

STUDIES ON CAROTENOIDS FROM LUPIN SEEDS

Shaofang Wang^{1,2}, Steve Errington³, Heng How Yap³

¹Chemistry Centre (WA), East Perth WA 6004

²Centre for Legumes in Mediterranean Agriculture, The University of Western Australia, Nedlands WA 6009

³Department of Applied Chemistry, Curtin University of Technology, Bentley, Western Australia

Corresponding author's email: swang@ccwa.wa.gov.au

ABSTRACT

Epidemiology studies show that carotenoids can prevent the development of some chronic diseases in humans, including cancers and cardiovascular diseases. Carotenoids have also many other biological activities, including antioxidant activity, influences on the immune system, control of cell growth and differentiation, and stimulant effects on gap junction communication. Therefore scientists paid much attention to carotenoids from fruits, vegetables and foods. However, little is known about the carotenoids in grain legumes such as lupins, chickpeas and field peas. The aim of this study was to examine the carotenoid profiles and estimate the amount in lupin seeds.

The carotenoids in pulse seeds, including four species of lupins grown in Australia (*Lupinus angustifolius*, *L. luteus*, *L. albus* and *L. mutabilis*) were studied by high performance liquid chromatography (HPLC). The various types of carotenoids have been identified in four species of lupins by comparison of retention times and UV spectra with those of standards. The carotenoid profiles of *L. angustifolius*, *L. luteus* and *L. albus* are similar, particularly the latter two species, whereas the carotenoid profile of *L. mutabilis* is somewhat different. Lutein, zeaxanthin and beta-carotene are present in all samples, with lutein as a major carotenoid. *L. angustifolius* contains the highest level of beta-carotene, whereas *L. mutabilis* and *L. angustifolius* have highest lutein content. Higher level of zeaxanthin was found in *L. albus* and *L. angustifolius*. For *L. mutabilis*, lutein was a major peak and other small peaks were detected. *L. angustifolius* has the highest level of carotenoids in all samples.

KEY WORDS

lupins, *Lupinus angustifolius*, *L. luteus*, *L. albus*, *L. mutabilis*, carotenoids

INTRODUCTION

Lupins are native to the Mediterranean region, Eastern Africa and the American. Lupins have a tap root system; they can survive in poor environment such as deep sandy, infertile soil and poor climate that soybean cannot survive (Smart *et al.* 1988, Cerletti *et al.* 1983).

These give lupins higher commercial potential than soybeans and they were established in Western Australia successfully. Three types of lupins- sweet narrow-leafed lupin (*Lupinus angustifolius*), white lupin (*L. albus*) and yellow lupin (*L. luteus*) have been cultivated in Western Australia. Recently, *L. mutabilis* was bred in Western Australia for its high protein and oil content. Forty percent of the lupin seed harvest has been used as feed for livestock and stock feed ingredients. They are also used as forage in the sheep industry.

The use of lupin grains as a raw material for industrial processing has been the subject of research in Australia (Petterson, 1996) and Europe (Bagger *et al.* 1998). Due the high protein content of lupins, they are used to produce dietary fibre product and a specific protein component with high whipping properties (Petterson, 1996). Southern Europe, Middle East and South American used high-alkaloid bitter lupins as food products, and Western Australia used low level alkaloid lupins for a wide range of food products (Grain Pool of Western Australia, 1991/92). Therefore, many researchers paid more attention towards the possibility of using lupins as a human food (Petterson *et al.* 1996).

Lupins have good commercial value due to the lupin seeds containing substantial amounts of protein and fat. The protein content ranges from 270-370 g/kg for *L. angustifolius*, 290-400 g/kg for *L. albus* and 230-380 g/kg for *L. luteus*, respectively. The oil content ranges from 49-70 g/kg *L. angustifolius*, 76-120 g/kg for *L. albus* and 27-42 g/kg for *L. luteus*. (Petterson *et al.* 1997).

