School of Public Health

The effect of germination and food processing on the concentration and activity of bioactive compounds in Australian Sweet Lupin

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This thesis is presented for the Degree of
Doctor of Philosophy of
Curtin University of Technology

October 2010

Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Signature: ……

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Acknowledgements

I am indebted to my supervisors, Dr. Antony James and Professor Vijay Jayasena for advice, guidance and ideas given to my research project of my study. Special thanks to Dr. Antony James for his assistance and patience in helping with the writing of my thesis. Thank you to Dr. Kathryn Sauer for chairing my thesis committee. I am also grateful to have been awarded the Endeavour International Postgraduate Research Scholarship (EIPRS) and Curtin Postgraduate Award (CUPS) which assisted greatly in my studies.

To all of the laboratory staff of School of Public Health, in particular, Nerissa Ho, Paul Dubois, Simon Warwick, Mala Seranata and Cheryl Cheng, thank you very much for your help and assistance during my laboratory work. Many thanks also to S.M. Nasar Abbas for his kind help and assistance in the muffin analysis. To Dr. Yun Zhao, thank you for helping me with my statistical analysis. To Prof. Rudi Appels, Prof. Wujun Ma, Mh. Shahidul Islam and Junhong Ma in State Agriculture and Biotechnology Centre, Murdoch University, thank you for the use of your equipment, advice and technical assistance for protein analysis using MALDI-TOF MS.

To Jun Chih, thank you very much for proofreading of my thesis and any help during my laboratory work. To Imelda Soriano, thank you for support and discussion during my laboratory work.

A special acknowledgement is to my husband, Aris, for love, patience and support which motivated me throughout my study. Also for my mum and dad in-law and sisters, thank you very much for praying and support. To my Dad, who was killed by lung cancer and passed away during my study, thank very much for teaching me how to work hard in every chance. My beloved children, Hasna and Navi, thank you for your patience for having a ‘busy mum”. This thesis is especially dedicated to you, Hasna and Navi. Hopefully, it will inspire you for studying hard in the future.

Abstract

Lupin is a grain legume which is high in protein and fibre, but low in fat and starch. Lupin also contains bioactive compounds such as phenolic compounds. Many studies have shown that a diet containing lupin has health benefits including reducing a number of risk factors associated with metabolic syndrome. However, studies examining the effect of germination of Australian Sweet Lupin (ASL) on its macronutrient, bioactive compounds and bioactivity are limited. Consequently the aim of the present study was to investigate the changes in the macronutrient composition and concentration of bioactive compounds in ASL during germination and how these changes were associated with *in vitro* bioactivity. The stability of bioactive compounds and their *in vitro* bioactivity was also investigated in muffins incorporated with germinated ASL flour (before and after baking).

In the present study, the macronutrient composition (protein, crude fibre and fat) and bioactive compound concentration (phenolic compounds and phytosterols) of ASL following germination at 25°C and 90-95% relative humidity for 9 days were determined. Total phenolic compounds (TPC) were extracted from germinated ASL flour using methanol and aqueous solvents and the concentration was determined using Folin-Ciocalteu reagent. Phytosterols in oil extracts were analyzed using gas-liquid chromatography. The radical scavenging activities toward 2, 2-diphenyl-1-picrylhydrazyl (DPPH) of the methanolic and oil extracts were also determined. Bioactivity related to bile acid binding *in vitro* of germinated ASL flour was also assessed. The changes in the pattern of ASL protein during germination were analysed using sodium dodecyl sulphate-polyachrilamide gel electrophoresis (SDS-PAGE) and matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS). The changes in the protein pattern were then compared with the result of Angiotensine I Converting Enzyme (ACE) inhibitory activity assay of the protein. Total protein extracts of germinated ASL flour were then fractionated into the protein isolate fraction and the soluble fraction. Both fractions were characterized for their activities including antioxidant activity, bile acid binding ability and ACE-inhibitory activity. ASL flour after germination for 7 days was chosen to be incorporated into muffin formulation at substitution levels of 2 - 8% of dried muffin weight. Physical characteristics of muffins including height,

diameter, colour and texture (hardness, cohesiveness, springiness and chewiness) were measured instrumentally. Stability of the bioactive compounds and the antiradical activity of the incorporated muffins before and after baking were also investigated.

Germination at day 9 resulted in significant increase in protein and crude fibre contents of ASL by 38% and 456% (db), respectively, and resulted in a substantial reduction in the lipid content of ASL by 71%. Germination also increased the concentration of total phenolic compounds in methanolic extracts of germinated ASL flours by about 700% compared to ASL flour of ungerminated seeds. The increased concentration of phenolic compounds was found to be associated with significant increased radical scavenging activity of the extracts. Concentration of the total phytosterols which was extracted from germinated ASL flour were also increased by 3 fold. The significant increase in phytosterol content in the oil may be associated with increasing in the antiradical activity of the oil. The main phytosterols in the oil extracted from germinated ASL flour are β-sitosterol (62%), campesterol (30%) and stigmasterol (8%). In the protein fraction, the high molecular weight proteins of ASL were not present following germination for 9 days. The change in the protein profile may be associated with the higher ACE inhibitory activity (antihypertensive *in vitro*) of germinated ASL flour. The protein isolated from germinated ASL flour had better antioxidant activity, while its soluble fraction was better in ACE inhibitory activity and bile acid binding property. The bile acid binding ability of germinated ASL flour had higher bile acid binding ability *in vitro* than ungerminated ASL flour. Furthermore, incorporation of germinated ASL flour up to 8% (db of muffins) into the muffin formulation influenced moisture, height, diameter, colour, hardness and cohesiveness of the muffins. This incorporation increased the total phenolic compounds, phytosterols content and antiradical activity of the muffins. The baking process at 190°C for 25 min did not substantially reduce the concentration of total phenolic compounds, phytosterols and the antiradical activity of the muffins.

The present study found that germination led to an increase in protein and fibre contents and concentration of bioactive compounds of ASL. Germination also increased *in vitro* antioxidant activity, ACE inhibitory activity and bile acid binding ability of ASL.

Abbreviations

ABC	ATP Binding Cassette
ABTS	2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)
ACE	Angiotensine I Converting Enzyme
ACN	Acetonitrile
ACAT	Acetyl Coenzyme A Acetyl Transferase
ASL	Australian Sweet Lupin
AOAC	Association of Official Agricultural Chemists
ANOVA	Analysis of Variance
ANFs	Antinutritional Factors
ATP	Adenosine Tri-Phosphate
*a**	Greenness
*b**	Yellowness
BA	Bile Acid
BCA	Bicinchoninic Acid
BMI	Body Mass Index
CVD	Cardiovascular Disease
COP	Cholesterol Oxidation Product
CA	Cholic Acid
CE	Chylomicrons
CDCA	Chenodeoxycholic acid
DPPH	2,2-di-phenyl-1-picrylhydrazyl
DM	Dry Matter
DTT	Dithiothreitol
db	Dry Basis
ET	Electron Transfer
EDTA	Ethylenediaminetetraacetate
EGTA	Ethyleneglycol-bis(aminoethyl ether)-N,N'-tetraacetic acid
FC	Folin-Ciocalteu
FFA	Free Fatty Acids
FPLC	Fast Protein Liquid Chromatography
FPAA	Free Protein Amino Acids

FNPAA	Free-non Protein Amino Acids
FID	Flame Ionization Detector
GABA	Gamma-aminobutyric-acid
GC	Gas Chromatography
GAE	Gallic Acid Equivalent
HA	Hippuric Acid
HAT	Hydrogen Atom Transfer
HHL	Hippuyl-L-Hystidyl-L-Leucine
HPLC	High Perfomance Liquid Chromatography
HDL	High Density Lipoprotein
HDL-C	High Density Lipoprotein Cholesterol
HMG-CoA	3-hydroxy-3-methylglutaryl Coenzyme A
IDF	International Diabetes Federation
kDa	KiloDalton
*L**	Lightness
LDL	Low Density Lipoprotein
LDL-C	Low Density Lipoprotein Cholesterol
LXR	Liver X Receptor
MeOH	Methanol
MC	Moisture Content
MW	Molecular Weight
MALDI-TOF	Matrix-assisted Laser Desorption/ionization-time of Flight
NSP	Non-Starch Polysaccharides
ORAC	Oxygen Radical Absorbance Capacity
PI	Protein Isolate
POPs	Phytosterol Oxidation Products
PBS	Phosphate Buffered Saline
PRTC	Peroxyl Radical Trapping Capacity
RSA	Radical Scavenging Activity
RH	Relative Humidity
RT	Retention Time
S	Svedberg (Sedimentation Unit)
SF	Soluble Fraction

SFA	Saturated Fatty Acid
SFE	Supercritical Fluid Extraction
SOP	Sterol Oxidation Product
SA	Sinapinic Acid
SREBP	Sterol Regulatory Element Binding protein
SDS-PAGE	Sodium Dodecyl Sulphate Polyachrilamide Gel Electrophoresis
TPA	Texture Profile Analyssis
TPC	Total Phenolic Compounds
TAG	Triacylglycerols
TEAC	Trolox Equivalent Antioxidant Capacity
TFA	Trifluoroacetyl
TRAP	Total Radical Trapping Antioxidant Parameter
UFA	Unsaturated Fatty Acid
UNSAP	Unsaponifiable
VLDL	Very Low Density Lipoprotein
WHO	World Health Organization
WR	Working reagent

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Chapter 1 Introduction

Lupin is a grain legume and an important crop in Australia. Australia produces more lupin than any other countries in the world. Lupin has been primarily used for animal feed. However, the superior compositions and the health benefits of lupin have led to its increased use in human food applications. The comparable nutritive value to soybean and its lower price compared to soybean are additional benefits for the use of lupin in the human diet.

Cardiovascular disease (CVD) is the leading cause of death worldwide and is responsible for approximately 30% of deaths each year (Bonow et al., 2002). Metabolic syndrome, a cluster of metabolic abnormalities including dyslipidemia, abdominal obesity, hypertension and insulin resistance, is a risk factor for cardiovascular disease. The prevalence of metabolic syndrome worldwide during the past two decades has increased due to an increase global epidemic of obesity as the main key factor for metabolic syndrome (Eckel et al., 2005). In Australia, during 1999-2000, when the International Diabetes Federation (IDF) definition was used as a criteria, one in three Australian aged at and above 25 years old suffered from metabolic syndrome. If based on the World Health Organization (WHO) and the National Cholesterol Education Program Adult Treatment Panel (NCEP ATP III) criteria, one in five of Australians aged at and above 25 years old was found to have metabolic syndrome (Cameron et al., 2007). Lifestyle, in particular a diet high in saturated fat and low physical activity, is the main contributor for occurrence of metabolic syndrome (Tuomilehto, 2005). In addition, a 'Western' dietary pattern has been associated with a higher risk of metabolic syndrome when compared to a more 'healthy' diet containing vegetables, fruits, legumes and whole grains. A change to a healthier diet is an effective way to prevent the development of metabolic syndrome.

Another global health burden is cancer. The data from WHO shows that mortality due to cancer was estimated to be about 7 million in 2001. The risk factors that are associated with death from cancer in low- to middle -income countries are smoking, alcohol drinking, low intake of fruits and vegetables; while in high-income countries, obesity is an important cause of cancer. Diets that are high in fat and sugar, but low

in fibre also contribute to incidence of cancer (Petti, 2009). Balancing diet with plant foods which contain a high concentration of antioxidants such as vitamin A, E, C, carotenoids, flavonoids and phytosterols is good to prevent occurrence of cancer.

It is necessary to investigate alternate ways to modify diet in order to reduce the prevalence of some chronic diseases especially cardiovascular disease and cancer. In this regard, lupins are a good alternative to be used as a food ingredient being high in protein, dietary fibre and bioactive compounds, but low in fat and starch. Human intervention studies using diets containing lupin have reported a reduction in some risk factors related to metabolic syndrome by acutely increasing satiety and reducing energy intake (Lee et al., 2006b, Archer et al., 2004). Using animal and human studies, lupin protein isolate of *L. albus* also have benefits in the management of diabetes (Magni et al., 2004), hypertension (Pilvi et al., 2006) and hypercholesterolemia (Hall et al., 2005, Martin et al., 2005). In addition, protein isolate derived from lupin flour has been reported to have bile acid binding ability which, when consumed following incorporation into food products, would be expected to promote a reduction in circulating cholesterol levels (Yoshie-Stark and Wasche, 2004). Some bioactive compounds have been found for example isoflavones (Duenas et al., 2009, Oomah et al., 2006), phytosterols (Hamama and Bhardwaj, 2004) and bioactive peptide with ACE inhibitory activity in *L. albus* (Yoshie-Stark et al., 2004). The bioactive compounds found in lupin may have an important role in lowering cholesterol levels, antioxidant activity, bile acid binding property and ACE inhibitory activity (antihypertensive). Moreover, lupins have been recently added into various food products to improve their health benefits. These products include noodles (Jayasena et al., 2008), pasta (Clark and Johnson, 2002), bread (Doxastakis et al., 2007) and muffins (Jayawardena, 2006).

Germination is a simple, low-cost method that can enhance the concentration and activity of bioactive compounds and consequently their health benefits. Phenolic compounds in lupin (Duenas et al., 2009) and soybean were improved by germination (Zielinski, 2003). Beneficial effects related to type 2 diabetes, Parkinson's disease and hypertension of velvet bean were also enhanced by germination (Randhir et al., 2009). Flours prepared from germinated yellow pea, lentil and faba bean had better nutritional value than the raw seeds and the flours

have been applied in bread and pasta production (Morad et al., 1980, Torres et al., 2007). However, information about the effect of germination on the chemical composition, the concentration and the activities of bioactive compounds such as bioactive peptides and phytosterols in particular ASL is not available. It is proposed that concentration of the bioactive compounds and their health benefits including antioxidant activity, ACE inhibitory activity and bile acid binding property can be increased during germination. This research was therefore conducted to determine the change in the concentration of bioactive compounds and their health benefits *in vitro* of ASL during germination process and to investigate the distribution of the bioactive compounds in the protein isolate fraction and the soluble fraction. An improvement in the concentration and activity of bioactive compounds in ASL during germination would be expected to further promote the advantages of use of ASL as an ingredient in a range of food applications. Germinated ASL flour and its protein isolate fraction would be an innovative food ingredient, which both would be more attractive ingredient than the ungerminated ASL.

Understanding of the effects of processing conditions on the stability of bioactive compounds contained in foods is important to maintain the bioactive compounds and health benefits in foods. No research has been conducted to examine the stability of bioactive compounds in a baked food (such as muffins) substituted with germinated ASL flour. In order to investigate the stability of bioactive compounds in muffins incorporated with germinated ASL flour, the stability of phenolic compounds, phytosterols and antioxidant activity *in vitro* in the muffins was also investigated in this study. A better understanding of the effects of processing on the *in vitro* bioactivities will further promote the health benefits of bioactive compounds in food applications. Overall the outcomes of this study will contribute positively to the development of germinated ASL as an alternate source to healthier diet.

1.1 Hypotheses

- The concentration of bioactive compounds and their bioactivities in ASL are affected by the duration of germination
- Food processing treatments involved in making muffins do not affect the concentration of bioactive compounds and their *in vitro* bioactivities in flour derived from germinated ASL

1.2 Objectives

- To determine the effects of germination of ASL on the concentration of bioactive compounds and their *in vitro* bioactivities
- To fractionate germinated lupin into protein isolate and soluble fraction and determine the distribution of bioactive compounds and *in vitro* bioactivities in each fraction
- To assess the stability of bioactive compounds and their *in vitro* bioactivities in muffins incorporated with germinated ASL flour

Chapter 2 Literature review

2.1 Lupin

Lupins are members of family *Leguminosae* and subfamily *Papilionoideae*. At present, this legume is mainly used for animal feed. However due to its unique nutritional value and chemical composition (El-Adawy et al., 2001, Erbas et al., 2005), lupins have recently been used in food for human consumption applications. The protein content of lupin is high and the protein has unique functional properties that are beneficial when applied in food applications (Jayasena and Quail, 2004). Lupin has a high content of dietary fibre, but a low content of starch which is beneficial in human diet. The high proportion of unsaturated fatty acids in lupin oil is in addition to the favourable nutritional composition of lupins. Lupins are also a great source of bioactive compounds including phytoestrogen, phytosterols, and bioactive peptides which are associated to protective against some diseases. The low antinutritional factors of lupin, in particular Australian Sweet Lupin (ASL), compared to other legumes also increase the acceptance of lupin in human diet.

2.1.1 History of lupins

Lupins were originally grown thousands of years ago in Mediterranean countries, East Africa and American. Lupins have diversity of species which the most species numbers are in the coastal and mountain regions of Western North America. The wild lupin species can be divided into American "New World" species and The Mediterranean and East African "Old World" species. Details of some lupin species are shown in Table 2-1. Wild lupins can be grown in a wide range of climatic conditions, for example, the sub-arctic climate of Alaska, semi-desert of the highlands of East Africa, Mexico and the Andes and the subtropical climate for Eastern South America. In addition, lupin plants prefer the soil characteristics such as acid to neutral, stony or ruby, low nitrogen and low water (Gladstones, 1998).

Table 2-1 : Botanical features and geographical distribution of some Lupinus species

Species	Synonyms	Common name	Characteristics	Distribution
New world species: *L. mutabilis* Sweet		Tarwi, Chocho, pearl lupin	White and small seeds, the wild species has high alkaloid contents, smaller and narrow leaf	South America, Andean Highlands
Old world species: *L. albus* L *var. albus var graecus*	*L. termis* Forsk *L. graceus* Boiss *L. jugoslavicus* Karim	White lupin, Albus lupin	White, blue flowers, the wild species has shattering pods, dark-coloured and impermeable seeds	Mediterranean
L . angustifolius L.	*L. varius* L. *L. linifolius* Roth *L. reticulates* Desv	Narrow-leaf lupin, blue lupin	Flower colour: blue, occasionally pink, white in domesticated plants; hard, medium-sized seeds	Mediterranean, Australia
L. micrantus Guss	*L hirsutus* L.	-	-	Mediterranean
L. luteus	-	Yellow lupin, Gelbe lupine, Altramuz Amarillo	Flowers: yellow, white in mutants	Mediterranean, West lberia
Old world, rough seeds:				
L. pilosus Murr	*L. hirsutus* L. *L varius* L., *L. anatolicus* Swiec	-	-	Nort east Meditterranean
L. cosentini Guss	-	Sandplain lupin	-	West Mediterranian, Morocco, Australia

Source: Gladstones (1998)

Wild lupins were cultivated to be used as a green fertilizer, ruminant feed, and human food (Gladstones, 1998). As human food, *Lupinus mutabilis* has been used since thousands of years ago in the Andean highlands and *Lupinus albus* in ancient Egypt (Petterson, 1998). In 1781, wild lupins were brought from their origin, Mediterranean into Northern Europe when some lupin seeds were sent into North Germany to improve the quality of soil. A German scientist, Reinhold von Sengbusch, then discovered the low-alkaloid cultivars of yellow lupin and narrow-leaf lupin in 1928. The new cultivars were suitable for a commercial cultivation. This period signified the beginning of breeding and development of lupins as crop plants. Three commonly grown lupin cultivars are shown in Figure 2-1.

L. angustifolius ***L. albus*** ***L. luteus***

Figure 2-1 : Lupin plants and their flower colours
(Anonym, 2010)

The sweet lupin species were introduced to Australia and developed as crop plants in the 1960s (Petterson, 1998). The sweet species in Australia is now known as Australian Sweet Lupin (ASL), the commercial name for *L. angustifolius.* There are some varieties of species of *L angustifolius* cultivated in Australia such as Danja, Gungurru and Yorrel (Nelson and Delane, 1991). Figure of ASL seeds is shown in Figure 2-2. ASL is the most popular lupin species cultivated in Western Australia due to its suitability to Western Australian climate (Nelson and Delane, 1991). Australia is largest producer and exporter (85%) of lupin in the world (Abare, 2007).

Figure 2-2 : Australian Sweet Lupin (*L. angustifolius*) seeds

2.1.2 Chemical composition of lupin

The main chemical compositions of lupin species are presented in Table 2-2.

Table 2-2 : The main chemical compositions of lupin species

Components	***L. albus* (%)**	***L. angustifolius* (%)**	***L. luteus* (%)**
Moisture	8.58	8.44	9.44
Protein	36.10	32.16	41.36
Crude fat	9.08	5.82	5.74
Crude fibre	10.27	14.89	12.74
Acid detergent fibre (ADF)	14.28	19.73	na
Neutral detergent fibre (NDF)	17.15	22.68	19.2
Lignin	0.65	0.7	0.5

Source: Petterson (1998), na: not available

2.1.2.1 Proteins

The protein level of lupin is affected by genotype and the location where the lupin is grown (Bhardwaj et al., 1998). As shown in Table 2-2, lupin species have a high protein content, varying from 32-42% (Petterson, 1998). The protein contents is similar to that in soybean (40%) (Kyle, 1994). The content is greater than that of the other legumes such as in chickpeas (28%), lentil (28.6%), and dry pea (23,3%), respectively (Roy et al., 2009).

Similar to other grain legumes, the composition of amino acids in lupin is not as balanced as animal proteins such as the egg protein due to lack in sulphur-containing amino acids including methionine and cysteine (Mandal and Mandal, 2000, Sujak et al., 2006, Chew et al., 2003). Furthermore, proteins contained in seeds can be classified into storage proteins, structural proteins and biologically active proteins. The active proteins include lectins, enzymes, and enzyme inhibitors. The storage proteins are non enzymes that provide the nitrogen and sulphur required during the germination and development of the plant. Another classification of seed proteins devised by Osborne is based on their solubility: water-soluble albumins (A), salt-soluble globulins (G), alcohol soluble prolamins (P) and acid/alkali-soluble glutelins (Gt) (Mandal and Mandal, 2000). According to Osborne fractionation, the main of storage proteins in lupin seeds consists of albumin and globulin in a ratio of one to nine (Blagrove and Gillespie, 1975). The other fractions, prolamine and gluteline were found in low proportion (Duranti et al., 2008). The globulins consist of 75% of the total protein and consist of α, β, γ and δ- conglutin (Duranti et al., 2008). The main characteristics of the type of globulins in different lupin seeds are summarised in Table 2-3.

The protein globulin of α - conglutin in lupin has sedimentation coefficients of 11-12S and is called "legume-like" globulins. The α - conglutin has similar chemical structure with glycinine in soybean and legumin A in pea. In lupin, it is present at about 35-37% of the total globulins. Some different cultivars of lupins show structural variation of α – conglutin. For example, α - conglutin in *L. albus* consists of four main types of subunits with molecular mass 53, 60, 66, and 70 kDa, respectively and a minor subunit of 19 kDa. Upon reduction during SDS PAGE, the

four main subunits are split into polypeptide chains (31,36, 42, and 46 kDa) and a polypeptide chain (19 kDa) (Melo et al., 1994). While in *L mutabilis*, α - conglutin is composed of four main type subunits with molecular masses of 50-65 kDa and two minor type of subunits (40-42 kDa) which upon reduction produce a number of polypeptide chains and 2 polypeptide chains of 18 and 19 kDa (Santos et al., 1997). On the other hand, Duranti et al. (2008) found that the quaternary structure of α – conglutin is a hexamer in which each monomeric unit is composed of two subunits, acidic subunit (42-52 kDa) and basic subunit (20-22 kDa). α - conglutin is located in the storage vacuoles of the cotyledonary cells. The extensive degradation during germination of this protein is evidence that the protein is a storage protein, which is important for the development of a plant (Duranti et al., 2008).

Table 2-3 : The general features of the globulins in lupin storage proteins

Protein	**Lupin species**	**Size**	**Molecular mass (kDa)**	**subunits**
α- conglutin	*L. albus*	11S	53-70	4 mayor
			19	1 minor
	L. mutabilis		50-65	4 mayor
			40-42	2 minor
β- conglutin	*L.albus*	7S	12-70	3
γ- conglutin	*L.albus*	7S	42-43	1
δ- conglutin	*L.albus*	2S	4&9	2

Another globulin of lupin, conglutin β is a "vicilin-like" protein which has a sedimentation coefficient of 7S. The conglutin β is structurally similar to β-conglycinin in soybean and vicilin in pea. In *Lupinus albus*, the protein is a trimeric protein in which monomers consist of several polypeptides with molecular weights

ranging from 12-70 kDa (Duranti et al., 2008). The protein can be broken down to smaller peptides during germination. It is thought that conglutin β is also a storage globulin providing a source of nitrogen and carbon for metabolism of germinating seeds (Freitas et al., 2007). Some studies also indicated that conglutin β may have a role in reducing serum cholesterol levels (Sirtori et al., 2004). Nevertheless, a study using proteomic analysis has shown that conglutin β is the major allergen of *L. angustifolius* (Danica et al., 2008). In another legumes, β-conglycinin was also found as potential food allergen in soybean (Krishnan et al., 2009).

The smallest globulin in lupin is conglutin δ. This globulin is a protomeric protein with a sedimentation coefficient of 2S, consisting of two polypeptide chains of 9 and 4 kDa linked by disulfide bonds. Conglutin δ is a major sulphur-rich protein found in mature lupin seeds (Lilley, 1986). It is not yet known what the biological function of this lupin protein is. It is thought that the 2S subunit is also a major allergen in lupins (Monsalve et al., 2004).

The last globulin, conglutin γ has a sedimentation coefficient of 7S. In *Lupinus albus* it consists of single of subunit with a molecular weight of 42-43 kDa. The subunit is composed of two polypeptide chains linked by disulphide bonds with a molecular mass 30 and 17 kDa (Santos et al., 1997). Conglutin γ is a glycosylated protein which has lectin activity and is contained in mature lupin cotyledons (Duranti et al., 1996). The protein accounts for approximately 6% of the total globulins. During the germination process conglutin γ is not degraded (Duranti et al., 2008), suggesting that conglutin γ is not a storage protein. In addition, an *in vitro* study showed that isolate type F, a fraction of lupin protein isolate that contains most conglutin γ, the isolate has ACE inhibitory activity, antioxidant activity, and bile acid binding property (Yoshie-Stark et al., 2004). Furthermore, Sirtori et al. (2004) reported that conglutin γ from *Lupinus albus* may have hypocholesterolemic properties by stimulation of LDL receptors as shown in animal studies and HepG2 cell lines. Conglutin γ extracted from *L. albus* was also able to reduce glucose levels in a hyperglycemic rat model (Magni et al., 2004). Therefore, some of lupin proteins appear to have a range of bioactive properties.

2.1.2.2 Oil

The oil content of lupin seeds varies from 5-9%, depending on the species (Table 2-2). Growth location is also a factor affecting oil content of lupin seeds, for example lupin seeds from a continental location have lower oil content (8.40%) than those from Mediterranean locations (11.08%) (Boschin et al., 2007). In particular *L. mutabilis*, it contains higher oil content (17%) than the other species (Fleetwood and Hudson, 1982) and the content is comparable with oil content of soybean (20%) (Kyle, 1994).

Lupin consists of high unsaturated fatty acids (UFA). The proportions of saturated fatty acid (SFA) and UFA in *L. albus* are 12% and 88%, respectively. The main component of SFA in *L. albus* was palmitic acid (9%) while the components in UFA were oleic acid (46%), linoleic acid (25%) and linolenic acid (12%) (Bhardwaj et al., 2004).

Another component of lupin oil is unsaponifiable compounds (UNSAP). The compounds consist of 2.1 to 2.8% of the total oil. The UNSAP have been known to contain mainly phytosterols (22.3±5.4%) and triterpene alcohols (19.4±5.1%). Furthermore, the major phytosterols identified in the lupin oil were ß-sitosterol (56±3.6%), campesterol (25.8±3.1%), and stigmasterol (10.7±1.9%), followed by Δ^5-avenasterol and Δ^{7-} stigmasterol (Hamama and Bhardwaj, 2004, Bradford and Awad, 2007). The other possible constituents of the UNSAP fraction are tocopherols (Gomaa and Ramadan, 2006) and minor phenolic compounds (Ramadan and Moersel, 2006, Ramadan et al., 2006, Miraliakbari and Shahidi, 2008). Two constituents of the UNSAP, phytosterols and phenolic compounds are bioactive compounds found in lipid fraction of lupins. Health benefits of the phenolic compounds and phytosterols are discussed in Section 2.2.3 and 2.2.4, respectively.

The high level of UFA in lupin oil further adds to the favourable nutritional composition of lupin. Overall, lupin has a favourable lipid profile with a high level of mono and polyunsaturated fatty acids and a range of sterols and other bioactive compounds.

2.1.2.3 Carbohydrates

The polysaccharides in lupin can be divided into structural polysaccharides such as cellulose, hemicellulose, lignin and pectins and non-structural polysaccharides such as galactose, and arabinose (Petterson, 1998). All the polysaccharides are non-starch polysaccharides (NSP), undigestible polysaccharides commonly known as dietary fibre. In lupin hulls, the proportion of cellulose, hemicellulose and lignin are 83-87%, 7-10%, and l7%, respectively. While, in the flour (cotyledone), hemicellulose is the major component (56%), followed by cellulose (36-44%) and lignin (3-8%), respectively (Gorecka et al., 2000). In cotyledone of lupin seeds, the amount of the NSP (dietary fibre) is around 30% per dry weight of the cotyledone (Petterson, 1998). The dietary fibre in lupin is observed higher than in soybean (Jayasena and Quail, 2004). On the other hand, the starch or digestible polysaccharide content of lupin is less that 1% (Martínez-Villaluenga et al., 2006, White et al., 2002).

In the analysis of dietary fibre, procedure for crude fibre analysis only measures a small part of components of total dietary fibre. Thus, results of crude fibre analysis do not provide information of all components of dietary fibre. Furthermore, the neutral detergent fibre (NDF) analysis only provides a measurement of insoluble dietary fibre (IDF) but it does not measure the soluble components of the dietary fibre. While the acid detergent fibre (ADF) analysis only measures the cellulose and lignin (Southgate, 1977, Asp et al., 1983). Table 2-2 shows the content of crude fibre and NDF and ADF of lupin species. As shown in the table, *L. angustifolius* has higher crude fibre, ADF and NDF than those in *L albus* and *L. luteus*.

Animal and human studies have found that the dietary fibre from lupin has an important role in appetite suppression (Lee et al., 2006b), blood glucose level reduction (Magni et al., 2004, Johnson et al., 2003), reduction of cardiovascular disease risks (Sirtori et al., 2004, Martin et al., 2005, Bettzieche et al., 2008, Hall et al., 2005) and improvement of bowel health (Johnson et al., 2006, Smith et al., 2006). Health benefits of lupin are discussed in Section 2.2.

2.1.2.4 Antinutritional factors

Antinutritional factors (ANFs) are antinutrient compounds found in legumes. The ANFs include alkaloids, phytate, saponins, tannins, and trypsine inhibitors. The main ANFs in lupin are alkaloids of which most are member of quinolozidine group, for example lupanine, 13-hydroxylupanine,and sparteine (Petterson, 1998). The alkaloid level varies among lupin species. Bitter lupin such as *L. termis* has higher alkaloid level rather than sweet species. Sweet lupin contains alkaloids of less than 0.02%, whereas the bitter contains 0.5 to 4 % alkaloid. Alkaloids cause unpalatable taste in legume seeds (Petterson, 1998). However, these components can be reduced during food processing such as boiling and soaking.

Other ANFs in lupins are phytates which are in a range of 0.79 – 0.96 %, tannins (< 0.01%), saponins (0.057%) and protease inhibitors (0.013 – 0.029%). ANFs in sweet lupins are lower than those in soybean as shown in Table 2-4 (Kyle, 1994, Petterson, 1998). In such, the low ANF of lupins increases the acceptance in human diet.

Table 2-4 : ANFs content in some lupin species

Components	***L. albus*** **(%)**	***L. angustifolius*** **(%)**	***L. luteus*** **(%)**	**Soybean (%)**
Total alkaloids	< 0.01	0.02	0.05	-
Saponin	nd	0.0573	-	1.9
Trypsin inhibitors	0.013	0.024	0.029	1.79
Phytate	0.79	0.58	0.96	1.59

Sources: Petterson (1998); Kyle (1994), nd: not detected

2.1.2.5 Bioactive compounds

Phenolic compounds and phytosterols are the main bioactive compounds in lupins. The bioactive compounds are discussed in this section.

2.1.2.5.1 Phenolic compounds

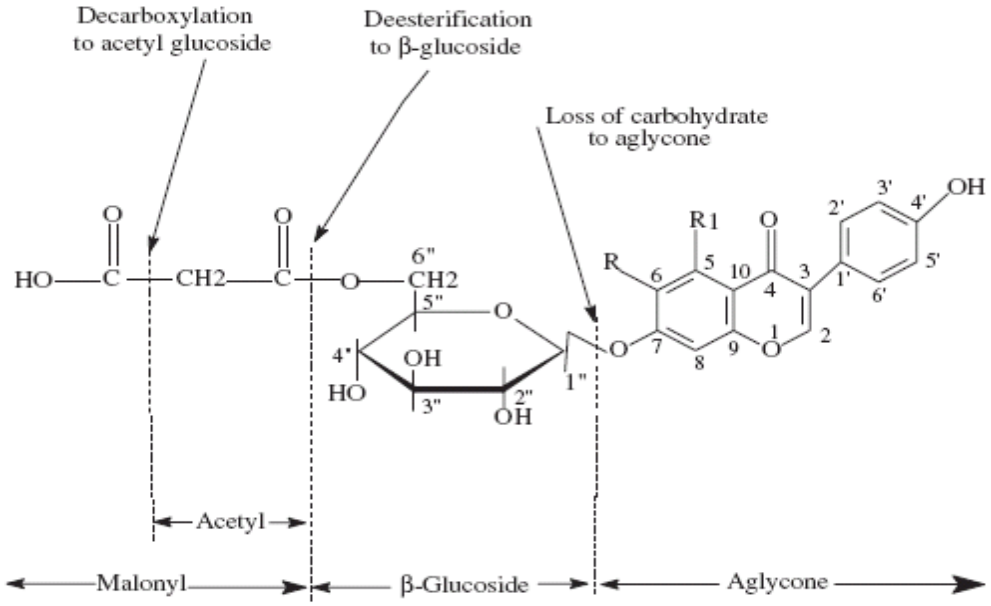
Phenolic compounds are plant derived compounds which have a hydroxyl group (-OH group) bound to an aromatic ring (Cseke, 2006). These compounds are secondary metabolites that are synthesized by plants in response to stress conditions such as infection, wounding and ultra-violet radiation (Naczk and Shahidi, 2004). The compounds can be categorized into simple phenols (p-hydroxybenzoic, vanillic, salicylic and gallic acids), phenol ethers (phenylpropanoids, caffeic and p-coumaric acids), flavonoids (chalcones, flavones, flavonols, flavanones, anthocyanins and isoflavones), tannins and quinone (Naczk and Shahidi, 2004). Many studies found that the phenolic compounds exhibit not only antioxidant activity but also insecticidal, antifungal, estrogenic activity, anti-inflammatory, antiallergic, antithrombrotic, antiviral, anticarcinogenetic and vasodilatory action (Soobrattee et al., 2005, Cseke, 2006).

Phenolic compounds are the most abundant groups of secondary metabolites in legumes (Amarowicz et al., 2004, Duenas et al., 2009). Their content depends on the species, variety, location and maturity of the legumes. *L. angustifolius* grown in France has total phenolic contents (TPCs) in a range 1190 to 1400 mg catechin equivalent/100 g lupin of which half are flavonoids (Oomah et al., 2006). While some lupin species in Western Australia including ASL have been reported to contain TPCs in a range of 374.4 to 2660.4 mg gallic acid equivalent/100g lupin, details of their chemical structure have not been determined yet (Wang and Clements, 2008). A Brazilian lupin cultivar, *L. mutabilis* has TPCs from 9.8 to 87, 16.1 to 30.8 and 1.3 to 6.1 mg/100g fresh weight for seed coat, cotyledon and hypocotyl, respectively (Ranilla et al., 2009). The studies showed that TPCs in lupin is comparable with TPCs in soybean (2200 - 6900 mg/100g) (Ok et al., 2009), suggesting that lupins are potential source of TPCs.

Understanding of the chemical structure of TPCs is important in their development as pure ingredient in functional foods and as pharmaceutical agent. Some studies have characterized phenolic compositions of lupins. Phenolic constituents of *L angustifolius* var Zapaton have been grouped into four different compound families, namely flavones (76% of identified phenolic), hydroxybenzoics (18%), isoflavones (4%), hydroxycinnamics (1.2%), and dihydroflavonols (0.4%), respectively (Duenas

et al., 2009). The main identified flavones in the groups are glycosides of luteolin, apigenin and diosmetin. In the isoflavone group, genistein derivatives and genistein aglycon are the main identified phenolic compounds that have also been observed in soybean (Duenas et al., 2009). Other phenolic compounds such as hydroxybenzoic acids, aldehydes, hydroxycinnamic acids, flavonol glycosides, flavan-3-ols and procyanidins were also found in other legumes such as beans, lentils and peas (Lopez-Amoros et al., 2006).

Isoflavones, flavones, flavonones, comestans, lignans, and stibens are examples phytoestrogen compounds which have estrogenic activity. Due to their health benefits, isoflavones in legumes have been the focus of many studies. The chemical structure of isoflavones varies in a wide range among legumes depending on their species, variety, and location. Some studies have reported chemical structure of isoflavones in lupin. White lupin contains isoflavones similar to soybean including genistin (7-O-glycoside), malonylgenistein (6"O –malonyl-7-O-glycoside), 4'-O-glycoside, 2'-hydroxygenistein and 2'-hydroxygensitin (Sirtori et al., 2003). Another study carried out by Berdarek et al. (2001), found that *L. albus* contains 4 isoflavonoid aglycones, 14 isoflavonoid glycosides, 4 flavonol glycosides and flavone glycoside (Bednarek et al., 2001). Chemical structure of legume-derived isoflavones is shown in Figure 2-3.



	R	R1
Daidzein	H	H
Genistein	H	OH
Gylcitein	OCH3	H

Figure 2-3 : Chemical structure of legume-derived isoflavones (Faraj and Vasanthan, 2004)

2.1.2.5.2 Phytosterol compounds

Phytosterols are bioactive compounds found in the UNSAP lipid fraction. The sterols are natural components of plant cell membranes that are abundant in vegetable oils, nuts, seeds and grains (Ryan et al., 2007, Quilez et al., 2003). Plant sterols and their saturated derivatives (stanols) are analogues with cholesterol due to the similarity of their chemical structure (Figure 2-4). Their chemical structure differs from cholesterols on a methyl or ethyl in the side chain at C24 and or a double bond at C22 (Chen et al., 2008, Brufaua et al., 2008, Weingartner et al., 2009). The main sterols of some plant oils and their proportion are β-sitosterol (50-60%), campesterol (10-40%) and stigmasterol (0-35%), respectively (Phillips et al., 2002, Lagarda et al., 2006, Bradford and Awad, 2007). In plants, they can occur as free sterols, steryl esters, steryl glycosides and acylated steryl glycosides (Figure 2-5).

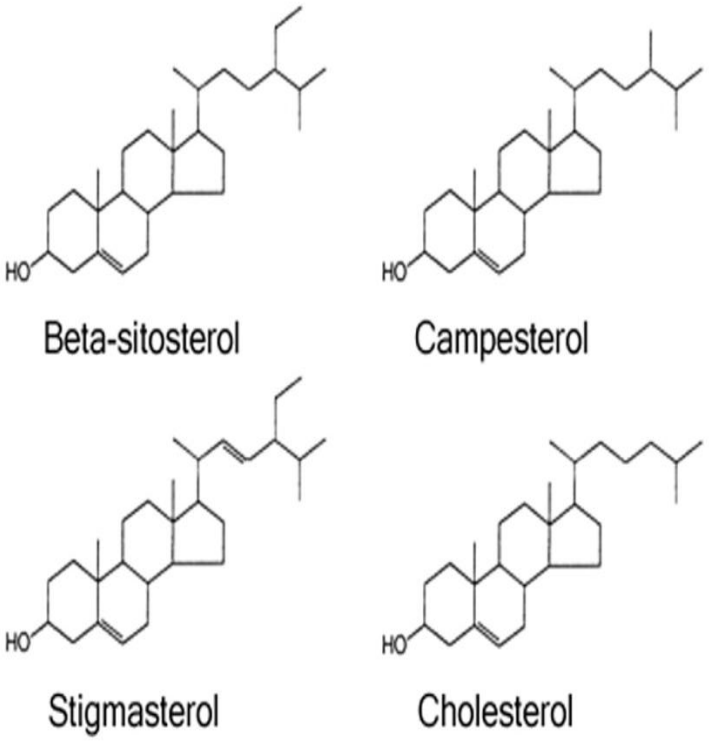


Figure 2-4 : Structure of phytosterols and cholesterol found in foods (Bradford and Awad, 2007)

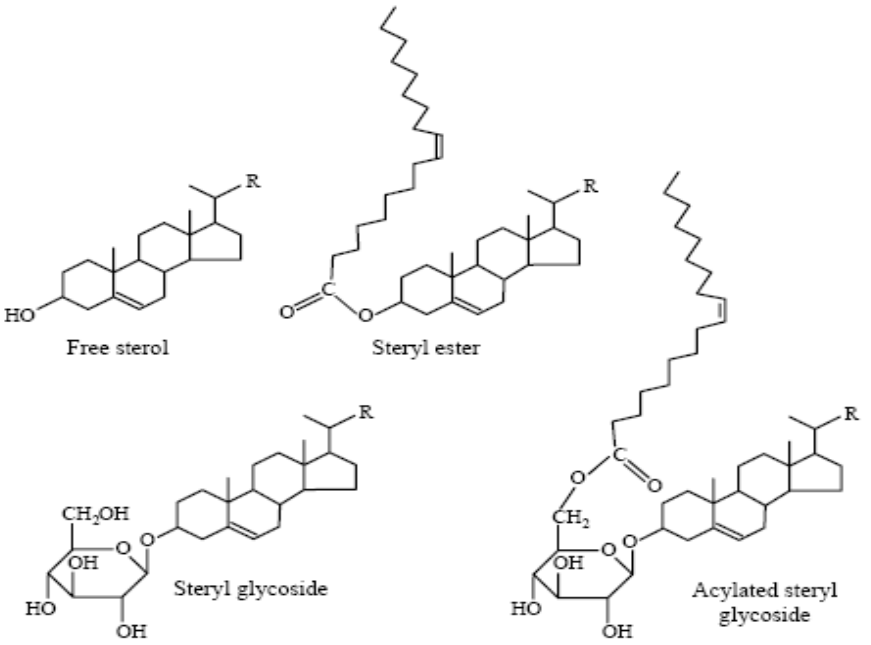


Figure 2-5 : Basic structure of free sterol and steryl conjugates. R (side chain) (Toivo et al., 2001)

The amount of oil present in plants depends on their cultivar and growing location. Therefore, it is likely that the content of the lipid soluble bioactive compounds such as phytosterols will differ (Hamama et al., 2003, Maatta et al., 1999). Table 2-5 shows a variety content of phytosterols in some edible oils from several plants.

Table 2-5 : Phytosterol contents of edible oils

Edible oils	Phytosterol content (mg/100g)
Corn oil	809-1557
Sunflower oil	374-725
Soybean oil	229-459
Olive oil	144-150
Palm oil	71-117

Source: Piironen et al. (2000)

There is limited information about phytosterol components in different lupin species, including the information about phytosterol compounds of ASL. However, composition of phytosterols present in white lupin has been reported by Hamama and Bhardwaj (2004). They found that sterols present in *L. albus* in descending order were β-sitosterol, campesterol, stigmasterol, Δ^5-avenasterol and Δ^7–stigmastenol.

As reviewed in many studies, the main health benefit of phytosterols of plant oils is hypocholesterolemic properties as discussed in Section 2.2.4. In addition, phytosterols have been reported to have a contribution in reducing the risks of some cancer such as colon, breast and prostate cancer (Bradford and Awad, 2007). Also, phytosterols have been found to have antioxidant activity in lipid oxidation process (Wang et al., 2002b, Hidalgo et al., 2009).

2.1.3 Application of lupin in food products

The high protein and dietary fibre and the low fat and starch contents of lupin make it an attractive food ingredient. Therefore there is growing interest in its use in food applications. In fact, lupin has been added into various foods in an effort to make them healthier and to improve the nutritional values of these lupin-incorporated food products.

Fermented foods such as tempe, miso and soy sauce are traditional foods commonly consumed in Asian countries. The main ingredient of these fermented foods is soybean. Tempe, originated from Indonesia is usually prepared by fermentation of soybean with mould *Rhizopus oligosporus* for 2 days at 30-38°C (Petterson, 1998). However, the use of soybean in making tempe has been able to be substituted by lupin and the resulted product was found to be accepted by both Indonesian and Australian panellists (Fudiyansyah et al., 1995). Substitution of soy to lupin in the ratio of 60:40 in preparing tempe was also comparable with the 100% soy tempe in term of the sensory qualities (Jayasena et al., 2007).

Noodles, pasta, and spaghetti can also be fortified with lupin flour, lupin protein isolate and lupin fibres. The nutritive value of instant noodles was increased by

addition of lupin flour up to 20% without any change in the sensory acceptances (Jayasena et al., 2008). When pasta was prepared by adding 6.1% lupin kernel fibre, the overall acceptability was not significantly different to the control (Clark and Johnson, 2002). The colour and rheological feature of spaghetti enriched by lupin protein isolate at 5% was also comparable to the control samples (Doxastakis et al., 2007).

Lupins also offer benefits to the bread making industries in producing products with higher nutritive values and health benefits. Substitution of wheat flour with lupin flour at level up to 5% did not affect the loaf volume and crumb structure of bread (Pollard et al., 2002). Another product, muffins incorporated with lupin flour at up to 20% were acceptable by panellists (Jayawardena, 2006), although according to Clark and Johnson (2002), addition at up to 6% of lupin kernel fibre reduced the overall acceptability of muffins.

Milk, milk-based products, ice-cream and tofu are other potential food applications for lupin. In tofu manufacturing, soybean can be replaced by up to 40% lupin, resulting in tofu with similar quality and acceptability as the control tofu containing 100% soybean (Jayasena et al., 2010). In ice cream formulation, lupin protein isolate has been incorporated to replace 100% skim milk powder and 50% egg in the standard ice cream formulation. Similarity between the sensory, chemical and physical quality of ice cream containing lupin protein isolate and control ice cream was also observed (Williams et al., 2007). In addition, yoghurt, made from *L. campestris* milk has been well accepted by panellists (Jimenez-Martinez et al., 2003).

Lupin protein isolate has been known to have similar functional properties with those of soy protein isolate (Jayasena et al., 2005). The unique functional properties of lupin protein isolate including emulsifying activity, emulsion stability, foaming capacity and foam stability make the protein isolate suitable for substitution in various food formulations. Some of the potential food applications of lupin protein isolate include dairy products, cream desserts, salad dressing and sausages (Sipsas, 2008, Jayasena and Quail, 2004).

Lupin-based biscuits and crisps have been developed to improve their nutritional values. By incorporating 70% lupin flour in the making of crisps, the content of protein and dietary fibre of lupin-based crisps is much higher than traditional potato chips. Biscuits containing 20% lupin flour also contain more protein and dietary fibre than in the standard formulation (Jayasena et al., 2009).

There are many evidences that lupin is a potential food ingredient that can be applied in a variety of food formulations. However, application of germinated ASL flour in food application is unavailable. It has been proposed in this study that the nutritive value and the bioactive compounds present in the germinated ASL flour can be enhanced by germination. The improvement in the nutritive value and bioactive compounds in the germinated ASL flour would make the flour more attractive than ungerminated lupin flour. These benefits will provide many opportunities for germinated ASL flour to be applied in a variety of food formulations.

2.1.4 Germination

Germination is the sprouting process of seeds and is the beginning of the development of seeds into a plant. The germination process has been used to overcome some disadvantages of legumes such as flavour and odour. The technique is low cost and simple way to improve the quality of legumes (Zielinski, 2002). The germinated legumes have been widely consumed as uncooked, boiled and fried snacks in some countries (Mostafa and Rahma, 1987). Furthermore, germinated legumes have been applied to substitute wheat flour in bread preparation (Morad et al., 1980) and to supplement semolina in pasta production (Torres et al., 2007).

The benefits of germination to enhance the quality of legumes have been confirmed by some studies. These include an increase in protein content of pigeon peas (Torres et al., 2007, Oloyo, 2004), soybean (Mostafa and Rahma, 1987), beans, lentils and peas (Vidal-Valverde et al., 2002) as well as the concentration of phenolic compounds and phytosterols of soybean, lentils, peas and lupins (Bau et al., 2000). Lipoxygenase activity and ANFs associated with the undesirable flavour and odour of legumes were reduced during the germination process (Zhu et al., 2005).

2.1.4.1 Stages of germination

Germination of seeds begins with the uptake of water by dry seeds under appropriate conditions followed by embryo expansion. The process of the uptake of water is triphasic with a rapid initial uptake or imbibition (phase I), followed by a plateau phase (phase II) and a further increase of uptake (phase III) (Bewley, 1997, Finch-Savage and Leubner-Metzger, 2006). As shown in Figure 2-6, following imbibition, the water causes solute leak in dry seeds and an increase in respiration. Metabolic activities such as Kreb's cycle enzymes, synthesis of protein/enzymes, repairing and synthesis of mitochondria are then activated. Proteins are synthesized using ribosome (mRNAs) or new mRNAs present within the cells of seeds. Proteins and enzymes produced at this stage are the proteins required for support of cellular metabolism. During the second phase, major metabolic events occur to prepare for radicle emergence. Emergence and elongation of radicle (Figure 2-7) completes germination process in phase III (Finch-Savage and Leubner-Metzger, 2006, Bewley, 1997).

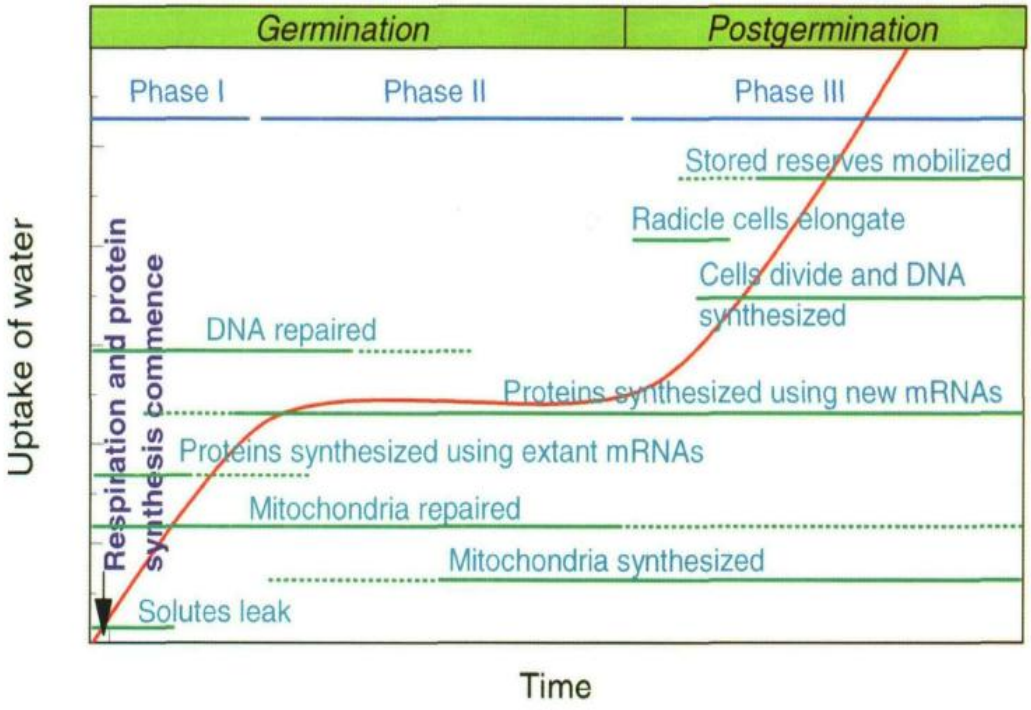


Figure 2-6: Triphasic of major activities in germination process.

