Phytosterols, Triterpene Alcohols, and Phospholipids in Seed Oil from White Lupin

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ABSTRACT: This study was conducted to determine effects of genotypes and growing environment on phytosterols, triterpene alcohols, and phospholipids (PL) in lupin (*Lupinus albus* L.) oil from seven genotypes grown in Maine and Virginia. The unsaponifiable lipid (UNSAP) and phospholipid (PL) fractions ranged from 2.1 to 2.8% and from 2.6 to 2.8% of oil, respectively. UNSAP in lupin oil contained 19.9 to 28.7% sterols and 17.3 to 22.0% triterpene alcohols. Growing location significantly affected contents of total PL, PS, phosphatidylglycerol, β-sitosterol, campesterol, and β-amyrin. Genotypic effects were significant for stigmasterol. PC (32.6 to 46.3% of PL), PE (21.6 to 32% of PL), and PS (11.2 to 17.9% of PL) were the major PL in lupin oil. The concentration of PL classes in lupin oil were in the following descending order: PC > PE > PS > PI > phosphatidic acid > lysophosphatidylcholine > phosphatidylglycerol > diphosphatidylglycerol. In descending order of abundance, the sterols present in lupin oil were: β-sitosterol > campesterol > stigmasterol > Δ^5-avenasterol > Δ^7-stigmastenol. Lupeol was the most prominent triterpene alcohol in lupin seed oil. In general, growing environment had a much greater influence on lupin oil characteristics than the genotypes.

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KEY WORDS: β-amyrin, Δ^5-Avenasterol, campestrol, lupeol, phosphatidylcholine, phosphatidylethanol-amine, phosphatidylinositol, phosphatidylserine, β-sitosterol, stigmasterol.

White lupin (*Lupinus albus* L.) is a legume crop gaining growing interest worldwide for its high potential as human food and animal feed (1–3). The nutritional characteristics of lupin seeds have been extensively reviewed (1–4). White lupin seeds contain 30 to 40% protein and 4 to 23% oil and lack protease inhibitors. Consumption of lupin diets lowers plasma glucose, cholesterol, and TG (5). In general, lupin oil is characterized by a balanced FA composition with total saturated FA ≤10% and total unsaturated FA ≤90%, of which 32 to 50% is oleic (18:1), 17 to 47% is linoleic (18:2), and 3 to 11% is linolenic (18:3) acid (1,3,4).

Plant sterols and phospholipids (PL) are minor bioactive lipid constituents that have positive effects on human health and are important by-products of the oil processing industry. PL have a wide array of food and nonfood applications, mainly as nontoxic biodegradable emulsifiers, industrial lubricants, and nutrition supplements. In addition, PC provides a natural precursor for the neurotransmitter acetylcholine. They have also shown antioxidant properties, particularly those with free amino groups (PE,PS) and consequently increase oil stability and shelf life (6). On the other hand, PL cause oil discoloration during the deodorization process. Phytosterols constitute a major portion of the unsaponifiable matter (UNSAP) in most vegetable oils (7). They possess a broad spectrum of therapeutic effects in animals and humans (8–10). In humans, consumption of plant-derived sterols, particularly β-sitosterol, reduces blood pressure (9), serum cholesterol levels, and the risk of chronic heart disease (8,9). Phytosterols also serve as intermediates for synthesis of hormonal sterols and other related pharmaceuticals (10). Furthermore, the triterpene alcohol lupeol (fagasterol) and phytosterols, especially β-sitosterol, exhibit significant anti-inflammatory effects and antitumor properties (8,9). In addition, phytosterols are known as antipolymerization factors and as antioxidants, especially those containing an ethylidene group in the aliphatic side chain (Δ^5- and Δ^7-avenasterols), in vegetable oil at frying temperature (11).

However, little is known about the UNSAP and PL composition of lupin oil. UNSAP and PL constitute up to 5 and 15% of lupin total lipids, respectively, and sterols and triterpene alcohols represent 25 and 22% of total UNSAP, respectively (1,4). The literature indicates that both genotype and growing location affect content and FA composition of oil from white lupin (3,4). We are interested in developing white lupin (*L. albus* L.) as an alternative grain crop in the mid-Atlantic region of the United States. However, information about the nutritional characteristics of lupin grains produced in this region is unavailable. This study was conducted to characterize phytosterols, triterpene alcohols, and PL in lupin oil and to determine effects of genotypes and growing environment on these constituents.

