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Nutritional Potential of Raw and Free α-Galactosides Lupin (*Lupinus albus* Var. *multolupa*) Seed Flours. Effect of Phytase Treatment on Nitrogen and Mineral Dialyzability

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The effect of the removal of α-galactosides from *Lupinus albus* L. var. *multolupa* on the chemical composition of the prepared flour and the dialyzability of N, total P, Ca, Mg, Fe, Zn, and Mn was studied. The extraction process caused a significant increase in total and insoluble nitrogen contents and decreased the amount of soluble protein nitrogen. However, neither these changes nor treatment with phytase seemed to considerably affect in vitro protein digestibility. Except for Ca and Cu, total mineral contents were significantly reduced by the extraction process. The process also caused a significant reduction in the dialyzability of all the minerals studied except P. The decrease in mineral dialyzability was partially counteracted by phytase treatment in amounts of 250−500 phytase units/kg of lupin flour. In the case of Fe, mineral dialyzability did not differ significantly between the two lupin flours studied with treatment with 500 phytase units/kg. Zinc dialyzability was the most efficiently improved by phytase treatment ($P < 0.0001$), followed by P, Fe, and Mn, and finally by Ca and Mg ($P < 0.05$).

KEYWORDS: ***Lupinus albus*; α-galactoside extraction; phytase; nutrient dialyzability**

INTRODUCTION

Lupins can survive in poor soils where the growth of other crop plants is limited (*1*). Lupin seeds (*Lupinus* spp.) have great potential for human and animal nutrition because of their high content of protein, minerals, vitamins, dietary fiber, and oil (*1*, *2*) and their low proportion of nonnutritive components, such as trypsin inhibitors and lectins (*3*). The content of α-galactoside oligosaccharides, tannins, and phytic acid in lupin seeds, however, is similar to or higher than that in other legumes (*4*). In addition to its good nutritional properties, lupin has important functional characteristics related to its hypocholesterolemic capacity (*5*) and its antioxidant (*6*) and antimicrobial (*7*) properties. Lupin is usually used in animal diets, where it replaces other protein sources in small proportions (*8*), and has started to be introduced in food products for human consumption (*9*, *10*). Among the major reasons that preclude a greater inclusion of lupin in human or animal diets are the production of flatulence, which results from the content of α-galactoside oligosaccharides, and the presence of phytic acid. Phytic acid decreases the availability of important dietary minerals because it forms insoluble complexes with di- and trivalent cations at the physiological pH of the small intestine of monogastric animals. With the aim of removing nonnutritional factors such as α-galactosides and phytic acid from legume seeds, treatments such as soaking in different pH solutions and cooking, germination, and fermentation have been developed (*11*−*13*). In addition, phytase supplementation has proved to be an efficient means of decreasing the content of phytic acid and reversing the phytate-dependent inhibition of mineral availability in foodstuffs intended for human or animal consumption, as has been shown by several in vitro and in vivo experiments (*14*−*16*).

With the aim of extracting α-galactoside oligosaccharides from legume seeds for use as functional food ingredients, Gulewicz et al. (*17*) developed an extraction procedure that combines initial water imbibition of the seeds followed by extraction in 50% ethanol at 40 °C. With the use of this procedure, those authors obtained a good yield of α-galactosides and a legume seed byproduct with a high protein content that can be used to prepare protein isolates or food products with improved nutritional value. However, prolonged soaking in different pH and temperature conditions may lead to important mineral losses as a result of leaching (*4*, *18*). Furthermore, the use of organic solvents such as ethanol may affect the structure of the food matrix and reduce protein quality.

In vitro techniques based on the diffusibility of nutrients through a dialysis membrane under conditions that resemble those found in the gastrointestinal tract can be a reliable indicator of the potential availability of these nutrients from different foods (*19*, *20*).

For the present study, we chose a lupin variety (*Lupinus albus* var. *multolupa*) with low alkaloid concentrations and medium

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α-galactoside content (*21*). Our objectives were (1) to assess N, P, Ca, Mg, Fe, Zn, and Mn availability from the free α-galactosides lupin flour that remains after α-galactoside extraction of the seed and (2) to test whether supplementation of a commercial phytase enzyme (*Aspergillus niger*) at inclusion rates similar to those usually found in human and animal nutrition (*15*, *16*) improves nitrogen and mineral availability.

MATERIALS AND METHODS

Lupins. *Raw Lupin*. Raw lupin flour was from *L. albus* var. *multolupa*. Seeds were ground to a fine powder (0.18 mm sieve) and lyophilized for chemical analysis and mineral dialyzability.

