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Effects of oligosaccharide removing procedure on the protein profiles of lupin seeds

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Abstract The effects on the protein pattern of raffinose family oligosaccharides ethanol extraction from intact lupin seeds (*Lupinus albus* and *Lupinus luteus*) has not been addressed so far. In this work, 1D and 2D electrophoretic techniques were used to detect changes of the protein profiles upon oligosaccharide removal procedure. Some differences between untreated and treated samples were clearly visible in 1D electrophoresis, where the decrease of some polypeptides was revealed. In addition, a dark zone at the top of each treated sample lane became visible, suggesting the formation of large-sized protein aggregates as a result of the extraction procedure. By using the greater resolution of 2D electrophoresis, the identification of some varying spots was made possible: In particular the bands corresponding to conglutin γ were significantly reduced in the processed samples. The leakage of this protein by ethanol treatment of the seeds was qualitatively and quantitatively confirmed with specific antibodies by Western and dot blotting techniques, respectively. Another polypeptide around 100 kDa undergoing decrease upon treatment was tentatively identified as lupin lipoxxygenase, according to literature data and direct lipoxxygenase activity measurements on treated and untreated seed extracts. The observed decrease of lipoxxygenase activity in the processed seeds was 37%. These results show that the extraction of α -galactosides, while maintaining the overall pattern of lupin storage proteins, led to the reduction of some critical proteins. In particular, conglutin γ and lipoxxygenase decrease could be desirable in view of their potential allergenicity

and effects on the flour organoleptic characteristics, respectively.

Keywords *Lupinus albus* · *Lupinus luteus* · Raffinose family oligosaccharides · Conglutin γ · Lipoxxygenase

Introduction

Lupin seeds are a good source of proteins, lipids and dietary fiber, and contain very little amounts of starch, trypsin inhibitor activity and other components with antinutritional effects [1–4]. Furthermore, sweet lupin varieties contain very low levels of toxic alkaloids. The main interest in lupin for food is related to its high protein content (30–40 g/100 g on a dry weight basis), which are a good source of lysine and other essential amino acids. As most legume seeds, lupins are relatively poor in sulphur-containing amino acids [5]. Lupin seeds have been used as food by people in the Mediterranean area and the Andean highlands [6] for centuries. Its better exploitation for food either as a whole seed or as protein concentrates/isolates is actively being investigated. In particular, lupin proteins are increasingly used as plant-derived ingredients in the formulation of food products such as dairy and meat analogues. Moreover, lupin proteins can beneficially be added to pasta, crisps, bread, biscuits and cakes as well as in the fine bakery and confectionery. In general, the addition of up to 10% lupin flour improves water binding, texture, shelf-life, aroma as well as the nutritive value. Therefore, lupin has attracted interest worldwide as a potential high-protein food ingredient suitable for human consumption [7–9].

One of the major constraints of the consumption of legume seeds is the presence of undigestible oligosaccharides, which may cause intestinal discomfort and flatulence [10, 11]. In particular, the raffinose family oligosaccharides (RFOs) represent undesirable components, since they are not hydrolysed by mucosal enzymes in the small intestine, but pass into the lower gut, where they are fermented by the intestinal flora with the liberation of gases [12]. RFOs are soluble low-molecular weight oligosaccharides represented

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by raffinose, and include stachyose, verbascose and other oligosaccharides formed by α -(1 $\rightarrow$ 6)-galactosides linked to the glucose moiety of sucrose [13]. These oligosaccharides are present in several legume seeds, but lupins are among the legumes with the greatest α -galactoside content (7–15 g/100 g on a dry weight basis) [14–17]. On the other hand, it has recently been claimed that pure RFOs can act as prebiotics [18, 19]. Prebiotics are defined as non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species in the colon [20]. RFOs have been shown to promote the growth of bifidobacteria population [21]. Consequently, there is great interest to identify and isolate oligosaccharides as prebiotic ingredients in functional foods, in order to positively affect the composition of the intestinal microflora [19, 22], which can contribute to human health in many ways.