Carotenoids are pigments widespread in nature with more than 600 different compounds, which have been identified in various organisms. Carotenoids include phytofluene, α -carotene, β -carotene, lycopene, lutein, β -cryptoxanthin, β -citaurin, zeaxanthin, violaxanthin, luteoxanthin, auroxanthin and 9-*cis*-violaxanthin and so on (Chang *et al.* 2006). β -Carotene is the most prominent representative of this very lipophilic class of compounds (Wilhelm, S., 1999). Carotenoids are synthesised and stored in the photosynthetic apparatus of higher plants (Wilhelm, S., 1999). The higher plants use carotenoids in the light-harvesting system and in antioxidant defense against photooxidation

(Demming-Adams, 1996). Animals and humans cannot synthesise carotenoids by themselves. Therefore, they need to absorb carotenoid from the diet and make use of provitamin A carotenoids for the vitamin A supply. B-Carotene and lutein are found in many different kinds of fruits and vegetables (Wilhelm, S., 1999). Only a few products contain important other carotenoids such as lycopene or zeaxanthin (Wilhelm., 1999). In the United States, tomatoes and tomato products provide about 90% dietary lycopene (Chug-Ahuja *et al.* 1993). The major source of zeaxanthin is corn. Most β -carotene is obtained from carrots followed by spinach, broccoli or green and red peppers.

The other main sources that provide carotenoid are fruits (Wilhelm, 1999). Fruits contain considerable amounts of the provitamin A carotenoids, β -carotene and β -cryptoxanthin (Wilhelm, 1999). They are the most important provitamin A in the developing countries. Lutein also plays an important role in the defensive system (Wilhelm, S., 1999). Hydroxylated carotenoids in fruits and vegetables may be present as parent carotenoids or esterified with various fatty acids (Khachik and Beecher, 1988). Besides that, carotenoids

are also found in various kinds of seafood including lobster and salmon (Liaaen-Jensen, 1990).

Recent epidemiology studies showed that carotenoids can prevent the development of some chronic diseases in humans, including cancers and cardiovascular diseases. Carotenoids have also many other biological activities, including antioxidant activity, influences on the immune system, control of cell growth and differentiation, and stimulant effects on gap junction communication (Krinsky, 1994, Wilhelm, 1999). Therefore scientists have paid much attention to carotenoids from fruits, vegetables and foods. However, little is known about the carotenoids in grain legumes such as lupins. The aim of this study was to examine the carotenoid profiles and estimate the amount in lupin seeds.

MATERIALS AND METHODS

Materials. Samples of *L. luteus* cv. Pootalong, *L. albus* cv. Andromeda, *L. mutabilis*, P26961, *L. angustifolius* cv. Kalya were obtained via the Western Australian Department of Agriculture and Food.

Table 1. Identification and separation of carotenoids in lupins.

Peak no.	Carotenoid	Rt (min)	UV Max (nm)	<i>L. luteus</i>	<i>L. albus</i>	<i>L. mutabilis</i>	<i>L. angustifolius</i>
				Ratio of area (peak/int)	Ratio of area (peak/int)	Ratio of area (peak/int)	Ratio of area (peak/int)
1	unidentified	11.444	406.0, 445.5, 487.5			0.20	
2	unidentified	12.557	368.6, 487.5	0.02	0.05	0.12	0.04
3	unidentified	13.293	368.6, 442.4, 470.4	0.12	0.006	0.005	0.11
4	Lutein	14.59	444.9, 472.8	1.45	1.16	1.93	1.12
5	Zeaxanthin	16.782	449.7, 480.1	0.46	1.001	0.17	0.83
6	Internal standard	18.015	460.7	1	1	1	1
7	unidentified	19.666	414.5, 437.6, 466.1	0.03	0.02	0.03	0.01
8	unidentified	20.892	277.9	0.02	0.07		0.02
9	unidentified	23.079	276.7, 448.5	0.02	0.01		0.05
10	unidentified	23.43	276.7, 472.8	0.04	0.003		
11	unidentified	24.954	276.7	0.01	0.01		
12	unidentified	26.722	277.9, 336.4, 368.4	0.04	0.05		
13	unidentified	27.182	235.1, 472.4	0.08	0.44		0.14
14	unidentified	28.156	410.9	0.04	0.02		0.004
15	Beta-carotene	29.401	449.7, 476.5	0.70	0.09	0.07	1.03
16	Alpha-carotene	30.875	444.9, 472.1	0.19	0.0007	0.02	0.28
17	unidentified	32.054	336.4, 449.7	0.12	0.06		0.18
18	unidentified	34.294	269.5, 435.1	?	?	?	?
19	Lycopene cis-isomer	35.962	438.8, 470.4	0.01	0.01		0.09
20	Lycopene cis-isomer	36.353	438.8, 470.4	0.01	0.01		
21	unidentified	36.987	302.9, 436.4	0.02	0.09		0.04
22	unidentified	37.392	348.4, 440.0	0.0004	0.21		
23	unidentified	37.885	458.2	0.03	0.11		0.05
24	unidentified	38.56	355.6, 446.2	0.06	0.07		0.19
25	unidentified	40.1	273.1, 366.2, 446.0, 476.5	0.06	0.10		0.28
26	Lycopene	41.755	449.7, 472.9	0.05	0.03		0.12