MATERIALS AND METHODS

All reagents were HPLC grade and were obtained from Sigma-Aldrich Corporation (St. Louis, MO) unless otherwise stated.

Plant material. Mature seeds of seven white lupin genotypes (L127N, L133N, L139N, L251N, L389N, PI-481545, and PI-483074) produced in Maine during 1990 and in Vir-

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ginia during 1994 were selected for this study. We compared the PL, unsaponifiable lipid, phytosterol, and triterpene alcohol characteristics of seed oil from these seven genotypes produced in Maine to that produced in Virginia. The growing environment in this study represented combined effects of both soil and climatic conditions.

Oil extraction. The oil was extracted from ground lupin seeds (5 g) at room temperature using hexane/isopropanol (3:2, vol/vol) and determined gravimetrically as described by Hamama *et al.* (12).

Analysis of PL. Total PL in lupin oil were determined spectrophotometrically (13) as described by Hamama *et al.* (12).

Separation and identification of PL. PL were precipitated from lupin seed oil (0.2 g in 2 mL $CHCl_3$) with cold acetone. The precipitate was separated by centrifugation, washed with cold acetone, and recentrifuged at $4000 \times g$ for 5 min. The precipitation of PL was repeated three times for each sample to ensure full PL recovery, and the three precipitates were dissolved in chloroform and combined (100 μL). The PL in chloroform (20 μL) were spotted on 0.25-mm layer silica gel plates (20 × 20 cm PE SIL G/UV; Whatman Ltd., Kent, England) and separated into individual PL classes using 2-D TLC techniques as described by Rouser *et al.* (14). Chloroform/ methanol/concentrated ammonium hydroxide (65:25:5, by vol) and chloroform/ acetone/methanol/acetic acid/water (30:40:10:10:5, by vol) were used as first and second developing solvent, respectively. The individual PL spots were visualized with both iodine vapor and UV light and identified by relative R_f using reference PL. The relative R_f to PE ratio of the second direction for standard lysoPC, PC, PS, PI, PE, phosphatidic acid (PA), phosphatidylglycerol (PG), and diphosphatidylglycerol (DPG), 50 μg each, fractionated under the same condition on separate TLC plates, were 0.06, 0.41, 0.83, 0.36, 1.00, 1.42, 1.44, and 1.67, respectively. The spot corresponding to each PL was scraped off separately into a 10-mL centrifuge tube and extracted twice with 2 mL chloroform/methanol (2:1, vol/vol) followed by 2 mL methanol to ensure total PL recovery. After centrifugation at $4000 \times g$ for 10 min, the supernatants containing PL were combined. The solvents were removed under reduced pressure and the individual PL classes redissolved in chloroform and quantitatively determined as previously described (13). The concentration of each PL was calculated from a PC standard curve prepared under the same conditions.

UNSAP. Lupin UNSAP was determined gravimetrically after saponification with 20% (wt/vol) of methanolic KOH, kept overnight at room temperature (12).

TLC of UNSAP. The unsaponifiable lipids of lupin oil were separated into subfractions on 0.25-mm layer silica gel plates (20 × 20 cm PE SIL G/UV; Whatman Ltd.) using 1-D TLC techniques with chloroform/diethyl ether (9:1, vol/vol) as the developing solvent (15). The UNSAP (5 mg in 100 μL of $CHCl_3$) containing 1% (w/w) each of stigmastanol and lanosterol as the internal standard for sterols (desmethyl sterols), and triterpene alcohols (4,4-dimethyl sterols), respectively, was applied on the silica gel plates as 3-cm bands. Stigmastanol and lanosterol, used as reference standards, were spotted (50 μg each) on the left and right sides of the TLC plates. The bands of developed UNSAP subfractions were located on the TLC plates by comparison of the R_f values with those of standards visualized with both iodine vapor and UV light. The bands corresponding to sterols (R_f 0.28) and triterpene alcohols (R_f 0.45) were scraped off separately and extracted three times with 2 mL methylene chloride. The sterol and triterpene alcohol fractions were dried under N_2 and stored at –10°C for further analysis.