Free α-galactosides flour obtention was performed according to the method of Gulewicz et al. (*17*). In brief, lupin seeds were soaked in distilled water at 4 °C for 10−12 h. α-Galactosides were extracted from the imbibed seeds with two consecutive extractions using 50% ethanol at 40 °C overnight. After the extraction process, the extracted seeds were homogenized and lyophilized, obtaining the free α-galactosides flour.

Analyses. *pH and titratable acidity* were determined as described by Barampama and Simard (*22*) and Frias et al. (*23*). Titratable acidity was expressed as milliequivalents of NaOH per 100 g of dry matter (DM).

Chemical Analysis. The moisture content of raw and free α-galactosides lupin flours was determined by drying to constant weight in an oven at 105 ± 1 °C. Total nitrogen was determined according to Kjeldahl's method. Crude protein was calculated as N × 6.25. Soluble protein and non-protein nitrogen were measured using the methodology described by Periago et al. (*24*) after extraction with 0.2% NaOH and precipitation with 30% trichloroacetic acid. Insoluble nitrogen was measured in the remaining flour after extraction. The ash content of the raw and free α-galactosides lupin flours was measured by calcination at 500 °C to a constant weight. Samples of ashed material were dissolved in 6 N HCl before analysis. Calcium, magnesium, iron, zinc, copper, and manganese contents were determined by atomic absorption spectrophotometry using a Perkin-Elmer AAnalyst 300 spectrophotometer. Lanthanum chloride was added to calcium and magnesium samples to prevent interferences caused by phosphate ions. Potassium and sodium were determined by atomic emission spectrophotometry using a Perkin-Elmer AAnalyst 300 spectrophotometer. Phosphorus was measured spectrophotometrically using the technique described by Chen et al. (*25*). Analytical results were validated by a standard reference wholemeal flour CRM-189 (Community Bureau of Reference. Commission of the European Communities). [Mean ± SD values of five independent replicates were as follows: Mn, 63.9 ± 1.30; Cu, 6.62 ± 0.28; Fe, 66.3 ± 0.14; Zn, 55.0 ± 1.11 (μg/g); P, 565.8 ± 0.90; Mg, 189.61 ± 5.81; and K, 600 ± 23.7 mg/100 g of dry matter. Certified values ± uncertainty ranges are for Mn, 63.3 ± 1.6; Cu, 6.4 ± 0.2; Fe, 68.3 ± 1.9; and Zn, 56.5 ± 1.7 μg/g. Indicative values are 530 for P, 190.0 for Mg, and 630 for K.] Phytic acid and free phosphorus were determined using the methodology described by Latta and Eskin (*26*) and Chen et al. (*25*).

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE). Proteins were extracted from the raw and free α-galactosides lupin flours in a two-step sequential extraction process as described by Melo et al. (*27*). The albumin fraction of the protein was extracted by stirring with bidistilled water (pH adjusted to 5) containing 1 mM phenylmethanesulfonyl fluoride The remaining flour was extracted with 100 mM phosphate buffer/1% SDS/2% β-mercaptoethanol/2% NaCl, pH 9; the residual flour was collected for nitrogen determination. SDS-PAGE was done according to the method of Laemmli (*28*). The final concentration of acrylamide in the running gel was 15%. The gels were fixed and stained with 0.2% Coomassie brilliant blue R-250 in methanol/acetic acid/water (5:4:1 v/v/v). The mixture of molecular weight markers (Merck) consisted of cytochrome *c* (12.3 kDa), myoglobin (16.9 kDa), carboanhydrase (30 kDa), ovalbumin (42.7 kDa), albumin (66.25 kDa), and ovotransferrin (78 kDa).

In vitro protein digestibility (IVPD) was determined using the pH-drop multienzyme system described by Hsu et al. (*29*) or the pH-stat multienzyme system described by McDonough et al. (*30*). For the pH-drop method, percent protein digestibility was calculated from the equation $y = 210.464 - 18.1X$, where X is the pH change after 10 min. In the pH-stat method, the enzyme mixture was added to the protein solution, and the pH value was kept constant at 7.98 by the addition of 0.1 M NaOH during 10 min exactly. Percent protein digestibility was calculated from the equation $y = 79.28 + 40.74B$, where B = mL of 0.1 M NaOH used during 5 min.