As far as lupin RFOs are concerned, Martínez-Villaluenga et al. [23] described an improved procedure for the purification of RFOs from intact lupin seeds, aimed at producing pure RFOs preparations for their use as prebiotics in functional food and developing RFOs-free lupin seeds, as a source of vegetable proteins for animal and human nutrition. This procedure, which utilizes the whole lupin seed, consists of a 50% ethanol extraction of RFOs at 40 °C. Although this method has been extended to the removal of alkaloids from bitter lupin seeds [24], the seeds used in this study did belong to the “sweet” varieties with alkaloid content around 0.03 g/100 g on a dry weight basis. Thus, this work did not include any evaluation of these compounds. Besides RFOs extraction, one of the aims of this process is to produce healthy seeds as a source of food proteins, therefore, we found it important to detect changes in the protein fraction upon this treatment. Aim of the present work was to evaluate the effect of raffinose family oligosaccharides extraction on the protein pattern of two lupin seed species.

Experimental

Material and methods

Seed treatment

Raffinose family oligosaccharides were extracted from seeds of two different varieties of *Lupinus albus* (Multolupa and Marta) and *Lupinus luteus* (LO-4486 and LO-4492) according to Gulewicz et al. [25] and purified according to Martínez-Villaluenga et al. [23]. The general scheme for obtaining pure RFOs is shown in Fig. 1. Briefly, 100 g of lupin seeds (A) were imbibed in distilled water at 4 °C overnight. The imbibed seeds were then extracted with 200 ml of 50% ethanol (v/v) at 40 °C overnight. After extraction, the supernatant was decanted. The seeds were re-extracted with 200 ml of fresh 50% ethanol solution under the same conditions. The supernatants from two cycles of extraction were concentrated under vacuum (C) and α -

galactosides were precipitated with 100% ethanol. RFOs precipitate was purified by filtration through diatomaceous earth and charcoal and, finally, through a Dowex 50WX8 column (12 cm $\times$ 1.5 cm i.d.) (D). RFOs-containing aqueous solutions from two different steps, namely (C) and (D), were eventually freeze-dried for their use in further experiments. The yield in RFO of the process was approximately 93% of the total RFOs seed content.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) Raw seeds were manually dehusked, and the kernels were ground to a meal. The resulting flour was suspended in cold acetone to solubilize the lipid fraction and centrifuged at $10,000 \times g$ for 30 min. This treatment was repeated three times. The flours were suspended in sample buffer at a final concentration of 10 mg/ml. The sample buffer contained 0.25 mol/l Tris-HCl, pH 6.8, 7.5% glycerol, 2% (w/v) sodium dodecyl sulfate (SDS) and 5% (v/v) 2-mercaptoethanol. After heating at 100 °C for 5 min, the slurries were centrifuged at $10,000 \times g$ for 15 min and 10 μ l of each supernatant were loaded onto the gel. The SDS-PAGE was performed in NuPAGE Novex Bis-Tris 10% (w/v) gels using an XCell SureLock Mini-Cell (Invitrogen, Milan, Italy). NuPAGE 2-(N-morpholino)ethanesulfonic acid (MES)–sodium dodecyl sulphate (SDS) Running Buffer and SeeBlue Plus2 Prestained Standard (Invitrogen, Milan, Italy) were used. The gels were stained with SimplyBlue SafeStain (Invitrogen, Milan, Italy).

Protein extraction for 2D electrophoretic analyses The defatted *L. albus* flours were extracted with a solution consisting of urea 7 mol/l, thiourea 2 mol/l, 2% (w/v) 3-[3-cholamidopropyl]dimethylamino]-

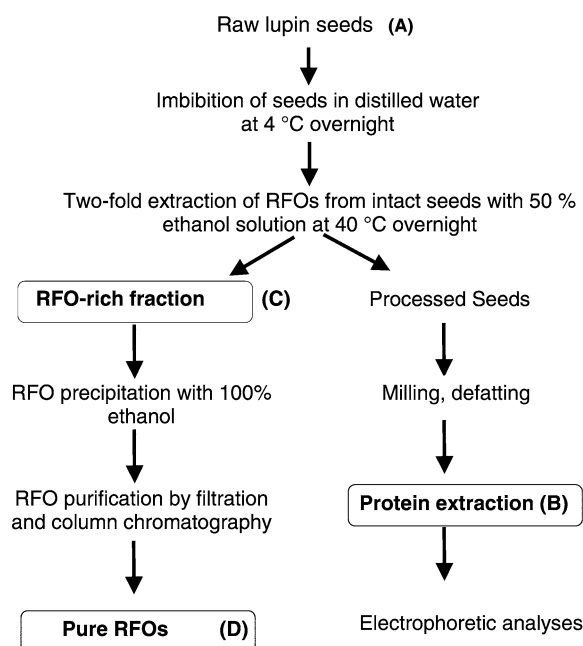


Fig. 1 General scheme of extraction of raffinose family oligosaccharides; see text for details

1-propanesulphonate (CHAPS) and 1,4-dithiothreitol (DTT) 0.065 mol/l in the ratio 1/30 (w/v) under stirring for 2 h. The slurry was centrifuged at $10,000 \times g$ for 30 min and the extracted proteins were immediately analysed or kept frozen at -80°C until use.

