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Chemical composition of a new *Lupinus* species found in Spain, *Lupinus mariae-josephi* H. Pascual (Fabaceae)

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Abstract

A new lupin from Valencia (Spain) has been described recently. The new species *Lupinus mariae-josephi* H. Pascual (Fabaceae) differs from any other Mediterranean and North American taxon in banner position, flower colour and inflorescence structure. This species was found in a calcareous area, and when it was grown in experimental fields it has always performed better in alkaline soils. This work attempts to establish the content of protein, oil and nutritionally active factors (alkaloids, α-galactosides and inositol phosphates) in this new species of lupin, to investigate differences with other *Lupinus* ssp. The analysed raw seed samples of this new species showed a total protein content of 329.4 g kg^{-1} and oil content of 54.0 g kg^{-1}. The total content of alkaloids, α-galactosides and phytate was similar to other bitter *Lupinus* species being lupanine, stachyose and myo-inositol hexaphosphate (IP_6) the main bioactive compounds. However, the profile of alkaloids and proteins, which is an important chemotaxonomic criterion, was characteristic of this species. It is worth to notice that this *Lupinus* is able to grow in calcareous soils what makes this species very promising for soil recovery.

Additional keywords: alkaloids; fatty acids; lupin; phytate; protein pattern; α-galactosides.

Resumen

Composición química de una nueva especie de *Lupinus* encontrada en España: *Lupinus mariae-josephi* H. Pascual (Fabaceae)

Recientemente se ha descrito una nueva especie de *Lupinus* en Valencia (España). Esta nueva especie, *Lupinus mariae-josephi* H. Pascual (Fabaceae) difiere de otras especies de *Lupinus* del área mediterránea o de Norteamérica en la posición del estandarte, el color de la flor y la estructura de la inflorescencia. Fue encontrada en un área calcárea, y en estos últimos años, cuando se ha cultivado, ha crecido en suelos calizos. El objetivo de este trabajo ha sido estudiar el contenido de proteína, grasa y factores nutricionalmente activos (alcaloides, α-galactósidos y fitatos) en esta nueva especie, para ver las diferencias que existen con otras especies de *Lupinus*. Los resultados han mostrado un contenido en proteína de 329,4 g kg^{-1} y un contenido en grasa de 54,0 g kg^{-1}. El contenido total de alcaloides, α-galactósidos y fitatos fue similar a otras especies amargas de *Lupinus* siendo la lupanina, la estaquiosa y el mioinositol hexafosfato (IP_6) los principales componentes bioactivos. Sin embargo, el perfil de alcaloides y el perfil proteico, que son un importante criterio taxonómico, fueron característicos de esta especie. Es importante resaltar que esta especie de *Lupinus* es capaz de crecer en suelos calizos, lo que la hace muy prometedora para la recuperación de suelos.

Palabras clave adicionales: ácidos grasos; alcaloides; altramuz; fitatos; perfil proteico; α-galactósidos.

Introduction

Lupin seeds are employed as a protein source for animal and human nutrition not only for their nutritional value (high in protein, lipids and dietary fibre), but also for their adaptability to marginal soils and climates.

Spain is one of the richest countries in lupin flora, with many species which could be used as genetic

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Abbreviations used: BSA (bovine serum albumin); FAME (fatty acid methyl esters); GC/MS (gas chromatography/mass spectrometry); IP6 (inositol hexaphosphate); NAF (nutritionally active factor); NPD (nitrogen phosphorus detector).

material. The potential exists for putting together a germplasm bank of great utility. Also, there are significant possibilities for the cultivation of this plant, especially in the West of the Iberian Peninsula, thus making use of siliceous, sandy and acidic soils. Until now it was considered that the genus *Lupinus* was represented in the Iberian Peninsula by eight species (Castroviejo and Pascual, 1999), usually living on acid or neutral substrates. Six are natives of the Iberian Peninsula and two, *Lupinus albus* and *Lupinus poliphylus*, often cultivated and sometimes naturalized. *Lupinus luteus* and *Lupinus angustifolius*, wild in our territory, often also grow as fodder, especially outside of Spain (Pascual, 2004). Recently a new lupin, from Valencia (Spain), has been described by Pascual (2004). The new species *L. mariae-josephi* H. Pascual (Fabaceae) differs from any other Mediterranean and North American taxons in banner position, flower colour and inflorescence structure (Figure 1). This species was found in a calcareous area, and moreover this plant can only grow in this type of soil under cultivation. According to Mahé *et al*. (2010) this species shows morphological similarities with the Mediterranean smooth seeded species and it shares the same chromosome number (2n = 52) with the Old Word taxa (*L. luteus, Lupinus hispanicus, Lupinus micranthus*), but also with unifoliate lupins from Florida (*Lupinus villosus*). Besides, *L. mariae-josephi* exhibited a seed coat micromorphology "intermediate" between the rough and the smooth seed coat types.

Figure 1. *Lupinus mariae-josephi* (H. Pascual).

The genus *Lupinus*, as other legumes, has the ability to fix atmospheric nitrogen through symbiosis with soil bacteria (*Rhizobium*) living inside nodules on the roots of this plant. Because of this, lupins have been grown since antiquity as important crops to improve soils and food.

Human consumption of lupins has increased in recent years, being regarded as a beneficial food ingredient. In fact, they are recommended as staple food by health organisations and dieticians (Leterme, 2002).

