ANTIOXIDANT CAPACITY AND PHENOLIC PROFILES OF LENTILS AS AFFECTED BY PROCESSING

by

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Abstract

Lentils are excellent sources of dietary fibre, carbohydrates, proteins, various vitamins, minerals, and bioactive compounds such as phenolic compounds. Epidemiological studies have reported their effects in lowering cholesterol and reducing the incidence of colon cancer, cardiovascular diseases, and type-2-diabetes. In the present study, effects of germination, hydrothermal (boiling) treatment, and dehulling on the antioxidant capacity of soluble- and insoluble-bound phenolics of lentils were examined. Upon germination, an increasing trend in the antioxidant capacity in the insoluble-bound phenolics (IBPs) and a declining trend in that of the soluble phenolics (except total phenolic contents) were observed during 4 days of germination. Based on the results, a new indicator, the ratio of insoluble-bound phenolics to soluble phenolics (SPs), was suggested as an effective means to monitor changes in the antioxidant activity of lentils during germination. The hydrothermal/boiling process also led to important findings. As expected, the hydrothermal process decreased the content of insoluble-bound phenolics in the lentil cultivars tested due to the release of bound phenolics from cell wall matrices. Interestingly, the decrease of bound phenolics was not proportionally reflected in the increase of soluble phenolics, possibly due to the loss of phenolics during the hydrothermal process via interaction with proteins and other seed components. Among lentil cultivars used, the hulls of 3494-6 showed the most effective antioxidant potential, while Maxim displayed the lowest in most measurements. Meanwhile, the dehulling process revealed the predominant distribution of phenolics in the hulls as compared to the dehulled grains, as observed for both soluble- and insoluble-bound phenolics of lentils in their antioxidant potential and inhibitory activities against oxidation of LDL cholesterol and DNA strand breakage. In the HPLC-ESI- MS^n analysis, different classes of soluble phenolics such as phenolic acids, flavonoids,

and proanthocyanidins were found in the hulls of all four tested lentil cultivars examined. The insoluble-bound phenolics has often been ignored in many evaluations by different research groups and hence this work has expanded the depth of knowledge in the field of food phenolics.

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LIST OF ABBREVIATIONS

DPPH	2,2-Diphenyl-1-picrylhydrazyl
ORAC	Oxygen radical absorption capacity
IBPs	Insoluble-bound phenolics
SPs	Soluble phenolics
LDL	Low-density lipoprotein
PAL	Phenylalanine ammonia lyase
CHS	Chalcone synthase
ABC	ATP-binding cassette
MATE	Multidrug and toxic compound extrusion
CFU	Colony forming units
HCV	Hepatitis C virus
HCC	Hepatocellular carcinoma
FAE	Ferulic acid esterases
DW	Dried weight
ABTS	2,2′-azino-bis (3- ethylbenzothiazoline-6-sulphonic acid)
AAPH	2,2′-azobis (2-methylpropionamidine) dihydrochloride

DMPO	5,5-dimethyl-l-pyrroline-N-oxide
TCA	Trichloroacetic acid
EDTA	Ethylenediaminetetraacetic acid
TPC	Total phenolic content
TFC	Total flavonoid content
AAE	Ascorbic acid equivalents
CE	Catechin equivalents
GAE	Gallic acid equivalents
TE	Trolox equivalents
EPR	Electron paramagnetic resonance
PNPG	*p*-Nitrophenyl-α-D-glucopyranoside
MSD	Mass selective detector
ESI	Electrospray ionization
ROS	Reactive oxygen species
DRSC	DPPH radical scavenging capacity

Chapter 1

Introduction and overview

Lentil is a convex lens-shaped bean on both sides. The long-flat lentil shell contains two lentil seeds inside. Lentil (*Lens culinaris* Medik) is an annual plant originating in the southern European and the Mediterranean coasts. They are sown in early May and can grow up to about 40 cm after about 110 days. Since they grow well in dry and harsh environments, it is easier to cultivate and store compared with other grains, so they are widely used as a staple food globally.

In India, lentil is called "Dal" and they are used as a good source of raw material for a variety of foods. For example, lentils are used in India to bake a bread called “Idli”. In addition, lentils are cooked in a “Khichdi” rice, “Dal makhni”, a vegetable stew called “Sambar”, and curry which is made with beans and herbs. Lentils are also enjoyed in many countries in South America, Middle East, and Europe. For example, lentils are used as the primary ingredient of pasta in Egypt as well as cooked in a stew and salad in the South American and European countries such as France, Italy, and Germany.

Lentils are rich in proteins and dietary fibres as well as other nutrients and antioxidant compounds, which lead to their beneficial health effects in preventing aging, strengthening immunity, and lowering cholesterol levels and hence cardiovascular diseases.

Among legumes, the production volume of lentils is the second largest after soybean. Table 1.1 shows the production of lentils in 2014. According to the report by the UN Food & Agriculture

Organization (FAO), Statistics Division, the global lentil production in 2014 was 4,885,271 metric tonnes. Canada ranked first by producing 1,987,000 metric tonnes a year, followed by India, Australia, Turkey and Nepal with 1,100,000, 348,080, 345,000 and 226,830 metric tonnes, respectively.

The nutritional value of raw lentils, including their compositions and contents is summarized in Table 1.2 based on the information that is provided by the United States Department of Agriculture (USDA) Database entry. Lentils contain carbohydrates (69%), lipids (1%) and proteins (25%) as their main energy source. They possess a relatively high content of proteins compared to the other legumes with a low lipid content.

In addition, lentils contain a variety of vitamin B such as thiamine (B1), riboflavin (B2), niacin (B3), pantothenic acid (B5), vitamin B6, and folate (B9) with contents of 0.87, 0.21, 2.60, 2.14, 0.54, and 0.49 mg/100 g, respectively, as well as 4.50 mg of vitamin C. Aside from the aforementioned vitamins, they are also rich in minerals such as calcium, iron, magnesium, phosphorus, potassium, sodium, and zinc. In short, the high protein and low lipid contents make lentils as a valuable food source for preventing diseases and conditions such as obesity.

In addition to their macro- and micronutrients, lentils contain a high level of phenolics including phenolics acids, flavonoids and proanthocyanidins, which are responsible for the efficient bioactivities such as anticancer and anti-hepatitis C virus (HCV), as well as reducing diabetes and cardiovascular disease (Hsu et al., 2015; Morgan et al., 2013; Narasimhan et al., 2015; Eitsuka et al., 2014; Mancuso et al., 2014).

Phenolic compounds, depending on their solubility in the extraction medium, can be mainly classified into two groups, namely soluble- and insoluble-bound phenolics. Most of the soluble

phenolics are localized in the vacuole of plant cells, in which they are trapped by weak interaction with other compounds (Li et al., 2012), whereas insoluble-bound phenolics are present in the cell wall matrices by forming covalent bonds with pectins, cellulose, hemicellulose and structural proteins.

Until now, a number of studies on the antioxidant potential and bioactivities of soluble phenolics in lentil cultivars have been conducted (Amarowicz et al., 2009; Alshikh et al., 2015). However, insoluble-bound phenolics have not been sufficiently investigated, especially with respect to changes in insoluble-bound phenolics during food processing such as germination, hydrothermal (boiling) processing and dehulling. Thus, the main objective of this study was to focus on the changes of insoluble-bound phenolics in lentils during processing.

Table 1.1 Lentil production (metric tonnes) in different countries of the world in 2014

Country	Production (metric tonnes)
Canada	1,987,000
India	1,100,000
Australia	348,080
Turkey	345,000
Nepal	226,830
World	**4,885,271**

From: http://www.fao.org/pulses-2014

Table 1.2 The contents of carbohydrate, fat, protein, vitamins and minerals in lentils

Nutrient	Content
Carbohydrates	69.0 g
Sucrose	2.0 g
Dietary fibre	10.7 g
Fat	1.0 g
Protein	25.0 g
Vitamins	
Thiamine (B1)	0.87 mg
Riboflavin (B2)	0.21 mg
Niacin (B3)	2.60 mg
Pantothenic acid (B5)	2.14 mg
Vitamin B6	0.54 mg
Folic acid (B9)	0.49 mg
Vitamin C	4.50 mg
Minerals	
Calcium	56 mg
Iron	6 mg
Magnesium	47 mg
Phosphorus	281 mg
Potassium	677 mg
Sodium	6 mg
Zinc	3 mg

Nutritional value per 100 g (3.5 oz)

From: http://www.fao.org/pulses-2016/blog/everything-you-need-to-know-about-lentils/en/

The specific objectives of this study are summarized below.