Dialyzability. The in vitro method of Miller et al. (*31*) that uses a pepsin digestion period of 2 h followed by pH equilibration and pancreatin digestion for another 2 h coupled with equilibrium dialysis (diameter = 14.3 mm, MWCO = 12000−14000 Da, Medicell International Ltd., London, U.K.) was adapted to assess nitrogen, phosphorus, calcium, magnesium, iron, zinc, and manganese dialyzability of raw and free α-galactosides lupin flours. At the end of pancreatin digestion, dialysis bags were removed from the digestion vials, and nitrogen, phosphorus, calcium, magnesium, iron, zinc, and manganese were assayed in the dialysate. Dialyzable nitrogen and minerals were expressed as percentage of the total present in each digestion vial assuming that the dialyzable component had equilibrated across the dialysis membrane by the time the dialysis bag was removed at the end of the digestion period.

Phytase Treatment. An amount of exogenous microbial phytase (Natuphos, BASF, Mt. Olive, NJ) equivalent to 250, 500, and 750 units/kg of lupin flour was added from a stock solution of 34 phytase units/mL at the beginning of the pepsin digestion dissolved in equal volumes of bidistilled water to raw and free α-galactosides lupin flour samples. The same amount of enzyme was added to blank samples to control for the presence of minor quantities of minerals in the phytase solution. Phytase activity of the exogenous microbial phytase used in the dialyzability experiments was assayed by measuring the free phosphorus released after incubation of the enzyme with sodium phytate during 15 min at 37 °C in 0.2 M citrate buffer (pH 5.0). One unit of phytase activity was defined as the amount of phytase activity that liberates 1 μmol of inorganic phosphorus from sodium phytate per minute at pH 5.0 and 37 °C.

For the determination of endogenous phytase activity in raw and free α-galactosides lupin seed flours, extraction was done in 0.2 M citrate buffer/phenylmethanesulfonyl fluoride/Triton X-100 (pH 5.0) (*32*) during 60 min at 4 °C with a flour-to-buffer ratio of 1:8 (w/v). The extract was spun down at 3000*g* for 10 min and the supernatant desalted using Sephadex G-25. The enzyme activity was assayed by measuring the free phosphorus released after incubation of the desalted fractions with sodium phytate during 30 min at 50 °C in 0.2 M citrate buffer (pH 5.0). One unit of phytase activity was defined as the amount of phytase activity that liberates 1 μmol of inorganic phosphorus from sodium phytate per minute at pH 5.0 and 50 °C.

Statistics. Analysis of the data was done using SAS (release 8.02, SAS Institute, Inc., Cary, NC). Data on chemical composition were analyzed using a two-tailed Student's *t* test. The effect of α-galactoside removal and supplementation of different phytase doses on mineral dialyzability from raw and free α-galactosides lupin flours was analyzed by a 2 × 4 factorial ANOVA with α-galactoside extraction and phytase (0, 250, 500, and 750 units/kg) as the main treatments. Tukey's test was used to detect differences between treatment means. Data on mineral dialyzability in response to α-galactoside extraction and phytase treatment was adjusted to a multiple lineal regression model. Multivariate analysis of variance was done among the estimated b parameters from the regression equations that predict Zn, P, Mn, Fe, Ca, and Mg dialyzability, with the aim of assessing the efficiency of response to phytase treatment by the different minerals studied. The level of significance was set at 0.05.

RESULTS

Chemical Analyses. No significant differences related to pH were observed as a result of the α-galactoside extraction process (**Table 1**), which, on the other hand, did cause a significant decrease in the titratable acidity of the free α-galactosides lupin flour (23%). Of the total nitrogen present in raw lupin flour, 81.2% corresponded to soluble protein nitrogen, 10.6% cor-

Table 1. Changes in pH, Titratable Acidity, and Nitrogen Content of *L. albus* Var. *multolupa* Flours as a Result of the α-Galactoside Extraction Process[a]

	raw lupin flour	free α-galactosides lupin flour
pH	5.4 ± 0.01	5.5 ± 0.01
titratable acidity (mequiv of NaOH/100 g)	16.67 ± 0.22B	12.83 ± 0.13A
total N (g/100 g)	5.58 ± 0.03A	6.24 ± 0.05B
insoluble N (g/100 g)	0.46 ± 0.01A	1.52 ± 0.06B
soluble protein N (g/100 g)	4.53 ± 0.04B	4.23 ± 0.05A
soluble non-protein N (g/100 g)	0.59 ± 0.01	0.58 ± 0.01
peptidic soluble non-protein N (g/100 g)	0.46 ± 0.004B	0.37 ± 0.007A

[a] Results (expressed in dry matter) are means ± SEM of five independent replicates. Means within the same line with different letters differ significantly ($P < 0.05$). Analysis of variance of the results was done using Student's *t* test.