Extraction. Lupin seeds were ground into fine powder. One gram of seed powder was extracted with 20 mL of co-solvent (1:1 dichloromethane: methanol) under ultrasonic conditions for 1 hour. The sample was centrifuged and the top clear solution was filtered with No. 541 filter paper and concentrated by evaporation. The residue was dissolved with 2 mL of ethyl acetate. One mL of the solution was mixed with 100 μ L of internal standard *trans*-apo-8- β -carotenal was transferred to an HPLC vial.

Saponification. Another one mL of solution was used for the saponification. The solution was evaporated to dryness. The residue was dissolved in 1 ml of diethyl ether, to which 5 ml of 6% w/v KOH in methanol was added. After it was mixed, the mixture was incubated at 60°C for 30 minutes in darkness. The mixture was dried by using a rotary evaporator. To the residue, 2 mL of water was added then, the mixture was extracted twice with 2 ml of ethyl acetate. The ethyl acetate solution was dried using nitrogen gas and the residue dissolved in 2 ml of ethyl acetate and 100 μ L of internal standard and placed in a vial for HPLC analysis.

INSTRUMENTATION

HPLC instrument. Chromatographic separation of carotenoids was performed using a Waters 2695 Separation Module. The HPLC was connected to Waters 2996 Photodiode Array Detector. An UV wave length between 210 nm to 550 nm was used for detection.

HPLC column. Column (YMCTM Carotenoid S-5, 4.6 x 250 mm, Massachusetts 01757-3646) was used for separation of carotenoids, which were identified on the basis of retention time and spectral characteristics of standards.

Solvents. Mobile phases A (methanol), B (80% methanol containing 0.2% ammonium acetate) and C (*tert*-butyl methyl ether) were applied as follows: 92% A, 8% B for 6 minutes, a linear gradient to 77% A, 8% B and 15% C by 7 min, held until 12 min, gradient changed to 27% A, 8% B and 65% C by 45 min, held until 46 min, then changed to 92% A and 8% B by 46.5 min and then held to the end of analysis (54min).

RESULTS AND DISCUSSION

HPLC profiles and identification of carotenoids in lupins. Lupin seed was extracted by organic solvent (1:1 methanol:dichloromethane). The residue obtained by concentration using a rotary evaporator was dissolved in 1 ml of ethyl acetate. A C30 column (YMCTM Carotenoid S-5, 4.6 x 250 mm, Massachusetts 01757-3646) was used to separate the mixture of carotenoids. The HPLC chromatograms for original extracts of four lupin species are shown in Fig. 1. *L. luteus* cv. Poofalong (a), *L. albus* cv. Andromeda (b) and *L. angustifolius* cv. Kalya (d) had similar profiles though the levels of carotenoids varied with the species.