Rapid water uptake (phase I) is followed by slower water uptake in lag phase (phase II), and a further increase in water uptake (phase III) (Bewley, 1997)

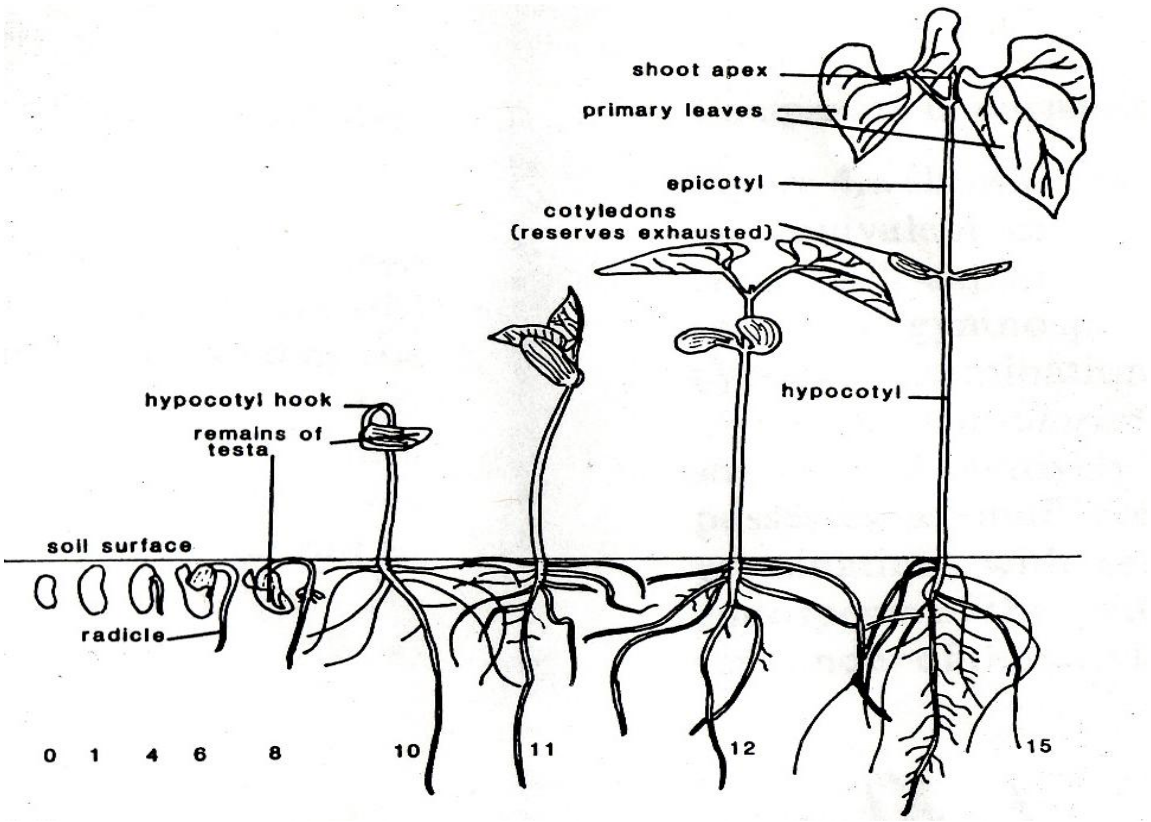


Figure 2-7 : The course of germination and seedling growth of a seed.

Following imbibition in early stage of germination, radicle emerges and hypocotyl grows. The hypocotyl then elongates, followed by opening out of cotyledon and appearance of leave (Bradbeer, 1988)

2.1.4.2 Factors affecting germination

2.1.4.2.1 Water

Water is required for imbibition of seeds during germination (Bradbeer, 1988). Steeping in water is an important treatment for germination (Briggs, 1998). Water stress with water potential between -0.25 to -1.00 MPa was found to affect the germination rate of *Prosopis caldenia* especially at high temperatures (37ºC or higher). Radicle and hypocotyle elongation of the seeds were diminished by the water stress (Villalobos and Pelaez, 2001). In another report, excessive amounts of water were found to inhibit germination rate (Bradbeer, 1988).

2.1.4.2.2 Oxygen

The uptake of oxygen and release of carbon dioxide occurs during respiration of germinating seeds. Oxygen plays an important role in the respiration process as an electron acceptor. The high respiration rate produces heat during germination, therefore air circulation and cooling equipment are necessary to manage temperature during germination. The supply of oxygen is essential because deficiency of the oxygen uptake will inhibit the germination process, and lead to seed death. Inhibition of respiration of the germinating seeds can release toxic products to human such as acetaldehyde, ethanol and lactate (Bradbeer, 1988).

2.1.4.2.3 Temperature

Temperature is another factor that determines the rate of seed germination (Villalobos and Pelaez, 2001, Kebreab and Murdoch, 1999). Germination of each species occurs optimally over a range of specific temperatures. For example *L. montanus* at temperature 15-20ºC (Acosta-PercÁStegui and RodrÍGuez-Trejo, 2005) and *Prosopis caldenia* at temperature between 25-30ºC (Villalobos and Pelaez, 2001). Low temperature can damage embryos or germinating seeds and prevent germination completion. On the other hand, high temperature inhibits the development of embryos and hypocotyle (Bradbeer, 1988, Villalobos and Pelaez, 2001).

2.1.4.2.4 Seed moisture and relative humidity

There is association between seed moisture content and relative humidity in the surrounding air (Figure 2-8). For example, once the relative humidity of seeds reaches 70%, the moisture content of seeds achieves about 13%. At this point, respiration of the seed increases, as consequently the seeds commence germination (McCormack, 2004). Therefore, germination of seeds is conducted at high relative humidity at about 90-99% (Lopez-Amoros et al., 2006, Kuo et al., 2004).

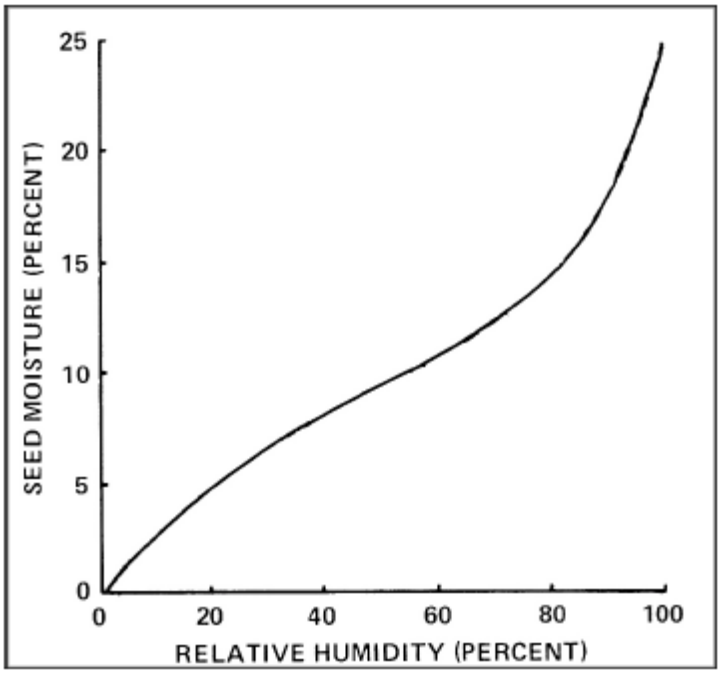


Figure 2-8 : Relationship of vegetable seed moisture content to relative humidity.

(McCormack, 2004)

2.1.4.3 Effects of germination on chemical compositions

Germination of seeds alters the chemical composition of seeds due to synthesis, degradation and transformation of biomolecules for the development of the plant. The compositional changes may affect the nutritive value, acceptability and health benefits of the germinated seeds.

2.1.4.3.1 Effects of germination on macronutrients and storage proteins

Effects of germination on protein, lipid and carbohydrate of legumes have been widely observed by some researchers. For example, total protein contents of soybean, fenugreek, lentils and peas have been reported to increase following a few days of germination (Mostafa and Rahma, 1987, Zielinski, 2003, Ghavidel and Prakash, 2007, Martinez-Villaluenga et al., 2008, Morad et al., 1980). Soluble protein, protein digestibility and essential amino acids of soybean, pigeon beans and peas were also improved by germination (Mostafa and Rahma, 1987, Martinez-Villaluenga et al., 2008, Sangronis and Machado, 2007). The improvement in protein

digestibility and essential amino acids results in enhancement of nutritive value and protein quality of the legumes.

The change in macronutrients of *L. angustifolius* following germination has been studied by Dagnia et al. (1992) as shown in Table 2-6. The study pointed out that the increase in protein contents did not affect the protein quality (indicated by its protein efficiency ratio) of the lupins.

Table 2-6 : Chemical composition and antinutritional factors of *L angustifolius* cv Gungurru kernels and sprouts (percentage of dry basis)

Components	Kernels	Sprouts (day 6)
Protein	39.50	45.31
Fat	8.20	4.10
Ash	2.55	3.98
Carbohydrate	38.55	33.21
Oligosaccharide	5.90	0.30
Phytate	0.47	0.16
Alkaloid (total)	0.072	0.016

Source: Dagnia et al. (1992)

Storage proteins are broken down to smaller molecular weight proteins during germination. Some of the storage proteins of *L. termes* have been known to breakdown and disappear within the 3 days of germination, yielding smaller peptides and amino acids that are important for the synthesis of enzymes during development of seeds. At the same time, some proteins appeared within 3 days of germination as a result of the synthesis. Meanwhile, some proteins remained stable during germination due to its importance in maintaining the structure of cells (Ahmed et al., 1995).

Among storage proteins of lupins, conglutin β is degraded first during germination, followed by conglutin α and δ. However, conglutin γ was not degraded during

germination period due to its resistence to proteolysis by trypsin and endopeptidase (Duranti et al., 1984). Furthermore, conglutin γ was found in high level of the roots of young germinating lupin seeds after 5 to 7 days of germination (Duranti et al., 1996). The studies suggested that germination may reduce two types of storage proteins, conglutin α, and β which were reported as the major allergens in lupins. On the other hand, germination may increase the bioactivity and health benefits of conglutin γ as will be discussed in Section 2.2.

Germination also reduces the lipid level in lupin. As shown in Table 2-6, lipid content in lupin sprouts of day 6 was reduced by about 50%. The reduction in fat content following germination was also observed in mung bean (Mubarak, 2005) and sunflowers seeds (Balasaraswathi and Sadasivam, 1997). The reduction could be attributed to utilization of fat as an energy source throughout germination.

The carbohydrate composition of legumes is also affected by germination. As shown in Table 2-6, oligosaccharide contents of legumes were reduced during germination (Donangelo et al., 1995, Dagnia et al., 1992, Cuadra et al., 1994). The oligosaccharides, in particular α-galactosides that are believed to contribute to flatulence after eating legumes, also diminished. Meanwhile, soluble carbohydrates such as glucose, fructose and sucrose in lupins (Donangelo et al., 1995) and pigeon pea (Torres et al., 2007) were increased by germination. Non-starch polysaccharides (NSP), dietary fibre in lupins and soybean, showed a small increase in content following 48-h germination. In addition, the starch content of lupins rose in low amount compared to black beans (Donangelo et al., 1995).

2.1.4.3.2 Effects of germination on bioactive compounds

The TPCs are also modified by germination. Germination resulted in an increase in TPCs in *L. angustifolius* L. var Zapaton (Fernandez-Orozco et al., 2006), soybean, beans, peas, lentils (López-Amorós et al., 2006, Kim et al., 2006) and black soybean (Lin and Lai, 2006). The maximum increase of TPCs of *L. agustifolius* L. var Zapaton was observed at day 9 of germination. The increase accounted for 53% when expressed as mg of catechin (Fernandez-Orozco et al., 2006) and 63 % when expressed as mg of gallic acid (Duenas et al., 2009).

Change in phenolic compounds of *L angustifolius* L. var Zapaton during germination has been studied intensively by Duenas et al. (2009). The hydroxycinnamic compounds (p-coumaric and ferulic acids) that represent 1.2% of TPCs in lupin were increased by 91% at day 9 of germination. The other phenolic compounds with flavone group such as luteolin and diometin derivates were also increased by 86% (Duenas et al., 2009). Isoflavones, the most known phenolic compounds in legumes, of the lupin were also increased during the germination process with the highest (95%) value at day 9 (Duenas et al., 2009). These compounds were identified as genistein derivates. Another study conducted on *L. albus* observed that isoflavones content increased following germination with the highest content occurring at day 2-4. The study suggested that there was an increase in the biosynthesis of isoflavonoids during 2-4 days of germination (Gagnon et al., 1995). Furthermore, Ferrer et al. (1990) observed that the main isoflavones in the hypocotyl of germinating *L. albus* were genistein, 2'-hydroxygenistein, lutein, wighteone and lupisoflavone.

Similarly, isoflavone composition of soybean was changed following soybean germination. The isoflavones which increased during germination were 6"O -malonylgenistein and 6"O-malonyldaidzin (Zhu et al., 2005). Meanwhile, based on Yu et al. (2007), the greatest isoflavone content in germinating soybean were β-glycosides (glycitin, daidzin) and three malonyl glycoside (such as 6"O –malonylgenistein and 6"O-malonyldaidzin). Most of the isoflavones are concentrated in the soybean hypocotyl (Faraj and Vasanthan, 2004).

The phenolic components were considered as an important group of bioactive compounds which play an important role in antioxidant activity. As a consequence, there was a significant increase in their antioxidant activity after germination in term of the DPPH radical scavenging capacity, total antioxidant capacity (TEAC), peroxyl radical-trapping capacity (PRTC), SOD-like activity and inhibition of phosphatidylcholine (PC) peroxidation (Zielinski, 2003, López-Amorós et al., 2006, Tsaliki et al., 1999, Lin and Lai, 2006, Duenas et al., 2009). All these studies confirmed that there wass great variation in the composition of phenolic compounds and their antioxidant activity during germination among different species of legumes, particularly in lupins. The studies also suggested that germination is a simple way to improve phenolic contents in lupin. Although changes in the levels of phenolic

compounds during germination of some *Lupinus* sp. have been investigated, no information was available about how these changes were associated with the bioactivity of ASL seeds throughout the germination process.

There is limited data on the effects of germination on phytosterol compounds in legumes, especially in lupins. However, a report by Zhang et al. (2007) showed that phytosterol contents in germinated Canola seeds increased after 20 days of germination. As phytosterols have hypocholesterolemic property, it is necessary to investigate the change in phytosterol contents during the germination period of ASL.

2.1.4.3.3 Effects of germination on sensory quality and ANFs

Germination negatively affects the overall acceptability of soybean sprouts due to the bitter taste caused by high phenolic contents after germination process. Consequently, pea sprouts were found to have better sensory quality than soybean (Troszynska et al., 2007). As reviewed by Petterson (1998), lupin sprouts have also been reported to have sensory acceptability comparable to soy and mung bean sprouts. The acceptance of lupin sprouts suggests lupin sprouts as source of ingredients in food applications. It is therefore necessary to investigate further on the health benefits of lupin sprouts.

In addition, the content of antinutrient compounds including phytic acids (Cuadra et al., 1994), trypsin inhibitors (Frias et al., 1995) and alkaloids (Dagnia et al., 1992) decreased following germination. Nevertheless, a study found that germination of lupin for more than 3 days resulted in the transformation of alkaloid to alkaloid esters which were toxic for human (Sanchez et al., 2005). This transformation is not likely to be a problem in ASL due to the low alkaloid content in ASL.

2.1.4.3.4 Effects of germination on bioactivities of bioactive compounds

The concentration of phenolic compounds and their antioxidant activity in grains and legumes can be increased by germination. The increase of phenolic compounds and antioxidant activity due to germination has been reported in soybeans (Zielinski, 2003), black soybeans, azuki beans, mungbeans (Lin and Lai, 2006) and common beans (Diaz-Batalla et al., 2006). However, information about the effect of germination on compounds such as bioactive peptides, phytosterols and other bioactive compounds in lupin that are associated with their bioactivities is unavailable. Nevertheless, some research using other legumes and grains has been conducted to investigate the potential of germination on improvement of bioactive compounds and their bioactivity. In soybean, lunasin protein associated with chemopreventive effect were found to increase significantly after germination for 2 days (Paucar-Menacho et al., 2010). In another studies, wheat, buckwheat, corn and oats sprouts treated with thermal processing were reported to have beneficial effects *in vitro* on type 2 diabetes by inhibition of α-amylase and α-glucosidase and other effects such as antihypertensive (by the ACE-inhibition) and antibacterial (Randhir et al., 2008). Furthermore, germination with elicitors of the pentose phosphate pathway on velvet bean (Randhir et al., 2009) and pea (Burguieres et al., 2008) had beneficial impacts on levo-dihydroxy phenylalanine (L-DOPA) which is important for the management of Parkinson's disease and inhibition of ACE and α- amylase (Randhir et al., 2009).

2.2 Health benefits of lupin

Components of lupin may help to prevent a wide range of risk factors associated with metabolic syndrome, cardiovascular disease, bowel diseases, and cancer.

2.2.1 Metabolic syndrome

There are different definitions of metabolic syndrome according to World Health Organization (WHO), the National Cholesterol Education Program Adult Treatment Panel (NCEP ATP III) and the International Diabetes Federation (IDF). However, based on the definitions, there are 4 key factors affecting the occurrence of metabolic

syndrome. They are dyslipidemia, abdominal obesity, hypertension (high blood pressure) and insulin resistance (Nagao and Yanagita, 2008, Sarti and Gallagher, 2006, Fonseca, 2005). Obesity is the major factor causing metabolic syndrome. Insulin resistance is then a consequence of an accumulation of fat caused by abdominal obesity. Abdominal obesity and insulin resistance subsequently induce development of type 2 diabetes and hypertension. All of those are risk factors that lead to occurrence of cardiovascular disease (CVD) (Sarti and Gallagher, 2006, Fonseca, 2005). People with the metabolic syndrome have higher cardiovascular mortality than people without the syndrome (Isomaa et al., 2001).

According to WHO (1999) obesity is classified based on the body mass index (BMI) and is defined as a BMI of or greater than 30 kg/m^2. Criteria for elevated blood pressure in metabolic syndrome equals to or more than 140/90 mm Hg. Whilst dyslipidaemia is categorized by elevated triglyceride equals to or more than 1.7 mmol/L and low HDL-cholesterol at less than 0.9 mmol/L for men and less than 1.0 mmol/L for women. In another classification based on NCEP ATP III, waist circumferences greater than 102 cm for men and greater than 88 cm for women and blood pressure greater than 130/85 mmHg are associated with obesity and high blood pressure. Insulin resistance is categorized as hyperinsulinaemia or impaired glucose regulation (Sarti and Gallagher, 2006, Eckel et al., 2005).

2.2.2 Effects of dietary lupin on metabolic syndrome

Prevalence of obesity in childhood and adolescence has increased significantly in the past two decades and is a major health problem worldwide (Gortmaker et al., 1993, Swallen et al., 2005). One of the approaches to prevent weight gain is by dietary intervention using foods containing high protein and fibre that increases satiety and reduces energy intake acutely (Hodgson and Lee, 2008).

Effects of diets containing lupin have been observed to increase satiety and to reduce energy intake acutely. For example fat replacement with lupin kernel fibre in a sausage patty was associated with increased post meal satiety compared to those consuming control (full fat sausage patty) despite the reduced energy content of the

meal (Archer et al., 2004). Furthermore, subjects consuming bread incorporated with 40% lupin flour for breakfast resulted in significantly higher satiety level and lower energy intake at lunch time compared to those consuming control (white bread) (Lee et al., 2006b). In addition, diets containing lupin have been found to reduce the risk of type 2 diabetes. For examples, in an animal study, purified conglutin γ of lupin protein reduced blood glucose levels in hyperglycaemic rats (Magni et al., 2004). In human studies, a reduction of insulinaemic response was found in subjects consuming bread with lupin fibre for breakfast when compared to those consuming white bread (Johnson et al., 2003).

Additionally, results from a number of animal and human studies have found that the intake of lupin in the diet leads to a lowering of serum cholesterol level. When high cholesterol rats were given 50 mg of lupin protein per day, plasma total cholesterol, very low density lipoprotein (VLDL) and low density lipoprotein (LDL) cholesterol were reduced by 21% and 30%, respectively. The mechanism may be mediated by upregulation of LDL receptor (Sirtori et al., 2004). High cholesterol pigs and chickens fed a diet containing *L. angustifolius* were found to have a reduction in LDL-cholesterol (Martin et al., 2005, Viveros et al., 2007). Supplementation of lupin protein in the diet of high cholesterol rabbits for 3 months also lowered cholesterol levels and reduced development of atherosclerosis when compared to those fed diet of milk protein (Marchesi et al., 2008). Furthermore, in human studies, consumption of diet containing lupin kernel fibre in healthy men was able to reduce total cholesterol, LDL-C and high density lipoprotein-cholesterol (HDL-C) compared to those consuming the control diet (Hall et al., 2005). In addition, an *in vitro* study also showed that lupin protein isolate and their hydrolysates have a property in reducing cholesterol by bile acid binding (Yoshie-Stark and Wasche, 2004).

All findings of the studies show that incorporation of lupin, its protein and fiber in foods may have beneficial effects to reduce risk factors related to metabolic syndrome and consequently the intake may also reduce the risk factors for CVD.

2.2.3 Phenolic compounds (isoflavones) and cholesterol lowering effects

Regulation of cholesterol homeostasis in human involves enzymes and transcriptional factors. It is known that 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (HMG-CoA R) is a crucial enzyme to synthesize cholesterol from acetyl CoA and acetoacetyl CoA in the liver. Up and down-regulation of expression of HMG-CoA R are regulated by sterol regulatory element binding protein (SREBP). The SREBPs are transcriptional factors that regulate not only HMG-CoA R but also the other genes such as LDL receptor, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase (Mullen et al., 2004). Expression of genes of LDL receptor and HMG-CoA R is controlled by the level of cellular free cholesterol. When the cellular free cholesterol is low, the genes are activated. On the other hand when cellular free cholesterol is high the genes are down regulated (Chen et al., 2008, Mullen et al., 2004).

Unlike lupin isoflavones, using HepG2 cells, soy isoflavones have been demonstrated to have a role in cholesterol homeostatis similar to statins (hypocholesterolemic agents) (Figure 2-9). Soy isoflavones may inhibit HMG-CoA R, resulting in low cholesterol concentration intracellular and thus inducing maturation of SREBP and up-regulating SREBP-regulated gene (Mullen et al., 2004). In another study, an incubation of HepG2 cells with genistein and daidzein was able to inhibit apoB secretion by mechanism of activation of LDL receptor (Borradaile et al., 2002). Due to the structural similarity of some isoflavones of lupin and soybean, it is suggested from the studies that lupin isoflavones may also have the similar mechanism in lowering cholesterol level with that found in soy isoflavones.

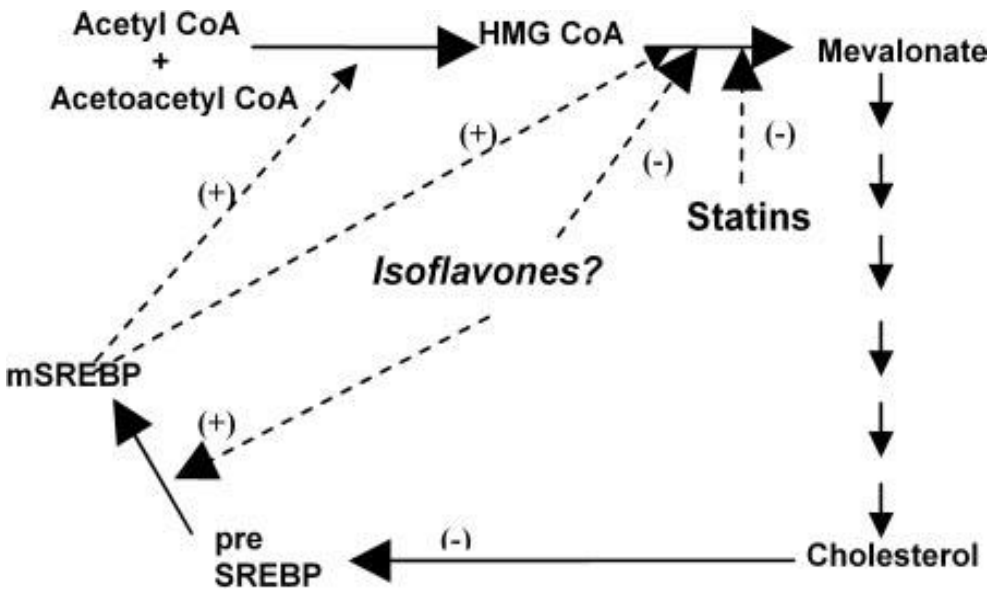


Figure 2-9 : Mechanism of cholesterol homeostasis and possible mechanism of statin and isoflavones

(Mullen et al., 2004)

2.2.4 Phytosterol compounds and cholesterol lowering effects

There is strong evidence that intake of plant phytosterols is beneficial in lowering cholesterol levels. However, the direct mechanism by which phytosterols may achieve this effect has not been completely elucidated. The possible mechanism of the health benefit is shown in Figure 2-10.

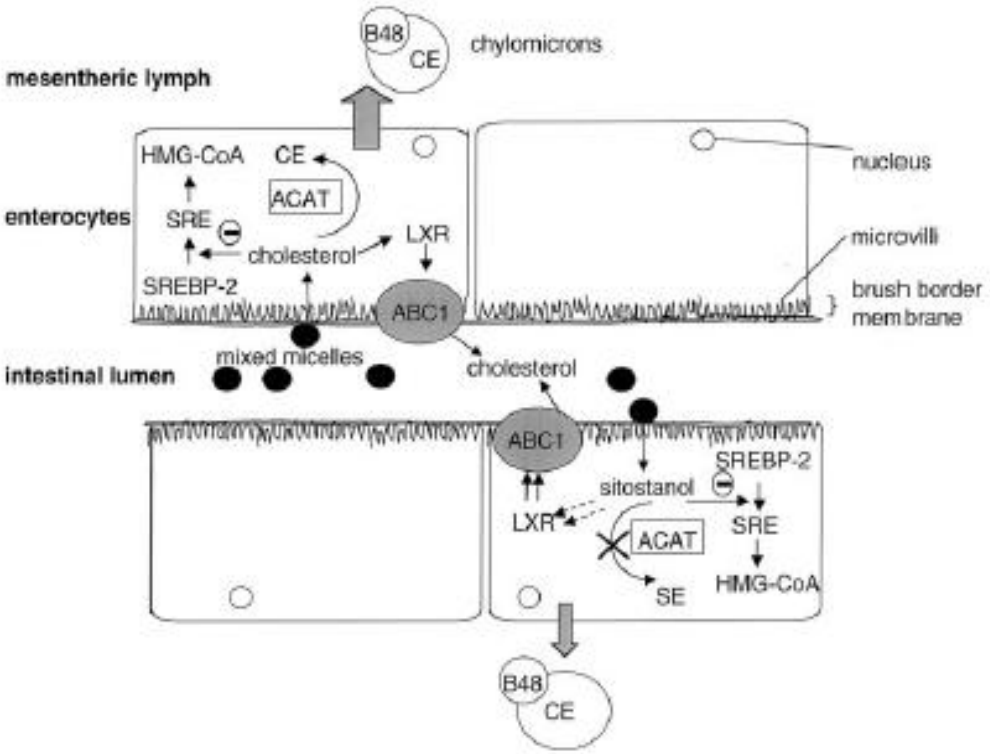


Figure 2-10 : Possible mechanism of effect of plant sterols on cholesterol metabolism

(Plat and Mensink, 2002)

The main mechanism by which phytosterols lower serum cholesterol level is by inhibition of cholesterol absorption from diet in the intestinal lumen. Normal diet contains about 200-500 mg cholesterol, 200-400 mg plant sterols and 50 mg plant stanols. Of these amount, half of cholesterol from the diet is absorbed, while only 5% of the plant sterols are absorbed (Weingartner et al., 2009, Chen et al., 2008). Cholesterol and plant sterols obtained from the diet are then taken up from micelles in the intestine lumen to be absorbed into enterocytes. This entry requires mediator Niemann-Pick C1 like 1 protein (NCP1L1) (Altmann et al., 2004). During interaction with micelles, phytosterols may competitively bind to micelles and consequently minimize cholesterol absorption (Ikeda et al., 1988, Plat et al., 2005, Jesch and Carr, 2006). In the enterocytes, cholesterol and phytosterols are esterified by the enzyme acetyl-coenzyme A acetyl transferase 2 (ACAT-2). The esterification is also possible to be inhibited by an ACAT inhibitor results in lower cholesterol absorption (Largis

et al., 1989). The esterified sterols then bind to chylomicrons (CE) to be delivered into the lymph system. Non esterified cholesterol and sterols are secreted back into the intestinal lumen via ATP binding cassette (ABC transporter), ABCA1 or via ABCG5/ABCG8 (Calpe-Berdiel et al., 2009, Plat and Mensink, 2002). ABCA1 and ABCG5/ABCG8 genes are regulated by liver X receptor (LXR) (Calpe-Berdiel et al., 2007, Plat and Mensink, 2002).

In addition to the mechanism, research has shown that intake of phytosterols may increase expression of ABCA1 (Plat and Mensink, 2002) or ABCG5/ABCG5 (Yu et al., 2002), which in turns, promotes the pumping back of cholesterol from enterocyte into intestinal lumen.

2.2.5 Angiotensin I Converting Enzyme (ACE)-inhibitor activity

ACE plays an important role in regulating blood pressure. The ACE is an enzyme that converts angiotensin I to angiotensin II. The converting reaction is by hydrolysis of the carboxyl terminal (His-Leu) of angiotensin I to produce a potent vasoconstrictor (angiotensin II). The result is narrowing of blood vessels. In addition to the activity, ACE has also a role in inactivation of bradykinin. Bradykinin is a peptide with vasodilator action that causes blood vessels to dilate (Figure 2-11) (Meng and Oparil, 1996, Fandino et al., 2006). When a component inhibits activity of ACE enzyme (ACE inhibitor), the formation of angiotensin II (vasoconstrictor) and inactivation of bradykinin are inhibited. As a result of the ACE inhibitor activity, blood pressure is lowered. Captopril, an antihypertensive drug is known to inhibit ACE activity.

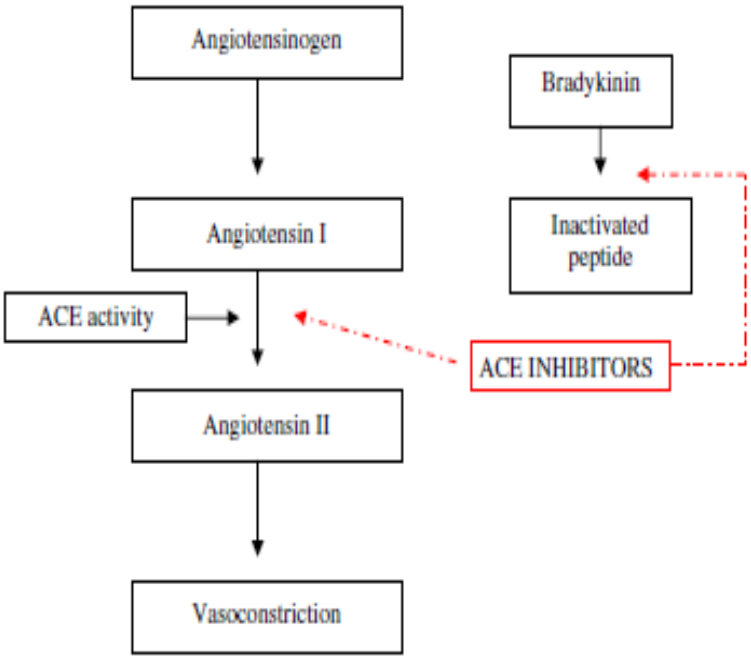


Figure 2-11 : Mechanism of ACE inhibitor activity
(Roy et al., 2009)

Peptides with the ACE inhibitory activity have been well known as a class of bioactive peptides derived from food proteins. To produce peptides with the ACE inhibitor, intact proteins from foods can be hydrolysed using intestine enzymes such as pepsin and alcalase *in vitro* (Erdmann et al., 2008). The presence of ACE inhibitor peptides has been found in various animal, and plant proteins as well as their hydrolysates. The examples include soy protein (Wu and Ding, 2002), fermented soybean (Zhang et al., 2006a, Rho et al., 2009), whey protein hydrolysate (Lourenço da Costa et al., 2007), lima and jamapa bean protein hydrolysate (Torruco-Uco et al., 2009), protein hydrolysate of hard clam (Tsai et al., 2008), oyster sauce (Je et al., 2005), potato protein (Pihlanto et al., 2008), egg white hydrolysate (Miguel et al., 2007) and shark meat hydrolysate (Wu et al., 2008). In addition to these findings, some of the ACE inhibitors have been characterized by their chemical structure and their activities *in vivo* or using animal models (Erdmann et al., 2008).

Results from *in vitro* study using *L. albus* also found that protein isolate and its hydrolysate has an ACE inhibitory activity (Yoshie-Stark et al., 2004). The study suggests that lupin is a potential source of ACE inhibitor. However, determination of the ACE inhibitor peptides in ASL has not been studied yet.

2.2.6 Bile acid binding property

Bile acid (BA) binding in the intestine is one possible mechanism leading to lowering of cholesterol levels. In humans, bile acids (BAs) are steroid metabolites synthesized in the liver from cholesterol. Their main function is acting as detergents for absorption of lipids and fat-soluble vitamins (Kahlon and Woodruff, 2002). The main BAs synthesized in the liver are cholic acid (CA) and chenodeoxycholic acid (CDCA) which are secreted into duodenum after conjugation with glycine or taurine. BAs are then reabsorbed in the intestine and transported back to liver (Kahlon and Woodruff, 2002, Debruyne et al., 2001). Inhibitors of BA absorption (sequestrants) in the intestine can bind BAs, preventing BA absorption and allowing them to go to faeces. Increase in excretion of BAs stimulates the liver to synthesise cholesterol and up-regulate expression of LDL receptor. As a result, cholesterol in the blood will be removed from circulation, thus lowering cholesterol concentration in the blood (Chen et al., 2008). Cholestyramine known as a cholesterol-reducing agent also has this BA binding property.

Dietary fibre have been reported as the component that is responsible for BA binding property (Eastwood and Hamilton, 1968). Furthermore, BA binding properties of grains, legumes and vegetables have been observed to have the properties as reported in soy, pinto beans, black beans and wheat gluten (Kahlon and Woodruff, 2002), spinach, kale, brussel sprouts, broccoli, mustard greens, green bell pepper, cabbage and collards (Kahlon et al., 2007). BA binding properties by protein was also reported in *L. albus* and soy protein (Yoshie-Stark and Wasche, 2004), and buckwheat protein (Ma and Xiong, 2009). Such observations suggest that legumes, grains and vegetables containing high fibre and protein (such as lupin) may have BA binding property. However, there is no report about this property in ASL and its sprouts. It is therefore necessary to investigate this property in ASL and its sprouts.

2.2.7 Antioxidant activity

The antioxidant properties of phenolic compounds are attributed by their functional group such as hydroxyl, glycosyl and methoxyl in their chemical structure. The functional group and the structural feature determines the antioxidant capacity of phenolic compounds (Rice-Evans et al., 1997, Cai et al., 2006, Soobrattee et al., 2005). For example or example derivatives of cinnamic acid are more active antioxidants than derivates of benzoic acid (Marinova and Yanishlieva, 2003).

In vitro studies showed that lupin and its products have antioxidant properties. Antioxidant capacity of three types of raw lupin seeds has been reported by Martinez-Villaluenga et al. (2009). They found that antioxidant activity of *L angustifolius* cv. Troll, *L. angustifolius* cv. Emir and *L. albus* cv. Multolupa measured by Trolox equivalent antioxidant capacity (TEAC) assay, DPPH radical-scavenging activity (DPPH RSA) assay and peroxyl radical traping capacity (PRTC) assay were 47 - 71, 2.83 - 3.09 and 0.71 - 1.26 μmol trolox/g dm, respectively (Martínez-Villaluenga et al., 2009). Another study carried out by Wang and Clements (2008) reported that antioxidant activity measured by DPPH RSA assay of lupin seeds also significantly varied with their variety. The study showed that *L. angustifolius* cv. Kalya had a higher antioxidant activity (0.438 mg trolox/g seeds) than other variety such as cv. Belara (0.21 mg trolox/g seeds) and Wongan Hills Telerack (0.163 mg trolox /g seeds) (Wang and Clements, 2008). Furthermore, Oomah (2006) reported that water-soluble substances of *L angustifolius* grown in Alberta exhibited antioxidant activity in a range 0.54 to 1.07 μmol trolox/g, while thet in the lipid-soluble substances range from1.9 to 3.3 μmol trolox/g (Oomah et al., 2006).

Antioxidant capacity of lupin is comparable with other legumes. It has been reported by Fernandez-Orozco et al. (2008) that TEAC of *Glycine max* cv. jutro was 37.3 μmol trolox/g dm and *Glycine max* cv. merit was 63.0 μmol trolox/g dm (Fernandez-Orozco et al., 2008). A study has also reported antioxidant activity from some legumes (Amarowicz et al., 2004). Their antioxidant activities in descending order are adzuki bean>red bean>faba bean>green lentil>red lentil>broad bean>pea, with values range from 30 – 176 μmol trolox/ g seeds.

Some studies have shown that antioxidant capacity has positive correlation with TPC content in lupin as measured by DPPH-RSA (Martínez-Villaluenga et al., 2009, Fernandez-Orozco et al., 2006, Tsaliki et al., 1999). However others have found that no such correlation existed (Wang and Clements, 2008). Antioxidant activity in lupin may be contributed by not only the phenolic compounds but also compounds such as vitamin C, tocopherols (Fernandez-Orozco et al., 2006, Fernandez-Orozco et al., 2008, Lampart-Szczapa et al., 2003) and low molecular weight peptides (Aluko and Monu, 2006). However, it is likely that most of the antioxidant activity in lupin is due to its phenolic compounds (Duenas et al., 2009, Fernandez-Orozco et al., 2006).

Phenolic compounds not only provide antioxidant activity but also provide various other activities such as chelating redox active metal ions, modulation of gene expression and interaction with the cell signalling pathways (Soobrattee et al., 2005). Such activities have strong association with their ability to prevent some diseases including cancer and cardiovascular diseases. In regards to anticancer activity, a study has shown that phenolic compounds from *L. campetris* seeds posses mutagenicity activity in 1-nitropyrene (1-NP) as a model of mutagen (Martinezy et al., 2003). This result was supported by another study using common bean that found that there was strong association between phenolic extract of the beans and their mutagenic and antioxidant activity (Cardaador-Martinez et al., 2006).

There is sufficient published information to confirm that lupins are good source of antioxidants, which are associated with protective against some degenerative diseases such as cancer and CVD. However, further characterisation of these compounds in ASL and its sprouts is required.

2.2.8 Beneficial effects in human bowel

Components of legume may stimulate growth of beneficial bacteria (prebiotics) which can modify the chemistry of the colon. As a result, the component may reduce the risk of bowel disease such as bowel cancer. Addition of lupin kernel fibre in the human diet for 4 weeks was able to improve bowel function and faecal chemistry (Johnson et al., 2006). The supplementation of the lupin kernel fibre in the diet also

increased the numbers of beneficial bacteria (*Bifidobacteria*) and reduced the number of pathogenic bacteria (*Clostridia*) (Smith et al., 2006).

2.3 Characterization and analysis of bioactive compounds in foods

2.3.1 Isolation, purification and characterization of protein

The unique functional properties and several claims of health benefits of lupin protein increase the interest of applying this protein in food formulations. Therefore, further investigation of ways to improve the yield, functional properties and health benefits whilst minimizing protein damage during protein isolation is required.

An industrial scale isolation has been developed by Wasche et al. (2001). The procedure involves protein solubilization at alkaline pH, followed by isoelectric precipitation at acidic pH. The α, β and δ –conglutin in the precipitate are separated by centrifugation, resulting in a "type E" fraction. The soluble proteins contained in the supernatant are recovered further by ultrafiltration, followed by spray drying to obtain a "type F" fraction. The F fraction mainly consists of γ-conglutin. Furthermore, a pilot scale of lupin protein isolation has been optimized to minimize thermal damage during spray drying. The study revealed that "type E" fraction yielded by the method has good emulsifying capacity, while "type F" fraction provides high foaming capacity (D'Agostina et al., 2005).

A laboratory scale fractionation of lupin protein has been developed by Sironi et al. (2005) to obtain higher purified protein level than the industrial scale. The protocol also involves alkaline extraction, but in this method, the δ –conglutin is able to be further separated from α, β-conglutin. Meanwhile, γ-conglutin contained in supernatant could be purified by selective precipitation with Zn^{2+} (Sironi et al., 2005).

Individual protein of α, β, δ and γ –conglutin can be separated further after isolation of the total globulin in seeds. A method to isolate the total globulin in seeds has been optimized by Franco et al. (1997). The method is adopted and modified from Blagrove and Gillespie (1978) and Melo (1994). In the optimized method,

ethylenediaminetetraacetate (EDTA) and ethyleneglycol-bis(aminoethyl ether)-N,N'-tetraacetic acid (EGTA) are added in the globulin extract solution in order to increase efficiency of extraction (Freitas et al., 2007). Individual globulin in the total protein extract of *L. albus* was able to be separated using Mono Q HR5/5 column of the fast-protein liquid chromatography (FPLC) (Melo et al., 1994).

Following protein isolation and purification, one-dimensional (1D) electrophoresis is a rapid, reproducible and inexpensive method applicable to determine and monitor protein in the isolated and purified proteins. Prior to electrophoresis, the protein in the sample is denatured by sodium dodecyl sulphate (SDS), an anionic detergent, to cleave non-covalent bounds in the native protein. Mercaptoethanol or dithiothreitol are agents that reduce disulphide bonds in the protein. The denatured proteins linked to SDS^- have net negative charge and under electric fields the proteins can move to positive charge through the gels of poliacrylamide. The movement is proportional with their molecular weight (Ahmed, 2005, Sem, 2007). This method has been employed for identification of protein in lupin seeds such as *L. mutabilis* (Santos et al., 1997), *L. albus* (Restani et al., 1981, Duranti et al., 1981, Melo et al., 1994), *L. angustifolius* (Blagrove and Gillespie, 1975) and germinating lupin species (Duranti et al., 1984, Gulewicz et al., 2008, Ahmed et al., 1995).

Two-dimensional (2-D) electrophoresis is another technique for protein analysis which is more sensitive than 1-D electrophoresis. 2-D electrophoresis is a combination of isoelectric focusing and SDS-PAGE sequentially. As a result, this technique is able to separate a complex protein mixture with the same molecular weight or same pI. The method is widely applied together with Matrix-assisted laser desorption/ionization (MALDI) coupled with time of flight mass spectrometer (MALDI- TOF-MS) in proteomic analysis. Following trypsin digestion *in situ* of protein spot in gel 2-D electrophorosis, subsequently protein can be analysed by mass spectrophotometer (Carbonaro, 2004).

MALDI-MS is a type of mass spectrometry that allows the characterisation of biomolecules such as proteins, nucleic acids and carbohydrates. The technique is accurate and sensitive in measuring molecular weight in a wide range of 1 to 300 kDa (Bonk and Humeny, 2001). Nowadays, it becomes a powerful tool not only in

biological science but also in food science and technology (Carbonaro, 2004, Han and Wang, 2008). In the MALDI analysis, protein analyte is dissolved in a volatile solvent such as trifluoroacetic acid (TFA) and then suspended in the matrix (Sinapinic acid (SA) or α-cyano-4-hydroxycinnamic). The analyte and matrix then form a solid crystal on a metal plate after evaporation. Bombardment of a laser light into the matrix causes energy to be transferred into protein, causing the protein to become excited and pass into the gaseous phase (Figure 2-12). A time-of-flight (TOF) then separates the protein ions bases on mass to charge ratio (m/z) (Sachon and Jensen, 2007, Carbonaro, 2004).

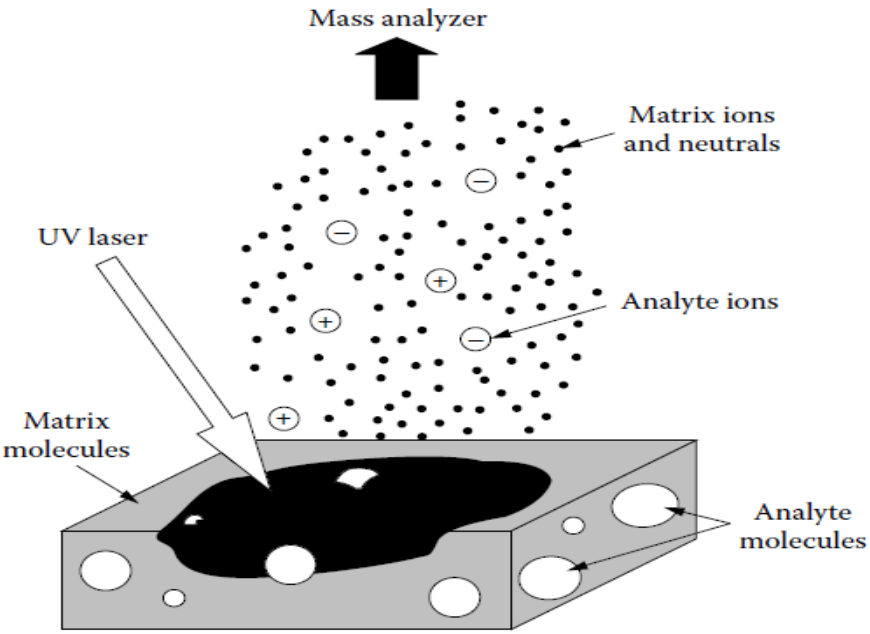


Figure 2-12 : Process of bombardment of a laser light into analyte in MALDI analysis

(Sachon and Jensen, 2007)

In a screening of bioactive proteins, 2-D electrophoresis and MALDI-MS have been used for analysis of storage proteins of *L. albus* (Wait et al., 2005), glycine soja

(Natarajan et al., 2006) and analysis purified γ-conglutin from *L. albus* (Mora et al., 2008). Proteomic analysis involving MALDI-MS and 2-D electrophoresis have been applied in the identification of allergen in *L. angustifolius* (Danica et al., 2008) and soybean (Krishnan et al., 2009). Furthermore, in controlling food quality, MALDI-TOF is useful for fingerprinting the quality of industrial soy protein isolate (Horneffer et al., 2007) and wheat gluten (Liu et al., 2009, Qian et al., 2008).

2.3.2 Phenolic compounds analysis

Determination of total phenolics in plant materials and foods is often done using Folin-Ciocalteu's reagent (FC). The reagent consists of phosphomolybdic-phosphotungstic acid. The method was first developed by Singleton and Rossi (1965) in purpose to determine phenolic substances of grapes and wines. The reaction involves oxidation of phenolic compounds by phosphomolybdic-phosphotungstic acid to produce a blue complex that can be measured as colorimetry assay at λ max 765 nm (Singleton and Rossi, 1965). The method is via reaction of the Folin – Ciocalteu's (FC) reagent, sodium bicarbonate and extract of sample. The method is simple, quick, sensitive and precise to determine TPC (Prior et al., 2005). However, some interference can affect the measurement due to their ability to reduce FC reagent. The interfering substances include sugars, aromatic amines, sulphur dioxide and ascorbic acid (Prior et al., 2005, Slinkard and Singleton, 1977). The oxidation reaction of phenols occurs in alkali condition to provide appropriate concentration of the phenolate ions. However, the FC reagent and the produced blue colour are not stable in alkaline condition. To overcome this problem, it is important to optimize FC concentration and alkalinity used in measurement. Sodium bicarbonate is preferable to sodium hydroxide and sodium cyanide in providing an alkaline condition as white precipitation that may appear during reaction can be minimised by sodium bicarbonate. In addition, sodium bicarbonate can delay the fading of the blue colour (Singleton and Rossi, 1965, Slinkard and Singleton, 1977).

Total phenolics are calculated using a calibration curve of known phenolics. The original method recommended the use of gallic acid as reference standard due to its stability, low price and ease of purification (Singleton and Rossi, 1965). Nowadays,

catechin, tannic acid, and caffeic acid were also used as the standard in some studies (Prior et al., 2005). The use of different phenolic standards is related to varying extracts yielded from different studies. Hence, the standards only provide an indication of amount of TPC.

To prepare phenolic extracts for analysis, phenolics from samples need to be extracted by solvent to produce a clear solution for colorimetry assay. Several solvents can be used in the extraction of phenolic compounds from samples. For example 80% (v/v) of aqueous methanol (Zielinski, 2003), 80% (v/v) of aqueous acetone (Amarowicz et al., 2004) and 50% (v/v) of aqueous methanol (Prakash et al., 2007) were applied in previous studies. The different solvents used for extraction might cause different results between studies.

In regards to identification of individual phenolic compounds, other methods such as high-performance liquid chromatography (HPLC) and gas chromatography (GC) can be used for determination and quantification of phenolic compounds. HPLC employing reserved-phase column and photodiode array (PDA) has been widely used for separation and quantification of phenolic compounds (Naczk and Shahidi, 2004, Katagiri et al., 2000). Furthermore, HPLC coupled by Liquid Chromatography/Electrospray Ionization Mass Spectrometry (LC-ESI-MS) has been used for structural characterization of phenolic compounds in some lupin species (D'Agostina et al., 2008, Stobiecki, 2000, Duenas et al., 2009). However, because the samples in GC analysis need more preparations including extraction, purification and derivatization, it seems that HPLC is a more preferable method to GC for phenolic analysis (Wang et al., 2002a, Naczk and Shahidi, 2004).

