifugation at 10,000 rpm and 4°C, the globulins and fibres were collected in the sediment and then resuspended in 1.0 M NaOH (1:10 ratio) to extract the globulins. The extract was centrifuged at 10,000 rpm at 4°C twice, adjusting the initial pH of 12 to 9.0, and the pH was then progressively decreased and adjusted to values of 6.5, 6.0, 5.5, 5.0, 4.5 and 4.0 using 1.0 M HCl. This solution was again centrifuged at 10,000 rpm and 4°C after attaining each desired pH value and the

protein pellet was recovered. The precipitate was washed with distilled water and again adjusted to the aforementioned pH values before freeze-drying each protein suspension at -50°C and 13 x 10⁻³ mbar. The preparation was weighed and stored for further protein studies (Schuster, R., 1988).

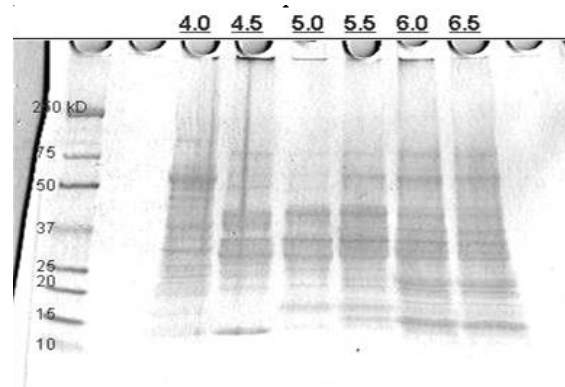


Fig. 1. SDS_PAGE *L. albus* globulins isolated at various pH ranges.

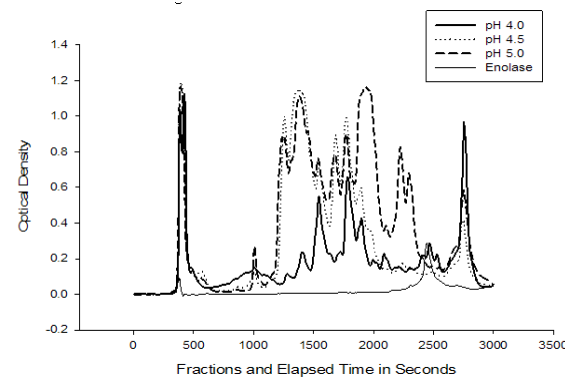


Fig. 2. *L. albus* globulins RPHPLC.

AMINO ACID ANALYSIS

Each protein sample (5 mg) was hydrolysed under a vacuum for 24 h at 108°C with 6 M HCl and then freeze-dried before the amino acid content was analysed on a Beckman 120C amino acid analyser.

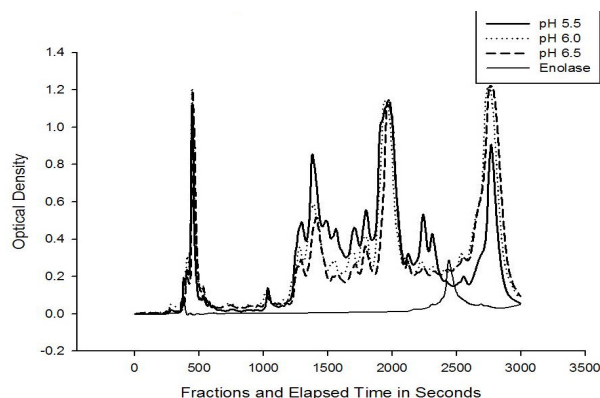


Fig. 3. *Lupinus albus* globulins RPHPLC.