Table 2. Changes in Protein Solubility in Water (pH 5) and 100 mM Phosphate Buffer/1% SDS/2% β-Mercaptoethanol/2% NaCl (pH 9) of *L. albus* Var. *multolupa* Flours Caused by the α-Galactoside Extraction Process[a]

	raw lupin flour	free α-galactosides lupin flour
total N	5.58 ± 0.03A	6.24 ± 0.05B
water-soluble N	0.61 ± 0.03B	0.34 ± 0.05A
buffer-soluble N	4.71 ± 0.12A	5.13 ± 0.08B
insoluble N	0.32 ± 0.02A	1.10 ± 0.03B

[a] Results (expressed in g/100 g of dry matter) are means ± SEM of five independent replicates. The factor 6.25 was used to calculate the crude protein content. Means within the same line with different letters differ significantly ($P < 0.05$). Analysis of variance of the results was done using Student's *t* test.

responded to soluble non-protein nitrogen, and the remaining 8.3% was not soluble at the basic pH used for nitrogen extraction. A considerable proportion of the soluble non-protein nitrogen (78%) was of peptidic nature and could be detected by a colorimetric method. The α-galactoside extraction process brought about a significant increase in the total (11.8%) and insoluble (3.3-fold) nitrogen contents compared with those in raw lupin flour, decreased the content of soluble protein nitrogen (6.6%; $P < 0.05$), and did not significantly affect the content of soluble non-protein nitrogen. With regard to the soluble non-protein nitrogen content of the free α-galactosides lupin flour, the percentage of peptidic nitrogen (63.8%) was lower than that found in the raw lupin flour.

The percentage of nitrogen soluble in water (pH 5) or buffer (pH 9) with respect to the total nitrogen content was higher in raw than in free α-galactosides lupin flour (10.6 vs 5% and 84.4 vs 82.2% in water and buffer solution, respectively) (**Table 2**). When the water-soluble proteins were separated in a polyacrylamide gel (**Figure 1**), the highest density of polypeptide bands corresponded with the raw lupin flour sample despite the similar amounts of nitrogen loaded in the gel for the raw and free α-galactosides flour samples. Regarding the buffer-soluble proteins, α-galactoside extraction led to the appearance of high molecular weight protein aggregates that remained at the top of the resolving gel and that were not present in the raw lupin sample.

The average total ash content of the free α-galactosides lupin flour decreased by 33% with respect to raw lupin flour as a result of the extraction process (**Table 3**). The minerals with the highest losses as a result of the extraction process were Mg (51.9%), Na (42%), and K (40.1%), whereas losses were lower

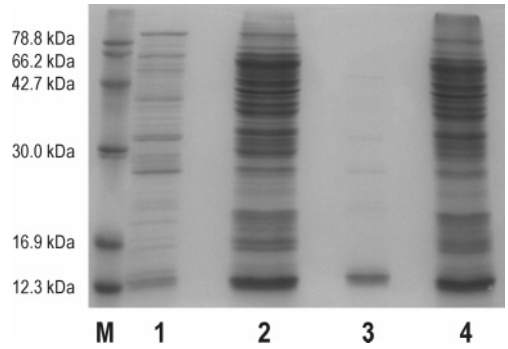

Figure 1. Lanes: M, molecular weight marker; 1, water-soluble proteins of raw lupin flour; 2, buffer-soluble proteins of raw lupin flour; 3, water-soluble proteins of extracted lupin flour; 4, buffer-soluble proteins of extracted lupin flour. Equal amounts of nitrogen (1.20 and 6.30 μg for the water- and buffer-soluble fractions, respectively) were loaded in each lane. The figure is representative of four independent analyses.

Table 3. Changes in Phytate and Mineral (P, Ca, Mg, Zn, Cu, Mn, Fe, K, Na) Contents of *L. albus* Var. *multolupa* Flours Caused by the α-Galactoside Extraction Process[a]

	raw lupin flour	free α-galactosides lupin flour
ash (%)	3.51 ± 0.02B	2.34 ± 0.01A
total P (mg/100 g)	332.15 ± 3.24B	301.60 ± 0.55A
phytate (mg/g)	8.31 ± 0.08	8.56 ± 0.12
free P (mg/100 g)	14.20 ± 0.22	14.27 ± 0.35
Ca (mg/100 g)	138.92 ± 1.21A	178.95 ± 0.77B
Mg (mg/100 g)	145.0 ± 0.74B	69.71 ± 1.06A
Zn (mg/100 g)	4.25 ± 0.04	4.20 ± 0.04
Cu (mg/100 g)	0.72 ± 0.03	0.78 ± 0.01
Mn (mg/100 g)	90.13 ± 1.27B	82.06 ± 0.47A
Fe (mg/100 g)	3.80 ± 0.02B	3.51 ± 0.03A
K (mg/100 g)	955.9 ± 7.90B	572.8 ± 3.51A
Na (mg/100 g)	112.3 ± 0.84B	65.18 ± 0.61A