2D Electrophoresis Isoelectric focusing was performed on 7 cm pH 3–10 non-linear Immobilized pH gradient (IPG) strips (Amersham Biosciences, Milan, Italy). The strips were rehydrated overnight in a solution consisting of urea 7 mol/l, thiourea 2 mol/l, 2% (w/v) CHAPS, 1,4-dithiothreitol (DTT) 0.065 mol/l, 2% (w/v) IPG buffer pH 3–10 (Amersham Biosciences, Milan, Italy) containing 70 μg of the protein sample. Strips were focused at 6,500 Vh, with a maximum of 3,000 V, at 20°C using the Multiphor II electrophoresis unit (Amersham Biosciences, Milan, Italy). Prior to the second dimension, strips were incubated in equilibration buffer (Tris-HCl 0.375 mol/l, pH 8.8, urea 6 mol/l, 2% (w/v) SDS, 20% (v/v) glycerol) containing DTT 0.065 mol/l for 15 min. Then strips were incubated with the equilibration buffer containing iodoacetamide 0.243 mol/l for 10 min. The separation was performed in 12% (w/v) SDS-PAGE gels using a mini-PROTEAN III cell (Bio-Rad, Milan, Italy). The polyacrylamide 2D gels were Coomassie Blue stained. Electrophoretic analyses of raw and treated samples were carried out in triplicate.

Western blot analysis One-dimensional gels were transferred to PVDF membranes (Millipore, Milan, Italy) by blotting according to Towbin et al. [26] on a Semi-Dry Electroblotter (Sartorius, Firenze, Italy).

Antibodies to conglutin γ were obtained according to Magni et al. [27] and were used also for dot blotting analysis. Primary antiserum (anti-conglutin γ) and secondary antiserum were diluted 1:1,000 (v/v) and 1:2,000 (v/v) in PBS, respectively.

Dot blot analysis The raw and processed seeds of *L. albus* var. Multolupa were milled and extracted with 80% (v/v) ethanol under stirring for 45 min. The slurry was centrifuged at $10,000 \times g$ for 30 min and a second extraction was performed with a solution consisting of Tris-HCl 0.5 mol/l, pH 6.8, 0.4% (w/v) SDS, 1,4-dithiothreitol (DTT) 0.001 mol/l during 45 min. After heating at 100°C for 5 min and sonicating for 10 min, the slurries were centrifuged at $10,000 \times g$ for 30 min.

The dot blotting assays were performed using the nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) on which proteins were directly immobilized by using a dot blotting apparatus (Sartorius, Firenze, Italy). Membranes were blocked with 3% (w/v) gelatin overnight, washed three times with PBS buffer (0.01 mol/l NaPi, pH 7.4, containing 0.15 mol/l NaCl), and then soaked for 2 h in PBS buffer containing rabbit anti-conglutin γ in the ratio 1000/1 (v/v). The dots were revealed by using horseradish peroxidase conjugate with goat-antirabbit antiserum 2000/1 (v/v) (Bio-Rad, Milan, Italy) and hydrogen peroxide with 4-chloronaphthol as substrate.

After dots revelation and image analyses by ImageMaster 2D Platinum Software (Amersham Biosciences, Milan, Italy), curves were drawn for the quantitative analysis of the sample. By applying this procedure, we have evaluated the linearity of the method using known amounts of purified conglutin γ as reference protein. A good linearity in the range between 0.01 and 0.1 μg of conglutin γ was established (data not shown).

Lipoxygenase (LOX) activity measurement LOX extracts were prepared following the method of Ridolfi et al. [28] and Clemente et al. [29] with the modification described by Yoshie-Stark and Wäsche [30]. LOX activity was determined spectrophotometrically at room temperature by measuring the increase in absorbance at 234 nm due to the oxidation of linoleic acid over 5 min [31]. The specific activity was expressed as units of enzyme per milligram of protein, where a unit of enzyme is defined as the amount of enzyme capable of causing an absorption increase of 0.001/min. The protein concentration was determined by Biorad protein assay (Biorad Laboratories, Hercules, CA) based on the Bradford-dye binding procedure using bovine serum albumin as standard.