2.3.3 Phytosterol analysis

There are some reference methods for the analysis of sterols from oil and or fat samples including ISO 6799, IUPAC methods 2.401 and AOAC Ch 6-91 (Lagarda et al., 2006, Laakso, 2005). The analytical methods principally consist of several steps that need to be carried out to prepare extracts containing sterols from samples for GC analysis. The first step involves the extraction of lipids from samples, followed by saponification reaction. The sterols in the unsaponifiable matter are then extracted by

organic solvent and continued by purification of sterol and or sterol derivatization. Finally, the purified sterol extracts can be analysed by GC.

There are a number of methods for extracting the lipids from samples such as solvent extraction and supercritical fluid extraction (SFE). Different solvents have been utilized to extract oil from various samples, such as hexane/isopropanol (Hamama and Bhardwaj, 2004), n-hexane (Ramadan et al., 2006), cyclohexane : diethylether (Maatta et al., 1999), petroleum ether (Ingram et al., 1968) and chloroform/methanol (Jiang and Wang, 2005). An alternative method, supercritical fluid extraction (SFE) has been reported to be effective, cheap and quick for vegetable oil extractions. Oil extraction using this method, followed by sterol extraction by solid phase extraction (SPE), was an efficient method for sterol analysis (Snyder et al., 1999).

The aim of saponification and acid hydrolysis are to release the esterified sterols and to remove glycerol lipids in the oil samples. Direct saponification reaction can be applied by mixing the oil samples and ethanolic potassium hydroxide either in room temperature or heat temperature (hot saponification). This direct saponification has been performed for sterol analysis in samples such as spread, milk, and yogurt. Glycosidic bond of food matrix and steryl glycosides can not be hydrolysed by the direct saponification. Therefore acid hydrolysis before the saponification step is required to break down the food matrix in samples especially cereal (Laakso, 2005).

After saponification, sterols contained in unsaponifiable material are extracted into organic solvent (diethyl ether or ether), followed by evaporation of solvent to dryness. This extraction requires a number of washing steps to reach neutral condition in organic solvent phase. The extraction is time consuming (Lagarda et al., 2006). SPE was found to be a better method than the solvent extraction due to its time-efficient and improved reproducibility (Toivo et al., 2001, Abidi, 2001).

Prior to determination of sterols using GC, sterols are usually derivatized into trimethylsilyl (TMS) derivatives with derivatizing agents such as N-methyl-N-trimethyl-silytrifluoroacetamide (MSTFA), bis (trimetylsilyl)-trifluoroacetamide (BSTFA) in order to improve peak shape, good resolution and sensitivity (Laakso, 2005). In order, to correct for the loss of sterol during saponification and extraction,

an internal standard (IS) with known concentration has to be applied in the samples. The IS is commonly betulin, cholestane or 5β-cholestan-3α-ol. The 5β-cholestan-3α-ol is preferable as the IS due to its similarity in structure to sterols and its absence in plant sterol samples (Lagarda et al., 2006).

Gas Chromatography-Flame Ionization Detector (GC-FID) is widely used for determination of phytosterols. For qualitative analysis, comparison in retention time of peak samples with peak of standards can be carried out. GC coupled with MS is usually used to determine structural analysis of phytosterols. GC method has been used for determination of phytosterols in olive oils (de Blas and del Valle González, 1996), cereal (Jiang and Wang, 2005), vegetable oils (Verleyen et al., 2002), white lupin oil (Hamama and Bhardwaj, 2004), canola oil (Hamama et al., 2003), oil of solanaceae plants (Ramadan and Moersel, 2006) and vicia faba (Takatsuto and Omote, 1989). It has been reported that GC has higher sensitivity, precision and separation than other methods such as HPLC (Lagarda et al., 2006, Laakso, 2005).

2.3.4 Antioxidant activity assay *in vitro*

The main types of classification of antioxidant activity assays are based on the reactions involved: hydrogen atom transfer (HAT) and electron transfer (ET) (Huang et al., 2005). HAT-based assays measure the ability of antioxidants in quenching free radicals by hydrogen donation. These methods include low-density lipoprotein autoxidation, oxygen radical absorbance capacity (ORAC), and total radical trapping antioxidant parameter (TRAP). Meanwhile, ET-based assays such as trolox equivalence antioxidant capacity (TEAC), ferric ion reducing antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, measure the ability of antioxidants to transfer an electron to reduce radicals, metals and carbonyls (Huang et al., 2005, Prior et al., 2005). To select which is the best assay for measurement of antioxidant capacity in a particular system, it is important to consider what mechanism is involved and the source of radical and oxidant. Some other factors to be consider include repeatability, reproducibility, analytical range and recognition of interfering substances of the assays (Prior et al., 2005).

The DPPH method has been used to measure phenolics in faba bean, broad bean, adzuki bean, red bean, pea, red and green lentil (Amarowicz et al., 2004), *L. angustifolius* (Oomah et al., 2006) and germinated legumes (Lopez-Amoros et al., 2006). In addition, the kinetic behaviour of the DPPH free radical scavenging of some polyphenols has been studied by Sanchez-Moreno et al. (1998) and Villano et al. (2007).

The DPPH method was developed about 50 years ago by Blois (1958). The DPPH molecule with spare electron (violet colour) in a solution absorbs ultraviolet light at 515 nm. When a substance that can donate an electron atom (for example, antioxidant) is mixed with a solution of DPPH, the violet colour will fade and change to pale yellow (Molyneux, 2004). The change in colour is proportional to the radical scavenging activity. This method is rapid, sensitive, simple, cheap and independent of sample polarity (Sanchez-Moreno, 2002). Thus, the method is very convenient to screen antioxidants from many samples. It is also highly reproducible compared to other methods such as 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay. However, this assay has some drawbacks for example, test compounds that have the same spectra with DPPH at 515 nm could also interfere the measurements.

2.3.5 ACE-inhibitory activity assay *in vitro*

A basic method that determines ACE-inhibitory activity *in vitro* is developed by Cushman and Cheung (Chusman, 1971). Some researchers then developed and modified the method with changes, for examples in buffer composition (Hernandez-Ledesma et al., 2003) and colour reagents (Chang et al., 2001). The modified Cushman and Cheung method has been applied to measure ACE activity *in vitro* of *L. albus* protein isolate (Yoshie-Stark et al., 2006, Yoshie-Stark et al., 2008) and bovine casein hydrolysate (Miguel et al., 2009).

The Cushman and Cheung method is based on the hydrolysis of substrate hippuryl-L-hystidyl-L-leusin (HHL) by ACE enzyme to produce hippuric acid (HA) and histidyl-leusin (HL). The product can be extracted using ethyl acetate. Absorbance is then measured at 228 nm as maximum absorbance of hippuric acid (HA). The release

of HA is related to the ACE activity. In the presence of ACE inhibitor, reduction in absorbance is proportional to the inhibition by assayed inhibitor.

2.3.6 Bile acid binding activity assay *in vitro*

Bile acid (BA) binding activity in the intestine is believed as a possible mechanism for the hypocholesterolemic effects of dietary fibre. *In vitro* studies have been carried out in order to screen any dietary components that may affect the BA binding property. *In vitro* assay is widely used since the assay is less costly and convenient than the *in vivo* animal study (Camire et al., 1993, Sayar et al., 2006).

Some *in vitro* methods have been developed for measurement of the BA binding property of dietary fibre. The methods include colorimetric method (Eastwood and Hamilton, 1968), gas-liquid chromatography method (Van Berge Henegouwen et al., 1974) and the Krichevsky and Story method (1974). The method of Krichevsky and Story used radioactive-labeled BAs in the estimation of bile acid binding. Camire et al. (1993) then modified the Krichevsky and Story method by adding a simulation of gastric and pancreatic digestion to the procedure (Camire et al., 1993, Camire and Dougerty, 2003). Based on the Camire method, unbound BAs can be predicted by colorimetric assay without the use of radioactive-labeled BAs. The colorimetric assay is specific for 3α-hydroxy bile acids and the enzymatic reaction is illustrated as follows:

$$\text{3α-Hydroxybile acids + NAD} \xrightarrow{\text{3α-HSD}} \text{3-Oxo bile acids + NADH}$$

$$\text{NADH + NBT} \xrightarrow{\text{Diaphorase}} \text{NAD + Formazan}$$

3α-Hydroxybile acids are oxidized to 3-Oxo bile acids by a catalyst of 3α-hydroxysteroid dehydrogenase (3α-HSD). Concomitantly, a reduction of NAD to NADH occurs. The hydrogen of NADH is subsequently transferred by a catalyst, diaphorase into nitrotetrazolium blue (NBT) to form a product called formazan at

wavelength between 530-540 nm. The absorbance of the product is proportional to unbound BAs in the sample. The enzymatic mechanism is originally adopted from Mashige et al 1981 (Mashige et al., 1981). A commercial kit from Trinity Biotech for analysis of bile acid binding is now available. The commercial kit would ensure standardisation in the measurement of BA binding property between laboratories.

The modified method of Camire et al (1993) has been widely used for the prediction of BA binding properties *in vitro* of soy protein, pinto beans, black beans, wheat gluten (Kahlon and Woodruff, 2002), buckwheat protein (Ma and Xiong, 2009), spinach, kale, brussels sprouts, broccoli, mustard greens, green bell pepper, cabbage, collards (Kahlon et al., 2007) and chitosan (Zhou et al., 2006). The method of BA binding assay *in vitro* has been used to measure the compounds that have been known to have BA binding activity *in vivo* such as cholestyramine and raisin (Camire and Dougerty, 2003). Therefore, results of the *in vitro* assay in this study are expected to have positively relevance with biological activity *in vivo*.

2.4 Effects of food processing on bioactive compounds

Frying, baking, boiling, steaming and microwaving are some domestic food processing methods. These processes are aimed not only to improve taste, flavour, and appearance but also to increase digestibility of foods. Choosing a suitable method for cooking food is important as the process can affect the chemical composition and bioactive compounds present in food (Ruiz-Rodriguez et al., 2007).

2.4.1 Effects of food processing on phenolic compounds and antioxidant activity

Some studies have been conducted to investigate the effect of food processing on TPCs and antioxidant activity of vegetables, fruits, grain and legumes. A significant increase in TPCs and antioxidant activity has been observed in thermal-processed grain sprouts (Randhir et al., 2008), heat-treated sweet corn (Dewanto et al., 2002b) and thermal-processed tomatoes (Dewanto et al., 2002a). The studies indicated that

the increase could be attributed by an increased release of free ferulic acid and bound phenolics from the cell matrix. A similar finding has been found in durum wheat pasta prepared with debranning fractions of wheat. Boiling the pasta in water was able to enhance the release of bound phenolic compounds from food matrix during extraction, resulting in an enhancement of their antioxidant activity. However, there was a decrease in the concentration of free phenolic compounds after boiling of the sample (Fares et al., 2010). In contrast, soaking, boiling and steaming processes reduced the TPCs and antioxidant activity of lentil and chickpeas. The effect depends on the type of legumes and the processing condition (Xu and Chang, 2008). Furthermore, a study also showed that boiling in water was able to diminish antioxidant compounds in *Chenopodium quinoa* seeds (Dini et al., 2010). The loss of phenolic compounds in green beans, broccoli, spinach after conventional cooking such as boiling, steaming and microwaving was due to phenolic breakdown during the cooking process (Turkmen et al., 2005).

In regards to the effects of food processing on isoflavone (phytoestrogen) content, extrusion of soy-corn mixture still retained the isoflavone content and the health benefit associated with antiproliferative activity (Singletary et al., 2000). The total isoflavones in soymilk were not reduced by autoclaving for 5 min. However, by increasing the duration of the autoclaving to 15 min, the total isoflavone concentration was decreased by 20%. The study also found that there was intra-conversion among the different forms of isoflavones to β-glycoside, acylglycoside or aglycon forms during autoclaving (Chiarello et al., 2006). Another study showed that baking and frying of cookies with soy flour did not change the total isoflavone content present in the cookies, but during the process β-glycoside form was increased (Shimoni, 2004).

Antioxidant activity of phenolic compounds, in particular the flavonoid group is affected by masking effect of proteins. The masking effect is due to binding between proteins and flavonoids resulting in polyphenol-protein complex, causing a reduction of free flavonoids. As a result, the scavenging capacity of the flavonoids was reduced. Therefore, food matrix is also an important factor that needs to be considered when processing foods containing phenolic compounds (Arts et al., 2002).

2.4.2 Effects of food processing on phytosterol stability

Another important bioactive compound in legumes is phytosterols. There is increasing interest in these compounds due to their role in the treatment of hypercholesterolemia. It has been found that intake of 2 g of sterol lowers LDL cholesterol by 10% (Ostlund, 2007). Therefore, the market of functional foods enriched with phytosterols are recently expanding. It is thus necessary to know the stability of phytosterols particularly after food processing.

Phytosterols as bioactive compounds in lipid are susceptible to oxidation during food processing and storage. The oxidation of phytosterol can occur by auto-oxidation in the presence of heat, light, sunlight and or reactive oxygen and by enzymatic oxidation. The steroid ring in pytosterol structure is subject to auto-oxidation. Whilst the side chain in the phytosterol structure is believed to be subject to enzymatic oxidation by some enzymes such as cytochrome P450 monooxygenases, dehydrogenases, hydroxylases (Hovenkamp et al., 2008). The oxidation leads to production of sterol oxidation products (SOPs) or oxidized phytosterols/oxyphytosterols (Ryan et al., 2009, Zhang et al., 2006b). Both the saturated and unsaturated, chemical structure, saturated or unsaturated, prolonged exposure to the above factors and processing medium also influence the stability of phytosterols (Soupas et al., 2004). Examples of oxidized derivatives of phytosterols (oxyphytosterols) mainly include 7-hydroperoxide, 7-keto, 5-hydroperoxide, 5,6 epoxy, and triol as shown in Figure 2-13 (Rudzinska et al., 2004, Ryan et al., 2009, Hovenkamp et al., 2008).

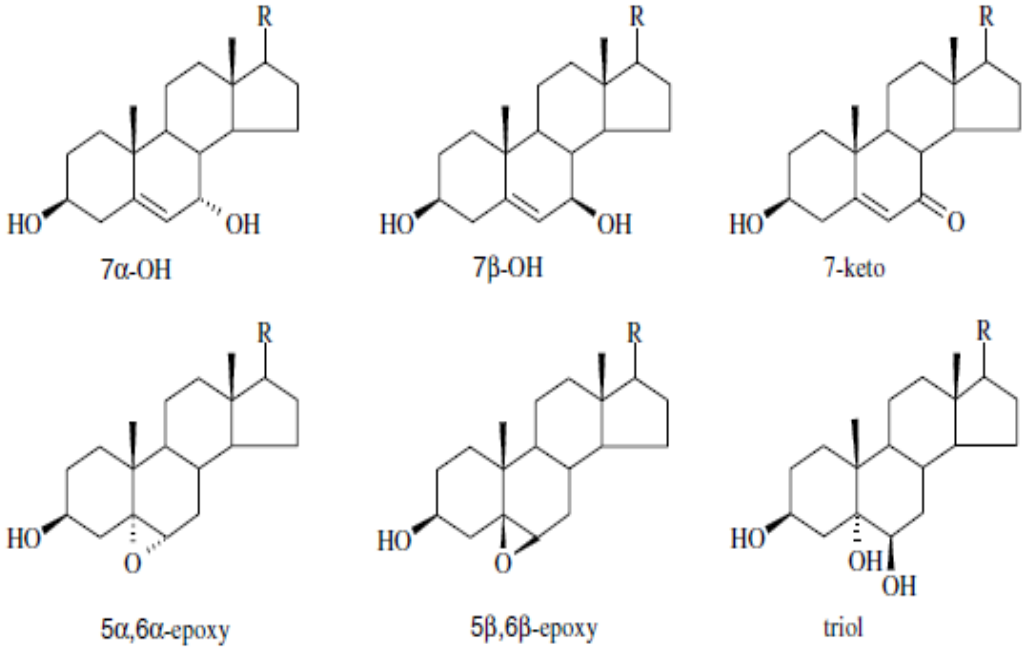


Figure 2-13 : Representative of chemical structure of oxiphytosterols. R is side chain in the phytosterol structure (Hovenkamp et al., 2008)

Pan-frying is one of the cooking processes that leads to oxidation of sterols present in food and oils. During the process, a wide range of derivatives from the oxidized phytosterols such as the oxy-, epoxy- and keto- sterol derivatives were formed (Soupas et al., 2007). Oxysterols have also been identified in stored vegetable oils (Bortolomeazzi et al., 2003), enriched spreads (Louter, 2004), and dairy products (Menendez-Carreno et al., 2008).

Microwaving phytosterol-enriched milk at 900W for 2 min and electrical heating at 90°C for 15 min has been observed to reduce the total phytosterol contents by 60%. The low percentages of phytosterols in the sample was due to degradation of their oxidized products (Menendez-Carreno et al., 2008). In contrast, the other study found that phytosterols contained in vegetables oils were stable at temperature up to their melting point of between 140 and 170°C. No oxidation and degradation was found in this temperature range. When heating was performed above 200°C for 30 min, the total phytosterols in the vegetable oils were reduced (Thanh et al., 2006). Furthermore, boiling beans and vegetables in the water for 30 min did not change

phytosterol content. However, free phytosterol contents in some beans and vegetables were increased by this treatment (Kaloustian et al., 2008).

Cholesterol has a similar structure to phytosterols and thus can also be affected by auto-oxidation and enzymatic oxidation to produce cholesterol oxidation products (COPs). The effect of COPs on health have been well studied and was found be associated with mutagenesis, carcinogenesis and atherosclerosis (Ryan et al., 2005, Lordan et al., 2009). As a consequence, phytosterol oxidation products (POPs) are also suspected to be able to promote toxicity as COPs (Ryan et al., 2009). Such observations suggest that understanding of phytosterol stability during food processing is important in maintaining the health benefit of the bioactive compounds and in reducing the adverse effects of oxidized products possibly occured during processing.

2.4.3 Effects of food processing on stability of bioactive protein

Only a few studies have been conducted on investigating the effect of processing on bioactive peptides in lupin. Boiling, autoclaving and microwaving have been performed onwhole lupin seeds in order to study the effect of processing on allergenicity of lupin. The results of the study suggested that autoclaving at 138ºC for 30 min can minimize the allergenicity of protein with molecular weight of 23 and 29 kDa (Alvarez-Alvarez et al., 2005). Moreover, a recent study observed that, under industrial thermal, mechanical, and high pressure treatments, β and δ-conglutin of *L. angustifolius* were stable to the processes (Sirtori et al., 2009).

In studying the effects of food processing on health benefits of proteins *in vitro*, it was found that autoclaving legume proteins for 30 min resulted in a decrease in their ACE inhibitory activity. Nevertheless, autoclaving for 50 min resulted in an increase in their ACE inhibitory activity. The results suggested that autoclaving for 30 min results in the formation of disulphide bond inter peptides that reduces the ACE inhibitory activity, while autoclaving for 50 min could be a maximum condition to release bioactive peptides that are responsible for the ACE inhibitory activity (Akillioglu and Karakaya, 2009). Meanwhile, pasteurization at 125°C has been

reported to reduce ACE inhibitory activity of protein isolate of *L. albus*, whitout affecting its bile acid binding property. However, an increased DPPH radical activity was observed in the study. Releasing small molecular weight protein during the heating could be the cause of a higher antioxidant activity following the treatmeant (Yoshie-Stark et al., 2006). A similar finding was found in the water-soluble protein of chickpea, where a higher free radical scavenging capacities was reported after a thermal processing at 121ºC (Arcan and Yemenicioglu, 2007).

2.5 Summary

Lupin is high in protein and some of the proteins appear to be bioactive compounds which are likely to have activities such as antihypertensive and lowering cholesterol level. In addition, lupin contains high dietary fibre but low starch. Lupin oil is rich in unsaturated fatty acids and phytosterols. It also contains high phenolic compounds which have an important role in antioxidant activity. Furthermore, studies have shown that lupin has a wide range of health benefits including reducing risk factors of CVD.

Germination is a simple method that improves the quality of legumes through enhancement of nutritive value, bioactive compounds, dietary fibre and their health benefits. In addition, germination can reduce allergenicity, flatulence factors and ANFs in some legumes. Although, the effects of germination on nutrient and bioactive compounds of lupin have been investigated to some extent, there has been no comprehensive investigation on the effects of germination of ASL on its. phenolic compounds, phytosterols, bioactive protein and their bioactivities during germination.

Food processing can alter phenolic compounds, phytosterols and protein and their bioactivities in food. Furthermore, the effects depend on the type of food and methods of processing. Therefore, an investigation of the effect of cooking process on bioactive compounds of ASL is important to understand the appropriate cooking method to maintain the health benefits of bioactive compounds present in germinated ASL.

Chapter 3 Materials and Methods

3.1 Materials

3.1.1 Lupin seeds

The ASL seeds (*Lupinus angustifolius*) investigated in this study were grown at Wongan Hills Research Station, Department of Agriculture and Food, Western Australia and harvested in 2005. The seeds after harvest were stored at 9 – 11ºC.

3.1.2 Chemicals and assay kits

Folin-Ciocalteu's phenol reagents, gallic acid, 2,2-di-phenyl-1-picrylhydrazyl radical (DPPH·), 6-hydroxy-2,5,7,8-tetramethyl chroman carboxylic acid (trolox), standards for phytosterol analysis (campesterol, stigmasterol, β-sitosterol), internal standard (5-β-cholestan-3α-ol, 5-α-cholestan-3β-ol), angiotensin-converting enzyme (ACE) from rabbit lung (3.92 units/mg protein), Hippuryl-L-Histidyl-L-Leucine (HHL), cholestyramine, bile acids (cholic acid, deoxycholic acid and chenodeoxycholic acid) and pancreatin (8x USP) were purchased from Sigma-Aldrich.

NuPAGE Novex [bis(2-hydroxyethyl)amino]tris(hydroxymethyl) methane 10% gels, NuPAGE MES SDS running buffer, NuPAGE reducing agent, NuPAGE LDS sample buffer and SeeBlue Plus2 prestained standard were purchased from Invitrogen, Milan, Italy. Bicinchoninic acid (BCA) protein assay kit was obtained from Pierce Kit. Bile acid testing kit, bile acid kit calibrators and bile acid stop reagent were obtained from Trinity Biotech.

3.1.3 Raw materials for muffin production

Wheat flour (Victory Premium Bakers flour, Weston milling), Farmland salt (Grocery Holding OTY Ltd.), sunflower oil (Coles Supermarket), baking powder (Anchor Food PTD Ltd.), caster sugar (CSR), eggs (Coles supermarket), skim milk, vanilla essence (Queen) were used in this study. Lupin flour was prepared from grounded-dehulled germinated ASL.

3.2 Definitions of seed germinations

It is important to clarify the criteria used to classify germinating lupin seeds applied in this study. Not all seeds were responsive to the germination conditions and some seeds remain in a non-germinated state. Seeds that do not have an emerging radicle observed after incubation for 1 day (24h), or have a radicle but of a size less than 1.0 cm are classified as non-germinated seeds. Whereas seeds that have a radical emerging from the embryo of size greater than 1.0 cm are classified as germinated seeds providing the radicle is not deemed to be rotten. Conversely rotten sprouts are defined as having a radicle greater than 1.0 cm, but that the radicle is either not fresh or exhibits a yellow/brown colour. Representative examples of lupin seeds that fit into these criteria are shown in Figure 3-1.

Figure 3-1 : Examples of germinated, non-germinated and rotten sprouts based on the criteria used in this study.

3.3 Methods

3.3.1 Germination of lupin seeds

Figure 3-2 shows the process of ASL germination and collection of samples for chemical analyses. Prior to germination, mature and unbroken lupin seeds (250g) were soaked in 1 L of water containing 0.07% (w/v) sodium hypochlorite (Fernandez-Orozco et al., 2008) for 30 min to limit microbial growth, followed by rinsing with distilled water until neutral pH. Dishes and trays used in the germination process were also treated with sodium hypochlorite. The disinfected ASL seeds were

soaked at room temperature, in dark, for16 h in 2 L distilled water. After pouring off the soaking water, the seeds were rinsed and spread on dishes lined with moistened paper towels. The dishes were covered with wet paper towels and placed on trays containing distilled water to ensure the paper towels on the top remained wet. The trays were placed in an incubator (drying oven) set at 25ºC and high relative humidity (RH). Three data loggers were placed in the top, middle and bottom trays of the incubator to record temperature and RH throughout the germination period. An example of temperature and RH recorded by data logger during germination period is given in Figure 3-3. The seeds in each dish were germinated for various lengths of time ranging from 1- 9 days. During the germination period, the seeds were watered twice per day with distilled water. At the appropriate time point, the seeds were harvested and separated by hand into dehulled sprouts, hulls, rotten sprouts and non germinated seeds. Weights of each fraction were recorded and the fractions were dried in an oven at 50ºC. The dried dehulled sprouts were then ground into powder using a coffee grinder (DeLonghi) and passed through a 0.5 mm sieve. The flour obtained was stored in plastic bags in darkness at 4ºC prior to chemical analyses and bioactivity measurements. Each of the germination period was repeated three times to produce 3 batches of flours for analyses.

ASL seeds

Soaked 16 h, put on plate lined with wet paper towels

plate

tray

Incubation at 25°C, RH 90%

Germination for 9 days

Lupin sprouts

hulls

Dehulled sprouts

Rotten sprouts& non germinated seeds

ASL sprout flour for analyses

Figure 3-2 : Flow chart of the germination process and collection of samples for chemical analyses

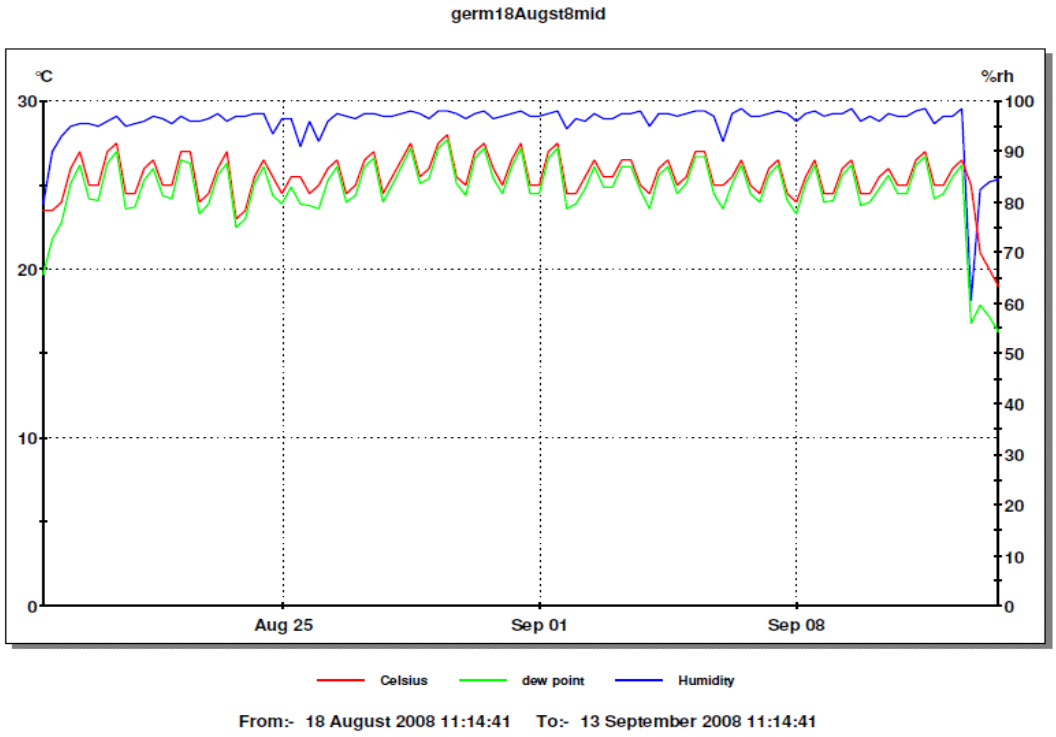


Figure 3-3 : An example of relative humidity (RH) and temperature recorded by data logger in incubator during the germination period

3.3.2 Moisture content and chemical composition analyses

3.3.2.1 Moisture content

The moisture content of the samples in this study was performed using a modification of AOAC (2000) method (925.10). The method has been modified based on standard laboratory methods that have been established and validated in the laboratory to ensure constant weight is achieved. Pre-weighed and dried dishes (W_0) were prepared for weighing of samples. Total weight of sample on the pre-weighed dish was recorded as W_1. The samples in the pre-weight dishes were dried in an oven set at 105ºC for 16 h. The dried samples in the pre-weight dishes were cooled down to room temperature in a dessicator and weighed as W_2. Moisture content in the samples was calculated as shown in Equation 1:

$$\text{Moisture content } (\%) = \frac{W_1 - W_2}{W_1 - W_0} \times 100 \text{ ---} \qquad \textbf{Equation 1}$$

Where:

W_0 = weight of empty dish (g)

W_1= weight of dish and sample (g)

W_2= weight of dried dish and sample (g)

3.3.2.2 Protein content

Protein content of the samples was determined by AOAC (2000) Kjeldahl method (950.36). The analysis was carried out using a Tecator Digestion System and a Kjeltec 1030 auto analyser (Figure 3-4).

Figure 3-4 : Kjeltec 1030 auto analyser.

Weighed samples were placed in a test tube, followed by addition of a catalyst (Kjeltab), a glass bead, 10 mL of digestion acid (sulphuric acid) and 5 mL of

hydrogen peroxide. The tubes were immediately placed into pre-heat digester set at 420ºC. The samples were digested until the solutions became clear or pale yellow. The digested samples were then cooled down to room temperature and then 75 mL of distilled water was added slowly. A receiver flask containing 25 mL of boric acid and indicator was placed under the condenser outlet of Kjeltec machine and 5 mL of 40% NaOH was poured into the solution. Subsequently, the steam distillation (Kjeltec) was started and continued until a 125 mL of final volume was collected. The ammonia content of boric acid was titrated against 0.1 M hydrochloric acid until a grey endpoint was reached. The amount of titrate is equivalent to the amount of ammonia present in the samples (%N). The percentage protein was calculated by multiplication of % N with a conversion factor (f) of 6.25 (Equation 2).

$$\%\,\text{protein} = \frac{(\text{sample titre mL} - \text{blank titre mL})}{\text{mg sample}} \times \text{M HCl} \times 14.1 \times \text{f} \times 100 \quad \textbf{--- Equation 2}$$

Where:

f = conversion factor = 6.25

3.3.2.3 Oil content

Oil content of the samples was determined by a modification method of the AOAC (2000) method (963.15) using a Buchi E-816 SOX extraction unit (Figure 3-5). Weighed samples (W_s) were placed into corresponding thimbles and subsequently was placed above pre-weighed extraction cups containing glass beads (W_e) in the extraction unit. The samples were extracted by petroleum ether for 90 min. Following the extraction, the solvent remained in the extraction cup was evaporated and the cups containing the oil extracted were then dried in an oven at 105°C for 24 h. The dried extraction cups were weighed and recorded as W_c. The oil content was calculated following Equation 3:

$$\%\,\text{Oil content} = \frac{Wc - We}{Ws} \times 100$$ **--- Equation 3**

Where:

W_s = weight of sample (g)

W_e = weight of empty cup (g)

W_c = weight of dried cup and oil after extraction (g)

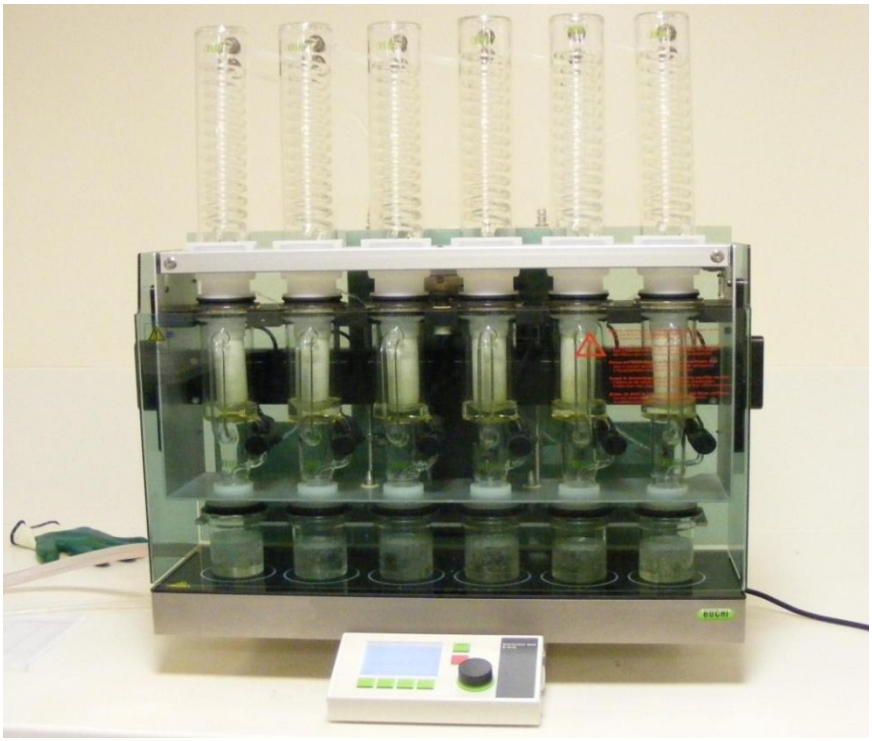


Figure 3-5 : Buchi E-816 SOX Extraction Unit

3.3.2.4 Crude fibre content

Crude fibre was determined using Fibrecap 2021 (Foss) as shown in Figure 3-6. The procedure based on the Weende method consists of three principle steps: (1) boiling in acid solution to remove free sugar and starch in the samples; (2) boiling in alkaline solution to remove protein and carbohydrate in the samples and (3) de-fatting of fibre residues with acetone.

Figure 3-6 : Fibrecap 2021 unit

Capsule and lid were prepared and weighed as W_1. The samples were put in the capsule and weighed as W_2. The capsule inserted into the capsule tray was placed on the boiling stand in the Fibrecap. The extraction beaker containing 350 mL of 1.25% sulphuric acid was placed on the hot plate. A condenser was placed on top of the extraction beaker and cold water tap was opened for the reflux system. The sample in the capsule was boiled for 30 min. Following the boiling, the condenser was removed and placed in the holder on the back of the hot plate. Subsequently, the extraction beaker and capsule were removed from the hot plate. The extraction beaker was later emptied and filled with 350 mL of boiling water. The sample in the capsule was then washed twice by partially lowering the capsule into fresh hot water. The capsule was agitated by raising and lowering the boiling stand.

The next step was boiling of the samples with 1.25% NaOH as was done during the acid boiling step. This step was then followed by washing with fresh hot water. Finally, the fibre residues in the capsule were de-fatted with acetone by agitation in the solvent in a small beaker for 30 sec. The capsule was then drained and dried in an oven for 5 h at 105ºC. After cooling down to room temperature in a desiccator, the fibre residue in the capsule was weighed (W_3). The samples were then placed in pre dried and pre weighed ashing crucible (W_4). The sample in the capsule was ashed in the ashing crucibles for at least 4 h at 600±10 ºC. The ashing crucibles were cooled slowly and put in a desiccators, weighed and their weight recorded (W_5). Calculation of % of crude fibre is shown in Equation 4:

$$\%\,\text{Crude fibre} = \frac{W_3 - (W_1 \text{ x } C) - (W_5 - W_4 - D)}{(W_2 - W_1 x C)} \times 100 \quad \textbf{--- Equation 4}$$

Where:

W_1 = initial capsule weight (mg)

W_2 = sample weight (mg)

W_3 = capsule and residual weight (mg)

W_4 = empty ashing crucibles (mg)

W_5 = total ash (mg)

C = blank correction for capsule solubility

C = blank capsule weight after extraction / blank capsule weight at start

D = capsule ash (mg)

3.3.3 Preparation of methanol, aqueous and oil extracts of the samples

A range of extracts were prepared from the germinated ASL flours and muffin samples to allow the separation of its different components. A methanol extract which contains the majority of the phenolic compounds was prepared and used for total phenolic analysis and antiradical assay. The aqueous extract (Tris buffer)

containing protein extracts was used for analyses related to protein pattern and protein bioactivity. The oil extract was prepared for the purpose of analyses related to phytosterols and their bioactivities.

TPCs in the samples were extracted according to the method used by Zielinski, (2003) with some modifications. Samples were extracted with 80% methanol (1/10; m/v) by shaking in waterbath for 2 h at room temperature. The extraction was repeated 2 times. The mixture was then centrifuged at 4000 rpm for 15 min. The supernatant was filtered and then evaporated in a rotary evaporator at 37ºC under vacuum to concentrate the content. The residue was cooled and frozen until further analyses.

Total proteins were extracted from samples using 0.1 M Tris-HCl buffer at pH 8.5 containing 0.1 M NaCl for 2 h with stirring. Insoluble materials were separated by centrifugation at 3000 rpm for 10 min. Supernatants containing protein were then collected and kept at -20ºC for determination of total protein, protein profile (SDS-PAGE) and their bioactivities.

Oil extracts were prepared using Buchi E-816 SOX extraction unit (Figure 3-5). Weighed samples (10 g) were placed into a thimble, and put on pre-weighed extraction cup containing glass beads. The extraction cup was placed on the extraction chamber in the soxhlet, and then extracted by petroleum ether for 90 min. Oils yielded from the extraction were kept for further analyses.

3.3.4 Measurement of Total Phenolic Compounds (TPC)

The TPC of germinated ASL flour and muffins incorporated with the flour were analysed using a Folin-Ciocalteau reagent (Singleton and Rossi, 1965) and gallic acid as a reference. A sample (0.5 mL) of extracted solutions (methanolic and aqueous extracts) was mixed with 2.5 mL of 10-fold diluted Folin-Ciocalteu reagents solution and the mixture was subsequently mixed with 2 mL of 7.5% of Na_2CO_3. After incubation at 45°C for 15 min for blue colour development, the absorbance was determined at 765 nm. A mixture of solvent (methanol) and reagents was used as the

blank. The TPC was expressed as gallic acid equivalent (mg of GAE/g dried sample) using the equation based on the calibration curve of gallic acid performed under the same experimental conditions. The standard curve was made in a range of concentration between 10 mg/L- 100 mg mg/L of gallic acid. For example the calibration equation is Y = 0.0103 X + 0.0148, (R^2 = 0.9991), where X was the absorbance and Y was the gallic acid concentration (mg/L). TPC was calculated using Equation 5.

Total phenolic compounds (mg of GAE/g DM) =

$$\frac{\text{Abs} - 0.0148}{0.0103} \times \text{vol. extract} \times 10^{-3} \times \text{dilution factors} \times \frac{1}{\text{weight sample (g)}}$$ **---Equation 5**

Where:
Abs = absorbance

3.3.5 Measurement of DPPH radical scavenging activity of methanolic, aqueous and oil extracts

DPPH radical activities of methanol and aqueous extracts of germinated ASL flour and muffins incorporated with germinated ASL flour were determined by using the Brand-Williams et al. method (1995). Sample extracts of 0.1 ml were added to 3.9 mL DPPH radical methanolic solution (25 mg/L). After incubation for 30 min, in darkness and at room temperature, the absorbance at 515 nm was recorded using spectrophotometer. The blank reference was methanol.

Ability to scavenge DPPH radical of the oil extracts was examined according to the Ramadan and Moersel method (2006). Oil samples (1 mg) were diluted in 100 µL toluene and then mixed with 390 µL toluenic solution of DPPH radicals (25 mg/L). Absorbance was measured after incubation for 30 min, in darkness at room temperature using spectrophotometer at 515 nm against a blank of toluene. The percent scavenging activity of the methanolic, aqueous and oil extracts was

calculated from the difference between absorbance with and without sample (control) following Equation 6.

$$\%\,\text{Scavenging activity} = \frac{\text{abs. of control - abs. of test sample}}{\text{abs. of control}} \times 100 \quad \textbf{--- Equation 6}$$

Where:

Abs = absorbance

3.3.6 Fractionation of germinated lupin flour into protein isolate and soluble fractions

Globulin in storage proteins were fractionated into fractions consisting of conglutin α, β and δ and the soluble conglutin γ. The fractionation was carried out according to method of Sironi et al. (2005). The procedure of fractionation consisted of alkaline extraction of all proteins, followed by acid precipitation and Zn precipitation of soluble protein in supernatant that is supposed to contain conglutin γ (Figure 3-7). For alkaline extraction, defatted germinated flour was suspended in water (1:20 w/v), then adjusted to pH 9.0 with NaOH solution and stirred for 2 h at room temperature. The slurry was centrifuged at 4500 rpm for 15 min and the residue (fibre) was collected. Acid precipitation was performed by addition of HCl solution to supernatant and pH reduction to pH 4.5. The slurry was centrifuged at 4500 rpm for 15 min. The precipitate was expected to consist of conglutin α, β and δ. Supernatant A were collected for further purification by readjusting the pH to 7.0 by addition of NaOH solution. Zinc chloride ($ZnCl_2$) was then added to the supernatant at a final concentration of 20mM. Following centrifugation, the precipitate (which should contain conglutin γ) was collected. The precipitate was washed to pH 7.0 with distilled water and then freeze-dried for further analyses.

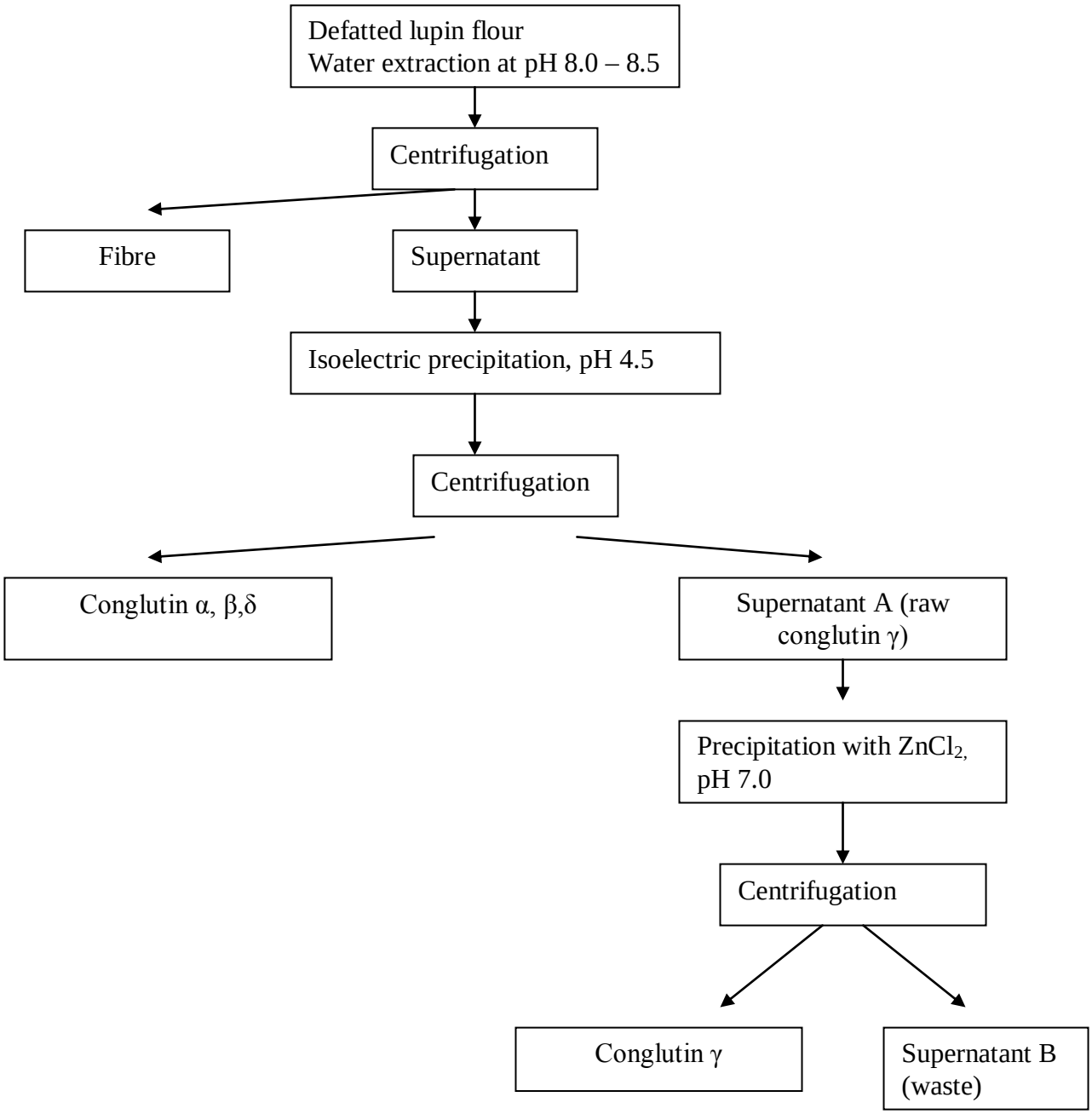


Figure 3-7 : Flow chart of procedure of lupin protein fractionation

3.3.7 Determination of protein concentration of protein extracts using Bicinchoninic acid (BCA) assay

The BCA assay was used for measurement of protein concentration in the protein extracts prior to bioactivity assay and analyses by SDS-PAGE and MALDI-TOF. The basic method is similar to Biuret reaction, but this method added BCA as chelator to form a complex with cuprous ion (Smith et al., 1985). The analysis was carried out using BCA Protein Assay Kit consisting of BCA reagent A and BCA reagent B. Reagent A contains sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate, while reagent B contains 4% cupric sulfate. Prior to the analysis, working reagent (WR) was prepared by mixing reagent A and reagent B in ratio 50:1. One hundred microliters of the protein samples were added with 2.0 mL of WR solution in test tubes and the mixture was incubated at 37ºC for 30 min for development of purple-coloured product. The absorbances were then read using spectrophotometer (Shimadzu Scientific) at 562 nm within 10 min after the incubation. Water was used as a blank to make the absorbance zero. The protein concentration was calculated using the standard curve of albumin in a range concentration of 0.25-1.5 mg/mL. The equation was Y= 1.1025X + 0.0081, where Y was absorbance and X was the concentration of albumin (mg/mL)

3.3.8 SDS PAGE of protein extracts

SDS PAGE was employed for determination of molecular weights of proteins in the germinated ASL flour samples, the protein isolate and the soluble fractions.

NuPAGE Novex [bis(2-hydroxyethyl)amino]tris(hydroxymethyl) methane 10% gels, NuPAGE MES SDS running buffer, NuPAGE reducing agent, NuPAGE LDS Sample buffer and SeeBlue Plus2 prestained standard (Invitrogen, Milan, Italy) were prepared for SDS PAGE. Electrophoresis was performed using XCell SureLock Mini-Cell (Invitrogen, Milan, Italy).

Prior to electrophoresis, protein samples were denatured by mixing 20 µL of protein solution (10 µg/ µL), 25 µl of NuPAGE sample buffer, 10 µL of NuPAGE reducing agent and 45 µl of double distilled water to a final volume of 100 µl. The mixture was heated at 70ºC for 10 min to reduce the protein. The reduced protein samples (10

µl) were loaded into the wells of the gels and electrophoresis was run at a voltage of 150 mV for 1 h. Once the dye front reached the bottom of the gel, the gel was stained with 0.15 % Coomassie Brilliant Blue in 50% (v/v) methanol and 7% acetic acid. After the staining, the gels were destained with solution composed of 7% of acetic acid and 20% methanol. The gels were then dried in gel dryer (model 543 Biorad) and scanned for documentation. The molecular weight of a protein was determined by comparing molecular weights of the proteins present in the sample against the protein standards (4-250 kDa).

3.3.9 Protein analysis using MALDI-TOF

Proteins in the samples used for MALDI-TOF analysis were extracted with 10 mM buffer Tris pH 8 for 1 h. The protein extracts were then reduced with dithiothreitol (DTT) at a final concentration of 1% by heating them at 65ºC for 30 min. One microliter of the reduced protein solution was mixed with 14 µl of sinapinic acid (SA) solution (10 mg/ml) as matrix in the analysis. The SA was prepared by dissolving it in 0.05% TFA and 59% ACN. The 1 µl of the mixture was loaded twice onto a MALDI –TOF plate and air dried thoroughly. Subsequently, the dried protein-matrix mixture was run in the MALDI-TOF equipment as shown in Figure 3-8.

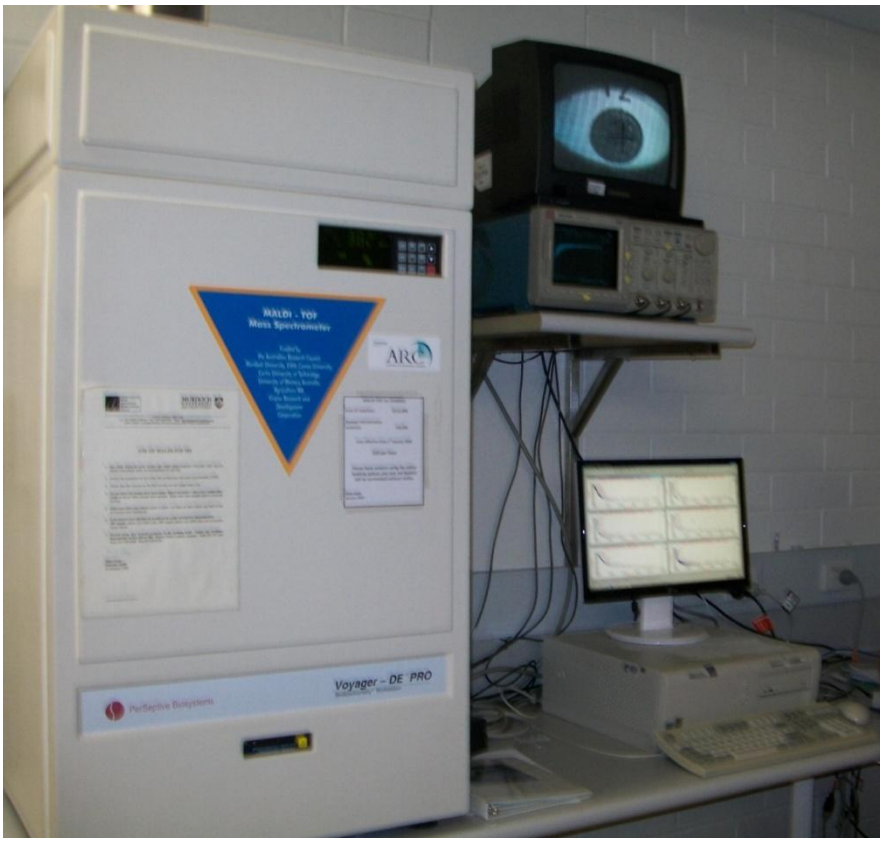


Figure 3-8 : MALDI-TOF equipment employed for protein characterization of protein extracts

3.3.10 Phytosterol analysis using GC

Phytosterol contents in sample oils were measured by a modified method of Jiang and Wang, (2005). The protocol consists of three main steps including saponification of the oils, extraction of unsaponifiable matter and analysis using GC. Saponification was done by mixing of oil (approximately 0.1-1 g) with 3 mL of 50% KOH, 3mL of ethanol absolute and internal standards (5-β-cholestan-3α-ol or 5-α-cholestan-3β-ol) in pre-weighed beaker, followed by heating the mixture in a 90°C for 2 h. The unsaponifiable matters were then extracted thrice with 15 mL of diethyl ether using funnels and the pooled diethyl ether extracts were washed several times with distilled water until they become neutral as illustrated in Figure 3-9. The washed diethyl ether extracts were dried over anhydrous sodium sulphate. The diethyl ether containing phytosterols was then evaporated under stream nitrogen to dryness.