Sterols. Sterol fractions were silylated by 1 mL *N*,*O*-bis (silyltrimethyl) trifluoroacetamide in 1% trimethylchlorosilane in glass vials having Teflon-lined caps at 80°C for 60 min and then analyzed using the same GC conditions as described by Hamama *et al.* (12). The peaks were tentatively identified by comparison of relative retention times (RRT) with trimethylsilane (Me_3Si) derivatives of standard desmethyl sterols prepared under the same conditions, and with RRT reported in the literature (12,15,16). Stigmastanol was used as the reference sterol and for determination of a response factor. Phytosterol concentrations were expressed as percentage (w/w) of total UNSAP.

Triterpene alcohols. Triterpene alcohols were silylated and analyzed by GC as just described. The peaks were tentatively identified by comparisons of RRT with Me_3Si derivatives of standard 4,4-dimethyl sterols prepared under the same conditions and with those previously reported (15,16). Lanosterol was used as the reference triterpene alcohol and for determination of a response factor. Triterpene alcohol concentrations were expressed as percentage (w/w) of total UNSAP.

All data were means of two determinations and were analyzed using the ANOVA procedure in SAS, v. 8 (17). The means of cultivars and growing locations were compared using Fisher's Protected Least Significant Difference at a 5% level of significance.

RESULTS AND DISCUSSION

In this study, we compared the PL and unsaponifiable lipids from mature seeds of seven lupin genotypes grown in Maine during 1990 to those from the same seven lines grown in Virginia during 1994.

Growing environment effects. Since low temperatures induce FA desaturation during maturation of oilseeds, and the reverse effects of high-growing temperature are known to occur (2,3,18,19), both genetic background of the plant and growing temperature conditions may affect the concentration and composition of PL and unsaponifiable lipids (12,18–20). In this study, we compared the mean of all seven genotypes grown in Maine to the mean of the same seven genotypes grown in Virginia. We observed that the growing location (Table 1) significantly affected contents of total PL in lupin oil (from 2.5 to 3.0% with a mean of 2.7% PL), PG (from 2.0 to 3.4% with a mean of 2.7% of PL), and PS (from 11.1 to 15.7% with a mean of 13.4% of PL). The seeds produced in Virginia had significantly higher contents of PG, a 70.0% increase over seeds produced in Maine (Table 1). On the other hand, the seeds produced in Maine had significantly higher contents of PS, a 41.4% increase over seed produced in Vir-

TABLE 1
Environmental Effects on the Contents and Compositions of Phospholipids in White Lupin Oil

	R_f/R_f PE[a]	Location		Mean[b]	LSD .05
		Maine	Virginia		
Phospholipids (% of oil)		3.0	2.5	2.7*	0.37
Phospholipid classes (wt% of total phospholipids)					
LysoPC	0.06	1.8	3.9	2.8	NS
Unknown	0.08	3.4	3.9	3.7	NS
PC	0.41	39.3	41.5	40.4	NS
PS	0.83	15.7	11.1	13.4*	3.39
PI	0.36	3.4	6.2	4.8	NS
PE	1.00	29.8	24.6	27.2	NS
Phosphatidic acid (PA)	1.42	2.8	3.9	3.4	NS
Phosphatidylglycerol (PG)	1.44	2.0	3.4	2.7*	0.95
Diphosphatidylglycerol (DPG)	1.67	1.9	1.4	1.6	NS

[a]R_f/R_f PE, the retention factor (R_f) of the given phospholipid compared to the retention factor (R_f) of PE, in the second direction of TLC.
[b]Significant differences existing between the two locations are indicated by an asterisk (*). LSD, least significant difference; NS, not significant.

ginia. We concluded that cooler temperature conditions during lupin growth, especially during seed development, in Maine (from 8 to 19°C with a mean of 13°C) as compared to Virginia (from 13 to 27°C with a mean of 20°C) induced higher content of PL (+20.0%).

