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Effects of hydroalcoholic α-galactoside extraction and phytase supplementation on the nutritive utilization of manganese, iron, zinc and potassium from lupin (*Lupinus albus* var. *multolupa*)-based diets in growing rats

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Abstract

The effects of α-galactoside removal, using a hydroalcoholic extraction process and phytase supplementation, on the digestive and metabolic utilization of total ash, Mn, Fe, Zn and K from *Lupinus albus* var. *multolupa*-based diets by growing rats were evaluated, using a balance technique, and compared to the results obtained using a casein–cystine control diet. The specific amount of minerals needed to complement those provided by the lupin flours and casein in order to reach the target requirements of the growing rat were supplemented as heme or non-heme iron sources or in the form of inorganic salts in the case of Zn or K. The nutritive utilization of total ash, Mn, Fe and Zn was higher from raw and α-galactoside-free lupin flour diets than from the casein–cystine control or the phytase-supplemented diets, whereas smaller differences were found regarding the nutritive utilization of K. Differences in mineral retention were reflected in changes of the mineral contents of some tissues, which varied among the different cations studied.

Keywords: Lupin; α-Galactoside extraction; Phytase; Nutritive utilization; Mn; Fe; Zn; K

1. Introduction

Lupins are important nutritional sources of protein, complex carbohydrates, minerals, vitamins and antioxidant compounds (Van Barneveld, 1999). The nutritive utilization of lupin protein is high, due to its good quality and to the absence, in this legume, of non-nutritional components with a potential inhibitory effect on protein digestibility (Martínez-Villaluenga, Urbano, Porres, Frías, & Vidal-Valverde, 2007; Porres, Aranda, López-Jurado, & Urbano, 2006). However, there are a number of compounds in lupin seed that prevent a wider use of this legume for human or animal nutrition; among such compounds are bitter-tasting alkaloids that affect the organoleptic properties of lupin and pose systemically harmful effects (Ruiz & Sotelo, 2001), flatulence-causing α-galactosides (Martinez-Villaluenga, Frias, & Vidal-Valverde, 2005), and phytic acid, which interferes with the nutritional availability of essential minerals (Porres, Aranda, López-Jurado, & Urbano, 2005). Furthermore, excessive Mn, accumulated by *Lupinus albus*, can exert a certain degree of toxicity and limit the use of this lupin species to the preparation of protein isolates in

Abbreviations: RLU, raw lupin flour diet; GFLU, α-galactoside-free lupin flour diet; C + C, casein–cystine control diet; PHYTS, α-galactoside-free lupin flour diet supplemented with phytase; AIN-93G, American Institute of Nutrition Standard experimental diet; SDF, soluble dietary fibre; IDF, insoluble dietary fibre; Ash_{fec}, amount of ash excreted in feces; Ash_{Int}, amount of ash ingested; K_{fec}, amount of K excreted in feces; K_{Int}, amount of K ingested; RBC, red blood cell count; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin content.

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which the levels of this mineral are reduced considerably (Martínez-Villaluenga et al., 2007), or dietetic products in which, due to mineral dilution caused by the lower inclusion levels of the legume, the accumulation of excessive Mn in the final food product is not to be expected. Nevertheless, few data exist, in the literature, concerning the availability of Mn from legumes or the accumulation and toxic effects of a moderately high dietary Mn intake for short-medium periods of time. As reported by Pappas et al. (1997), procedural details, such as species, dose, duration of the experiment or age, can induce substantial variations among different studies, making it difficult to interpret the variety of reported effects. In this regard, studies dealing with Mn toxicity are usually carried out via intragastric administration of the mineral dissolved in water (Öner & Sentürk, 1995), administration of high doses dissolved in sucrose solution (Tran, Chowanadisai, Crinella, Chicz-DeMet, & Lönnerdal, 2002) or in the drinking water (Pappas et al., 1997; Torrente, Colomina, & Domingo, 2005), inhalation exposure (Normandin et al., 2004), or inhalation exposure in combination with dietary administration of the mineral in a semisynthetic diet (Dorman, Struve, & Wong, 2002), with experimental periods ranging from 20 to 91 days.

To avoid the negative effects on palatability and the development of toxicity associated with alkaloids, sweet lupin varieties have been established with low levels of these non-nutritional components. In addition, the levels of α-galactosides can be substantially reduced by the use of different technological processes, such as germination, fermentation or hydroalcoholic extraction (Granito et al., 2002; Gulewicz et al., 2000; Vidal-Valverde et al., 2002). Using the latter treatment, α-galactoside-rich preparations with improved functional value are obtained and these can be used in the development of prebiotics (Martinez-Villaluenga, Frias, Vidal-Valverde, & Gomez, 2005). Moreover, the seed residue remaining after the extraction process is a good source of high-quality protein, minerals and dietary fibre with promising nutritional value (Porres et al., 2005, 2006).

The phytase-catalyzed hydrolysis of phytic acid is an efficient means for improving the digestibility of essential minerals such as Fe and Zn, assessed by either *in vitro* techniques or animal models, such as poultry or swine (Porres et al., 2005; Stahl, Han, Roneker, House, & Lei, 1999; Viveros, Brenes, Arija, & Centeno, 2002). Other important dietary factors that may play an important role in the availability of these minerals are the source of dietary protein (vegetable, meat, milk), mineral speciation, chemical form in which the mineral is present in the foodstuff (heme or non-heme iron), and interaction with other minerals or non-nutritional components, e.g. tannins, that may interfere with mineral availability (Fairweather-Tait, 1996).