Figure 3-9 : Extraction of unsaponifiable fraction using funnels

The residue resulted from unsaponifiable extraction was dissolved with hexane for GC analysis. Analysis of phytosterols in the residue was carried out using a Perkin-Elmer Autosystem XL GC with Phenomenex ZB-1, size 530 µm x 30 m, 1.5 µm film thicknesses. The column was equipped with a flame ionization detector (FID) and helium gas as carrier at a rate of 1 mL/min. The temperature of detector and injector were set at 300ºC. The initial oven temperature was held at 70ºC for 4 min and at 270ºC for 30 min.

Phytosterol standards (campesterol, sigmasterol and β-sitosterol) were used to detrmine phytosterols present in the samples. For quantification, a calibration curve for each standard was created by plotting between concentrations (x) against area (y). The linear regression obtained from the plot of each standard was employed for calculation of phytosterol concentration present in the samples. Recovery of spiked internal standard in each sample was applied to correct the calculation. For example, standard curve of campesterol was $Y = 2^{E+07x} - 415856$, $R^2 = 0.998$. The correspondening calculation was shown in Equation 7:

Phytosterol concentration (mg/g sample) =

$$\frac{(Y + 415856)}{2^{\,E+07x}} \times \frac{1}{(\%\ \text{recovery x}\ 0.01)} \times \frac{1}{\text{weight oil sample (g)}} \quad \text{---} \quad \textbf{Equation 7}$$

Where:

Y= area of the standard

X = concentration of standards (mg/mL)

3.3.11*In vitro* ACE inhibitory assay

ACE-inhibitory activity of the samples was determined by a modification of the Cushman and Cheung method (1971). The type and quantity of the chemicals were summarized in Table 3-1. The ACE from rabbit lung (3.92 units/mg protein) and substrate of Hippuryl-L-Histidyl-L-Leucine (HHL) were purchased from Sigma (St. Louis, MO). The substrate, 5 mM of HHL and ACE solution of 100 mU/mL were prepared by dissolving them in 100 mM boric acid-borax buffer containing 0.5 M of NaCl at pH 8.3. In each assay, a sample solution (10 µl) and 40 µl of 5mM HHL were pre-incubated in test tube at 37 ºC for 5 min. Following addition of 40 µl of ACE solution (100mU/ml), the mixture was incubated at 37 ºC for 30 min. The reaction was terminated by addition of 100 µl of HCl solution. Sodium borate buffer was then added to a final volume of 0.25 ml of the mixture. The hippuric acid produced was extracted using 1.5 mL of ethyl acetate and then centrifuged at 3500 rpm for 10 min. The ethyl acetate was evaporated at 95ºC and the residue was redissolved in 1 mL of the distilled water. The absorbance was measured at 228 nm to estimate ACE- inhibitory activity which was expressed as a percentage of inhibition. The activity of each sample was measured in triplicate.

Table 3-1: Details of chemicals used for the assay of ACE inhibitory activity

	Sample (A)	**Control (C)**	**Reaction Blank (B)**	**Sample blank (D)**
100mU/ml ACE (µl)	0	0	40	40
1 N HCl **(**µl)	0	0	100	100
5mM HHL **(**µl)	40	40	40	40
ACE inhibitor/sample (µl)	10	0	0	10
100mM sodium borate buffer (µl)	0	10	10	0
Incubated at 37ºC, 5'				
100 mU/ml ACE (µl)	40	40	0	0
Incubated at 37°C, 30'				
1 N HCL (µl)	100	100	0	0
100mM sodium borate buffer (µl)	60	60	60	60

To correct for the interference of compounds in the samples that can be extracted by ethyl acetate with absorbance at the same wavelength, the sample absorbance was corrected with absorbance of a blank sample. In this case, sample blank of germinated lupin extract of day 5, 7 and 9 showed a higher absorbance (0.4 mean value) compared to those of sample blank for day 1 and 3. The same results have been shown by Hernandez-Ledesma et al. (2003).

The control (C) was prepared in the same manner, but the sample was replaced with sodium borate buffer. Reaction blank (B) was prepared by adding the HCl after adding the ACE enzyme and by replacing sample with buffer. Sample blank (D) was prepared in the same way as the reaction blank, but replacing buffer with sample. The details of preparation of these samples are presented in Table 3-1. Percentage of the ACE inhibition was calculated following Equation 8.

$$\text{ACE inhibitory activity (\%)} = \frac{[(C - B) - (A - D)]}{(C - B)} \times 100 \quad \text{---} \quad \textbf{Equation 8}$$

Where:

A= absorbance of sample

B= absorbance of reaction blank

C= absorbance of control

D= absorbance of sample blank

3.3.12 *In vitro* bile acid (BA) binding assay

The procedure to measure *in vitro* BA binding activity was carried out as previously reported by Kahlon and Woodruff (2002) and Camire & Dougherty (2003). About 50 mg samples in a tube test were digested with 1 ml of 0.01 N HCl for 1 h at 37ºC in a shaker bath to simulate gastric condition. Following incubation, the pH of the mixture was adjusted to pH 6.3 with 0.1 N NaOH and added with 5 ml of porcine pancreatine (8XUSP, 1 mg/ml, in 0.01M phosphate buffer, pH 6.3) and 4 ml of 0.5 mM bile acid solution (cholic acid, deoxycholic acid and sodium salt of chenodeoxycholic acid) in 0.1 M phosphate buffer pH 6.3. The mixture was incubated for 1 h at 37 ºC in a shaker bath which simulates intestinal digestion. The mixture was centrifuged at 4000 rpm for 20 min and the supernatant containing bile acids was collected for analyses.

The unbound BAs in the supernatant were analysed using BA kit (Kit 450, Trinity Biotech.) consisting of Reagent A (2.5 mM nicotinamide adenine dinucleotide, 0.61 mM nitroblue tetrazolium chromaphore, and 625 U/L of diaphorase, pH 7.0) and Reagent B (3α-hydroxysteroid dehydrogenase). The test reagent was prepared by mixing 4 mL of Reagent A and 1 mL of Reagent B. The blank reagent was a mixture of 4 mL of Reagent A and 1 mL of deionized water. Supernatant (0.2 mL) of samples containing free bile acids from the reaction above was mixed with 0.5 mL of test reagent, while the blank sample was mixed with 0.5 mL of blank reagent. All the mixtures were incubated at 37°C for 5 min. The reaction was stopped by addition of 0.1 mL of Bile Acids Stop Reagent (catalog 450-3) and the absorbance was

measured at 530 nm. Concentration of unbound (free) BA was determined using a standard curve obtained from BA calibrators (Trinity 450-10) at 10, 20, 40, 60, 80 and 100 µmol/L. Details of sample preparations are presented below in Table 3-2.

Table 3-2 : Procedure for assay of bile acid binding

	Sample (A)	**Control (C)**	**Sample blank (Bs)**	**Control blank (Bc)**
Sample (mg)	50	0	50	0
0.01 N HCL **(**ml)	1	1	1	1
Incubation in shaking water bath (digestion)	37°C, 1 h	37°C, 1 h	37°C, 1 h	37°C, 1 h
Addition of NaOH to pH 6.3 (duodenal physiological) (mL)	1	1	1	1
Pancreatin (1mg/mL) (gastric digestion) (mL)	5	5	5	5
Bile acid solution (0.5mM) in 0.1 M phosphate buffer pH 6.3	4	4	4	4
Incubation in shaking water bath	37°C, 1 h	37°C, 1 h	37°C, 1 h	37°C, 1 h
Centrifugation	4000 rpm, 15’	4000 rpm, 15’	4000 rpm, 15’	4000 rpm, 15 ‘
Supernatant (µl)	200	200	200	200
Test reagent kit (µl)	500	500		
Blank reagent kit (µl)			500	500
Incubation	37°C, 5 min	37°C, 5 min	37°C, 5 min	37°C, 5 min
Stoper (µl)	100	100	100	100
Read absorbance at 530 nm				

Cholestyramine (as positive control) and a control (without sample) were also performed following the reaction above. Each absorbance in the samples, the positive control and the control was corrected with their blank. Bound BA was determined as the difference between the amount of BA added (control) and in the sample. BA binding activity was calculated using Equation 9 and presented as µmol/g sample.

$$\text{BA binding} = (\text{unbound BA of control} - \text{unbound BA of sample}) \times \frac{1}{\text{weight sample}} \quad \text{--- Equation 9}$$

Where:
BA= bile acid

3.3.13 Preparation of muffin

Control muffin formula was based on a standard muffin recipe developed by Department of Food Science and Technology, School of Public Health, Faculty of Health Science, Curtin University of Technology. The muffins incorporated with germinated lupin flour (day 7) were prepared by modification of the control muffin recipe. Germinated lupin flour of 2, 4, 6 and 8% (as % of dried muffin weight based on the assumption that 1000 g batter results in 670 g dried muffin) was incorporated into the control formula as shown in Table 3-3.

Table 3-3 : Muffin formulations

Ingredients (g)	Control	2%	4%	6%	8%
Flour	372	359	345	332	318
Germinated-lupin flour	0	13	27	40	54
Caster sugar	182	182	182	182	182
Salt	2	2	2	2	2
Baking powder	17	17	17	17	17
Egg	99	99	99	99	99
Milk	199	199	199	199	199
Vegetable oil	124	124	124	124	124
Vanilla essence	5	5	5	5	5
Total weight (g)	1000	1000	1000	1000	1000

The dry ingredients (flour, sugar, baking powder, and salt) were scaled and mixed together manually for 1 min until homogenous. The eggs were beaten manually and added to other liquid ingredients (milk and vegetable oil) and mixed until uniform distribution was established. The wet ingredients were then added to the dry ingredients and mixed manually using a big spoon. Some of the batter was collected and freeze-dried for further analyses and approximately 80 g of batter was poured into each muffin paper case on muffin tray and baked in pre-heated oven at 190°C for 25 min. After baking, the muffins were cooled down to room temperature, packed in plastic containers and stored at room temperature for the physical measurements. Some muffins were kept in freezer overnight and freeze dried in the next day for further chemical analyses and bioactivity analyses.

3.3.14 Physical measurements of muffins

For physical analysis of muffins, 3 muffins from each of 3 different batches of flour was used. Moisture content of muffins was determined according to the method described in Section 3.2.3.1. The crumb colour of muffins was measured using

Spectrophotometer Minolta CM-508i (Hunterlab Ultrascan Sphere Spectrofotometer-Hunter Assoociates Laboratory Inc., Reston, VA) that employ CIELAB colour system (*L** *a** *b**). The *L** represents black to white colours with a range value 0 to 100. The positive *a** represents red colour and negative *a** represents green colour, while positive *b** represents yellow colour and negative *b** represents blue colour. The muffins were cut across and the *L**, *a** and *b** value of the crumb were then measured at 3 to 5 different spots. Height and diameter of muffin samples were also determined by measuring height and width at three different points of the muffins.

Texture profile analysis (TPA) of muffins was determined by using a TA-XT2i Texture Analyzer (Stable Micro System Ltd., Surrey, England, UK). Cubes of space 2.5 cm height from the center of muffins were determined with pretest speed at 10.0 mm/s, test speed at 5.0 mm/s, posttest speed at 5.0 mm/s, compression distance 50% of muffin's height, 2 sec delay between two bites, trigger force at 5.0 g and data acquisition rate at 200 pps. Hardness, cohesiveness, springiness and chewiness of the muffins were calculated as described by Bourne (2002). Hardness (N) is the peak force during the first compression cycle. Cohesiveness is the ratio of positive force area during the second compression to that during the first compression. Springiness (cm) is the height that the sample recovers during the time that elapses between the end of the first bite and the start of second bite. Chewiness (N.cm) was calculated by multiplying hardness, cohesiveness and springiness.

3.3.15 Chemical analysis and measurements bioactive compounds in the muffins

Batter and baked muffins were extracted following the method mentioned in Section 3.3.3. Analysis of TPC, DPPH radical scavenging activity, pytosterols and BA binding activity were based the methods previously described in Section 3.3.4, 3.3.5, 3.3.10 and 3.3.12, respectively. For these analysis, in each analysis, 3 muffins from each of 3 different batches of flour was used.

3.4 Statistical Analysis

Data were analysed using SPSS for Windows version 18. One-way ANOVA was applied to determine the differences of data among the different days of germination or treatments. Following Levene's homogeneity test of variance, Brown-Forsythe and Games-Howell and the Tukey's post hoc tests were later used to determine the differences between the samples. Data which did not meet the assumption of one way ANOVA test were analyzed by non parametric Kruskal-Wallis test followed by Mann-Whitney U test. To analyze the differences between 2 groups of samples, student's independent t-test was performed. A 0.5% level of significance was applied in the statistical tests, where p-value of less than 0.05 indicated the presence of significant differences.

Chapter 4
Effects of germination of ASL on the concentration of bioactive compounds and their bioactivities *in vitro*

4.1 Introduction

Germination of seeds modifies its chemical composition. The germination process involves a variety of reactions including synthesis, degradation and transformation of biomolecules during transformation of the seeds into a plant. The concentration of main macronutrients such as protein, lipid and carbohydrate in some legumes were modified by the germination process (Mostafa and Rahma, 1987, Zielinski, 2003). Furthermore, the concentration of ANFs such as phytate, trypsin inhibition, α-galactoside and alkaloids in lupin and other legumes are diminished by germination (Cuadra et al., 1994, Frias et al., 1995, Dagnia et al., 1992). On the other hand, the concentration of bioactive compounds such as phenolic compounds was increased by the germination process (Duenas et al., 2009, Fernandez-Orozco et al., 2006). However, little is known about the relationship between the changes in the concentration of phenolic compounds, phytosterols, bioactive proteins and their bioactivities *in vitro* in ASL during germination. The aim of this study was to determine the changes in the chemical composition and bioactive components in ASL during germination and how these changes are associated with their *in vitro* bioactivities. This chapter presents and discusses the changes in the macronutrients, total phenolic compounds related to their scavenging radical activity, phytosterol compounds related to their scavenging radical activity, BA binding property, protein pattern associated with ACE inhibitory activity, and profile of protein isolate and soluble fraction analysed by SDS-PAGE and MALDI TOF of ASL during the germination period.

4.2 Results and Discussion

4.2.1 Effect of germination of ASL on the yield of ASL sprouts

Laboratory scale germination has been conducted by some researchers using different methods. Moisture is needed by the seeds for germination. In the laboratory environment for the purpose of providing moisture, seeds can be spread on a wet

filter paper or cheesecloth (Zhang et al., 2007, Zielinski, 2002, Estevez and Luh, 1985) placed on a plastic container (Mostafa and Rahma, 1987), petri dishes (Zhang et al., 2007) or glass jar (Sawyer et al., 1985). Germination can be carried out at room temperature (Sawyer et al., 1985) or in an incubator set at a constant temperature (Zielinski, 2002). Specific equipment to control temperature and humidity such as seed germinator (Lopez-Amoros et al., 2006), climatic cabinet (Frias et al., 2005) and greenhouse (Zhang et al., 2007) have been also used for laboratory scale germination.

The optimal conditions such as relative humidity (RH) and temperature for germination of ASL were studied in the present study using an oven. The oven was conditioned at high RH (95-99%) by putting water on each tray inside the oven. As previously shown in Figure 3-3, the RH inside the oven recorded by the data logger was stable, ranging from 95 to 99%. During optimization of the germination condition, two different temperatures (25°C and 28°C) were set up in the oven. However, when germination was performed at 28 °C (Appendix A1), the proportion of rotten sprouts and non germinated seeds at day 8 was higher (13% of total wet weight sprouts) than when germination was carried out at 25° C (1.5% of total wet weight sprouts). Prolonged germination period to more that 9 days for germination at 28°C caused about 30% of sprouts to rot (Appendix A1). Therefore, germination at 25°C in dark at high RH condition using a drying oven for 9 days was chosen for germination conditions in this study. The temperature at 25°C used in this study was the same as the temperature used by Zielinski (2002) and Zielinska et al. (2008) in the germination of *L. albus* seeds (Zielinska et al., 2008). However, other studies by Fernandez-Orosco et al. (2006) and Duenas et al. (2009) applied a different temperature of 20°C for germination of *L. angustifolius* var. Zapaton.

The weight of germinated seeds per 100 g of lupin seeds at 25°C is shown in Table 4-1 (wet basis) and Table 4-2 (dry basis). The whole germinated seeds (that is the sprouts) were separated manually into de-hulled sprouts, hulls, rotten sprouts and non-germinated seeds (Figure 3-2). All of the parts were then weighed and dried. The dried de-hulled sprout flours were then used for further analyses and were referred as germinated ASL flour. Based on the fresh weight of sprouts, 100 g of lupin seeds were able to produce 667 g de-hulled lupin sprouts following germination for 9 days

(Table 4-1 and Figure 4-1). This finding was higher than that observed in a study examining the yield of alfafa sprouts where 432-484 g fresh sprouts were produced per 100 g seeds after 3 days of germination (Sawyer et al., 1985).

Table 4-1 : Lupin yield during germination (wet weight basis)

Sample	Dehulled sprouts	Hulls	Rotten and non germinated	Total sprouts	MC of total sprouts
Day 1	205.57 ± 4.57	49.39 ± 0.74	9.73 ± 2.32	264.69 ± 5.09**a**	67.41 ± 1.68**a**
Day 2	246.86 ± 5.43	44.06 ± 0.29	13.95 ± 7.98	304.87 ± 3.34**b**	73.78 ± 1.09**a**
Day 3	301.21 ± 10.66	50.78 ± 2.32	10.95 ± 10.58	362.94 ± 2.69**c**	77.72 ± 1.54**a**
Day 4	360.35 ± 8.50	59.13 ± 2.92	14.57 ± 1.74	434.05 ± 9.00**d**	84.67 ± 1.23**b**
Day 5	426.17 ± 38.50	73.50 ± 11.39	17.26 ± 9.68	516.93 ± 39.88**e**	86.91± 2.64**bc**
Day 6	522.45 ± 16.64	87.90 ± 5.29	12.29 ± 4.26	622.63 ± 16.39**f**	89.56 ± 0.88**dc**
Day 7	606.50 ± 23.02	93.58 ± 5.28	10.98 ± 6.46	711.05 ± 18.98**g**	91.41 ± 0.32**d**
Day 8	650.99 ± 23.48	97.70 ± 4.91	24.27 ± 8.90	772.96 ± 18.31**h**	91.61 ± 0.87**d**
Day 9	666.99 ± 46.77	99.66 ± 6.04	40.48 ± 24.70	807.13 ± 37.25**h**	91.87 ± 0.93**d**

Shown are the yields of lupin expressed as wet weight (g/100g lupin seeds) during germination. Results are expressed as means ± standard deviations of measurements of three independent germinations. Different letter in the same column are significantly different ($P \leq 0.05$)

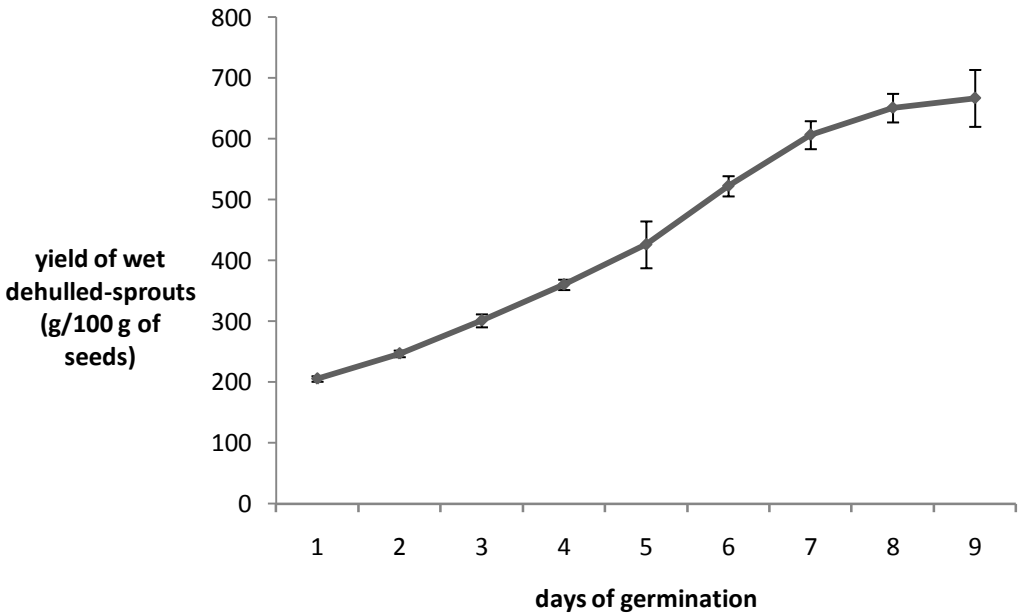


Figure 4-1 : Yield of dehulled ASL sprouts during germination (wet weight basis).

Shown are the yields of dehulled sprouts during germination expressed on a wet weight basis (g/100 g of seeds). Values are means ± standard deviations of measurements of three independent germinations.

Table 4-2 : Lupin yield during germination (dry weight basis)

Sample	Dehulled sprouts	Hulls	Rotten and non germinated	Total sprouts	MC of total sprouts
Day 1	63.48±1.92	20.45±0.68	3.56±0.75	87.66±2.35**ab**	3.04±0.73a**b**
Day 2	59.66±0.48	19.69±0.09	4.92±2.92	84.43±2.42**abc**	4.78±0.39a**b**
Day 3	58.28±2.79	19.79±0.81	3.31±3.11	81.53±1.59**bc**	4.15±0.84**ab**
Day 4	56.32±2.05	19.74±0.77	3.95±0.43	80.14±2.49**bc**	4.81±0.71**ab**
Day 5	54.44±1.78	19.68±0.74	4.70±2.74	78.94±1.28**bcde**	4.39±0.58**ab**
Day 6	52.32±1.69	19.34±0.53	2.57±0.97	74.50±1.33**de**	5.32±0.58**abc**
Day 7	52.88±1.98	19.85±0.44	2.30±1.13	75.13±1.55**de**	7.59±1.50**bc**
Day 8	47.65±2.13	19.30±0.73	4.35±1.59	71.41±2.39**ef**	7.12±0.90**bc**
Day 9	44.67±3.34	19.23±0.65	4.54±2.85	68.55±1.68**f**	7.08±0.44**bc**

Shown are the yields of lupin expressed as dry weight (g/100g lupin seeds) during germination. Results are expressed as means ± standard deviations of measurements of three independent germinations. Different letter in the same column are significantly different ($P < 0.05$).

Germination resulted in a significant increase in the weight of all parts of sprouts when expressed on the fresh weight basis. As shown in Table 4-1, following germination, the fresh weight of sprouts increased with the length of germination period. The total wet weight of sprouts increased significantly from 264.6 g at day 1 to 807.1 g at day 9 ($P \leq 0.05$). The increase in fresh weight may be caused by the high water content (about 91% at day 9) in the sprouts following germination. This was confirmed by the result of measurement of moisture content of the sprouts as shown in Table 4-1. These results are in agreement with the findings of other research where it was observed that the moisture content of alfafa sprouts (Hamilton and Vanderstoep, 1979), soybean and mungbean sprouts (Abdullah and Baldwin, 1984) and amaranth sprouts (Ruiz and Bressani, 1990) were also significantly increased following germination.

When expressed on a dry weight basis (db), the yield of ASL sprouts decreased during germination (Table 4-2 and Figure 4-2). As can be seen in Table 4-2, 32% of the initial weight of ASL seeds was lost during germination at day 9. In other words, at day 9 of germination, 100 g of ASL seeds resulted in 68 g of total ASL sprouts (db) or 44.67 g of dehulled ASL sprouts (db). The significant reduction ($P < 0.05$) in the yield of total sprout per dry weight basis may be caused by breakdown of major macromolecules such as lipids, proteins and carbohydrate during the germination process. A similar reduction was also found in sprouting of flaxseed where 35 % dry matter was lost after germination for 8 days (Wanasundara et al., 1999).

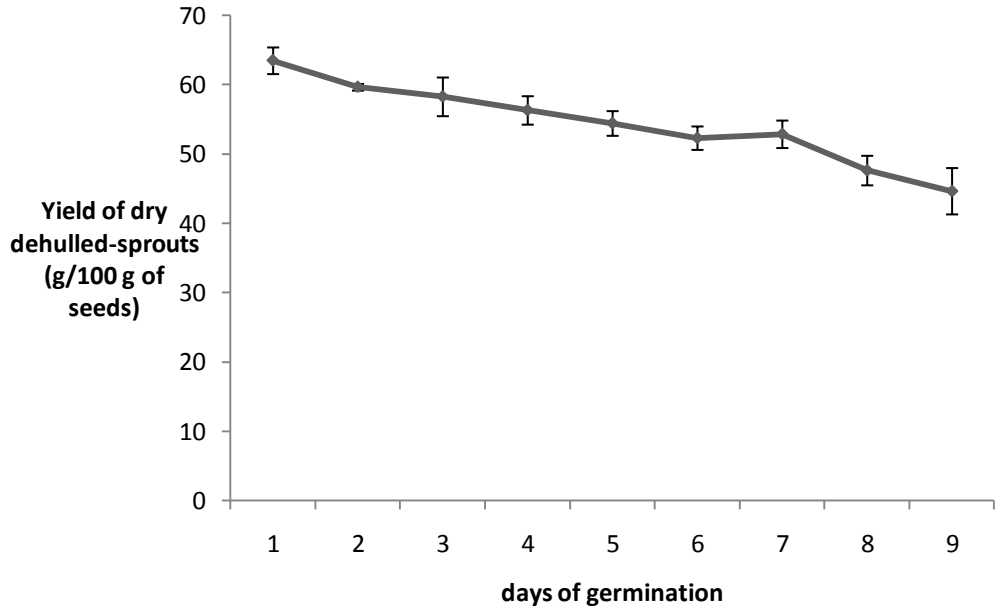


Figure 4-2 : Yield of dehulled ASL sprouts during germination (dry weight).

Shown are the yields of dehulled sprouts during germination expressed on a dry weight basis (g/100 g of seeds). Values are means ± standard deviations of measurements of three independent germinations.

During germination the appearance of fresh ASL sprouts and dried ASL sprout flours was different as illustrated in Figure 4-3 and Figure 4-4, respectively.

Figure 4-3 : Appearance of fresh dehulled ASL sprouts during germination.
Shown are representative samples of ASLsprouts at different days of germination.

Figure 4-4 : Appearance of dry dehulled ASL sprout flour samples
Shown are representative samples of dried-dehulled ASL sprout flours obtained from different days of germination

As shown in Figure 4-3, the radicle grew to approximately 1 cm at day 1 and 15 cm at day 9 and the leaf appeared at day 7 of the germination period. The differences of physical characteristic of sprouts and their flours across the different length of germination period, may be attributed by the changes of their chemical composition during germination.

4.2.2 Effect of germination on the chemical compositions of ASL

Germination affects the protein, crude fibre and lipid contents of ASL (Table 4-3 and Figure 4-5). The protein and crude fibre increased significantly ($P \leq 0.05$). and lipid content decreased significantly ($P < 0.05$).per dry weight basis during germination.

Table 4-3 : Change in chemical compositions during germination

Sample	Protein (%)	Fat (%)	Crude fibre (%)
Raw	44.1 ± 1.7**a**	6.4 ± 0.1**a**	1.6 ± 0.02**a**
Day 1	48.3 ± 1.6**b**	6.7 ± 0.1**b**	2.2 ± 0.7**b**
Day 3	50.4 ± 1.4**b**	4.6 ± 0.2**c**	3.5 ± 0.3**c**
Day 5	54.0 ± 1.6**c**	3.1 ± 0.3**d**	5.4 ± 0.2**d**
Day 7	56.7 ± 2.1**c**	2.3 ± 0.1**e**	7.4 ± 0.2**e**
Day 9	61.0 ± 2.0**d**	1.9 ± 0.1**f**	8.9 ± 0.3**f**

Shown are protein, fat and crude fibre content during germination expressed on a dry weight basis (g/100 g DM of dehulled ASL sprout flour).Values are means ± standard deviations of three independent analysis from three independent germinations. Different letter in the same column indicates a significant difference ($P \leq 0.05$)

Lupin was found to have high protein content. The ungerminated seeds had 44% (db) protein and following germination for up to 9 days, the protein content significantly increased ($P \leq 0.05$) to 61% db (Table 4-3). The increase was higher than that found by Dagnia et al (1992) where it was observed that germination for 6 days of *L. angustifolius* increased the protein content by about 10%. This effect of germination on protein content was also found in other legumes such as soybean (Mostafa and Rahma, 1987), mungbean (Mubarak, 2005), fenugreek (El-Mahdy and El-Sebaiy, 1982) and dry bean, lentils, faba beans (Hsu et al., 1980). The previous studies suggested that the utilisation of lipid and carbohydrates as energy sources during germination result in the loss of dry matter from germinated seeds. The increase in the protein content of germinated ASL per g dry weight basis compared to the protein content in the raw ASL may therefore be a result of the loss of dry matter in the germinated ASL. In other studies, it has been reported that during germination amino acids, small molecular weight proteins and enzymes are formed in *L. albus* (Duranti et al., 1984). Germination also led to a significant increase in free protein amino acids (FPAAs) and free non-protein amino acids (FNPAAs) in soybean and lupin (Martinez-Villaluenga et al., 2006). In addition, during germination, non-protein amino acids (FNPAAs) such as GABA, trigonelline, β-alanine have been reported to be synthesized in soybean and lupin sprouts as metabolic intermediates or

plant hormones in the seedling seeds (Martinez-Villaluenga et al., 2006). In the present study, determination of protein concentration by Kjeldahl may also determine the concentration of FPAAs and FNPAAs. Therefore, the increases in these components may also contribute to the increased estimation of protein contents.

A study carried out by Dagnia et al. (1992) showed that the quality of the protein and the total content of essential amino acids of germinated *L. angustifolius* were lower than the ungerminatedseed. The low quality of protein in sprouts is likely to be due to the decrease of sulphur-containing amino acids such as methionine and cysteine during germination. The total essential amino acid contents increase during germinating legumes such as in soybean (Mostafa and Rahma, 1987) and in mungbean (Mubarak, 2005). The digestibility of protein is increased following germination as found in germinated white beans, black beans, pigeon beans (Sangronis and Machado, 2007) and in germinated cowpea, lentil and chickpea (Ghavidel and Prakash, 2007).

Another major chemical component of lupin is the oil. It is a source of nutritional components and bioactive compounds such as mono- and polyunsaturated fatty acids, tocopherols and phytosterols. As shown in Table 4-3, following germination for 9 days, a significant decline in ASL total lipid content from 6.4 % to 1.9 % (70% decrease; $P \leq 0.05$) was observed. This result is higher than that reported by Dagnia et al. (1992) where germination of *L. angustifolius* for 6 days was found to decrease the lipid content by 50%. The decline is likely to be due to the use of the lipid as energy source during germination. Lipid content has also been reported to decrease during germination in soybean (Mostafa and Rahma, 1987) and flax seeds (Wanasundara et al., 1999). Many nutritional and bioactive compounds are found in the lipid component in legumes. Therefore, the gradual decrease in the lipid content in this study would be expected to affect the concentration of these nutritional and bioactive compounds.

The dietary fibre content in lupin is high and is important in its hypocholesterolemic properties. In this study, the dietary fibre content during germination was estimated based on the crude fibre content measured using Fibrecap 2021 unit. A disadvantage of crude fibre measurement is that the measurement only determines a fraction of the

total dietary fibre (Asp et al., 1983). Therefore, the crude fibre content observed in the samples in this present study (Table 4-3) was much lower than the actual dietary fibre content of raw lupin (30%) (Petterson, 1998). Nevertheless, the data on crude fibre content during germination in the present study has indicated that germination could significantly increase the content of crude fibre (Table 4-3). The concentration of crude fibre following germination for 9 days was significantly ($P \leq 0.05$) greater (456% more) than that present in raw seeds. In other studies, a high concentration (36%) of dietary fibre has been reported in *L. albus* following germination for 2 days (Trugo et al., 2000). The effect of germination on dietary fibre has also been studied in peas where it was found that the total dietary fibre was substantially increased by about 100% (Martin-Cabrejas et al., 2003). The increase in dietary fibre was found to be mostly due to changes in the polysaccharides found in the cell wall such as cellulose, glucose and mannose, suggesting that the changes were due to an increase in the cellular structure of the plant during germination. Therefore, germination of ASL seeds in the present study appears to be an effective way to improve fibre content in ASL.

Germination improved the protein and fibre content of ASL and reduced the fat content of ASL (Figure 4-5). Although the protein and fibre content increased during germination, the loss of dry matter of germinated ASL (db) was significant. Therefore, the loss of the fat during germination may predominantly affect the loss of dry matter of the germinated ASL (db).

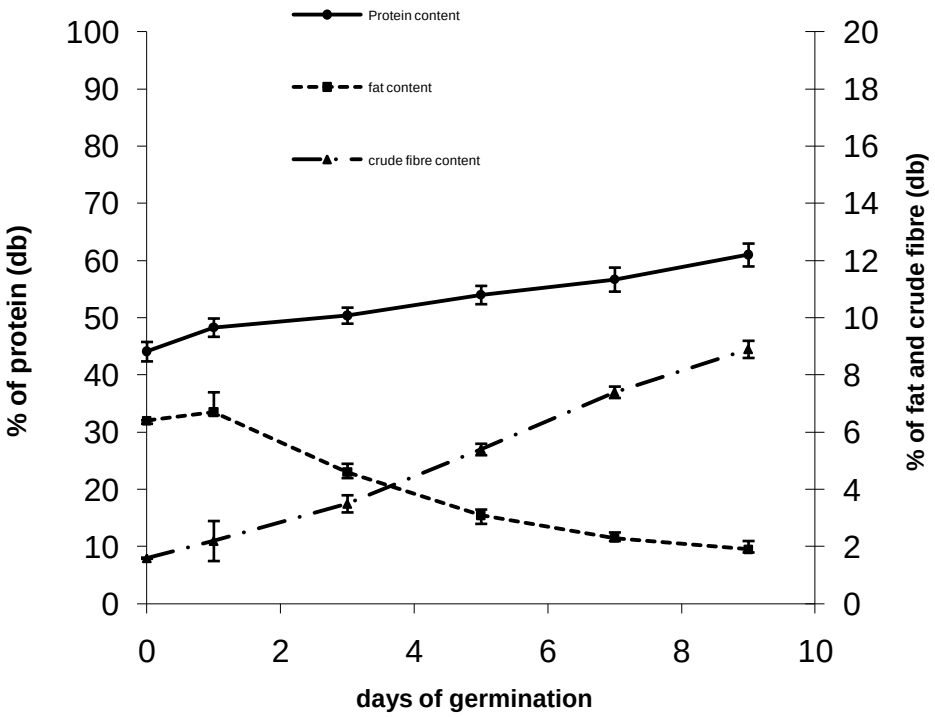


Figure 4-5 : Changes in macronutrients of ASL during germination.

Shown are the changes in protein, fat and crude fibre during germination expressed on a dry weight basis (g/100 g DM of dehulled sprout flour). Values are means ± standard deviations of three independent analysis from three independent germinations. The left vertical axis shows scale for content of protein and crude fibre, while the right vertical axis shows scale for content of fat (% g/100 g DM)

4.2.3 Effect of germination on total phenolic compounds (TPCs) and DPPH radical scavenging activity

In the present study the concentration and activity of phenolic compounds in both the aqueous (Tris buffer) and 80% MeOH extracts were determined in order to investigate the most effective solvent to extract these compounds with high antiradical activity. The 80% MeOH has been used to extract phenolic compounds in lupin in other studies (Fernandez-Orozco et al., 2006, Martínez-Villaluenga et al., 2009). The TPC in the present study was expressed as gallic acid equivalent (mg of GAE/100g dried sprout flour). The results of measurements of TPC in the extracts is shown in Table 4-4. As shown in the table, the spread of the data could be caused by

the samples that came from 3 different extractions of 3 different batches of germination

In the aqueous extracts, the TPCs at day 3-9 was found to have higher TPCs than in the raw and day 1, whereas in the MeOH extracts, the TPCs at day 7 and 9 was the highest among the other samples (Table 4-4). Up to day 5 of the germination, the TPCs in the aqueous extracts was higher than in the methanolic extracts, however at day 7, the TPCs in the methanol extract was higher than that in the water extracts (Table 4-4, Figure 4-6). The amount of the TPCs in the aqueous extracts was significantly higher ($P< 0.05$) than in the methanolic extracts until day 5 of germination. The results suggest that phenolic compounds produced in the early stage of germination are more water soluble, whereas in the later stages, a greater number of methanol soluble phenolic compounds are produced. Another possible reason was that that components such as vitamin C present in the aqueous extract may interfere with the determination of TPCs, resulting in higher TPCs concentrations than that actually present in the sample. On the other hand, results from another study have shown that phenolic compounds in germinated *L. angustifolius* were more extractable in phosphate buffered saline (PBS) than in methanol (Fernandez-Orozco et al., 2006).

Table 4-4 : Change in TPCs during germination

Samples	TPC mg gallic acid/100 g DM	
	Aqueous extract	**MeOH extract**
Raw	256.62 ± 45.0**a**	95.4 ± 18.9**a***
Day 1	245.74 ± 30.23**a**	166.7 ± 27.4**b***
Day 3	556.29 ± 127.98**b**	366.9 ± 126.2**c***
Day 5	544.14 ± 141.24**b**	410.9 ± 97.1**c***
Day 7	513.50 ± 16.24**b**	671.5 ± 134.4**d***
Day 9	543.89 ± 78.55**b**	788.0 ± 147.3**d***

Shown are the changes in concentration of TPCs during germination expressed as mg equivalent gallic acid/100 g DM of dehulled ASL sprout flour. Values are means ± standard deviations of measurements of three independent extractions from each of three independent germinations. Different letter in the same column indicates a significant difference ($P < 0.05$). * indicates significant differences between aqueous and MeOH extracts using student t-test ($P < 0.05$)

When looking at the change in the TPCs at 9 days of germination, the TPCs of the MeOH extract increased sharply by 700% compared to ungerminated seeds whereas the TPCs in the aqueous extracts only increased by about 100% ($P \leq 0.05$, Table 4-4). The significant increase of the TPCs during germination could be explained by a complex mechanism of metabolism of seeds during the germination period. Results of this study showed that the increase in the TPCs in the methanol extract (700%) was greater than that reported by Fernandez-Orosco et al. (2006) and Duenas et al. (2009) who found an increase of 53% (expressed as mg of catechin) and 63% (expressed as mg of gallic acid) of TPCs after 9 days of germination of *L. angustifolius*. The different germination conditions such as temperature could be a reason for the differences in the findings among studies. An increase in the TPCs has been also reported during germination of beans, lentils and peas (Lopez-Amoros et al., 2006). Furthermore, the change in the TPCs after germination has been shown to affect the composition of individual phenolic compounds of beans, lentils and peas (Lopez-Amoros et al., 2006) and *L. angustifolius* var. Zapaton (Duenas et al., 2009). Isoflavones such as genistein derivatives have been reported as the main isoflavones in lupin sprouts at day 9 (Duenas et al., 2009). Genisteins are phytoestrogens that are

thought to play an important role in preventing some diseases including CVD and cancer. The significant change in the TPCs found in this study (700% increase) is a promising finding suggesting the importance of lupin sprouts as a source of phenolic compounds.

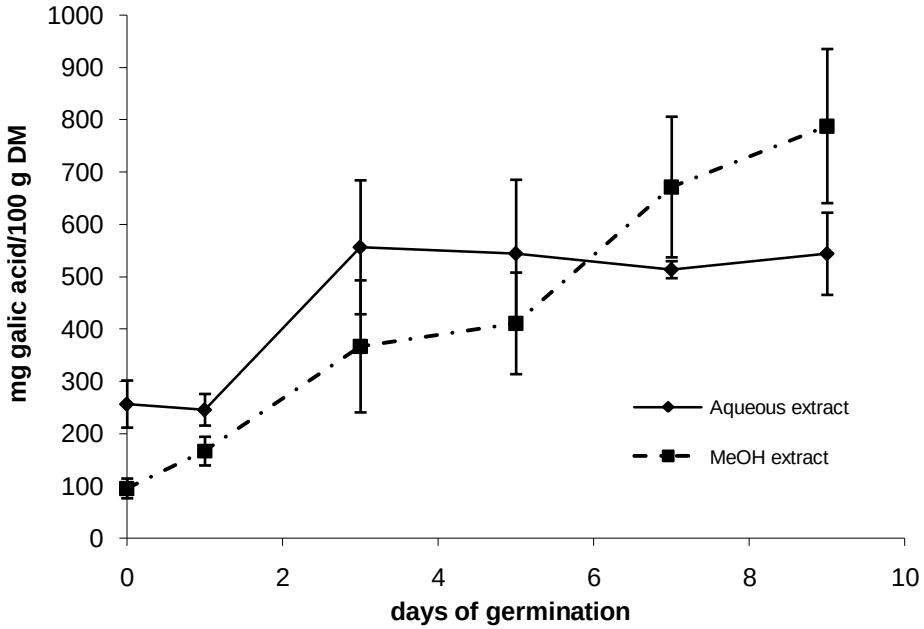


Figure 4-6 : Changes in TPC of ASL during germination.

Shown are the changes in the concentration of TPC during germination expressed as mg gallic acid/100 g DM of dehulled ASL sprout flour. Values are means ± standard deviations of measurements of three independent extractions from each of three independent germinations.

Many phenolic compounds are natural antioxidants. The change in levels and structure of phenolic compounds in lupin during germination may influence their antioxidant activities. In regards to the evaluation of antioxidant activity of germinated lupin in this study, the DPPH free radical scavenging activity was used to determine the antioxidant activity and its activity is expressed as mg trolox/g DM sample flour.

The DPPH radical scavenging activity of the aqueous and methanolic extracts increased with the days of germination (Table 4-5 and Figure 4-7). At day 3, 5, 7 and

9 of the germination, the DPPH radical scavenging activity (DPPH-RSA) of the MeOH extracts was significantly higher ($P < 0.05$) than that observed in the aqueous extracts (Table 4-5 and Figure 4-7). During germination of up to day 7, a slight but significant increase in the DPPH-RSA of the methanolic extracts was observed. However, at day 7 to 9 of the germination, there was a sharp increase which accounts for 138% of the scavenging activity. This result indicates that germination enhances compounds with radical scavenging activity higher in the MeOH than in aqueous extracts. The enhancement of the DPPH-RSA of the aqueous extracts from day 1 to day 9 was about 138% compared to 443% in the MeOH extracts. It appears that the majority of the phenolic compounds with radical scavenging activity are present in the MeOH extract.

Table 4-5 : Change in DPPH radical scavenging activity during germination

Samples	DPPH radical scavenging activity (mg trolox/g DM)	
	Aqueous extract	**MeOH extract**
Raw	0.22 ± 0.08**a**	0.25 ± 0.03**a**
Day 1	0.65 ± 0.29**b**	0.70 ± 0.03 **b**
Day 3	0.81 ± 0.35**b**	1.30 ± 0.3**cd***
Day 5	0.79 ± 0.25**b**	1.10 ± 0.2**c***
Day 7	1.20 ± 0.26**c**	1.60 ± 0.2**d***
Day 9	1.55 ± 0.24**c**	3.80 ± 0.5**e***

Shown are the changes in DPPH radical scavenging activity during germination expressed as mg equivalent trolox/100 g DM of dehulled ASL sprout flour. Values are means ± standard deviations of measurements of three independent extractions from each of three independent germinations.. Different letter in the same column indicates a significant difference ($P \leq 0.05$). * indicates significant differences between aqueous and MeOH extracts using student t-test ($P < 0.05$)

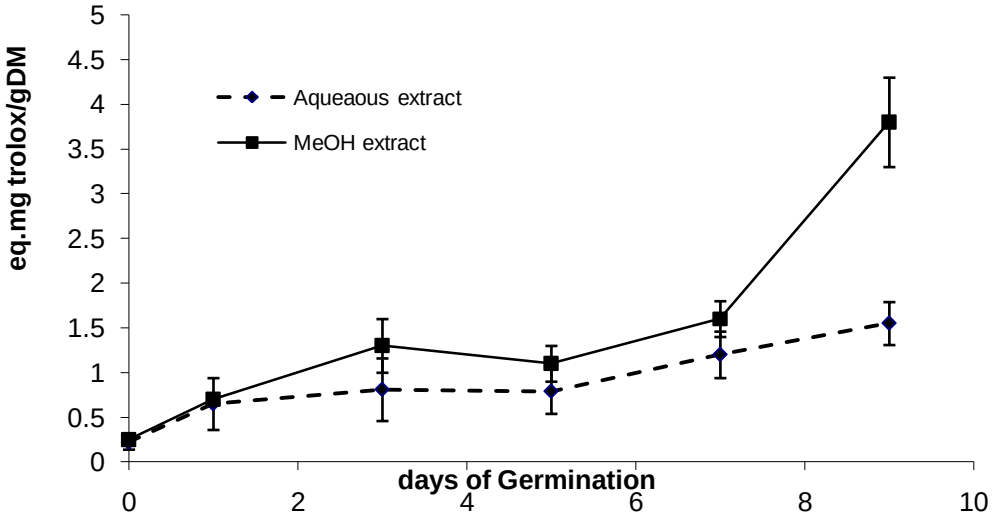


Figure 4-7 : Changes in DPPH radical scavenging activity of ASL during germination

Shown are the changes in the DPPH radical scavenging activity during germination expressed as mg trolox/100 g DM of dehulled sprout flour. Values are means ± standard deviations of measurements of three independent extractions from each of three independent germinations.

Germination increases the TPCs of ASL in the MeOH and aqueous extracts. The significant increase in the phenolic content in the extracts did not correlate well with the increase of the DPPH-RSA (Appendix A11). For instance, the high phenolic compounds determined in aqueous extracts at days 1-7 were not associated with the high antioxidant activity in the extracts. The results indicated that not all phenolic compounds may exhibit antioxidant activity by mechanism of free radical scavenging activity or there could be other compounds present in the MeOH extracts that have contributed to the radical scavenging activity. The correlation between the concentration of phenolic compounds and antioxidant activity in the MeOH and the aqueous extracts was obtained: R^2 = 0.78 and R^2 = 0.49, respectively (Appendix A11). The low correlation between the TPC and antioxidant capacity of MeOH extracts of *L. angustifolius* was also observed Oomah et al. (2006). It is suggested that MeOH is preferable for extraction of phenolic compounds with high antiradical activity in the raw and germinated lupin samples.

4.2.4 Effect of germination on phytosterol content and antioxidant activity of the oil fraction

Phytosterols are compounds that have been known to have cholesterol lowering properties and antioxidant activities. As described in Section 2.1.2.5.2, phytosterols in plants can be found in the variuos forms of free sterol, sterol ester, steryl glycosides and acylated steryl glycoside. During analysis, esterified sterols can be hydrolyzed by saponification to release free sterols, while the glycosidic bonds in the glycosylated form can be hydrolyzed by acid hydrolysis. In this study, free sterols in the oils of raw and germinated ASL flours were analyzed after hydrolysis of the samples by direct hot saponification, followed by GC analysis as described by Hamama and Bhardwaj (2004) and Hamama et al. (2003).

Phytosterols in the chromatograms were determined by comparing the retention time (RT) with that of the phytosterol standards. Examples of the GC chromatograms of oils extracted from raw seeds and germinated ASL flour (day 7) are given Figure 4-8.

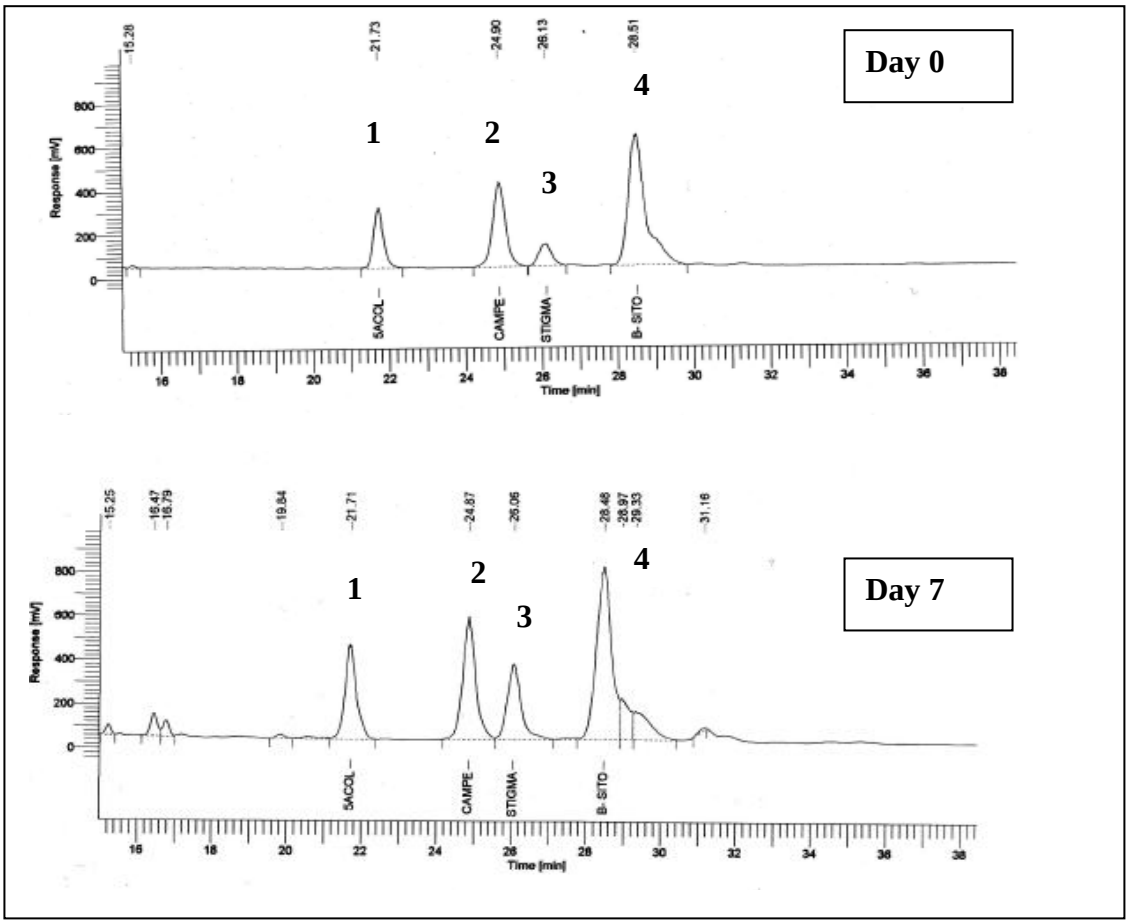


Figure 4-8 : GC Chromatogram of phytosterols from oil extracted from raw seeds (day 0) and germinated ASL (day 7)

The chromatograms show peaks of phytosterols separated with GC condition in this study. The following identified peaks (from left to right) are : (1) internal standard (IS) : 5-α-cholestan-3β-ol, (2) campesterol, (3) stigmasterol, (4) β-sitosterol.