Since PL are known as the principal class of lipid found in most cellular membranes, they play an important role in cell metabolism and physiologic functions. PC, PE, PS and PE are the most important membrane building blocks in the synthesis of membrane lipid bilayer and liposome formation in the living cells. It has been reported that low growing temperatures increase FA desaturation level in plant lipids in general and in the membrane PL in particular. It is believed that such changes are made to ensure full function of the membrane and to prevent the membrane from damage under chilling temperature, whereas at higher growing temperatures the opposite trend is known to occur (18,19,21). The present results suggest that growing temperature may induce changes in PL composition and consequently the structure of the membrane bilayer, especially during seed development.

Effects of growing environment on the level of total unsaponifiable lipids, phytosterols, and triterpene alcohols in lupin oil were not significant (Table 2). Growing location clearly induced significant changes in the level of β-sitosterol (52.3 to 58.7%, with a mean of 56.0%) and campesterol (25.6 to 26.1%, with a mean of 25.8%) in lupin oil (Table 2). The lupin seeds grown in Maine contained 12.2% more β-sitosterol and 2% more campesterol than seeds produced in Virginia. The β-sitosterol/stigmasterol ratios in lupin produced in Maine and Virginia were 6.5 and 4.3, respectively (Table 2). The lupin seed grown in Maine contained 51.2% higher β-sitosterol/stigmasterol ratio than in Virginia. The seeds grown in Virginia had a significantly higher content of β-amyrin (7.6 times more) than those grown in Maine. The present data are in close agreement with previous observations on the biosynthesis of phytosterols in soybean seeds and in other plant tissues (20–24).

We speculate that lower temperatures during lupin growth stimulated β-sitosterol synthesis and inhibited the synthesis of stigmasterol. On the other hand, high growing temperatures reversed this trend (22). Apparently, owing to its planar structure as compared to stigmasterol, β-sitosterol plays an important role in minimizing membrane water permeability (23). Therefore, a higher ratio of β-sitosterol/stigmasterol should maintain membrane fluidity under low temperatures by lowering water loss under chilling conditions (24). In fact, phytosterols are important structural components of plant membranes, and their free forms serve as stabilizer for PL bilayers in plant cell membranes (8). Therefore, modification of the membrane structure in response to low growing temperature may affect the biosynthesis of plant sterol in oilseeds. We suggest that further studies be conducted to clarify the role of growing environment on phytosterol biosynthesis and dimethyl sterol accumulation in lupin seeds.

Genotypic effects. Variation among lupin genotypes in contents of total PL and PL classes in lupin oil was not significant (Table 3). Total PL content in lupin oil ranged from 2.6 to 2.8% with a mean of 2.7% of lupin oil (Table 3). These concentrations are within the range of those previously reported (1). In addition to the eight PL classes, tentatively identified, one unknown compound with R_f/R_f PE of 0.08 was detected in the PL fraction. PC, PE, PS, and PI were the abundant PL, with respective means ± SD of 40.4 ± 7.8, 27.2 ± 5.4, 13.4 ± 2.6, and 4.8 ± 3.1%. PL classes in lupin oil, in descending order of abundance, were: PC > PE > PS > PI > PA > lysoPC > PG > DPG.

Effects of genotypes on the level of lupin UNSAP were not significant (Table 4). Unsaponifiable lipids were 2.1 to 2.8% of lupin oil with a mean of 2.5%. Phytosterols (22.3 ± 5.4%) and triterpene alcohols (19.4 ± 5.1%) were the major subfractions of lupin UNSAP (Table 4). Of the eight phytosterols detected, five were tentatively identified, and β-sitosterol was the most abundant sterol. β-Sitosterol (56.0 ± 3.6%), campes-

TABLE 2
Environmental Effects on the Content of Unsaponifiable Lipid and Compositions of Phytosterol (wt% of total desmethyl sterols) and Triterpene Alcohol (wt% of total 4,4-dimethyl sterols) Molecular Species in White Lupin Oil