[a] Results (expressed in dry matter) are means ± SEM of five independent replicates. Means within the same line with different letters differ significantly ($P < 0.05$). Analysis of variance of the results was done using Student's *t* test.

Table 4. In Vitro Protein Digestibility (IVPD) of Raw and Free α-Galactosides *L. albus* Var. *multolupa* Flours[a]

	raw lupin flour	free α-galactosides lupin flour
IVPD pH-stat (%)	93.34 ± 0.20B	91.50 ± 0.41A
IVPD pH-drop (%)	84.95 ± 0.50	86.1 ± 0.49

[a] Results (expressed in dry matter) are means ± SEM of four independent replicates. Means within the same line with different letters differ significantly ($P < 0.05$). Analysis of variance of the results was done using Student's *t* test.

for Mn (9.0%) and Fe (7.6%). With regard to Ca, the extraction process brought about a 28.8% increase in content. Total P losses as a result of the extraction process were 9.2%, whereas no significant differences between the two lupin flours studied were observed in phytate, free P, or Cu content.

In Vitro Protein Digestibility. IVPD (**Table 4**), measured with the use of a pH-stat method, gave significantly higher values in raw than in free α-galactosides lupin flour ($P < 0.05$), whereas no significant differences were apparent when a pH-drop method was used.

Nitrogen and Mineral Dialyzability. No significant differences were found in N or P dialyzability between the two lupin flours studied (**Table 5**). In contrast, a negative effect of the α-galactoside extraction process was observed on the dialyz-

Table 5. Changes in Nutrient Dialyzability of *L. albus* Var. *multolupa* Flours Caused by α-Galactoside Extraction Process and Treatment with Different Phytase Doses[a]

	raw lupin flour, phytase dose of				free α-galactosides lupin flour, phytase dose of							
	0 units/kg	25 units/kg0	500 units/kg	750 units/kg	0 units/kg	250 units/kg	500 units/kg	750 units/kg	SEM	extraction effect	phytase effect	extraction × phytase
N	61.2A	61.4A	59.6A	60.0A	58.0A	58.6A	58.8A	59.4A	1.98	0.20	0.98	0.88
total P	8.8A	31.8B	42.9C	50.2D	7.6A	30.3B	43.7C	54.0D	1.17	0.55	<0.0001	0.11
Ca	91.5A	107.3B	106.9B	102.9B	33.8C	45.1D	49.0D	47.2D	2.30	<0.0001	<0.0001	0.55
Mg	68.1A	74.0AB	78.5B	74.3AB	53.8CD	63.7A	68.0AB	62.8AD	2.24	<0.0001	<0.0001	0.79
Zn	27.5A	78.9B	80.1B	80.7B	5.5C	25.9A	47.0D	45.7D	2.61	<0.0001	<0.0001	<0.0001
Mn	36.1A	60.5B	62.3B	61.7B	19.4C	35.7A	45.8D	45.8D	1.09	<0.0001	<0.0001	0.0002
Fe	27.8A	45.0B	49.1B	48.3B	13.3C	34.8A	50.5B	52.8B	2.28	0.0050	<0.0001	0.0001

*Results (expressed in dry matter) are means of five independent experiments. SEM, pooled standard error of the mean. Means within the same row with different letters differ significantly ($P < 0.05$).

ability of Ca, Mg, Fe, Zn, and Mn, for which dialyzability was significantly higher in the raw than in the free α-galactosides lupin flour. This was reflected in a significant effect ($P < 0.0001$) of the extraction process on the dialyzability of the above-mentioned cations.

The treatment with 250 phytase units (PU)/kg significantly increased the P, Ca, Mg, Fe, Zn, and Mn dialyzability of the two lupin flours studied, which was reflected in a significant effect of the phytase treatment on the dialyzability of these minerals. Addition of higher phytase doses (500 and 750 PU/kg) did not lead to any further improvement in mineral dialyzability from the raw lupin flour except for P. In contrast, a gradual and significant improvement in Fe, Zn, and Mn dialyzability was observed in the free α-galactosides lupin flour with increasing phytase doses up to 500 PU/kg. This improvement was reflected in a significant statistical interaction between the α-galactoside extraction process and phytase treatment in relation to Fe, Zn, and Mn dialyzability. The highest phytase dose (750 PU/kg) had a significant effect on only the total P dialyzability of the two lupin flours studied.