Results and discussion

Various investigations have been carried out to develop treatments aimed at removing or decreasing the content of α -galactosides and to enhance the nutritional quality of lupin seeds. Fermentation, germination, enzymatic treatment, dehulling, soaking and cooking were among the processes applied to the seed [32–34]. Our research group developed a simple and effective procedure to extract α -galactosides from lupin, which provided low α -galactosides lupin seeds at low costs [1]. Other advantages of this procedure are that it is not seed destructive and the processed lupin seeds obtained can be used in the design of novel protein products as α -galactoside extraction increases the protein content of the seeds [1]. In addition, this procedure allowed to obtain α -galactosides extracts which can be further purified [23] and used as prebiotic ingredients [19, 35]. The procedure for the extraction of raffinose family oligosaccharides and proteins for subsequent analyses is outlined in Fig. 1.

The overall effect of the RFOs removal treatment on the protein profile of the processed seeds has never been considered so far. Therefore, 1D and 2D electrophoretic patterns of the lupin species and varieties utilized in this study were performed and the results are shown in Figs. 2 and 3, respectively. In particular, the use of NuPAGE (Fig. 2) has allowed the analysis of a wide M_r range of polypeptides, while the 2D analysis, with its higher resolution, has allowed the identification of specific protein components from *L. albus* extracts as affected by the treatment. Figure 2 reports the denatured and reduced protein patterns of two white lupin varieties, namely Multolupa and Marta, and two yellow lupin cultivars, namely LO-4492 and LO-4486. The profiles show the typical complexity of lupin

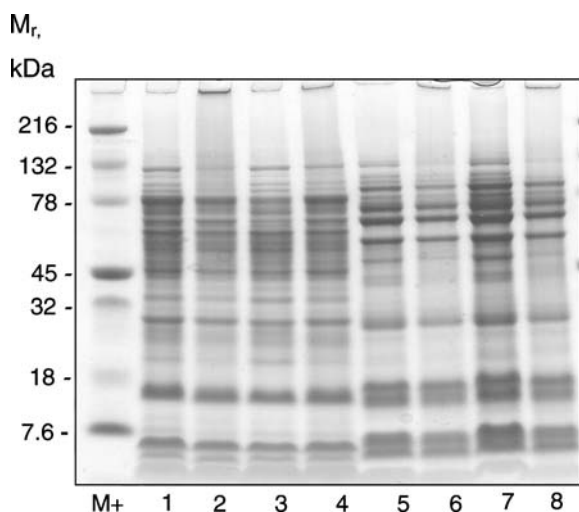
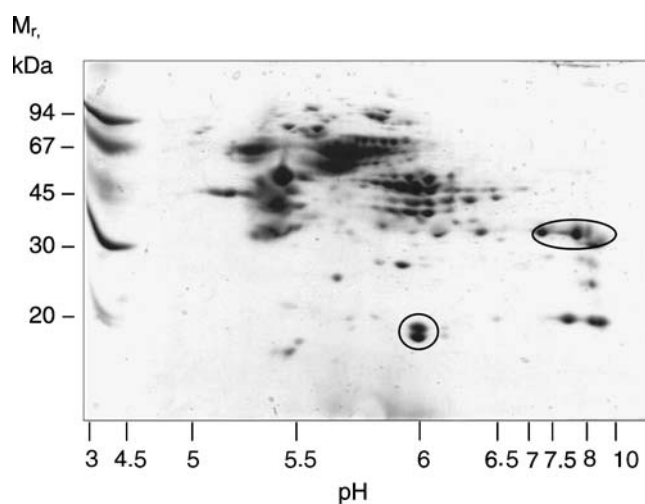


Fig. 2 SDS-PAGE electrophoresis under reducing conditions (SimplyBlue Safe Stain). Lanes 1, 3, 5, 7: Protein extracts of raw seeds from *L. albus* var. Multolupa, *L. albus* var. Marta, *L. luteus* LO-4492 and *L. luteus* LO-4486, respectively. Lanes 2, 4, 6, 8: Protein extract of processed seeds from *L. albus* var. Multolupa, *L. albus* var. Marta, *L. luteus* cv. 4492 and *L. luteus* cv. 4486, respectively. Lane M+: Molecular weight markers. Same amounts of lupin proteins were loaded in the gel