1) Measuring the changes of antioxidant potential using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging ability, reducing power, hydroxyl radical scavenging ability, and oxygen radical absorbance capacity (ORAC) of soluble- and insoluble-bound phenolics during germination, hydrothermal (boiling) processing, and dehulling.

2) Monitoring bioactivities such as inhibitory activities against LDL cholesterol oxidation and DNA strand breakage of soluble- and insoluble-bound phenolics in lentils during food processing

3) Identification and quantification of soluble- and insoluble-bound phenolics in lentil cultivars.

Chapter 2

Literature review

2.1 The role of phenolics in plants

Phenolic compounds play a multifunctional role in plants by regulating growth as an internal physiological regulator or a chemical messenger, among others (Cheynier et al., 2013). They also affect the growth hormone auxin such as indole-3-acetic acid (IAA) in which monohydroxy B-ring flavonoids act as a cofactor of peroxidase causing degradation of IAA while dihydroxy B-ring flavonoids inhibit the degradation process of IAA (Stafford, 1991; Mathesius, 2001). In addition, quercetin, apigenin, and kaempferol can bind to plasma membrane proteins (receptors), interfering with the transport of polar auxin compounds through the membrane, thus affecting plant architecture and growth (Cheynier et al., 2013).

Phenolic compounds are also responsible for the protection of plants from sunlight by absorbing harmful short high energy wavelengths at the electron-rich parts such as π-bond of the aromatic rings, leading to reduced oxidative stress (Shahidi and Yeo, 2016). They can also protect the plant from external predators such as insects with their possible toxic effects or protein precipitation and hence puckering effects in the mouth.

2.2 Classification of phenolics

Phenolics can be classified into several groups such as phenolic acids, flavonoids, stilbenes, coumarins, lignins and tannins (Figure 2.1). Phenolic acids include hydroxybenzoic acids (C6–C1)

such as *p*-hydroxybenzoic, vanillic, syringic, protocatechuic and ellagic acids as well as hydroxycinnamic acids (C6–C3) such as *p*-coumaric, caffeic, ferulic, and sinapic acids. Flavonoids are composed of a three-ring structure in the C6–C3–C6 form, which constitutes different classes of compounds such as flavones, flavanones, flavonols, flavanonols, isoflavones, flavanols, and anthocyanidins, based on different substitution patterns such as hydroxyl and methoxy groups. Stilbenes are also a group of phenolics that include resveratrol. Coumarins, as a benzopyrone group, are also present in different foods. Moreover, phenolics are also present in the polymeric form in plants; lignins are the polymer of monolignols such as *p*-coumaric acid and sinapic acid, while tannins are polymeric phenolics. Tannins exist as hydrolyzable tannins such as ellagitannins and as condensed tannins, also known as a proanthocyanidin. The latter group is further divided into several classes such as A, B, and C, depending on the type of linkage between the flavonoids, namely single or double linkages.

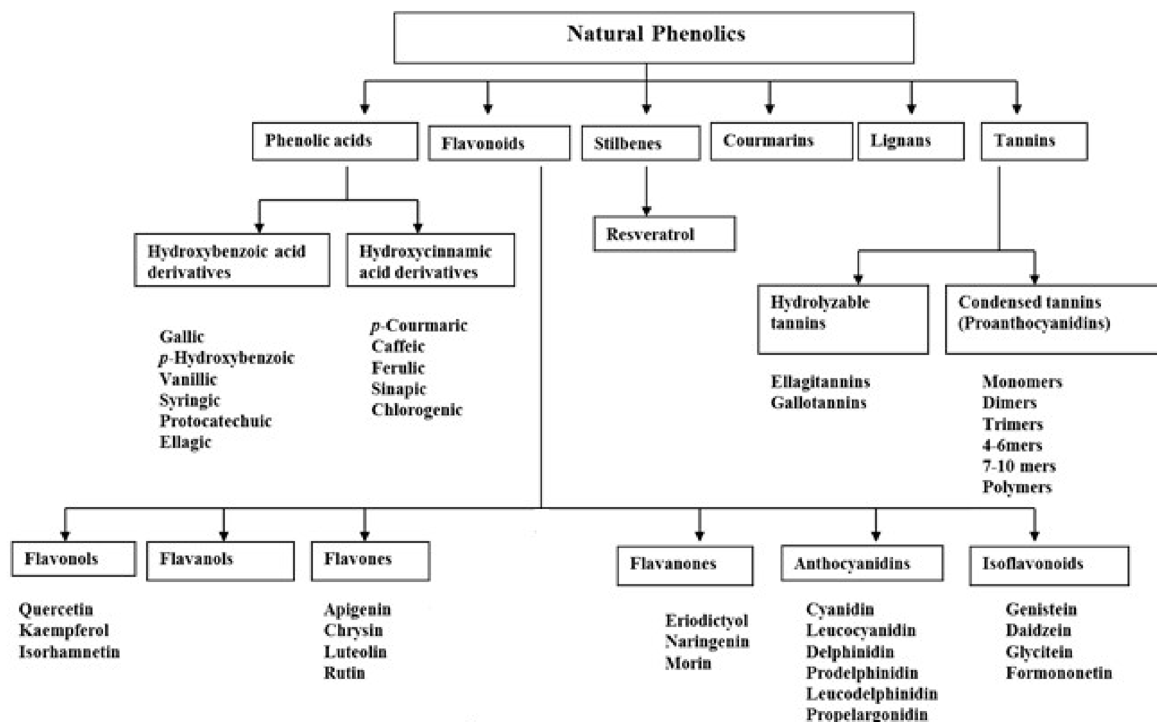


Figure 2.1 Classification of phenolic compounds from natural sources.

2.2.1 Phenolic acids

Phenolic acids are the primary phenolic compounds in natural sources such as cereals, legumes, and other seeds, where they act as the building material of cell wall matrices by connecting insoluble macromolecules such as cellulose, hemicellulose, and pectin, supporting the formation of rigid cell wall structures. Thus, they generally occur in a various conjugated form than the free type.

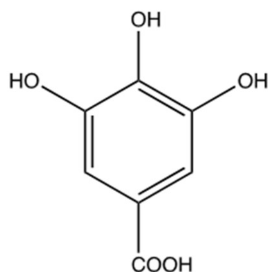
Phenolic acids are divided into two groups, namely hydroxycinnamic acids and hydroxybenzoic acids. Hydroxycinnamic acids include *p*-coumaric, caffeic, ferulic and sinapic acids, while hydroxybenzoic acids encompass *p*-hydroxybenzoic, protocatechuic, vanillic, syringic and gallic acids. The differentiation in substituted functional groups such as hydrogen, hydroxyl and methoxy groups decides individual differences of each phenolic acid.

Phenolic acids are present in almost all plant-based foods as their secondary metabolites. Thus we are exposed to the phenolic acids on a daily basis. The ingested phenolic acids are absorbed in the gastrointestinal tract and then circulate in the blood system after methylation, sulfation and glucuronidation in the liver (Manach et al., 2004). For example, hydroxycinnamic acids in wines are absorbed in the gastrointestinal tract after ingestion and transformed into glucuronide and sulfate conjugates, followed by circulation in the blood (Nardini et al., 2009). This conjugation process increases the hydrophilicity of the phenolic compounds and helps remove them via the biliary or the urinary route.

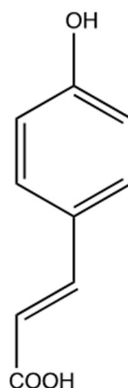
The absorbed phenolic acids in the digestive tract exhibit strong bioactivities. For example, ferulic acid possesses inhibitory activity against neurodegenerative disease, cardiovascular ailments, and

diabetes (Narasimhan et al., 2015; Eitsuka et al., 2014; Mancuso et al. 2014). In particular, ferulic acids serve as robust anticancer agents in many different cancer cell lines such as those of breast, colon, and pancreas (Eitsuka et al., 2014; Choi and Park, 2015; Janicke et al., 2011). In addition, coumaric and caffeic acids show anticancer activity in human lung (A549) and colon adenocarcinoma (HT29-D4) cancer cell lines (Bouzaiene et al., 2015).

