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Nutritional evaluation of protein, phosphorus, calcium and magnesium bioavailability from lupin (*Lupinus albus* var. *multolupa*)-based diets in growing rats: effect of α-galactoside oligosaccharide extraction and phytase supplementation

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The nutritional composition of the legume *Lupinus albus* var. *multolupa*, raw or after α-galactoside extraction, and its effect on the bioavailability of protein, P, Ca, and Mg by growing rats was evaluated using a balance technique. The protein and dietary fibre content of the lupin flours studied was high, and 89–94 % of the dietary fibre was present as insoluble dietary fibre. The α-galactoside extraction process did not disrupt the nutritional quality of protein, and the digestive and metabolic utilisation of this nutrient was high and comparable with that obtained from a casein–cystine control diet (pair-fed to the average daily food intake of the experimental groups fed the different lupin diets). Bioavailability of P, Ca, and Mg from the lupin diets tested was high, and supplementation of an exogenous microbial phytase (750 phytase units/kg) did not cause any further improvement. Mineral content in the bone tissue (femur and sternum) did not correlate to mineral balance, which, on the other hand, was related to the mineral content of other tissues such as blood, plasma, liver and kidney. Due to its ability to grow under adverse edaphic and climatic conditions and to its good nutritional quality, α-galactoside-free lupin flour supplemented with the required amounts of minerals and vitamins to meet nutrient requirements can be used as an excellent dietary source for the preparation of dietetic products.

***Lupinus albus* var. *multolupa*: Protein: Minerals: Bioavailability: Rats: α-Galactosides**

The importance of lupin (*Lupinus* spp.) as a valuable source of nutrients to be used in human or animal nutrition (Farrel *et al.* 1999; De Penna *et al.* 2003; Hall & Johnson, 2004) has increased in recent years. This is due to its high content of protein, minerals, dietary fibre and fat in certain species, as well as to its low levels of non-nutritional components such as trypsin inhibitors, lectins or alkaloids in the sweet varieties (Gueguen & Cerletti, 1994; Dupont *et al.* 1994; van Barneveld, 1999). In addition to these nutritional properties, lupin also features beneficial functional properties such as antioxidant, antimicrobial or hypocholesterolaemic effects (Tsaliki *et al.* 1999; Lampart-Szczapa *et al.* 2003; Sirtori *et al.* 2004; Hall *et al.* 2005). Another factor of interest is that lupins have the capacity to grow under environmental and edaphic conditions that are not tolerated by other crops (Hill, 1977). Among the major reasons that preclude a wider inclusion of lupin in food products intended for human or animal consumption are the flatulence associated with its high α-galactoside-oligosaccharide content (Suarez *et al.* 1999; Martinez-Villaluenga *et al.* 2005*a*), the inhibitory effect of phytic acid on mineral absorption (Urbano *et al.* 2000), and the presence in lupin of substantial amounts of NSP. High amounts of NSP may interfere with an adequate growth performance in pigs or depress weight gain and increase the viscosity of intestinal content in poultry, leading to the appearance of liquid depositions when a high level of lupin is included in animal feed (Donovan *et al.* 1993; Farrel *et al.* 1999; Steenfeldt *et al.* 2003).

With the aim of decreasing the amount of non-nutritional components that interfere with an optimal nutritive utilisation of legumes, different technological processes have been assayed, including fermentation, germination, soaking at different pH levels, cooking and enzyme supplementation (Vidal-Valverde *et al.* 1998, 2002; Urbano *et al.* 2003; Porres *et al.* 2003*a*). Gulewicz *et al.* (2000) have developed a method for the extraction of flatulence-causing α-galactoside oligosaccharides, which in turn can be used for the preparation of prebiotics (Bouhnik *et al.* 1999; Gulewicz *et al.* 2002; Martinez-Villaluenga *et al.* 2005*b*). An α-galactoside-free seed by-product is obtained that can be used for the preparation of protein isolates or food products with high nutritional value oriented to human or animal consumption. In previous experiments with an α-galactoside-free by-product obtained from the seeds of *Lupinus albus* var. *multolupa*

Abbreviations: ADC, apparent digestibility coefficient; PHYTS, phytase-supplemented α-galactoside-free lupin; %R/A, percentage of retention in relation to absorption.

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(Porres *et al.* 2005), it was observed that the extraction process yielded a high-quality food product with good nutritional composition and availability of N, Ca, and Mg, as assessed by an *in vitro* dialysability assay, which improved significantly for Ca and Mg in response to phytase addition.

Mineral supplementation is a widely extended practice to compensate for the limited amount and bioavailability of certain minerals in legumes, and to enrich a variety of foods intended for specific population groups such as children, the elderly or athletes. Mineral and vitamin supplementation is also recommended in certain physiological or pathological situations that require consumption of these nutrients exceeding the RDA (Clarkson & Haymes, 1994; Barringer *et al.* 2003; Mitchell *et al.* 2003).

In the present study, we sought to use raw lupin flour or the seed by-product remaining after an α-galactoside oligosaccharide extraction process aimed to reduce the flatulence caused by that seed component, to prepare feed products with high nutritional value oriented to animal nutrition, or integral dietetic food products that provide a substantial amount of dietary fibre oriented to human nutrition. It was also a major objective of this research work to confirm the excellent mineral availability from the raw and α-galactoside-free lupin flours previously observed *in vitro* (Porres *et al.* 2005) using an *in vivo* model system such as the growing rat. Taking into consideration the small amount of certain minerals provided by lupin flour, a fact that is further aggravated by the α-galactoside extraction process, we decided to complement these minerals by the exact amount required to meet the recommended nutrient levels for the growing rat.