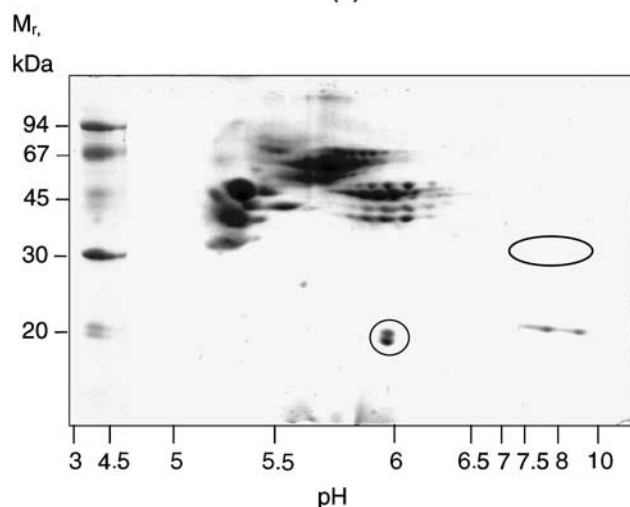
protein patterns, specially for *L. albus* proteins samples. This can be, at least in part, attributed to the extensive endogenous proteolysis of globulin fractions, such as the vicilin-like globulins, observed in these seeds [36]. Some notable differences between untreated and treated samples were clearly visible. In particular, a decrease of polypeptides with M_r 100, 90, 32.5 and 20 kDa was qualitatively detectable in treated *L. albus* seeds. In treated *L. luteus* seeds again 100 and 90 kDa polypeptide decreased, as well as two polypeptides of 50 and 45 kDa. Noteworthy, a subtle, but evident dark zone at the top of all treated sample lanes became visible, suggesting the formation of protein aggregates, despite the reducing conditions used for the electrophoresis. The formation of these large protein aggregates may also explain the less intense global staining in all treated samples with respect to the untreated ones, also observed in the 2D analysis of Fig. 3 (panel b versus panel a). A similar finding was described by Porres et al. [37] in lupin seeds from *L. albus* var. Multolupa, which were submitted to ethanol extraction for removing α -galactosides and, also in this case, it was attributed to the appearance of high M_r protein aggregates independent from the disulphide bond formation/interchange.

Due to the lower resolution power of 1D electrophoresis techniques as compared to 2D techniques, we also adopted this latter approach to attempt the identification of some decreasing polypeptides after the RFO removal treatment. Since a detailed analysis of *L. albus* 2D maps, but not of *L. luteus*, was available [27, 38], the use of this technique was restricted to the treated and untreated white lupin seed extracts.

By using 2D electrophoretic techniques (Fig. 3), more differences between treated and untreated samples became visible. High molecular weight polypeptides of 90 and



(a)



(b)

Fig. 3 Two-dimensional IEF/SDS-PAGE under reducing conditions (Coomassie Blue Stain). Raw (a) and processed seeds (b) from *L. albus* var. Multolupa. Same amounts of proteins were loaded on the first dimension (IEF). Ellipses enclose the large and small subunits of conglutin γ

67 kDa, with pI 5.0–6.0, and 45–30 kDa with pI between 6.0 and 6.5 are not visible after the process of RFOs extraction. Similarly, this occurred to polypeptides with basic and acidic pIs and molecular weights between 30 and 20 kDa. The overall reduced staining of the treated samples with respect to the untreated one could be attributed to the lack of separation of the large protein aggregates, according to the findings obtained with 1D electrophoresis (Fig. 2), and to the decrease of specific polypeptides, as it was the case of conglutin γ . Therefore, in order to confirm this finding, we used specific polyclonal antibodies to conglutin γ in Western blot analysis to detect the presence of conglutin γ in raw, processed seeds and RFOs preparation from *L. albus* var. Multolupa. In addition, a quantitative estimation of the residual conglutin γ in treated and untreated *L. albus* seeds was carried out. The results shown in Fig. 4, despite the apparently similar behaviour of conglutin γ in