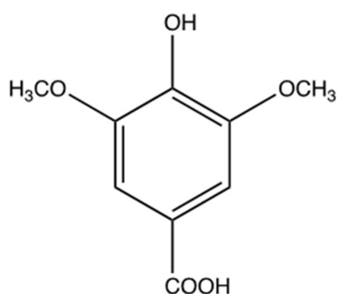
A hydroxybenzoic acid **A hydroxycinnamic acid**



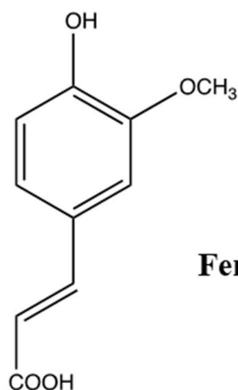
Gallic acid



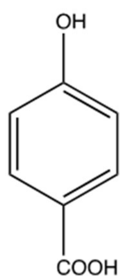
p-Coumaric acid



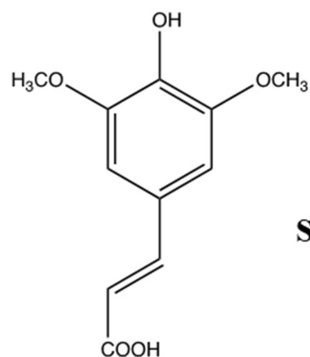
Syringic acid



Ferulic acid



p-Hydroxybenzoic acid



Sinapic acid

Figure 2.2 Chemical structures of representative phenolic acids.

2.2.2 Flavonoids

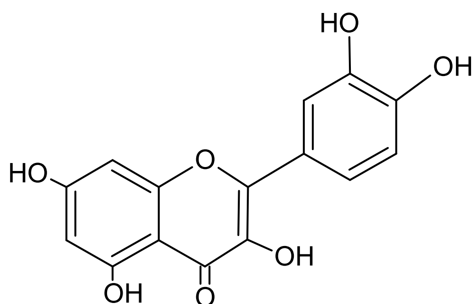
Flavonoids have a C6-C3-C6 carbon skeleton in their chemical structure, in which two phenyl groups are joined via a C3 chain, and this is often present in the form of glucoside through different molecules. In plant life, flavonoids perform various roles such as UV-light protection, defense from external predators such as insects and controlling auxin transport (Kitamura, 2006). More than 7000 flavonoids have been found in plant-based foods, many of which contribute to the attractive colours of flowers, fruits, and leaves.

In their chemical structures, the different substitution patterns of hydroxyl and methoxy groups dictate the different classes of flavonoids such as flavonols, flavanols, flavones, flavan-3-ol, flavanones, anthocyanidins, and isoflavones. The chemical structures of the representative flavonoids of each group are shown in Figure 2.3.

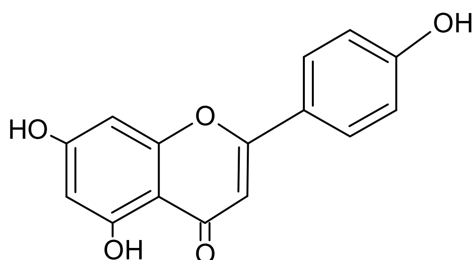
Flavonoids possess remarkable bioactivities such as anticancer, anti-inflammation and anti-virus effects as well as reducing cardiovascular diseases and type-2-diabetes. For example, myricetin inhibits cancer cells and colon carcinogenesis (Harnly et al., 2006; Nirmala and Ramanathan, 2011). Naringenin also exhibits anti-inflammatory activity and cytotoxicity in carcinoma cells (Orjala et al., 1994; Tamogami and Kodama, 2000). In addition, bioactivities such as anti-inflammation, analgesic, antiallergic, cardioprotective, antidiabetic and osteoporotic activities were also found for kaempferol (Calderon-Montano et al., 2011; Hamalainen et al., 2007; Toyoda et al., 1997; Trivedi et al., 2008).

Aside from bioactivities, many studies have been conducted on the antioxidant capacities of flavonoids *in vitro*. For instance, flavonoids show strong DPPH radical scavenging ability,

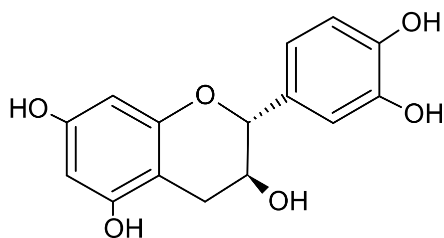
reducing power, hydroxyl radical scavenging activity, and oxygen radical absorbance capacity (ORAC) as well as metal chelation and enzyme inhibition activity (Alshikh et al., 2015; Xu and Chang, 2008; Yeo and Shahidi, 2015; Zhang et al., 2015).



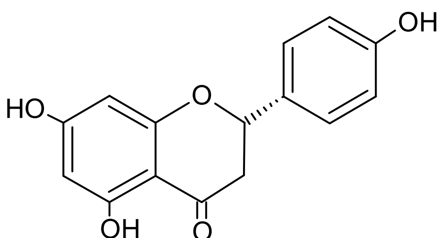
Quercetin (flavonol)



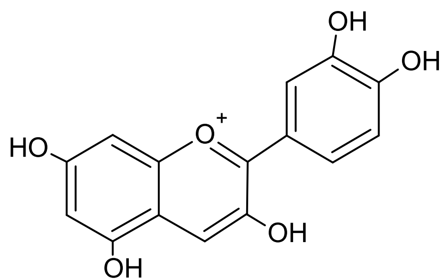
Apigenin (flavone)



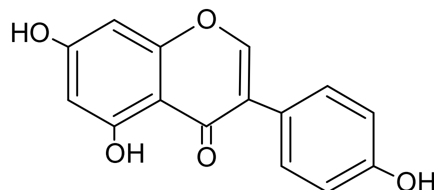
Catechin (flavan-3-ol)



Naringenin (flavanone)



Cyanidin (anthocyanidin)



Genistein (isoflavone)

Figure 2.3 Chemical structures of representative flavonoids.

2.2.3. Stilbenes and Coumarins

Stilbenes

Stilbene is also known as 1, 2-diphenylethene, which possesses a carbon skeleton of C6-C2-C6 (Figure 2.4). The substituted hydroxyl group are responsible for the creation of a wide variety of oligomeric structures of stilbenes by forming covalent bonds. Thus, they can be classified into several categories based on the level of polymerization, namely monomers, dimers, trimers, tetramers, and hexamers.

There are two stilbene isomeric forms, *cis* and *trans*, with somewhat different properties. For example, the *trans* form shows better stability than the corresponding *cis* form. The structure of stilbene can be isomerized from *cis* to *trans* or vice versa based on the environment of the reaction medium; for example, the presence of heat, light, and proton can isomerize the *cis* to the *trans* form and vice versa.

Stilbenes show strong bioactivities such as anti-bacterial effects, among others. For example, tetrastilbenes such as kobophenol-A and -B display anti-bacterial activity, against *Staphylococcus aureus* (Kawabata et al., 1991). Stilbenes are also known as an active inhibitor of topoisomerase II, which causes unwinding of coiled DNA during cellular transcription (Yamada et al., 2006).

Resveratrol, which is abundant in grapes and wines, is a representative example of stilbenes. The average content of resveratrol in red wine and white wine is 14.0 and 0.1 mg/L, respectively (Baur and Sinclair, 2006). In contrast, a relatively lower level of resveratrol is found in grape juice and whole grapes compared to the wines at 0.05-0.5 mg/L. Resveratrol has received a great deal of attention from both scientists and consumers since its bioactivities such as anti-inflammation, anti-

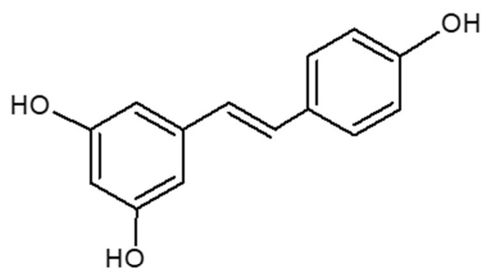
tumorigenesis, and cardioprotective effects have been reported (Baur and Sinclair, 2006). In addition, resveratrol is effective in the inhibition of Parkinson's and Alzheimer's diseases by moderating hemeoxygenase activity since the incorrect operation of hemeoxygenase is associated such ailments (Salem et al., 2006; Han et al., 2006).