Changes in the percentage of dialyzable P, Ca, Mg, Fe, Zn, and Mn in response to the α-galactoside extraction process and phytase treatment were fitted to a multiple linear regression model that was satisfied by the equation $y = a + b$[phytase dose] $+ c$[phytase dose]$^2 + d$[α-galactoside extraction] (**Table 6A**), where $y =$ percentage of dialyzable mineral. Multivariate analysis of the *b* terms (corresponding to the phytase dose), intended to determine which of the minerals had a more efficient response to phytase treatment (**Table 6B**), showed significant differences among the minerals studied. The highest *b* term value corresponded to Zn ($P < 0.0001$), followed by P, Fe, and Mn, among which no significant differences could be found, and finally by Ca and Mg ($P < 0.05$).

Table 6. Relationship between the Percentage of Dialyzable Minerals and Phytase Treatment in Raw and Free α-Galactosides Flour of *L. albus* Var. *multolupa*

A. Multiple Regression Model[a]

mineral	*a*	*b*	*c*	*d*	adjusted r^2	*P*
P	7.83	0.09954	–0.00005600		0.9490	<0.0001
Ca	150.62	0.06490	–0.00006572	–58.41	0.9451	<0.0001
Mg	78.16	0.04870	–0.00005041	–11.67	0.5168	<0.0001
Zn	70.82	0.16926	–0.00014497	–35.79	0.8749	<0.0001
Mn	55.87	0.09549	–0.00008264	–18.46	0.9273	<0.0001
Fe	27.54	0.09581	–0.00007436	–4.668	0.6986	<0.0001

B. Multivariate Test of Differences among the Different *b* Terms

	Zn	P	Fe	Mn	Ca	Mg
Zn		$F = 28.82$ $P < 0.0001$	$F = 23.09$ $P < 0.0001$	$F = 28.00$ $P < 0.0001$	$F = 72.75$ $P < 0.0001$	$F = 44.07$ $P < 0.0001$
P	$F = 28.82$ $P < 0.0001$		$F = 0.11$ $P = 0.7439$	$F = 0.33$ $P = 0.5663$	$F = 12.29$ $P = 0.0008$	$F = 22.12$ $P < 0.0001$
Fe	$F = 23.09$ $P < 0.0001$	$F = 0.11$ $P = 0.7439$		$F = 0.00$ $P = 0.9806$	$F = 5.37$ $P = 0.0232$	$F = 10.42$ $P = 0.0018$
Mn	$F = 28.00$ $P < 0.0001$	$F = 0.33$ $P = 0.5663$	$F = 0.00$ $P = 0.9806$		$F = 8.76$ $P = 0.0041$	$F = 17.88$ $P < 0.0001$
Ca	$F = 72.75$ $P < 0.0001$	$F = 12.29$ $P = 0.0008$	$F = 5.37$ $P = 0.0232$	$F = 8.76$ $P = 0.0041$		$F = 1.32$ $P = 0.2539$

[a] The multiple regression model applied was satisfied by the following equation: mineral dialyzability $= a + b$[phytase dose] $+ c$[phytase dose]$^2 + d$[extraction treatment].

DISCUSSION

Chemical Analyses. The lupin flour used in the present study had a high protein content similar to that described by other authors for this lupin variety and for soybeans (*33*) and higher than that of other legumes, such as peas, beans, and lentils (*13*, *34*, *35*). The proportion of insoluble nitrogen or soluble protein and non-protein nitrogen with respect to the total nitrogen content was similar to that in other legumes, such as peas and beans (*13*, *34*). The increase in total nitrogen content resulting from the α-galactoside extraction process was due not only to the mineral losses caused by leaching but also to the removal of α-galactosides (*36*) and the decrease in dietary fiber content (Martinez-Villaluenga, unpublished results). The increase in insoluble nitrogen content was related to the appearance of high molecular weight protein aggregates in the salt-soluble protein fraction that hardly migrated into the resolving gel (**Figure 1**). Nevertheless, the increase in insoluble nitrogen content was lower than reported by Sanz et al. (*37*) after extraction of lentil flour with 80% ethanol for 3 h at 50 °C. The lower proportion of ethanol added to the extraction solution in the present study (50%), the lower temperature used during the extraction process (40 °C), and the use of whole lupin seed rather than legume flour may explain the lower degree of protein denaturation under our experimental conditions.