		Location			
Lipid fraction	RRT	Maine	Virginia	Mean[a]	LSD .05
UNSAP (% of oil)		2.6	2.4	2.5	NS
UNSAP subfraction (wt% of total UNSAP)					
Phytosterols		24.2	20.5	22.3	NS
Triterpene alcohols		19.9	19.9	19.4	NS
Phytosterol molecular species[b]					
Unknown-1	0.65	2.9	4.2	3.4	NS
Campesterol	0.82	26.1	25.6	25.8*	1.12
Stigmasterol	0.87	9.1	12.3	10.7	NS
Unknown-2	0.89	0.1	1.1	0.5	NS
Unknown-3	0.94	0.8	0.6	0.7	NS
β-Sitosterol	1.00	58.7	52.3	56.0*	2.33
Δ^5-Avenasterol	1.03	1.2	2.2	1.6	NS
Δ^7-Stigmastenol	1.10	1.2	1.6	1.3	NS
β-Sitosterol/stigmasterol		6.5	4.3	5.2	
Triterpene alcohol molecular species[c]					
β-Amyrin	0.91	1.7	12.9	6.8*	0.70
Lupeol	1.00	92.4	80.2	86.8	NS
Cycloartenol	1.02	0.5	1.1	0.8	NS
Unknown-4	1.08	1.7	2.3	2.0	NS
Methylenecycloartenol	1.23	1.8	1.5	1.6	NS
Unknown-5	1.58	2.0	2.1	2.1	NS

[a]Significant differences existing between the two locations are indicated by an asterisk (*); UNSAP, unsaponifiable matter; for other abbreviations see Table 1.
[b]Relative retention times (RRT) compared with β-sitosterol (RT = 25.5 cm/min).
[c]RRT compared with lupeol (RT = 27.66 cm/min).

terol (25.8 ± 3.1%), and stigmasterol (10.7 ± 1.9%) were the major sterols in lupin oil (Table 4). The concentrations of these phytosterols were in the range of most vegetable edible oils (7) and are in close agreement with those of Hudson *et al.* (4). In addition, two unidentified minor components with RRT to β-sitosterol of 0.89 (with a mean of 0.5%) and 0.94 (with a mean of 0.7%) were detected in desmethyl sterols fraction of L127N, L251N, L389N, and PI-481545; and L127N, L139N, L251N, L389N, and PI-481545, respectively.

In descending order of abundance, the sterols present in lupin oil were: β-sitosterol > campesterol > stigmasterol > Δ^5-avenasterol > Δ^7-stigmastenol. GC analysis revealed that the first emerged peak of the desmethylsterol (Unknown = 1) fraction has the same RRT to β-sitosterol (0.65) as that of authentic cholesterol. The presence of cholesterol (from 0.0 to 200 μg/g oil) in vegetable oils has been reported (4,7,8,25), and its presence in higher plants is now largely accepted (8). Variation among lupin genotypes for the contents of phytosterol molecular species (Table 4) was significant only for stigmasterol (7.1 to 14.0% with a mean of 10.7%). The accumulation of stigmasterol among lupin genotypes was in the following decreasing order: L251N > L389N > L139N > PI-481545 = PI-482074 > L133N > L127 N. Among the seven genotypes, the content of stigmasterol differed significantly only between L251N (14.0%) and L127N (7.1%).

The genotypic effects on the content and the level of triterpene alcohol classes were not significant (Table 4). Lupeol was the most predominant (73.8 to 92.0% with a mean of 86.8% of total dimethyl sterols) and β-amyrin (4.8 to 11.1% with a mean of 6.8% of total dimethyl sterols) was the second-most abundant triterpene alcohol (Table 4). These data are in close agreement with those reported by Hudson *et al.* (4). Cycloartenol (0.1–2.5%) and methylenecycloartenol (0.8–4.4%) were tentatively identified and present in minor quantities in lupin seed oil (Table 4). Based on retention times relative to lupeol, we detected two unknown components in total dimethyl sterols, one with RRT of 1.08 (0.3 to 5.3% with a mean of 2.0% of total dimethyl sterols), and a second with RRT of 1.58 (0.0 to 6.3% with a mean of 2.1% of total dimethyl sterols).

In conclusion, this study indicates that the growing environment had a much greater influence on lupin oil characteristics than the genotypes. However, significant differences among four lupin species for the content of sterols and triterpene alcohols have been previously observed (4). Based on the characteristics of phytosterols and PL, lupin oil may be dietetically recommended. Moreover, the antioxidant properties of phytosterols and PL may increase the oil's oxidative stability and shelf life.