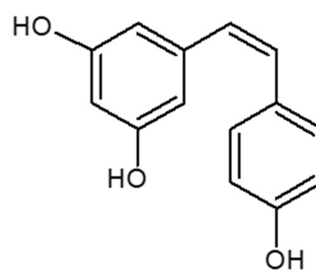
Coumarins

Coumarins are referred to as benzopyrone with a C6-C3 skeleton and an oxygen heterocycle in the C3 unit as shown in Figure 2.5 (Lacy and O´Kennedy, 2004). They are found in a wide variety of plants such as tonka bean (*Dipteryx odorata*), sweet woodruff (*Galium odoratum*), sweet grass (*Hierochloe odorata*), deertongue (*Dichanthelium clandestinum*), vanilla grass (*Anthoxanthum odoratum*), mullein (*Verbascum spp*.), and sweet-clover (*Melilotus sp*.). Aside from plants, coumarins are present as the metabolites of microorganisms such as *Streptomyces* and *Aspergillus* species, namely novobiocin and coumermycin (Lacy and O´Kennedy, 2004; Jain and Joshi, 2012).

Coumarins show a variety of bioactivities such as anti-inflammatory, antimicrobial, antiviral, antioxidant, antinociceptive, antitumor, antiasthmatic, antidepressant, anti-HIV, antituberculosis, and anti-Alzheimer activities (Lee et al., 2011; Nitiema et al., 2012; Xu et al., 2014; Kostova et al., 2011; Anand et al., 2012; Sanchez-Recillas et al., 2014; Kudo et al., 2013; Manvar et al., 2011).

Moreover, coumarin possesses anti-coagulant activity which inhibits blood coagulation by suppressing the hepatic synthesis of vitamin K-dependent coagulation factors. Thus, coumarin is also used as an anticoagulant for the treatment of diseases such as thrombotic phlebitis and pulmonary embolism.





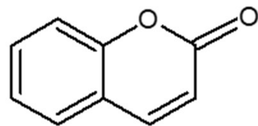
***trans*-resveratrol**

(Stilbene)

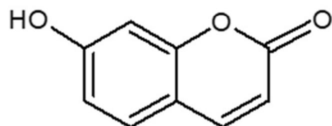
***cis*-resveratrol**

(Stilbene)

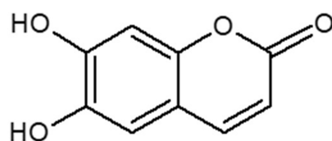
Figure 2.4 Chemical structures of *trans*- and *cis*-resveratrol.



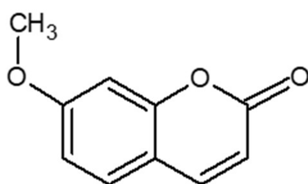
Coumarin



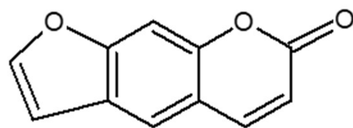
Umbelliferone



Aesculetin



Herniarin



Psoralen

Figure 2.5 Chemical structures of coumarin and its derivatives.

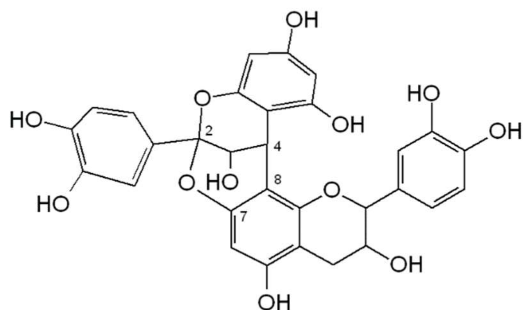
2.2.4 Tannins

Tannins are divided into hydrolyzable and condensed tannins. The hydrolyzable tannins may further be classified into gallotannins and ellagitannins. A representative gallotannin is hexagalloylated compound 2-O-digalloyl-1,3,4,6-tetra-O-galloyl-β-D-glucopyranose in which the additional gallic acid is attached to C2 of the glucopyranose ring. Ellagitannins, on the other hand, contain C-C bonds between adjacent galloyl moieties in the pentagalloylglucose molecule.

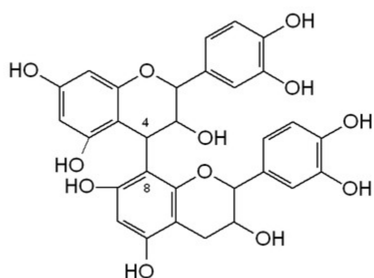
The condensed tannins, also referred to the proanthocyanidins, can be classified into A, B, and C groups based on the type of linkage between the flavonoids (Figure 2.6), namely procyanidin A which has double covalent bond in its dimer structure, while proanthocyanidin B (dimer) and C (trimer) are bound with a single linkage. Each group is further separated into the individual proanthocyanidins depending on the position of the coupling.

Proanthocyanidins are widely distributed in plant-based foods such as lentils, grape seed, cocoa seeds, and apples, among others (Alshikh et al., 2015; De Freitas and Glories, 1999; Pérez-Ilzarbe, 1992). The structural properties of proanthocyanidin, especially two or three hydroxyl groups in the B-ring of flavan-3-ol unit, confers them their bioactivity; for instance, proanthocyanidin showed not only inhibitory activity against the oxidation of low density lipoprotein (LDL) cholesterol, stomach mucosa injury, and radioprotective effects against chromosomal damage, but also a practical bacterial anti-adhesion effect (Saito et al., 1998; Castillo et al., 2000; Amy and Howell, 2002). In addition, they possess a robust antioxidant potential such as oxygen radical scavenging capacity (Ricardo da Silva et al., 1991).

A type proanthocyanidin dimer (4→8 bonds)



B type proanthocyanidin dimer (4→8 bonds)



B type proanthocyanidin dimer (4→6 bonds)

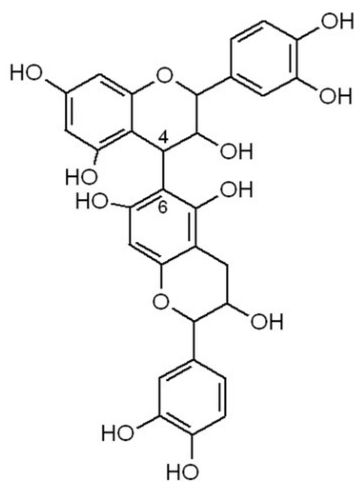


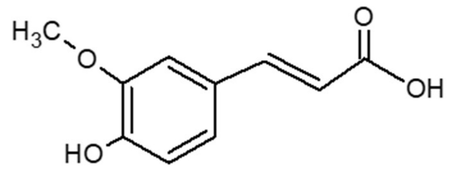
Figure 2.6 Chemical structures of A and B type proanthocyanidin dimers.

2.3 Soluble- and insoluble-bound phenolics

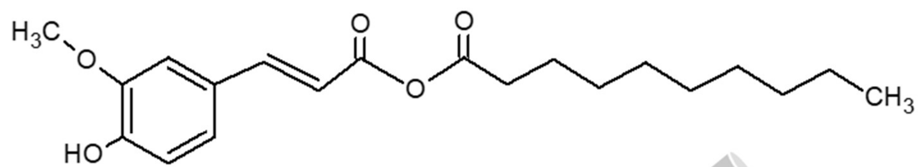
Phenolic compounds can be divided into free, esterified and insoluble-bound types, depending on whether they occur in a free or bound form. Soluble phenolics include free phenolics, which do not form a chemical bond with other molecules, and esters that are associated through ester bond with fatty acids and glucuronic acid (Figure 2.7). These soluble phenolics are readily extracted by extraction medium, hence are also referred to as extractable phenolics. On the other hand, insoluble-bound phenolics are bound with macromolecules that are not extracted by extraction medium such as cellulose, hemicellulose, pectin, and structural protein. Thus, the chemical bond must first be broken via hydrolysis, chemically or enzymatically, to extract insoluble-bound phenolics from foods.

As already mentioned, most insoluble-bound phenolics are chemically and covalently bonded to cell wall components and these account for a relatively large proportion (20–60% in vegetable, fruits, and legume/seeds) compared to the soluble phenolics in foods (Nayak et al., 2015).

(a)



(b)



(c)

Cellulose, pectin, or structural protein

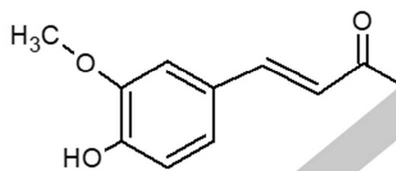


Figure 2.7 Free (a), ester (b), and bound form (c) of ferulic acid.