The ash content of the raw lupin flour used in the present study was within the range of values found in the literature (*1*) for this variety and was higher than that of other legumes, such as chickpeas (*38*). The high solubility of Mg, K, and Na was responsible for the decrease in ash content observed as a result of the extraction process. The losses of total P, Mn, and Fe also contributed, albeit to a lesser extent. Furthermore, total mineral losses were higher than what is inferred from the results, given that total mineral content was expected to increase as a result of the removal of α-galactoside oligosaccharides and the

decrease in dietary fiber content as a result of the extraction process. In the case of manganese, the losses resulting from the extraction of α-galactosides are beneficial, because the seeds of *L. albus* are efficient accumulators of this mineral. Nevertheless, the manganese content of the lupin variety used for the present study was considerably lower than the upper range of values reported in the literature, which may be as high as 447.9 mg/100 g of DM (*39*).

Other authors have described mineral losses higher than those reported in the present study after soaking and cooking various legume seeds at different pH values (*4*, *18*). In general, the amount of mineral that leaches into the soaking solution during the course of processing depends on the legume species, the thickness of the seed coat, the time and temperature of the soaking or extraction process, and the pH of the soaking or extracting solution.

The calcium over-concentration observed after the α-galactoside extraction process was due to the above-mentioned loss of other components of the seed by solubilization and the fact that most of the calcium in the seed is usually present in a poorly soluble form associated with phytate in the cotyledons (*40*) or in complexes with oxalate and pectins in the seed hull (*41*, *42*).

In Vitro Protein Digestibility. The IVPD of raw lupin flour assessed by the use of either the pH-drop or pH-stat method was high and was similar to that of other soy-based and animal protein sources (*29*, *30*). The higher levels of insoluble nitrogen present in the free α-galactosides lupin flour did not seem to affect its protein digestibility measured by the pH-drop method. This was in contrast with the results obtained with the pH-stat method, in which a small but significant decrease in digestibility was associated with higher insoluble nitrogen content. These differences are probably related to the different buffering capacities of the two legume flours, which may have affected the results obtained by the pH-drop but not the pH-stat method.

Nitrogen and Mineral Dialyzability. The slight reduction in the percentage of dialyzable nitrogen found for the free α-galactosides lupin flour was related to its higher content of insoluble nitrogen. On the other hand, the percentage of dialyzable nitrogen in both lupin flours was lower than expected from the sum of their soluble protein and non-protein nitrogen contents. However, this index of protein digestibility was significantly higher in the lupin flours used in the present study than in beans (*13*) and was similar to percentages found in legumes with good protein quality, such as peas (*34*).

The detrimental effect of phytic acid on protein digestibility arises from its ability to form poorly digested complexes with dietary protein (*43*) and to inhibit the activity of proteolytic enzymes (*44*). Under our experimental conditions, such an inhibitory effect was not observed, and supplementation with increasing phytase doses did not result in a significant increment in nitrogen dialyzability.

Phosphorus dialyzability from legumes is highly correlated with the amount of free inorganic P (*13*). This was confirmed in the present study by the low percentage dialyzability of total P from both flours. The lack of effect of the α-galactoside extraction process on the content of phytic acid, and thus on the release of free inorganic P, may have resulted from the pH and temperature conditions of the extraction process not being optimal for the efficient degradation of phytic acid by the endogenous phytase of lupin seed (40 units/kg at pH 5.0 and 50 °C). Furthermore, the extraction process was performed with whole legume seeds, which may have prevented phytic acid from leaching into the extraction media. The addition of increasing doses of a commercial phytase (*A. niger*) that was active at the pH conditions used was responsible for the significant improvement in phosphorus dialyzability. However, the rise in phosphorus dialyzability observed for both lupin seed flours after supplementation with increasing doses of phytase did not correlate well with the amount of enzyme added. Furthermore, the dialyzability of total P did not exceed 50−55% upon addition of the highest phytase dose (750 PU/kg). These results suggest that a higher amount of phytase is necessary to achieve complete dephosphorylation of phytic acid. In addition, product-related inhibition of the phytase enzyme or a lower affinity of *A. niger* phytase for inositol phosphates with a lower degree of phosphorylation (*45*) may play a role in the final amount of dialyzable phosphorus. Nevertheless, the observed increase in total P availability resulting from phytase treatment represents an important nutritional improvement for the development of lupin products intended for human or animal consumption.