Health benefits have been ascribed to three main groups of components in legume seed meals: no-nutritional factors, proteins and carbohydrates. The presence of certain amounts of some no-nutritional factors or nutritionally active factors (NAF_s) such as inositol phosphates, alkaloids, oligosaccharides, etc, have been linked over the last two decades to health promoting properties, and they are at present considered natural bioactive substances. The scientific interest in these factors is now also turning to studies of their possible useful and beneficial applications as gut, metabolic and hormonal regulators and as prebiotic agents (Pusztai *et al*., 2004).

This work attempts to establish the content of protein, fatty acids and NAF_s (alkaloids, α-galactosides and phytic acid) in the new species of lupin, *L. mariae-josephi* from Valencia (Spain), to investigate differences with other *Lupinus* ssp.

Material and methods

Plant material

Seeds of *L. mariae-josephi* from soils of eight spots of native plant populations of Montserrat de Alcalá and Llombai (Valencia, Spain) were collected at random. Seeds of *L. villosus* (K 36b, Florida, USA) were kindly provided by Dr. Abdelkader Ainouche (Université de Rennes, France) and *L. micranthus*, *L. luteus*, *L. hispanicus*, *L. angustifolius* and *L. albus* by Dr. Andrés Gil (Junta de Extremadura, Badajoz, Spain).

Chemical analysis

Raw samples (100 seeds) were ground to pass through a 1 mm sieve (Tecator, Cyclotec 1093). The flour was analysed for ash, moisture, crude protein and oil using the AOAC (Association of Official Analytical

Chemists) methods (1990). The soluble protein was determined by the dye-binding assay of Bradford (1976), using BSA as standard.

Lupin protein extracts and protein electrophoresis

Cotyledons were ground to pass through a 1-mm sieve (Tecator, Cyclotec 1093), and the flour was defatted with n-hexane (34 mL g^{-1} of flour) for 4 h, shaken and air-dried after filtration. The resulting defatted flours were extracted twice in a solution of 0.05 M Tris-HCl (pH 8.0) buffer with salt (0.5 M NaCl) at a 1:10 w/v ratio for 1 h at 4°C by stirring according to the method described by Álvarez-Álvarez *et al*. (2005). The extracts were centrifuged at 27000 *g* for 20 min at 4°C, and the combined supernatants were dialyzed against distilled water for 48 h at 4°C using dialysis membranes with a cutoff of 3.5 kDa (Spectra/Por, Serva, Heidelberg, Germany) and then freeze-dried.

The soluble protein content of the extracts was determined by the Bradford dye binding assay (Bio-Rad, Hercules, CA, USA) using BSA (Sigma, St. Louis, MO, USA) as a standard.

SDS-PAGE was carried out as described by Laemmli (1970) on 15% polyacrylamide gels using MINI PROTEAN III system (Bio-Rad, California, USA) under reducing conditions. Previously samples were incubated at 95°C for 5 min in Laemmli Sample Buffer (Bio-Rad, California, USA) containing 2% (v/v) of 2-betamercaptoethanol. Proteins were visualized with Coomassie brilliant blue R250 staining.

Coomassie-stained gel was scanned, and the molecular weight of the bands was assessed using Quantity One software (Bio-Rad) and the Precision Plus Protein™ Unstained molecular weight marker (Bio-Rad) as standard.

Analysis of fatty acid methyl esters

A 0.5 g sample of flour was weighed and transferred into a glass test tube (Falcon) with screw-cap and homogenized for 2 min with 1 mL of a BHT/methanol:chloroform:methanol mixture (1:10:5 v/v/v) using a tissue homogenizer (Ultra-Turrax). Chloroform (10 mL) was then added and the sample was homogenized for one more minute. Tubes were then centrifuged for 10 min at 480 g and 0°C and the chloroform layer was removed, filtered through anhydrous sodium sulphate and collected into a 100 mL round-bottom flask. Finally, the dehydrated and filtered chloroform was removed under vacuum and the oil stored at –20°C for further fatty acid analysis.

Fatty acid methyl esters (FAME) were prepared as follows: 100 mg of each lupin oil containing 1 mg mL^{-1} nonadecanoic acid (C19:0) as internal standard (Sigma, St. Louis, MO, USA) were placed into a 10 mL glass tube with screw-cap. Tubes were flushed with nitrogen to dryness and 1 mL of 5 N KOH in methanol and 1 mL of 14% boron trifluoride in methanol were added. All tubes were then sealed under nitrogen, mixed for 10 sec and heated for 1 h at 100°C. After cooling, 2 mL of hexane was added to each tube, re-capped, mixed and left standing to allow the hexane layer to separate (Morrison and Smith, 1964). One millilitre of each hexane layer was diluted 10-fold with hexane and 1 mL was transferred to autosampler vials.

Analysis of FAME was performed in a 10 µL sample using a Perkin-Elmer Autosystem 1-A gas chromatograph equipped with a flame ionization detector, a split-splitless injector, a 25 m × 0.25 mm i.d. SGL 1000 column (Sugelabor, Madrid, Spain) and helium C-50 as carrier gas. The initial oven temperature was 150°C, with a temperature ramp of 10°C min^{-1} to 200°C and 5°C min^{-1} to 210°C. FAME were identified by comparing their retention times with those of authenticated and known concentrations of methyl ester standards including nonadecanoic methyl esters (Sigma, St. Louis, MO, USA) that were analyzed under similar conditions. The percentage fatty acid content was calculated using factors obtained from peak areas of the known mixture of methyl esters.

