Fractionation and Characterization of Proteins from *Gevuina avellana* and *Rosa rubiginosa* Seeds

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ABSTRACT: *Gevuina avellana* and *Rosa rubiginosa* proteins were evaluated for their potential food use. The proteins were sequentially separated into five fractions according to their solubilities in deionized water, 0.5 M NaCl, 70% (vol/vol) isopropyl alcohol, 50% (vol/vol) glacial acetic acid, and 0.1 M NaOH. The five fractionated protein groups were then characterized by SDS-PAGE and gel filtration chromatography to determine their M.W. profiles. Ninety-six percent of *G. avellana* total protein was solubilized in three extraction stages, and 88% of *R. rubiginosa* total protein was solubilized in one extraction stage. Albumins were the major protein fraction in *G. avellana* and glutelins-1 the most abundant in *R. rubiginosa*. The protein solubility profile determined over the pH range 1–12 showed minimal solubilities at pH 3–5 and pH 3–7 for *G. avellana* and *R. rubiginosa*, respectively. Electrophoretic studies revealed the existence of proteins composed of two major kinds of polypeptides linked together *via* disulfide bonds and with molecular masses ranging from 13 to 119 kDa. Gel filtration chromatography profiles of globulins and albumins were studied for both seeds. Isoelectric focusing showed an isoelectric point in the ranges of 4.5–6 and 3–6.5 for *G. avellana* and *R. rubiginosa* proteins, respectively.

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KEY WORDS: *Gevuina avellana*, protein characterization, protein fractionation, *Rosa rubiginosa*, solubilization.

Oils from the Chilean seeds *Gevuina avellana* and *Rosa rubiginosa* are recognized as having excellent properties for cosmetics and pharmaceutical products. Although these seeds are still underexploited, their commercial production and processing is being developed (1–3). Oil extraction from these two seeds can be performed in equipment conventionally used for extracting other oilseeds. The resulting defatted meals could be used for feed or for food either as whole meal or, after fractionation, as protein and fiber. High value-added ingredients such as oil, protein, and dietary fiber isolated from crops can improve the economy of agricultural production areas.

Chilean hazelnut, *G. avellana* Mol. (Proteaceae), is native to Chile and Argentina and its fruits may be consumed after roasting or used for their extracted components, oil, protein, and fiber. The oil has valuable properties for edible use and for cosmetics, owing to its good skin-penetrating ability and UV-radiation protective properties. The defatted meal of *G. avellana* contains 18–21% protein, which can be used for food and feed purposes because of its balanced composition and biological value (4).

Dog rose (*R. rubiginosa*) is a European species of the Rosaceae family, with important expansion in the Andean area. *Rosa rubiginosa* is a low-oil-content seed with economical relevance for the excellent skin-regenerating properties of its oil (5). The residual defatted meal from *R. rubiginosa* potentially could be used as a source of both low-cost protein and dietary fiber, since it contains 10% protein and more than 50% crude fiber (6).

Research on the characterization of the protein from these seeds is required for both fundamental and applied studies, i.e., for incorporation into processed food and/or feed products. In this work, the proteins from *G. avellana* and from *R. rubiginosa* were fractionated according to their solubility in different solvent systems. Characterization of the extracted fractions was undertaken with regard to the M.W. distribution and isoelectric point (pI).

EXPERIMENTAL PROCEDURES

Materials. *Gevuina avellana* and *R. rubiginosa* seeds were supplied by Forestal Casino Ltda. (Santiago, Chile). The seeds were ground in a coffee grinder (Taurus, Lleida, Spain), sieved to select particles smaller than 0.6 mm, and defatted overnight at room temperature with hexane using a solvent/seed ratio of 15 (g/g). After extraction, the two phases were separated by filtration and the solid was reextracted twice under the same conditions. The defatted flours were air-dried and stored at 4°C. On average, *G. avellana* defatted flour contained 7% moisture, 18.2% protein, and 45.4% neutral detergent fiber (NDF) (7). *Rosa rubiginosa* defatted flour contained 5% moisture, 8.2% protein, and 76.2% NDF (8).

Coomassie Brillant Blue R-250 and molecular mass (Mms) markers for gel filtration chromatography (GFC) were purchase from Sigma (St. Louis, MO). Sephacryl S200 and S100 columns were purchase from Pharmacia Biotech (Uppsala, Sweden). Mms markers for electrophoresis were obtained from Bio-Rad (Richmond, CA).