The analysis of primary and secondary amino acids was carried out by the automated pre-column derivatisation technique in which the amino acids were

derivatised in a two-step reaction using OPA for the primary amino acids and FMOC for the secondary amino acids. The derivatisation is performed within the injection cycle. A Hewlett-Packard HP 1090 Series M liquid chromatograph fitted with a DR5 solvent delivery system, a variable volume auto injector, a cooled auto-sampler (4°C), a temperature controlled column compartment, a solvent-preheating device and a 5 µm Hypersil ODS (20 cm x 2.1 mm I.D.) The amino acid analysis of the protein hydrolysate was performed with a diode array detector at a wavelength 338 nm for primary and 266 nm for secondary amino acids. For trace levels in the low picomole range, an HP 1046A programmable fluorescence detector was used with excitation and emission wavelengths of 230 and 455 nm for the primary amino acids. This detector was switched to 266 and 310 nm for secondary amino acids (Schuster R. 1988).

REVERSE PHASE HPLC

To separate and identify the globulin fractions isolated at pH 6.5, 6.0, 5.5, 5.0, 4.5 and 4.0, 10 mg of each globulin fraction was solubilised in 0.25 M Na₂HPO₄/NaH₂PO₄ buffer at pH 8.5 and sonicated for 5 min to complete the solubilisation. Turbid samples were centrifuged at 3,000 rpm to remove the sediment and the supernatant was collected for protein measurement (Bradford) and HPLC separation of the proteins. The latter was carried out using a 300SB-C3, 9.4 mm x 25 cm Zorbax analytical column equipped with a 0.5 mL extension loop attached to a Beckman System Gold with a 126 programmable solvent module and a 166 programmable detector module, as well as a uv detector set at 280 nm. The column was eluted with 10 mM TFA in HPLC water (A) and 10 mM TFA in acetonitrile (B) gradients at a flow rate of 3.5 mL/min starting with a gradient from 10 to 60 or 70% acetonitrile in water (with 10 mM TFA in both solvents) over about 30 minutes followed by ramping to 95% to remove more hydrophobic components from the column.

After finding where the proteins of interest eluted, the gradient was modified so that the acetonitrile concentration increased at a rate of about 1% per minute, and the concentration at the start and at the end of the gradient was adjusted to optimise separation of the proteins of interest. Protein fractions obtained at pH 5.0, 5.5 and 6.0 were solubilised in a solvent mixture containing 300 µL of HPLC water, 100 µL acetonitrile, 5 µL trifluoroacetic acid and 30% acetonitrile in 10 mM trifluoroacetic acid (TFA). Although the 45 Kda enolase does not identify γ -conglutins, as occurred in size exclusion chromatography, we used it as a molecule that might migrate close to conglutin- γ . Once the protein profiles were recorded and the fractions collected, an aliquot was prepared for mass spectrometry analysis (Szepesi, 1992).

GEL-FILTRATION CHROMATOGRAPHY

Size exclusion chromatography was performed using a Biosep-SEC- S 2000, 300 x 7.8 mm column containing Superose 12 resin in 0.25 mM PO₄ buffer (pH 7.5) and using the Beckman System Gold fitted with a 126 programmable solvent module and a 166 programmable detector module set at 280 nm. Samples were eluted in a 0.25 mM PO₄ buffer (pH 7.5) mobile phase at a flow rate of 1 ml/min. Samples from selected peaks were collected manually after the exclusion volume was discarded.

All gel filtrations were carried out at room temperature and two sample solutions were prepared for gel filtration using the Superose 12 resin. The first was the gel-filtration elution buffer containing the *L. albus* seed globulins and the second was the gel-filtration elution buffer containing the 46-kDa enolase.