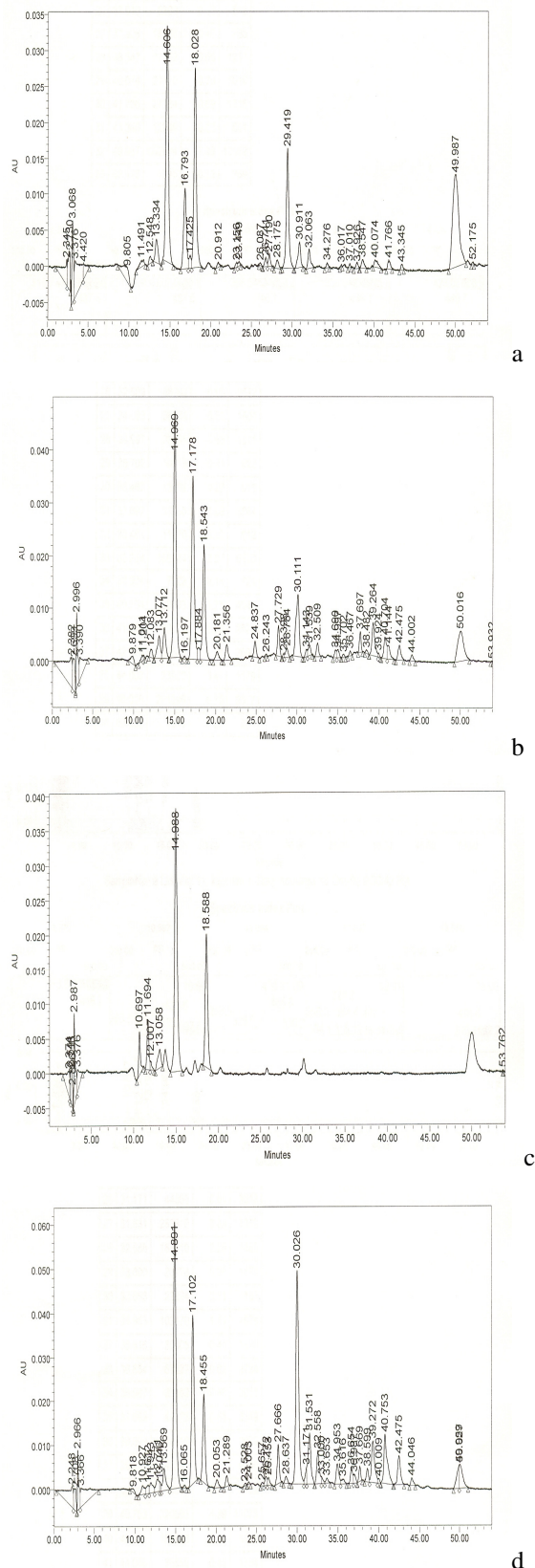


Fig. 1. HPLC chromatogram of original sample for *L. luteus* cv. Poofalong (a), *L. albus* cv. Andromeda (b), *L. mutabilis* (c); and *L. angustifolius* cv. Kalya (d).

L. mutabilis (c) contained less peaks than the other three species: only a few peaks were detected with lutein and β -carotene dominant. Several compounds including some *cis-trans* isomers were identified based on their UV spectra and retention time compared with those of standards. Three major carotenoids namely lutein (14.59 min) zeaxanthin (16.782 min) and beta-carotene (29.401 min) are present in lupin seeds. Other minor carotenoids including violaxanthin, lycopene *cis*-isomer, lycopene and α -carotene were also detected in lupin seeds (Table 1).

Saponification of original extracts of lupin seeds. Carotenoids occur in nature in both free and esterified forms. Saponification is used to hydrolyse the ester group to release the free carotenoids. In this case, the total carotenoids are calculated. The methanol and dichloromethane extract was saponified in the presence of 6% w/v KOH in methanol. A HPLC chromatogram for saponificated extract of *L. luteus* cv. Poofalong is shown in Fig. 2. Because most of ester carotenoids were converted to free carotenoids, the peaks numbers in saponified HPLC were reduced. Only five peaks were found in the saponified sample compared with 26 in the original organic extract. These peaks are unknown (11.444 min), lutein (14.565 min), zeaxanthin (16.726 min), β -carotene (29.602 min) and α -carotene (31.080 min).

QUANTIFICATION OF CAROTENOIDS IN LUPIN SEEDS

The carotenoid amount was calculated using lutein, zeaxanthin and β -carotene as standards via individual calibration curve with four different concentration using *trans*-apo-8- ζ -carotenal as internal standards. All samples were analysed in triplicate.

Lutein in lupin seeds. There is no significant difference in lutein levels between the four species. *L. mutabilis* (18.61 $\mu\text{g/g}$.) and *L. angustifolis* cv. Kalya (24.11 $\mu\text{g/g}$.) have the highest amount of lutein compared to other lupines. The amount in *L. albus* cv. Andromeda is a little bit lower than those in other three species (Fig. 3).

Zeaxanthin in lupin seeds. There are big differences in lutein levels between species with a range of 16.23 to 135.04 $\mu\text{g/g}$. *L. albus* cv. Andromeda and *L. angustifolis* cv. Kalya have the highest level of zeaxanthin at 135.04 and 134.44 $\mu\text{g/g}$, respectively. *L. luteus* cv. Poofalong seeds yielded 65.68 $\mu\text{g/g}$ of zeaxanthin, and *L. mutabilis* had the lowest level of zeaxanthin (Fig. 3).

Beta-carotene in lupins. The range of β -carotene presents in the samples was 11.98-50.43 $\mu\text{g/g}$. *L. angustifolis* cv. Kalya had the highest content of β -carotene. *L. luteus* cv. Poofalong and *L. albus* cv. Andromeda contain only half the amount (Fig. 3).

Other carotenoids in lupin seeds. Lupin seeds also contained carotenoids other than lutein, zeaxanthin and β -carotene, most of them esters of lutein. This was confirmed from samples after saponification.