Protein extractability. The effect of pH on protein extractability in distilled water was studied using 1 g of defatted flour suspended in 20 mL of distilled water. The distilled water was adjusted from pH 1 to pH 12 using either 0.5 M HCl or 0.5

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M NaOH before extraction and readjusted as needed during extraction. The suspension was shaken at 250 rpm for 90 min at room temperature in an orbital shaker (Innova 4000; New Brunswick Scientific, Edison, NJ) and then centrifuged at 2680 × *g* for 15 min in a Rotixa 50 RS centrifuge (Hettich-Zentrifugen, Tuttlingen, Germany). The protein content in the supernatant was determined by Kjeldahl (9), using the factor 6.25, and by the method of Lowry *et al.* (10). For each pH point a triplicate estimation was performed. The protein content in the defatted flour was determined by Kjeldahl, and a 100% protein extraction corresponds to the protein content of each seed. The protein extractability was the ratio (expressed in percentage) between the protein extracted at each pH value and the total protein determined by Kjeldahl.

Protein fractionation. Seed proteins were fractionated according to their solubilities following the Osborne classification (11), involving a successive extraction with five different solvents: deionized distilled water, 0.5 M NaCl, 70% (vol/vol) isopropyl alcohol, 50% (vol/vol) glacial acetic acid, and 0.1 M NaOH. These solvents were used sequentially to extract the solid residue resulting from the extraction with the previous solvent. Extraction assays were performed in duplicate. After both the isopropyl alcohol and the acetic acid extractions, the solid was rinsed with water and the liquid was discarded. During extraction, the temperature was maintained at 22–25°C, and the stirring rate was 250 rpm. The soluble and insoluble fractions obtained with each solvent were separated by centrifugation at 2680 × *g* for 15 min and further vacuum filtration through filter paper (model 235; Albet, Barcelona, Spain). When more than one extraction stage was needed, the supernatants from each solvent were pooled. Samples of each extraction were freeze-dried and kept at −20°C until needed.

GFC. GFC was performed in a fast protein liquid chromatography system equipped with Sephacryl® S200 and Sephacryl® S100 columns, both purchased from Pharmacia Biotech. The freeze-dried albumin and globulin fractions from both seeds were separately dissolved in 25 mM Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl and applied to the column previously equilibrated with the same buffer. The column was calibrated with the following standards: BSA (66 kDa), carbonic anhydrase (29 kDa), cytochrome C (12.4 kDa), and aprotinin (6.5 kDa). Mms markers were obtained from Bio-Rad. Samples were loaded *via* a 500-μL loop, and the column was eluted at 0.3 mL/min, collecting fractions of 1.5 mL.

Gel electrophoresis. SDS-PAGE was carried out in 12% acrylamide gels according to Laemmli (12) in either the absence or presence of β-mercaptoethanol. The electrophoresis was run at 40 mA for 2 h in a mini Protean II dual slab cell (Model SE 250; Pharmacia Biotech). Phosphorylase B (97.4 kDa), BSA (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa) were used as Mms markers. Gels were stained for protein detection with Coomassie Brilliant Blue R-250 (Sigma). The freeze-dried powders obtained from the protein fractions of both seeds were directly dissolved into the electrophoresis sample buffer, and a volume containing 10 μg of protein was loaded to the gel.

Isoelectric focusing (IEF). IEF was performed at 4°C using a Multiphor II electrophoresis system (Pharmacia Biotech) according to the manufacturer's recommendations using Ampholine PAGplates (Pharmacia Biotech) with a pH range of 3.5 to 9.5. Freeze-dried albumin and globulin fractions from both seeds were dissolved in distilled water to reach a protein concentration of 1 μg/μL. The samples were centrifuged at 3250 × *g* for 5 min, and 8-μL aliquots of the supernatants were applied to the IEF gel. The pH gradient of the focused gels was monitored immediately at the end of each run by cutting a control gel into pieces of 1 cm width. After shaking with distilled water for 1 h, the pH was measured. The pH gradients were typically from 3.5 to 8.5.

Analytical methods. The nitrogen content of each of the defatted meals was determined by Kjeldahl, using the factor 6.25 to convert the nitrogen value to protein. Liquid samples were assayed for protein content according to the method of Lowry *et al.* (10) using BSA (Sigma) as the standard.