Materials and methods

Legume and α-galactosides extraction process

Raw lupin flour was from *L. albus* var. *multolupa* seeds, 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 lyophilised for chemical analysis and diet preparation (raw lupin). Extraction of α-galactoside oligosaccharides was performed according to Gulewicz *et al.* (2000) In brief, lupin seeds were imbibed 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 homogenised and lyophilised, obtaining the α-galactoside-free flour for chemical analysis and diet preparation. The composition of the raw and α-galactoside-free lupin flours is shown in Table 1.

Experimental diets

Raw and α-galactoside-free lupin diets. Raw and α-galactoside-free lupin diets were formulated to meet the nutrient requirements of the growing rat following the guidelines provided by the American Institute of Nutrition (Reeves *et al.* 1993) with lupin flour as the sole source of protein. L-Cystine (0·6 %) was added with the aim of compensating the well-known deficiency of S-containing amino acids in legumes (Table 2). The amount of Ca, P, Mg, Zn, Fe, Mn and Cu provided by the lupin flours was taken into consideration for the final concentration present in the diet. Additional amounts of these cations needed to bring the dietary levels up to target requirements of the growing rat were supplied with organic and inorganic sources as described by Reeves *et al.* (1993). For Ca, P, and Mg, the dietary sources of minerals used were $CaCO_3$, $CaHPO_4$, and MgO. The rest of the minerals were included in the AIN-93G mineral premix. In order to meet the requirements of the growing rat, the amount of fat provided by the lupin flours was complemented with olive oil up to the required levels. Of the total amount of fat present in the lupin diets, 87 % came from the lupin flour, and the remaining 13 % was supplemented by means of olive oil. The total amount of fat present in the casein–cystine control diet was supplemented as olive oil. The amount of dietary fibre (insoluble dietary fibre,

Table 1. Composition of raw and α-galactoside-free lupin flours*

	Raw lupin flour	α-Galactoside-free lupin flour
Energy (kJ/kg)	21 100	22 500
N (g/kg)	55·8	62·4
N × 6·25 (g/kg)	344	395
Fat (g/kg)	146·4	159·8
Ash (g/kg)	35·1	23·4
P (mg/kg)	3321·5	3016·0
Ca (mg/kg)	1389·2	1789·5
Mg (mg/kg)	1450	697·1
IDF (g/kg)	402·5	382·5
SDF (g/kg)	52·1	25·3

IDF, insoluble dietary fibre; SDF, soluble dietary fibre.
* Results are expressed on a DM basis. Values are means of five independent replicates.

Table 2. Composition of the experimental diets*

Diet	C+C	LFU	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
CO_3Ca	6·1	6·1	4·95
$CaHPO_4$	8·00	7·05	8·25
MgO	1·14	–	0·66
Nutritional composition			
Energy (kJ/kg)	19 510	18 670	18 910
N (g/kg)	28·1	26·0	27·1
Protein (g/kg)	176·0	163·0	169·0
Fat (g/kg)	79·0	79·0	77·0
Total P (mg/kg)	3434	3203	3221
Ca (mg/kg)	4341	5080	4880
Mg (mg/kg)	691·4	693·7	712·7
IDF (g/kg)	190·0	190·0	159·5
SDF (g/kg)	22·0	24·6	10·6

C+C, casein–cystine control diet; RLU, raw lupin diet; GFLU, α-galactoside-free lupin diet; IDF, insoluble dietary fibre; SDF, soluble dietary fibre.
* Results are expressed on a DM basis. Values are means of five independent replicates.

382·5–402·5 g/kg; soluble dietary fibre, 25·3–52·1 g/kg) provided by the lupin flours was not modified.

Casein–cystine control diet. This diet was 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 content. In order to adjust the amount and type of dietary fibre to similar values as 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 (Procter and Gamble, Madrid, Spain).

Phytase supplementation. An amount of exogenous microbial phytase (*Aspergillus niger*) (Natuphos, BASF, Mount Olive, NJ, USA) equivalent to 750 phytase units/kg lupin diet was added in powder form to the α-galactoside-free lupin diet. The final amount of phytase activity present in the diet was measured after enzyme supplementation, and corresponded to a value of 865 phytase units/kg.

All the experimental diets tested in the present experiment were formulated to be isoenergetic and isonitrogenous.

Experimental design

We used a biological balance technique that records changes in body weight and food intake and then calculates N, P, Ca, and Mg intake and faecal and urinary N, P, Ca, and Mg excretion. A total of forty young albino Wistar rats were divided into four experimental groups (ten per group; five male and five female). 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 metabolism cages designed for the separate collection of faeces and urine; the cages were located in a well-ventilated thermostatically controlled room (21 ± 2°C) with a 12 h light–dark cycle. Throughout the experimental period all rats had free access to double-distilled water.