Nutritionally active factors

Alkaloids

Extraction of the milled seed was done as described by Muzquiz *et al*. (1993). Finely ground lupin seed (0.5 g) was homogenized in 5% trichloroacetic acid (3×5 mL) with an Ultra-Turrax and centrifuged at 12100 g for 5 min. After centrifugation, 1 mL of 10 M NaOH was added to the supernatant. The alkaloids were then extracted with dichloromethane (3×5 mL). The dichloromethane extract was evaporated to dryness, and the alkaloids were dissolved in 1 mL of methanol. A 0.5 mL aliquot of the extract was added to 0.5 mL

of a solution of codeine in methanol (2 mg mL^{-1}), which was the internal standard.

A Perkin-Elmer gas chromatograph equipped with a nitrogen-phosphorous detector (NPD) and operated by a Turbochrom program was used. The column used was a SPB-1 capillary column (30 m × 0.25 mm i.d.; Teknokroma, Bellefonte, PA, USA) and helium was the carrier gas. The temperatures of the injector and detector were 240°C and 300°C, respectively. The oven temperature was 150°C, increased by 5°C min^{-1} to 235°C and final hold time of 23 min at 235°C. Calibration curve was performed for lupanine with a linear response over the range 0-1.250 mg mL^{-1} and correlation coefficients of above 0.99.

For identification of the alkaloids, capillary CG-MS was applied. A Perkin-Elmer Autosystem XL gas chromatograph (working with the same column and conditions as above) was coupled with a mass selective detector (Perkin-Elmer Turbomass Gold) that was combined with the Turbomass software for the identification of alkaloids in the samples.

Inositol phosphates

Individual inositol phosphates (IP_3-IP_6) were extracted according to Burbano *et al*. (1995) and determined according to Lehrfeld (1994). The ground sample (0.5 g) was extracted with 5 mL of 0.5 M HCl using an Ultra-Turrax homogenizer for 2 min. The mixture was centrifuged for 5 min at 27000 g. The supernatant (2.5 mL) was diluted with 25 mL of distilled water and placed onto a SAX column (Varian, Palo Alto, CA, USA). The column was washed with 2 mL of water and then, the inositol phosphates were eluted with 2 mL of 2 M HCl. The eluate was evaporated to dryness and the residue was dissolved in a buffer solution. The solution was centrifuged at 12200 g for 6 min to remove any suspended material prior to injection into the HPLC system. The column (150 × 4.1 mm i.d.) consisted of a macroporous polymer PRP-1 (5 µm) (Teknokroma, Reno, Nevada, USA) was used at 45°C. A Beckman HPLC system gold (Fullerton, CA, USA) with a refractive index detector and a fixed-loop (10 µL) injection valve was used. The mobile phase consisted of 515 mL of methanol added to 485 mL of water. Eight millilitres of tetrabutylammonium hydroxide (Fluka, 40% in water), 1 mL 5 M sulfuric acid, 0.5 mL 91% formic acid (Fluka) and 100 µL of a phytic acid hydrolysate (6 mg mL^{-1}) were sequentially added. The mobile phase was filtered through a Millipore filter (0.45 µm) and degassed under vacuum.

Oligosaccharides

Oligosaccharides (sucrose, oligosaccharides of the raffinose family and ciceritol) were determined by HPLC according to Muzquiz *et al.* (1992), with some modifications. Ground material (0.1 g) was homogenized with aqueous ethanol (50%, 5 mL) for 1 min at 4°C. The mixture was centrifuged for 5 min at 12100 g and the supernatant decanted. The procedure was repeated twice and the combined supernatants passed through a Waters C_{18} minicolumn (Milford, MA, USA) with a Supelco vacuum system. Aqueous ethanol (50%; 3 mL) was added to flush the column and the combined extracts and washings were evaporated to dryness. The residue was dissolved in double-deionised water (1 mL) and then centrifuged (12100 g × 8 min) and filtered (Millipore 0.45 µm). Samples (20 µL) were analysed using a Spherisorb-5-NH_2 (250 × 4.5 mm i.d.) column (Waters, Milford, MA, USA) with acetonitrile:water (65:35, v/v) as the mobile phase. A Beckman HPLC system Gold with a refractive index detector was used.

Results and discussion

Chemical analysis

The chemical proximate analysis for *L. mariae-josephi*, showed contents of 892.1 g kg^{-1} and 26.6 g kg^{-1} for dry matter and ash respectively. The oil content (54.0 g kg^{-1}) was similar to those of *L. luteus* (45.0 g kg^{-1}), *L. hispanicus* (36.0 g kg^{-1}) and *L. angustifolius* (49.0 g kg^{-1}) (Muzquiz *et al*., 1989) and lower than those of *L. mutabilis* (184.0 g kg^{-1}) and *L. albus* (148.0 g kg^{-1}) (Fuentes and Planchuelo, 2006). García-Lopez *et al*. (2001) found that the oil content fluctuated from 57.0 g kg^{-1} for *Lupinus mexicanus* to 115.0 g kg^{-1} for *Lupinus montanus*.

The protein content (329.4 g kg^{-1}) was lower than those of *L. luteus* (417.0 g kg^{-1}), *L. hispanicus* (412.0 g kg^{-1}) and *L. albus* (378.0 g kg^{-1}) and similar to *L. angustifolius* (329.0 g kg^{-1}), *Lupinus exaltatus* (334.0 g kg^{-1}) and *L. montanus* (312.0 g kg^{-1}) (Muzquiz *et al*., 1989; García-Lopez *et al.,* 2001). Barrientos *et al*. (2006) found a total amount of protein of 368.0 g kg^{-1} in *L. mexicanus*.