L. angustifolis cv. Kalya had the highest value of other carotenoids, *L. mutabilis* the lowest. All lupines revealed reasonably high content of other carotenoids (Fig. 3).

Total carotenoids in lupin seeds. The total carotenoids in the samples were calculated for the sum of lutein, zeaxanthin, β -carotene and others. There are significant differences in total carotenoids between these four species with a range of 53 $\mu\text{g/g}$ to 229.73 $\mu\text{g/g}$. *L. angustifolis* cv. Kalya contained the highest value of total carotenoids (229 $\mu\text{g/g}$). The total carotenoids in *L. albus* are close to *L. angustifolis* cv. Kalya. *L. mutabilis* only contained one fourth of total carotenoids of *L. angustifolis* cv. Kalya (Fig. 3).

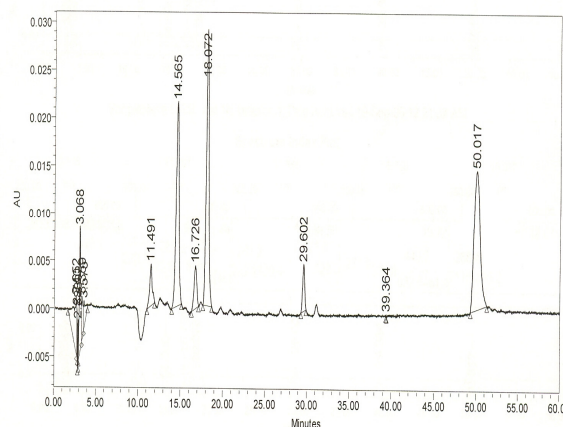


Fig. 2. HPLC chromatogram of saponified sample for *L. luteus* Poofalong.

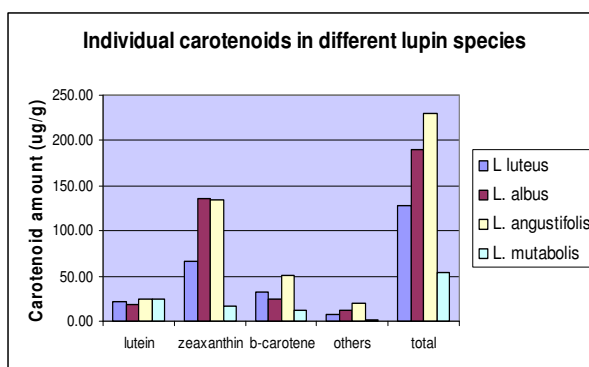


Fig.3. The individual carotenoids in different species of lupins (note: should read β -carotene and *L. angustifolis*).

It was reported that β -carotene and lutein are found in many different kinds of fruits and vegetables like red, yellow, orange, and leafy dark green vegetables, corn, egg yolks, squash, broccoli and peas (Wilhelm, S., 1999). Corn (Scott and Eldridge, 2005; Moros, 2002) contains high levels of lutein and zeaxanthin with 20 $\mu\text{g/g}$ for whole corn. Three species of lupins namely *L. luteus* cv. Poofalong, *L. albus* cv. Andromeda, and *L. angustifolis* cv. Kalya contained more zeaxanthin than lutein and β -carotene. *L. mutabilis* yielded more lutein, than zeaxanthin (Fig. 4). It was found that lutein and zeaxanthin are the only carotenoids in nearly equal

amount in the macula of the eye (the macula retina is about 5% of the total retina). Previous studies have suggested a potential link between age-related macular degeneration (AMD) and lutein and zeaxanthin. These compounds may reduce the risk of AMD by absorbing blue light that could damage the macula, by preventing free radicals from damaging eye cells and by strengthening eye cell membranes. Recently, some research work suggested that dietary with high level lutein may help reduce the risk of developing colon cancer (Slattery *et al.* 2000). The high levels of carotenoids in lupin seeds indicated that people have dietary with lupin may help them prevent from some diseases like eyes and colon diseases.

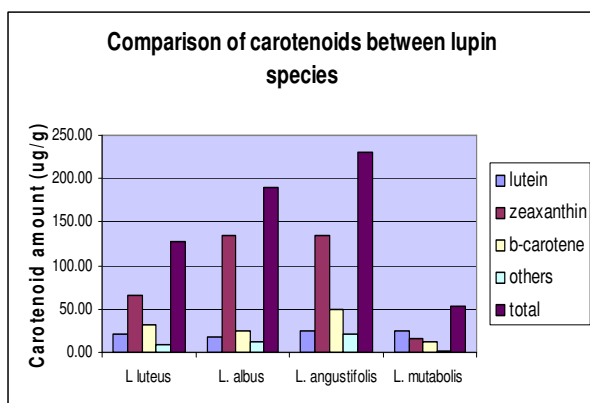


Fig. 4. The comparison of carotenoids between four lupin species (note: should read β -carotene and *L. angustifolius*).