Legumes usually contain reasonably high amounts of potassium; this cation is of the utmost nutritional importance, due to its participation in numerous cell functions, and its essential role in bone health and in the regulation of blood pressure. Because of its physiological importance, the homeostatic regulation of potassium must be closely regulated. Nevertheless, no major dietary factors appear to have a significant influence on the intestinal absorption of this mineral, and its metabolic regulation takes place at the renal level, in contrast to what has been reported for other cations, e.g., Mn, Fe or Zn, that exhibit intestinal regulation (Windisch, 2002).

The objectives of the present study were (1) to study the effect of an α-galactoside extraction process and recombinant phytase supplementation on the digestive and metabolic utilization of total ash, Mn, Fe, Zn and K from lupin seed meal in growing rats, and (2) to supplement a lupin protein of good nutritional quality with two highly available dietary Fe sources (heme and non-heme) with the aim of assessing the influence of lupin protein and moderately high Mn levels on the availability of Fe.

2. Materials and methods

2.1. Plant material

Lupin seeds (*Lupinus albus*, var. *multolupa*) were provided by the Agrarian Research and Technology Development Service from the Agriculture and Commerce Council of the Junta de Extremadura (Spain). Seeds were cleaned, ground to a fine powder (0.18 mm sieve) and lyophilized for chemical analysis and diet preparation (RLU).

2.2. α-Galactosides extraction process

Hydroalcoholic extraction of α-galactosides was done according to Gulewicz et al. (2000). Briefly, lupin seeds were imbibed in distilled water at 4 °C for 10–12 h. Alpha-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 α-galactoside-free flour for chemical analysis and diet preparation (GFLU).

2.3. Experimental diets

2.3.1. Raw and α-galactoside-free lupin diets (RLU, GFLU)

The different experimental diets were formulated to meet the nutrient requirements of the growing rat, following the guidelines provided by the American Institute of Nutrition (Reeves, Nielsen, & Fahey, 1993) with lupin flour as the sole source of protein. L-Cystine (0.6%) was added to compensate the well known deficiency of sulfur-containing amino acids in legumes. The amounts of Mn, Fe and Zn provided by the lupin flours (Porres et al., 2005) were taken into consideration for the final concentration present in the diet; additional amounts of Fe and Zn, needed to bring the dietary levels up to target requirements of the growing rat, were supplied using Fe-citrate, hemoglobin, and $ZnCO_3$,

whereas no Mn was added to the lupin-containing diets. The rest of the minerals were included in the AIN-93G mineral premix. The amount of dietary fibre (insoluble dietary fibre, 382.5–402.5; soluble dietary fibre, 25.3–52.1 g/kg) provided by lupin flours was not modified.

2.3.2. Casein–cystine control diet (C + C)

The diet formulated to meet the nutrient requirements of the growing rat, following the guidelines of the American Institute of Nutrition (Reeves et al., 1993) with the exception of dietary fibre and Mn contents. In order to adjust the amount and type of dietary fibre to values similar to those of the lupin diets, cellulose was supplemented as the dietary source of insoluble fibre. Soluble dietary fibre (*Plantago ovata*) was added in powder form as commercialized by a pharmaceutical company (Procter & Gamble, Spain). The dietary sources used to supplement Mn, Fe, and Zn were $MnCO_3$, Fe-citrate, hemoglobin, and $ZnCO_3$.

2.3.3. Phytase supplementation (PHYTS)

An amount of exogenous microbial phytase (*Aspergillus niger*) (Natuphos, BASF, Mt. Olive, NJ, USA), equivalent to 750 phytase units/kg lupin diet, was added in powder form to the α-galactoside-free lupin diet (PHYTS). Phytase activity was assayed in the phytase-supplemented diet as described by Porres et al. (2005). 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. The final amount of phytase activity present in the diet, which comprised the endogenous phytase activity present in lupin flour and the exogenously added microbial activity, was 865 PU/kg.

2.4. Chemical analysis of lupin diets, feces, urine and tissues

Moisture content was determined by drying to constant weight in an oven at 105 ± 1 °C. Ash was measured by calcination at 500 °C to a constant weight. Manganese, iron, and zinc contents were determined by atomic absorption spectrophotometry, using a Perkin–Elmer AAnalyst 300 spectrophotometer. Potassium content was determined by atomic emission spectrophotometry, using a Perkin–Elmer AAnalyst 300 spectrophotometer. Analytical results were validated by the following standard reference materials: whole-meal flour # CRM-189, lyophilized green beans # RM-383, and synthetic feed for growing pigs # BCR-709 (Community Bureau of Reference. Commission of the European Communities). Mean ± SD values of five independent CRM-189 replicates were Mn 64.9 ± 0.6, Fe 74.4 ± 1.7, Zn 57.6 ± 1.0 μg/g vs certified values ± uncertainty range declared by the Community Bureau of Reference for Mn 63.3 ± 1.6, Fe 68.3 ± 1.9, Zn 56.5 ± 1.7 μg/g; RM-383, ash 2.48 ± 0.01 g/100 g, K 7.89 ± 0.05 mg/g vs certified values ± uncertainty range of ash 2.4 ± 0.1, K 7.8 ± 0.2; BCR-709, ash 4.29 ± 0.07 g/100 g vs certified value ± uncertainty range of ash 4.2 ± 0.4.

2.5. Biological methods

2.5.1. Experimental design

We used a biological balance technique that records changes in body weight and food intake and then calculates total mineral, manganese, iron, zinc, and potassium intakes, fecal total mineral excretion, and fecal and urinary manganese, iron, zinc and potassium excretion. In total, 40 young albino Wistar rats, divided into four experimental groups ($n = 10$ per group, five male and five female), were used. The growing animals (recently weaned) with an initial body weight of 64 ± 1.5 g, were housed from day 0 of the experiment in individual stainless steel metabolic cages designed for the separate collection of feces and urine; the cages were located in a well ventilated thermostatically controlled room (21 ± 2 °C) with 12 h light/dark period. Throughout the experimental period, all rats had free access to double-distilled water.