Protein electrophoresis

The gel presented in Figure 2 shows the polypeptide patterns of seed protein extract from *L. mariae-josephi* seeds compared to patterns of different lupin species (*L. albus*, *L. luteus, L. angustifoliu*s, *L. micranthus* and *L. villosus)*. *L. mariae-josephi* exhibited a different electrophoretic pattern as compared to these other lupin species. Its SDS-PAGE pattern consisted of over 27 polypeptides chains, with molecular mass ranging from 96 to 10 kDa, with major bands of 65, 55, 48, 34 and 30 kDa, being the two last the most characteristic of this species. The patterns of the other lupin species were constituted by different number of polypeptides with molecular mass between 95 and 9 kDa. Three major proteins of 58, 46 and 42 kDa in *L. albus* and 65, 51 and 37 kDa in *L. luteus* were detected. *L. villosus* presented four main proteins bands (60, 47, 17 and 14 kDa) as well as *L. micranthus* (from 67 to 40 kDa) and up to six major bands, ranging from 72 to 36 kDa, were detected in *L. angustifolius*.

Total seed proteins electrophoresis has been considered valid for addressing taxonomic studies (Ladizinsky and Hymowitz, 1979), and some of them have determined genetic variability in several legumes (Jha and Ohri, 2002; Vaz *et al.,* 2004; Emre *et al.*, 2006). Interestingly, *L. mariae-josephi* exhibited marked different protein pattern when it was compared with Old World and New World lupins (Figure 2).

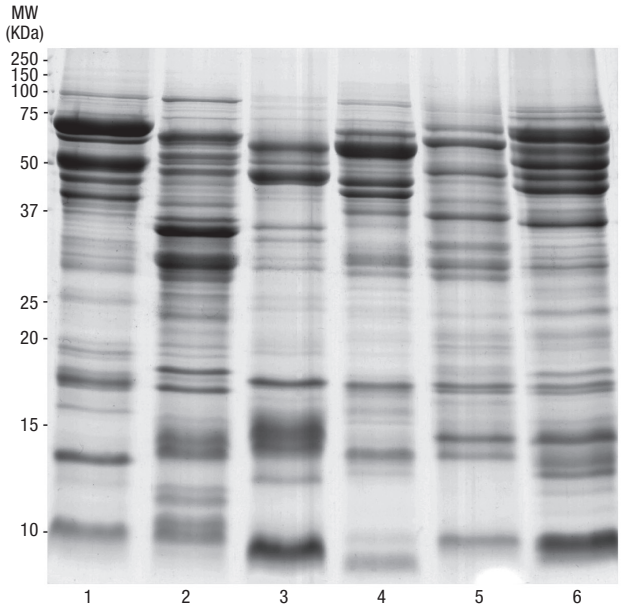

Figure 2. SDS-PAGE analysis of protein extracts from different lupins: *L. micranthus* (lane 1); *L. mariae-josephi* (lane 2); *L. villosus* (lane 3); *L. albus* (lane 4); *L. luteus* (lane 5) and *L. angustifolius* (lane 6). MW: molecular weight marker (kDa).

In lupins, albumins and three globulins fractions: β-conglutin, α-conglutin and γ-conglutin, have been characterized as the main storage proteins (Blagrove and Gillespie, 1975; Mossé and Pernollet, 1983; Esnault *et al*., 1991; Melo *et al*., 1994). β-conglutin, usually the major component, presents the greatest heterogeneity between species showing numerous polypeptide chains with molecular masses from 15 to 72 kDa. The α-conglutin fraction is composed of some heavy polypeptide chains (from 31 to 63 kDa) and a lighter polypeptide chain (20 kDa). And γ-conglutin, generally the minor component, contains two polypeptide chains (one of around 17 kDa; and another of around 27 – 30 kDa) (Blagrove and Gillespie, 1975; Esnault *et al*., 1991; Melo *et al*., 1994). According to the SDS-PAGE pattern and distribution of storage protein fractions previously reported for other lupin species, probably the storage proteins of *L. mariae-josephi* are distributed similarly to these species.

Fatty acids

It is worth to notice that the major fatty acids are the unsaturated, oleic acid (C18:1) (284.4 g kg^{-1}), linoleic acid (C18:2) (338.9 g kg^{-1}), and γ-linolenic acid (C18:3) (125.2 g kg^{-1}) (Table 1). The ratio of oleic to linoleic acids, found (0.84) in this species was higher than *L. luteus* (0.67) and lower than this of *L. mutabilis* (1.07) (García-Lopez *et al*., 2001). The high content of essential unsaturated fatty acids (linoleic and γ-linolenic acids) provides heart-healthy fat features, is also essential for growth and proper functioning of tissues and when incorporated in the diet of monogastric are precursors of other polyunsaturated fatty acids. A lower ω-6 /ω-3 ratio exert suppressive effects in the prevention of cardiovascular disease although the optimal ratio may vary with the disease and it is quite possible that the therapeutic dose of ω -3 fatty acids will depend on the degree of severity of disease (Simopoulos, 2006).