The first peak was internal standard, while the following three peaks belonged to campesterol, stigmasterol and ß-sitosterol. Therefore, they were the main phytosterols obtained in the oil extracts from the raw and germinated ASL. The RTs for campesterol, stigmasterol and ß-sitosterol were 24.40, 14.13 and 28.51 min, respectively. The chromatograms between day 0 (raw ASL seeds) and day 7 of germinated ASL flours had a similar pattern. However, weak peaks emerged following the peak of ß-sitosterol of chromatogram on day 7 were observed. The weak peaks could not be identified due to unavailability of the standards for these peaks. In another study, eight phytosterols have been identified in *L. albus*. The five most abundant phytosterols in *L. albus* were β-sitosterol (56 %), campesterol (25.8

%), stigmasterol (10.7%), Δ^5-avenasterol (1.6%), and Δ^7-stigmastenol (1.3%) (Hamama and Bhardwaj, 2004). Therefore, the weak peaks emerged on Figure 4-8 on day 7 after the peak of β-sitosterol could be the peaks of Δ^5-avenasterol and or Δ^7-stigmastenol. The peaks could not be separated by the conditions of GC used in this analysis.

Quantitatively, the β-sitosterol was found to be the major phytosterols in the oils of both of raw and germinated ASL flours, followed by campesterol and stigmasterol (Table 4-6). The total phytosterols calculated based on these three phytosterols in the raw and germinated ASL flours varied from 1.7 to 6.1 % of the weight of oils. These amounts were higher than those observed in another seed such as canola oil (0.8%) (Hamama et al., 2003).

Table 4-6 : Phytosterol contents in the oil fraction of germinated ASL flour (g/100g oils)

Sample	g/ 100g oils (%)			Total (g/100g oils) (%)
	campesterol	stigmasterol	β-sitosterol	
Raw	0.536 ± 0.038**a**	0.118 ± 0.011**a**	1.077 ± 0.074**a**	1.732 ± 0.123**a**
Day 1	0.561± 0.123**a**	0.169 ± 0.089**ab**	1.172 ± 0.335**a**	1.903 ± 0.548**a**
Day 3	0.886 ± 0.078**b**	0.358 ± 0.0096**b**	1.802 ± 0.059**b**	3.045 ± 0.145**b**
Day 5	1.197 ± 0.048**c**	0.559 ± 0.065**c**	2.439 ± 0.221**c**	4.146 ± 0.324**c**
Day 7	1.412 ± 0.085**d**	0.865 ± 0.086**d**	2.964 ± 0.165**d**	5.241 ± 0.326**d**
Day 9	1.631± 0.282**d**	1.281 ± 0.235**e**	3.155 ± 0.457**d**	6.067 ± 0.958**d**

Shown are the phytosterol contents in the oil fraction of germinated ASL flour expressed as g/100 g oils. Values are means ± standard deviations of measurements of three independent germinations. Different letter in the same column indicate a significant difference ($P \leq 0.05$)

When looking at the change in phytosterols at day 9 of germination, the total phytosterol concentration increased by about 3 fold (expressed per weight of oil basis) compared to the raw seeds (Table 4-6). However, when expressed per dry matter of the germinated ASL flour, the total phytosterols remained constant (Table 4-7). The significant increase in the phytosterol content (expressed by oil weight

basis) was due to the marked reduction of the total lipid content (71% decrease) in the germinated ASL flours. As a result of the reduced lipid content, the lipid soluble components such as phytosterols are concentrated in the germinated ASL flour samples. The reduction of oil amount in the germinated ASL concomitant with the improvement of phytosterol concentration therefore has benefits for the use of germinated ASL flours as source of bioactive compounds.

Table 4-7 : Phytosterol contents in the oil fraction of germinated ASL flour (g/100g DM flours)

Sample	g/100 g flours (%)			Total (g/100g DM)
	campesterol	stigmasterol	β-sitosterol	
Raw	0.045 ± 0.01**a**	0.0099 ± 0.002**a**	0.091 ± 0.020**a**	0.147 ± 0.032**a**
Day 1	0.049 ± 0.018**a**	0.015 ± 0.01**a**	0.104 ± 0.046**a**	0.170 ± 0.074**a**
Day 3	0.034 ± 0.014**a**	0.014 ± 0.007**a**	0.070 ± 0.034**a**	0.118 ± 0.054**a**
Day 5	0.031 ± 0.019**a**	0.015 ± 0.009**a**	0.064 ± 0.039**a**	0.109 ± 0.067**a**
Day 7	0.029 ± 0.004**a**	0.018 ± 0.004**a**	0.062 ± 0.011**a**	0.109 ± 0.019**a**
Day 9	0.034 ± 0.008**a**	0.026 ± 0.006**a**	0.065 ± 0.016**a**	0.126 ± 0.029**a**

Shown are the phytosterol contents in the oil fraction of germinated ASL flour expressed as g/100 g DM flours. Values are means ± standard deviations of measurements of three independent germinations. Different letter in the same column indicates a significant difference ($P \leq 0.05$)

Furthermore, the increase in the total phytosterol content during germination (per weight oils) did not affect the composition and relative proportion of the phytosterols as shown in Figure 4-9. As germination proceeded, the amount of individual phytosterol increased. However, the proportion among them remained relatively constant; β-sitosterol (60%); campesterol (30%) and stigmasterol (10%). These proportions were in agreement with the results of Hamama and Bhardwaj (2004) who observed phytosterol content in *L. albus* with the following proportions; ß-sitosterol (56±3.6%), campesterol (25.8±3.1%), and stigmasterol (10.7±1.9%) with small amount of the others (Δ^5-avenasterol and Δ^{7-} stigmasterol). There was also no change in the composition of phytosterols during the germination period of alfafa (Huang and Grunwald, 1988) and soybean (Mostafa and Rahma, 1987). As the

phytosterol composition in the present study remained unchanged during germination until day 9, it appeared that there were no new phytosterols synthesized throughout the germination period. Whereas in other plants such as the cruciferae family germination has been shown to result in the biosynthesis of sterols or interconvertions of sterols to another form (Ingram et al., 1968).

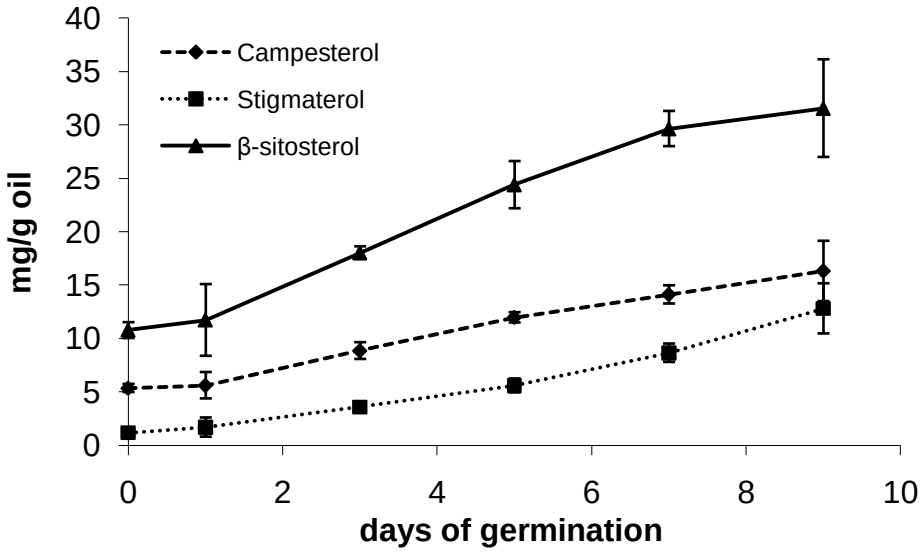


Figure 4-9 : Concentration of individual phytosterols of oils extracted from germinated ASL flour.

Shown are the changes in the individual phytosterols of oils extracted from germinated ASL flour expressed as mg /g oils. Values are means ± standard deviations of measurements of three independent germinations. .

There is limited information about the effect of germination on the phytosterols in lupin. However, some studies have observed the effects of germination on phytosterol content in canola seeds (Zhang et al., 2007) and alfafa seeds (Huang and Grunwald, 1988). For example, oil extracted from 20 days of seedling canola seeds had a 4-5 fold higher concentration of phytosterols than that of ungerminated canola. The 3 fold increase in the phytosterol content as observed in the present study appeared to be comparable with results observed in the previous study.

Phytosterols have been shown to have a range of bioactivities including hypocholesterolemic properties and antioxidant activity. A study has shown that phytosterols contribute to the antioxidant activity by their ability to inhibit oil polymerisation and to prevent peroxide formation (Wang et al., 2002b). In this present study, crude oils extracted from germinated ASL flour at the same weight basis exhibited a greater DPPH radical scavenging activity (RSA) than that of raw ASL seeds (Figure 4-10). Following the germination period, the RSA of the oil obtained from day 9 increased about 9 fold compared to ungerminated ASL seeds. The increase may be attributed to the significant increase in phytosterol content in the oil. The other components in the oil such as tocopherols, and phenolics may have also contributed to the RSA activity of the extracted oils (Ramadan and Moersel, 2006, Miraliakbari and Shahidi, 2008).

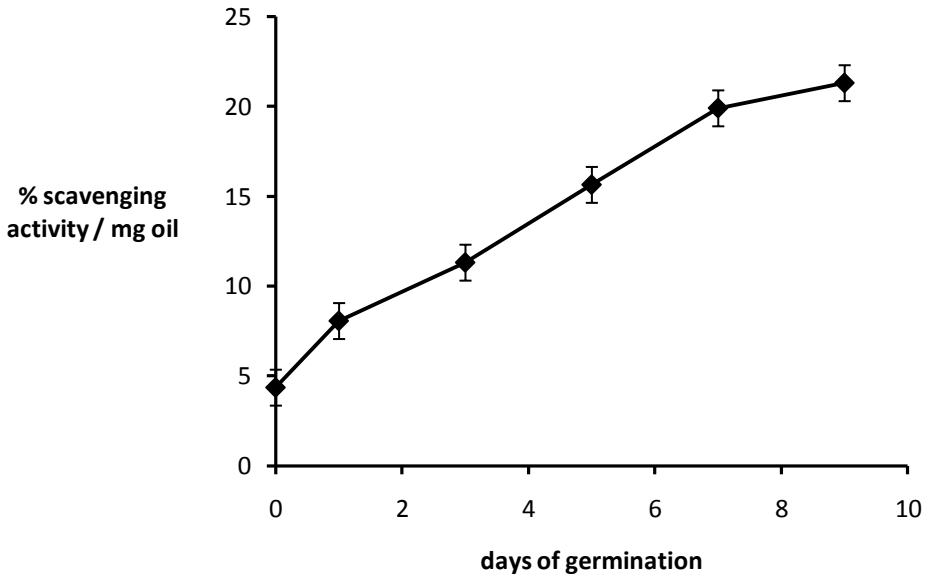


Figure 4-10 : Antioxidant activity of oils extracted from germinated ASL flour.
Shown are the changes in the antioxidant activity of oils extracted from germinated ASL flour expressed as % scavenging activity/mg oil. Values are means ± standard deviations of measurements of three independent germinations.

It is clear that germination results in an improvement in the concentration of phytosterol in the oils extracted from germinated ASL flours. The high concentration

of phytosterols in the oils of germinated ASL flours may have contributed to the observed radical scavenging activity.

4.2.5 Bile acid binding property of germinated ASL flours

To investigate BA binding property of the raw seed and germinated ASL flours, an equal weight dry matter of the samples (50 mg) was assayed for BA binding property *in vitro* using the primary BA (cholic acid and sodium chenodeoxycholic acid) and a secondary BA (deoxycholic acid). Cholestyramine, a drug that lowers cholesterol by BA binding activity was used as the positive control. The results of the BA binding assay are shown in Table 4-8.

Table 4-8 : BA binding property of germinated ASL flour

Samples	bound cholic acid (µmol/100 mgDM)	bound deoxycholic acid (µmol/100 mgDM)	bound sodium chenodeoxy cholate (µmol/100 mgDM)
raw	0.621 ± 0.108**a**	0.138 ± 0.033**a**	0.656 ± 0.146**a**
Day1	0.625 ± 0.092 **a**	0.322 ± 0.098**b**	0.927 ± 0.135**a**
Day 3	0.727 ± 0.209 **ab**	0.494 ± 0.065**c**	1.300 ± 0.120**b**
Day 5	0.862 ± 0.077 **b**	0.570 ± 0.032**c**	1.406 ± 0.109**b**
Day 7	0.941 ± 0.031 **b**	0.615 ± 0.033 **c**	1.293 ± 0.076**b**
Day 9	1.040 ± 0.072**c**	0.670 ± 0.099 **c**	1.223 ± 0.069**c**
Cholestyramine	5.136 ± 0.268**d**	4.860 ± 0.611**d**	7.680 ± 0.63**d**

Shown are the changes in BA binding property of germinated ASL flour expressed as µmol/100 mg DM. Values are means ± standard deviations of measurements of three independent germinations. Different letter in the same column indicate a significant difference ($P \leq 0.05$)

Cholestyramine bound to cholic acid at an amount of 5.136 µmol/100 mg DM. Meanwhile, raw seed and germinated ASL flours bound to cholic acid at a various amounts, ranging from 0.621 to 1.04 µmol/100 mg DM. As expected, cholestyramine

had a higher cholic acid binding activity than that of raw and germinated ASL flours. As germination proceeded, the cholic acid binding activity of germinated ASL flours tended to increase significantly until day 9 of germination (Table 4-8). At day 9 of germination, the binding was at its highest. No other studies have determined the BA binding ability of lupin flour, but a few studies have reported significant BA binding ability of protein isolates from lupin.

Deoxycholic acid was bound by cholestyramine at an amount of 4.86 μmol/100 mg DM and at a range of 0.138 to 0.670 μmol/100 mg DM by raw seed and germinated ASL flours (Table 4-8). The amount of cholestyramine bound to deoxycholic acid was lower than that bound to cholic acid and sodium of chenodeoxycholic acid. During germination, the deoxycholic acid binding activity of the germinated ASL flours increased significantly from 0.138 μmol/100 mg DM (raw seed) to 0.670 μmol/100 mg DM (day 9). Furthermore, sodium of chenodeoxycholic acid was bound by cholestyramine at a level of 7.68 μmol/100 mg DM and by the raw and germinated ASL flours at a range of 0.65 to 1.40 μmol/100 mg DM (Table 4-8). These results showed that the components in lupin that bind bile acids exhibited a different affinity for different type of bile acids.

Some studies have investigated the BA binding property of a range of legumes, grains and vegetables using BA mixtures. The bile acid binding properties of soybean, pinto beans, black beans and wheat gluten (determined by mixture of BAs) were in the range of 0.60 to 1.58 μmol/100 mg DM (Kahlon and Woodruff, 2002) whereas spinach, brussels sprouts, broccoli, mustard green, cabbage, collards had the BA binding properties recorded as between 0.21 to 0.87 μmol/100 mg DM (Kahlon et al., 2007) with black eye bean, garbanzo, lima bean had BA binding properties of between 2.5 to 8.88 μmol/100 mg DM (Kahlon and Shao, 2004). Meanwhile, the binding of cholestyramine to BA mixtures was 10.91 to 11.1 μmol/100 mg DM (Kahlon and Shao, 2004, Kahlon and Woodruff, 2002). Thus, the findings of the present study are comparable with those reported by the other studies, although the cholestyramine binding to BA mixture seems to be much lower than those observed in the studies.

The variability in BA binding among studies could be affected by the different assay conditions used. For example, in the present study, a BA concentration of 0.5 mM was used. However, Story and Krichevsky (1976) used a cholic concentration of 10 mM while Camire and Dougerty (2003) applied a cholic concentration of 12.5 mM. In addition, the amount of sample used varied in different studies. The amount of cholestyramine used in this study was 10 mg, while in the study conducted by Camire et al. (1993) was 25 mg.

Furthermore the use of different units among studies can also make it difficult to compare the results between studies. In this regard, BA binding properties of *L. albus* protein isolate has been reported by a number of studies. However, different units were used to quantitate the BA binding properties in comparison to that used in the present study. For example in one study, the percentage of binding to the BAs was expressed as percentage bound that represents differences between free BA concentration in the control and in the samples. The cholic acid was reported to be bound to a level of 53.1% by cholestyramine, 34.7 to 41.3% by de-oiled lupin and its hydrolysate, 14.8 to 29.4% by lupin protein isolate E and its hydrolysate, 54.4 to 58.3% by lupin protein isolate F and its hydrolysate (Yoshie-Stark and Wasche, 2004).

The greater BA binding ability of germinated ASL found in this study shows that germination is a simple method to enhance the BA binding ability of ASL. The higher binding property of germinated ASL flour samples may be attributed to the significant change in crude fibre and protein content during germination period. As reported by Cornfine et al. (2010), dietary fibre of *L. angustifolius* L. has a taurocholic acid binding property at level of 19% compared to cholestyramine (100%) when the dietary fibre was assayed using an *in vitro* digestion model.

4.2.6 ACE inhibitory activity of total protein extracts of raw and germinated ASL flour in association with their protein pattern

Total protein extracted from raw and germinated ASL flours may consist of storage proteins, structural proteins and biologically active enzymes. The storage proteins can be categorized into globulins, albumin, prolamin and gluteline. Globulins in

lupins consist of α, β, γ and δ- conglutin (Duranti et al., 2008). The total protein extracts of lupin can be further fractionated to separate protein globulins into protein isolate E (α, β, δ-conglutin) and soluble fraction/isolate F (γ-conglutin) as reported by Sironi et al. (2005). Additionally, protein isolates of legumes have been widely used in food applications. Proteins of legumes may have an important role in managing blood pressure by inhibition of ACE activity that is similar with mechanism of captopril as antihypertensive agent. To screen proteins extracted from the raw and germinated ASL flours that have the ACE inhibitory activity, the total protein extracts obtained from those samples were investigated in their *in vitro* ACE inhibitory activity. The ACE inhibitory activity of the protein extracts was then compared to their SDS-PAGE pattern in order to determine which proteins are responsible for the ACE inhibitory activity.

4.2.6.1 ACE inhibitory activity of total protein extracts of raw and germinated ASL flour

The percentages of ACE-inhibition of raw and germinated ASL proteins are shown in Table 4-9. The ACE inhibitory activity ranged from 38.36 to 88.25 %. Compared to the ACE inhibitory activity of raw ASL protein, all germinated ASL samples had significantly higher ACE inhibitory activity ($P \leq 0.05$). The high ACE inhibitory activity of the germinated ASL proteins could be due to the releasing of peptides or amino acids by enzymatic hydrolysis through germination. This has been confirmed by the results of SDS PAGE which showed that the amount of high molecular weight of proteins reduced following germination (Figure 4-11). Other studies have found that enzymatic hydrolysis and fermentation tends to generate ACE inhibitory peptides from native proteins (Hernandez-Ledesma et al., 2003, Li et al., 2005, Yoshie-Stark et al., 2008). The study by Li et al. (2005) on mungbean found that unhydrolyzed and its hydrolysate showed inhibition to ACE at level of 2.49% and 76.42%, respectively. The results suggest that enzymatic hydrolysis is a key factor in increasing ACE inhibitory activity. It is suggested that enzymatic hydrolysis occurring during germination may be a natural way to obtain ACE inhibitory peptides which have contributed to increased antihypertensive activity.

Table 4-9 : Change in ACE inhibitory activity of protein extracts during germination

Samples	% inhibition of ACE per 100 µg protein
Raw	38.36 ± 6.88**a**
Day 1	78.17 ± 6.02**b**
Day 3	88.25 ± 8.85**b**
Day 5	78.13 ± 15.64**b**
Day 7	72.94 ± 14.8 **b**
Day 9	84.13 ± 7.57**b**

Shown are the changes in the ACE inhibitory activity during germination expressed as percentage of inhibition. Values are means ± standard deviations of measurements of three independent germinations. Different letter in the same column indicates a significant difference ($P \leq 0.05$)

The range of different conditions used in the assays including amount of sample, substrate and enzyme complicates the comparison amongst the different studies. Nevertheless, the percentage of the ACE inhibition activity of the germinated ASL proteins in this study was comparable compared to that conducted by Hernandez-Ledesma et al. (2003). They found that the percentage of ACE inhibitory activities were 43.4, 39.5, 87.2, 31, 1 and 79.7 % for full-fat yogurt whey, skimmed yogurt whey, infant formulas whey, white wine whey and red wine, respectively. Furthermore, de-oiled lupin, lupin protein isolate E and F of *L. albus* and their hydrolysate have been reported to have ACE inhibitory activity ranging from 0.7 to 59% (Yoshie-Stark et al., 2004).

4.2.6.2 Pattern of total protein extracts based on SDS-PAGE

The pattern of total proteins extracted from the raw and germinated ASL flour samples were analysed using SDS-PAGE. The changes in the patterns were then compared with the changes in the ACE inhibitory activity during the germination process. As shown in Figure 4-11, the proteins present in raw ASL have a molecular weight ranging from 15-90 kDa (lane 1). The major proteins found in legume seed

are storage proteins, making up to approximately 75% of the total protein content (Duranti et al., 2008). The total protein profile of ASL was dramatically changed by germination after day 3 as shown in lane in Figure 4-11. The identity of the proteins present at the different stages of germination could be determined by comparison to the protein profile observed in previous studies using *L. albus* seeds. According to Melo et al. (1994), the reduced α – conglutin of *L. albus* is composed of several subunits with molecular weights of 31, 36, 42, 46 kDa and a minor subunit of 19 kDa. Meanwhile, Duranti et al. (2008) reported that α- conglutin was composed of acidic and basic protein subunit with molecular weight (MW) of 42-54 kDa and 20-22kDa, respectively. Furthermore, β conglutin has a more complex composition with several polypeptides ranging from 12-70 kDa (Duranti et al., 2008) or 15 to 65 kDa (Santos et al., 1997). δ- conglutin consists of two small polypeptides with MW of 9 and 4 kDa. Finally, γ –conglutin contains a main subunit with MW of 42-43 kDa containing 2 polypeptides of 30 and 17 kDa linked with disulfide bond (Melo et al., 1994). In comparison with the previous studies and the SDS-PAGE results of protein isolate and soluble fraction, it can be considered that the proteins which have the molecular weight of approximately 19, 38, 40, 46, 58, 50-65 and 90 kDa are likely to belong to the subunits of the α and β conglutin, whereas proteins with molecular weight of 35, 30 and 19 kDa are probably polypeptides of γ-conglutin.

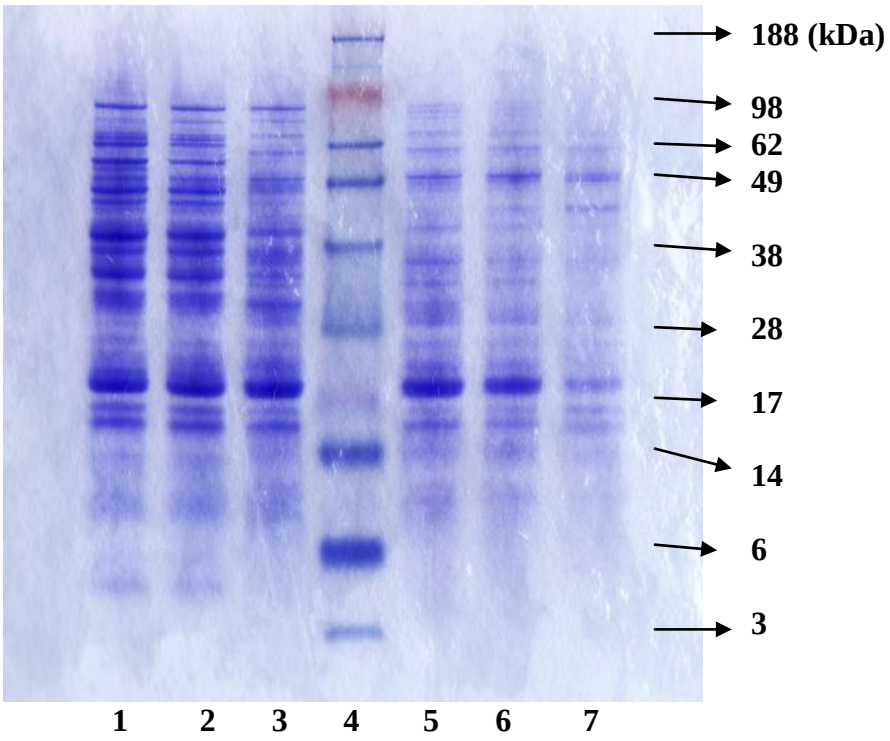


Figure 4-11 : Changes in total protein profile of extracts from germinated ASL flour

The electrophoresis gel shows bands of proteins extracted from germinated ASL flour separated by SDS-PAGE. The following lanes (from left to right) are protein extracts of: (1) raw seeds, (2) day 1, (3) day 3, (4) protein molecular weight marker, (5) day 5, (6) day 7, (7) day 9. The molecular weights of the protein standards (lane 4) are indicated on the right of the electrophoresis gel. Coomassie Blue was used for staining of the gel.

During the germination process, it was observed that there were some marked changes in the protein composition. For example, proteins with molecular weight of around 40, 50, 65 and 90 kDa began to disappear at day 5 of germination (Figure 4-11 lane 5). Whilst, a single band with molecular weight at around 46 kDa remained until day 9 of germination (Figure 4-11 lane 7). Two bands with molecular weight at around 16 kDa were still present following 9 days of germination. Normally during the process of germination, protein breakdown occurs as the plant uses the storage proteins for source of nitrogen and carbon for biomolecule synthesis (Duranti et al., 1984). However despite this there was no evidence of low molecular weight polypeptides being formed during germination. It was therefore suggested that, the

high molecular weight of proteins may have broken down to short chain peptides (3-5 peptides) and amino acids which were not detectable by SDS PAGE. The short peptides produced during germination and the undegraded protein may have contributed to the ACE inhibitory activity. In other studies using non-lupin protein, it was showed that the ACE inhibitory activity was due to the short chain peptides such as Tyr-Pro-Lys from broccoli (Lee et al., 2006a) and Glu-Tyr and Phe-Glu from shark meat hydrolysate (Wu et al., 2008).

It was shown that the total protein extracts of germinated ASL flour samples have the higher ACE inhibitory activity than the protein of the raw ASL seed. The short chain peptides and/or free amino acids which were not detectable by SDS-PAGE may have contributed to the high activity. It is therefore necessary to perform further analyses to investigate the type of proteins which posses the ACE inhibitory activity. Utilization of MALDI-TOF which is a powerful tool to characterise the proteins would give more information about the components that exhibit this activity.

4.2.7 Profile of Protein Isolate and Soluble Fraction based on SDS-PAGE and MALDI-TOF and its association with their bioactivities

The total proteins in the raw and germinated ASL were fractionated into protein isolate and soluble fraction. In order to investigate their beneficial effects, the protein isolate and soluble fraction yielded were analysed in their health benefits *in vitro*.

The yields of protein isolates isolated from germinated ASL flour were found to decrease significantly ($P \leq 0.05$) following germination (Table 4-10). In the raw ASL seeds, protein accounts for 21% (dry weight) whereas following germination for 9 days the protein only accounts for 4.7% (dry weight). Following germination the yields of protein in the soluble fraction were significantly reduced by about 76% ($P \leq 0.05$). The yields of protein isolate and soluble fraction were in contrast with the total protein contents (Table 4-3), where it was observed that protein content increased after germination. This result suggests that the high protein content after germination was due to an increase in non-protein nitrogen.

Table 4-10 : Yields of protein isolate and soluble fraction isolated from germinated ASL flours

Samples	% yields (db)	
	Protein Isolate	**Soluble Fraction**
Raw	21.6 ± 1.8**a**	5.9 ± 1.1**a**
Day 3	17.6 ± 3.8**ab**	3.8 ± 0.03**b**
Day 5	14.7 ± 3.2**b**	3.2 ± 0.33**c**
Day7	6.0 ± 0.5**c**	2.1 ± 0.27**d**
Day 9	4.7 ± 0.4**d**	1.4 ± 0.42**e**

Shown are yields of the protein isolate and the soluble fractions isolated from germinated ASL flour expressed as % (g/100g db). Values are means ± standard deviations of three independent isolations from three independent germinations. Different letter in the same column indicates a significant difference ($P \leq 0.05$)

It has been reported that proteins isolated by the same procedure by Sironi et al. (2005) were predominantly composed of α and β conglutin and the isolate was termed protein isolate E. Whereas the soluble fraction was referred to as protein isolate F which consists mainly of γ-conglutin. Figure 4-12 and Figure 4-13 show the electrophoresis gels following SDS PAGE to confirm the proteins present in protein isolate and soluble fraction. As shown in Figure 4-12, polypeptides of protein isolate which may be α and β conglutin with molecular weight of around 50, 40 and 19 kDa diminished following germination for more than 3 days. These results are in agreement with the previous results of SDS PAGE for total proteins (Figure 4-11). Furthermore, the bands with MW of 30, 19 and 16 kDa may be γ- conglutin and a low molecular weight protein present in the soluble fraction (Figure 4-13).

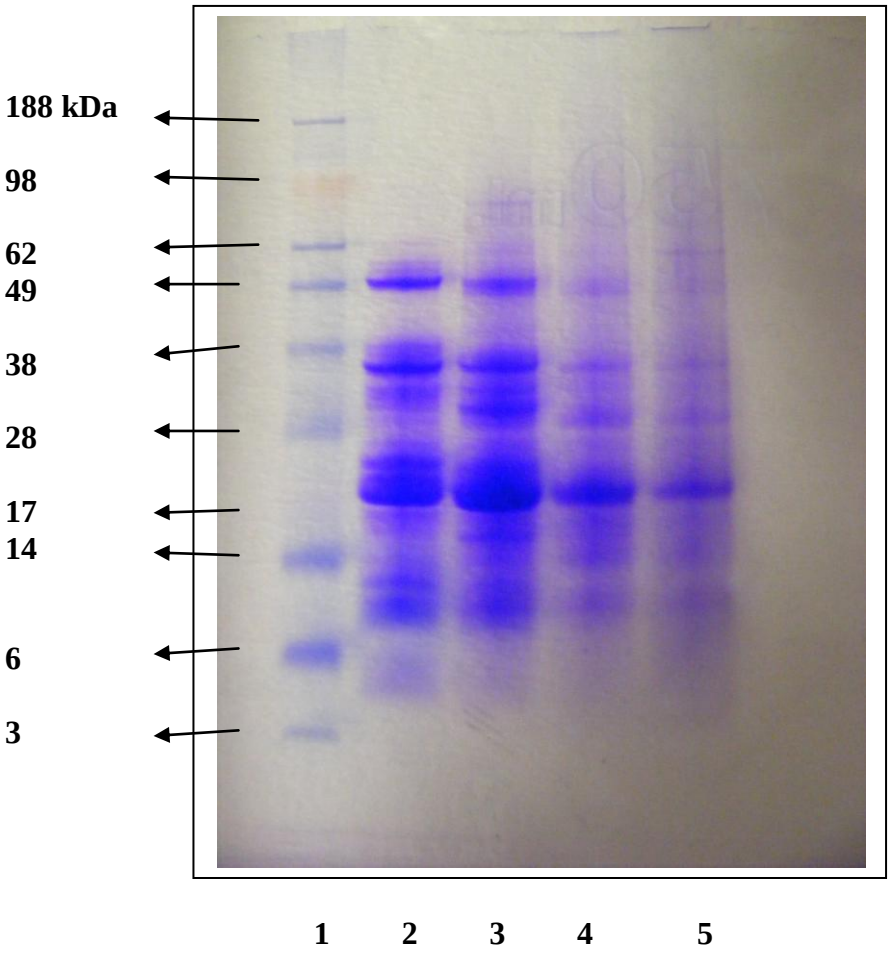


Figure 4-12 : Changes in the protein profile of protein isolate (PI) from germinated ASL flour

The electrophoresis gel shows bands of the protein isolate from germinated ASL flour separated using SDS-PAGE. The following lanes (from left to right) are: (1) protein molecular weight marker (2) raw seeds, (3) day 3, (4) day 7, (5) day 9. The molecular weights of the protein standards (lane 1) are indicated on the left of electrophoresis gel. Coomassie Blue was used for staining of the gel.

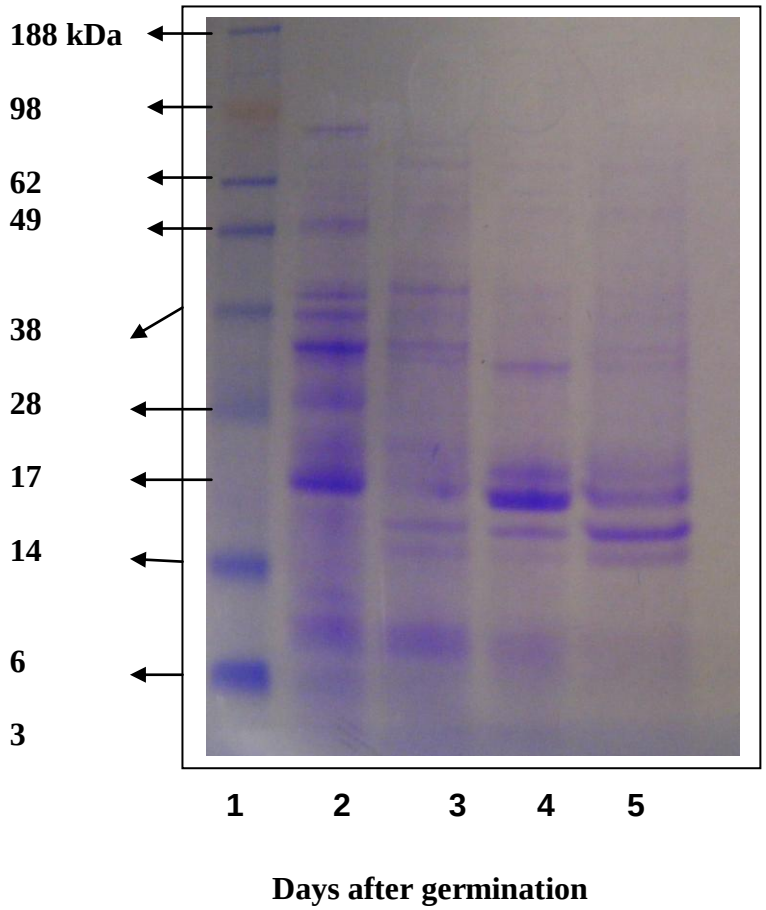


Figure 4-13 : Changes in protein profile of the soluble fraction (SF) from germinated ASL flour

The electrophoresis gel shows bands of the soluble fractions from germinated ASL flour separated using SDS-PAGE. The following lanes (from left to right) are: (1) protein molecular weight marker (2) raw seeds, (3) day 3, (4) day 7 and (5) day 9. The molecular weights of the protein standards (lane 1) are indicated on the left of electrophoresis gel.

The molecular weight of proteins in the protein isolate and soluble fraction were determined by MALDI-TOF as shown in Figure 4-14 to Figure 4-16. Surprisingly as shown in the Figures, the high molecular weight of proteins (around 65, 50, 40 kDa) which appeared in the SDS-PAGE analysis, did not appear in the MALDI-TOF spectra. However, a sharp peak with molecular weight of 19 kDa and a small peak of 38 kDa in the total protein isolate appeared in the spectra (Figure 4-14). The peaks could be polypeptides of conglutin α. Furthermore, the peaks with molecular weight of 20 and 30 kDa in protein isolate may be polypeptides of conglutin γ (Figure 4-15). Figure 4-16 shows the profile of soluble fraction of day 7. The results of MALDI-TOF showed that there was inconsistency between the results obtained by SDS-PAGE and the spectra of MALDI-TOF. Such discrepancy may be due to the difficulty in dissolving the freeze-dried protein isolate prior to the MALDI-TOF analysis. The low concentration of protein globulin in the isolates may be another reason for difficulties in finding peaks of the specific protein globulin such as β conglutin in the MALDI-TOF spectra. A study by Mora et al. (2008) suggested that a specific globulin required specific extraction conditions to be isolated. For example, conglutin γ precipitates better during lupin protein isolation at pH 5-6. Therefore, an optimization of isolation conditions and further purification of protein isolate may be necessary to completely elucidate the protein patterns in lupin protein globulins using the MALDI-TOF analysis

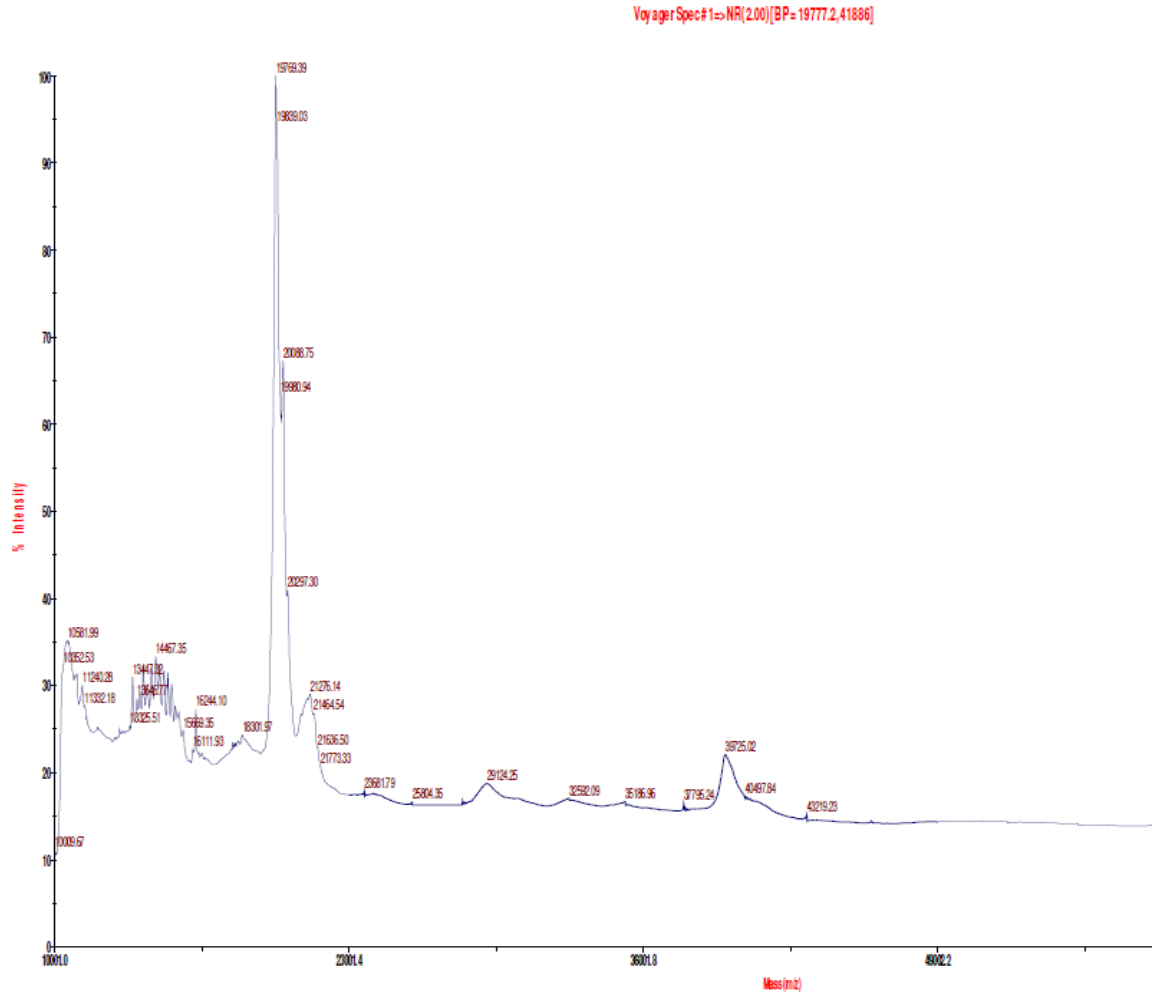


Figure 4-14 : Total protein extracts extracted from the raw ASL seeds using MALDI-TOF

Shown is MALDI-TOF spectra of total protein extracts extracted from the raw ASL seeds. The spectra shows peaks of protein separated based on their ratio m/z.

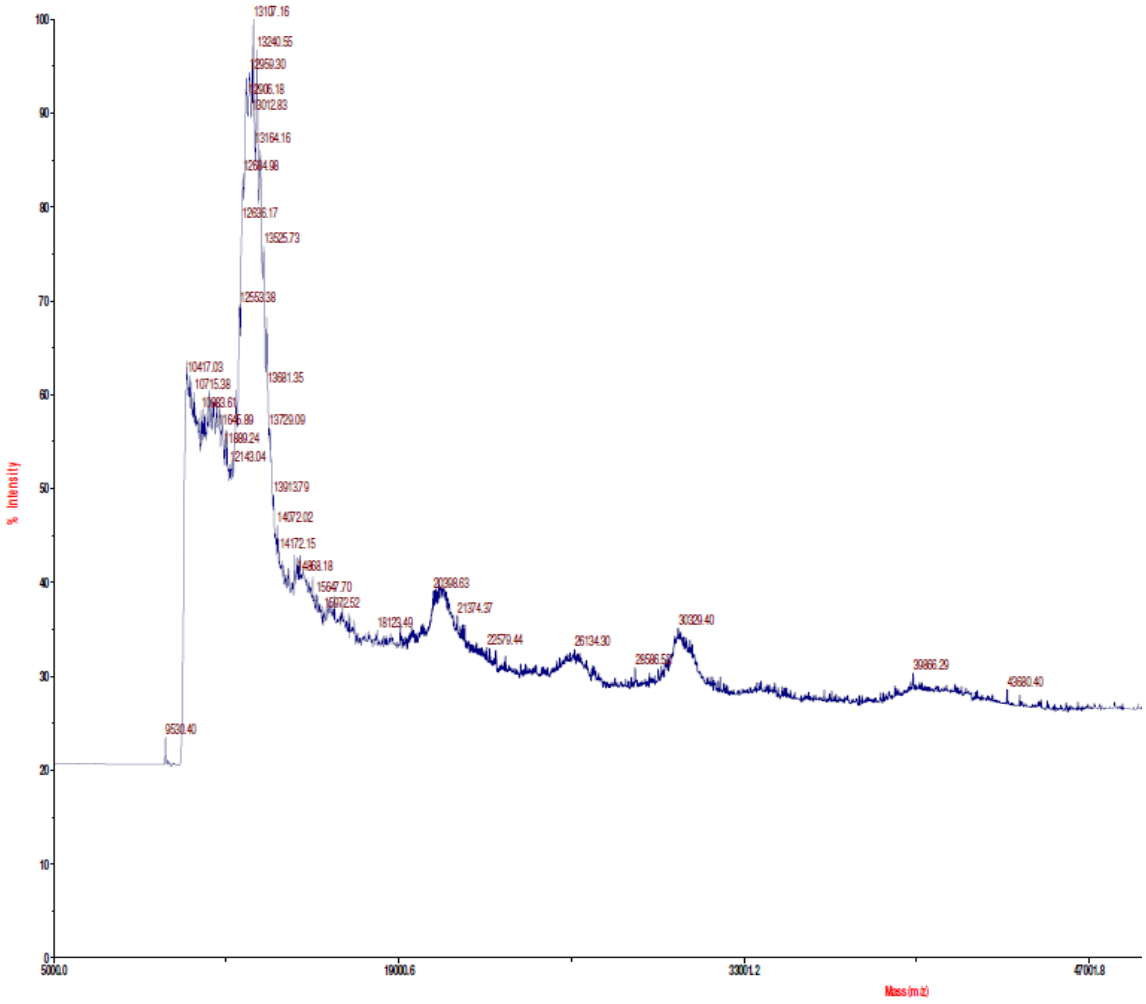


Figure 4-15 : Analysis of the protein isolate isolated from germinated ASL flour (day 7) by MALDI-TOF

Shown is MALDI-TOF spectra of the protein isolate isolated from germinated ASL flour (day 7). The spectra shows peaks of protein separated based on their ratio m/z.

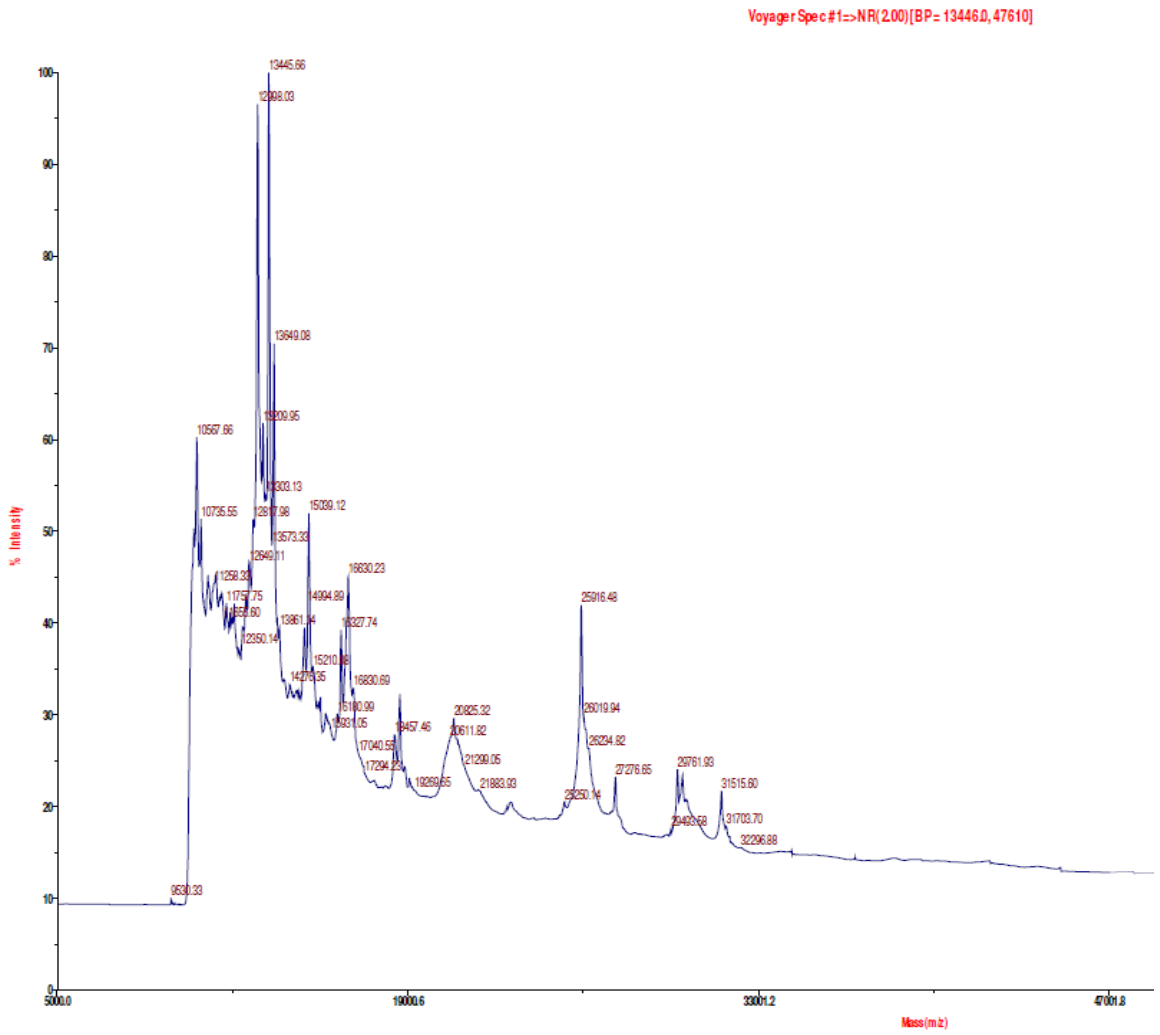


Figure 4-16 : Analysis of the soluble fraction isolated from germinated ASL flours (day 7) using MALDI-TOF

Shown is MALDI-TOF spectra of the soluble fraction extracted from germinated ASL flour (day 7). The spectra shows peaks of protein separated based on their ratio m/z.

The results of bioactivities assay *in vitro* of scavenging radical activity, ACE inhibitory activity and bile acid binding property of the protein isolate and soluble fraction are presented in Table 4-11. During the germination process, the scavenging radical activity to DPPH of both protein isolate and soluble fraction increased significantly until day 9. The antiradical activity of protein isolate was 2 fold higher than the activity in the soluble fraction at day 9. The antiradical activity of the

protein isolate was also higher than that of the activity of the crude methanolic extracts as described in Table 4-5. The higher DPPH radical scavenging activity in the protein isolate compared to soluble fraction could be due to the presence of a high phenolic content (antioxidant) in the protein isolate as found in a study by Yoshie-Stark et al. (2004).

Table 4-11 : Activities *in vitro* of protein isolate and soluble fraction isolated from germinated ASL

Samples	Scavenging DPPH radical activity (mg trolox/gDM of isolate)	ACE inhibitory activity (%) per 100 µg protein	Bound cholic acid (umol/100 mg DM of isolate)
Protein Isolate			
Raw	0.80 ± 0.02 **a**	44.69 ± 13.17 **a**	0.67 ± 0.35 **a**
Day 3	3.76 ± 0.76 **e**	40.78 ± 2.47 **ab**	0.87 ± 0.14 **a**
Day 7	6.56 ± 0.68 **f**	59.97 ± 13.23 **ac**	0.82 ±0.27 **a**
Day 9	8.39 ± 0.70 **g**	51.40 ± 8.08 **ac**	0.58 ± 0.17 **a**
Soluble fraction			
Raw	0.89 ± 0.01 **b**	85.67 ± 1.63 **d**	0.88 ± 0.17 **a**
Day 3	1.41 ± 0.12 **c**	93.16 ± 6.34 **df**	1.29 ± 0.20 **b**
Day 7	2.24 ± 0.21 **d**	82.21 ± 3.16 **ed**	1.23 ± 0.26 **b**
Day 9	3.32 ± 0.18 **e**	96.89 ± 2.94 **f**	1.48 ± 0.18 **b**

Shown are bioactivities *in vitro* of protein isolate and soluble fraction isolated from germinated ASL flour. Values are means ± standard deviations of three independent measurements from three independent germinations. Different letter in the same column indicates a significant difference ($P \leq 0.05$)

In regards to the ACE inhibitory activity of the protein isolate and soluble fractions, it was observed that the soluble fraction demonstrated a 2 fold greater ACE inhibitory activity than that observed in the protein isolate across all days of germination (Table 4-11). This result was not in agreement with the results of

Yoshie-Stark et al. (2004) who observed that protein isolate E of *L. albus* was more active as an ACE inhibitor than protein isolate F. It is thought that protein hydrolysis was required for the ACE inhibitory activity. It is therefore likely that proteolysis occured during germination, resulting in short peptides and/or free amino acids possessing ACE inhibitory activity. The proteins observed following SDS-PAGE analysis with molecular weight of 30, 19 and 16 kDa (Figure 4-13) may have contributed to the ACE inhibitory activity as they were found in the soluble fraction.