RESULTS AND DISCUSSION

Effect of pH on protein extractability. The protein extractability profiles in distilled water for *G. avellana* and for *R. rubiginosa* defatted flour are shown in Figure 1.

In the case of *G. avellana*, at acidic pH values of 1–2 and at pH 9, similar protein solubilization levels were achieved (around 65%). Nonetheless, at more alkaline pH values of 10–11, the efficiency of the protein extraction was reduced to 60% of the initial protein content, probably owing to denaturation and precipitation of the protein at the alkaline pH values.

Apart from some minor differences, results obtained for *R. rubiginosa* were similar; the extractability in the pH range 9–11 was more efficient (yields higher than 90%), and the extraction yields decreased for pH values greater than 11 and reached a minimum in the range of pH 3 to 7 (3 to 5 for *G. avellana*).

Protein fractions. For the characterization of the protein fractions from *G. avellana*, a slight modification of the sequential extraction proposed by Osborne (11) was needed. Thus, in using a liquid/solid ratio (LSR) of 20:1 and only one extraction, 40%

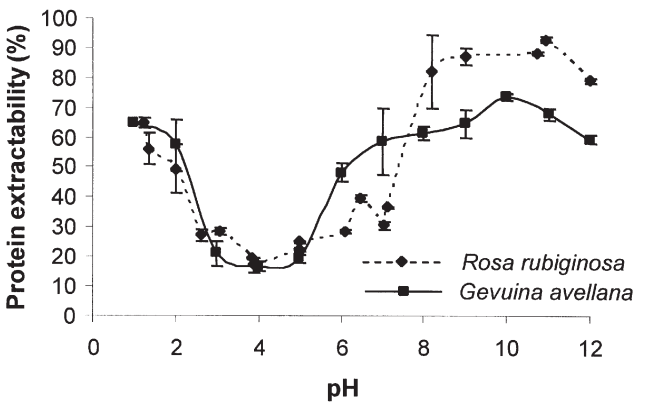

FIG. 1. Effect of pH on protein extractability (%) in distilled water of *Gevuina avellana* and *Rosa rubiginosa* defatted seeds.

TABLE 1
Effect of the Liquid/Solid Ratio (LSR) and the Number of Stages on the Extractability (%) of *Gevuina avellana* Protein During Protein Fractionation According to the Osborne Method

	LSR 20 stages	LSR 50 stages			LSR 100 stages		
Fraction stage	1	1	2	3	1	2	3
	Protein extractability (%)						
Albumins	24.1	59.0	58.8	49.7	46.3	47	42.5
Globulins	14.3	5.0	8.1	7.7	8.7	17	20.2
Prolamines	3.5	3.3	2.6	2.3	2.5	11.8	8.1
Glutelins-1	16.2	9.4	11.5	19.7	22.3	10.7	16.1
Glutelins-2	1.5	5.3	6.9	7.5	8.2	7.6	9.4
Total	59.6	82	87.9	86.9	88.0	94.1	96.3
Residue	40.4	18	12.1	13.1	12.0	5.9	3.7

unextracted protein was left in the solid fraction (Table 1). Higher LSR and up to three extraction stages were performed to identify whether protein extraction was limited by the operational factors. Extraction with each solvent was repeated until no further protein was extracted. Table 1 shows the effect of the LSR on the total protein solubilized and the extractability of different fractions, respectively. The use of more than two extraction stages with LSR of 50 or 100 did allow the extraction of significantly more proteins. The yield of the major protein fraction (albumins) increased when an LSR of 50 was used, but no further increases occurred after increasing the LSR to 100. The best results in total protein extraction were obtained with an LSR of 100 and three extraction stages (only 3.7% in the residue) mainly as a result of the increase in the content in of the globulin, prolamine, and glutelin-2 fractions compared with the yields obtained with LSR 20 or LSR 50.

The amount of protein extracted with each solvent is shown as a percentage of the total extractable protein in Table 2. The five soluble fractions obtained from the defatted *R. rubiginosa* meal contained 86.7% of the initial protein in the seed, as inferred from the Kjeldahl determinations. The major fraction corresponded to glutelins-1 (38.8%) followed by globulins (18.6%) and albumins (13.8%). Ninety-six percent of the initial protein was extracted from *G. avellana* with an LSR of 100 and using three extraction stages. Albumins were the major fraction at 42.5%, and globulins and glutelins-1 represented 20.2 and 16.1%, respectively, of the total protein content in *G. avellana*. A good correlation was found between the protein content analyzed by the method of Lowry *et al.* (10) in the liquid extracts and the protein calculated from the nitrogen content of the residues analyzed by Kjeldahl.