Three 10-day experiments, in which the animals consumed *ad libitum* the three different lupin diets (raw lupin, α-galactoside-free lupin, and phytase-supplemented α-galactoside-free lupin), were carried out in the first place. An additional casein–cystine control group was *pair-fed* with the average daily intake of rats given the three lupin diets. During the first 3 days of experiments, the rats were allowed to adapt to the diet and experimental conditions, and the main experimental period comprised the next 7 days, during which body weight and food intake were recorded and feces and urine were collected for analysis. After completion of the feeding experiments, the rats were deprived of food for 16 h, weighed, anaesthetized with CO_2, and sacrificed. Blood was collected (with heparin as an anticoagulant) and taken for digestion and quantification of blood parameters (KX-21 Automated Hematology Analyzer, Sysmex Corporation, Kobe, Japan). The femur, sternum, liver, kidney, brain, and heart were collected for analysis and stored at −20 °C. All experiments were undertaken according to Directional Guides Related to Animal Housing and Care (European Community Council, 1986).

2.5.2. Biological indices

The following indices and parameters were determined for each group according to the formulas given below: apparent digestibility coefficient (ADC) (1) for total ash, manganese, iron, zinc, and potassium; manganese, iron, zinc and potassium retention (balance) (2), and percent manganese, iron, zinc or potassium retention/manganese, iron, zinc or potassium absorption (%R/A) (3):

$$\mathrm{ADC} = [(I - F)/I] \times 100 \quad (1)$$

$$\mathrm{Balance} = I - (F + U) \quad (2)$$

$$\%\mathrm{R/A} = [I - (F + U)]/(I - F) \times 100 \quad (3)$$

where I is the intake, F is the fecal excretion, and U is the urinary excretion.

2.6. Statistics

One-way analysis of variance was applied to the data with the use of SAS version 8.02. Differences between means were compared with Tukey's test. The level of significance was set at $P < 0.05$. Fecal excretion of total ash, manganese, iron, zinc and potassium was adjusted to a multiple linear regression model with total fecal weight and fecal excretion of phosphorus as regressors. The regression model was adjusted stepwise with the aim of maximizing the fit of the model at each step. Simple linear regression was also applied to test for relationships between several different experimental parameters and indices studied.

3. Results and discussion

3.1. Chemical composition

The chemical composition of lupin flours used in the present study with regard to Mn, Fe, Zn, K, SDF and IDF, and the effect of α-galactoside extraction on the contents of these nutrients has been described elsewhere (Porres et al., 2005, 2006). Diet formulation was adequate to meet the nutrient requirements of the growing rat (National Research Council, 1995) with the exception of dietary fibre and Mn contents, which were superior due to the high levels of these components originally present in the lupin flours used for the present study. All the experimental diets tested in the present study were isocaloric and isonitrogenous, and the chemical composition of the α-galactoside-free lupin flour diet supplemented with phytase (PHYTS) was similar to that of its unsupplemented control (GFLU).

All the Mn present in the lupin diets tested was from the lupin flours, whereas the amounts of Fe and Zn provided by the lupin flours corresponded to 34.2% and 52.3% of the total mineral content, respectively, for the raw lupin diet, and 29.1% and 43.2% of the total mineral content, respectively, for the α-galactoside-free lupin diet. Taking into consideration the content and potential availability of Fe present in the lupin flours, highly available non-heme and heme Fe sources, such as ferric citrate (48–53% of total dietary Fe content) and hemoglobin (14–15% of total dietary Fe content) were added to all of the experimental diets in order to meet the target requirements of the growing rat. With regard to the amount of Zn needed to reach the nutritional requirements, this was added in the form of $ZnCO_3$.

Of the total K analytically determined in lupin diets, 59.5% and 43.6% was from raw and α-galactoside-free lupin flours, respectively (Table 1), whereas the remaining amount up to the total mineral content was from different sources present in the mineral mix (Reeves et al., 1993).

Nearly all the Mn, Fe, and K present in the casein–cystine control diet was added exogenously as Fe citrate, hemoglobin, $MnCO_3$, or different K sources in the mineral premix, whereas 26.5% of the total Zn content was from casein and the rest, up to target requirements, was from $ZnCO_3$.

3.2. Mineral intake, fecal weight, and fecal P excretion

Daily Mn, Fe, Zn and K intakes in all the experimental groups tested were a reflection of daily food intake and the chemical composition of the diets (Tables 1–3). Because of the high Mn content of the experimental diets, the daily intake of this mineral was well above the nutrient recommendations for the growing rat (National Research Council, 1995). However, due to the short experimental period used and the moderate excess of Mn provided by the diets, in comparison with the values reported in other studies (National Research Council, 1995; Torrente et al., 2005), no apparent deleterious effects on the health status of the animals elicited by an excessive dietary Mn level were observed under our experimental conditions.

Fecal weight was significantly higher for the casein–cystine experimental group than for the rest of the experimental groups tested (RLU, GFLU, PHYTS), among which only minor differences were observed (Tables 2 and 3), whereas the fecal P excretion was significantly higher in the experimental group fed the phytase-supplemented α-galactoside-free lupin diet compared to the other experimental diets tested (Tables 2 and 3), despite a similar P intake (Porres et al., 2006). Differences in fecal weight can be attributed to the different intrinsic natures of dietary fibre present in the lupin diets (fermentable non-starch polysaccharides) and the casein–cystine control diet (non-fermentable cellulose), whereas the higher fecal excretion of P derived from the phytase-catalyzed hydrolysis of phytate, along the gastrointestinal tract of the rat, can be attributed to mineral interactions that lead to a significant decrease in P bioavailability under the experimental conditions of the present study, in contrast to what has been reported in the literature for other animal species, e.g. swine or poultry (Lei & Porres, 2005).