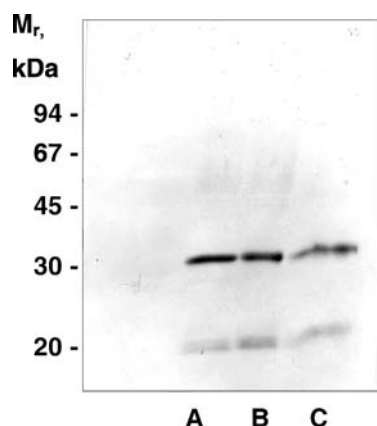


Fig. 4 Western blotting using anti-conglutin γ as primary antibody. Lanes: A, protein extract of raw seeds from *L. albus* var. Multolupa; B, protein extract of processed seeds from *L. albus* var. Multolupa; C, RFOs extract from *L. albus* var. Multolupa seeds

untreated and treated seeds, evidence the presence of conglutin γ in the RFO preparation; therefore, this indicates that 50% ethanol extraction at 40 °C was capable of extracting detectable amounts of conglutin γ from the seeds. The finding could be related to previous data showing that conglutin γ is secreted also by hot water treatment of the whole seeds [39]. The quantitative analysis of conglutin γ leakage during ethanol treatment of the seed was carried out by dot blotting technique by using the same antibodies as above. Assays were carried out with raw and treated seed extracts (not shown). Quantitative results showed that the average amount of conglutin γ in treated seeds was 79% of the untreated ones.

Another band that significantly decreased in the electrophoretic profiles of Fig. 2 had an M_r around 100 kDa. Various attempts aimed at identifying this band were carried out. The first one was to determine its N-terminal amino acid sequence after blotting of the band to PVDF membranes (not shown). Unfortunately, the N-terminus was blocked and no further sequencing attempts, involving proteolytic fragmentation of the polypeptide, were done. However, since other authors have described a lupin lipoxigenase of 90–100 kDa [30], we decided to measure the lipoxigenase activity of treated and untreated seed extracts in order to monitor a change of this enzyme concentration/activity during the treatment. With this approach we were able to measure a specific activity of 782 and 628 U/mg protein in the untreated and treated samples, respectively, corresponding to a decrease in lipoxigenase activity of 37% in the processed seeds. Whether this decrease was due to the extraction of this enzyme by hot ethanol or to enzyme inactivation due to the treatment itself is not clear as yet. However, the significant decrease of this band in the lanes of Fig. 2 speaks in favour of a physical removal of this protein from the treated seeds.

The results of this work, mainly consisting in the identification of two decreasing proteins upon the RFOs removal treatment, can be of interest for two main reasons: One is that the process can be optimised in relation to the desired levels of protein in the final product; the second one

is that both proteins shown to decrease upon the treatment displayed relevant biological activities. In particular, conglutin γ is suspected of being a new putative allergen [38], and lipoxigenase, by catalysing the fatty acid hydroperoxidation, is involved in the development of off-flavours in foods during storage [30]. Therefore, both the proteins are related with the quality of lupin flours and derived protein ingredients for human food uses.

Conclusions

In this work, the changes of the protein profiles in lupin seeds submitted to raffinose family oligosaccharides removal were monitored by 1D and 2D electrophoresis, Western and dot blotting analyses and enzymatic activity measurements. The overall pattern of lupin storage proteins did not change significantly, but various individual bands decreased significantly. In addition, aggregated protein material became visible after hot ethanol treatment. The analytical methods used in this work allowed the identification of two main proteins decreasing upon the RFOs extraction procedure. These two proteins, namely conglutin γ and lipoxigenase, are both strongly related to the quality of lupin flours and their presence in food formulas should be monitored and adapted according to specific food uses.