SDS-PAGE AND PAGE

SDS-PAGE was performed on 12 and 15% polyacrylamide gels using a Minigel Protean II system (BioRad). Samples of 10 µg of both native and p-mercaptoethanol reduced protein were resolved per lane. SDS-PAGE and PAGE were performed in Tris-HCl buffer along with 17 KDa to 250 KDa protein standards including myoglobin (17 KDa), and ovalbumin (45 KDa) to calibrate the molecular weights. To analyse conglutin- γ , the proteins from *L. albus* were size fractionated by SDS-PAGE, loading 12 µg of protein in each lane. The *L. albus* proteins were solubilised in 2% SDS and 0.1M dithiothreitol, and the gels were stained with Coomassie brilliant blue

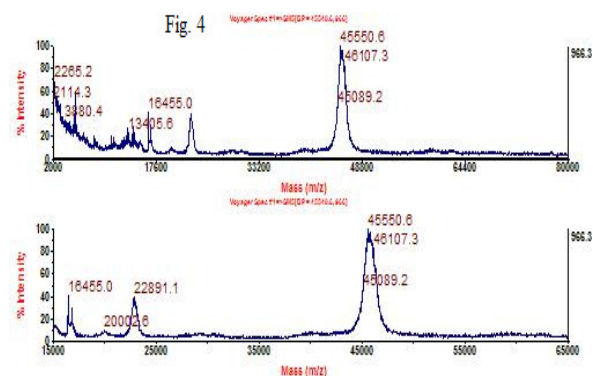


Fig. 4. Mass and intensity spectrum.

R250. Gels were washed three times with 50% acetonitrile/50 mM Tris-HCl (pH 8.0) for 15 min to remove the Coomassie blue dye and then soaked in 100% acetonitrile for 5 min before removing the acetonitrile. The relative molecular masses (Mr) of the protein bands were determined by comparison with the protein standards (Sigma). The gel bands were digitalised using a Studio Scan II scanner (Agfa) and the data was quantitatively processed with Cream software (Kem-En-Tec, Copenhagen, Denmark: Costa J. *et al.* 2005).

MASS SPECTROSCOPY

Positive ion mass spectra were obtained by matrix-assisted laser desorption/ionisation delayed extraction time-of-flight (MALDI-TOF) mass spectrometry using a Voyager DE STR Biospectrometry Work Station (Foster City, CA). The spectrometer was operating in reflector mode using the manufacturer's default settings. The nitrogen laser excitation frequency was set at 3 Hz and the laser power was optimised to obtain a good signal-to-noise ratio after averaging 200–400 single-shot spectra. Mass calibrations were performed over several m/z ranges, using a ProteoMass TMPep tide Protein MALDIMS Calibration Kit (Sigma–Aldrich, USA). Each sample obtained from each peak, or two globulins isolated from *L. albus* at different pHs, were separated in the reverse phase HPLC or size exclusion HPLC after mixing with 100 μ L of a solvent mixture of 30% acetonitrile containing 0.1% trifluoroacetic acid (TFA). The sample solution (1 μ L) was mixed with 1 μ L of a 10 μ g/ μ L solution of 2,5-dihydroxy benzoic acid (DHB) in TFA, and this preparation (1.4 μ L) was placed onto a MALDI-sample plate. The enolase migrating peak of each fraction was dried under N_2 and layered to record the molecular weight of each fraction by Matrix-assisted laser-desorption ionisation (MALDITOF-MS) analysis of the recovered 46 KDa migrating enolase fraction (Lin, D. *et al.* 2003).

RESULTS AND DISCUSSION

The results of the proximal analysis are shown in Table 1 and as can be seen, crude protein (37.7%) and fibre (18.34%) were the major components of the flour. The protein content in *L. albus* was lower than the 44.36% content reported for the domesticated *L. mutabilis* variety (Millán *et al.* 1995). However, the fibre content was similar to that reported for other domesticated varieties (Muzquiz *et al.* 1989). Alkaline protein extraction at pH 12 followed by rapid adjustment to pH 9 at 4°C resulted in a high protein yield (94%) and the removal of 90% of the unwanted compounds (fibre, sugars, etc.). This procedure also avoids possible disruptions of protein structure and amino acid degradation (Sgarbieri *et al.* 1978; Goncalves *et al.* 1997). In this study only 20% of the protein was recovered by precipitation at pH 4.5. After alkaline extraction, the proteins were precipitated at different isoelectric points. The solubility of *L. albus* proteins was minimal at pH 5.0, 5.5 and 6.0 and in fact, the solubility was best in 2.5% NaCl in a 0.25 M Na_2HPO_4/NaH_2PO_4 , pH 9.0 buffer instead of using the usual mixture of 300 μ L of HPLC water, 100 μ L acetonitrile, 5 μ L trifluoroacetic acid and 30% acetonitrile in 10 mM trifluoroacetic acid (TFA) before injection into the HPLC column. Also, globulins isolated at pH 5.0, 5.5 and 6.0 displayed a more intense signal in the fraction that ran with a Rt of 16.2 min where the γ -conglutin peak (45.6 KDa) was identified.