2.4 Localization of soluble- and insoluble-bound phenolics

Phenolic compounds occur in both soluble and insoluble-bound forms. Most of the soluble phenolics are localized in the vacuoles of plant cells where they are trapped (Martinoia and Ratajczak, 1997) (Figure 2.8). On the other hand, insoluble-bound phenolics are localized in cell wall matrix of the plant cells. The phenolic compounds synthesized in the intracellular organs, mainly the endoplasmic reticulum, are released and transported into the vacuole or cell wall matrix through the vesicle transfer system that is a small lipid bilayer system, which can contain phenolics and facilitates their migration into the cell wall matrix (Meyer et al., 2009). The transported phenolic compounds are bound to macromolecules such as structural proteins, cellulose, and pectin, among other molecules, through covalent bonds via ether, ester and carbon-carbon bonds in the cell wall matrix, thus forming insoluble-bound phenolics (Figure 2.9). Therefore, most of the soluble and insoluble-bound phenolics are localized in the intracellular organs/sites of plant cells. Seeds/legumes consist of the outer part, known as seed coat/hull and the inner part, including endosperm, epicotyl, hypocotyl, and radicle. The endosperm of legumes accounts for a considerable weight of the seeds, acts as a nutrition storage organ and is consumed during the germination process. Most nutritional compounds of endosperms are starch, protein, and lipid, accounting for 7, 12 and 5% of their total dry weight, respectively (Bradford and Bewley, 2003). Thus, most of the endosperm consists of storage cells, implying a low content of phenolic-containing cells. On the other hand, the seed coat/hull is composed of epidermis, hypodermis, chlorenchyma, palisade, parenchyma and endothelium cells, all of which constitute most of the organs such as vacuoles and cell walls, thus containing high amounts of phenolics in both the soluble and insoluble-bound forms.

Leaves and stems, as well as other essential parts of plants, also possess phenolic-containing cells such as epidermal cells, guard cells, subsidiary cells and epidermal hairs (trichomes) in the leaf and epidermal cells, parenchyma cell, chlorenchyma cell and collenchyma cell in the stem (Jung et al., 2012). Therefore, phenolics are ubiquitous secondary plant metabolites of natural sources primarily in the cell concentrated portion of plants.

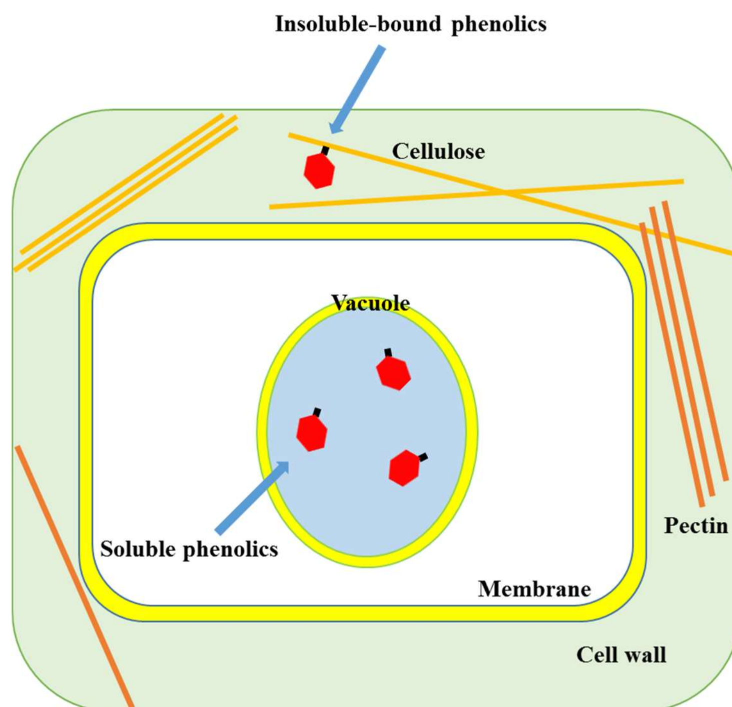


Figure 2.8 Localization of soluble and insoluble-bound phenolics in a plant cell (Shahidi and Yeo, 2016).

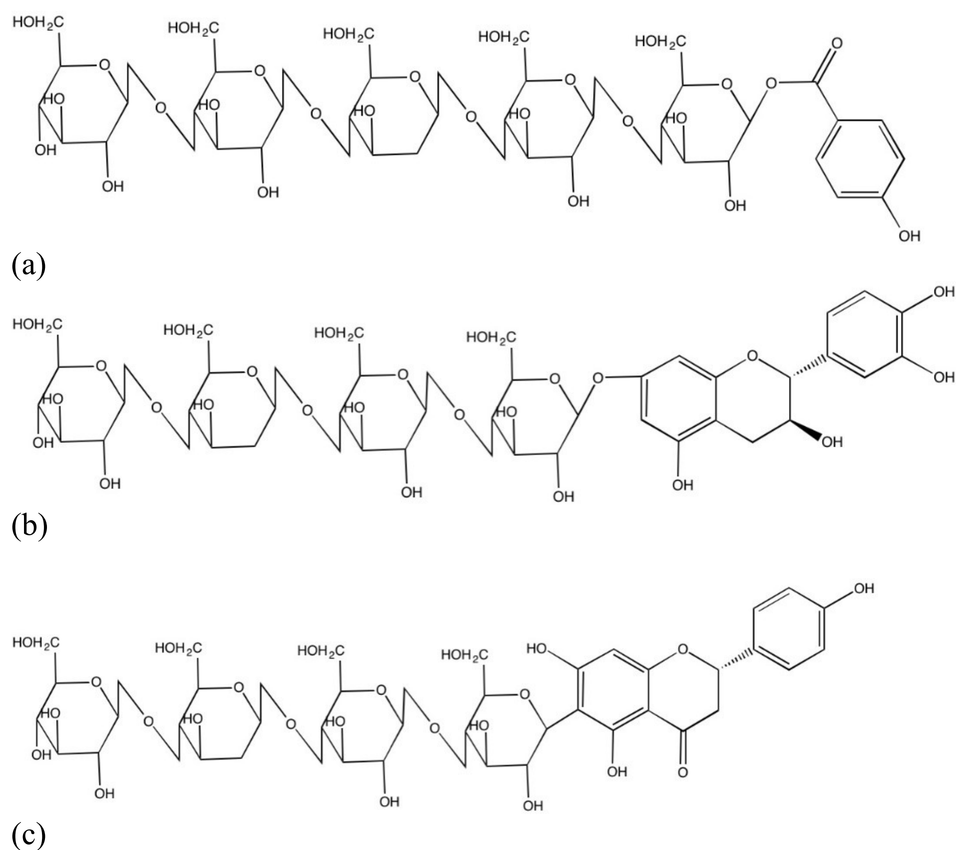


Figure 2.9 Representative covalent bonds found in insoluble-bound phenolics; (a) ester bond of 4-hydroxybenzoic acid, (b) ether bond of catechin, and (c) carbon-carbon bond of naringenin attached to the cellulose molecule (Shahidi and Yeo, 2016).

2.5 Synthesis, transport and formation of insoluble-bound phenolics

Synthesis

The synthesis of phenolic compounds takes place mostly at the cytoplasmic surface of endoplasmic reticulum, which is continuous with the outer nucleus of the plant cell. Many types of enzymes are involved in transforming the structure of phenolics, leading to different classes, namely phenolic acids, flavonoids, including anthocyanidins and other kinds of phenolics.

Phenylalanine, an essential amino acid synthesized through the shikimate pathway of erythrose-4-phosphate and phosphoenolpyruvate, is a precursor for the synthesis of phenolic compounds and also tyrosine, albeit to a lesser extent (Figure 2.10). Phenylalanine releases ammonia via the action of phenylalanine ammonia lyase (PAL), yielding trans-cinnamic acid with the formation of a double bond. The trans-cinnamic acid is transformed into *p*-coumaric acid by the action of P450 monooxygenase, followed by caffeic acid with hydroxylase and then, ferulic acid by o-methyl transferase. Ferulic acid is further altered into sinapic acid by hydroxylase and o-methyl transferase to add a methoxy group to the molecule. Benzoic acid derivatives can be formed by the loss of two carbon atom moiety from phenylpropanoids.

Flavonoids' synthesis, known as the flavonoid branch pathway, is initiated by combining *p*-coumaroyl CoA and three molecules of malonyl CoA, converting them into naringenin chalcone by the action of chalcone synthase (CHS), followed by flavonone through chalcone isomerase (Figure 2.11). The resultant flavonone may be converted into different types of flavonoids such as flavononol, flavonol, isoflavone, flavone, anthocyanins and catechins via individual enzymatic reactions. For example, genistein, kaempferol, and apigenin can be synthesized by isoflavone

synthase, flavonol synthase/flavanone-3-hydroxylase, and flavone synthase, respectively, from the flavonone.