These data, along with positive results from production re-

TABLE 3
Genotype Effects[a] on the Contents and Compositions of Phospholipids in White Lupin Oil

	Genotypes								
Lipid fraction	L127N	L133N	L139N	L251N	L389N	PI-481545	PI-48-2074	Mean	LSD .05
Phospholipids (% of oil)	2.8	2.7	2.6	2.7	2.6	2.8	2.8	2.7	NS
Phospholipid classes (wt% of total phospholipids)									
LysoPC	3.5	2.1	2.4	1.9	2.7	2.2	5.1	2.8	NS
Unknown	4.2	2.7	5.4	1.6	1.4	5.9	4.5	3.7	NS
PC	35.5	46.3	43.4	32.6	43.0	43.5	38.7	40.4	NS
PS	11.5	14.3	11.6	15.1	17.8	11.5	12.1	13.4	NS
PI	6.5	3.5	1.8	5.9	5.8	4.3	5.9	4.8	NS
PE	31.9	23.7	25.7	37.5	21.4	22.9	27.0	27.2	NS
PA	2.8	2.9	4.1	2.8	4.6	3.7	2.6	3.4	NS
PG	2.8	2.4	3.6	1.9	1.9	3.7	2.8	2.7	NS
DPG	1.4	2.2	1.9	0.9	1.5	2.3	1.4	1.6	NS

[a]For abbreviations see Tables 1 and 2.

TABLE 4
Genotype Effects on the Content of Unsaponifiable[a] Lipid and Compositions of Phytosterol (wt% of total desmethyl sterols) and Triterpene Alcohol (wt% of total 4,4-dimethyl sterols) Molecular Species in White Lupin Oil

	Genotypes								
Lipid fraction	L127N	L133N	L139N	L251N	L389N	PI-481545	PI-48-2074	Mean	LSD .05
UNSAP (% of oil)	2.8	2.4	2.4	2.8	2.6	2.1	2.3	2.5	NS
UNSAP subfraction (wt% of total UNSAP)									
Phytosterols	24.9	20.3	20.7	19.9	20.4	21.6	28.7	22.3	NS
Triterpene alcohols	19.9	22.0	18.7	17.3	19.0	18.6	20.5	19.4	NS
Phytosterol molecular species									
Unknown-1	3.2	3.1	4.4	2.9	3.9	4.9	2.9	3.4	NS
Campesterol	24.2	29.0	27.4	22.5	24.0	27.0	26.5	25.8	NS
Stigmasterol	7.1	9.6	10.8	14.0	12.6	9.8	9.8	10.7*	0.70
Unknown-2	1.5	0.0	0.0	0.8	1.3	0.1	0.0	0.5	NS
Unknown-3	0.7	0.0	0.8	0.3	3.3	1.3	0.0	0.7	NS
β-sitosterol	60.0	56.5	54.5	56.6	50.0	52.0	58.5	56.0	NS
Δ^5-Avenasterol	2.2	0.8	1.1	1.9	2.2	3.0	1.4	1.6	NS
Δ^7-Stigmastenol	1.2	1.1	1.1	1.2	2.7	2.0	0.9	1.3	NS
Triterpene alcohol molecular species									
β-amyrin	4.8	6.9	9.4	6.0	6.0	11.1	5.4	6.8	NS
Lupeol	82.4	90.2	85.7	92.0	85.4	73.8	88.5	86.8	NS
Cycloartenol	1.2	0.6	0.4	0.1	0.9	2.5	0.7	0.8	NS
Unknown-4	2.5	1.2	1.1	0.3	5.3	1.9	1.9	2.0	NS
Methylenecycloartenol	3.2	1.0	1.4	0.8	1.4	4.4	1.1	1.6	NS
Unknown-5	6.0	0.0	2.0	0.8	1.1	6.3	2.5	2.1	NS

[a]UNSAP, unsaponifiable matter. For other abbreviations see Table 1.

search (2), indicate that white lupin has tremendous potential as an alternative grain crop in the mid-Atlantic region of the United States.

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