SDS-PAGE. The size distribution of the three major protein fractions (albumins, globulins, and glutelins-2) was analyzed (Figs, 2, 3). We had difficulties in dissolving the powders obtained after freeze-drying the Osborne protein fractions, particularly the albumin fractions, probably because of protein precipitation induced by the drying treatment.

On the other hand, gel electrophoresis revealed the heterogeneity of the protein complexes of the Osborne fractions as shown in the patterns obtained with and without added β-mercaptoethanol. Thus, the high-M.W. protein bands visualized in the unreduced state (Figs. 2a, 3a) tended to disappear after reduction with β-mercaptoethanol and, in general, bands with smaller sizes appeared (Figs. 2b, 3b), suggesting that the proteins were probably extracted as high-M.W. aggregates consisting of several polypeptide chains linked by disulfide bonds.

Albumins, globulins, and glutelins from *G. avellana* showed very similar protein distribution in both the unreduced and reduced state. Concerning the first, the most representative groups had the following Mms: 62, 50, 31, 29, 25, 19, and 14 kDa (Fig. 2a). In addition, two faint bands at 43 and 35 kDa were visible in the globulins. After the reducing treatment (Fig. 2b), two bands at 43 and 35 kDa appeared in the three fractions. Bands at 25 and 19 kDa were still visible in the albumins and globulins but disappeared in the glutelins.

Similarities in the protein patterns among the three fractions extracted from *G. avellana* may indicate a contamination problem. A similar behavior was reported for chickpea albumins purified following the classical water extraction of the Osborne method, because a fraction of chickpea globulins is soluble in water and extracted together with albumins (13). The presence of the same proteins in the glutelin fractions may indicate the incomplete extraction achieved with the saline solution (globulins).

The unreduced Osborne fractions extracted from *R. rubiginosa* shared a protein band at 60 kDa and protein aggregates with Mms higher that 100 kDa (Fig. 3a). The intensity of the latter bands increased from albumins to glutelins and, in the case of glutelins, an important percentage did not even enter the stacking gel.

TABLE 2
Composition of Proteins from *Gevuina avellana* and *Rosa rubiginosa*

Solvent	Fraction	*Gevuina avellana*[a] (%)	*Rosa rubiginosa* (%)
Distilled water	Albumins	42.5	13.8
0.5 N NaCl	Globulins	20.2	18.6
70% IPA	Prolamines	8.1	6.0
50% AcH	Glutelins-1	16.1	38.8
0.1 N NaOH	Glutelins-2	9.4	9.7
	Residue	3.7	13.3

[a]Protein extractability using an LSR 100. IPA, isopropyl alcohol; AcH, acetic acid.

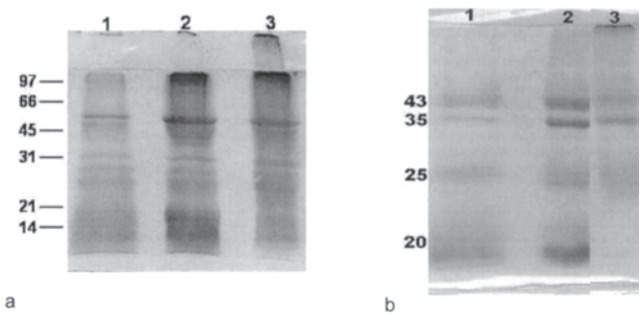

FIG. 2. SDS-PAGE of *Gevuina avellana* proteins: lane 1, albumins; lane 2, globulins; lane 3, glutelins-1. Samples (a) without β-mercaptoethanol treatment and (b) with β-mercaptoethanol treatment. The 12% acrylamide gels were stained for protein with Coomassie Blue R-250.