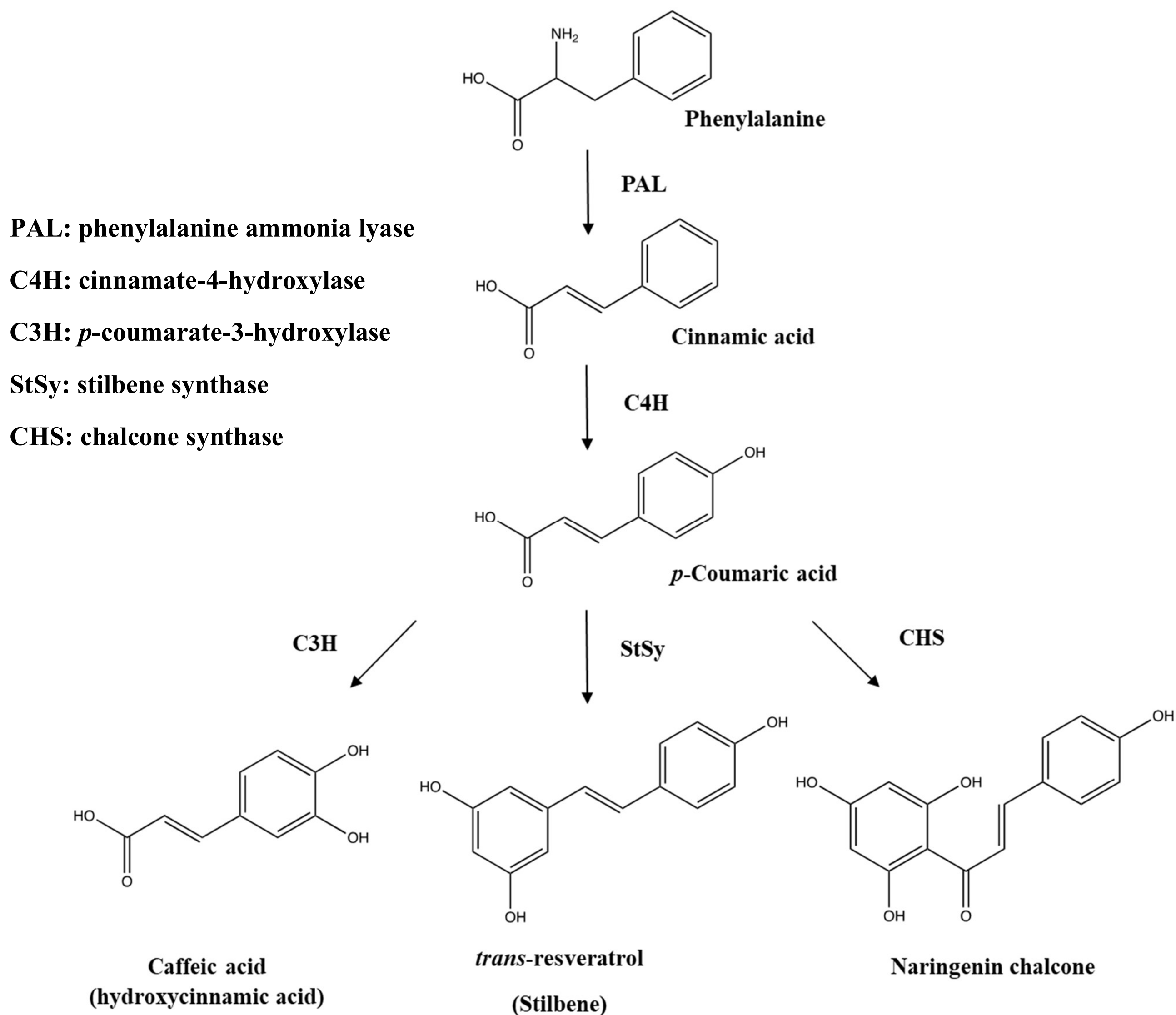
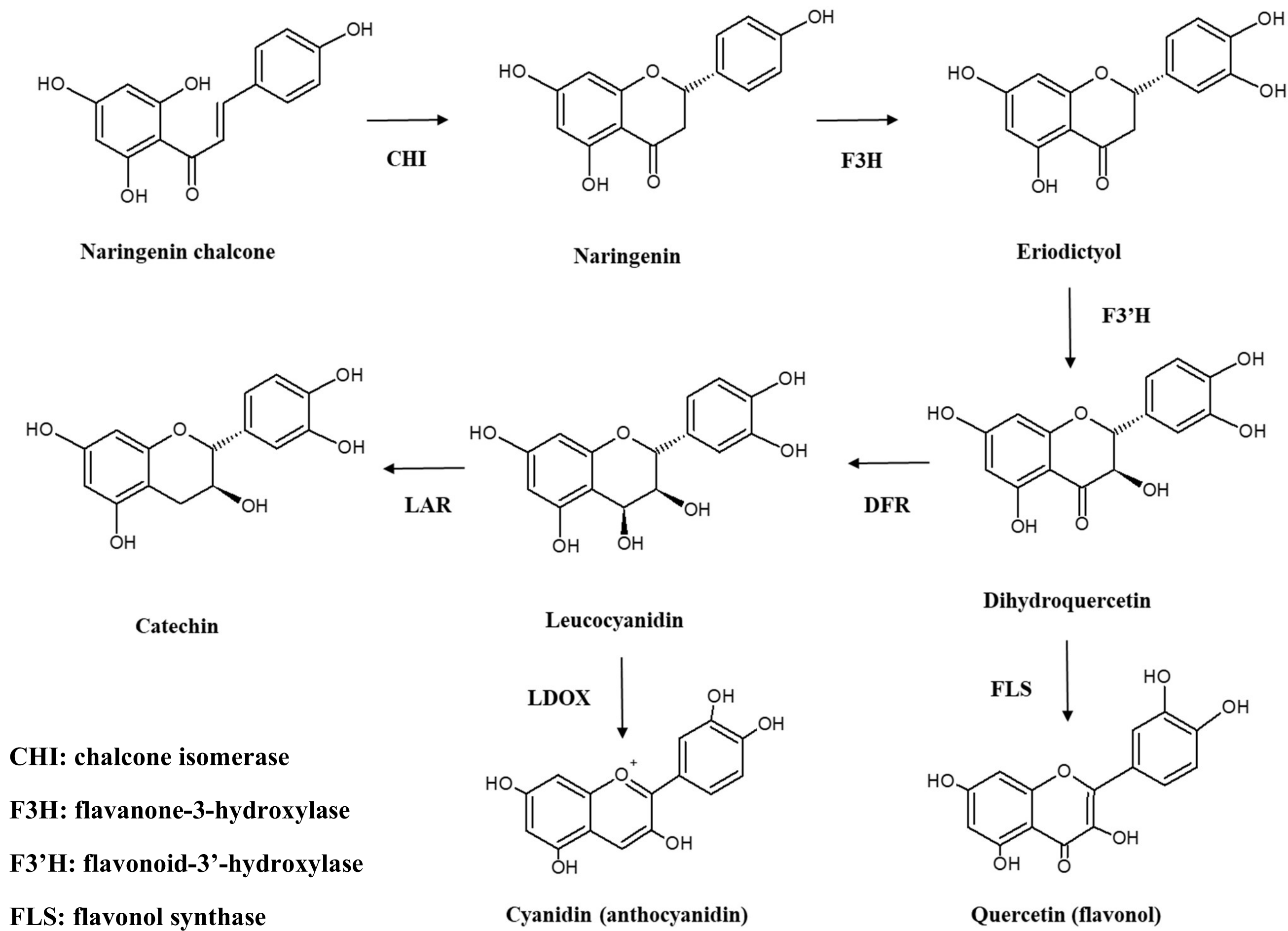


Figure 2.10 Biosynthesis pathway of phenolic acids and stilbene from phenylalanine.



CHI: chalcone isomerase

F3H: flavanone-3-hydroxylase

F3'H: flavonoid-3'-hydroxylase

FLS: flavonol synthase

DFR: dihydroflavonol-4-reductase

LAR: leucoanthocyanidin reductase

LDOX: leucoanthocyanidin dioxygenase

Figure 2.11 Biosynthesis pathway of flavonoid from naringenin chalcone.

Transport of phenolics in plant cells

The synthesized phenolic compounds in the cytoplasmic surface of endoplasmic reticulum are transferred to other organs of plant cells through vesicle transfer system and membrane-mediated transport (Grotewold, 2001; Grotewold, 2004; Kitamura, 2006; Zhao and Dixon, 2009), as shown in Figure 2.12.

