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Evaluation of the antioxidant activity of lupin seed flour and derivatives (*Lupinus albus* ssp. Graecus)

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Abstract

In order to investigate natural sources of nutritive and non-nutritive antioxidants, the methanol extracts of lupin (*Lupinus albus* spp. Graecus) flour (with and without alkaloids) and lupin protein isolate were first examined for their antioxidant activity. The antioxidant activity of these extracts was determined by a rapid spectrophotometric method based on the coupled oxidation of β-carotene and linoleic acid and also by carrying out stability tests at 63°C with cottonseed oil. The results showed that the methanol extracts of lupin exhibit a marked antioxidant activity, higher than that of soya flour extracts. In a second step, an attempt was made to detect some of the components that may contribute to the overall antioxidant activity such as phenols, flavonoids, amino acids and peptides by using thin-layer chromatography. Finally, the pronounced antioxidant activity was correlated to the presence of total phenolics and phospholipids at high concentrations in the lupin extracts.

Keywords: *Lupinus albus*; Antioxidant activity of lupin

1. Introduction

Antioxidants retard the development of unpleasant flavour brought about by oxidation of unsaturated fatty acids, usually present as triacylglycones and/or polar lipids. Nowadays there is a general trend towards replacing the use of synthetic antioxidants in food processing by natural oxidation inhibitors or by the preferential use of ingredients that naturally possess antioxidant activity. It should be kept in mind, however, that all naturally occurring antioxidant substances are not necessarily less toxic than synthetic compounds (Pratt, 1992).

In the food industry, synthetic antioxidants are often used because they are effective and less expensive than natural antioxidants. They can increase the shelf life of foods by 15–200%, allowing food to be transported and stored for long periods.

However, naturally occurring nutritive and non-nutritive antioxidants have recently become a major area of scientific research (Lagouri & Boskou, 1996). Researchers concentrate on vitamins C, E and carotenoids (Frankel, 1996; Kamal-Eldin & Appelqvist, 1996; Mallet et al., 1994; Miller & Rice-Evans, 1997; Miller et al., 1996; Palozza & Krinsky, 1992; Tsuchihashi et al., 1995) as well as plant extracts containing natural non-nutritive antioxidant flavonoids such as quercetin, kaempferol and myricetin (Finger et al., 1992; Gordon & An, 1995; Huang & Frankel, 1997; Vekiari et al., 1993) or phenolic diterpenes and phenolic acids such as carnosol, carnosic acid and rosmarinic acid (Chen et al., 1992; Cuvellier et al., 1992, 1996; Frankel et al., 1996; Richheimer et al., 1996; Wu et al., 1982).

Among the best investigated sources of natural antioxidants are the legume seeds. The soybean and derivatives (flour, concentrate and isolate) have attracted much attention for their antioxidant activity (flavonoids, tocopherols, phospholipids, amino acids and peptides) (Hayes et al., 1977; Pratt & Birac, 1979; Pratt et al., 1981).

Lupin is a legume with a very high protein content and important dietary minerals. It can be grown in more temperate climates and is tolerant to poor soils. Although many substances, such as modified isoflavonoids, flavan-3-ols (Stobiecki & Popenda, 1994), phenols and proanthocyanidins (Ricardo-da-Silva et al., 1993) and carotenoids (El-Difrawi & Hudson, 1979; Feldeim,

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1990) have been isolated from *Lupinus* varieties, no studies are published so far on their antioxidant activity.

The present study was initiated to investigate methanol extracts of *Lupinus albus* spp. Graecus (flour and derivatives) for their antioxidant activity in model systems and compare the results with those of soya derivatives.

2. Materials and methods

2.1. Samples

Lupin seeds (*Lupinus albus* spp. Graecus) were provided by a Greek grower (Lasithi, Crete). Soya seeds (93446) were provided by the Institute of Cotton and Industrial Plants (Sindos, Thessaloniki).

2.2. Preparation of extracts

Methanol (pro analysi 99.8%, Riedel de-Haën)-soluble components were extracted from lupin seeds flour (LSF) and lupin seed protein isolate (LSPI). LSF and soya flour were obtained from ground dry seeds in a Tomas–Wiley mill (model ED-5, USA) to pass a 100 mesh screen. The LSPI were obtained by isoelectric precipitation, as described by Alamanou and Doxastakis (1995a).