Three 10 d experiments, in which the animals consumed the three different lupin diets *ad libitum* (raw lupin, α-galactoside-free lupin, and phytase-supplemented α-galactoside-free lupin (PHYTS)), were carried out. 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 d of the experiment, the rats were allowed to adapt to the diet and experimental conditions. The main experimental period was conducted over the next 7 d during which body weight and food intake were recorded and faeces and urine were collected for analysis. At the end of the experimental period the animals were anaesthetised with CO_2 and killed. Blood was collected (with heparin as an anticoagulant) and centrifuged at 3000 ***g*** for 15 min to separate plasma that was frozen and stored (−20°C) until analysis; samples of whole blood were taken for digestion before centrifuging. The femur, sternum, liver and kidney 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).

Composition analysis of lupin flours, diets, faeces, urine and tissues

Energy was measured with an adiabatic bomb calorimeter (Gallenkamp, Loughborough, Leics., UK). 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. Total N was determined according to Kjeldahl's method. Crude protein was calculated as N × 6·25. Total fat was determined by gravimetry of the diethyl ether extract after acid hydrolysis of the sample. Ca and Mg contents were determined by atomic absorption spectrophotometry using a PerkinElmer Analyst 300 spectrophotometer (PerkinElmer, Wellesley, MA, USA). Lanthanum chloride was added to Ca and Mg samples in order to prevent interferences caused by phosphate ions. Total P was measured spectrophotometrically using the technique described by Chen *et al.* (1956). Analytical results were validated by standard references CRM-189 (wholemeal flour; Community Bureau of Reference, Geel, Belgium), CRM-383 (haricot beans; Community Bureau of Reference), and CRM-709 (pig feed; Community Bureau of Reference). Mean values and standard errors of the mean of five independent values for ash, P, Ca and Mg content were as follows: ash, CRM-383 = 2·48 (SEM 0·006) % *v.* certified value of 2·4 (uncertainty 0·1) %, CRM-709 = 4·29 (SEM 0·03) % *v.* certified value of 4·2 (uncertainty 0·4) %; P, CRM-709 = 5·42 (SEM 0·006) mg/g *v.* certified value of 5·4 (uncertainty 0·7) mg/g; Ca, CRM-383 = 2·78 (SEM 0·02) mg/g *v.* certified value of 2·9 (uncertainty 0·2) mg/g; Mg, CRM-189 = 1·89 (SEM 0·06) mg/g *v.* indicative value of 1·9 mg/g, CRM-383 = 1·04 (SEM 0·005) mg/g *v.* indicative value of 0·9 (SD 0·1) mg/g, CRM-709 = 1·96 (SEM 0·005) mg/g *v.* certified value of 1·89 (uncertainty 0·30) mg/g). The concentration of Ca in plasma samples was measured using an analytical kit (Linear Chemicals, Barcelona, Spain). Soluble and insoluble dietary fibre of the samples was quantified according to Prosky *et al.* (1992). Phytase activity of the diet was determined as described by Porres *et al.* (2005).

In vitro *protein digestibility*. *In vitro* protein digestibility was determined in each of the four experimental diets tested using the pH-drop multi-enzyme system described by Hsu *et al.* (1977) and the pH-stat multi-enzyme system described by McDonough *et al.* (1990).

Biological indices

The following indices and parameters were determined for each group according to the formulas:

Protein efficiency ratio (g weight gain per d/g protein intake per d);

Food transformation index (g total DM intake per d/g increase in body weight per rat per d);

Apparent digestibility coefficient (ADC) for N, P, Ca and Mg;

N, P, Ca and Mg retention (balance);

Percentage N, P, Ca or Mg retention relative to N, P, Ca or Mg absorption (percentage of retention in relation to absorption; %R/A).

ADC, balance and %RA are calculated as follows:

$$\text{ADC} = ((I - F)/I) \times 100;$$

$$\text{Balance} = I - (F + U);$$

$$\%\text{R/A} = ((I - (F + U))/(I - F)) \times 100,$$

where I is intake, F is faecal excretion, and U is urinary excretion.

Statistics

One-way ANOVA was applied to the data with the use of SAS version 8.02 (SAS Institute, Cary, NC, USA). Differences between means were compared with Tukey's test. The level of significance was set at $P<0{\cdot}05$. Faecal excretion of P, Ca, and Mg was adjusted to a multiple linear regression model with total faecal weight and faecal excretion of P, Ca or Mg as regressors. The regression model was adjusted stepwise with the aim of maximising 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.

Results

Chemical composition

The chemical composition of the lupins used in the present study, with regard to N, ash, Ca, P and Mg, and the effect of the α-galactoside oligosaccharide extraction process on the content of the these nutrients has been described previously (Porres *et al.* 2005). The process of α-galactoside removal resulted in a 9 % increase in the fat content of the remaining α-galactoside-free seed by-product, whereas a 5 % reduction in insoluble dietary fibre and a 52 % reduction in soluble dietary fibre content were observed (Table 1).

All the experimental diets tested were isoenergetic and isonitrogenous (Table 2). Of the total P analytically determined in lupin diets, 48·9 and 39·1 % was from raw and α-galactoside-free lupin flour respectively, whereas in the case of Ca, the amount of mineral provided by the former lupin flours was 12·9 and 15·3 % of the total diet content. As for the casein–cystine control diet, 4·9 and 47 % of the total Ca and P contents respectively, were from casein; the remaining amounts of Ca and P needed to meet the nutrient recommendations were added using $CaCO_3$ or $CaHPO_4$. The total content or 47 % of the total Mg measured in the raw and α-galactoside-free lupin diets respectively, was from lupin flour. The remaining Mg, up to the target requirements, was supplemented as MgO in the α-galactoside-free lupin diet. With regard to the casein–cystine control diet, only 2·5 % of the total Mg content was supplied by casein, the rest being provided as MgO.