3.3. Effect of α-galactoside extraction on the digestive utilization of total ash, Mn, Fe, Zn, and K

The digestive utilization of total mineral content assessed as ash digestibility was highest ($P < 0.05$) in the groups of animals fed the RLU and GFLU diets, among which no significant differences were found (Tables 2 and 3), and significantly lower in the experimental groups fed the C + C diet, and the PHYTS diet for which the lowest values of all experimental groups were obtained ($P < 0.05$). These results suggest that digestive utilization of the mineral component from the diets was better in the animals that consumed the raw and α-galactoside-free lupin flour diets than in the animals fed the casein–cystine or the phytase-supplemented lupin diets, and corroborate the results previously reported by our group in reference to Ca, P and Mg (Porres et al., 2006).

Table 1
Composition of the experimental diets

Diets	C + C	RLU	GFLU
Ingredients (g/kg)			
Casein	194	–	–
Raw lupin flour	–	472	–
α-galactoside-free lupin flour	–	–	417
L-Cystine	6	6	6
Olive oil	79	10	10
Starch	348	348	403
Sucrose	100	100	100
Cellulose	190	–	–
Mineral mix	35	35	35
Vitamin mix	10	10	10
Choline chloride	1.5	1.5	1.5
$MnCO_3$	0.76	–	–
$ZnCO_3$	0.055	0.04	0.045
Fe citrate	0.19	0.10	0.11
Hemoglobin	2	1.95	2.15
Nutritional composition			
Ash (g/100 g DM)[a]	3.04 ± 0.02	4.19 ± 0.01	3.68 ± 0.02
K (mg/100 g DM)[a]	387.4 ± 6.1	758.6 ± 7.3	547.7 ± 8.33
Fe (mg/100 g DM)[a]	5.8 ± 0.07	5.25 ± 0.09	5.03 ± 0.03
Mn (mg/100 g DM)[a]	32.7 ± 0.03	36.3 ± 0.58	31.4 ± 0.30
Zn (mg/100 g DM)[a]	3.85 ± 0.07	3.82 ± 0.03	4.03 ± 0.05
IDF (g/100 g DM)[b]	19.00	19.00	15.95
SDF (g/100 g DM)[b]	2.20	2.46	1.06

[a] Results are means ± SEM of five independent replicates.
[b] Results taken from Porres et al. (2006).

Mn digestibility by the group of animals fed the casein–cystine (C + C) control diet, in which nearly all the Mn was supplied as $MnCO_3$, was significantly lower (4.3–4.7-fold) than the values exhibited by the animals fed the raw and α-galactoside-free lupin flour diets (Table 2). Moreover, in all the experimental groups tested, Mn digestibility was lower than the 40% retention values reported by Keen, Bell, and Lönnerdal (1986) for 21-day suckling rats fed infant formula, although the amount of net mineral absorbed would be considerably higher under the experimental conditions of the present study, due to a considerably higher dietary Mn intake. The lower digestive utilization of Mn found in the experimental groups of rats fed the raw and α-galactoside-free lupin diets, despite a similar age period (21 days), can be attributed to the high amounts of Mn provided by our experimental diets and, to a lesser extent, to the solid type of diet used in the present study or the presence, in lupin diets, of phytic acid and vegetal protein, which are considered strong inhibitors of Mn absorption (Fairweather-Tait, 1996; Hurley & Keen, 1987; Lönnerdal, 2002). The additional reduction in Mn digestibility with the casein–cystine control diet was probably related to the higher fecal weight in the former group of animals compared to the other experimental groups tested. Other dietary factors, such as the interaction with Ca + Fe, could have affected Mn digestibility (Fairweather-Tait, 1996). Nevertheless, the comparative importance of such interaction in this study would be considerably diminished, since all the experimental diets provided similar concentrations of the three minerals.

Compared to the animals that consumed the casein–cystine control diet, the digestive utilization of Fe was 1.5-fold higher in the animals that consumed the raw and α-galactoside-free lupin diets (Table 2). The lower digestive utilization of Fe with the casein–cystine control

Table 2
Effect of α-galactoside extraction process and phytase supplementation on the digestive and metabolic utilization of manganese and iron[A]