Both pulverized samples were mixed with double the quantity of methanol in a blender (Multi Quick System 100 of Braun) for 5 min. Then they were placed in a water bath at 50°C for 1 h. Half the quantity was filtered and the residue was discarded and the filtrate concentrated to dryness using a rotary evaporator (Buchi 011, Switzerland). The other half quantity was boiled for 5 min and then had the same treatments and was concentrated as before. All the extracts were stored at −20°C until used.

The lupin seeds have alkaloids (1–2.5%) which are water soluble (Wing, 1993) and thus can easily be removed. A quantity of lupin seeds was boiled for 2 h, to inactivate the enzymes and denature the proteins, and then was mixed with tap water 30 times the weight of the seeds. The presence of alkaloids was determined by using TLC plates and the Dragengroff reagent (Clarke, 1978; Theodoridis, 1994). The water was changed every day until all the water-soluble alkaloids were removed. The alkaloids were removed after 10 days' washings. These lupin seeds were used to produce LSF without alkaloids.

2.3. Methods

Antioxidant activity of the extracts was determined by measuring the changes of absorbance during co-oxidation of β-carotene in the presence of linoleic acid (Hammerschmidt & Pratt, 1978; Lagouri & Boskou, 1994) and calculation of the Antioxidant Activity Coefficient (AAC) as described by Chevolleau et al. (1992). In each sample, solvent was added in order to have 20 mg of the dried extract in every ml. For this determination, the spectrophotometer (Spectronic 20 of Bausch & Lomb, Rochester, NY) was operated at 470 nm.

To determine antioxidant activity against autoxidation, for LSF and soya flour, 10 ml of the extract (10% w/v) were mixed with 10 g of refined cottonseed oil free from additives with antioxidant activity (peroxide value 1 meq kg^{-1}). For the LSPI the concentrated extract was diluted with methanol to 3% (w/v) and then 10 ml of this extract was mixed with 10 g of the above-mentioned cottonseed oil. The solvent was removed from the oil using a rotary evaporator (temperature 50°C) and the samples of the oil (1 g each) were transferred to a series of glass bottles (cross section 12 cm^2, volume 36 cm^3). The bottles were stored in an oven at 63°C in the dark. The peroxide value was determined until it reached 70 meq/kg. Then we calculated the Protection Factor (PF) as the ratio of time that was needed for peroxide value to reach 70 meq kg^{-1} to the time that was required for the blank to reach the same value (Nergiz, 1991; Papadopoulos & Boskou, 1991). The repeatability of the method was satisfactory (CV% = 9.1, $n = 7$).

Phenolics content in methanolic extracts of LSF and LSPI was determined by using the Folin Ciocalteau reagent (Gutfinger, 1981) and a U-2000 spectrometer (Hitachi, Japan). Also the oil content was determined (AOAC 17.014-16,1984) and the phosphorus content on fat extracted from LSF and LSPI (IUPAC, 1987).

Finally, TLC plates precoated with silica gel (DC-Karten Kieselgel 60, with pore diameter 0.2 mm, Riedel-de Haën, Seelze, Germany) were sprayed to indentify some components that may be responsible for the antioxidant activity. The sprays that were used for spot identification were: 1% $FeCl_3$-$K_3Fe(CN)_6$ in H_2O for phenols (blue); 1% $AlCl_3$ in ethanol for flavonoids (fluorescence in UV); 0.2 g ninhydrin in ethanol for peptides, amino acids (red/brown) (Duve & White, 1991; Stahl, 1969; Tian & White, 1994). The TLC plates were activated at 100°C for one hour, and were then developed in methanol:chloroform (50:50, v/v). The plates that were sprayed with ninhydrin were placed in an oven at 110°C until the colour (red/brown) appeared.

The detection of the alkaloids in all extracts was done by using thin-layer chromatography and the Dragengroff reagent as previously described.