Protein isolated at all pHs showed similar amino acid patterns and contained equal amounts of Met, more Gly and Arg, and less Lys and Ala than in bovine serum albumin (BSA, Table 2). The Gln, Glu and Gly concentration increased as the acidity rose, reaching a maximum of 23% at pH 4.5. Contrary to cereals, lupin proteins contain large amounts of lysine and fewer sulphur-containing amino acids (Dervas *et al.* 1999). Evaluation of proteins isolated at various pH values in SDS-PAGE gels identified similar bands at 30 Kda. The protein bands at 17 KDa and 45 KDa were more intense when the protein was isolated at pH 5.0 and 5.5, while the band at 45KDa ran parallel to ovoalbumin and possibly contained the conglutin- γ fraction.

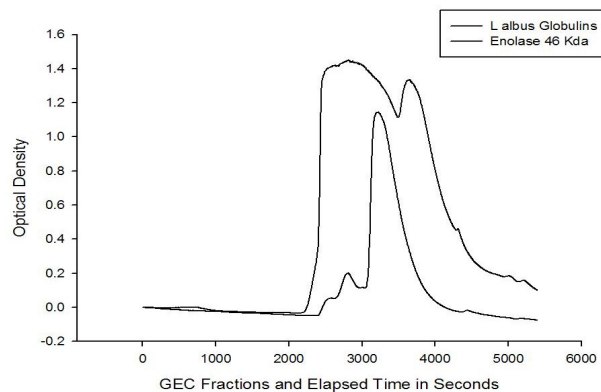


Fig. 5. *L. albus* globulins and enolase.

The reverse phase gradient partition HPLC analysis revealed that a fraction with a Rt of 16.5 min contained conglutin- γ . The signal was more intense for globulins isolated and solubilised at pH 5.0 and 5.5, and they migrated before enolase whose MWt corresponded to 45 KDa. It seems that the significant amount of Gln and Glu determined a clear cut isoelectric point at pH 4.5. Moreover, γ -conglutin containing fractions might be obtained at pH values ranging from between 5.0 and 5.5. The remaining fractions were dried under vacuum and stored at -80°C for mass spectrometry analysis. The time of flight of these fractions was diverse as was the hydrophobic globulin affinity for the hydrocarbon column packing. In fact, conglutin- γ seems to be very hydrophobic because it elutes when the mobile phase becomes sufficiently non-polar to accommodate the non-polar nature of protein. Matrix-assisted laser-desorption ionisation (MALDITOF-MS) analysis of the globulin fraction with a Rt of 16.1 min had the expected molecular weight of conglutin- γ (45.6 Kda). Purified globulins and enolase were best solubilised in NaCl containing phosphate buffer at pH 9.0 and they could be separated by size exclusion, with the phosphate buffer at pH 8.5 containing conglutin- γ . Although, the protein fractions where the enolase migrated were parallel to the 45 KDa, we still needed to resolve the globulin peak into sharper and more defined 45.5 KDa peak.

The results of this work indicate that the less soluble globulins are those that precipitated at pH values of 5.5, 6.0 and 6.5, and that conglutin- γ accumulate in these fractions. Protein solubilisation was enhanced at pH values of 8.5 and above. However, the purification and isolation of specific conglutins requires more detailed information about their size, solubility, and the interactions they undergo when in solution.