	C + C	RLU	GFLU	PHYTS
Fecal weight (g DM/day)[B]	1.9 ± 0.02[b]	0.9 ± 0.06[a]	0.8 ± 0.04[a]	0.9 ± 0.07[a]
Fecal P (mg/day)[B]	11.0 ± 0.33[a]	10.6 ± 0.87[a]	10.0 ± 0.58[a]	14.4 ± 0.76[b]
Mineral digestibility (%)	66.1 ± 0.98[b]	70.7 ± 1.40[c]	70.3 ± 0.69[c]	60.7 ± 2.13[a]
Body weight (g)	94.9 ± 1.91[c]	73.2 ± 2.03[a]	70.3 ± 1.39[a]	82.4 ± 2.61[b]
Mn				
Mn intake (μg/day)	2860[a]	3221 ± 155[b]	2685 ± 85.8[a]	2865 ± 10.6[a]
Fecal Mn (μg/day)	2783 ± 26.2[b]	2683 ± 130[b]	2187 ± 58.1[a]	2775 ± 116[b]
Urinary (μg/day)	43.4 ± 4.42[a]	27.0 ± 4.23[a]	41.1 ± 9.83[a]	35.9 ± 5.44[a]
Absorbed Mn (μg/day)	112 ± 15.4[a]	538.0 ± 46.8[b]	498 ± 43.1[b]	89.9 ± 114.9[a]
ADC (%)	3.9 ± 0.54[a]	16.6 ± 1.19[b]	18.4 ± 1.25[b]	3.14 ± 3.99[a]
Retained Mn (μg/day)	69.3 ± 14.8[a]	511 ± 48.1[b]	457 ± 40.3[b]	54.0 ± 114.7[a]
% R/A	57.1 ± 7.34[a]	94.4 ± 1.06[b]	91.9 ± 1.73[b]	–
Fe				
Fe intake (μg/day)	524[b]	466 ± 22.4[a]	431 ± 13.8[a]	436 ± 1.61[a]
Fecal Fe (μg/day)	373.6 ± 7.0[c]	264 ± 12.6[a]	256 ± 9.9[a]	316 ± 19.6[b]
Urinary Fe (μg/day)	43.7 ± 2.86[b]	24.5 ± 1.40[a]	27.3 ± 2.65[a]	43.8 ± 4.26[b]
Absorbed Fe (μg/day)	151 ± 7.04[ab]	202 ± 13.0[c]	174 ± 7.97[bc]	122 ± 7.24[a]
ADC (%)	28.7 ± 1.34[a]	43.2 ± 1.41[b]	40.5 ± 1.29[b]	28.0 ± 1.80[a]
Retained Fe (μg/day)	106.7 ± 7.7[a]	177 ± 13.0[b]	147 ± 7.26[b]	78.0 ± 10.5[a]
% R/A	70.3 ± 2.37[a]	87.4 ± 1.16[b]	84.3 ± 1.34[b]	62.4 ± 5.78[a]

[a–c] Means within the same line with different superscripts differ significantly ($P < 0.05$).
[A] Results are means ± SEM of 10 Wistar rats.
[B] Results taken from Porres et al. (2006).

Table 3
Effect of α-galactoside extraction process and phytase supplementation on the digestive and metabolic utilization of zinc and potassium[A]

	C + C	RLU	GFLU	PHYTS
Fecal weight (g DM/day)[B]	1.9 ± 0.02	0.9 ± 0.06	0.8 ± 0.04	0.9 ± 0.07
Fecal P (mg/day)[B]	11.0 ± 0.33[a]	10.6 ± 0.87[a]	10.0 ± 0.58[a]	14.4 ± 0.76[b]
Mineral digestibility (%)	66.1 ± 0.98[b]	70.7 ± 1.40[c]	70.3 ± 0.69[c]	60.7 ± 2.13[a]
Body weight (g)	94.9 ± ± 1.91[c]	73.2 ± 2.03[a]	70.3 ± 1.39[a]	82.4 ± 2.61[b]
Zn				
Zn intake (μg/day)	336.6[a]	341 ± 16.3[ab]	345 ± 11.0[ab]	377 ± 1.4[b]
Fecal Zn (μg/day)	233 ± 6.8[b]	209 ± 9.8[a]	231 ± 6.5[b]	279 ± 11.2[c]
Urinary Zn (μg/day)	28.4 ± 2.1[a]	28.0 ± 2.6[a]	26.3 ± 2.8[a]	30.8 ± 2.2[a]
Absorbed Zn (μg/day)	104 ± 6.8[ab]	132 ± 8.5[b]	114 ± 6.5 a[b]	98.0 ± 12.6[a]
ADC (%)	30.8 ± 2.01[b]	38.6 ± 1.3[c]	32.8 ± 1.1[b]	23.6 ± 1.97[a]
Retained Zn (μg/day)	78.7 ± 7.4[ab]	104 ± 6.6[c]	87.4 ± 5.4[bc]	59.5 ± 6.6[a]
% R/A	72.9 ± 2.9[b]	78.9 ± 1.1[b]	76.8 ± 1.9[b]	66.0 ± 2.2[a]
K				
K intake (mg/day)	33.86[a]	67.4 ± 3.23[c]	46.9 ± 1.50[b]	47.5 ± 0.18[b]
Fecal K (mg/day)	2.93 ± 0.50[a]	9.36 ± 0.90[c]	4.41 ± 0.46[a]	7.29 ± 0.49[b]
Urinary K (mg/day)	18.0 ± 1.27[a]	32.0 ± 3.68[b]	25.3 ± 1.22[b]	30.9 ± 2.04[b]
Absorbed K (mg/day)	30.9 ± 0.50[a]	58.0 ± 2.95[c]	42.5 ± 1.26[b]	40.2 ± 0.54[b]
ADC (%)	91.4 ± 1.47[b]	86.1 ± 1.29[a]	90.7 ± 0.85[b]	84.6 ± 1.03[a]
Retained K (mg/day)	13.0 ± 0.85[ab]	26 ± 2.58[c]	17.1 ± 1.18[b]	9.3 ± 1.65[a]
% R/A	42.3 ± 3.27[b]	38.7 ± 1.83[b]	40.2 ± 2.54[b]	23.5 ± 4.38[a]

[a–c] Means within the same line with different superscripts differ significantly ($P < 0.05$).
[A] Results are means ± SEM of 10 Wistar rats.
[B] Results taken from Porres et al. (2006).