3. Results and discussion

Antioxidant activity was determined by measuring the coupled oxidation of carotene and linoleic acid

as previously described. The rate of bleaching of β-carotene solution was determined by the difference in spectral absorbance reading, at 470 nm, between the initial reading (0 time) and the last reading for which the bleaching remained essentially linear (usually 180 min). The absorbance was evaluated every 30 min and the results are presented in Fig. 1. According to the changes of absorbance, the AAC was determined (Fig. 2). AAC values clearly showed that LSF and LSPI extracts have higher antioxidant activity than soya flour. Soya flour only had higher antioxidant activity than that of LSF without alkaloids. The same conclusions were also obvious from PF values (Fig. 3). In all cases, hot methanol extracts had higher antioxidant activity than cold methanol extracts.

An attempt was made to relate the observed antioxidant activity to the content of phenolics and phospholipids of the extracts and also to detect some of the components, such as phenolics and peptides/amino acids, that may be contribute to the antioxidant activity.

At first, TLC plates, when sprayed with $FeCl_3$-$K_3Fe(CN)_6$ in H_2O, showed the presence of phenols and then the content of polyphenols was determined (Table 1). There was an important reduction in the high polyphenol content of LSF after the removal of its alkaloids. This phenol reduction may cause the significantly low AAC and PF, as previously described (Figs. 2 and 3). This was an expected result because it is known that polyphenols are water soluble and so they were removed during the washings.

In Table 2, the phosphorus content on fat extracted LSF and LSPI are presented. The determination of phosphorus was used as an indication of phospholipids. Phospholipids are often synergistic in combination with phenolic antioxidants (Koya & Terao, 1995; Pokorny, 1991) and tocopherols (Hudson & Ghavanmi, 1984; Kamal-Eldin & Appelqvist, 1996).

Finally, TLC plates sprayed with appropriate reagents showed the presence of phenols, as well as the presence of

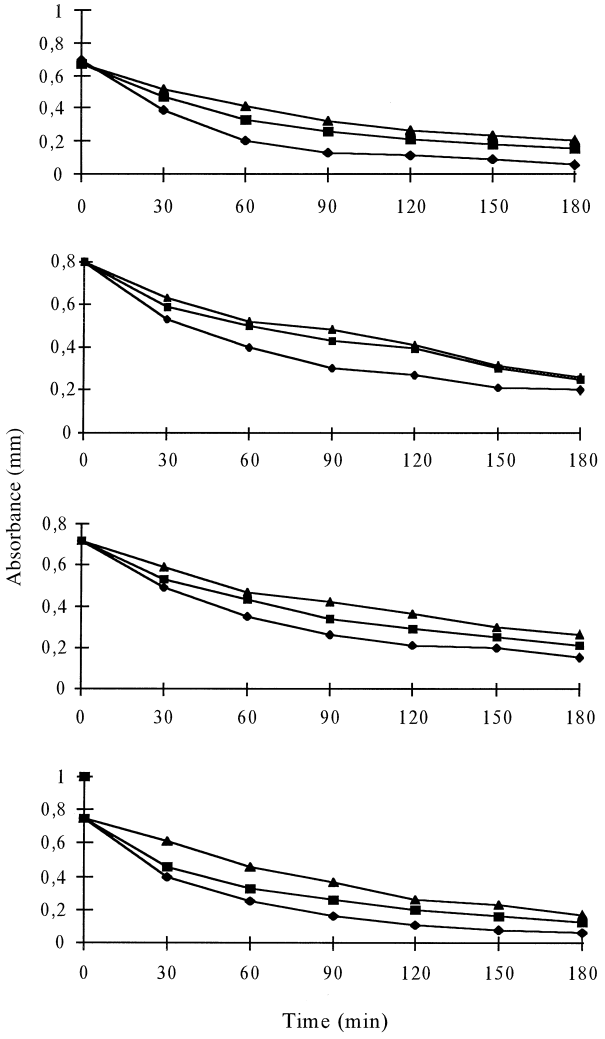

Fig. 1. Changes of absorbance during co-oxidation of β-carotene in the presence of linoleic acid, of LSF, LSF without alkaloids, LSPI and soya flour, respectively. ◆, Blank; ■, cold methanol extract; ▲, hot methanol extract.