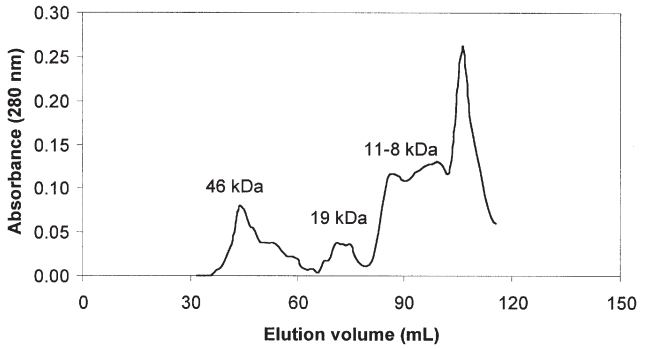

FIG. 4. Gel filtration chromatography on Sephacryl S100. Samples of *Gevuina avellana* albumins were loaded on a Sephacryl S100 column (1.6 × 60 cm) equilibrated in 25 mM Tris/HCl (pH 7.5) containing 150 mM NaCl. Flow rate, 0.3 mL/min. Fractions: 1.5 mL.

The globulin fraction was characterized by the presence of very intense bands at low Mms (25, 20, and 17 kDa) whose intensity increased as the Mms decreased. The same bands were observed in the albumin fraction, although their intensities were much lower. The bands might represent a small fraction of globulins that were extracted with the albumins. Finally, the glutelins contained a number of very faint bands at Mms lower than 60 kDa.

In the reduced samples (Fig. 3b), the albumins were integrated by very faint bands with Mms of 63 and 40 kDa. In the other fractions, the protein aggregates tended to disappear and, as for *G. avellana*, split into bands with lower Mms. Thus, for the globulins, with the exception of the band at 60 kDa, we detected two very intense bands at 52 and 40 kDa and a broad band with Mms in the interval 15–10 kDa together with very faint bands at 25–22 kDa. The reduced glutelins-1 were characterized by the appearance of intense bands at 42, 35, and 26 kDa and other minor peaks at 63, 45, 19, and 14 kDa.

Molecular weights in the range of those found in the present work have been reported for protein fractions for other seeds. Bejosano and Corke (14) fractionated proteins from *Amaranthus* spp. and buckwheat according to the methodology of Osborne and found subunits of M.W. under 67 kDa for globulins and glutelins, and under 14.3 kDa for subunits of albumins and prolamines. Rizkalla *et al*. (15) reported the presence of subunits of M.W. between 82.2 and 13.9 kDa for albumins and between 53.1 and 16.8 kDa for globulins isolated from *Nigella sativa*.

Gel filtration. Figures 4 and 5 show the gel filtration chromatograms obtained from native globulins and albumins from both seeds using Sephacryl S100 and S200 columns, respectively. *Gevuina avellana* albumins (Fig. 4) presented two main peaks: a sharp one of approximately 6 kDa, in the limit of the separation range of the Sephacryl S100 column, preceded by a

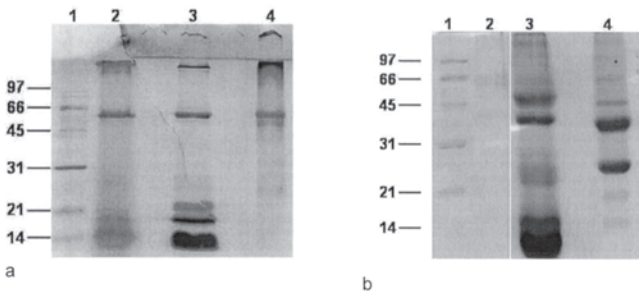

FIG. 3. SDS-PAGE of *Rosa rubiginosa* proteins: lane 1, molecular mass standards; lane 2, albumins; lane 3, globulins; lane 4, glutelins-1. Samples (a) without β-mercaptoethanol treatment and (b) with β-mercaptoethanol treatment. The 12% acrylamide gels were stained for protein with Coomassie Blue R-250.