Food intake, faecal weight, and digestive and metabolic utilisation of protein

Neither the α-galactoside extraction process, nor the additional supplementation with phytase, gave rise to any significant modification in daily food intake, compared with the group of animals fed the raw lupin flour diet (Table 3). With regard to the experimental group fed the casein–cystine control diet, daily food intake was pair-fed to the average value of the other three experimental groups studied. Dietary intake of energy, protein, and soluble or insoluble dietary fibre reflected the daily food intake and chemical composition of the experimental diets. Faecal weight was significantly higher for the casein–cystine experimental group when compared with the rest of the experimental groups tested (raw lupin, α-galactoside-free lupin, PHYTS), among which only minor differences were observed.

Table 3. Food intake, faecal excretion, weight gain and digestive and metabolic utilisation of protein

(Mean values of ten Wistar rats and pooled standard errors of the mean)

Diet	C+C	RLU	GFLU	PHYTS	Pooled SEM
Intake (g DM/d)	8·7	8·9	8·6	8·7	0·3
Energy intake (kJ/d)	170·5	165·8	161·8	163·9	4·8
Protein intake (g/d)	1·5	1·4	1·4	1·5	0·0
IDF intake (g/d)	1·7	1·7	1·4	1·4	0·1
Weight gain (g/d)	2·8^{b}	2·5^{b}	2·0^{a}	2·0^{a}	0·2
PER*	1·8^{b}	1·6^{b}	1·4^{a}	1·4^{a}	0·1
FTI*	3·3^{a}	3·7^{a}	4·4^{b}	4·4^{b}	0·3
Faecal weight (g DM/d)	1·9^{c}	0·9^{b}	0·8^{a}	0·9^{a,b}	0·1
N intake (mg/d)	246·0	230·8	231·6	234·5	6·7
Faecal N (mg/d)	24·2^{a}	39·6^{c}	32·4^{b}	40·6^{c}	2·4
Urinary N (mg/d)	90·1^{a}	78·9^{a}	87·3^{a}	94·9^{a}	6·6
Absorbed N (mg/d)	221·8^{b}	191·2^{a}	199·2^{a}	193·9^{a}	5·9
ADC (%)*	91·1^{b}	83·9^{a}	86·1^{a}	83·4^{a}	0·6
Balance (mg/d)*	131·7^{b}	112·3^{a}	111·9^{a}	99·0^{a}	5·7
%R/A*	59·5^{a}	59·1^{a}	56·1^{a}	52·5^{a}	2·8

C+C, casein–cystine control diet; RLU, raw lupin diet; GFLU, α-galactoside-free lupin diet; PHYTS, α-galactoside-free lupin diet supplemented with exogenous microbial phytase; PER, protein efficiency ratio; FTI, food transformation index; ADC, apparent digestibility coefficient; %R/A, percentage of retention in relation to absorption.

* For calculations, see p. 1104.

^{a,b,c} Mean values within the same row with unlike superscript letters were significantly different ($P<0{\cdot}05$).

The highest digestive utilisation of protein, judged by either net absorption of N or ADC, was observed in the group of animals fed the casein–cystine control diet ($P<0{\cdot}05$), related to a lower faecal excretion of N ($P<0{\cdot}05$), with no significant differences being found among the groups of animals fed the different lupin diets.

In vivo results obtained for protein digestibility in the present experiment were in agreement with the *in vitro* protein digestibility measured by pH-drop and pH-stat techniques, which showed high protein digestibility values for the different lupin diets, which were close to those obtained for casein (pH-drop, 85·2 % in casein *v.* 79·7 and 80·3 % in raw and α-galactoside-free lupin diets respectively; pH-stat, 97·6 % in casein *v.* 96·7 and 96·8 % in raw and α-galactoside-free lupin diets respectively).

The highest N balance of all the experimental groups studied was obtained for the animals fed the casein–cystine control diet ($P<0{\cdot}05$), with no significant differences being found among the three groups fed raw or processed lupin diets. The metabolic utilisation of protein, expressed as a percentage of retained relative to absorbed N (%R/A), was similar in all the experimental groups studied.

Digestive and metabolic utilisation of phosphorus, calcium, and magnesium

Daily P, Ca, and Mg intake in all the experimental groups tested was a reflection of daily food intake and the chemical composition of the diets (Table 4). The process of α-galactoside oligosaccharide removal did not elicit any significant

Table 4. Digestive and metabolic utilisation of phosphorus, calcium and magnesium

(Mean values of ten Wistar rats and pooled standard errors of the mean)