The first possible transfer mechanism is the membrane-mediated transport. After synthesis at the endoplasmic reticulum, phenolics are released to the cytoplasmic space and can enter the organ's interior by penetrating the compartment membrane through a transporter protein (Agati et al., 2012). For example, the ATP-binding cassette (ABC) transporter and multidrug and toxic compound extrusion (MATE) proteins are the main routes for the incorporation of glucosides of anthocyanins, apigenin, and catechins to the vacuole tonoplast (Yazaki, 2005).

Another transfer mechanism is via a cytoplasmic vesicle. The phenolics synthesized are secreted into the cytoplasmic space and are incorporated into a lipid bilayer membrane, called cytoplasmic vesicle or sac, followed by their transfer to each cell organ such as vacuoles, nucleus and cell wall, which is known as a vesicle transfer system, facilitating the delivery of phenolics to the target site by protecting them from other reactive compounds and enzymes present in plant cells. When the cytoplasmic vesicles arrive at the surface of organs, they release their phenolic contents to the organ inside by fusing to their membrane (Grotewold, 2001).

In addition, the cytoplasmic vesicles can also be absorbed into the Golgi apparatus, leading to the transformation of the phenolic structures due to the presence of multiple enzymes there which is

then followed by their release to the cytoplasmic space by Golgi vesicles that transfer the phenolics to each organ.

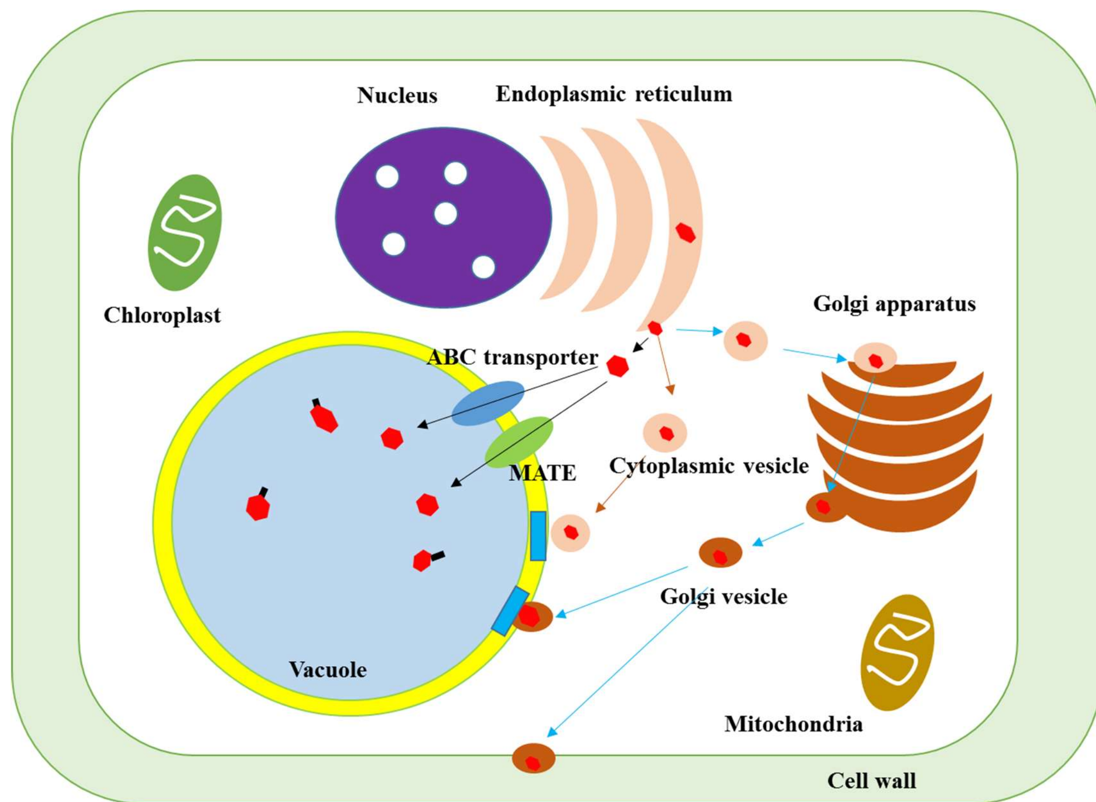


Figure 2.12 Transfer mechanisms of phenolics after synthesis at the endoplasmic reticulum in a plant cell. (ABC, ATP-binding cassette; MATE, multidrug and toxic compound extrusion) (Shahidi and Yeo, 2016).

Formation of insoluble-bound phenolics in the cell wall

The exact mechanism for the transfer of phenolics to the cell wall and formation of insoluble-bound phenolics has not yet been fully established. However, there is some evidence about the transfer of phenolics to the cell wall matrix (Zhao and Dixon, 2009; Strack et al., 1988; Markham et al., 2000). The cytoplasmic and Golgi vesicles containing phenolics can move to the plasma membrane and secrete the phenolics into the cell wall matrix (Meyer et al., 2009). In addition, the phenolics in the cytoplasm can be transported to the plasma membrane through ABC transporters and across the plasma membrane, reaching the cell wall matrix (Zhao and Dixon, 2009).

In the cell wall, the released phenolic compounds can form covalent bonds with cell wall substances such as cellulose, hemicellulose, arabinoxylans, structural proteins and pectin through ester, ether and C-C bonds (Figure 2.13). The carboxyl group of phenolic acids such as benzoic and cinnamic acids can form ester bonds with hydroxyl groups of cell wall substances (McLusky et al., 2009) and can also yield ether bonds by binding between hydroxyl groups of phenolic compounds and hydroxyl groups of cell wall substances and C-C bonds as well when they directly create covalent bond between a carbon atom of phenolics and a carbon atom of cell wall substances. The covalent bonds so formed play a major role in connecting cell wall substances, enhancing the rigid structure of the cell wall matrix. In addition, cell wall phenolics protect them from a number of pathogens and penetration of fungi as well as UV damage (Nicholson et al., 2009; Jansen et al., 2009).

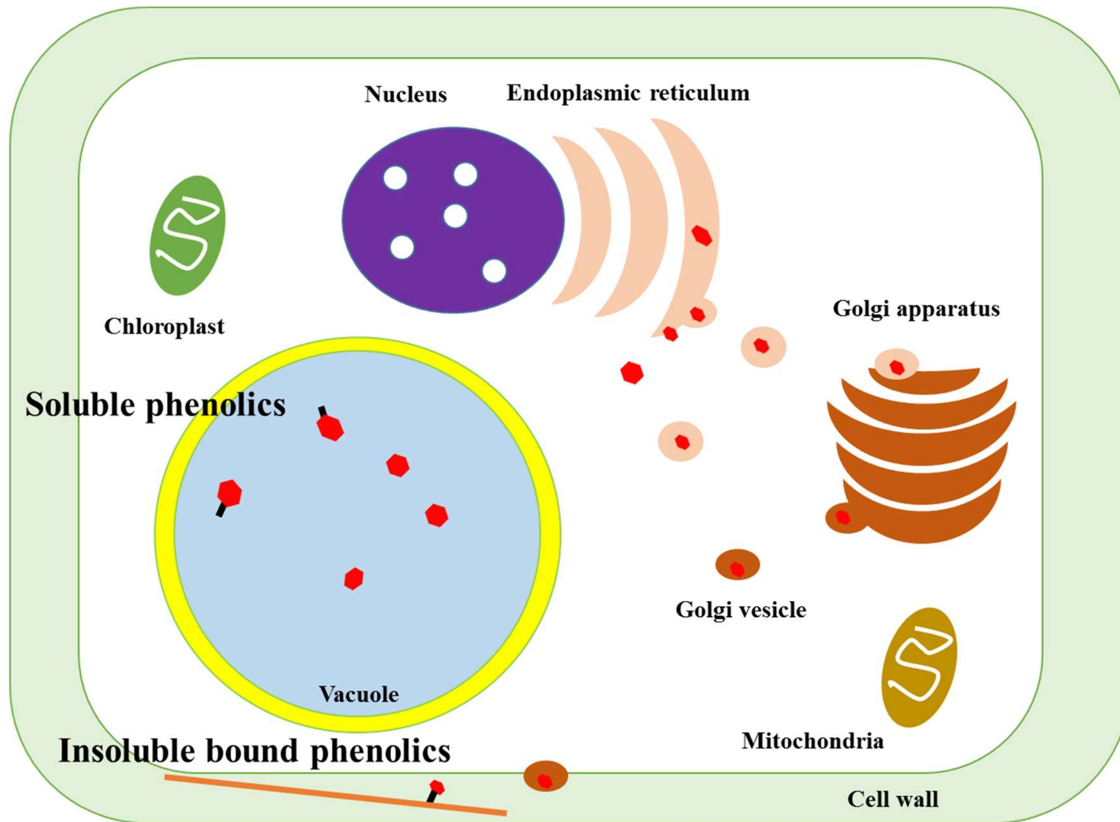


Figure 2.13 Formation of insoluble-bound phenolics (Shahidi and Yeo, 2016).