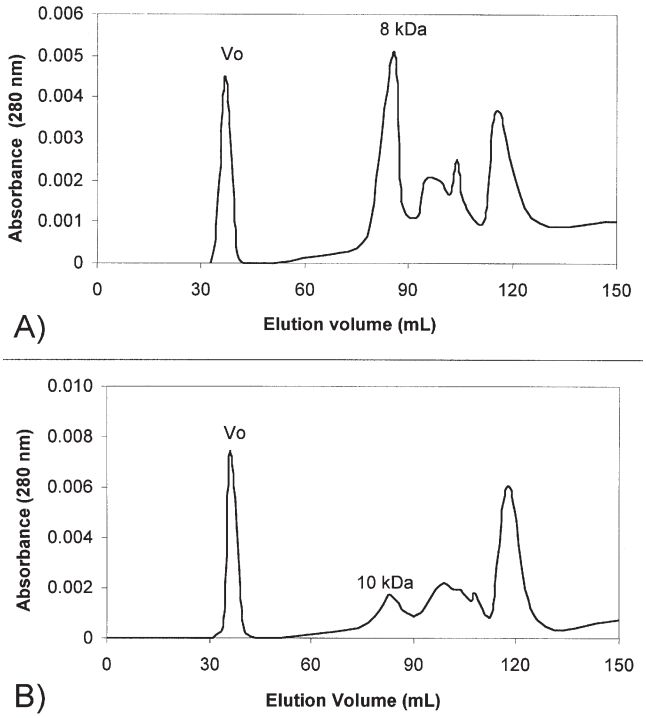

FIG. 5. Gel filtration chromatography on Sephacryl S200. Samples of *Rosa rubiginosa* (A) albumins and (B) globulins were separately loaded on a Sephacryl S200 column (1.6 × 60 cm) equilibrated in 25 mM Tris/HCl (pH 7.5) containing 150 mM NaCl. Flow rate, 0.3 mL/min. Fractions, 1.5 mL.

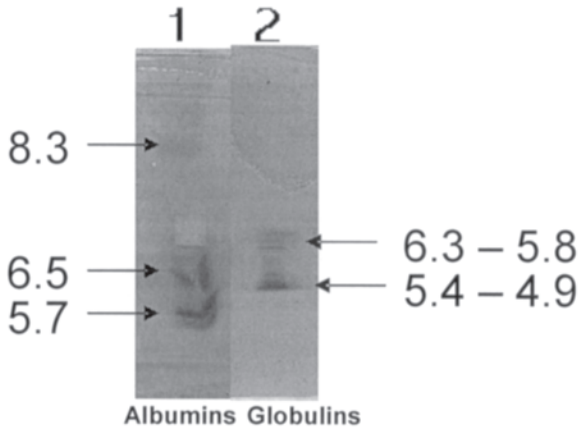

FIG. 6. Isoelectric focusing of *Gevuina avellana* protein. Lane 1, albumins; lane 2. globulins.

wide protein peak with an absorbance maximum at 10 kDa. In addition, two minor peaks were detected at lower elution volumes with Mms around 45 and 20 kDa, respectively. The protein with the highest Mms might correspond to the subunit found by SDS-PAGE under nonreducing conditions.

For the *G. avellana* globulin fraction, the elution profile obtained from Sephacryl S100 showed extremely low absorbance levels and did not allow differentiation of any major group of proteins (not shown).

Both the albumin and globulin fractions isolated from *R. rubiginosa* (Fig. 5) have a clear protein peak in the void volume of the Sephacryl S200 column (V_0), indicating that an important percentage of the native protein was constituted by molecular aggregates of M.W. over 200 kDa. The analysis of both heat-denatured protein fractions by SDS-PAGE also indicated, as previously discussed, the presence of aggregates. Only the treatment with β-mercaptoethanol promoted, to a certain extent, the deaggregation, indicating that stabilization of the aggregates involved disulfide bonds.

In addition to the aggregated components, both the albumin and globulin fractions contained the same low-M.W. components: a peak at approximately 10 kDa and a second, wider peak corresponding to a protein of approximately 5 kDa (determined in the center of the peak) (Figs. 5a, 5b). Indeed, below the lower separation range of the column (5 kDa), a new protein peak eluted from the column in both Osborne fractions.

IEF. The pI values of the Osborne fractions separated from *R. rubiginosa* could not be determined because, in these experimental conditions, the proteins precipitated in the gels at the beginning of the electrophoresis. This result may have occurred because the native proteins of the seed were mainly high-M.W. aggregates as observed by GFC.

Figure 6 shows the IEF gels from albumin (lane 1) and globulin (lane 2) extracts from *G. avellana*. The albumin fractions were solvated into three groups of proteins, each containing two bands. The pI values were centered in the minor group at 8.3 and in the two major groups at 5.7 and 6.5. In the case of globulins (lane 2), only the acidic protein groups were present, but each group formed at least three bands whose intensity decreased inversely to the pI. The pI was centered in the interval 5.4–4.9 and 6.3–5.8 for the first and second protein groups, respectively.

The pI values estimated by IEF for *G. avellana* albumin and globulin fractions were coincident with the protein extractability curve of this seed (Fig. 1), which showed, as expected from the pI values, a minimum at acidic pH values (3–5).

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