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References

- Martínez-Villaluenga C, Frías J, Vidal-Valverde C (2005) Food Chem (Accepted for publication)
- Faluyi MA, Zhou XM, Zhang F, Leibovitch S, Migner P, Smith DL (2000) Eur J Agron 13:7–37
- Huyghe C (1997) Fields Crops Res 53:147–160
- Petterson DS, Mackintosh J (1994) The chemical composition of lupin seed grown in Australia. In: Dracup M, Palta J (eds) Proceedings of the First Australian Lupin Technical Symp Perth, Western Australia, 17–21 October 1994. Department of Agriculture, WA, pp 39–48
- Lampart-Szczapa E (1996) Nahrung/Food 40:71–74
- Derbas G, Doxastakis G, Hadjisavva-Zinoviada S, Triantafyllakos N (1999) Food Chem 66:67–73
- Fudiyansyah N, Petterson DS, Bell RR, Fairbrother AHA (1995) Int J Food Sci Technol 30:297–305
- Johnson SK, Gray DM (1993) Int Food Inged 5:18–23
- Petterson DS, Crosbie GB (1990) Food Aust 42:266–268
- Cristofaro E, Mottli F, Whurmann JJ (1974) In: Seppel HI, McNut KW (eds) Sugars in nutrition. Academic Press, New York, pp 313–363
- Price KR, Lewis J, Wyatt GM, Fenwick RG (1988) Nahrung/Food 32:609–626
- Saini HS, Gladstones JS (1986) Aust J Agric Res 37:157–166
- Dey PM (1985) In: Dey PM (ed) Biochemistry of storage carbohydrates in green plants. Academic Press, London, pp 5–129
- Frías J, Vidal-Valverde C, Kozłowska H, Gorecki R, Honke J, Hedley CL (1996) Z Lebens Unters Forsch 203:27–32
- Muzquiz M, Burbano C, Pedrosa MM, Folkman WY, Gulewicz K (1999) Ind Crops Prod 9:183–188

16. Ruiz-López MA, García-López PM, Castañeda-Vazquez H, Zamora NJF, Garzón-De la Mora P, Bañuelos Pineda J, Burbano C, Pedrosa MM, Cuadrado C, Muzquiz M (2000) *J Food Comp Anal* 13:193–199
17. Martínez-Villaluenga C, Frías J, Vidal-Valverde C (2005) *Food Chem* 91:645–649
18. Gulewicz P, Szymaniec S, Bubak B, Frias J, Vidal-Valverde C, Trojanowska J, Gulewicz K (2002) *J Agric Food Chem* 50:384–389
19. Villaluenga CM, Wardeńska M, Pilarski R, Bednarczyk M, Gulewicz K (2004) *Folia Biologica-Krakow* 52:135–142
20. Gibson GR, Roberfroid MD (1995) *J Nutr* 125:1401–1412
21. Tomomatsu H (1994) *Food Technol* 48:61–65
22. Kliber A, Chichłowska J, Martínez-Villaluenga C, Gulewicz K (2005) *Int J Food Technol* (Submitted for publication)
23. Martínez-Villaluenga C, Frías J, Gulewicz K, Vidal-Valverde C (2004) *J Agric Food Chem* 52:6920–6922
24. Gulewicz K (1988) Habilitation dissertation. Polish Academy of Science, Poznan, Poland
25. Gulewicz P, Ciesiolka D, Frías J, Vidal-Valverde C, Frejnagel S, Trojanowska K, Gulewicz K (2000) *J Agric Food Chem* 48:3120–3123
26. Towbin M, Staehelin T, Gordon J (1979) *Proc Natl Acad Sci USA* 77:509–520
27. Magni C, Ballabio C, Restani P, Sironi E, Scarafoni A, Poiesi C, Duranti M (2005) *J Agric Food Chem* 53:2275–2281
28. Ridolfi M, Terenziani S, Patumi M, Fontanazza G (2002) *J Agric Food Chem* 50:835–839
29. Clemente A, Olias R, Olias JM (2000) *J Agric Food Chem* 48:1070–1075
30. Yoshie-Stark Y, Wäsche A (2004) *Food Chem* 88:287–292
31. Axelrod B, Cheesborough TM, Laakso S (1981) *Meth Enzymol* 71:441–451
32. Doblado R, Frias J, Muñoz R, Vidal-Valverde C (2003) *J Food Prot* 66:2313–2330
33. Vidal-Valverde C, Frías J, Valverde S (1993) *J Am Diet Assoc* 93:547–550
34. Khokhar S, Frias J, Price KR (1996) *J Sci Food Agric* 70:487–492
35. Martínez-Villaluenga C, Frias J, Vidal-Valverde C, Gómez R (2005) *J Food Prot* 68:1246–1252
36. Duranti M, Sessa F, Carpen A (1992) *J Exp Bot* 43:1373–1378
37. Porres JM, Aranda P, López-Jurado M, Urbano G (2005) *J Agric Food Chem* 53:3088–3094
38. Magni C, Herndl A, Sironi E, Scarafoni A, Ballabio C, Restani P, Bernardini R, Novembre E, Vierucci A, Duranti M (2005) *J Agric Food Chem* 53:4567–4571
39. Duranti M, Scarafoni A, Gius C, Negri A, Faoro N (1994) *Eur J Biochem* 222:387–393