Diet	C+C	RLU	GFLU	PHYTS	Pooled SEM
Faecal weight (g DM/d)	1·9^{c}	0·9^{b}	0·8^{a}	0·9^{a,b}	0·1
P					
P intake (mg/d)	30·0	28·5	27·8	29·7	0·8
Faecal P (mg/d)	11·0^{a}	10·6^{a}	10·0^{a}	14·4^{b}	0·7
Urinary P (mg/d)	1·4^{c}	0·3^{a}	0·6^{a,b}	0·9^{b}	0·1
Absorbed P (mg/d)	19·0^{b}	17·9^{b}	17·7^{b}	15·2^{a}	0·6
ADC (%)*	63·4^{b}	63·1^{b}	64·1^{b}	51·4^{a}	1·8
Retained P (mg/d)	17·6^{b}	17·6^{b}	17·2^{b}	14·3^{a}	0·7
%R/A*	92·5^{a}	98·5^{b}	96·9^{b}	93·7^{a}	0·9
Ca					
Ca intake (mg/d)	37·9	44·7	41·2	42·3	1·3
Faecal Ca (mg/d)	15·4^{a}	15·1^{a}	13·6^{a}	22·3^{b}	1·2
Urinary Ca (mg/d)	4·3^{a}	5·8^{b}	4·7^{a,b}	4·8^{a,b}	0·5
Absorbed Ca (mg/d)	22·5^{a}	29·6^{b}	27·6^{b}	20·0^{a}	1·2
ADC (%)*	59·3^{b}	66·5^{c}	67·2^{c}	50·0^{a}	2·1
Retained Ca (mg/d)	18·2^{b}	23·8^{c}	22·9^{c}	15·2^{a}	1·0
%R/A*	80·8^{b}	80·6^{b}	83·1^{b}	75·9^{a}	1·6
Mg					
Mg intake (mg/d)	6·1	6·6	6·5	6·5	0·2
Faecal Mg (mg/d)	2·5^{b}	1·8^{a}	1·7^{a}	2·0^{a}	0·1
Urinary Mg (mg/d)	2·4	2·3	2·4	2·9	0·2
Absorbed Mg (mg/d)	3·6^{a}	4·8^{b}	4·8^{b}	4·5^{b}	0·2
ADC (%)*	59·1^{a}	72·8^{b}	74·2^{b}	70·0^{b}	1·7
Retained Mg (mg/d)	1·3^{a}	2·5^{b}	2·4^{b}	1·6^{a}	0·2
%R/A*	35·2^{a}	47·1^{b}	47·6^{b}	35·4^{a}	2·6

C+C, casein–cystine control diet; RLU, raw lupin diet; GFLU, α-galactoside-free lupin diet; PHYTS, α-galactoside-free lupin diet supplemented with exogenous microbial phytase; ADC, apparent digestibility coefficient; %R/A, percentage of retention to absorption.

* For calculations, see p. 1104.

[a,b,c] Mean values within the same row with unlike superscript letters were significantly different ($P<0·05$).

change in net absorption or the ADC of P when compared with the experimental groups fed the raw lupin or casein–cystine control diet. However, the PHYTS diet significantly increased faecal P excretion with a subsequent decrease in the digestive utilisation of this mineral expressed as net absorption or ADC ($P<0·05$) when compared with the other experimental diets tested (casein–cystine control; raw lupin; α-galactoside-free lupin). No significant differences in P balance were found among the experimental groups fed the casein–cystine control, raw, or α-galactoside-free lupin diets, while the former index was significantly reduced in the group of animals fed the PHYTS diet.

The digestive utilisation of Ca and Mg, expressed as the ADC, was lower in the casein–cystine control when compared with the raw and α-galactoside-free lupin diets ($P<0·05$). A further reduction in Ca digestibility was obtained for the experimental group fed the PHYTS diet ($P<0·05$), whereas no significant differences were found for Mg digestibility. The metabolic utilisation of Ca expressed as the %R/A value was similar in the casein–cystine control, raw lupin and α-galactoside-free lupin groups, with a slight reduction ($P<0·05$) being found for the PHYTS experimental group. The metabolic utilisation of Mg was significantly lower for the animals fed the casein–cystine control or PHYTS diets when compared with those fed the raw lupin and α-galactoside-free lupin diets, with no significant differences being found between the two former or the two latter experimental groups.

Mineral content in tissues

The P, Ca and Mg content of plasma, blood and different organs is presented in Table 5. The levels of P and Ca in plasma were not substantially affected by the dietary

Table 5. Mineral content of different tissues

(Mean values of ten Wistar rats and pooled standard errors of the mean)