In vitro calcium digestibility may be affected by dietary components such as phytic acid, dietary fiber, and oxalate (*46*). However, these components did not seem to play an important role in the raw lupin seed meal used in the present study, as shown by the high calcium dialyzability obtained, which was higher than that found for other legumes (*13*, *46*). Phytic acid appeared to be the only factor that may have affected calcium dialyzability, given that supplementation with 250 PU/kg was enough to achieve complete dialyzability of this mineral.

In contrast with what was described by other authors (*47*), the decrease in calcium dialyzability was found to correlate with the removal of α-galactosides from the lupin seed meal. The lower calcium dialyzability was also related to the observed decrease in titratable acidity of the free α-galactosides lupin flour, which may have resulted from the leaching of organic acids into the extraction solution (*48*). Other factors that could contribute to lower calcium dialyzability are changes in dietary fiber composition and phytic acid, although an increase in dialyzability similar to that observed for raw lupin flour was found in free α-galactosides lupin flour after supplementation with 250 PU/kg.

Magnesium dialyzability from raw lupin flour was lower than expected in view of the high amount of soluble, and thus potentially dialyzable, magnesium lost from the seed during the α-galactoside extraction process. This could be attributed to the high dietary fiber in lupin seeds and, to a lesser extent, the presence of phytic acid, given that treatment with the lowest phytase dose (250 PU/kg) was sufficient to slightly improve magnesium dialyzability, which did not continue to improve with increasing phytase doses.

Despite the 50% reduction in total magnesium content caused by the α-galactoside extraction treatment, the dialyzability of the remaining magnesium was efficient compared with raw lupin flour. In a similar way to what was observed with calcium, the improvement in dialyzability caused by phytase treatment (250 PU/kg) of extracted lupin flour was analogous to that observed with the raw lupin flour and did not continue to increase with higher phytase doses.

The higher percentage of dialyzable iron from raw lupin flour than from other legumes such as beans (*13*) may be related to the lupin flour's lower phytic acid content, higher titratable acidity, and different protein structure. The loss of soluble iron and endogenous ligands responsible for titratable acidity during α-galactoside extraction is a probable reason for the lower proportion of iron capable of diffusing through the dialysis membrane at the pH conditions used to resemble digestion in the small intestine than was the case for the raw lupin flour.

Nevertheless, under our experimental conditions, phytic acid seemed to be the most significant component related to iron dialyzability in the two lupin flours studied, given that supplementation with the lowest phytase dose studied (250 PU/kg) led to a considerable improvement in the percentage of dialyzable iron, whereas a maximum dialyzability value of 50% was reached when 500 PU/kg was supplemented.

The low percentage of dialyzable zinc found in raw lupin flour was mainly correlated under our experimental conditions with the presence of phytic acid, as seen by the huge increase in dialyzability caused by supplementation with 250 PU/kg. Other components of raw lupin flour, such as dietary fiber, may also play a role and would be responsible for the lower zinc dialyzability of raw lupin flour than what has been reported for another legumes such as beans (*13*, *49*).

The significant reduction in Zn and Mn dialyzability caused by the loss of soluble cations and changes in the chemical composition of the lupin seed that took place during the α-galactoside extraction process was parallel to that previously reported for Ca and Fe. Zn and Mn dialyzability differed between the two lupin flours studied in response to phytase treatment, and dialyzability values were significantly lower for the free α-galactosides lupin flour even at the highest supplemented dose (750 PU/kg). This is in contrast with what was observed for Fe and suggests that changes in certain food components that can interact with phytic acid and thus affect mineral dialyzability may alter the response of the cations to phytate hydrolysis by phytase during the digestive process.

The different affinities of phytic acid for divalent and trivalent cations may be of great importance to research into the inhibitory effect of this food component on mineral absorption. In the multivariate analysis, we found that the dialyzability of zinc was most efficiently improved by phytase treatment. The nutritional importance of phytase was intermediate for Fe and Mn and much lower for Ca and Mg.

In conclusion, the flour of *L. albus* var. *multolupa* is a valuable dietary source of protein and minerals (Ca, P, Mg, Zn, Fe, Cu, K, and Na). Neither the removal of α-galactosides nor supplementation with increasing phytase doses had a significant effect on IVPD. Mineral losses caused by leaching into the extraction solution of cations with higher solubility and potentially higher dialyzability may be partially compensated for by phytase treatment in amounts of 250−500 PU/kg. In that sense, the best response is obtained for Fe. The dialyzability of this cation is high, and treatment with 500 PU/kg totally compensates for the reduction in dialyzability caused by the α-galactoside extraction process.

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