The predominant saturated fatty acids in this species were palmitic (C16:0) (17.5 g kg^{-1}) and estearic acids (C18:0) (54.8 g kg^{-1}). Palmitic acid was found to be the predominant in all lupin populations. Muzquiz *et al*. (1989) found levels ranging from 11.6 g kg^{-1} in *L. mutabilis* to 109.6 g kg^{-1} in *L. angustifolius*. The average palmitic acid content found for all populations of *L. mexicanus* (207 g kg^{-1}), *L. montanus* (263 g kg^{-1})

Table 1. Saturated and unsaturated fatty acid content (g kg^{-1}) of *Lupinus mariae-josephi*

Fatty acid	g kg^{-1}
C12:0	1.3 ± 0.0[a]
C13:0	39.4 ± 0.4
C14:0	1.8 ± 0.1
C15:0	1.9 ± 0.1
C15:1	0.9 ± 0.1
C16:0	17.5 ± 0.8
C16:1	0.0 ± 0.0
C17:0	1.8 ± 0.0
C17:1	0.5 ± 0.1
C18:0	54.8 ± 1.3
C18:1n9	284.4 ± 0.8
C18:2n6	338.9 ± 1.3
C18:3n3	125.2 ± 0.6
C20:0	3.8 ± 0.2
C20:1	1.3 ± 0.0
C20:3n3	0.2 ± 0.0
C22:0	15.8 ± 0.2
C24:0	3.0 ± 0.4
C24:1	7.9 ± 0.4

[a]mean ± standard error; n = 4

and *L. exaltatus* (203 g kg^{-1}) was higher than those of the domesticated species (Garcia-Lopez *et al*., 2001).

The fatty acid profile, oleic-linoleic ratio and the unsaturated-saturated acid ratio in *L. mariae-josephi* were similar to that reported for other lupin species.

Nutritionally active factors

Lupin seed, compared with other legumes, is relatively devoid of protein antinutrients such as lectins and protease inhibitors, but, in the wild, it contains high concentration of quinolizidine alkaloids, α-galactosides, and inositol phosphates (Scarafoni *et al.*, 2004).

Alkaloids

The alkaloid composition of the raw seeds of *L. mariae-josephi* samples, as obtained by capillary gas chromatography, is shown in Table 2. *L. mariae-josephi* as other bitter species of lupine has a high content of alkaloids (13.3 g kg^{-1}) similar to the values found in bitter seeds of *L. luteus* (4-13 g kg^{-1}) and *L. angustifolius* (13-16 g kg^{-1}) and lower than those found in *L. hispanicus* (14-24 g kg^{-1}) and *L. mutabilis* (31-33 g kg^{-1}) (Muzquiz *et al*., 1989). Muzquiz *et al*. (1994) studied 49 genotypes of *L. albus* of which 29 were bitter with a range of alkaloids of 15-27 g kg^{-1}. Ruiz-Lopez *et al*. (2000) studied the alkaloid content in three *Lupinus* species from Jalisco (Mexico) and the total alkaloid content ranged from 14 to 44 g kg^{-1}.

Table 2. Alkaloids composition and content (g kg^{-1}) of *Lupinus mariae-josephi*

Alkaloid	g kg^{-1}
Sparteine	0.022 ± 0.004[a]
Tetrahydrorhombifoline	0.270 ± 0.062
5,6-Dehydro- α-isolupanine	0.165 ± 0.023
α-Isolupanine	2.631 ± 0.521
5,6-Dehydrolupanine	0.120 ± 0.030
Lupanine	8.731 ± 0.841
11,12-Dehydrolupanine	0.598 ± 0.107
Multiflorine	0.046 ± 0.006
10,17-Dioxosparteine	0.036 ± 0.006
17-Oxolupanine	0.534 ± 0.055
13- α-Hydroxylupanine	0.184 ± 0.020
Total alkaloids	13.337 ± 1.806

[a]mean ± standard error; n = 4

In *L. mariae-josephi* sparteine, tetrahydrorhombifoline, 5, 6-dehydro-α-isolupanine, α-isolupanine, 5, 6- dehydrolupanine, lupanine, 11, 12-dehydrolupanine, multiflorine, 10, 17-dioxosparteine, 17-oxolupanine and 13-OH-lupanine were present (Fig. 3). In these seeds,

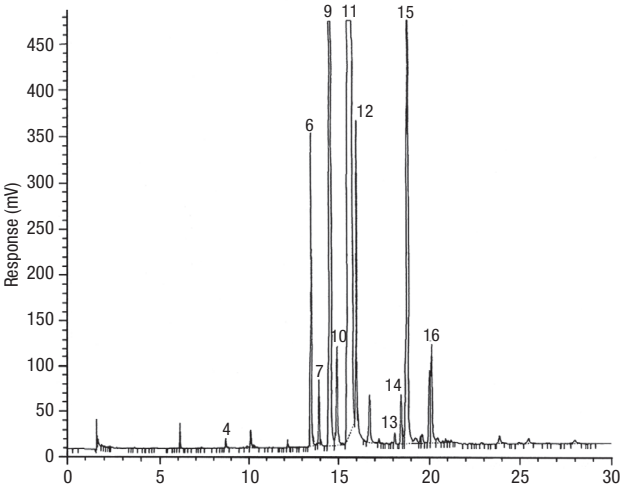

Figure 3. Gas chromatogram of crude alkaloids extracts of *L. mariae-josephi*. Injector, 240°C; detector 300°C; oven 150-235°C, 5°C min^{-1}; carrier gas, helium; detection of alkaloids by nitrogen-specific detector (NPD) and mass-selective detector. Alkaloids: 4) Sparteine; 6) Tetrahydrorhombifoline; 7) 5,6-Dehydro-α-Isolupanine; 9) α-Isolupanine; 10) 5,6-Dehydrolupanine; 11) Lupanine; 12) 11,12-Dehydrolupanine; 13) Multiflorine; 14) 10,17-Dioxosparteine; 15) 17-Oxolupanine; 16) 13-OH-Lupanine.