Diet	C+C	RLU	GFLU	PHYTS	Pooled SEM
P					
Plasma (mg/l)	88·0^{b}	84·4^{a,b}	80·4^{a}	78·8^{a}	2·4
Blood (mg/l)	475^{c}	371^{a}	357^{a}	439^{b}	9·7
Femur (mg/g ash)	195·2^{c}	164·0^{a}	168·3^{a}	182·4^{b}	1·9
Sternum (mg/g ash)	188·9^{c}	173·6^{a}	174·0^{a}	182·5^{b}	1·9
Kidney (mg/g ash)	246·1^{b}	221·6^{a}	224·8^{a}	234·8^{a,b}	5·1
Liver (mg/g ash)	259·1^{b}	228·9^{a}	228·8^{a}	261·9^{b}	2·9
Ca					
Plasma (mg/l)	81·7^{a,b}	84·7^{b}	77·6^{a}	76·2^{a}	2·1
Blood (mg/l)	26·3^{a}	56·6^{b}	60·5^{b}	24·8^{a}	2·7
Femur (mg/g ash)	327·0^{b}	302·2^{a}	306·8^{a}	326·6^{b}	2·5
Sternum (mg/g ash)	268·1^{b}	259·0^{a}	258·6^{a}	262·3^{a,b}	3·0
Kidney (mg/g ash)	1·3^{a}	3·2^{b}	3·4^{b}	1·4^{a}	1·5
Liver (mg/g ash)	1·1^{a}	1·4^{b}	1·4^{b}	1·1^{a}	0·1
Mg					
Plasma (mg/l)	18·7^{a}	21·9^{b}	22·2^{b}	17·9^{a}	0·5
Blood (mg/l)	36·6^{a}	42·2^{b}	41·3^{b}	36·2^{a}	0·7
Femur (mg/g ash)	8·0^{b}	6·2^{a}	6·2^{a}	7·9^{b}	0·1
Sternum (mg/g ash)	8·7^{b}	7·9^{a}	8·1^{a}	8·2^{a,b}	0·2
Kidney (mg/g ash)	16·0^{a}	15·7^{a}	16·0^{a}	15·9^{a}	0·3
Liver (mg/g ash)	17·3^{b}	16·5^{a}	16·4^{a}	17·6^{b}	0·2

C+C, casein–cystine control diet; RLU, raw lupin diet; GFLU, α-galactoside-free lupin diet; PHYTS, α-galactoside-free lupin diet supplemented with exogenous microbial phytase.

[a,b,c] Mean values within the same row with unlike superscript letters were significantly different ($P<0·05$).

treatments studied, whereas Mg content was lower in the experimental groups fed the casein–cystine control or the PHYTS diet when compared with the raw lupin or α-galactoside-free lupin diets ($P<0·05$). Under our experimental conditions, a higher accumulation of Ca and Mg in the femur and sternum was observed in the experimental groups of animals with a lower balance of these minerals (casein–cystine control; PHYTS), whereas a certain degree of correlation between tissue mineral content and mineral retention was observed in the other tissues studied (blood, liver, kidney). The higher accumulation of Ca and Mg in the femur, and, to a lesser extent, in the sternum, was achieved at the expense of other tissues such as the kidney, blood and the liver, in the case of Ca, or blood, plasma and erythrocytes (data not shown) in the case of Mg. The accumulation of Ca and Mg in the bone tissue was related to the amount of P stored, as shown by the high correlation found for the femur (Ca *v.* P, r 0·78; Mg *v.* P, r 0·86; $P<0·05$) and sternum (Ca *v.* P, r 0·61; Mg *v.* P, r 0·72; $P<0·05$).

Discussion

Chemical composition of flours and experimental diets

Chemical composition of the raw lupin flour used for the present experiment was within the range of values found in the literature (Hill, 1977; Donangelo *et al.* 1995; van Barneveld, 1999). The increased energy and fat content of lupin flour after α-galactoside removal was due to the concentration of these nutrients caused by the loss of other seed components such as α-galactosides and other soluble sugars, ash or dietary fibre. In spite of the fact that α-galactoside extraction was done using the whole lupin seed, considerable losses in soluble dietary fibre content were observed (48·6 %), which could be due to the prolonged extraction time used.

Diet formulation was adequate to meet the nutrient requirements of the growing rat (National Research Council, 1995) with the exception of dietary fibre content which was superior due to the high levels of this component originally present in the lupin flours used for the present study.

Food intake, faecal weight and digestive and metabolic utilisation of protein

Dietary intake was inferior for the animals fed the different lupin diets assayed in the present experiment when compared with daily food intake obtained for other food legumes previously studied by our group such as the pea (Urbano *et al.* 2003) or the faba bean (Fernández *et al.* 1996). This was despite the fact that a sweet lupin variety with a low alkaloid content was selected, and the fact that the lupin diets of the present study were formulated to meet all the requirements of the growing rat with the aim of avoiding any possible nutrient imbalance. This lower daily food intake could be attributed to the development of rancidity, which may significantly affect the organoleptic properties of lupin and its palatability, as has been described by Lamghari *et al.* (1997). This reduction in palatability would be reflected in a significantly lower daily food intake. The fat content of the lupin variety used for the present experiment was substantially higher than that of other legumes such as the lentil, pea or faba bean, although lower than soyabean (Donangelo *et al.* 1995; Perez-Maldonado *et al.* 1999; El-Adawy *et al.* 2000; Porres *et al.* 2003*b*), and has the potential to play a major role in the development of rancidity. Furthermore, a high proportion of the lipid content in lupin is of the unsaturated type, which is more susceptible to becoming rancid. Treatment of a lupin protein isolate with ascorbic acid, with the aim of inhibiting the development of rancidity during isolate storage, resulted in a significant increase in daily food intake compared with the same lupin protein isolates that had not been treated with this compound (C Martínez-Villaluenga, G Urbano, JM Porres, J Frias & C Vidal-Valverde, unpublished results).

The high content of dietary fibre present in both the lupin flours studied may have contributed to the lower daily food intake, due to its satiating effect. Satiation produced by dietary fibre, with the subsequent reduction in food intake, is the basis of many dietary treatments aimed at weight control (Burton-Freeman, 2000; Koh-Banerjee & Rimm, 2003; Slavin, 2005).