diet can be attributed to dietary factors, such as the inclusion in the casein–cystine control diet of a considerable amount of an insoluble, non-fermentable dietary fibre source, cellulose, that caused a significant increase in fecal weight (Tables 1 and 2), the potential inhibitory effects of high Mn levels added in inorganic form, or the strong inhibitory effect of casein on non-heme Fe absorption (Fairweather-Tait, 1996). The higher digestive utilization of Fe with the raw and α-galactoside-free lupin flour diets, associated with a lower fecal weight, compared to the casein–cystine control diet, could be indicative of the lack of deleterious effects on Fe absorption of the good-quality lupin protein used in the present study, in an inverse manner to the well-known inhibitory effects of casein. On the other hand, the potential inhibitory effect of certain components of the lupin seed flour, such as phytic acid or tannins, on Fe absorption cannot be disregarded (Fairweather-Tait, 1996; Stahl et al., 1999) although, due to the dilution of phytic acid and tannin content during the course of semi-synthetic diet preparation, it does not appear that the inhibitory effect on Fe absorption of the above-mentioned compounds was very pronounced under the experimental conditions of the present study. Furthermore, the intrinsic nature of lupin fibre that is readily fermented during its passage through the large intestine, in contrast to cellulose, may have contributed to a higher Fe absorption by the lupin-fed animals. In this regard, Hayasi, Hara, Asvarujanon, Aoyama, and Luangpituksa (2001) have reported the enhancing effect of various fermentable insoluble dietary fibre sources on Fe and Zn absorption compared to cellulose, and suggested that cecal fermentation is a possible, though not sole factor involved in such an enhancing effect. The authors also reported that the addition of sodium phytate to the diets enhanced the fermentative production of short chain fatty acids and caused a substantial reduction in cecal pH and fecal weight. In a different set of experiments, Hara, Konishi, and Kasai (2000) and Yonekura and Suzuki (2005) have reported the ability of the cecum and colon to absorb moderate amounts of Zn in the rat. Moreover, the absorption of this mineral can be significantly enhanced by microbial fermentation processes that take place in these segments of the large intestine and cause an important reduction in the pH of the intestinal lumen.

The results of the present study suggest that supplementation of the two selected Fe sources to a lupin meal with good protein quality can be an efficient means to obtain dietetic products with reasonably high availability of this essential cation.

The similar Fe availability attained by the experimental groups fed the raw and α-galactoside-free lupin flour diets did not agree with the results obtained using an *in vitro* dialyzability technique, for which a significant reduction in the potential availability of Fe has been reported, as a result of the α-galactoside extraction process (Porres et al., 2005). Such differences indicate that most of the Fe absorbed by the animals was probably derived from hemoglobin or Fe-citrate, thus masking the inhibitory effect of the extraction process on endogenous legume Fe availability under our experimental conditions.

The digestive utilization of Zn was highest for the experimental group fed the raw lupin flour diet (RLU) ($P < 0.05$), followed by the groups fed the α-galactoside-

free lupin flour (GFLU) and the casein–cystine (C + C), groups among which no significant differences were observed, and finally by the experimental group that consumed the phytase-supplemented diet (PHYTS) for which the lowest value of all the experimental groups was found ($P < 0.05$) (Table 3). Differences in Zn digestibility among the different experimental diets tested were due to differences in the fecal excretion of this mineral, given that the amount of Zn ingested was similar among the various experimental groups. The lower digestive utilization of Zn found in the experimental group that consumed the casein–cystine control diet, compared to the raw lupin flour diet or to a casein-methionine diet with similar protein content, previously assayed by our group (Urbano et al., 2006), can be attributed to the inclusion of high non-fermentable cellulose amounts in the former diet, as has been discussed for Mn and Fe. Other dietary factors with known influence on Zn digestibility, such as protein quality or mineral interaction, did not appear to play an essential role under the experimental conditions of the present study.

The higher digestive utilization of Zn found in the experimental group that consumed the raw lupin flour (RLU), compared to the α-galactoside-free lupin diet (GFLU), can be attributed to a loss of soluble and potentially more available mineral from the legume during the hydroalcoholic extraction process, since the exogenous Zn, added to the diets as $ZnCO_3$ was supplemented after the α-galactoside extraction process, and no major differences regarding other dietary factors that could have affected Zn digestibility (e.g. phytic acid content or protein quality) were apparent between the RLU and GFLU diets (Porres et al., 2005, 2006).

In general, the digestive utilization of K assessed by the apparent digestibility coefficient was high in all the experimental groups (84.6–91.4%) (Table 3), and within the range of values found in the literature for a semi-synthetic basal diet (Kaup, Behling, & Greger, 1991). In the group of animals that consumed the raw lupin flour diet (RLU), only 27.4 mg of K, from the total 58 mg absorbed daily, came from the mineral premix added to the diet; this finding points to an efficient digestive use of the mineral provided by the lupin flour. The lower digestibility values found in the rats that consumed the raw lupin flour diet compared to the animals fed the α-galactoside-free or the casein–cystine control diet can be attributed to the higher amounts of K supplied by the raw lupin flour diet, which doubled the amount supplied by the casein–cystine control.

3.4. Effect of phytase supplementation on the digestive utilization of total ash, Mn, Fe, Zn, and K

Supplementation of an α-galactoside-free lupin diet with exogenous microbial phytase (PHYTS) led to a significant increase in fecal excretion of total ash, Mn, Fe, Zn, and K compared to its unsupplemented control (GFLU), and resulted in a considerable reduction in the digestibility of all the minerals studied, causing the net Mn absorption and retention to drop to values close to zero, considering the great variability observed in the experimental results (Table 2). Due to the similar composition of the GLFU and the PHYTS diets, the most important factor to take into account, when trying to explain the increase in fecal mineral excretion caused by phytase supplementation, is probably the increase in fecal P excretion, caused by phytase-catalyzed phytate hydrolysis in the gastrointestinal tract of the growing rat under our experimental conditions. This higher fecal P excretion affected both the endogenous minerals present in the lupin seed flour and the mineral sources exogenously added to the diet. The ability of phosphate to interfere with the intestinal absorption of numerous cations, such as Mg, Zn, Fe or Mn, due to the formation of insoluble complexes that will be finally excreted in the feces, has been consistently reported in the literature (Brink, Beynen, Dekker, van Beresteijn, & van der Meer, 1992; Fairweather-Tait, 1996). Furthermore, Hallberg, Rossander, & Skanberg, 1987 have reported a significant improvement in Fe availability from a bran fraction dephytinized by endogenous phytase action, after water-washing removal of inorganic phosphate formed during the dephytinization process, compared to the enzymatically dephytinized bran that was not washed.