Meanwhile, the cholic acid binding activity of soluble fractions attained from day 3 to 9 of germination were higher than the binding activity observed in the protein isolate. This finding was in agreement with that found by Yoshie-Stark et al. (2004) who observed that the cholate binding property of protein isolate F was higher than that of the protein isolate E. The present study provides evidence that low molecular weight protein has greater BA binding property.

Collectively, it appears that germination leads to a reduction in protein isolate and soluble fraction of ASL. The decline may be caused by breakdown of storage protein to short chain peptides and/or free amino acids which were concentrated in the soluble fraction. The soluble fractions showed greater the ACE inhibitory activity and bile acid binding property than the protein isolates. It is likely that these greater bioactivities are due to a greater presence of lower molecular weight proteins in the soluble fraction. This finding was supported by other studies (Yoshie-Stark et al., 2004, Yoshie-Stark and Wasche, 2004). Whereas protein isolates appeared to have more antioxidant activity by scavenging radical activity than the soluble fractions. Therefore, soluble fractions of germinated ASL need to be further examined to better understand their health benefits. It would be desirable to determine the components which are responsible for such bioactivities in the soluble fractions.

4.3 Summary

Germination of ASL leads to a large increase in sprout wet weight (7 fold). However, a reduction in the dry weight was observed. The increase in the wet weight is caused by the high content of water in the ASL sprouts. The reduction in dry weight could

be due to the breakdown of some macromolecules such as protein, lipid and carbohydrates during the germination process.

Protein, which account for 44 % (db) in the raw ASL seeds, was increased to 61% (db) after 9 days of germination. The SDS-PAGE analysis of protein isolate extracted from the germinated ASL flours showed that the proteins might be broken down into short chain peptides and free amino acids. The short protein and/or free amino acids are thought to be concentrated in the soluble fraction, causing the fraction to be more active in regards to ACE inhibition and BA binding property. The protein isolate demonstrated higher antiradical activity than the soluble fraction.

The concentration of lipids was reduced by 71% (db) during germination up to 9 days. The lipids may be broken down into smaller molecules for use as an energy source. However, reduction in the amount of lipid present has affected the concentration of phytosterols dissolved in the lipids. The concentration of total phytosterols in the oil extracted from germinated ASL flours increased about 3 fold when expressed per oil weight. However, when it was expressed per weight of the germinated lupin flour, the concentration of total phytosterols remained stable. The main phytosterols in the oil of germinated ASL were found to be β-sitosterol (62%), campesterol (30%) and stigmasterol (8%). The oil of germinated ASL flour had a higher antiradical activity than the raw ASL seeds, suggesting the high concentration of phytosterols in the oil could have contributed to antiradical activity.

The fibre content, which is measured as crude fibre, increased significantly by about 456% (db) at day 9 of germination. The increased amount of the fibre may have a contribution to the high BA binding property of the germinated ASL flour.

Germination leads to increased TPC in the methanol extracts (700%) and in aqueous extracts (100%) of germinated ASL flour (db). The improvement in the TPC in both extracts was associated with the increases in the DPPH radical scavenging activity of the extracts. Methanol appeared to be a better solvent for extraction of compounds with antiradical activity in germinated ASL flours than aqueous solvents.

Overall, germination affected the amount of protein, lipid, fibre and bioactive compounds of ASL. The changes may have subsequently led to an increase in the *in vitro* bioactivities of germinated ASL.

Chapter 5
Stability of bioactive compounds and their bioactivities in muffins incorporated with germinated Australian Sweet Lupin flour

5.1 Introduction

Lupin flour is an ideal food ingredient as it is high in protein, dietary fibre and bioactive compounds but is low in oil and starch. Due to its nutritional and health benefits, lupin, in particular ASL has been recently used in a number of food applications such as tempe, tofu, muffins and biscuits. To further improve the quality and health benefits of lupin, germination, a simple and low cost method, has been carried out on ASL in this study. It was observed in the present study that germination resulted in significant increase in the protein and crude fibre contents of ASL by 38%, and 456% (db), respectively, with a substantial reduction in the lipid content (71%). In addition to the changes in the chemical composition, germination also resulted in significant increament in the concentration of TPC in the methanolic extracts of germinated ASL flours by about 700% compared to that of the ungerminated ASL seeds. Total phytosterols in the oil extracted from germinated ASL flour were increased by 3 fold. The increase was associated with an increase in the antiradical activity of the oil. Furthermore, it was found that germination resulted in a higher ACE inhibitory activity (antihypertensive *in vitro*) in the protein fraction when compared to the ungerminated ASL flour. Greater BA binding property of germinated ASL flour compared to ungerminated ASL was also observed in this study. These improvements in the concentration of bioactive compounds and their health benefits of germinated ASL flours could improve the application of germinated ASL flours as an ingredient in a range of food formulations.

A poor diet, in particular high in saturated fat and sugar but low in dietary fibre is thought to be an important contributing factor for the development of metabolic syndrome (Tuomilehto, 2005) and other diseases related to cancer (Petti, 2009). The healthier diet containing high fibre, bioactive compounds such as antioxidants, but low in fat is recommended to reduce the risks of developing health problems related

to metabolic syndrome and cancer. Consequently, there is an increasing demand for healthy foods with high fibre, protein and low fat, such as lupin.

Muffins are widely consumed in western countries. They are easy to prepare from batter consisting of sugar, fat, flour, eggs, milk and baking powder. The standard muffins are composed of a high level of sugar and fat (Baixauli et al., 2008). A number of studies have then been conducted to improve the nutritional and functional properties of muffins. For example, muffins have been incorporated with lupin flour at up to 20% with acceptable sensory properties (Jayawardena, 2006). A number of other studies have investigated the other means to increase the content of dietary fibre and bioactive compound in muffins. In a study by Grigelmo-Miguel et al. (2001), addition of peach dietary fibre up to 4% in reduced-fat muffins had a similar acceptability to the control muffins. Muffins incorporated with dried apple skin powder resulted in increased contents of the total dietary fibre, TPC and antioxidant capacity of the muffins (Rupasinghe et al., 2008). However, no study has been conducted in regard to muffins incorporated with germinated ASL flours and the associated health benefits.

Processing conditions are important to maintain the activity of bioactive compounds and their health benefits as mixing and baking processes may affect the chemical composition and/or activity of the bioactive compounds. Bioactive compounds such as phenolic compounds and phytosterols contained in foods are susceptible to oxidation during food processing. In addition, the heating process involved with baking muffins could degrade the bioactive compounds in the muffins. Meanwhile, the physical characteristics of muffins are an important factor affecting consumer acceptability. Therefore, incorporation of germinated ASL flours in the muffins, analysis of instrumental physical characteristics of muffins and stability of bioactive compounds during the processing (both before and after baking) needed to be investigated in this study.

5.2 Results and Discussion

5.2.1 Chemical and physical properties of muffins prepared with germinated ASL flour

Consumer acceptability of food products is affected by their physical characteristics. To determine the effect of germinated ASL flour on physical characteristics of muffins, height, diameter, colour and texture (hardness, cohesiveness, springiness and chewiness) of the muffins were measured. ASL flour at day 7 of germination was incorporated into the control muffin formula at 2, 4, 6, and 8% (as percentage of dried muffin weight). The appearance of the muffins with the varying percentage of germinated ASL flour is shown in Figure 5-1.

0% **2%** **4%** **6%** **8%**

Figure 5-1 : Appearance of representative muffins prepared with different amount of germinated ASL flours (percentage of dried muffin weight)

5.2.1.1 Moisture content

Moisture content of the muffins are shown in Figure 5-2. Muffins incorporated with germinated ASL flour at various amounts (2-8% of dried muffin weight) had significant higher moisture content (22.70 to 23.29%) than the control muffins (20.73%). However, the difference in the moisture contents among muffins made with germinated ASL flour (2-8%) was not significant ($P>0.05$). The increased moisture content in muffins containing germinated ASL flour could be due to the higher content of dietary fibre in the treated muffins. The crude fibre content in the

germinated ASL flour (day 7) was higher (7.4%) than that in the raw flour (Table 4-3). The fibre may affect water retention in the muffins by preventing water evaporation during baking. Grigelmo-Miguel et al. (2001) reported that addition of peach dietary fibre in the reduced-fat muffins resulted in an increase of moisture content due to the high water-holding capacity of the peach dietary fibre. Lupin protein was also reported to increase the water retention capacity of breads incorporated with lupin protein isolate, resulting the higher moisture content of the incorporated breads than the control breads (Paraskevopoulou et al., 2010). Therefore, it is suggested that the high fibre and protein contents in the germinated ASL flour may result in an increase in the moisture content of the incorporated muffins. These findings are in contrast to the findings of a study by Jayawardena (2006) who observed no significant differences in moisture contents between control muffins and muffins incorporated with lupin flour (ungerminated) up to 30%. The moisture content of foods is associated with the shelf life and consumer acceptability (Smith et al., 2004).

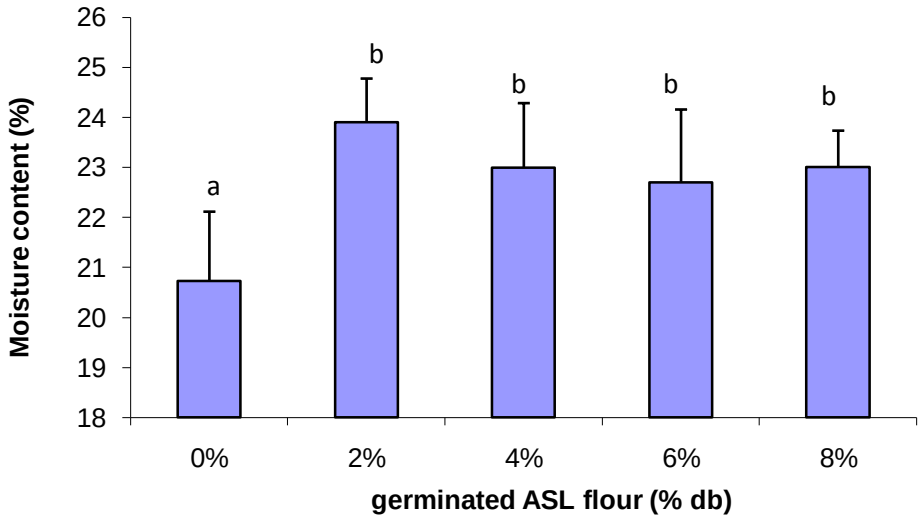


Figure 5-2 : Moisture contents of muffins prepared with different amounts of germinated ASL flours (percentage of dried muffin weight)

Shown are the moisture contents of muffins prepared with different amount of germinated ASL flour expressed as % dried muffin weight. Values are means ± standard deviations of measurements of 3 muffins from each of 3 different batches of flour. Different letter in each bar indicates a significant difference ($P < 0.05$)

5.2.1.2 Height and diameter

Heights of the muffins are shown in Figure 5-3. The height of muffins containing germinated ASL flour up to 2% was similar to that of the control muffins. However, the height of the muffins was significantly lower at 4% and above of the substitution ($P < 0.05$). Meanwhile, the heights of muffins with germinated ASL flour at 6% and 8% were similar and were the lowest among the treated muffins. The high fibre content in the germinated ASL flour may have contributed to the reduction in height as reported by Grigelmo-Miguel et al. (2001). They found that addition of peach dietary fibre in the reduced-fat muffins reduced air pockets, resulting in lower height of the muffins. Figure 5-4 shows images of the air cells of muffins at different substitution level of germinated ASL flour. The crumb of muffins incorporated with germinated ASL flour was more compact compared to the control muffins. The low amount of air cells in the crumb of the muffins may have consequently affected the height of the muffins.

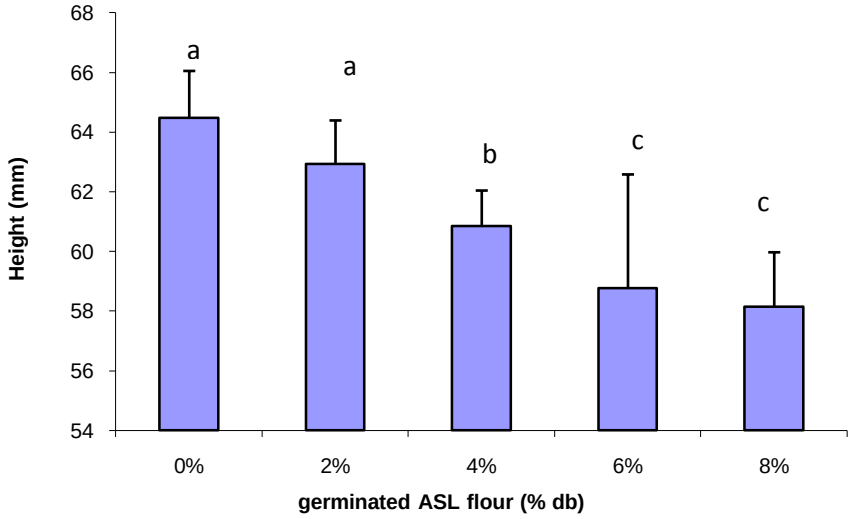


Figure 5-3 : Height of muffins prepared with different amount of germinated ASL flours (percentage of dried muffin weight)

Shown are the heights of muffins prepared with different amount of germinated ASL flour expressed as mm. Values are means ± standard deviations of measurements of 3 muffins from each of 3 different batches of flour. Different letter in each bar indicates a significant difference ($P < 0.05$)

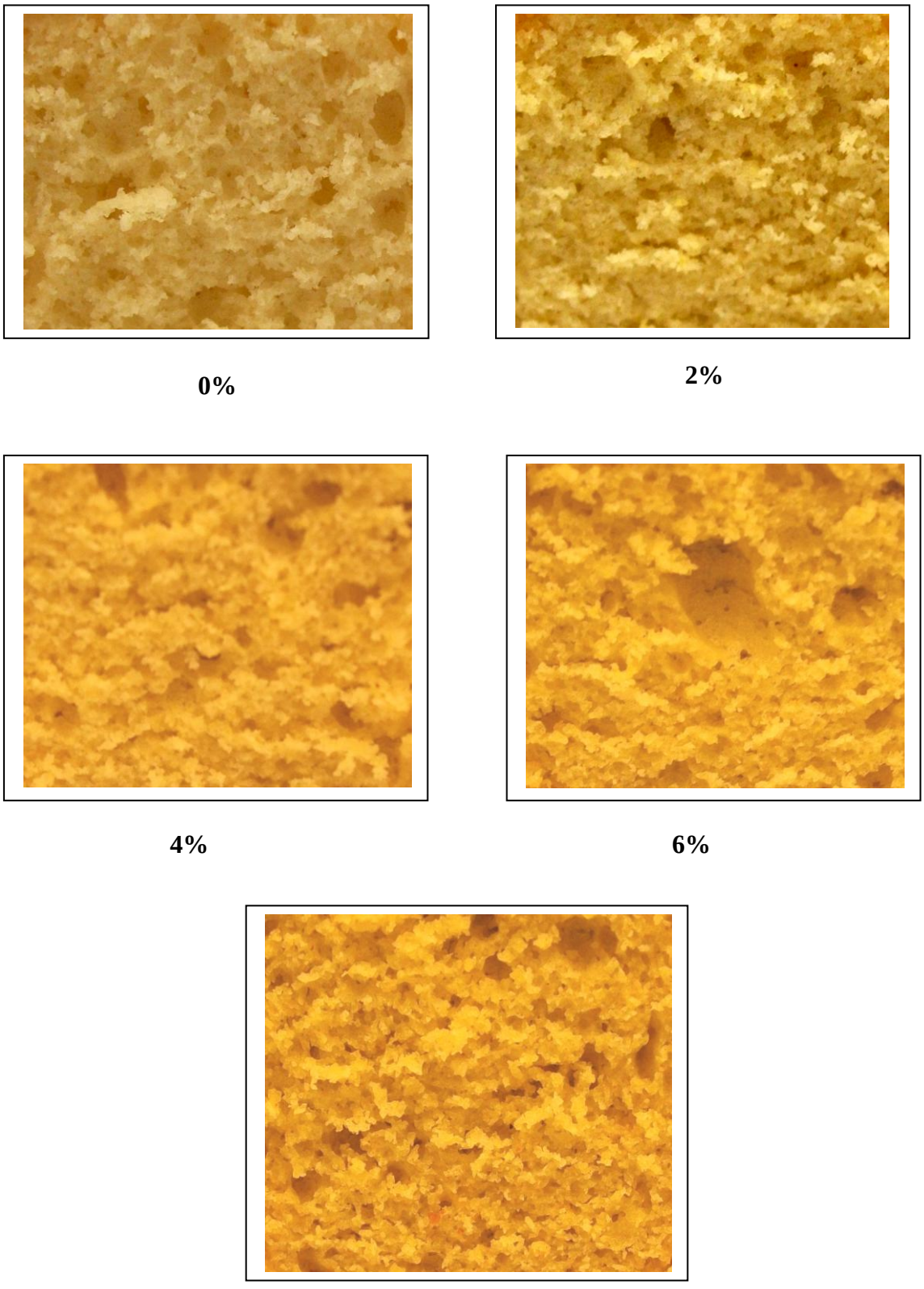


Figure 5-4 : Images of muffins prepared with different amount of germinated ASL flours (percentage of dried muffin weight)

Incorporation of germinated ASL flour up to 4% had no significant effects on diameters of the muffins (Figure 5-5). The height and diameter measurements of muffins in the present study suggested that incorporation of germinated ASL flour at 4% reduced the height of the muffins, without affecting the diameter. The decrease in volume following incorporation of ungerminated ASL flours (30% as flour basis) in the muffins has also been observed in a study by Jayawardena (2006). Addition of lupin flour and lupin protein isolates to wheat flour doughs in bread making was reported to affect the rheological properties of the doughs. The bread volume was reduced as the level of incorporation was increased. The protein contained in lupin has been reported to have a role in dilution of the gluten, affecting the viscoelastic properties of wheat gluten. This reduces the ability of viscoelastic network of gluten to retain gases in the batter, which subsequently would have reduced volume of the bread (Paraskevopoulou et al., 2010, Dervas et al., 1999, Doxastakis et al., 2002). On the other hand, addition of germinated soybean flour at a low level (up to 1.5%) to wheat flour in bread making was reported to improve loaf volume of the bread. The improvement of the bread volume was due to an increment of enzymatic activities such as alpha amylase and lipase in the dough generated from the germinated soybean (Rosales-Juarez et al., 2008).

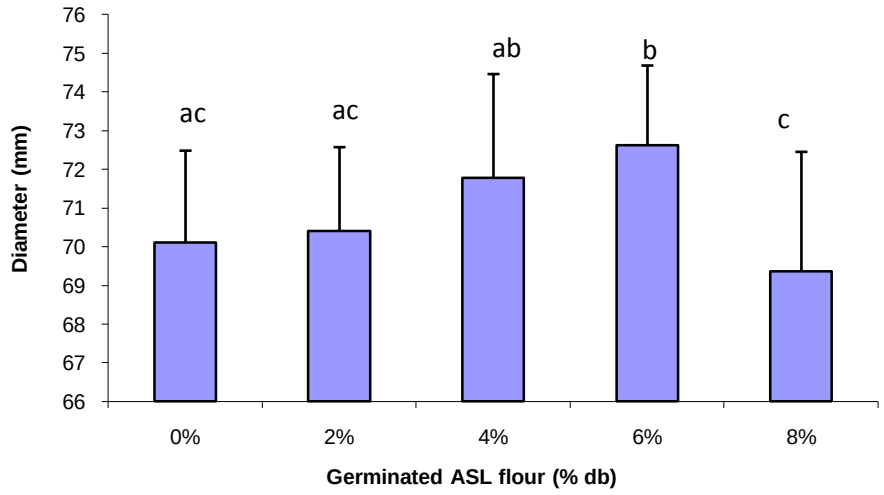


Figure 5-5 : Diameters of muffins prepared with different amount of germinated ASL flours (percentage of dried muffin weight)

Shown are the diameters of muffins prepared with different amount of germinated ASL flour expressed as mm. Values are means ± standard deviations of measurements of 3 muffins from each of 3 different batches of flour. Different letter in each bar indicates a significant difference ($P < 0.05$)

5.2.1.3 Colour

Colour of the muffin crumbs was measured using Minolta spectrophotometer CM-508i (Minolta Co. Ltd. Japan) and the colour was expressed in terms of *L**, *a** and *b**. The *L**, *a**, and *b** values represent lightness, redness and yellowness of the crumbs, respectively as reflected from the surface of the crumbs. The results of colour measurement are shown in Table 5-1.

Table 5-1 : Instrumental colours of ASL flours and muffins incorporated with germinated ASL flour at various levels (percentage of dried muffin weight)

Samples	*L**	*b**	*a**
Raw ASL flour	90.63±0.65	28.46±0.52	-1.31±0.11
Germinated ASL flour (day 7)	84.59±1.23	22.13±0.88	-1.61±0.46
% incorporation:			
0%	71.43 ± 3.88**a**	25.00 ± 1.06**a**	-0.89 ± 0.50**a**
2%	70.32 ± 1.87**ab**	31.80 ± 0.9**b**	-0.66 ± 0.54**a**
4%	67.48 ± 2.03**bc**	35.83 ± 1.31**c**	0.27 ± 0.21**b**
6%	66.22 ± 2.26**cd**	41.26 ± 1.7**d**	0.79 ± 0.33**b**
8%	64.06 ± 1.73**d**	42.58 ± 1.4**d**	2.45 ± 0.95**c**

Shown are the colours of the raw and germinated ASL flour and muffins prepared with different amount of germinated ASL flour expressed as L* (lightness, extending from 0 (black) to 100 (white), b* (yellowness, positive value is yellow and negative value is blue) and a* (redness, positive value is red and negative value is green). Values are means ± standard deviations of measurements of 3 muffins from each of 3 different batches of flour. Different letter in the same column indicates a significant difference ($P < 0.05$).

The value of lightness (*L**) of muffins was reduced by increasing the level of germinated ASL flour incorporation. Only muffins with 2% substitution level had similar *L** level to the control muffins. The yellowness (*b**) of muffins increased with increasing substitution level of germinated ASL flour. No significant difference of redness (*a**) was observed between the control muffins and 2% substitution. Substitution of germinated ASL flour up to 8% resulted in an increase in redness (*a**) compared to the control muffins. Overall, the lightness of the muffins was reduced, but yellowness and redness were increased by substitution of germinated ASL flour. The redder, yellower and darker colours of muffins substituted with germinated ASL flour was predominantly due to the intrinsic colour of the ASL (Table 5-1). A lower

value of *L**, but higher values of *a** and *b** of the muffins compared to the original colour of the raw flours suggested that the baking process could have also affected the overall colour of the muffins. As discussed in Chapter 4, germinated ASL flour contained higher amount of bioactive compounds such as phenolic compounds and phytosterols. The phenolic compounds are susceptible to the browning reaction during the baking process (Randhir et al., 2008), thus affecting lightness, yellowness and redness of the muffins incorporated with germinated ASL flour.

The results shown in the present study were similar to the results reported by Jayawardena (2006) who observed yellower, redder and darker muffins after incorporation of ASL flour (ungerminated) up to 30% (as flour basis) in the muffins. The yellower colour of the lupin-incorporated muffins had a better acceptability than the control muffins. In the other studies, the substitution of lupin flour in noodle (20%) and tofu (40%) also tended to result in redder and yellower products (Jayasena et al., 2008, Jayasena et al., 2010). The high fibre content in germinated ASL flour could have affected the colour of the muffins as observed in the present study. Similar effects on the colour of muffins have been observed by Grigelmo-Miguel et al. (2001) who reported higher substitution of peach dietary fibre into muffins resulted in higher redness and yellowness, but lower lightness. A darker pound cakes incorporated with 30% amaranth flour which is rich in dietary fibre and protein was also observed in a study by Capriles et al. (2008). The darker cakes have been reported to have a negative impact on the overall acceptability of the cakes (Capriles et al., 2008).

5.2.1.4 Texture

Textural properties of muffins have an important role in determining the quality and sensory acceptability of the muffins. In this study, the textural properties of muffins incorporated with germinated ASL flour were evaluated on hardness, cohesiveness, springiness and chewiness as analyzed by a TA-XT2i Texture Analyzer. The results of the texture analysis are shown in Table 5-2. The results indicated that the hardness of muffins incorporated with germinated ASL flour were not linear with the amount of germinated ASL flours substituted. Substitution of germinated ASL flour at levels

between 2-6% resulted in muffins with reduced hardness. Interestingly, addition of germinated ASL flour at 8% did not affect the hardness of the muffins. The higher moisture content in the muffins incorporated with germinated ASL flour found in the present study may have a correlation with the lower hardness (greater softness) of the muffins. These findings were different from the findings reported by Jayawardena (2006) who stated no significant differences between muffins incorporated with ASL flour (ungerminated) up to 20% (as flour basis) and the control muffins. The study also showed that the moderate moistness of the muffins was more preferable by the panelists (Jayawardena, 2006).

Table 5-2 : Texture profile of muffins incorporated with various levels of germinated ASL flour at (as percentage of dried muffin weight)

Percentage of germinated ASL flour	Hardness (N)	Cohesiveness (N.mm)	Springiness (mm)	Chewiness
0%	981.64 ± 107.13**a**	0.54 ± 0.03**a**	0.92 ± 0.02**a**	489.24 ± 0.04**a**
2%	719.92 ± 126.60**b**	0.63 ± 0.03**b**	0.93 ± 0.01**a**	421.76 ± 0.02**a**
4%	696.82 ± 105.01**b**	0.62 ± 0.02**b**	0.93 ± 0.01**a**	401.98 ± 0.01**a**
6%	796.49 ± 112.66**ab**	0.64 ± 0.02**b**	0.93 ± 0.02**a**	471.20 ± 0.06**a**
8%	974.27 ± 163.99**a**	0.61 ± 0.03**b**	0.85 ± 0.30**b**	506.92 ± 0.25**a**

Shown is the texture profile of muffins prepared with different amount of germinated ASL flour expressed as hardness (N), cohesiveness (N.mm), springiness (mm) and chewiness. Values are means ± standard deviations of measurements of 3 muffins from each of 3 different batches of flour. Different letter in the same column indicates a significant difference ($P < 0.05$).

Cohesiveness of a product describes how well the product resists at a second deformation compared to that in the first deformation. Cohesiveness parameter of muffins is related to the denseness of muffins. All muffins incorporated with germinated ASL flour had similar cohesiveness value, ranging from 0.61 to 0.64 N.mm (Table 5-2). The value across all levels of substitution was significant higher compared to that of the control muffins. Meanwhile, incorporation of ungerminated ASL flour up to 10 % (as flour basis) did not significantly affect the cohesiveness of

the muffins (Jayawardena, 2006). The increase in cohesiveness of muffins incorporated with germinated ASL flour in the present study could affect their sensory acceptability. The low cohesiveness in muffins added with resistant starch was associated with crumbly texture of muffins, resulting in low sensory acceptability (Sanz et al., 2009)

The ability of a product to spring back after the first compression applied is referred to as springiness. Springiness has been associated with freshness of a food product, thus the high value of springiness of a muffin is associated with a high quality of the muffin (Sanz et al., 2009). Generally, higher values of springiness are associated with less hard and less chewy samples (Meullenet et al., 1998). In the present study, springiness of the control muffins and muffins treated with germinated ASL flours up to 6% was not significantly different. Muffins with 8% substitution had significantly lower springiness ($P < 0.05$) than the other samples (Table 5-2). In another study, the value of springiness of muffins incorporated with ASL flour (ungerminated) up to 5-20% (as flour basis) was reduced significantly (Jayawardena, 2006). The lower springiness of muffins with 8% substitution level in the present study may reduce the acceptability of the muffins as suggested by Sanz et al. (2009). They reported that, in muffins added with resistant starch, the low springiness had a negative impact on acceptability of the muffins (Sanz et al., 2009).

Chewiness (hardness x cohesiveness x springiness) describes the length of time required to chew the muffins to a state ready for swallowing. The hardness of muffins in the present study tended to decrease as the level of substitution was increased (except for 8%), while chewiness in across all treatments and the control muffins was found to be not significantly different (Table 5-2). In terms of sensory evaluation, the low chewiness of muffins incorporated with ungerminated ASL flour was associated with greater acceptability of the muffins (Jayawardena, 2006). In addition, the low hardness and low chewiness of the reduced-fat high-dietary fibre muffins were also more preferred by the sensory panelists (Grigelmo-Miguel et al., 2001).

5.2.2 Stability of phenolic compounds and scavenging radical activity of muffins incorporated with various levels of germinated ASL flours

Results from the present study show that germinated ASL flour contains a higher amount of phenolic compounds compared to the ungerminated ASL flour (Table 4-4). The phenolic compounds in the germinated ASL flour could have a key role in antioxidant activity. Due to the health benefits and concerns regarding adverse effects of oxidized phenolics in health, it is important to study the stability of phenolic compounds in germinated ASL flour during processing. In order to investigate the effects of processing (mixing and baking) of muffins incorporated with germinated ASL flour on TPCs and the antiradical activity, TPCs and the antiradical activity both in the batter (before baking) and muffins (after baking) were measured. Values of TPCs and the antiradical activity of the batter and the muffins are shown in Table 5-3.

Table 5-3 : TPCs and antiradical activity of muffins incorporated with various levels of germinated ASL flours (**as percentage of dried muffin weight).**

Percentage of germinated ASL flour (db)	TPC (mg gallic acid/100g DM)		Scavenging radical activity (mg trolox/g DM)	
	before baking	after baking	before baking	after baking
0%	44.96 ± 2.43**a**	32.56 ± 2.67**a***	0.033 ± 0.012**a**	0.148 ± 0.013a*
2%	62.76 ± 3.12**b**	61.09 ± 3.03**b**	0.091 ± 0.004**b**	0.265 ± 0.011**b***
4%	74.08 ± 4.39**c**	81.42 ± 2.37**c***	0.148 ± 0.017**c**	0.328 ± 0.008**c***
6%	85.94 ± 3.92**d**	90.71 ± 4.78**d***	0.241 ± 0.005**d**	0.322 ± 0.026**c***
8%	97.05 ± 2.48**e**	94.44 ± 9.56**e**	0.256 ± 0.013**d**	0.335 ± 0.031**c***

Shown are TPCs and scavenging radical activity for muffin samples before and after baking expressed as mg equivalent gallic acid/100 g DM and mg trolox/g DM. Values are means ± standard deviations of measurements of 3 muffins from each of 3 different batches of flour. Different letter in the same column indicates a significant difference ($P < 0.05$). * indicates significant differences between before and after baking using student t-test ($P < 0.05$)

Phenolic compounds in the batter and muffins were extracted using 80% MeOH. The TPCs in the extracts were then measured and expressed as gallic acid equivalents (mg of GAE/100g dried muffins). When compared with the control muffins, there was a significant increase in TPCs of the batter and the muffins treated with germinated ASL flours. The TPCs found at 8% germinated ASL flour substitution (both of pre and post baking) was 200% more than that found in the control muffins. Phenolic compounds were detected in the control muffins as well. As reported by Alvarez-Jubete et al. (2009), wheat grain contains polyphenol constituents which have antioxidant activity. The detected TPCs in the control muffins may have come from the compounds contained in wheat and vegetable oil and other ingredients used in muffin formulation.

Muffins baked at 190°C for 25 min at 4% and 6% substitution levels were found to have significantly higher TPC contents than their batters ($P < 0.05$). Thermal processing has previously been reported to increase the concentration of phenolic compounds. It was believed that the high temperature facilitated the release of phenolic compounds from matrix cells (Randhir et al., 2008). Therefore, the higher concentration of TPCs in muffins incorporated with some levels of germinated ASL flour found in the present study could be due to both the release of phenolic compounds from the cellular structures and the formation of phenolic products from thermal degradation. In contrast to these findings, degradation of phenolic compounds in quinoa-substituted bread has been reported to reduce the concentration of phenolic compounds following baking at 220-225°C for 20 min (Alvarez-Jubete et al., 2009). Boiling sweet and bitter quinoa seeds for 20 min also led to a significant loss of phenolic compounds and antioxidant capacity of the seeds (Dini et al., 2010). The different results among these studies could be due to the differences in cellular structure and compositions of the seeds used in the studies.

The chemical structure of phenolic compounds in some legumes and the stability of these compounds during processing have been reported by some studies. Results from a study on *L. angustifolius* var Zapaton reported the structure of phenolic compounds as flavones, hydroxybenzoics, isoflavones, hydroxycinnamics, and dihydroflavonols (Duenas et al., 2009). Meanwhile, hydroxybenzoic acids, hydroxycinnamic acids, flavonol glycosides, flavan-3-ols and procyanidins were also

found in beans, lentils and peas (Lopez-Amoros et al., 2006). Flavonols and flavan-3-ols in apple skin powder were revealed to be more resistant to thermal degradation during the baking process compared to anthocyanins and phenolic acids (Rupasinghe et al., 2008). On the other hand, isoflavones (a phytoestrogen) of soybean were susceptible to thermal degradation. In particular, daidzein is more degradable than genistein (Ungar et al., 2003). Intra-conversions among the different forms of isoflavones to β-glycoside, acylglycoside or aglycon forms also occurred during the heat treatment (Chiarello et al., 2006). Maillard browning reaction reaction, polymerization and oxidation are other possible reactions of phenolic compounds caused by the heat treatments (Randhir et al., 2008).

In order to investigate the effect of change in the concentration of TPCs occurred during the production of muffin on their antioxidant activity, scavenging radical activity to DPPH radicals of the batter and baked muffin were measured and expressed as mg trolox/g DM of the muffins. The results of the antioxidant assay are shown in Table 5-3. As expected, the addition of germinated ASL flour was associated with an increasing radical scavenging ability of both the batter and muffins compared to the controls. The higher substitution level of germinated ASL flour resulted in a greater radical scavenging activity (Table 5-3). Baking was found to increase the radical scavenging activity of the muffins across all levels of lupin incorporation (Table 5-3). The increased antiradical activity in the muffins may be due, at least in part, to the increased concentration of TPCs, as previously discussed. In this regard, the higher antiradical activity of muffins could be due to the formation of products during the baking process that possess antiradical scavenging activity. A similar increase in antioxidant activity was also reported in muffins incorporated with apple skin powder (Rupasinghe et al., 2008). Meanwhile, pronyl-L-lysine produced during the Maillard reaction was reported to have antioxidant properties in bread (Lindenmeier and Hofmann, 2004).

Baking of muffins at 190°C for 25 min did not reduce the concentration of TPCs in muffins incorporated with germinated ASL flour. The TPCs and their antiradical activity were increased following the baking process in some levels of substitution. Therefore, incorporation of germinated ASL flour in muffins would provide more

health benefits due to the increase in phenolic compounds and their antiradical activity in the muffins.

5.2.3 Stability of phytosterols in muffins incorporated with various levels of germinated ASL flours

Phytosterols have been reported to have cholesterol-lowering effects when consumed. Consequently an understanding of their stability in foods during processing would help in maximising these health benefits. In this study, muffins incorporated with germinated ASL flour were used to study the stability of phytosterols contained in germinated ASL flour.

The double bond present in phytosterol structure is susceptible to oxidation during food processing in the presence of oxygen. Heat, light, free radicals and other factors influence the rate of the oxidation. Oxidized products of phytosterol are thus produced following processing of food. The degraded products of phytosterols were reported to have adverse health effects such as cytotoxicity in macrophage-derived cell lines (Adcox et al., 2001). As baking is a common food processing technique, it is important to understand its effect on the stability of phytosterols in baked muffins. In order to investigate the stability of phytosterols during baking, oils were extracted from the batter and muffins to determine the extent of any degradation of campesterol, stigmasterol and ß-sitosterol in the present study.

Examples of the chromatograms obtained from batter and muffins samples are illustrated in Figure 5-6. The GC chromatograms of oils extracted from the batter and muffins (baked at 190 °C for 25 min) incorporated with germinated ASL flours were found to have similar patterns. Five main peaks were identified in the chromatograms of both the batter and the muffins when compared against the peaks of phytosterol standards. The first peak was the internal standard (5-β-cholestan-3α-ol). The other peaks were cholesterol (5-cholesten-3β-ol), campesterol, stigmasterol and ß-sitosterol, respectively. Raw and germinated ASL flour had no cholesterol content (Table 4-6). Therefore, it is likely that the cholesterol identified in the muffin samples was from eggs and milk added during the production of muffin. Phytosterols

detected in the control muffins are likely to be from vegetable oil added to the muffin formulation. As found in oils of the raw seeds and germinated ASL flours (Table 4-6), β-sitosterol was the major phytosterols found in the muffin samples, followed by campesterol and stigmasterol. According to this qualitative analysis of the chromatograms, similarity in the chromatogram of the batter and the muffin indicated no substantial change in the composition of phytosterol during the baking process. Since there were no new peaks observed after baking, phytosterols were not degraded during the muffin baking process at 190°C for 25 min.

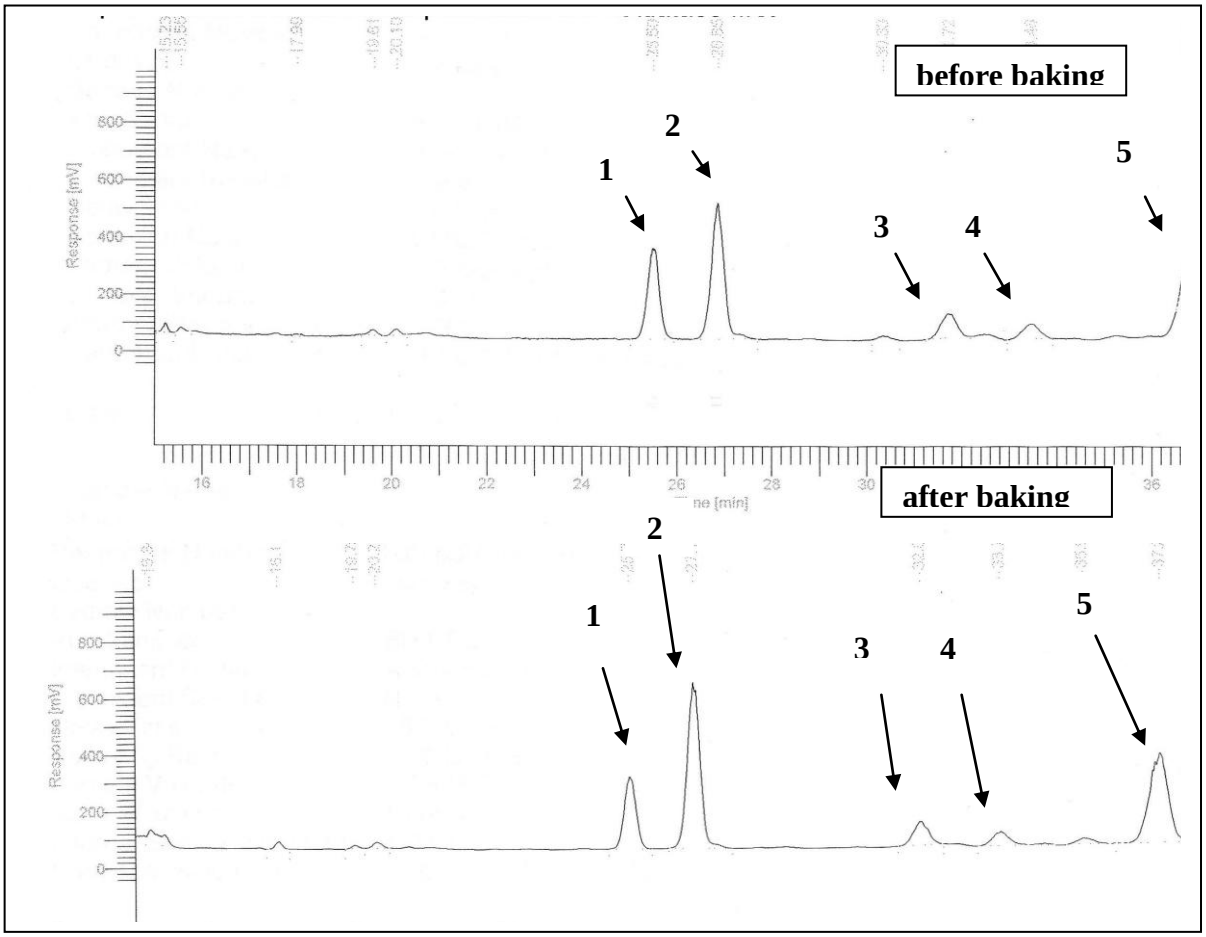


Figure 5-6 : GC chromatogram of oils extracted from muffins incorporated with 6% germinated ASL flour before and after baking process

The chromatograms show peaks of cholesterol and phytosterols in the batter and muffins incorporated with 6% of germinated ASL flour separated by GC. The following identified peaks (from left to right) are : (1) internal standard (IS) :5-β-cholestan-3α-ol, (2) cholesterol, (3) campesterol, (4) stigmasterol, (5) β-sitosterol.

The quantity of phytosterols in the batter and muffins is illustrated in Figure 5-7. After baking at 190°C for 25 min, 0%, 6% and 8% substitution of germinated ASL flour significantly increased the amount of campesterol in the muffins (Table 5-4). Stigmasterol in muffins incorporated at 0% and 6% substitution was found to be significantly higher than in the batter. Furthermore, β-sitosterol in the muffins incorporated with of 4-8% substitution levels had higher amount than the batter. Generally, the amount of phytosterols in the muffins was found to be higher than that in the batter.

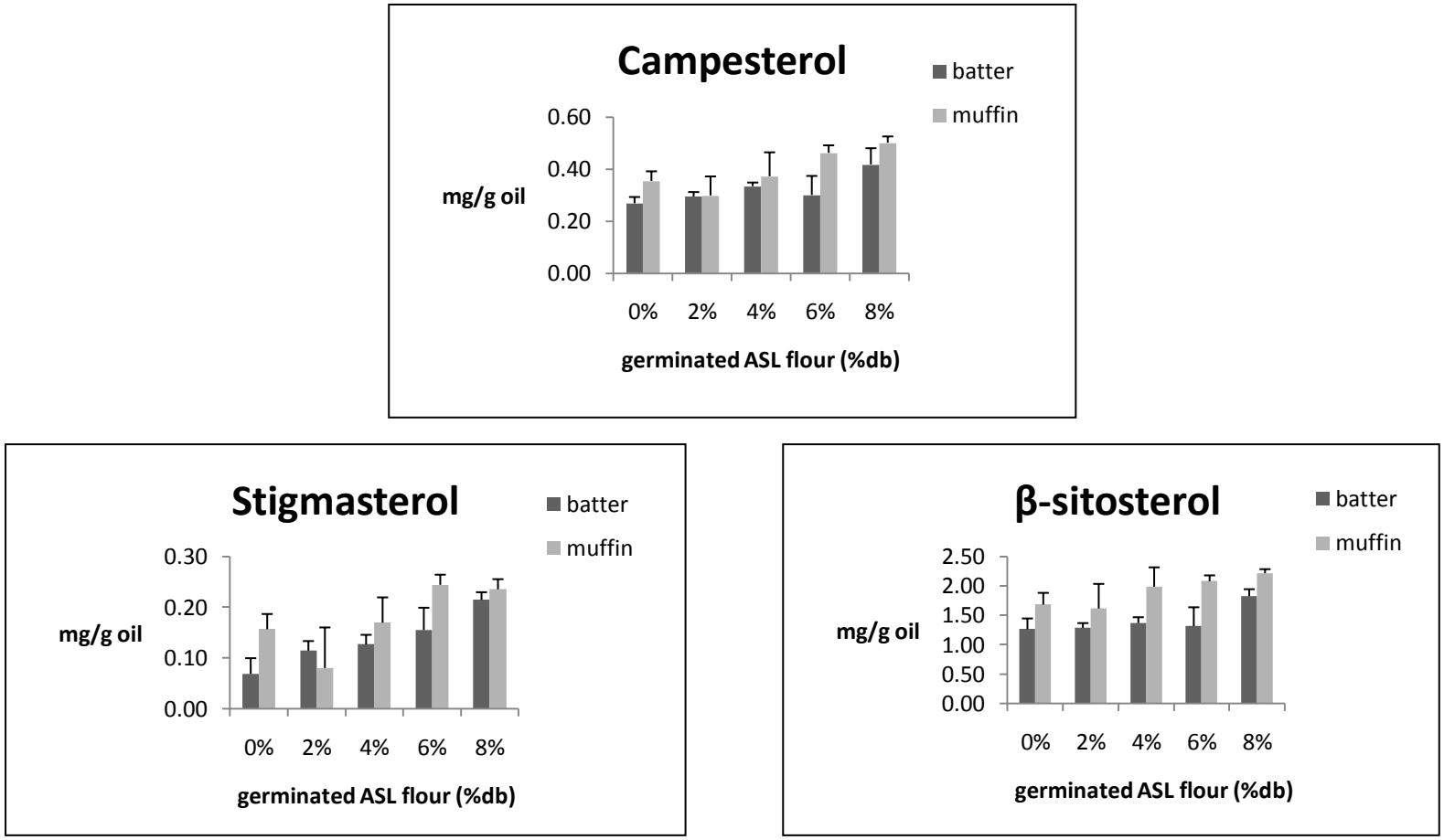


Figure 5-7 : Contents of phtosterols in batters and muffins incorporated with various level of germinated ASL flour

Shown are contents of phytosterols in batters (before baking) and muffins (after baking) incorporated with germinated ASL flour expressed as mg/g oil. Values are means ± standard deviations of measurements of 3 muffins from 3 different batches of flour.

Table 5-4 : Content of phytosterols in batters and muffins incorporated with various levels of germinated ASL flours (as percentage of dried muffin weight)

Sample	Campesterol (mg/g oil)		Stigmasterol (mg/g oil)		β-sitosterol (mg/g oil)	
	batter	muffin	batter	muffin	batter	muffin
0%	0.27 ± 0.024**a**	0.35 ± 0.037**a***	0.07 ± 0.03**a**	0.16 ± 0.03**a***	1.26 ± 0.18**a**	1.68 ± 0.20**a**
2%	0.29 ± 0.017**a**	0.30 ± 0.073**a**	0.11 ± 0.02**a**	0.08 ± 0.08**ac**	1.29 ± 0.08**a**	1.62 ± 0.42**a**
4%	0.33 ± 0.014**b**	0.37 ± 0.092**ab**	0.13 ± 0.02**ab**	0.17 ± 0.05**bc**	1.37 ± 0.10**a**	1.99 ± 0.33**a** *
6%	0.30 ± 0.073**ab**	0.46 ± 0.029**bc***	0.16 ± 0.04**b**	0.24 ± 0.02**d***	1.32 ± 0.32**a**	2.08 ± 0.10**a** *
8%	0.42 ± 0.062**c**	0.50 ±0.024 **c***	0.21 ± 0.01	0.24 ± 0.02**d**	1.82 ± 0.12**a**	2.22 ± 0.07**a** *

Shown are phytosterol contents in batters (before baking) and muffins (after baking) incorporated with germinated ASL flour expressed as mg/g oil. Values are means ± standard deviations of measurements of 3 muffins from 3 different batches of flour. Different letter in the same column indicates a significant difference ($P < 0.05$). * indicates a significant difference before and after baking using student t-test ($P < 0.05$)

A similar effect following heat treatment has also been reported following boiling the beans and vegetables for 30 min (Kaloustian et al., 2008). They proposed that cooking resulted in the release of free phytosterols from steryl glycosides in the food matrix. As a result the total phytosterols which were extractable in the unsaponifiable fraction in the muffins was higher than those in the batter.

The stability of phytosterols has been investigated in a number of studies. For example, heat treatment of phytosterols in vegetable oils at 100°C for 1 h was found to not affect the compounds. When the heating was carried out at 200°C for 0.5 - 1 h, the heat treatment degraded the compounds (Thanh et al., 2006). The amount of degraded phytosterols was found to increase with increasing temperature and time of heat treatment (Rudzinska et al., 2009). When heat treatment was applied at 60°C for 1 and 24 h on phytosterol standards, the treatment resulted in 7% and 16% disappearance of phytosterols, respectively. Meanwhile, heating at 120°C for the same period resulted in a loss 14% and 63% of phytosterols, respectively. Eleven oxidized phytosterols, were found following the heat treatment (Rudzinska et al., 2009). Epoxy-, 7-hydroxy- and 7-keto- derivatives were the main products of phytosterol oxidation. The loss of phytosterols after heat treatment should be equal to the amount of oxyphytosterols formed (Rudzinska et al., 2009). Similar studies have shown that temperature, heating time, sterol structure, lipid medium and type of processing techniques are key factors affecting oxidation of phytosterol (Soupas et al., 2004, Soupas et al., 2007). Although baking at 190°C for 25 min did not degrade the phytosterol in muffins prepared in the present study, baking at a greater temperature or for prolonged time may affect the stability of phytosterols in muffins.

When the effects of the level of germinated ASL flour substitution on the amount of phytosterols was examined (Table 5-4), substitution of the germinated ASL flour more than 4% resulted in a significant increase in the amount of campesterol, stigmasterol in the batter and the muffins. However, the amount of β-sitosterol in both the batter and the muffins remained unchanged across the different levels of substitution of germinated ASL flour.

5.3 Summary

Substitution of germinated ASL flour up to 8% (db of muffins) affected the physical characteristics of the muffins. Muffins substituted with germinated ASL flour up to 8% had significantly higher moisture content than the control muffins. The increased amount of germinated ASL flours resulted in a reduced height while the diameter remained unchanged. Muffins with germinated ASL flour was yellower, redder and darker than those of the control muffins. The hardness and cohesiveness were affected by the incorporation of germinated ASL flour. The low hardness (softer) of the muffins incorporated with germinated ASL flour could be due to the increased moisture content of the muffins. Based on these findings, it appears that there is a great potential to produce acceptable muffins containing germinated ASL flour up to 8%.

Incorporation of germinated ASL flour up to 8% also resulted in an increase in TPCs and phytosterol contents of the muffins. Ability of the muffins to scavenge radical activity was also increased with increasing level of incorporation of germinated ASL flour. Furthermore, the baking process at 190°C for 25 min did not substantially affect the concentration of TPCs and their antiradical activity. With some levels of germinated ASL flour substitution, the TPCs and their antiradical activity were actually increased following the baking process. Furthermorer, the concentration and relative composition of the phytosterols contained in the muffins incorporated with germinated ASL flour were not altered by the baking process.

Incorporation of germinated ASL flours up to 8% positively affected hardness, cohesiveness, colour and volume of the muffins. The incorporation would provide more health benefits to the muffins baked at 190°C for 25 min with increased contents of phenolic compounds and phytosterols as well as improved antiradical activity.