Under our experimental conditions, the presence of α-galactoside oligosaccharides in raw lupin flour did not appear to play a major role in daily food intake, as shown by the lack of effect of α-galactoside oligosaccharide removal by the extraction process. However, changes in chemical composition and food matrix could have taken place during the extraction process in addition to the loss of α-galactosides that would affect the organoleptic properties of lupin flour. Consequently, daily food intake would be affected by changes in several food constituents, rather than in a single one, and these changes could offset the benefits of α-galactoside removal.

The lower faecal weight observed in the experimental groups fed raw or α-galactoside-free lupin diets, compared with the casein–cystine control (2-fold higher in the casein–cystine control diet), can be attributed to the different nature of the insoluble dietary fibre fraction of the diets. In the casein–cystine control diet, the fraction is mainly composed of insoluble non-fermentable cellulose that leaves the gastrointestinal tract of the animals largely undegraded. In contrast, a considerable proportion of the insoluble dietary fibre present in lupins (44·3–61·0 %) is present as non-cellulose polysaccharides (Donangelo *et al.* 1995; Górecka *et al.* 2000; Trugo *et al.* 2000) that are potentially fermentable during their passage along the large intestine of the rat and could result in a lower amount of faecal residue. The remaining insoluble dietary fibre fraction of lupins is mainly composed of cellulose (39·0–52·6 %), which does not suffer any major degradation during its passage through the gastrointestinal tract and ends up excreted in the faeces.

In general, the digestive utilisation of lupin protein by growing rats was high, although slightly lower than that of the casein–cystine control group. It was similar to that of other important dietary legumes with good protein digestibility studied by our group, such as lentils (79 %; Porres *et al.* 2003*b*), faba beans (82 %; Fernández *et al.* 1996) or peas (82 %; Urbano *et al.* 2003). This efficient protein digestibility found for lupins can be attributed to the absence or low levels of certain non-nutritional components such as trypsin inhibitors, lectins or tannins, which may interfere with the digestive utilisation of protein, and to the specific structure of lupin proteins, which makes them highly susceptible to degradation by digestive proteinases. The process of α-galactoside removal

did not cause any major change in protein digestibility despite a higher amount of insoluble N with potentially lower digestibility present in the α-galactoside-free lupin flour (Porres *et al.* 2005).

The lower N balance attained by rats fed the lupin-based diets compared with the casein–cystine control diet can be attributed to a lower net absorption of this nutrient in the former animals, given that the metabolic utilisation of protein expressed as percentage of retained relative to absorbed N (%R/A) was similar in all the experimental groups studied. Differences in N balance between the casein–cystine control and the raw lupin experimental group could justify the higher weight gain ($P<0·05$) of the animals fed the casein–cystine diet in the exponential growth period assayed, given that similar amounts of fat and starch were supplied by both diets. In contrast, significant differences in weight gain were found among the animals fed raw or α-galactoside-free lupin diets, despite a similar N balance. These differences could be related to a poorer nutritive utilisation of protein and carbohydrates by the animals fed the α-galactoside-free lupin diets. The latter diets were formulated to contain a higher amount of starch in order to make them isonitrogenous and isoenergetic with the raw lupin diet. The poorer nutritive utilisation of the α-galactoside-free lupin diet was reflected in a lower protein efficiency ratio and food transformation index ($P<0·05$) compared with the raw lupin diet.

Digestive utilisation of phosphorus, calcium, and magnesium

Under the experimental conditions of the present study, the dietary P, Ca and Mg supplied by the legume or else provided as mineral supplements in the raw lupin and α-galactoside-free lupin diets was efficiently absorbed by the animals, as shown by the high digestive utilisation expressed as the ADC, which was similar to that observed in the casein–cystine control group for P and superior in case of Ca and Mg.

Taking into consideration the numerous factors which may affect P, Ca and Mg absorption from the diet (Schaafsma, 1997), it appears that under our experimental conditions, the digestive utilisation of these minerals from the experimental diets tested was related to faecal weight and the faecal excretion of P. Faecal weight was not related to the amount of insoluble dietary fibre, which was similar in all the experimental diets tested, but rather to the intrinsic nature of this food component. Insoluble dietary fibre in the casein–cystine control diet was predominantly cellulose, which is not susceptible to degradation by fermentative processes in the large intestine and is capable of causing significant faecal losses of Mg by means of a solvent-drag mechanism (Hardwick *et al.* 1991). This was corroborated by the high correlation found between the faecal weight and the faecal excretion of Mg by rats fed the casein–cystine control, raw or α-galactoside-free lupin diets (r 0·81; $P<0·05$).

With regard to Ca availability, the reduction observed in the digestive utilisation of this mineral by the group of rats fed the casein–cystine control diet could be attributed to adsorption of the cation to cellulose caused by the high levels of this polysaccharide present in the diet, as well as to possible interactions among Ca, dietary fibre and other dietary components, but not to ionic interactions between Ca and cellulose, given that a very low ionic-binding capacity has been described for cellulose by several authors (Torre *et al.* 1992; Blaney *et al.* 1996; Claye *et al.* 1998; Luccia & Kunkel, 2002).

A significant amount of the insoluble dietary fibre fraction from lupins is present as non-cellulosic polysaccharides, which are susceptible to degradation during their passage through the large intestine. The degradation of insoluble dietary fibre would release an important amount of dietary Ca and Mg and create optimal conditions for the absorption of these minerals in the colonic environment, all of which will significantly contribute to enhancing their absorption by rats (Younes *et al.* 1996; Lopez *et al.* 1998).