From the results of the present study, it can be concluded that the main factors that affected the digestive utilization of total ash, Mn, Fe and Zn were the fecal weight and fecal P excretion, as shown by the multiple linear regression model assayed ($Ash_{fec}/Ash_{Int} = 0.09029 + 0.00002140$[Fecal weight] $+ 0.01892$[Fecal P], Adj $- R^2 = 0.8819$, $Pr > F < 0.0001$; Fecal Mn $= 1164.15 + 336.72$[Fecal weight] $+ 92.73$[Fecal P], Adj $- R^2 = 0.6722$, $Pr > F < 0.0001$; Fecal Fe $= 123.61 + 88.04$[Fecal weight] $+ 7.07$[Fecal P], Adj $- R^2 = 0.7547$, $Pr > F < 0.0001$; Fecal Zn $= 101.25 + 11.91$[Fecal P], Adj $- R^2 = 0.7347$, $Pr > F < 0.0001$). However, the effect of these regressors on the digestive utilization of K was not so apparent, as seen by the lower correlation coefficient obtained when the same multiple linear regression model was applied ($K_{Fec}/K_{Int} = 0.03870 - 0.02736$[Fecal weight] $+ 0.00958$[Fecal P]; Adj $- R^2 = 0.3037$; $Pr > F = 0.0005$).

3.5. Metabolic utilization of Mn, Fe, Zn, and K

The net retention of Mn and Zn maintained a direct relationship with the net amount of mineral absorbed, with no major differences in urinary excretion being found among the different experimental groups tested. These results are in agreement with the intestinal regulation of Mn and Zn metabolism that has been suggested by numerous authors (Windisch, 2002). Nevertheless, due to the short length of the experimental period used, no signs of mineral accumulation in any of the tissues selected were apparent despite the considerable differences in the retention of the above mentioned cations among the different experimental groups, with the exception of blood Zn (Table 4). Regarding the Mn and Zn contents of liver

and Mn content of the kidney, these appeared to be more related to the final weight of the animals than to the amount of mineral retained.

Urinary excretion of Fe was significantly higher in the experimental groups that consumed the casein–cystine or phytase-supplemented diets than in those that consumed the raw and α-galactoside-free lupin flour diets. These results, together with the net mineral absorption values in the different experimental groups, led to a significantly lower metabolic utilization of Fe, expressed as the percentage of retained to absorbed mineral (%R/A), by the animals fed the casein–cystine control and the phytase-supplemented diet compared to the raw and α-galactoside-free lupin flour diets. Such differences in Fe retention were not reflected in the content of this mineral in the heart or brain or in different hematological parameters studied (RBC, hemoglobin, hematocrit, MCV, MCH, MCHC) with the exception of total blood (Table 5). Differences in the total blood content among the different experimental groups point to differing amounts of plasma-transported Fe, since hematocrit and RBC counts are similar. In contrast, the higher Fe retention by the animals fed the raw and α- galactoside-free lupin flour diets was reflected in the amount of Fe stored in the kidney and sternum. Regarding the liver, the Fe content of that tissue appeared to be more related to the final weight of the animals than to the amount of mineral retained under the experimental conditions of the present study.

Platelet levels were within the normal range of values described in the literature for the Wistar rat (Charles River Technical Bulletin, 1998). However, counts were considerably higher ($P < 0.05$) in the blood of the animals that consumed the legume diets than in the casein-fed animals (Table 5).

The urinary excretion of K was considerably higher than the fecal excretion of this mineral in all the diet groups (Table 3) in a similar way to what has been reported by Sabboh et al. (2006). On the other hand, K retention by the animals that consumed raw or α-galactoside-free lupin flour, as well as by those fed the casein–cystine control diet, was related to the intestinal absorption of the mineral, since no apparent differences were found in the proportion of retained to absorbed cation. The metabolic utilization of K was significantly lower in the animals that consumed the phytase-supplemented lupin diet (PHYTS) than in its unsupplemented control (GFLU), leading to a lower net retention of the mineral ($P < 0.05$). Nevertheless, the amount of retained mineral did not maintain any correlation with its accumulation in the different tissues studied, with the exception of the femur (Table 4) ($r = 0.4876$; $P < 0.05$).

In conclusion, the α-galactoside extraction process did not modify the availability of minerals provided by the lupin diet, whereas phytase supplementation produced a considerable reduction in the nutritive utilization of Mn, Fe, and Zn under our experimental conditions due to the interaction of these minerals with the phosphate released at the gastrointestinal level by phytase. In relation to the high Mn content of the experimental diets, this could be a beneficial effect, since it decreases its absorption. Supplementation of a good quality vegetal protein, such as lupin, with Fe and Zn, in quantities sufficient to meet the nutrient requirements, gave rise to a food product with

Table 4
Effect of α-galactoside extraction process and phytase supplementation on manganese, zinc and potassium contents of different tissues[A]