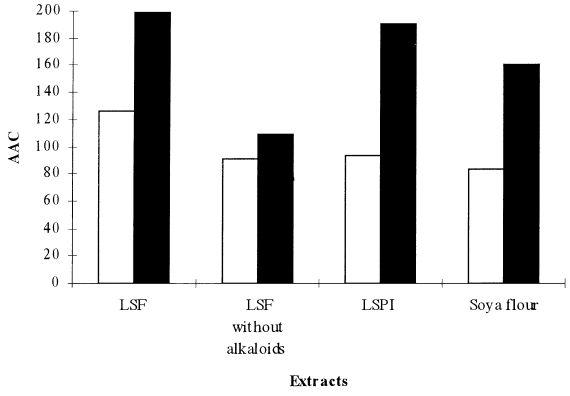

Fig. 2. Antioxidant activity coefficient (AAC) of LSF, LSF without alkaloids, LSPI and soya flour. □, Cold methanol extracts; ■, hot methanol extracts.

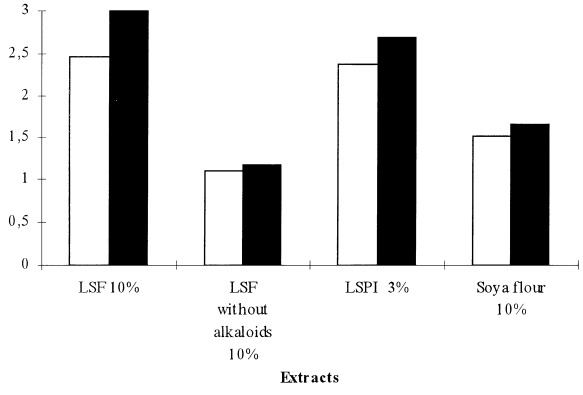

Fig. 3. Protection factors of LSF, LSF without alkaloids, LSPI and soya flour. □, Cold methanol extracts; ■; hot methanol extracts.

Table 1
Polyphenol content (%) in extracts of LSF (lupin seed flour) and LSPI (lupin seed protein isolate)

Lupin derivatives	Polyphenol content (% of extract)
LSF extracts with:	
cold methanol	13.6
hot methanol	20.7
LSPI extracts with:	
cold methanol	11.8
hot methanol	28.7
LSF without alkaloids:	
cold methanol	7.4
hot methanol	9.7

Table 2
Oil (g 100 g^{-1}) and phosphorus (mg 100 g^{-1}) content in LSF (lupin seed flour) and LSPI (lupin seed protein isolate)

Content	LSF	LSPI
Phosphorus (mg)	33.5	2.26
Oil (g)	10.5	0.48

flavonoids, amino acids and peptides. Considerable research work has been concluded regarding the antioxidant activity of flavonoids, especially in combination with synergists such as citric acid, ascorbic acid or phosphoric acid (Frankel, 1996; Miller & Rice-Evans, 1997; Schuler, 1990). The antioxidant activity of the amino acids had investigated in model systems (Farag et al., 1978a, b) and in real systems, e.g. sunflower oil and cottonseed oil (Ahmad et al., 1983), safflower oil (Riisom et al., 1980), and milk fat (Chen & Nawar, 1991). Our results show that the amino acids and peptides, that were present in relatively high concentrations in lupin seeds (Gross, 1989), may contribute to the antioxidant activity.

In lupins, a major hindrance to their widespread utilisation in human and animal nutrition is the presence of quinolizidine alkaloids which are toxic and bitter. Laboratory experiments clearly indicate that aqueous alcohols, such as methanol, ethanol and isopropanol, can remove the alkaloids from defatted lupin extracted by hexane to produce debittered protein concentrate (Chajuss, 1989). Spraying of the TLC plates with the Dragengroff reagent showed that the continual washing of LSF with water (10 days) resulted in reduction of alkaloids and also that LSPI had a lower content of alkaloids than LSF. Lucisano et al. (1984) proved that the removal of the oil with hexane, after four stages, removed 60% of the alkaloids, and especially in the first stage there was a 40% reduction in the content of alkaloids.

Finally, because the methods of production of soya protein fractions can be used also for lupins, the lupin products can be used or incorporated in foods systems in order to provide both functional properties (Alamanou & Doxastakis, 1995a, b, 1997; Alamanou et al., 1996) and antioxidant activity.

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