An increase in the digestive utilisation of P was expected to result from phytase supplementation of the α-galactoside-free lupin diet due to the ability of phytase to release phytic acid-P in the acidic stomach environment, and to efficiently improve the digestive utilisation of this mineral by single-stomached animals (Lei & Porres, 2005; Porres *et al.* 2005). Therefore, the observed increase in faecal P excretion in response to phytase supplementation could be attributed, taking into consideration the formulation of experimental lupin diets that led to a relatively low proportion of phytic acid-P in the α-galactoside-free lupin diet (32 %), to the need to add a considerable amount of inorganic P in the form of $CaHPO_4$ in order to meet the requirements of this nutrient for the growing rat. During the course of digestion, the release of an additional phosphate amount by phytase, far from exerting a beneficial effect on the nutritive utilisation of this mineral, led to insoluble complex formation among phosphate, Ca and Mg (Brink *et al.* 1992) that interfered with the absorption of the above-mentioned cations. This was reflected in a high correlation among the faecal excretion of the three minerals, as shown by the multiple linear regression model applied (faecal excretion of P = 2·479 + 1·5228 (faecal excretion of Mg) + 0·4227 (faecal excretion of Ca) – 0·95 082 (faecal weight); adjusted R^2 0·8743; $P<0·0001$).

The dietary factors previously described as having an individual effect on the faecal excretion and thus the digestive utilisation of Ca and P interacted under our experimental conditions in the *in vivo* model assayed, as highlighted by the multiple linear regression model applied to the data (Ca_{faecal}/Ca_{intake} = 0·03640 (faecal weight) + 0·03567 (faecal excretion of P), adjusted R^2 0·7807, $P<0·0001$; faecal excretion of Mg = 0·7461 (faecal weight) + 0·09238 (faecal excretion of P), adjusted R^2 0·7983, $P<0·0001$).

Metabolic utilisation of phosphorus, calcium and magnesium

Overall, the animals efficiently preserved the absorbed P and Ca, as is reflected in the high metabolic utilisation of these minerals expressed as net retention or %R/A. The slight reduction in metabolic utilisation of the above-mentioned minerals by the groups of rats fed the casein–cystine control or the PHYTS diet when compared with the raw and α-galactoside-free experimental groups was due to the higher urinary excretion of P by the animals. However, in the case of Ca, the lower retention was due to the lower net absorption of this mineral by the former two groups, as no major differences in urinary excretion or metabolic utilisation of Ca were found among any of the four experimental groups tested. The lower amount of Mg retained by the animals fed the casein–cystine control diet was due not only to the lower net absorption of

this mineral by the experimental animals, but also to the lower metabolic utilisation, expressed as %R/A. This reduction cannot be justified on the basis of the parameters usually described as having an effect on the metabolic utilisation of Mg, such as protein quality of the diet or the weight gain of the animals. The reduction appeared to be related to the low net absorption of the mineral, given that urinary excretion of Mg was similar among the four experimental groups studied despite the higher net absorption of Mg obtained for the animals fed the raw or α-galactoside-free lupin diets.

Although the experimental period used for the present study was short (10 d), it should be pointed out that the Mg balance in the animals fed the casein–cystine control diet and the PHYTS diet was half that obtained for the other two experimental groups studied. These lower Mg balances may contribute to a lower general status of this mineral in the body, which would be reflected in lower contents of this cation in plasma, erythrocyte and, possibly, in muscle tissue, which has been described as one of the first tissues to show signs of Mg depletion when the dietary levels of this mineral are reduced (Lerma *et al.* 1993).

Mineral content in tissues

In all the experimental groups studied, plasma levels of P, Ca and Mg were within the normal range of values for the growing rat (Fernández *et al.* 1997; Aranda *et al.* 2004) and correlated well to the amount of retained mineral, with the exception of Ca, as was to be expected from the strict regulation of this mineral in order to maintain homeostasis of the internal media. P retention was not related to the amount of this mineral stored in any of the different tissues studied, with the exception of plasma, although the differences were not statistically significant. Thus, if P balance is to be considered a reference index for the nutritional status of this mineral, the latter would be much lower in the animals fed the PHYTS diet. In contrast, this experimental group would present a higher nutritional status of P if the tissue content (femur, sternum, liver or blood) were considered as the reference index.

Conclusions

The present study of growing rats shows that α-galactoside extraction from the seeds of *L. albus* var. *multolupa* results in a seed by-product that contains a substantial amount of good-quality protein. Such seed by-product, supplemented with the necessary amounts of minerals and vitamins to compensate for the nutritional losses that take place during the extraction process, can be successfully used for animal feed preparation and for the development of integral dietetic products with a high nutritional and functional value intended for human nutrition and suitable for utilisation in different physiological or pathological situations. The total dietary fibre content of the lupin flour used in the present experiment was high, and a significant proportion was found to be insoluble. Nevertheless, the bioavailability of Ca and Mg was not negatively affected by this food component in the lupin diets tested. This can be attributed to the complex nature of such insoluble dietary fibre, which contains a substantial proportion of non-cellulosic polysaccharides susceptible to being fermented in the large intestine.

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