lupanine was the main alkaloid, representing 67% of total alkaloids, and α-isolupanine, 11, 12-dehydrolupanine and 17-oxolupanine were other major components. All the alkaloids of *L. mariae-josephi* were identified by their mass fragmentation pattern and compared with literature (Table 3). A low proportion of alkaloid esters was found in these seeds, and only 13-OH-17-oxolupanine was identified (data not shown). Other esters that were reported in *L. albus* and *L. angustifolius* by Mühlbauer *et al*. (1987) do not appear in this species. Lupanine is also the major alkaloid in *L. albus, L. angustifolius* and *L. mutabilis* (Muzquiz *et al.*, 1989; 1994). Przybylak *et al.* (2005) also found lupanine as major alkaloid in *Lupinus rotundiflorus, L. exaltatus and L. mexicanus*. In *L. micranthus* albine, multiflorine and 13-α-hydroxi-multiflorine were the most abundant alkaloids. In this species appear the alkaloid characteristic of *L. albus*, albine (Wink *et al*., 1995). In the American species, *L. villosus,* with the same chromosome number (2n = 52) that *L. mariae-josephi*, the main alkaloids were angustifoline, α-isolupanine, lupanine, multiflorine and 13-OH-lupanine (Fig. 4). Meiβner and Wink (1992) studied 31 different taxa of 75 North American lupins, leading to a complex pattern of alkaloid profiles analysed by GC/MS. Comparing the profile of alkaloids in *L. mariae-josephi* with these North American species we can say that this new species of lupin presents a similar profile to that reported by these authors for *Lupinus kingii,* which share the American area origin with *L. villosus*.

Figure 4 shows the alkaloids profile of the lupin species, analysed in this work, with the same chromosome number than *L. mariae-josephi* (2n=52). *L. villosus* contains the alkaloids α-isolupanine, 5, 6-dehydrolupanine, lupanine, multiflorine and 13-OH-lupanine that also appear in *L. mariae-josephi* although *L. villosus* presents, as majority, the alkaloid angustifoline characteristic of *L. angustifolius*.

An important chemotaxonomic criterion is alkaloid composition which also confirms distinctness between species. Wink *et al*. (1995) indicated that the lupins of the lupinine and of the multiflorine complex are genetically close related and probably share a common ancestry.

Compared to the Old World smooth seeded *Lupinus,* Mahé *et al*. (2010) indicated that *L. mariae-josephi* shares more similarities, in general in morphology and habit, with member of sections Lutei (*L. luteus, L. hispanicus)* and Micranthi (*L. micranthus*) than with *L. albus* and *L. angustifolius*. However, according to our results, the alkaloids composition present in *L. mariae-josephi* is not similar to those found by Muzquiz *et al*. (1989) in *L. luteus* and *L. hispanicus* but, this species presents more similar alkaloids with *L. micranthus* (Wink *et al*., 1995)*,* although the alkaloid albine, characteristic of *L. albus,* does not appear in *L. mariae-josephi*.

The smooth seeded wild species *L. micranthus*, the most widespread of the lupins around the Mediterranean Basin, has been identified as an important taxon for the understanding of the relationships between Old World lupins. Naganowska *et al*. (2003) indicated its intermediate position among lupin species, which could be a result of hybridization and introgression between smooth and rough-seeded types or reflect a remainder of the transitional lineage from which the smooth-seeded species were derived.

Table 3. Mass spectral data of *Lupinus mariae-josephi* alkaloids by capillary GC/Mass spectrometry. Reference: Meiβner and Wink (1992)

Peak nº	Alkaloid	M^+	Characteristic ions (% relative abundance)
1	Sparteine	234	137 (100), 98 (94), 193 (25),110 (19), 234 (17), 84 (15), 122 (12)
2	Tetrahydrorhombifoline	248	28 (100), 207 (83), 112 (26), 108 (13), 84 (5), 148 (2)
3	5,6-Dehydro-α-isolupanine	246	98 (100), 97 (52), 246 (21), 84 (11), 134 (7), 148 (4)
4	α-Isolupanine	248	136 (100), 149 (52), 248 (33), 98 (32), 150 (31), 110 (19), 84 (16)
5	5,6-Dehydrolupanine	246	98 (100), 97 (38), 246 (20), 134 (11), 84 (10)
6	Lupanine	248	136 (100), 149 (52), 98 (28), 150 (34), 248 (32), 110 (12)
7	11,12-Dehydrolupanine	246	134 (100), 246 (67), 148 (39), 55 (27), 231 (18), 112 (18)
8	Multiflorine	246	134 (100), 246 (35), 136 (26), 110 (22), 149 (21), 97 (18)
9	10,17-Dioxosparteine	262	84 (100), 150 (58), 152 (47), 262 (47), 84 (40), 110 (30), 97 (25)
10	17-Oxolupanine	262	150 (100), 110 (40), 112 (30), 97 (30), 262 (29), 84 (28)
11	13-α-Hydroxylupanine	264	152 (100), 165 (41), 134 (36), 112 (32), 246 (25), 264 (22), 148 (19)

M^+: molecular ion.