CONCLUSION

In conclusion, the carotenoids in four species of lupins were studied by HPLC. A number of peaks were detected in original extracts including a number of ester carotenoids and several free carotenoids. Some of them including lutein, zeaxanthin and β -carotene were identified in lupin seeds based on their UV spectra and retention time compared to those of standards. The carotenoid profiles and levels varied with species.

ACKNOWLEDGMENTS

The authors would like to thank Drs Mark Sweetingham and Leigh Smith, Department of Agriculture and Food, Western Australia for providing lupin seeds for this study.

LITERATURE CITED

- Cerletti, P, P. Restani and M. Duranti. 1983. Potential for Lupine Proteins Relevant to their Nutritional Performance. 1998. Qual Plant Foods Nutrition. 32: 145-154.
- Chang, J.X, P.D. Fraser, W.J. Wang and P.M. Bramley. 2006. Differences in the Carotenoid Content of Ordinary Citrus and Lycopene-Accumulating Mutants.

- Chug-Ahuja, J. K., J.M. Holden, M.R. Forman, A.R. Mangels, G.R. Beecher and E. Lanza. 1993. The Development and Application of a Carotenoid Database for Fruits, Vegetables and Selected Multicomponent Foods. J. Am. Diet. Assoc. 93: 318-323.
- Demming-Adams, B., A.M. Gilmore and W.W. Adams. 1996. In vitro Function of Carotenoids in Higher Plants. FASEB J. 10: 403-412.
- Edlenbos, M., L.P. Christensen and K. Grevsen. 2001. HPLC Determination of Chlorophyll and Carotenoid Pigments in Processed green pea Cultivars (*Pisum sativum* L). J. Agric. Food Chem. 49(10): 4768-4774.
- Khachik, F. and G.R. Beecher. 1988. Separation and Identification of Carotenoids and Carotenoid Fatty Acid Esters in some Squash Products by Liquid Chromatography. 1 Quantification of Carotenoids and Related Esters by HPLC. J. Agric. Food Chem. 36: 929-937.
- Krinsky, N.I. 1994. Carotenoids and Cancer: Basic Research Studies. In 'Natural Antioxidants in Human health and Disease'. Academic Press, San Diego.
- Liaaen-Jensen. 1990. Marine Carotenoids-Selected Topics. New J. Chem. 14: 747-759.
- Slattery, Martha, L., Joan Benson, Karen Curtin, Khe-Ni Ma, Donna Schaeffer and John D Potter. 2000. Carotenoids and colon cancer. American Journal of Clinical Nutrition 71: 575-582.
- Moros, E.E., D. Darnoko and M. Cheryan. 2002. Analysis of xanthophylls in corn by HPLC. Journal of Agricultural and Food Chemistry 50: 5787-5790.
- Petterson, D.S. 1996. Final Report to the Grain Research and Development Corporation.
- Petterson, D.S., S. Sipsas and J.B. Mackintosh. 1997. The Chemical Composition and Nutritive Value of Australia Grain Legume. Grains Research and Development Corporation, Canberra.
- Scott, C.E. and A.L. Eldridge. 2005. Comparison of carotenoid content in fresh, frozen and canned corn. Journal of Food Composition and Analysis 18(66): 551-559.
- Smart, W.L., M.M. Ralph, J.M. Lidale, R.D. Ramma, C.J. Robinson and E.W. Armstrong. 1988. Looking at Lupins Published at Department of Agriculture Lake Grace.
- Ski Kantha, S. and J.W. Erdman. 1987. Legume Carotenoids. CRC Critical Reviews in Food Sciences and Nutrition 26: 137-155.
- Weber, E.J. Carotenoids and tocopherols of corn grain determined by HPLC. Journal of the American Oil Chemists Society, 1987, 64: 1129-1134.
- Wilhelm, S and H. Sies. 1999. Carotenoids: Occurrence, Biochemical Activities, and Bioavailability. Academic Press, United States of America.
- Pigments In Corn, Squash And Other Vegetables May Help Protect Against Age-related Vision Loss, Science Daily August 16, 2006.