	C + C	RLU	GFLU	PHYTS
Mn				
Femur (μg/g DM)	3.85 ± 0.08^{a}	4.41 ± 0.10^{b}	3.85 ± 0.09^{a}	3.76 ± 0.09^{a}
Kidney (μg/g DM)	5.99 ± 0.26^{c}	4.11 ± 0.13^{a}	4.76 ± 0.17^{b}	6.14 ± 0.20^{c}
Liver (μg/g DM)	15.4 ± 0.63^{b}	10.2 ± 0.85^{a}	10.6 ± 0.26^{a}	11.5 ± 0.27^{a}
Heart (μg/g DM)	3.39 ± 0.25^{a}	4.31 ± 0.33^{b}	3.70 ± 0.31^{ab}	3.48 ± 0.24^{ab}
Brain (μg/g DM)	2.82 ± 0.11^{a}	3.14 ± 0.08^{b}	2.59 ± 0.07^{a}	2.69 ± 0.06^{a}
Zn				
Blood (μg/dl)	0.37 ± 0.01^{a}	0.70 ± 0.06^{c}	0.52 ± 0.04^{b}	0.31 ± 0.01^{a}
Femur (μg/g DM)	254 ± 6.0^{c}	239 ± 5.4^{b}	222 ± 5.3^{a}	255 ± 3.4^{c}
Sternum (μg/g DM)	101 ± 4.7^{a}	111 ± 5.2^{ab}	109 ± 2.6^{ab}	119 ± 4.1^{b}
Kidney (μg/g DM)	104 ± 3.7^{b}	106 ± 2.5^{b}	102 ± 1.9^{b}	94.2 ± 1.7^{a}
Liver (μg/g DM)	139 ± 6.8^{b}	127 ± 6.1^{b}	109 ± 3.4^{a}	125 ± 1.5^{b}
Heart (μg/g DM)	81.9 ± 2.1^{a}	79.2 ± 1.4^{a}	79.4 ± 1.2^{a}	79.5 ± 0.9^{a}
Brain (μg/g DM)	63.3 ± 0.7^{a}	68.1 ± 0.7^{c}	65.6 ± 0.4^{b}	64.6 ± 0.5^{ab}
K				
Blood (mg/dl)	147 ± 2.4^{a}	130 ± 3.8^{a}	132 ± 3.1^{a}	135 ± 2.4^{a}
Femur (mg/g DM)	5.89 ± 0.21^{b}	6.76 ± 0.12^{c}	6.68 ± 0.14^{c}	5.25 ± 0.14^{a}
Sternum (mg/g DM)	9.00 ± 0.25^{ab}	9.30 ± 0.16^{b}	9.30 ± 0.24^{b}	8.45 ± 0.20^{a}
Kidney (mg/g DM)	12.2 ± 0.13^{b}	12.1 ± 0.25^{b}	11.7 ± 0.18^{b}	11.1 ± 0.14^{a}
Liver (mg/g DM)	12.2 ± 0.10^{a}	11.4 ± 0.83^{a}	11.2 ± 0.11^{a}	11.6 ± 0.04^{a}

[a–c] Means within the same line with different superscripts differ significantly ($P < 0.05$).
[A] Results are means ± SEM of 10 Wistar rats.

Table 5
Effect of α-galactoside extraction process and phytase supplementation on iron content of different tissues and hematological indices[A]

	C + C	RLU	GFLU	PHYTS
Fe				
Femur (μg/g DM)	17.4 ± 0.42[a]	18.4 ± 0.57[a]	18.1 ± 0.46[a]	18.1 ± 0.43[a]
Sternum (μg/g DM)	372 ± 19.1[ab]	443 ± 34.9[b]	625 ± 40.8[c]	288 ± 14.6[a]
Kidney (μg/g DM)	248 ± 11.5[a]	378 ± 17.8[b]	378 ± 10.6[b]	250 ± 14.0[a]
Liver (μg/g DM)	473 ± 36.7[b]	310 ± 20.7[a]	318 ± 17.2[a]	384 ± 27.2[a]
Heart (μg/g DM)	49.5 ± 2.12[a]	56.1 ± 2.67[a]	52.6 ± 1.47[a]	52.4 ± 2.34[a]
Brain (μg/g DM)	109 ± 1.56[b]	102 ± 4.72[ab]	103 ± 2.79[ab]	97.3 ± 2.39[a]
Blood (mg/dL)	44.6 ± 1.01[b]	33.0 ± 0.95[a]	31.9 ± 0.60[a]	46.3 ± 1.20[b]
RBC (106/mm^3)	5.60 ± 0.25[a]	5.33 ± 0.23[a]	5.39 ± 0.21[a]	5.43 ± 0.34[a]
Hemoglobin (g/dL)	10.2 ± 0.48[a]	10.2 ± 0.31[a]	10.8 ± 0.42[a]	10.8 ± 0.39[a]
Hematocrit (%)	32.2 ± 1.11[a]	32.0 ± 0.79[a]	32.8 ± 0.92[a]	34.2 ± 1.30[a]
MCV (fL)	57.8 ± 1.17[a]	60.5 ± 1.79[a]	61.2 ± 1.26[a]	57.9 ± 0.69[a]
MCH (pg)	18.3 ± 0.46[a]	19.3 ± 0.46[ab]	20.0 ± 0.25[b]	18.3 ± 0.28[a]
MCHC (g/dL)	31.6 ± 0.59[a]	32.0 ± 0.56[a]	32.7 ± 0.57[a]	31.7 ± 0.56[a]
PLT (103/mm^3)	405 ± 15.3[a]	590 ± 14.5[b]	688 ± 30.8[c]	603 ± 24.7[b]

[a–c] Means within the same line with different superscripts differ significantly ($P < 0.05$).
[A] Results are means ± SEM of 10 Wistar rats.

high availability of Fe and Zn. In addition, the endogenous K provided by the legume exhibited good levels of availability and could be recommended in situations where the needs for this mineral are high.

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