Chapter 6 General Discussion

Cardiovascular disease and cancer are global health burdens. Metabolic syndrome is a cluster of abnormalities including obesity, hypertension, dyslipidemia, insulin resistance and represents an important risk factor for cardiovascular disease (Nagao and Yanagita, 2008, Sarti and Gallagher, 2006). Obesity is the key factor for occurrence of metabolic syndrome (Eckel et al., 2005). The macronutrient composition in the diet including high saturated fat and sugar and less physical activity appear to be the main causes of obesity. Many strategies have been implemented to reduce the prevalence of obesity. Some of the approaches in preventing weight gain include behavioural changes by reducing energy intake and increasing the regular level of physical activity (Eckel et al., 2005). Consumption of diets consisting of plant foods which contain high antioxidants such as vitamin A, E, C, carotenoids, flavonoids and phytosterols is also encouraged to improve health (Petti, 2009). Consequently, the demand for healthy foods containing high protein, fibre and bioactive compounds but low in fat such as lupin to promote health is increasing.

Legumes represent an ideal food ingredient for management of risk factors associated with metabolic syndrome and cancer as they contain high level of protein, fibre and bioactive compounds. Consumption of lupin, in particular, has been found to reduce some risk factors related to metabolic syndrome. A diet containing lupin has been demonstrated to reduce prevalence of obesity in humans by increasing satiety and reducing energy intake acutely (Lee et al., 2006b, Archer et al., 2004). In animal and human studies, the consumption of lupin protein isolate of *L. albus* also showed benefits in management of diabetes (Magni et al., 2004), hypertension (Pilvi et al., 2006, Lee et al., 2009) and hypercholesterolemia (Hall et al., 2005, Martin et al., 2005).

Germination is a simple method for improving the nutritional value and concentration of bioactive compounds in legumes. As a result, consumption of germinated legumes would be more beneficial than that of ungerminated seeds. Use of germinated ASL flour obtained in the present study as a food ingredient in diets would provide many benefits

for managing some risk factors of CVD such as obesity, hypercholesterolemic and high blood pressure level. The higher protein and fibre content, but lower fat content of germinated ASL flour compared to those in ungerminated ASL flour may result in an improvement in beneficial effects of germinated ASL flour on its weight reducing effects. A diet high in protein and fibre has been reported to have potential benefits for reduction of weight by affecting satiety and energy intake. Ungerminated lupin kernel flour which is rich in protein and fibre has been incorporated into breads and its consumption has been reported to improve satiety acutely (Lee et al., 2006b). The satiating effect of protein and fibre is reported to be due to its affect on secretion of ghrelin, an appetite-regulating hormone (Horvath et al., 2001).

In addition to the possible improvement in weight reducing effects, germinated ASL flour possibly has an improvement in cholesterol and blood pressure lowering effects. As found in the present study, germinated ASL flour demonstrated the BA binding properties and ACE inhibitory activity *in vitro*. The BA binding property is likely to result in lowering of cholesterol levels. Meanwhile, ACE inhibitory activity may result in blood pressure lowering effects when germinated ASL flour is consumed. Despite the low fat content of germinated ASL flour, the present study found that it still contains high levels of phytosterols. Phytosterols are also known to lower serum cholesterol level by inhibition of cholesterol absorption from diet in the intestinal lumen. Hence, the high content of protein, fibre and bioactive compounds in germinated ASL flour would play an important role in lowering cholesterol and blood pressure when germinated ASL flour is applied in diets. Furthermore, the soluble fraction extracted from germinated ASL flour in the present study showed high ACE inhibitory activity and BA binding property suggesting that soluble fraction can be further developed in food applications. In particular, functional foods enriched with soluble fraction could be developed for blood pressure and cholesterol reduction purpose.

In addition to the health benefits, germinated ASL flour may have more potential benefits in antioxidant activity and other various health benefits than ungerminated ASL flour. The high concentration of phenolic compounds which have antiradical scavenging

activity in germinated ASL flour may contribute to the high antioxidant activity of the flour. Phenolic compounds have been reported to exhibit not only antioxidant activity but also a wide range of bioactivities such as insecticidal, antifungal, estrogenic activity, anti-inflammatory, anti-allergic, antithrombotic, antiviral, anti-carcinogen and vasodilatory action (Soobrattee et al., 2005, Cseke, 2006). Furthermore, phytosterols contained in germinated ASL flour may also have contributed to its high antioxidant activity. Phytosterols have been reported to have a contribution against some cancer (Bradford and Awad, 2007) and antioxidant activity in lipid oxidation process (Wang et al., 2002b, Hidalgo et al., 2009). High concentration of these bioactive compounds found in germinated ASL flour suggests it may have antioxidant activity and other various health benefits. Collectively it appears that the application of germinated ASL flour in diets could reduce weight, lower cholesterol and blood pressure levels and prevent occurrence of cancer.

Incorporation of germinated ASL flour into food products is likely to be a good approach to improve the health benefits. From the present study, incorporation of germinated ASL flour in muffins at up to 8% (db of muffins) resulted in an increase in its phenolic compounds and phytosterols and its antiradical activity *in vitro*. These changes would make muffin incorporated with germinated ASL flour an attractive healthy food with benefits above and beyond both traditional muffins and muffins incorporated with ungerminated ASL flour. In turn, it would also assist in the prevention of diseases related to cancer and other degenerative diseases. Incorporation up to 8% of germinated ASL flour had no significant effect on the volume, colour, hardness and moisture content. Incorporation of more than 8% is likely to negatively affect the quality of muffins, making them unacceptable. Incorporation of germinated ASL flour up to 8% is recommended as it improves the level of bioactive compounds and antioxidant activity of muffins.

Phenolic compounds, phytosterol contents and bioactivities related to antioxidant activity in the muffins incorporated with ASL flour in the present study were not reduced by the baking process at 190°C for 25 min. The antiradical activity of the

compounds in the muffins was increased after the baking process. Therefore, this baking conditions had no adverse effect on the bioactive compounds and the antioxidant in the muffins. The present processing conditions could therefore be applied to other food products such as bread, biscuits and cakes, without affecting the concentration of bioactive compounds or their bioactivities.

Germinated ASL flour therefore represents an attractive food ingredient with a wide range of health benefits for prevention of some risk factors related to cardiovascular diseases and cancer. Incorporation of germinated ASL flour in a variety of food products seems to be a good approach to increase the availability of healthy foods.

Chapter 7 Conclusions

In the present study, changes in the composition and concentration of bioactive compounds, such as phenolic compounds, phytosterols and bioactive proteins were investigated during the course of germination of ASL for nine days. Association between these germination and changes in the *in vitro* bioactivities was also examined. In order to investigate the stability of the bioactive compounds and their activity *in vitro* during the production of muffins incorporated with germinated ASL flour, the stability of bioactive compounds and their activity *in vitro* in the batter and the muffins were also investigated.

Germination of ASL yielded approximately 650 g of wet dehulled ASL sprouts per 100 g of ASL seeds after 9 days of germination. The high weight was caused by the high water content of the wet sprouts (~90%) at day 9 of germination. On a dry weight basis, 100 g of ASL seeds could produce around 45 g of dehulled ASL sprouts after 9 days of germination.

Germination significantly increased in the protein and crude fibre contents by about 38% (db), and 450% (db), respectively at day 9 of germination. The lipid content was reduced substantially by about 70% (db) at day 9 of germination.

Germination also increased in the TPCs in the methanol extracts (700%) and in aqueous extracts (100%) of germinated ASL flours (db) at day 9 of germination. The methanol extracts exhibited a higher DPPH radical scavenging activity than the aqueous extracts. Methanol is a better solvent when compared to aqueous solvent for extraction of phenolic compounds with antiradical activity in germinated ASL flours.

The concentration of total phytosterols in the oil extracted from germinated ASL flours increased by about 3 fold (per weight oil) during germination. However, when expressed per weight of germinated lupin flour, the concentration of total phytosterols remained unchanged. The main phytosterols in the oil extracted from germinated ASL were β-

sitosterol (62%), campesterol (30%) and stigmasterol (8%). The oil of the germinated ASL flour had a higher antiradical activity than the oil of the raw ASL seeds.

Germination enhanced the BA binding property of the flour. Germination also increased the ACE inhibitory activity of the proteins extracted from germinated ASL flour. The soluble fraction isolated from germinated ASL flour exhibited a greater extent of ACE inhibition and BA binding property than the protein isolate.

Incorporation of germinated ASL flour up to 8 % resulted in an increase in the moisture content of the muffins. The incorporation of germinated ASL flour up to 8% affected the height and diameter of the muffins. Muffins with germinated ASL flour were yellower, redder and darker than the control muffins. The level of incorporation up to 8% positively affected the hardness and cohesiveness of the muffins.

Incorporation of germinated ASL flour up to 8% also resulted in an increase in total phenolic compounds and phytosterols content in the muffins. The radical scavenging activity of the muffins was increased with increased level of incorporation. The baking process at190°C for25 min did not substantially reduce the concentration of TPCs, phytosterols and the antiradical activity in muffins.

Chapter 8 Future perspectives and recommendations for further studies

The increase in bioactive compounds and the bioactivities of the compounds found in germinated ASL flour in the present study are important findings that support the use of germinated ASL flour as an ingredient in food formulations. These foods, when consumed as part of a diet, are likely to reduce the risk of CVD and cancer. However, the activity of bioactive compounds measured in the present study was only determined using *in vitro* methods. It is therefore important to determine the *in vivo* effects of a diet containing germinated ASL flour. For example, the effects of a diet containing germinated ASL flour on serum lipids, satiety and energy intake of human should be investigated.

The large increase in crude fibre (around 450%) in germinated lupin found in this study is an interesting finding and the beneficial effects of dietary fibre on prevention of some risk factors related to metabolic syndrome would therefore promote the use of germinated lupin in food products. However, the analysis in the present study was only based on the crude fibre content, which is only a part of the dietary fibre. Understanding how all components of dietary fibre including soluble polysaccharides, insoluble polysaccharides, cellulose, and lignin change during germination of ASL should also be further investigated.

The present study found that the concentration of TPCs in ASL increased significantly by 700% after 9 days of germination. The significant increase in the TPCs is a promising finding suggesting the importance of germinated lupin as a source of phenolic compounds. Phenolic compounds are an important source of antioxidants present in legumes and other plants. A high intake of antioxidants is associated with a reduced incidence of diseases related to cardiovascular and cancer. Therefore, it would be valuable to characterize the individual phenolic compounds and their bioactivities present in germinated ASL.

The findings from the present study also showed that protein isolate and soluble fraction isolated from germinated ASL flour possess some bioactivities *in vitro*. The protein isolate had higher antiradical activity than the soluble fraction. Soluble fraction appears to be active in ACE inhibitory activity and BA binding activity. However, the present study was not able to characterize the compounds which are responsible for the bioactivities. Therefore, it would be interesting to determine which bioactive compounds were responsible for these bioactivities. Protein isolate is an important product in the food industry due to its wide range of functional properties. Further investigation on the bioactivities of soluble fraction as established from the present study can therefore support the use of soluble fraction of germinated ASL flour as a valuable ingredient in the food industry instead of dairy-derived protein isolate.

Based on the findings on the physical characteristics of muffins incorporated with germinated ASL flour, there is a possibility to produce acceptable muffins containing ASL flour up to 8%. However, sensory evaluation involving panellists should be performed to accurately assess the acceptability of the muffins. The increased concentration of bioactive compounds and their bioactivity *in vitro* of muffins incorporated with germinated ASL flour found in the present study would provide evidence to support incorporation of these muffins as part of a healthy diet. A study investigating the health benefits of consumption of germinated ASL flour incorporated muffins should also be conducted on human in the future.

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Appendices

Appendix A: Data of effects of germination of ASL on chemical composition, bioactive compounds and *in vitro* bioactivities and the statistical analysis.

A1: Rotten and non-germinated seeds during germination at 25°C and 28°C

Samples	Wet weight of rotten&non-germinated seeds (g/100g seeds)		Total wet sprouts (g/100g seeds)		Proportion of rotten&non-germinated seeds to total wet sprouts(%)	
	25°C	28°C	25°C	28°C	25°C	28°C
Day1	4.45±0.61	8.54±0.45	242.41±9.15	231.42±6.33	1.84±0.30	3.69±0.11
Day2	7.57±0.60	8.62±1.84	317.70±2.95	299.37±5.01	2.38±0.17	2.88±0.59
Day3	20.01±1.14	12.09±0.73	396.32±5.00	381.12±16.58	5.05±0.23	3.17±0.07
Day4	43.06±5.73	18.32±2.86	485.67±4.90	452.00±11.96	8.86±1.09	4.05±0.54
Day5	50.35±3.17	14.82±3.11	557.12±11.97	554.29±7.74	9.05±0.75	2.68±0.60
Day6	25.53±3.17	20.68±1.81	662.17±10.28	595.72±13.30	3.85±0.43	3.48±0.35
Day7	13.8±3.58	49.96±2.08	779.56±16.24	672.49±15.73	1.78±0.50	7.43±0.44
Day8	12.46±7.58	93.94±9.08	825.71±4.66	700.54±1.17	1.51±0.92	13.41±1.31
Day9	29.41±4.10	220.65±9.89	856.71±9.00	715.65±5.03	3.41±0.46	30.83±1.35

A2-1: Data of lupin yield during germination (wet weight)

Sample	Replication	Whole Sprouts (g)	non-germinated (g)	Dehulled (g)	Hulls (g)	MC of whole sprouts (%)
Day 1	1	262.408	11.832	202.032	48.544	67.971
	2	261.136	7.236	203.948	49.952	65.521
	3	270.512	10.128	210.724	49.66	68.745
Day 2	1	305.096	10.224	251.144	43.728	72.979
	2	308.096	23.112	240.752	44.232	75.014
	3	301.428	8.512	248.692	44.224	73.336
Day 3	1	362.46	23.068	289.58	49.812	76.465
	2	365.84	6.232	310.508	49.1	79.439
	3	360.524	3.552	303.54	53.432	77.252
Day 4	1	430.996	15.74	358.956	56.3	85.311
	2	426.976	15.384	352.632	58.96	85.438
	3	444.172	12.572	369.46	62.14	83.245
Day 5	1	502.596	16.652	411.136	74.808	86.120
	2	486.192	27.228	397.46	61.504	84.758
	3	561.992	7.896	469.92	84.176	89.852
Day 6	1	624.204	15.272	516.812	92.12	88.567
	2	605.512	14.188	509.36	81.964	90.231
	3	638.184	7.404	541.172	89.608	89.886
Day 7	1	730.712	13.66	629.02	88.032	91.627
	2	692.828	15.668	583.016	94.144	91.551
	3	709.62	3.608	607.46	98.552	91.04
Day 8	1	772.98	15.708	664.264	93.008	92.205
	2	754.64	33.48	623.88	97.28	92.024
	3	791.26	23.632	664.824	102.804	90.61
Day 9	1	805.976	67.156	638.892	99.928	92.104
	2	770.464	35.884	641.084	93.496	90.85
	3	844.936	18.392	720.98	105.564	92.66

A 2-2: Examples of statistical analysis of moisture content (MC) of whole sprouts

Descriptives

MCwetsprouts

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1.0000	3	67.412700	1.6834300	.9719288	63.230828	71.594572	65.5210	68.7459
2.0000	3	73.776567	1.0867629	.6274429	71.076898	76.476235	72.9791	75.0144
3.0000	3	77.719167	1.5407292	.8895404	73.891783	81.546550	76.4655	79.4392
4.0000	3	84.665233	1.2315097	.7110125	81.605994	87.724473	83.2451	85.4388
5.0000	3	86.910500	2.6374546	1.5227351	80.358700	93.462300	84.7584	89.8527
6.0000	3	89.561500	.8783019	.5070879	87.379677	91.743323	88.5671	90.2313
7.0000	3	91.406367	.3195207	.1844753	90.612633	92.200100	91.0400	91.6273
8.0000	3	91.613367	.8736774	.5044179	89.443032	93.783702	90.6100	92.2059
9.0000	3	91.871367	.9271724	.5353032	89.568143	94.174591	90.8500	92.6600
Total	27	83.881863	8.6280332	1.6604658	80.468727	87.294999	65.5210	92.6600

Test of Homogeneity of variances

MCwetsprouts

Levene Statistic	df1	df2	Sig.
2.338	8	18	.064

Tests of Normality

	Sample	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
MCwetsprouts	1.0000	.297	3	.	.917	3	.443
	2.0000	.324	3	.	.877	3	.315
	3.0000	.286	3	.	.931	3	.493
	4.0000	.367	3	.	.793	3	.099
	5.0000	.284	3	.	.933	3	.499
	6.0000	.311	3	.	.898	3	.378
	7.0000	.342	3	.	.845	3	.226
	8.0000	.348	3	.	.834	3	.199
	9.0000	.266	3	.	.953	3	.581

a. Lilliefors Significance Correction

ANOVA

MCwetsprouts

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1900.801	8	237.600	123.193	.000
Within Groups	34.716	18	1.929		
Total	1935.517	26			

Post Hoc tests

Multiple Comparisons

MCwetsprouts
Tukey HSD

(I) Sample	(J) Sample	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.0000	2.0000	-6.3638667*	1.1339243	.001	-10.336981	-2.390752
	3.0000	-10.3064667*	1.1339243	.000	-14.279581	-6.333352
	4.0000	-17.2525333*	1.1339243	.000	-21.225648	-13.279419
	5.0000	-19.4978000*	1.1339243	.000	-23.470914	-15.524686
	6.0000	-22.1488000*	1.1339243	.000	-26.121914	-18.175686
	7.0000	-23.9936667*	1.1339243	.000	-27.966781	-20.020552
	8.0000	-24.2006667*	1.1339243	.000	-28.173781	-20.227552
	9.0000	-24.4586667*	1.1339243	.000	-28.431781	-20.485552
2.0000	1.0000	6.3638667*	1.1339243	.001	2.390752	10.336981
	3.0000	-3.9426000	1.1339243	.053	-7.915714	.030514
	4.0000	-10.8886667*	1.1339243	.000	-14.861781	-6.915552
	5.0000	-13.1339333*	1.1339243	.000	-17.107048	-9.160819
	6.0000	-15.7849333*	1.1339243	.000	-19.758048	-11.811819
	7.0000	-17.6298000*	1.1339243	.000	-21.602914	-13.656686
	8.0000	-17.8368000*	1.1339243	.000	-21.809914	-13.863686
	9.0000	-18.0948000*	1.1339243	.000	-22.067914	-14.121686
3.0000	1.0000	10.3064667*	1.1339243	.000	6.333352	14.279581
	2.0000	3.9426000	1.1339243	.053	-.030514	7.915714
	4.0000	-6.9460667*	1.1339243	.000	-10.919181	-2.972952
	5.0000	-9.1913333*	1.1339243	.000	-13.164448	-5.218219
	6.0000	-11.8423333*	1.1339243	.000	-15.815448	-7.869219
	7.0000	-13.6872000*	1.1339243	.000	-17.660314	-9.714086
	8.0000	-13.8942000*	1.1339243	.000	-17.867314	-9.921086
	9.0000	-14.1522000*	1.1339243	.000	-18.125314	-10.179086
4.0000	1.0000	17.2525333*	1.1339243	.000	13.279419	21.225648
	2.0000	10.8886667*	1.1339243	.000	6.915552	14.861781
	3.0000	6.9460667*	1.1339243	.000	2.972952	10.919181
	5.0000	-2.2452667	1.1339243	.574	-6.218381	1.727848
	6.0000	-4.8962667*	1.1339243	.010	-8.869381	-.923152
	7.0000	-6.7411333*	1.1339243	.000	-10.714248	-2.768019
	8.0000	-6.9481333*	1.1339243	.000	-10.921248	-2.975019
	9.0000	-7.2061333*	1.1339243	.000	-11.179248	-3.233019

*. The mean difference is significant at the 0.05 level.

Multiple Comparisons

MCwetsprouts
Tukey HSD

(I) Sample	(J) Sample	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
5.0000	1.0000	19.4978000*	1.1339243	.000	15.524686	23.470914
	2.0000	13.1339333*	1.1339243	.000	9.160819	17.107048
	3.0000	9.1913333*	1.1339243	.000	5.218219	13.164448
	4.0000	2.2452667	1.1339243	.574	-1.727848	6.218381
	6.0000	-2.6510000	1.1339243	.371	-6.624114	1.322114
	7.0000	-4.4958667*	1.1339243	.020	-8.468981	-.522752
	8.0000	-4.7028667*	1.1339243	.014	-8.675981	-.729752
	9.0000	-4.9608667*	1.1339243	.009	-8.933981	-.987752
6.0000	1.0000	22.1488000*	1.1339243	.000	18.175686	26.121914
	2.0000	15.7849333*	1.1339243	.000	11.811819	19.758048
	3.0000	11.8423333*	1.1339243	.000	7.869219	15.815448
	4.0000	4.8962667*	1.1339243	.010	.923152	8.869381
	5.0000	2.6510000	1.1339243	.371	-1.322114	6.624114
	7.0000	-1.8448667	1.1339243	.779	-5.817981	2.128248
	8.0000	-2.0518667	1.1339243	.676	-6.024981	1.921248
	9.0000	-2.3098667	1.1339243	.540	-6.282981	1.663248
7.0000	1.0000	23.9936667*	1.1339243	.000	20.020552	27.966781
	2.0000	17.6298000*	1.1339243	.000	13.656686	21.602914
	3.0000	13.6872000*	1.1339243	.000	9.714086	17.660314
	4.0000	6.7411333*	1.1339243	.000	2.768019	10.714248
	5.0000	4.4958667*	1.1339243	.020	.522752	8.468981
	6.0000	1.8448667	1.1339243	.779	-2.128248	5.817981
	8.0000	-.2070000	1.1339243	1.000	-4.180114	3.766114
	9.0000	-.4650000	1.1339243	1.000	-4.438114	3.508114
8.0000	1.0000	24.2006667*	1.1339243	.000	20.227552	28.173781
	2.0000	17.8368000*	1.1339243	.000	13.863686	21.809914
	3.0000	13.8942000*	1.1339243	.000	9.921086	17.867314
	4.0000	6.9481333*	1.1339243	.000	2.975019	10.921248
	5.0000	4.7028667*	1.1339243	.014	.729752	8.675981
	6.0000	2.0518667	1.1339243	.676	-1.921248	6.024981
	7.0000	.2070000	1.1339243	1.000	-3.766114	4.180114
	9.0000	-.2580000	1.1339243	1.000	-4.231114	3.715114

*. The mean difference is significant at the 0.05 level.

Multiple Comparisons

MCwetsprouts
Tukey HSD

(I) Sample	(J) Sample	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
9.0000	1.0000	24.4586667*	1.1339243	.000	20.485552	28.431781
	2.0000	18.0948000*	1.1339243	.000	14.121686	22.067914
	3.0000	14.1522000*	1.1339243	.000	10.179086	18.125314
	4.0000	7.2061333*	1.1339243	.000	3.233019	11.179248
	5.0000	4.9608667*	1.1339243	.009	.987752	8.933981
	6.0000	2.3098667	1.1339243	.540	-1.663248	6.282981
	7.0000	.4650000	1.1339243	1.000	-3.508114	4.438114
	8.0000	.2580000	1.1339243	1.000	-3.715114	4.231114

*. The mean difference is significant at the 0.05 level.

Homogeneous subsets

MCwetsprouts

Tukey HSD[a]

Sample	N	Subset for alpha = 0.05				
		1	2	3	4	5
1.0000	3	67.412700				
2.0000	3		73.776567			
3.0000	3		77.719167			
4.0000	3			84.665233		
5.0000	3			86.910500	86.910500	
6.0000	3				89.561500	89.561500
7.0000	3					91.406367
8.0000	3					91.613367
9.0000	3					91.871367
Sig.		1.000	.053	.574	.371	.540

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

A3: Data of lupin yield during germination (dry weight)

Sample	Replication	Whole Sprouts (g)	non-germinated (g)	Dehulled (g)	Hulls (g)	MC of whole sprouts (%)
Day 1	1	85.3788	4.1558	61.3189	19.7459	2.327
	2	87.5116	2.7184	64.1155	20.4894	2.999
	3	90.0807	3.8177	64.9966	21.1125	3.789
Day 2	1	83.3851	3.3843	60.0551	19.788	4.709
	2	87.1894	8.2784	59.1324	19.6052	4.421
	3	82.7035	3.0854	59.7977	19.6689	5.195
Day 3	1	81.6314	6.8818	55.5577	19.0382	3.181
	2	79.8902	1.8738	58.205	19.6862	4.611
	3	83.0738	1.170	61.07	20.6569	4.643
Day 4	1	80.5518	4.169	56.5519	19.7215	4.899
	2	77.4669	4.2231	54.1604	18.9821	4.061
	3	82.3893	3.4493	58.2449	20.5268	5.463
Day 5	1	80.2691	4.5942	55.3772	20.1874	3.997
	2	78.8398	7.4904	52.3789	18.8338	4.114
	3	77.7226	2.0058	55.5508	20.0269	5.046
Day 6	1	74.9939	2.6894	52.3504	19.4025	5.435
	2	72.9923	3.4746	50.6182	18.787	5.822
	3	75.51	1.5438	53.9913	19.833	4.69
Day 7	1	76.0864	2.6942	53.2822	20.0237	6.237
	2	73.3419	3.1688	50.7319	19.3461	7.332
	3	75.9686	1.0256	54.6273	20.1805	9.21
Day 8	1	70.9262	2.5298	49.0223	19.2816	6.563
	2	69.3011	5.4424	45.1888	18.5798	6.63
	3	74.0044	5.0734	48.732	20.0421	8.16
Day 9	1	67.9096	7.7724	41.267	18.783	6.677
	2	67.2825	3.4556	44.7952	18.9376	7.55
	3	70.4605	2.3946	47.9507	19.979	7.0

A4-1 : Raw data of protein content analysis

Sample	Replication	WEIGHT (g)				Titran volume	% Protein
		Dish	Dish+sample	Dish+rem	Sample		
Day 0	1	0.5361	1.0148	0.5361	0.4787	24.0	44.00
	2	0.5289	0.9959	0.5292	0.4667	24.4	45.88
	3	1.0805	1.6502	1.0807	0.5695	27.5	42.40
Day 1	1	0.4965	1.0915	0.4966	0.5949	33.5	49.48
	2	0.5608	1.1065	0.5612	0.5453	30.4	48.97
	3	0.7791	1.2873	0.7803	0.507	26.8	46.41
Day 3	1	0.4996	1.0158	0.5005	0.5153	30.3	51.65
	2	0.5120	0.9629	0.5126	0.4503	26.0	50.69
	3	1.0803	1.5945	1.0815	0.513	28.6	48.96
Day 5	1	0.5189	1.017	0.5192	0.4978	30.8	54.35
	2	0.5643	1.1161	0.5649	0.5512	34.8	55.48
	3	1.1066	1.5555	1.1071	0.4484	26.7	52.28
Day7	1	0.5100	0.966	0.5108	0.4552	30.2	58.27
	2	0.5131	1.0396	0.5139	0.5257	34.5	57.67
	3	1.0699	1.5920	1.0712	0.5208	32.2	54.32
Day 9	1	0.4272	1.0721	0.4288	0.6433	46.2	63.15
	2	1.4609	1.8717	1.4631	0.4086	28.3	60.82
	3	1.0855	1.6354	1.0880	0.5474	36.8	59.08

A4-2: Examples of statistical analysis of protein content using Non-parametric test (Kruskal-Wallis Test)

NPar Tests
Kruskal-Wallis test

Ranks

	SAMPLE	N	Mean Rank
PROTEIN	1	3	2.00
	2	3	5.67
	3	3	7.33
	4	3	11.67
	5	3	13.33
	6	3	17.00
	Total	18	

Test Statistics[a,b]

	PROTEIN
Chi-Square	15.924
df	5
Asymp. Sig.	.007

a. Kruskal Wallis Test

b. Grouping Variable: SAMPLE

Mann-Whitney Test

Ranks

	SAMPLE	N	Mean Rank	Sum of Ranks
PROTEIN	1	3	2.00	6.00
	2	3	5.00	15.00
	Total	6		

Test Statistics[b]

	PROTEIN
Mann-Whitney U	.000
Wilcoxon W	6.000
Z	-1.964
Asymp. Sig. (2-tailed)	.050
Exact Sig. [2*(1-tailed Sig.)]	.100[a]

a. Not corrected for ties.

b. Grouping Variable: SAMPLE

Mann-Whitney Test

Ranks

	SAMPLE	N	Mean Rank	Sum of Ranks
PROTEIN	1	3	2.00	6.00
	3	3	5.00	15.00
	Total	6		

Test Statistics[b]

	PROTEIN
Mann-Whitney U	.000
Wilcoxon W	6.000
Z	-1.964
Asymp. Sig. (2-tailed)	.050
Exact Sig. [2*(1-tailed Sig.)]	.100[a]

a. Not corrected for ties.

b. Grouping Variable: SAMPLE

Mann-Whitney Test

Ranks

	SAMPLE	N	Mean Rank	Sum of Ranks
PROTEIN	1	3	2.00	6.00
	4	3	5.00	15.00
	Total	6		

Test Statistics[b]

	PROTEIN
Mann-Whitney U	.000
Wilcoxon W	6.000
Z	-1.964
Asymp. Sig. (2-tailed)	.050
Exact Sig. [2*(1-tailed Sig.)]	.100[a]

a. Not corrected for ties.

b. Grouping Variable: SAMPLE

Mann-Whitney Test

Ranks

	SAMPLE	N	Mean Rank	Sum of Ranks
PROTEIN	1	3	2.00	6.00
	5	3	5.00	15.00
	Total	6		

Test Statistics[b]

	PROTEIN
Mann-Whitney U	.000
Wilcoxon W	6.000
Z	-1.964
Asymp. Sig. (2-tailed)	.050
Exact Sig. [2*(1-tailed Sig.)]	.100[a]

a. Not corrected for ties.

b. Grouping Variable: SAMPLE

Mann-Whitney Test

Ranks

	SAMPLE	N	Mean Rank	Sum of Ranks
PROTEIN	1	3	2.00	6.00
	6	3	5.00	15.00
	Total	6		

Test Statistics[b]

	PROTEIN
Mann-Whitney U	.000
Wilcoxon W	6.000
Z	-1.964
Asymp. Sig. (2-tailed)	.050
Exact Sig. [2*(1-tailed Sig.)]	.100[a]

a. Not corrected for ties.

b. Grouping Variable: SAMPLE

A5: Raw data of oil content analysis

Sample	Replication	WEIGHT(g)						% oil (w/w)
		Cup +stone	Cup+stone +timble	Cup+stone+timble +sample	Dried cup+stone +oil	Oil extracted	Sample	
Day 0	1	99.74	103.04	104.42	99.82	0.0862	1.3823	6.24
	2	98.27	101.44	102.49	98.34	0.0676	1.0488	6.45
	3	96.85	99.89	101.07	96.92	0.0765	1.1846	6.46
Day 1	1	97.77	100.88	102.09	97.85	0.0816	1.2172	6.70
	2	96.85	99.73	101.70	96.98	0.1377	1.9761	6.97
	3	98.58	104.42	106.98	98.75	0.1751	2.5653	6.83
Day 3	1	102.04	105.03	106.28	102.099	0.0561	1.2532	4.48
	2	97.86	101.12	103.88	97.98	0.125	2.7586	4.53
	3	99.77	105.60	108.008	99.89	0.1156	2.404	4.81
Day 5	1	99.09	102.13	103.302	99.12	0.0331	1.1724	2.82
	2	102.65	105.68	107.09	102.70	0.0436	1.4158	3.08
	3	100.73	106.34	108.74	100.81	0.0804	2.398	3.35
Day 7	1	99.73	102.83	104.72	99.774	0.0418	1.8938	2.21
	2	98.52	101.39	103.44	98.574	0.0476	2.0518	2.32
	3	103.01	108.69	110.76	103.059	0.0471	2.0688	2.28
Day 9	1	99.61	102.64	103.98	99.63	0.0237	1.3391	1.77
	2	99.16	104.761	120.42	99.47	0.3109	15.661	1.99
	3	99.64	105.39	108.12	99.68	0.0498	2.7245	1.83

A6: Raw data of analysis of crude fibre content

Sample	W1(g) (capsl+lid)	W2(g) (capsl+lid+sample)	W3(g) (caps+lid+residu)	W4(g) (crucible)	W5(g) (Cruc+ash)	total ash	amount fibre	%crude fibre
G1								
Day 0	1.5074	2.00	1.5134	41.4002	41.4014	0.0012	0.0080	1.61
Day 1	1.5193	2.1803	1.5298	42.1144	42.1155	0.0011	0.0126	1.90
Day 3	1.5015	2.1815	1.5214	39.7401	39.7412	0.0011	0.0220	3.22
Day 5	1.5169	2.0976	1.5472	44.091	44.0921	0.0011	0.0324	5.56
Day 7	1.5085	2.2211	1.5574	44.7814	44.7824	0.001	0.0511	7.15
Day 9	1.5139	2.0328	1.5566	47.6695	47.6705	0.001	0.0449	8.61
GII								
Day 0	1.5058	2.0016	1.5119	43.6636	43.6647	0.0011	0.0080	1.61
Day 1	1.5128	2.1024	1.5212	41.1528	41.1537	0.0009	0.0105	1.78
Day 3	1.5092	2.0311	1.5266	40.1646	40.1655	0.0009	0.0195	3.73
Day 4	1.5204	1.9761	1.5419	43.0154	43.0162	0.0008	0.0237	5.20
Day 7	1.5083	2.2971	1.5648	39.395	39.3958	0.0008	0.0587	7.44
Day 9	1.5198	2.3169	1.5908	39.409	39.4099	0.0009	0.0731	9.17
GIII								
Day 0	1.5258	1.9824	1.531	41.9038	41.9048	0.001	0.0072	1.58
Day 1	1.5135	2.074	1.5288	41.1404	41.1414	0.001	0.0173	3.08
Day 3	1.5202	2.0852	1.538	40.2657	40.2666	0.0009	0.0199	3.52
Day 5	1.5245	2.2464	1.5621	42.9199	42.9208	0.0009	0.0397	5.50
Day 7	1.5227	2.3475	1.5832	40.263	40.2639	0.0009	0.0626	7.59
Day 9	1.5097	2.0798	1.5596	47.7765	47.7774	0.0009	0.0520	9.12

G=Germination; W= weight; C= 0.0022; D=0.001

A7-1: Raw data of total phenolic compound (TPC) analysis of methanol extracts

Sample	Germination	Weight of tube	Weight tube+sample	ABS	Conc (mg/L)	Vol.of extract	mg GAE/gDM sample	mg GAE/100g DM sample
Day 0	1	10.3943	11.3972	0.113	9.534	8.50	0.808	80.80
		10.2043	11.2682	0.142	12.350	6.70	0.778	77.77
		10.3838	11.5517	0.179	15.942	8.50	1.160	116.02
	2	10.4078	11.0224	0.119	10.117	8.00	1.317	131.68
		10.2221	11.0224	0.107	8.951	8.00	0.895	89.48
		10.7011	11.7996	0.168	14.874	8.00	1.083	108.32
	3	10.6414	11.2917	0.094	7.689	7.50	0.887	88.68
		10.5671	11.4373	0.112	9.437	8.00	0.868	86.76
		10.6407	11.7737	0.13	11.184	8.00	0.790	78.97
Day 1	1	10.2179	11.3892	0.239	21.767	6.00	1.115	111.50
		10.237	11.5258	0.258	23.612	8.00	1.466	146.57
		10.6492	11.9229	0.352	32.738	6.50	1.671	167.07
	2	10.2305	11.8865	0.407	38.078	6.60	1.518	151.76
		10.2469	11.536	0.304	28.078	7.70	1.677	167.71
		10.5691	11.7246	0.295	27.204	7.50	1.766	176.57
	3	10.2098	11.7193	0.481	45.262	6.70	2.009	200.90
		10.2303	11.6352	0.361	33.612	7.60	1.818	181.83
		10.6517	11.8132	0.315	29.146	7.80	1.957	195.73
Day 3	1	10.3916	11.789	0.625	59.243	8.00	3.392	339.16
		10.4108	11.865	0.674	64.000	7.00	3.081	308.07
		10.5514	11.7552	0.638	60.505	6.00	3.016	301.57
	2	10.3881	11.9455	0.735	69.922	6.70	3.008	300.81
		10.407	11.6897	0.604	57.204	7.00	3.122	312.18
		10.5539	11.3713	0.625	59.243	7.20	5.218	521.83
	3	10.2335	11.6608	0.635	60.214	6.70	2.827	282.65
		10.2528	11.2074	0.46	43.223	6.50	2.943	294.31
		10.6483	11.466	0.65	61.670	8.50	6.411	641.06

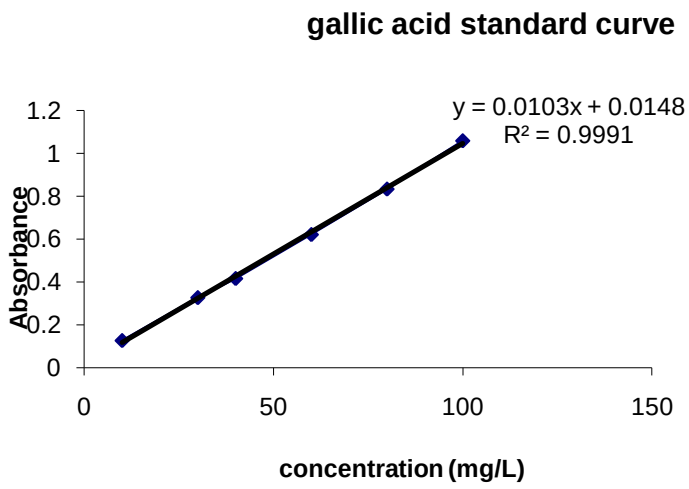
ABS:Absorbance; GAE : gallic acid equivalen; Conc : concentration

Continuation of A7

Sample	Germination	Weight of tube	Weight tube+sample	ABS	Conc (mg/L)	Vol. of extract	mg GAE/gDM sample	mgGAE/100g DM sample
Day 5	1	10.2309	11.2309	0.137	11.864	8.00	4.746	474.56
		8.0506	9.0387	0.123	10.505	7.70	4.093	409.31
		10.5528	11.618	0.182	16.233	5.00	3.810	380.99
	2	10.1736	11.1563	0.121	10.311	6.70	3.515	351.49
		10.1925	11.2572	0.119	10.117	6.50	3.088	308.81
		10.5741	11.8428	0.137	11.864	6.00	2.805	280.54
	3	10.1973	11.3715	0.177	15.748	8.50	5.700	569.98
		10.4067	11.3504	0.173	15.359	6.50	5.290	528.95
		10.5524	11.6527	0.177	15.748	5.50	3.936	393.58
Day 7	1	10.2545	11.4172	0.231	20.990	6.70	6.048	604.78
		10.2725	11.7749	0.315	29.146	7.00	6.790	678.98
		10.6275	11.861	0.274	25.165	7.50	7.651	765.05
	2	10.2381	11.7807	0.222	20.117	7.00	4.564	456.42
		10.2578	11.6459	0.314	29.049	7.00	7.324	732.44
		10.6479	11.6438	0.232	21.087	7.50	7.940	794.03
	3	10.3854	11.6687	0.201	18.078	7.00	4.930	493.04
		10.4067	11.6624	0.243	22.155	7.50	6.616	661.64
		10.5523	11.5598	0.224	20.311	8.50	8.568	856.78
Day 9	1	10.2071	11.556	0.26	23.806	6.70	5.912	591.22
		10.2255	11.531	0.282	25.942	6.00	5.961	596.13
		10.5773	11.8106	0.32	29.631	7.80	9.370	937.01
	2	10.3846	11.5542	0.286	26.330	7.00	7.879	787.92
		10.4041	11.4901	0.317	29.340	7.00	9.456	945.57
		10.5339	11.4987	0.305	28.175	6.70	9.783	978.29
	3	10.3948	11.7802	0.303	27.981	7.00	7.069	706.89
		10.4081	11.5747	0.243	22.155	7.50	7.122	712.18
		10.5461	11.6836	0.295	27.203	7.00	8.370	837.04

ABS: Absorbance; GAE : gallic acid equivalen; Conc : concentration

A7-2: An example of standard curve of gallic acid



A8: Data of TPC analysis in aqueous/Tris extracts

	TPC (mg GAE/100 g DM)					
Replication	**Day 0**	**Day 1**	**Day 3**	**Day 5**	**Day 7**	**Day 9**
GI - 1	234.84	234.89	381.17	562.28	350.17	590.91
2	230.02	245.73	666.29	374.27	568.74	577.60
3	247.65	195.30	491.94	497.38	439.29	388.56
GII - 1	231.97	228.56	321.19	504.43	356.65	605.19
2	236.77	245.26	661.84	375.23	590.94	599.99
3	230.58	216.32	509.61	534.15	458.97	418.97
GIII- 1	373.22	286.72	680.42	845.53	751.73	617.34
2	233.59	264.49	643.94	500.88	466.35	540.46
3	290.92	294.43	650.23	703.08	638.57	555.97

A 9-1: Raw data of DPPH scavenging activity of methanol extracts

Replication I	ABS 30'	% Inhibition	Conc. (mg/ml)	Vol. extract (ml)	Weight of sample (g)	mg eq trolox/g DM sample
GI						
Day 0	0.75	7.52	0.02	8	0.6503	0.27
Day 1	0.513	36.74	0.13	6.5	1.2737	0.64
Day 3	0.365	54.99	0.19	6	1.2038	0.95
Day 5	0.356	56.10	0.19	5	1.0652	0.91
Day 7	0.257	68.31	0.24	7.5	1.2335	1.45
Day 9 (dill 10x)	0.672	17.14	0.56	7.8	1.2333	3.55
	DPPH 0.811					
GII						
Day 0	0.75	7.52	0.02	8	0.8702	0.20
Day 1	0.568	29.96	0.10	7.5	1.1555	0.66
Day 3	0.477	41.18	0.14	7.2	0.8174	1.25
Day 5	0.36	55.61	0.19	6	1.2687	0.91
Day 7	0.326	59.80	0.21	7.5	0.9959	1.57
Day 9 (dill 10x)	0.734	9.49	0.29	6.7	0.9648	2.01
	DPPH 0.811					
GIII						
Day 0	0.758	10.51	0.03	8	1.133	0.23
Day 1	0.574	32.23	0.11	7.8	1.1615	0.74
Day 3	0.541	36.13	0.12	8.5	0.8177	1.29
Day 5	0.449	46.99	0.16	5.5	1.1003	0.81
Day 7	0.362	57.26	0.20	8.5	1.0075	1.68
Day 9 (dill 10x)	0.714	15.70	0.51	6.8	1.1375	3.05
	DPPH 0.847					

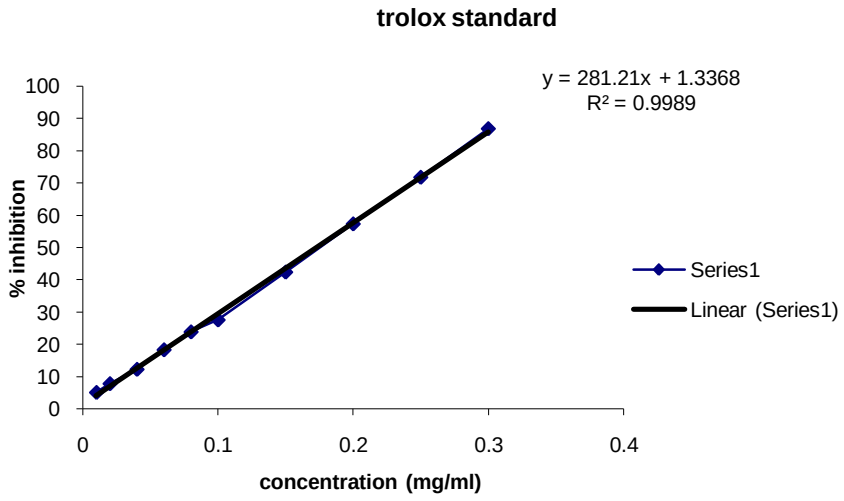
ABS:Absorbance; G: Germination; Dill : dilution

Continuation of A 9-1, summary of replication

Sample & replication	mg eq trolox/g DM germinated flour		
GI			
Day 0	0.27	0.19	0.39
Day 1	0.64	0.60	0.58
Day 3	0.95	0.95	1.33
Day 5	0.91	1.41	1.47
Day 7	1.45	1.30	1.37
Day9	3.55	2.40	3.86
GII			
Day 0	0.20	0.25	0.22
Day 1	0.66	0.64	0.75
Day 3	1.25	1.01	1.70
Day 5	0.91	1.12	0.87
Day 7	1.57	1.35	2.06
Day 9	2.01	3.67	4.68
GIII			
Day 0	0.23	0.21	0.24
Day 1	0.74	0.78	0.84
Day 3	1.29	1.10	1.74
Day 5	0.81	1.11	1.64
Day 7	1.68	1.50	1.85
Day 9	3.05	3.75	3.34

G: Germination

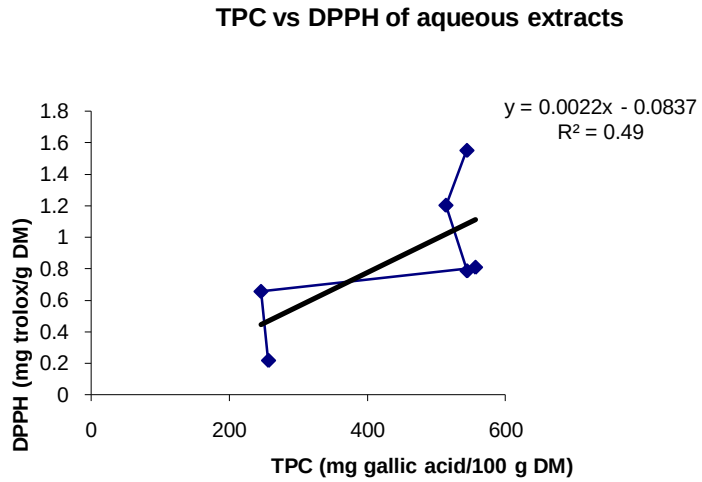
A 9-2: An example of standard curve of trolox

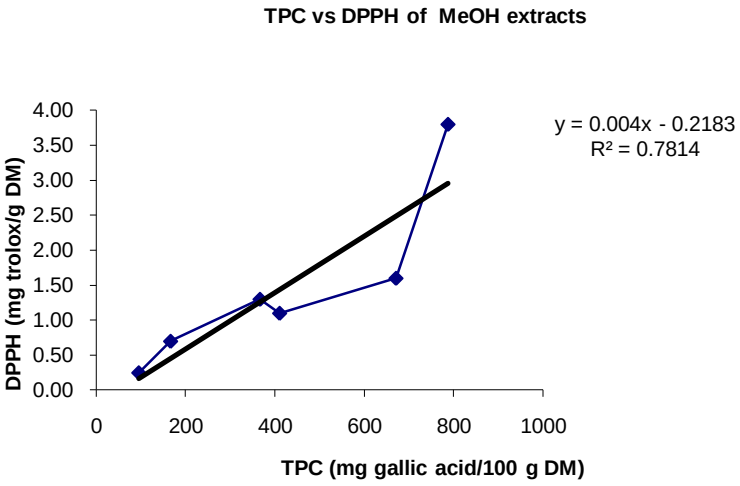


A10: Summary of replication data of DPPH radical scavenging activity of aqueous extracts

Sample& replication	mg eq trolox/g DM germinated flour		
GI			
Day 0	0.21	0.06	0.29
Day 1	0.45	0.42	0.57
Day 3	0.56	0.84	0.50
Day 5	0.97	1.01	0.73
Day 7	1.11	1.43	1.08
Day9	1.70	1.84	1.53
GII			
Day 0	0.16	0.21	0.24
Day 1	1.03	0.91	1.21
Day 3	1.27	1.32	0.96
Day 5	0.90	0.52	0.37
Day 7	1.54	1.58	1.00
Day 9	1.74	1.88	1.53
GIII			
Day 0	0.18	0.24	0.38
Day 1	0.45	0.42	0.45
Day 3	0.87	0.85	0.12
Day 5	1.07	1.03	0.48
Day 7	1.11	1.23	0.75
Day 9	1.18	1.33	1.24

A 11: Relationship between total phenolic compounds (TPC) and DPPH radical scavenging activity of aqueous and MeOH extracts





A12: Raw data of phytosterol content analysis

Samples	Replication	mg/g oils			mg/g flours		
		Campesterol	Stigmasterol	β-sitosterol	Campesterol	Stigmasterol	β-sitosterol
Day 0	1	5.7380	1.3036	11.5556	0.3655	0.0830	0.7361
	2	5.3637	1.1635	10.6842	0.4392	0.0953	0.8748
	3	4.9794	1.0800	10.0845	0.5588	0.1212	1.1318
Day 1	1	5.0045	1.2741	10.1017	0.4240	0.1079	0.8558
	2	4.8028	1.0883	9.4852	0.3614	0.0819	0.7137
	3	7.0357	2.7262	15.5828	0.7075	0.2741	1.5670
Day 3	1	9.4498	3.6900	18.5760	0.2065	0.0806	0.4059
	2	9.1552	3.5503	18.0931	0.3180	0.1233	0.6285
	3	7.9674	3.5060	17.3914	0.4881	0.2148	1.0654
Day 5	1	12.5020	6.0050	25.7697	0.5250	0.2522	1.0821
	2	11.5620	4.8496	21.8414	0.1765	0.0740	0.3335
	3	11.8420	5.9382	25.5573	0.2306	0.1156	0.4976
Day 7	1	15.0137	9.6273	31.5403	0.3000	0.1924	0.6302
	2	13.3138	8.3488	28.8189	0.3350	0.2101	0.7252
	3	14.0256	7.9867	28.5676	0.2461	0.1401	0.5013
Day 9	1	18.8537	15.3510	35.1086	0.3552	0.2892	0.6615
	2	16.8083	12.3770	33.1565	0.4116	0.3031	0.8120
	3	13.2761	10.6967	26.3973	0.2469	0.1990	0.4910