Table 1. Chemical composition of *L. albus* defatted flour.

Component	Mean value (%)
Moisture	7.8 \pm 0.05
Crude protein	37.7 \pm 0.94
³ Crude fibre	18.4 \pm 1.34
³ Lipids	6.34 \pm 0.09
³ Ash	2.72 \pm 0.15
Acidity	0.15 \pm 0.02
Soluble Sugars	2.8 \pm 0.2

³Data are reported on a dry mater basis.

LITERATURE CITED

- Faluyi, M. A., X.M. Zhou, F. Zhang, S. Leibovitch, P. Migner and D.L. Smith. 2000. Seed quality of sweet white lupin (*Lupinus albus*) and management practice in eastern Canada. *European Journal of Agronomy* 13: 7.
- Huyghe, C. 1997. White lupin (*Lupinus albus* L.). *Field Crops Research* 53: 147.
- Knight, R. 2000. Linking Research and Marketing Opportunities for Pulses in the 21st Century. Proceedings of third international food legumes research conference (pp. 161–164). Dordrecht: Kluwer Academic Publishers.
- Cerletti, P., A. Fumagalli and D. Venturin. 1978. Protein composition of seeds of *Lupinus albus*. *Journal of Food Science* 43: 1409.
- AOAC. 1975. Official methods of analysis (12th ed.), (No. 985.28, 975.44E and 988.1531, AOAC). Washington D.C.: Association of Official Analytical Chemists.
- AOAC. 1984. Official Methods of analysis (14th ed). Washington, DC: Association of Official Analytical Chemists.
- Skoog, D.A., D.M. West and F.J. Holler. 1996. *Fundamentals of Analytical chemistry*. Florida: Sanders College Publishing.
- Rainer, Schuster. 1988. Determination Of Amino Acids In Biological, Pharmaceutical, Plant And Food Samples By Automated Precolumn Derivatisation And High-Performance Liquid Chromatography. *J. of Chromatography* 431: 271.
- Szepesi, G. 1992. How to use Reverse-Phase HPLC, VCH Publishers, NY.
- Barth, H.G., B.E. Boyes and C. Jackson. 1994. Size exclusion chromatography. *Anal Chem.* 66: 595R.
- Costa, J., D.A. Ashford and C.P. Ricardo. 2005. One- and two-dimensional electrophoretic identification of IgE-binding polypeptides of *Lupinus albus* and other legume seeds. *J. Agric. Food Chem.* 53: 4567.
- Lin, D., D.L. Tabb and John R. Yates III. 2003. Large-scale protein identification using mass spectrometry. *Biochimica et Biophysica Acta.* 1646: 1.
- Sgarbieri, C., M. Antonia and M. Galeazzi. 1978. Some physicochemical and nutritional properties of a sweet lupin (*Lupinus albus* var. *Multolupa*) protein. *J. of Agricultural and Food Chemistry* 26: 1438.
- Millán, F., M. Alaiz, I. Hernandez-Pinzon, R. Sánchez and J. Bautista. 1995. Study of neutral lipids of *Lupinus mutabilis* meal and isolates. *J. of the American Oil Chemists Soc.* 72: 7471.
- Músquiz, M., C. Burbano, C. Rey and M. Cassinello. 1989. A chemical study of *Lupinus hispanicus* seed nutrition components. *Journal of the Sci. Food and Agric.* 47: 197.
- Gonalves, N., J. Vioque, A. Clemente, R. Sánchez-Vioque, J. Bautista and F. Millán. 1997. Obtención y caracterización de aislados proteicos de colza. *Grasas y aceites* 48: 282.
- Dervas, G., G. Doxastakis, S. Zinoviadi and N. Triandatafillakos. 1999. Lupin flour addition to wheat flour doughs and effect on rheological properties. *Food Chem.* 66: 67.