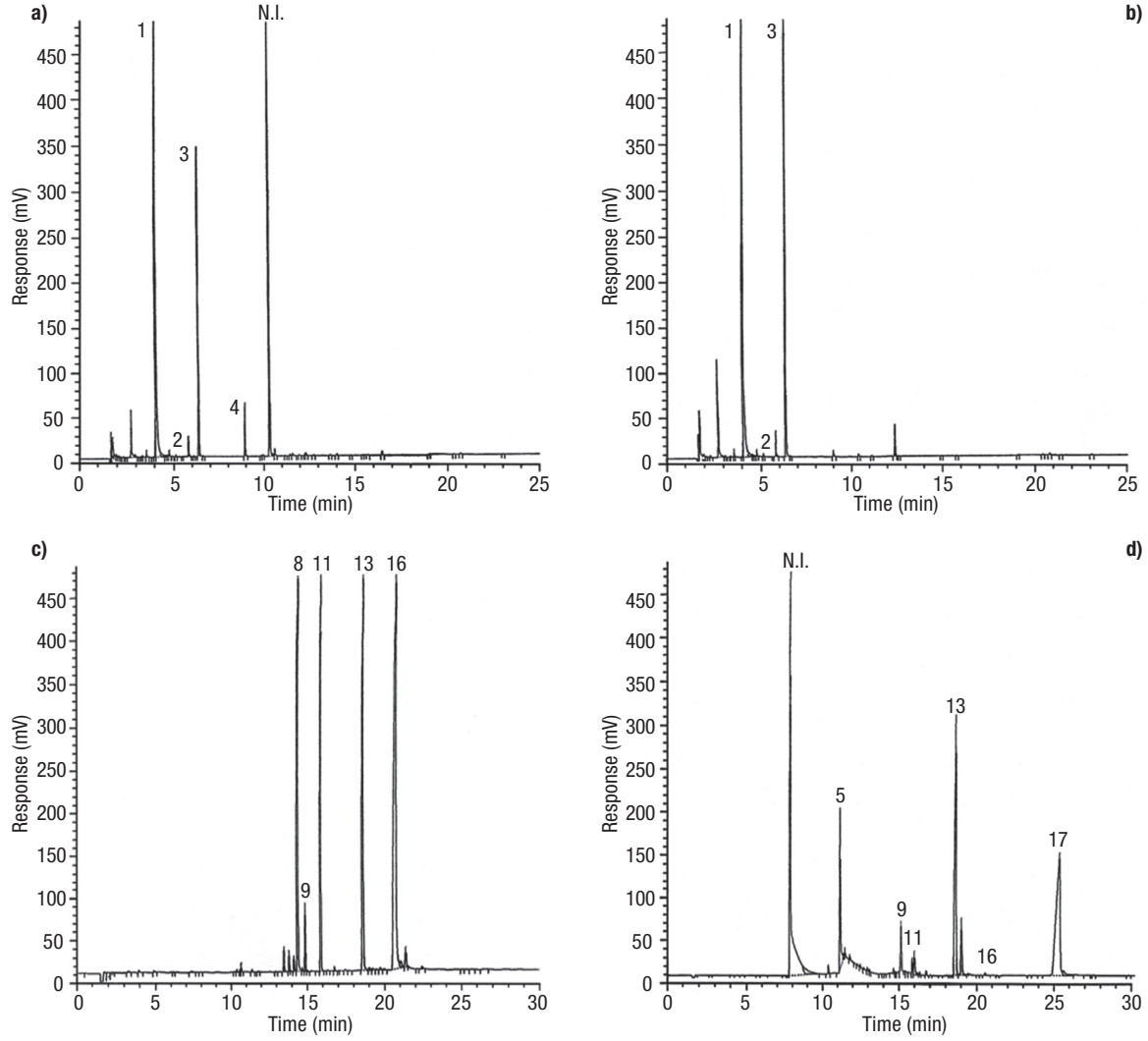

Figure 4. Separation of an alkaloid extract from *L. luteus* (a), *L. hispanicus* (b), *L. villosus* (c) and *L. micranthus* (d) bitter seeds by capillary GC. Injector, 240°C; detector 300°C; oven 150-235°C, 5°C min^{-1}; carrier gas, helium; detection of alkaloids by nitrogen-specific detector (NPD) and mass-selective detector. Alkaloids: 1) Lupinine; 2) Tryptophol; 3) Gramine; 4) Sparteine; 5) Albine; 8) Angustifoline; 9) α-Isolupanine; 11) Lupanine; 13) Multiflorine; 16) 13-OH-Lupanine; 17) 13-α-OH-Multflorine; N.I.) not identified.

Crossing experiments, involving *L. mariae-josephi* and its congeners having the same chromosomes number (2n = 52), should provide additional insights to make a final conclusion on its species status.

From a nutritional point of view it is important to know both the amount of alkaloid present and the toxicity levels of individual alkaloids since they do not all have the same toxicity. The toxic effects of quinolizidine alkaloids are not cumulative and are rapidly excreted from the body by the kidney, provided that the total amount of alkaloids does not exceed 0.02% (Muzquiz and Burbano, 2005).

Oligosaccharides

The total oligosaccharides content found in *L. mariae-josephi* seeds was 79.1 g kg^{-1}. The main α-galactosides found in dry seeds were stachyose, raffinose and verbascose. Stachyose, as in other species of *Lupinus,* was the predominant sugar (39.2 g kg^{-1}) (Quemener, 1988; Trugo *et al.*, 1988; Muzquiz *et al*., 1992; de la Cuadra *et al*., 1994; Jimenez-Martinez *et al*., 2004). Raffinose content (33.2 g kg^{-1}) appeared in higher concentration than verbascose (8.4 g kg^{-1}). Stachyose and verbascose values found in *L. mariae-josephi* are

similar to those of other species of *Lupinus*, however raffinose content was much higher that in other European or American species as *L. albus, L. luteus, L. angustifolius, L. hispanicus, L. exaltatus, L. reflexus* and *L. mexicanus* (de la Cuadra *et al*., 1994; Muzquiz *et al*., 1999a; Ruiz-Lopez *et al*., 2000).