A13: Raw data of bile acid binding (Cholic acid) activity analysis

Sample	Weight of sample (g)	Abs test	Abs blank	test-blank	Conc of bile acid (umol/L)	Supernatant (11ml)	Binding	umol/50 mgDM
Germination I								
Cholesteramin	0.0121	0.149	0.015	0.134	29.7297	0.3270	0.6570	2.7150
Day 0	0.0508	0.35	0.084	0.266	65.4054	0.7195	0.2646	0.2604
Day 1	0.0516	0.343	0.114	0.229	55.4054	0.6095	0.3746	0.3630
Day 3	0.0518	0.248	0.058	0.19	44.8649	0.4935	0.4905	0.4735
Day 5	0.0524	0.248	0.04	0.208	49.7297	0.5470	0.4370	0.4170
Day 7	0.0518	0.244	0.049	0.195	46.2162	0.5084	0.4757	0.4591
Day 9	0.0516	0.241	0.081	0.16	36.7568	0.4043	0.5797	0.5618
Control		0.396	0.041	0.355	89.4595	0.9841		
Germination II								
Cholesteramin	0.0123	0.16	0.013	0.147	33.2432	0.3657	0.6243	2.5379
Day 0	0.0513	0.35	0.12	0.23	55.6757	0.6124	0.3776	0.3680
Day 1	0.0538	0.338	0.091	0.247	60.2703	0.6630	0.3270	0.3039
Day 3	0.0517	0.313	0.048	0.265	65.1351	0.7165	0.2735	0.2645
Day 5	0.0544	0.244	0.034	0.21	50.2703	0.5530	0.4370	0.4017
Day 7	0.0518	0.233	0.038	0.195	46.2162	0.5084	0.4816	0.4649
Day 9	0.053	0.224	0.043	0.181	42.4324	0.4668	0.5232	0.4936
Control		0.37	0.013	0.357	90.0000	0.9900		
Germination III								
Cholesteramin	0.0134	0.149	0.009	0.14	31.3514	0.3449	0.6570	2.4516
Day 0	0.0503	0.316	0.058	0.258	63.2432	0.6957	0.3062	0.3044
Day 1	0.0542	0.323	0.061	0.262	64.3243	0.7076	0.2943	0.2715
Day 3	0.0513	0.283	0.044	0.239	58.1081	0.6392	0.3627	0.3535
Day 5	0.0501	0.242	0.041	0.201	47.8378	0.5262	0.4757	0.4747
Day 7	0.0533	0.222	0.036	0.186	43.7838	0.4816	0.5203	0.4881
Day 9	0.055	0.216	0.042	0.174	40.5405	0.4459	0.5559	0.5054
Control		0.375	0.014	0.361	91.0811	1.0019		

Abs: absorbance; Conc: concentration

A14: Raw data of bile acid binding (Deoxycholic acid) activity analysis

Sample	Weight of sample	Abs test	Abs blank	test-blank	Conc of bile acid (umol/L)	Supernatant (11ml)	Binding	umol/50 mgDM
Germination I								
Cholesteramin	0.0053	0.152	0.032	0.12	25.9459	0.2854	0.2557	2.4120
Day 0	0.0503	0.283	0.094	0.189	44.5946	0.4905	0.0505	0.0502
Day 1	0.055	0.249	0.121	0.128	28.1081	0.3092	0.2319	0.2108
Day 3	0.0521	0.194	0.086	0.108	22.7027	0.2497	0.2914	0.2796
Day 5	0.0523	0.16	0.052	0.108	22.7027	0.2497	0.2914	0.2785
Day 7	0.0556	0.159	0.075	0.084	16.2162	0.1784	0.3627	0.3262
Day 9	0.055	0.15	0.075	0.075	13.7838	0.1516	0.3895	0.3541
Control		0.236	0.03	0.206	49.1892	0.5411		
Germination II								
Cholesteramin	0.0052	0.143	0.033	0.11	23.2432	0.2557	0.2854	2.7443
Day 0	0.0512	0.263	0.085	0.178	41.6216	0.4578	0.0832	0.0813
Day 1	0.0501	0.259	0.091	0.168	38.9189	0.4281	0.1130	0.1127
Day 3	0.0514	0.182	0.05	0.132	29.1892	0.3211	0.2200	0.2140
Day 5	0.0505	0.156	0.043	0.113	24.0541	0.2646	0.2765	0.2737
Day 7	0.055	0.147	0.05	0.097	19.7297	0.2170	0.3241	0.2946
Day 9	0.0538	0.15	0.045	0.105	21.8919	0.2408	0.3003	0.2791
Control		0.236	0.03	0.206	49.1892	0.5411		
Germination III								
Cholesteramin	0.0062	0.163	0.035	0.128	28.1081	0.3092	0.2646	2.1338
Day 0	0.0531	0.257	0.078	0.179	41.8919	0.4608	0.0803	0.0756
Day 1	0.0501	0.247	0.095	0.152	34.5946	0.3805	0.1605	0.1602
Day 3	0.0529	0.181	0.063	0.118	25.4054	0.2795	0.2616	0.2473
Day 5	0.0553	0.152	0.059	0.093	18.6486	0.2051	0.3359	0.3037
Day 7	0.0512	0.155	0.053	0.102	21.0811	0.2319	0.3092	0.3019
Day 9	0.053	0.11	0.037	0.073	13.2432	0.1457	0.3954	0.3730
Control		0.253	0.036	0.217	52.1622	0.5738		

Abs: absorbance; Conc: concentration

A15:Raw data of bile acid binding (Sod CDCA) activity analysis

Sample	Weight of sample	Abs test	Abs blank	test-blank	Conc of bile acid (umol/L)	Supernatant (11ml)	Binding	umol/50 mgDM
Germination I								
Cholesteramin	0.0068	0.144	0.013	0.131	28.9189	0.3181	0.4846	3.5632
Day 0	0.0494	0.26	0.056	0.204	48.6486	0.5351	0.2676	0.2708
Day 1	0.04	0.272	0.082	0.19	44.8649	0.4935	0.3092	0.3865
Day 3	0.0425	0.205	0.08	0.125	27.2973	0.3003	0.5024	0.5911
Day 5	0.0424	0.191	0.081	0.11	23.2432	0.2557	0.5470	0.6451
Day 7	0.0372	0.194	0.059	0.135	30.0000	0.3300	0.4727	0.6354
Day 9	0.031	0.221	0.06	0.161	37.0270	0.4073	0.3954	0.6378
Control		0.314	0.02	0.294	72.9730	0.8027		
Germination II								
Cholesteramin	0.0068	0.144	0.013	0.131	28.9189	0.3181	0.5084	3.7381
Day 0	0.0489	0.256	0.089	0.167	38.6486	0.4251	0.4014	0.4104
Day 1	0.0392	0.249	0.077	0.172	40.0000	0.4400	0.3865	0.4930
Day 3	0.0401	0.219	0.109	0.11	23.2432	0.2557	0.5708	0.7117
Day 5	0.0402	0.185	0.087	0.098	20.0000	0.2200	0.6065	0.7543
Day 7	0.036	0.187	0.052	0.135	30.0000	0.3300	0.4965	0.6896
Day 9	0.0309	0.225	0.053	0.172	40.0000	0.4400	0.3865	0.6254
Control		0.343	0.041	0.302	75.1351	0.8265		
Germination III								
Cholesteramin	0.0051	0.156	0.016	0.14	31.3514	0.3449	0.4281	4.1971
Day 0	0.05	0.262	0.08	0.182	42.7027	0.4697	0.3032	0.3032
Day 1	0.0395	0.24	0.092	0.148	33.5135	0.3686	0.4043	0.5118
Day 3	0.039	0.198	0.084	0.114	24.3243	0.2676	0.5054	0.6480
Day 5	0.0358	0.202	0.089	0.113	24.0541	0.2646	0.5084	0.7100
Day 7	0.036	0.197	0.062	0.135	30.0000	0.3300	0.4430	0.6152
Day 9	0.0309	0.222	0.057	0.165	38.1081	0.4192	0.3538	0.5725
Control		0.307	0.023	0.284	70.2703	0.7730		

Abs: absorbance; Conc: concentration

A16: Data of ACE inhibitory activity assay of protein extracts of germinated ASL flour

Sample	Replication of ACE inhibitory activity (%)		
	I	II	III
Day 0	41.23	28.36	39.88
Day 1	79.96	70.44	84.90
Day 3	96.15	78.69	89.92
Day 5	67.82	70.44	96.13
Day 7	83.62	79.28	55.93
Day 9	89.60	87.31	75.49

A17: Raw data of protein isolate (PI) and soluble fraction (SF) yields

Sample	Repli-cation	Weight of isolate+container (g)	Container (g)	Weigt of isolate (g)	Weight of flour (g)	% yields (db)
PI day 0	1	17.172	13.27	3.902	20.08	19.43
	2	17.818	13.21	4.608	20.09	22.94
	3	17.68	13.2	4.48	20.09	22.30
PI day 3	1	17.004	13.2	3.804	20.34	18.70
	2	17.4	13.2	4.2	20.23	20.76
	3	16	13.3	2.7	20.2	13.37
PI day 5	1	16.6693	13.2	3.4693	25.23	13.75
	2	16.255	13.2	3.055	25.16	12.14
	3	18.2756	13.2	5.0756	27.7	18.32
PI day 7	1	15.0717	13.2	1.8717	28.6	6.54
	2	14.6747	13.2	1.4747	27.2	5.42
	3	14.7779	13.2	1.5779	25.8	6.12
PI day 9	1	14.409	13.28	1.129	26	4.34
	2	14.1757	13.24	0.9357	20.33	4.60
	3	14.567	13.2	1.367	26.5	5.16
SF day 0	1	14.6635	13.2	1.4635	20.08	7.29
	2	14.276	13.2	1.076	20.09	5.36
	3	14.2716	13.2	1.0716	20.09	5.33
SF day 3	1	13.9758	13.2	0.7758	20.34	3.81
	2	13.9713	13.2	0.7713	20.23	3.81
	3	13.9564	13.2	0.7564	20.2	3.74
SF day 5	1	14.0109	13.2	0.8109	25.23	3.21
	2	14.0737	13.2	0.8737	25.16	3.47
	3	13.979	13.2	0.779	27.7	2.81
SFday 7	1	13.889	13.2	0.689	28.6	2.41
	2	13.708	13.2	0.508	27.2	1.87
	3	13.74	13.2	0.54	25.8	2.09
SF day 9	1	13.5854	13.2	0.3854	26	1.48
	2	13.5657	13.2	0.3657	20.33	1.80
	3	13.457	13.2	0.257	26.5	0.97

A18: Raw data of DPPH radical scavenging activity of protein isolate and soluble fraction

Sample		Weight of sample	Absorbance	% inhibition	Conc (mg/L)	vol. of extract	mg trolox/g DM flour	mg trolox/100g DM flour
PI								
Day 0	1	0.5112	1.007	7.88	0.05	9.00	0.82	81.8881
	2	0.5078	1.011	7.51	0.04	9.00	0.78	77.8237
	3	0.5028	1.01	7.60	0.04	9.00	0.80	79.7623
Day 3	1	0.5	0.803	26.54	0.18	9.00	3.23	322.6366
	2	0.501	0.786	28.09	0.19	9.00	3.42	341.8624
	3	0.527	0.661	39.53	0.27	9.00	4.64	463.8896
Day 7	1	0.51	0.544	50.23	0.35	9.00	6.14	613.6902
	2	0.448	0.604	44.74	0.31	9.00	6.20	620.1954
	3	0.495	0.457	58.19	0.40	9.00	7.35	735.2059
Day 9	1	0.508	0.28	74.38	0.52	9.00	9.20	920.4204
	2	0.5146	0.374	65.79	0.46	9.00	8.02	801.6510
	3	0.5146	0.379	65.33	0.46	9.00	7.96	795.9614
			DPPH 1.0931					
SF								
Day 0	1	0.25	0.908	16.93	0.06	4.00	0.89	88.7405
	2	0.257	0.899	17.76	0.06	4.00	0.91	90.8804
	3	0.252	0.908	16.93	0.06	4.00	0.88	88.0362
							0.89	
Day 3	1	0.26	0.771	29.47	0.10	4.00	1.54	153.8946
	2	0.25	0.829	24.16	0.08	4.00	1.30	129.8608
	3	0.264	0.793	27.45	0.09	4.00	1.41	140.7189
Day 7	1	0.256	0.66	39.62	0.14	4.00	2.13	212.7217
	2	0.227	0.646	40.90	0.14	4.00	2.48	247.9231
	3	0.227	0.71	35.05	0.12	4.00	2.11	211.2351
Day 9	1	0.167	0.626	42.73	0.15	4.00	3.53	352.5814
	2	0.152	0.697	36.24	0.12	4.00	3.27	326.5923
	3	0.152	0.707	35.32	0.12	4.00	3.18	318.0313
			DPPH 1.0931					

A19: Data of ACE inhibitory activity assay of protein isolate (PI) and soluble fraction (SF)

Sample & replication	% Inhibition
PI	
Day 0-1	49.62
Day 0-2	54.71
Day 0-3	29.77
Day 3-1	40.16
Day 3-2	43.51
Day 3-3	38.68
Day 7-1	54.45
Day 7-2	50.38
Day 7-3	75.06
Day 9-1	60.31
Day 9-2	49.36
Day 9-3	44.53
SF	
Day 0-1	87.13
Day 0-2	83.92
Day 0-3	85.96
Day 3-1	85.96
Day 3-2	95.60
Day 3-3	97.93
Day 7-1	82.90
Day 7-2	84.97
Day 7-3	78.76
Day 9-1	98.19
Day 9-2	98.96
Day 9-3	93.52

A 20: Data of analysis of bile acid binding activity of protein isolate (PI) and soluble fraction (SF)

Sample& replication	Weight of sample	abs test	abs blank	test-blank	Conc. of bile acid (umol/L)	supernatant (11ml)	binding	umol/50 mgDM
PI								
Day 0- 1	0.051	0.22	0.05	0.17	39.46	0.434	0.533	0.523
Day 0 - 2	0.052	0.262	0.024	0.238	57.84	0.636	0.331	0.318
Day 0 - 3	0.0528	0.309	0.021	0.288	71.35	0.785	0.182	0.173
Day 3- 1	0.0507	0.253	0.076	0.177	41.35	0.455	0.512	0.505
Day 3 - 2	0.0517	0.282	0.06	0.222	53.51	0.589	0.379	0.366
Day 3 - 3	0.0517	0.289	0.093	0.196	46.49	0.511	0.456	0.441
Day 7-1	0.0529	0.249	0.063	0.186	43.78	0.482	0.486	0.459
Day 7- 2	0.0505	0.235	0.062	0.173	40.27	0.443	0.524	0.519
Day 7- 3	0.0544	0.309	0.054	0.255	62.43	0.687	0.280	0.258
Day 9-1	0.0569	0.272	0.071	0.201	47.84	0.526	0.441	0.388
Day 9-2	0.0512	0.324	0.056	0.268	65.95	0.725	0.242	0.236
Day 9-3	0.0514	0.313	0.05	0.263	64.59	0.711	0.257	0.250
SF								
Day 0-1	0.0567	0.262	0.046	0.216	51.89	0.571	0.396	0.350
Day 0-2	0.0519	0.218	0.027	0.191	45.14	0.496	0.471	0.453
Day 0-3	0.051	0.228	0.057	0.171	39.73	0.437	0.530	0.520
Day 3-1	0.0526	0.182	0.088	0.094	18.92	0.208	0.759	0.722
Day 3-2	0.0525	0.186	0.077	0.109	22.97	0.253	0.715	0.680
Day 3-3	0.0593	0.176	0.037	0.139	31.08	0.342	0.625	0.527
Day 7-1	0.0523	0.311	0.148	0.163	37.57	0.413	0.554	0.530
Day 7-2	0.0514	0.282	0.123	0.159	36.49	0.401	0.566	0.550
Day 7-3	0.052	0.241	0.16	0.081	15.41	0.169	0.798	0.767
Day 9-1	0.0513	0.362	0.234	0.128	28.11	0.309	0.658	0.641
Day 9-2	0.0541	0.491	0.415	0.076	14.05	0.155	0.813	0.751
Day 9-3	0.0541	0.255	0.205	0.05	7.03	0.077	0.890	0.822

Abs: absorbance; conc : concentration

Appendix B: Data of physical characteristics, bioactive compounds, *in vitro* bioactivities and statistical analysis of muffins incorporated with germinated ASL flour

B1-1: Raw data of moisture content of muffins

Sample	Dishes (g)	Dishes+ sample (g)	Dish+ sample after drying(g)	Moitsure content (%)
Control				
B1				
1	22.3995	25.6817	25.0442	19.42
2	19.4655	21.6452	21.2154	19.72
3	22.546	25.1517	24.634	19.87
B2				
1	21.5016	24.1666	23.5535	23.01
2	21.4788	24.7369	24.0029	22.53
3	17.1314	20.1796	19.5598	20.33
B3				
1	19.7704	22.6485	22.0036	22.41
2	19.485	22.9435	22.266	19.59
3	19.5229	24.2691	23.3357	19.67
5%				
B1				
1	23.4748	26.1194	25.4514	25.26
2	17.0593	19.5737	18.9615	24.35
3	22.5125	24.5071	24.0063	25.11
B2				
1	19.3987	21.5054	21.0087	23.58
2	20.2132	22.3525	21.8397	23.97
3	17.8864	19.7746	19.347	22.65
B3				
1	18.1116	21.2095	20.5012	22.86
2	19.2181	21.8788	21.2928	22.02
3	22.8999	25.4439	24.8166	24.66

Continuation of B1

Sample	Dishes (g)	Dishes+ sample (g)	Dish+sample after drying (g)	Moisture content (%)
10%				
B1				
1	21.1679	22.5326	22.2197	22.93
2	18.4511	20.3267	19.9029	22.60
3	22.1347	23.647	23.2776	24.43
B2				
1	17.8931	19.2785	18.975	21.91
2	20.9606	22.6855	22.2872	23.09
3	19.5122	21.2738	20.9146	20.39
B3				
1	16.6575	18.2674	17.8977	22.96
2	21.5689	23.9391	23.3441	25.10
3	20.6116	22.043	21.706	23.54
15%				
B1				
1	21.5958	22.832	22.5712	21.10
2	19.3988	21.054	20.6887	22.07
3	22.022	23.7881	23.3988	22.04
B2				
1	18.3507	19.2693	19.0693	21.77
2	21.864	24.3993	23.8578	21.36
3	18.1972	19.2799	19.041	22.07
B3				
1	18.4994	20.336	19.8756	25.07
2	14.7464	17.3948	16.7276	25.19
3	22.5668	25.1085	24.5083	23.61
20%				
B1				
1	23.9179	25.3536	25.0226	23.05
2	23.0917	25.5317	24.9479	23.93
3	17.914	19.4736	19.1086	23.40
B2				
1	22.6345	23.764	23.5133	22.20
2	21.0805	22.6282	22.2796	22.52
3	23.4252	24.9178	24.5531	24.43
B3				
1	22.1454	23.4125	23.1252	22.67
2	22.8614	24.7448	24.3238	22.35
3	23.3818	25.2843	24.8562	22.50

B= Batch of muffin preparation

B1-2: Examples of statistical analysis of moisture content of muffins

Descriptives

MC

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1.0000	9	20.727778	1.4713835	.4904612	19.596772	21.858783	19.4200	23.0100
2.0000	9	23.900783	.9273672	.3091224	23.187945	24.613620	22.6459	25.2600
3.0000	9	22.994401	1.3663775	.4554592	21.944111	24.044692	20.3906	25.1034
4.0000	9	22.697812	1.5458990	.5152997	21.509528	23.886095	21.0969	25.1926
5.0000	9	23.007432	.7685819	.2561940	22.416648	23.598216	22.1957	24.4339
Total	45	22.665641	1.5992993	.2384095	22.185158	23.146124	19.4200	25.2600

Test of Homogeneity of Variances

MC

Levene Statistic	df1	df2	Sig.
1.998	4	40	.113

ANOVA

MC

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	49.561	4	12.390	7.869	.000
Within Groups	62.980	40	1.574		
Total	112.541	44			

Post hoc tests

Multiple Comparisons

MC

Tukey HSD

(I) Sample	(J) Sample	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.0000	2.0000	-3.1730050*	.5915136	.000	-4.862422	-1.483588
	3.0000	-2.2666234*	.5915136	.004	-3.956040	-.577207
	4.0000	-1.9700338*	.5915136	.015	-3.659451	-.280617
	5.0000	-2.2796541*	.5915136	.004	-3.969071	-.590237
2.0000	1.0000	3.1730050*	.5915136	.000	1.483588	4.862422
	3.0000	.9063815	.5915136	.548	-.783035	2.595798
	4.0000	1.2029711	.5915136	.269	-.486446	2.892388
	5.0000	.8933509	.5915136	.562	-.796066	2.582768
3.0000	1.0000	2.2666234*	.5915136	.004	.577207	3.956040
	2.0000	-.9063815	.5915136	.548	-2.595798	.783035
	4.0000	.2965896	.5915136	.987	-1.392827	1.986007
	5.0000	-.0130306	.5915136	1.000	-1.702448	1.676386
4.0000	1.0000	1.9700338*	.5915136	.015	.280617	3.659451
	2.0000	-1.2029711	.5915136	.269	-2.892388	.486446
	3.0000	-.2965896	.5915136	.987	-1.986007	1.392827
	5.0000	-.3096202	.5915136	.984	-1.999037	1.379797
5.0000	1.0000	2.2796541*	.5915136	.004	.590237	3.969071
	2.0000	-.8933509	.5915136	.562	-2.582768	.796066
	3.0000	.0130306	.5915136	1.000	-1.676386	1.702448
	4.0000	.3096202	.5915136	.984	-1.379797	1.999037

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

MC

Tukey HSD[a]

Sample	N	Subset for alpha = 0.05	
		1	2
1.0000	9	20.727778	
4.0000	9		22.697812
3.0000	9		22.994401
5.0000	9		23.007432
2.0000	9		23.900783
Sig.		1.000	.269

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 9.000.

B2: Raw data of height and diameter of muffins

Samples	Control		5%		10%		15%		20%	
	Height (mm)	Dia-meter (mm)	Height (mm)	Dia-meter (mm)	Height (mm)	Dia-meter (mm)	Height (mm)	Dia-meter (mm)	Height (mm)	Dia-meter (mm)
B1										
1	67	71	62	70	60	70	55	75	56	63
	66	71	63	70	61	73	56	74	56	63
	66	71	63	71	62	71	56	74	56	63
2	66	72	63	73	58	76	60	72	55	70
	67	71	64	71	60	70	60	74	55	68
	65	71	64	71	60	70	60	74	55	68
3	63	66	63	71	60	73	60	75	58	66
	63	67	64	72	61	72	59	74	57	66
	62	67	63	72	60	71	59	74	58	66
B2										
1	64	71	65	73	61	72	55	72	58	74
	64	72	64	67	62	73	56	73	58	70
	65	71	64	69	62	73	56	73	58	70
2	64	77	61	74	59	72	57	74	58	70
	63	71	62	71	60	75	59	73	58	72
	63	72	62	71	60	75	59	73	58	72
3	63	71	64	72	62	72	59	74	58	71
	64	72	65	72	60	73	58	69	60	73
	64	72	64	71	60	73	58	69	60	73
B3										
1	67	69	65	71	62	74	55	76	62	72
	67	70	64	66	63	75	57	74	60	70
	67	71	64	67	63	75	57	74	60	70
2	64	68	60	65	61	70	60	69	58	70
	64	69	60	71	62	71	62	70	60	70
	64	69	61	70	62	71	62	70	60	70
3	63	67	61	72	60	66	58	72	58	73
	63	67	62	68	61	66	59	70	60	70
	63	67	62	70	61	66	75	70	60	70

B= Batch of muffin preparation

B3: Raw data of colour of muffins

Sample	Control			5%			10%			15%			20%		
	L*	***b****	***a****	***L****	***b****	***a****	***L****	***b****	***a****	***L****	***b****	***a****	***L****	***b****	***a****
B1															
1	63.28	24.78	0	70.81	31.85	-1.13	65.02	34.09	0.4	64.61	41.56	1.03	64.38	41.05	1.67
2	72.58	25.59	-1.18	71.94	32.17	-1.09	68.35	36.56	0.28	67.72	42.11	0.72	68.06	42.19	1.67
3	71.94	23.89	-1.29	72.21	31.52	-1.06	63.82	34.89	0.45	64.5	41.83	0.98	62.54	40.7	1.99
B2															
1	75.06	23.11	-1.53	69.75	32.78	-0.76	68.98	35.94	0.14	63.62	40.29	1.29	64.6	42.91	2.15
2	74.29	25.43	-0.88	68.10	31.85	-0.81	67.27	35.1	0.2	63.32	37.49	0.4	62.56	43.14	2.32
3	66.62	24.46	-0.22	70.43	33.31	-0.55	68.72	34.61	0.26	67.27	40.18	0.97	63.48	43.35	1.97
B3															
1	72.95	25.28	-1.23	69.42	30.28	-0.82	67.47	35.81	-0.19	67.36	42.85	0.67	64.37	41.28	2.37
2	72.86	25.92	-0.91	67.35	31.1	0.58	67.28	37.84	0.55	67.77	42.59	0.82	64.08	45.04	4.58
3	73.27	26.55	-0.74	72.89	31.35	-0.3	70.45	37.65	0.3	69.84	42.44	0.23	62.46	43.56	3.37

B= Batch of muffin preparation

B4: Raw data of texture analysis of muffins

Samples	Force 1 (g)	Force 2 (g)	Force 3 (g)	Area-FT 1:2 (g.s)	Time-diff. 1:2 (s)	Area-FT 1:3 (g.s)	Area-FT 2:3 (g.s)	Area-FT 3:4 (g.s)	Time-diff. 4:5 (s)	Area-FT 4:6 (g.s)
Control										
B1-1	649.03	806.54	806.54	1264.38	2.9	1556.4	296.01	0.32	2.69	807.22
B1-2	940.38	1128.32	1128.32	1344.67	2.41	1699.27	360.2	0.46	2.26	985.28
B1-3	805.13	965.29	965.29	1009.88	2.18	1274.45	269.34	0.35	2.05	746.05
B2-1	856.81	1056.22	1056.22	1416.24	2.84	1765.84	354.84	0.68	2.56	965.11
B2-2	841.7	1025.54	1025.54	1527.61	3	1890.36	367.81	0.21	2.75	1033.29
B2-3	812.58	997.42	997.42	1467.96	3.05	1816.91	353.89	0.53	2.77	989.81
B3-1	792.02	984.75	984.75	1571.05	3.05	1922.92	356.73	0.34	2.77	984.78
B3-2	861.6	1052.25	1052.25	1605.14	3.05	1975.33	375.4	0.3	2.76	1053.56
B3-3	667.92	818.41	818.41	1248.72	3.1	1538.58	293.92	0.53	2.79	813.79
5%										
B1-1	757.24	887.06	887.06	1327.55	3.16	1709.25	386.11	0.13	2.93	1039.56
B1-2	583.22	689.56	689.56	934.89	2.94	1207.24	275.77	0.45	2.74	736.26
B1-3	595.15	694.09	694.09	916.19	3.05	1194.72	281.98	0.34	2.85	765.8
B2-1	684.33	788	788	1061.26	3.03	1397.16	339.81	0.48	2.82	914.99
B2-2	473.97	557.76	557.76	792.82	3.01	1023.19	233.14	-0.04	2.83	634.22
B2-3	476.93	560.08	560.08	706.9	3.01	927.1	222.99	0.5	2.81	591.08
B3-1	633.09	740.33	740.33	1002.78	2.93	1301.8	302.69	0.58	2.71	819.49
B3-2	560.78	650.26	650.26	796.54	2.87	1047.94	254.63	0.68	2.66	679.04
B3-3	771.96	912.11	912.11	1403.15	3.23	1812.97	414.35	0.49	3.06	1091.98

Continuation of B4

Samples	Force 1 (g)	Force 2 (g)	Force 3 (g)	Area-FT 1:2 (g.s)	Time-diff. 1:2 (s)	Area-FT 1:3 (g.s)	Area-FT 2:3 (g.s)	Area-FT 3:4 (g.s)	Time-diff. 4:5 (s)	Area-FT 4:6 (g.s)
10%										
B1-1	602.98	705.3	705.3	854.01	2.68	1111.32	260.81	0.24	2.46	701.61
B1-2	585.28	689.27	689.27	783.53	2.53	1028.08	247.97	0.36	2.34	655.67
B1-3	666.31	791.08	791.08	1120.86	3.0	1444.54	327.6	0.25	2.76	877.21
B2-1	563.48	666.38	666.38	927.67	3.08	1199.36	275	0.31	2.83	726.59
B2-2	585.02	689.93	689.93	892.58	3.0	1165.85	276.7	0.42	2.79	734.19
B2-3	484.07	573.65	573.65	763.91	3.08	991.34	230.28	0.45	2.86	614.43
B3-1	614.36	733.69	733.69	1121.01	3.23	1435.42	318.04	0.32	3.04	858.26
B3-2	463.1	535.98	535.98	711.64	3.22	934.82	225.84	0.06	3.0	614.94
B3-3	754.28	886.11	886.11	1254.64	3.13	1637.43	387.19	0.44	2.87	1018.52
15%										
B1-1	494.88	582.7	582.7	698.18	2.78	915.56	220.27	0.41	2.65	581.64
B1-2	682.64	814.37	814.37	1179.08	3.23	1514.8	339.76	-5.02	3.14	899.75
B1-3	744.72	885.6	885.6	1252.31	3.11	1614.32	366.41	0.46	2.84	973
B2-1	706.86	827.1	827.1	996.55	2.95	1312.87	320.43	0.37	2.7	848.26
B2-2	609.59	708.85	708.85	981.06	3.15	1284.76	307.22	0.41	2.91	822.2
B2-3	672.63	783.92	783.92	1092.55	3.23	1423.05	334.4	0.01	3.04	896.24
B3-1	623.63	722.43	722.43	919.9	3.1	1212.42	296.11	0.43	2.8	791.96
B3-2	821.54	952.85	952.85	1147.86	2.8	1522.99	379.87	0.52	2.58	1003.37
B3-3	771.88	890.59	890.59	997.82	2.73	1331.73	338.34	0.25	2.52	889.1

Continuation of B4

Sample	Force 1 (g)	Force 2 (g)	Force 3 (g)	Area-FT 1:2 (g.s)	Time-diff. 1:2 (s)	Area-FT 1:3 (g.s)	Area-FT 2:3 (g.s)	Area-FT 3:4 (g.s)	Time-diff. 4:5 (s)	Area-FT 4:6 (g.s)
20%										
B1-1	1030.11	1238.25	1238.25	1244.63	2.67	1611.18	372.69	-0.13	2.27	986.11
B1-2	1106.4	1349.6	1349.6	1334.35	2.54	1707.18	379.54	0.29	2.14	998.21
B1-3	1051.45	1293.16	1293.16	1451.62	2.82	1839.31	394.11	-0.23	2.25	1053.06
B2-1	503.62	622.23	622.23	800.86	2.86	1022.82	225.05	0.55	2.56	584.36
B2-2	858.13	1018	1018	1056.79	2.55	1380.61	328.86	0.32	2.25	865.28
B2-3	658.46	771.72	771.72	824.7	2.79	1092.27	271.4	0.53	2.48	704.99
B3-1	711.65	845.6	845.6	975.62	2.85	1275.99	304.57	0.33	2.3	795.37
B3-2	622.81	740.51	740.51	901.6	3.17	1166.14	268.21	0.56	2.66	719.61
B3-3	754.63	889.34	889.34	1084.91	2.99	1422.8	342.31	0.6	2.61	906.72

B= Batch of muffin preparation
Hardness= Peak force during the first compression cycle (force 2 in the table)
Cohesiveness= Area-FT 4:6/Area-FT 2:3
Springiness= time-diff 4:5/ time-diff 1:2
Chewiness=hardness x cohesiveness x springiness

B5:Raw data of total phenolic compounds (TPC) analysis of batter

Sample	Replication	Weight of sample	MC	weight corrected with MC (g)	Absorbance	Conc (mg/L)	vol.of extract (ml)	mgGAE/gDM batter	mgGAE/100g DM batter
BATTER									
0%	1	2.10	8.75	1.92	0.22	19.83	8.70	0.45	44.99
					0.24	21.38	8.70	0.49	48.52
					0.22	19.83	8.70	0.45	44.99
	2	2.12	7.04	1.97	0.22	19.83	8.50	0.44	43.96
					0.21	18.66	8.50	0.41	41.37
					0.23	20.89	8.50	0.46	46.33
	3	2.04	7.41	1.89	0.20	18.27	8.70	0.41	41.47
					0.23	20.89	8.70	0.47	47.42
					0.22	20.12	8.70	0.46	45.65
5%	1	2.04	10.38	1.83	0.31	28.37	8.20	0.61	60.68
					0.33	30.80	8.20	0.66	65.87
					0.32	30.02	8.20	0.64	64.21
	2	2.04	10.19	1.83	0.33	30.41	8.00	0.63	63.46
					0.31	28.56	8.00	0.60	59.61
					0.30	27.30	8.00	0.57	56.97
	3	2.09	9.93	1.89	0.32	29.15	8.50	0.65	64.62
					0.31	28.37	8.50	0.63	62.90
					0.32	30.02	8.50	0.67	66.56

GAE=Gallic acid equivalent; Conc= concentration

Continuation of B5

Sample	Replication	Weight of sample	MC	Weight with corrected MC (g)	Absorbance	Conc (mg/L)	Vol. of extract (ml)	mg GAE/gDM batter	mg GAE/100g DM batter
10%	1	2.00	10.04	1.80	0.34	31.67	8.30	0.69	68.57
					0.36	33.42	8.30	0.72	72.35
					0.34	31.48	8.30	0.68	68.15
	2	2.12	8.71	1.94	0.40	37.20	8.40	0.82	81.52
					0.36	33.22	8.40	0.73	72.80
					0.39	35.94	8.40	0.79	78.75
	3	2.02	8.29	1.85	0.37	34.49	8.30	0.75	74.66
					0.36	33.90	8.30	0.73	73.40
					0.38	35.36	8.30	0.77	76.56
15%	1	2.10	8.05	1.93	0.42	39.73	8.40	0.87	87.05
					0.43	40.41	8.40	0.89	88.54
					0.42	39.34	8.40	0.86	86.20
	2	2.05	8.31	1.88	0.43	40.12	8.30	0.87	86.86
					0.45	42.35	8.30	0.92	91.69
					0.44	41.28	8.30	0.89	89.38
	3	2.01	7.40	1.86	0.41	38.17	8.30	0.83	82.65
					0.40	37.01	8.30	0.80	80.13
					0.40	37.40	8.30	0.81	80.97
20%	1	2.00	8.52	1.83	0.46	42.93	8.40	0.94	94.07
					0.46	42.83	8.40	0.94	93.86
					0.47	44.00	8.40	0.96	96.41
	2	2.02	8.53	1.85	0.47	44.39	8.40	0.97	97.26
					0.47	44.39	8.40	0.97	97.26
					0.46	43.32	8.40	0.95	94.92
	3	2.09	6.81	1.95	0.48	45.46	8.40	1.00	99.60
					0.49	45.94	8.40	1.01	100.67
					0.48	45.36	8.40	0.99	99.39

GAE=Gallic acid equivalent; Conc=concentration

B6: Raw data of total phenolic compounds (TPC) analysis of muffins

Sample	Replication	Weight of sample	MC	Weight corrected with MC	Absorbance	Conc (mg/L)	Vol. of extract (ml)	mg GAE/gDM	mg GAE/100g sample
MUFFIN									
0%	1	2.07	4.82	1.97	0.15	13.03	8.30	0.27	27.43
					0.19	16.72	8.30	0.35	35.20
					0.16	14.10	8.30	0.30	29.68
	2	2.02	5.04	1.91	0.17	14.87	8.30	0.31	31.32
					0.18	15.75	8.30	0.33	33.16
					0.17	15.36	8.30	0.32	32.34
	3	2.05	5.06	1.95	0.18	16.33	8.30	0.34	34.38
					0.19	16.72	8.30	0.35	35.20
					0.18	16.33	8.30	0.34	34.38
5%	1	2.07	4.25	1.98	0.28	26.14	8.30	0.55	55.03
					0.31	28.27	8.30	0.60	59.53
					0.31	28.17	8.30	0.59	59.32
	2	2.04	4.29	1.96	0.31	28.85	8.30	0.61	60.75
					0.32	29.63	8.30	0.62	62.39
					0.32	29.63	8.30	0.62	62.39
	3	2.08	3.82	2.00	0.32	29.24	8.30	0.62	61.57
					0.34	31.38	8.30	0.66	66.07
					0.32	29.83	8.30	0.63	62.80

GAE=Gallic acid equivalent; Conc=concentration

Continuation of B6

Sample	Replication	Weight of sample	MC	Weight with corrected MC	Absorbance	Conc (mg/L)	Vol. of extract	mg GAE/gDM	mg GAE/100g sample
10%	1	2.08	4.69	1.98	0.40	37.11	8.30	0.78	78.13
					0.41	37.88	8.30	0.80	79.77
					0.40	37.40	8.30	0.79	78.74
	2	2.14	4.60	2.04	0.42	39.05	8.30	0.82	82.22
					0.44	40.80	8.30	0.86	85.90
					0.41	38.37	8.30	0.81	80.79
	3	2.07	5.20	1.96	0.42	39.34	8.30	0.83	82.83
					0.42	39.05	8.30	0.82	82.22
					0.42	39.05	8.30	0.82	82.22
15%	1	2.07	4.40	1.98	0.44	41.18	8.30	0.87	86.72
					0.44	40.89	8.30	0.86	86.10
					0.43	40.60	8.30	0.85	85.49
	2	2.04	3.57	1.97	0.50	47.30	8.30	1.00	99.59
					0.45	42.45	8.30	0.89	89.37
					0.45	42.25	8.30	0.89	88.96
	3	2.20	5.18	2.08	0.47	44.19	8.30	0.93	93.05
					0.49	45.65	8.30	0.96	96.12
					0.46	43.22	8.30	0.91	91.01
20%	1	2.05	4.99	1.95	0.54	50.60	8.30	1.07	106.54
					0.54	51.18	8.30	1.08	107.77
					0.54	50.60	8.30	1.07	106.54
	2	2.02	4.74	1.92	0.45	42.64	8.30	0.90	89.78
					0.43	40.21	8.30	0.85	84.67
					0.45	42.64	8.30	0.90	89.78
	3	2.05	5.17	1.94	0.45	42.35	8.30	0.89	89.17
					0.44	40.80	8.30	0.86	85.90
					0.45	42.64	8.30	0.90	89.78

GAE=Gallic acid equivalent; Conc=concentration

B7: Raw data of DPPH radical scavenging activity of batter

Sample	Replication	Weight of sample	Abs 30'	% Inhibition	Conc (mg/ml)	Volume of extract (ml)	Weight of sample (corrected with MC)	mg eq trolox/g DM batter
BATTER								
0%	1	2.1006	0.78	1.64	0.0011	8.700	1.9168	0.0049
			0.77	2.90	0.0056	8.700	1.9168	0.0252
			0.765	3.53	0.0078	8.700	1.9168	0.0354
	2	2.1218	0.763	3.78	0.0087	8.500	1.9724	0.0375
			0.764	3.66	0.0083	8.500	1.9724	0.0356
			0.765	3.53	0.0078	8.500	1.9724	0.0336
	3	2.0405	0.763	3.78	0.0087	8.700	1.8893	0.0401
			0.765	3.53	0.0078	8.700	1.8893	0.0359
			0.76	4.16	0.0100	8.700	1.8893	0.0463
5%	1	2.0406	0.738	6.94	0.0199	8.200	1.8288	0.0893
			0.738	6.94	0.0199	8.200	1.8288	0.0893
			0.737	7.06	0.0204	8.200	1.8288	0.0913
	2	2.0392	0.735	7.31	0.0213	8.000	1.8314	0.0928
			0.737	7.06	0.0204	8.000	1.8314	0.0889
			0.738	6.94	0.0199	8.000	1.8314	0.0870
	3	2.0944	0.736	7.19	0.0208	8.500	1.8864	0.0938
			0.733	7.57	0.0222	8.500	1.8864	0.0998
			0.74	6.68	0.0190	8.500	1.8864	0.0857

Abs= absorbance; Conc=concentration

Continuation of B7

Sample	Replication	Weight of sample	Abs 30'	% Inhibition	Conc (mg/ml)	Volume of extract (ml)	Weight of sample (corrected with MC)	mg eq trolox/g DM batter
10%	1	2.0041	0.726	8.45	0.0253	8.300	1.8029	0.1164
			0.722	8.95	0.0271	8.300	1.8029	0.1247
			0.71	10.47	0.0325	8.300	1.8029	0.1495
	2	2.1202	0.704	11.22	0.0352	8.400	1.9355	0.1526
			0.704	11.22	0.0352	8.400	1.9355	0.1526
			0.707	10.84	0.0338	8.400	1.9355	0.1467
	3	2.0197	0.702	11.48	0.0361	8.300	1.8523	0.1616
			0.7	11.73	0.0370	8.300	1.8523	0.1656
			0.7	11.73	0.0370	8.300	1.8523	0.1656
15%	1	2.0992	0.661	16.65	0.0544	8.400	1.9302	0.2369
			0.657	17.15	0.0562	8.400	1.9302	0.2447
			0.656	17.28	0.0567	8.400	1.9302	0.2467
	2	2.047	0.657	17.15	0.0562	8.300	1.8769	0.2487
			0.661	16.65	0.0544	8.300	1.8769	0.2407
			0.661	16.65	0.0544	8.300	1.8769	0.2407
	3	2.01	0.667	15.89	0.0517	8.300	1.8613	0.2308
			0.661	16.65	0.0544	8.300	1.8613	0.2428
			0.663	16.39	0.0535	8.300	1.8613	0.2388
20%	1	2.0038	0.654	17.53	0.0576	8.400	1.8331	0.2638
			0.652	17.78	0.0585	8.400	1.8331	0.2680
			0.653	17.65	0.0580	8.400	1.8331	0.2659
	2	2.0234	0.653	17.65	0.0580	8.400	1.8508	0.2634
			0.654	17.53	0.0576	8.400	1.8508	0.2613
			0.652	17.78	0.0585	8.400	1.8508	0.2654
	3	2.0874	0.661	16.65	0.0544	8.400	1.9452	0.2351
			0.659	16.90	0.0553	8.400	1.9452	0.2390
			0.658	17.02	0.0558	8.400	1.9452	0.2409

Abs= absorbance; Conc=concentration

B8: Raw data of DPPH radical scavenging activity of muffin

Sample	Replication	Weight of sample	Abs 30'	% Inhibition	Conc (mg/ml)	Vol. of extract(ml)	Weight of sample (corrected with MC)	mg eq trolox/g DM muffin
Muffin								
0%	1	2.0708	0.715	10.96	0.0342	8.3	1.9710	0.1441
			0.71	11.58	0.0364	8.3	1.9710	0.1534
			0.71	11.58	0.0364	8.3	1.9710	0.1534
	2	2.015	0.719	10.46	0.0324	8.3	1.9134	0.1407
			0.724	9.84	0.0302	8.3	1.9134	0.1311
			0.724	9.84	0.0302	8.3	1.9134	0.1311
	3	2.0502	0.712	11.33	0.0355	8.3	1.9465	0.1516
			0.704	12.33	0.0391	8.3	1.9465	0.1667
			0.706	12.08	0.0382	8.3	1.9465	0.1629
5%	1	2.0708	0.662	17.56	0.0577	8.3	1.9828	0.2415
			0.655	18.43	0.0608	8.3	1.9828	0.2545
			0.653	18.68	0.0617	8.3	1.9828	0.2582
	2	2.0448	0.648	19.30	0.0639	8.3	1.9571	0.2709
			0.646	19.55	0.0648	8.3	1.9571	0.2747
			0.646	19.55	0.0648	8.3	1.9571	0.2747
	3	2.0755	0.648	19.30	0.0639	8.3	1.9962	0.2656
			0.644	19.80	0.0657	8.3	1.9962	0.2730
			0.646	19.55	0.0648	8.3	1.9962	0.2693

Abs= absorbance; Conc=concentration

Continuation of B8

Sample	Replication	Weight of sample	Abs 30'	% Inhibition	Conc (mg/ml)	Vol extract (ml)	Weight of sample (corrected with MC)	mg eq trolox/g DM muffin
10%	1	2.0774	0.621	22.67	0.0758	8.3	1.9800	0.3179
			0.607	24.41	0.0820	8.3	1.9800	0.3439
			0.614	23.54	0.0789	8.3	1.9800	0.3309
	2	2.1371	0.616	23.29	0.0781	8.3	2.0388	0.3178
			0.613	23.66	0.0794	8.3	2.0388	0.3232
			0.607	24.41	0.0820	8.3	2.0388	0.3340
	3	2.0661	0.621	22.67	0.0758	8.3	1.9587	0.3214
			0.616	23.29	0.0781	8.3	1.9587	0.3308
			0.615	23.41	0.0785	8.3	1.9587	0.3327
15%	1	2.072	0.637	20.67	0.0688	8.3	1.9808	0.2881
			0.635	20.92	0.0696	8.3	1.9808	0.2918
			0.631	21.42	0.0714	8.3	1.9808	0.2992
	2	2.0383	0.605	24.66	0.0829	8.3	1.9655	0.3502
			0.605	24.66	0.0829	8.3	1.9655	0.3502
			0.601	25.16	0.0847	8.3	1.9655	0.3577
	3	2.1983	0.612	23.79	0.0798	8.3	2.0844	0.3179
			0.613	23.66	0.0794	8.3	2.0844	0.3161
			0.608	24.28	0.0816	8.3	2.0844	0.3249
20%	1	2.0506	0.594	26.03	0.0878	8.3	1.9483	0.3740
			0.598	25.53	0.0860	8.3	1.9483	0.3665
			0.597	25.65	0.0865	8.3	1.9483	0.3684
	2	2.019	0.617	23.16	0.0776	8.3	1.9233	0.3350
			0.636	20.80	0.0692	8.3	1.9233	0.2986
			0.63	21.54	0.0719	8.3	1.9233	0.3101
	3	2.0481	0.637	20.67	0.0688	8.3	1.9422	0.2938
			0.622	22.54	0.0754	8.3	1.9422	0.3222
			0.611	23.91	0.0803	8.3	1.9422	0.3430

Abs= absorbance; Conc=concentration

B9: Raw data of phytosterol analysis of batter

Sample (Batter)		Oil sample (g)	Area of peaks				Conctration (mg/ml)				Reco-very	Actual concentration (mg/ml)			mg/g oils		
			campe	stigma	β-sito	IS(epico)	campe	stigma	β-sito	IS		campe	stigma	β-sito	campe	stigma	β-sito
Control	1	0.2896	677465	373910	3962465	4370902	0.055	0.016	0.267	0.310	77.51	0.071	0.020	0.344	0.244	0.070	1.18
	2	0.211	577879	234753	2977840	4835132	0.048	0.007	0.203	0.340	85.06	0.057	0.008	0.238	0.269	0.037	1.13
	3	0.3063	784300	458176	4641980	3843834	0.062	0.021	0.311	0.276	68.95	0.089	0.031	0.451	0.292	0.100	1.47
5%	1	0.2984	994222	559427	4592404	4826778	0.075	0.028	0.308	0.340	84.92	0.089	0.033	0.362	0.297	0.109	1.21
	2	0.2966	1168150	790554	6508728	6119984	0.087	0.043	0.432	0.424	105.94	0.082	0.040	0.408	0.276	0.136	1.37
	3	0.239	1053968	520493	4856417	6185068	0.079	0.025	0.325	0.428	106.99	0.074	0.023	0.304	0.310	0.098	1.27
10%	1	0.3894	1085708	600248	5601617	3622817	0.081	0.030	0.373	0.261	65.36	0.124	0.046	0.571	0.319	0.119	1.46
	2	0.2996	1187564	607635	4771053	4781565	0.088	0.031	0.319	0.337	84.19	0.104	0.037	0.379	0.348	0.122	1.26
	3	0.266	986462	612842	4585465	4785402	0.075	0.031	0.307	0.337	84.25	0.089	0.037	0.365	0.334	0.139	1.37
15%	1	0.3026	1077411	858169	5486934	5400025	0.081	0.047	0.366	0.377	94.24	0.086	0.050	0.388	0.283	0.165	1.28
	2	0.3796	1261341	773978	5939188	5939188	0.093	0.042	0.395	0.412	103.00	0.090	0.040	0.384	0.237	0.106	1.01
	3	0.3742	1688982	1078624	7933082	4807817	0.120	0.061	0.525	0.338	84.61	0.142	0.073	0.620	0.381	0.194	1.65
20%	1	0.3674	1938504	1330793	9097689	5572244	0.137	0.078	0.601	0.388	97.03	0.141	0.080	0.619	0.383	0.218	1.68
	2	0.3639	2050232	1150540	9481286	5244098	0.144	0.066	0.626	0.367	91.70	0.157	0.072	0.682	0.431	0.198	1.87
	3	0.3417	1838927	1177448	8623015	4957581	0.130	0.068	0.570	0.348	87.05	0.150	0.078	0.655	0.438	0.228	1.91

IS=Internal standard

B10: Raw data of phytosterol analysis of muffin

Sample (muffins)	Oil sample (g)	Area of peaks				Concentration (mg/ml)				Reco-very	Actual concentration (mg/ml)			mg/g oils		
		campe	stigma	β-sito	IS	campe	stigma	β-sito	IS		campe	stigma	β-sito	campe	stigma	β-sito
Control 1	0.5207	2363729	1377334	12055627	4494782	0.164	0.081	0.793	0.318	79.53	0.207	0.102	0.997	0.397	0.195	1.91
2	0.3748	1135621	613183	5960029	3726605	0.085	0.031	0.397	0.268	67.04	0.126	0.046	0.592	0.336	0.124	1.58
3	0.4456	1063007	699130	5668194	2955194	0.080	0.037	0.378	0.218	54.51	0.146	0.067	0.693	0.329	0.151	1.56
5%																
1	0.2623	1106064	698103	5808900	4663564	0.083	0.037	0.387	0.329	82.27	0.100	0.045	0.470	0.383	0.170	1.79
2	0.3845	958500	105524	4912670	4246293	0.073	-0.002	0.329	0.302	75.49	0.097	-0.002	0.435	0.252	-0.006	1.13
3	0.3192	712332	393404	6328676	3820812	0.057	0.017	0.421	0.274	68.57	0.083	0.025	0.613	0.260	0.077	1.92
10%																
1	0.2539	1149940	764712	6734872	4172784	0.085	0.041	0.447	0.297	74.29	0.115	0.055	0.602	0.453	0.217	2.37
2	0.2486	1055861	685157	5429766	4629941	0.079	0.036	0.362	0.327	81.72	0.097	0.044	0.443	0.390	0.176	1.78
3	0.3092	980428	617816	7469760	5046400	0.074	0.031	0.495	0.354	88.49	0.084	0.036	0.559	0.272	0.115	1.81
15%																
1	0.406	2756536	1761817	13580655	6301629	0.190	0.106	0.892	0.436	108.89	0.174	0.097	0.819	0.429	0.239	2.02
2	0.3698	1940550	1174190	8930973	4446958	0.137	0.068	0.590	0.315	78.75	0.174	0.086	0.749	0.470	0.232	2.03
3	0.4481	3171229	1929054	14958295	5730523	0.217	0.117	0.982	0.398	99.61	0.218	0.117	0.985	0.486	0.261	2.20
20%																
1	0.3424	1884359	1224127	8885610	4548199	0.133	0.071	0.587	0.322	80.39	0.166	0.088	0.730	0.484	0.257	2.13
2	0.3433	1805104	1100112	8848660	4287300	0.128	0.063	0.584	0.305	76.15	0.168	0.082	0.767	0.490	0.240	2.24
3	0.3515	1593858	828240	7445378	3385714	0.114	0.045	0.493	0.246	61.50	0.186	0.073	0.802	0.529	0.209	2.28

IS=Internal standard

B11: Examples of standard curve of β-sitosterol and stigmasterol