Lupin seeds also contain cyclitols and methyl-cyclitols as it has been reported by Kadlec (2000) and Piotrowicz-Cieslak (2004) being ciceritol the most studied. Ciceritol content found in *L. mariae-josephi* (10.3 g kg^{-1}) was higher than the galactosyl cyclitols that were studied by Piotrowicz-Cieslak *et al*. (2003) in different species of *Lupinus* from Mexico [the highest value appears in *L. rotundiflorus* (3.4 g kg^{-1})].

The carbohydrate composition in lupins may show differences between lines, species or varieties (Muzquiz *et al.*, 1999a; Piotrowicz-Cieslak *et al*., 1999). Similar results were found in beans (Muzquiz *et al*., 1999b), lentils (Frias *et al.,* 1996), peas (Kvasnicka *et al*., 1994) and soybeans (Lowell and Kuo, 1989). Seed soluble carbohydrates are required for the acquisition of desiccation tolerance during seed development and maturation (Obendorf, 1997). The reported differences in the sucrose and raffinose family oligosaccharides (RFOs) content in the seeds may determine and indicate their storing capacity. The sucrose-RFO_s ratio, specifically, reflects seeds storing capacity (Horbowicz and Obendorf, 1994). Seeds with the ratio < 1 can be stored longer than 10 years. The greatest storage capacity was found for the seeds of the Mexican species *L. montanus* with the ratio < 0.09.

Humidity and temperature during seed maturation are the factors which particularly modify the content of RFO_s. The lupin seeds cultivated at a temperature of 15°C have 30% greater level of RFO_s than those seeds cultivated at 28°C (Piotrowicz-Cieslak, 2004). The chemical composition of seeds, although it is determined genetically, may be strongly modified by a wide variety of environmental factors. An increased accumulation of RFO_s was observed in the seeds subject to natural or forced desiccation (Lahuta *et al.*, 1998).

The α-galactosides are often considered to be antinutritional factors, because they are not hydrolysed in the small intestine of monogastrics and pass to the lower gut where they are fermented with gas production, possibly causing flatulence, diarrhoea, nausea and cramps. Conversely, their ingestion as pure compounds in the diet increases the bifidobacteria population of the colon through the production of short-chain fatty acids. This contributes positively to human health in many ways (Tomomatsu, 1994; Crittenden and Playne, 1996; Grizard and Barthomeu, 1999).

Inositol phosphates

The total content of inositol phosphates present in the raw seeds of *L. mariae-josephi* was 3.6 g kg^{-1} and only IP_4 (0.2 g kg^{-1}) and IP_6 (3.4 g kg^{-1}) were found. The phytate content was similar to those reported by de la Cuadra *et al*. (1994) in *L. albus*. Total inositol phosphates in *L. luteus, L. albus* and *L. mutabilis* varied from 1 to 8 g kg^{-1} (Burbano *et al*., 1995) and in Mexican lupins: *L. exaltatus, L. reflexus* and *L. mexicanus* ranged between 11 and 17 g kg^{-1} (Ruiz-Lopez *et al.*, 2000) although in all these species IP_3, IP_4 and IP_5 were detected**.**

The major inositol phosphate in different *Lupinus* especies was always IP_6. In many cases, the phytic acid content can vary with variety, climate, type of soil, and year.

In most legume seeds the phosphorus of phytic acid is about 80% of total phosphorus. In *Vicia faba* phytic acid levels ranged from 7.1 to 11 g kg^{-1}, *Pisum sativum* between 7.5 and 9.4 g kg^{-1} and in *Lens culinaris* and *Cicer arietinum* levels were similar and approximately 7.0 g kg^{-1} (Martinez-Dominguez *et al*., 2002).

Phytic acid has been considered an antinutrient. Excessive phytic acid in the diet can have a negative effect on mineral balance because of the insoluble complexes formed with essential minerals (Cu^{2+}, Zn^{2+}, Fe^{2+} and Ca^{2+}) which cause poor mineral bioavailability (Cosgrove, 1980). The phytic acid capacity to complex with proteins decreases protein solubility and therefore, impact enzyme activity (Urbano *et al*., 2000).

Some inositol phosphates, including IP_6 from soybean, have been suggested to have beneficial health effects, such as amelioration of heart diseases by controlling hypercholesterolemia and atherosclerosis, prevention of kidney stone formation and a reduced risk of colon cancer (Greiner *et al*., 2002).

Conclusions

The seed of *L. mariae-josephi* is a good protein and unsaturated fatty acid source. The chemical composition is similar to other lupin species, however this

species, compared to the other *Lupinus* species of Old and New World which has the same number of chromosomes (2n = 52), shows a protein and an alkaloids profile characteristic of this species but sharing features with other lupins. This could support that this new species could be the result of hybridization or introgression between others lupins.

Moreover, the main goal of this new species is that *L. mariae-josephi* is able to grow in high pH soils, unlike other *Lupinus* species growing in acid soils, which makes this species very promising for soil recovery as well as a starting material to extend the use of *Lupinus* to areas of calcareous soils in Spain and in other countries.

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