2.6 Insoluble-phenolic acids and -flavonoids contents in cereals, legumes, and other seeds

2.6.1 Phenolic Acids

Recently, a variety of insoluble-bound phenolics in different plant sources have been reported, as shown in Table 2.3. In cereals, bound phenolic acids such as protocatechuic, *p*-hydroxybenzoic, vanillic, *p*-coumaric and ferulic acid were found in black rice and their levels were 162.1, 21.2, 27.7, 17.6 and 64.7 µg/g, respectively (Alves et al., 2016). Das and Singh (2016) reported that maize possessed bound phenolic acids such as *p*-hydroxybenzoic, vanillic, syringic, caffeic, *p*-coumaric, ferulic and isoferulic acids and their levels depended on the part of maize grain, thus pericarp portion was more abundant in bound phenolics than the germ and endosperm. Aside from the above investigations, a number of studies have been conducted on the identification and quantification of bound phenolics acids in cereals such as durum wheat, bread wheat, barley, oat, rye, rice, triticale and millet, revealing similar bound phenolic composition with cereals discussed above (Irakli et al., 2012; Chandrasekara and Shahidi, 2010; Alshikh et al., 2015).

The bound phenolic acids are also abundant in legumes. Alshikh et al. (2015) studied six lentil cultivars and reported the composition and levels of bound phenolic acids such as gallic (0.0–1.8 µg/g), protocatechuic (0.0–4.4 µg/g), *p*-coumaric (0.0–3.1 µg/g) and ferulic acid (0.0–0.3 µg/g). Chen et al. (2015) reported that cranberry beans contained *p*-hydroxybenzoic, *p*-coumaric, ferulic and sinapic acid in the bound form and their levels were 4.4–71.2, 4.9–18.4, 11.0–41.7 and 0.03–3.55 µg/g of the whole bean in seven different cultivars, respectively. Other legumes such as mung bean, pinto bean, black bean, kidney bean, cow bean, chickpea were also abundant in bound phenolic acids as shown in Table 2.3. In addition, protocatechuic acids were found in cow gram,

flower waist bean, hyacinth bean and pearl bean as the main bound phenolic acids, ranged from 110 to 172 μg/g of dry weight (DW) (Wang et al., 2016).

Bound phenolic acids are also present in oilseeds. Sunflower seeds contain gallic, protocatechuic, caffeic, *p*-coumaric, ferulic and sinapic acids and their contents ranged from 2.5 to 50.8 μg/g of DW (Pajak et al., 2014). Naczk and Shahidi (1989) investigated the bound phenolic acids in rapeseed meal and found sinapic, *p*-coumaric and trans-ferulic acids as the main insoluble-bound phenolics. Aside from major oilseeds, flaxseed, moringa oleifera seed flour and soybean were also studied for their bound phenolic acids and showed high contents of gallic and *p*-coumaric acids as shown in Table 2.3.

According to the literature, fruit seeds also serve as valuable sources of bound phenolic acids such as protocatechuic, *p*-coumaric, gallic, caffeic and syringic acids. Those of phenolic acids were found in blackberry, black raspberry, blueberry seed meals and the range of individual phenolics acids were 39–221, 25–243, 242–356, 25–217 and 0–93 μg/100 g, respectively (Ayoub et al., 2016).

Table 2.3 Insoluble-bound phenolic acids in cereals, legumes, and other seeds

Sources	Bound phenolic acids (μg/g, DW)	Reference
Cereals		
Black rice	Protocatechuic (162.1), 4-hydroxybenzoic (21.2), vanillic (27.7), *p*-coumaric (17.6), and ferulic (64.7) acids	Alves et al., 2016
Maize (Pericarp)	*p*-Hydroxybenzoic (499.0), vanillic (1788.0), syringic (83.0), *p*-coumaric (3.0), ferulic (3247.0), and isoferulic (842.0) acids	
Maize (Germ)	*p*-Hydroxybenzoic (21.0), vanillic (522.0), syringic (897.0), caffeic (4.0), *p*-coumaric (12.0), ferulic (247.0), and isoferulic (58.0) acids	Das and Singh., 2016
Maize (endosperm)	*p*-Hydroxybenzoic (10.0), vanillic (6.0), caffeic (0.6), *p*-coumaric (0.3), ferulic (27.0), and isoferulic (1.0) acids	
Durum wheat	Gallic (6.0), 4-hydroxybenzoic (4.4), vanillic (2.9), syringic (2.9), *p*-coumaric (11.8), *trans*-ferulic (216.3), sinapic (41.2), and *trans*-cinnamic (5.4) acids	
Bread wheat	Gallic (5.9), 4-hydroxybenzoic (4.7), vanillic (2.9), Syringic (3.7), *p*-coumaric (13.9), *trans*-ferulic (253.1), sinapic (45.5), and *trans*-cinnamic (5.2) acids	
Barley	Gallic (5.5), protocatechuic (4.9), 4-hydroxybenzoic (5.6), vanillic (5.4), caffeic (4.6), syringic (4.6), *p*-coumaric (75.9), *trans*-ferulic (320. 8), sinapic (41.9), salicylic (9.7), and *trans*-cinnamic (5.9) acids	
Oat	4-Hydroxybenzoic (6.3), vanillic (12.9), caffeic (6.5), syringic (8.4), *p*-coumaric (603.0), *trans*-ferulic (1032.8), sinapic (100.6), and trans-cinnamic (5.4) acids	Irakli et al., 2012
Rye	Gallic (7.1), protocatechuic (4.2), 4-hydroxybenzoic (5.3), vanillic (4.1), caffeic (5.1), syringic (3.4), *p*-coumaric (20.7), *trans*-ferulic (216.7), sinapic (44.7), and *trans*-cinnamic (4.6) acids	
Rice	Gallic (5.1), 4-hydroxybenzoic (4.5), vanillic (1.7), syringic (2.8), *p*-coumaric (20.9), *trans*-ferulic (65.5), sinapic (22.8), and *trans*-cinnamic (4.9) acids	
Triticale	Gallic (4.8), protocatechuic (3.8), 4-hydroxybenzoic (5.4), vanillic (5.4), caffeic (4.3), syringic (4.1), *p*-coumaric (19.9), *trans*-ferulic (334.1), sinapic (58.4), salicylic (10.9), and *trans*-cinnamic (5.6) acids	
Corn	4-Hydroxybenzoic (4.7), vanillic (4.9), caffeic (4.9), syringic (4.2), *p*-coumaric (95.4), *trans*-ferulic (954.4), sinapic (78.8), and *trans*-cinnamic (5.2) acids	
Millet (7cultivars)	Ferulic (178.0-1685.0) and *p*-coumaric (20.0-1139.0) acids	Chandrasekara and Shahidi, 2010
Legumes		

Lentils (6 cultivars)	Gallic acid (0.0-1.8), protocatechuic acid (0.0-4.4), *p*-coumaric acid (0.0-3.1), and ferulic acid (0.0-0.3) acids	Alshikh et al., 2015
Lentils	Gallic acid (8.54-11.56), protocatechuic acid (6.39-8.17)	Zhang et al., 2014
Cranberry beans (7 cultivars)	*p*-Hydroxybenzoic (4.4-71.2), *p*-coumaric (4.9-18.4), ferulic (11.0-41.7), and sinapic (0.03-3.55) acids	Chen et al., 2015
Soy isoflavone concentrate	*p*-Hydroxybenzoic (12.0), vanillic (62.0), syringic (262.0), *p*-coumaric (61.0), ferulic (55.0), and sinapic (25.0) acids	Verardo et al., 2015
Mung bean	Gallic (3.0), caffeic (0.3), *p*-coumaric (0.2), ferulic (2.2), and sinapic (1.5) acid	Pajak et al., 2014
Pinto bean	Protocatechuic (25.6), *p*-hydroxybenzoic (42.7), *p*-coumaric (26.8), ferulic (34.7), and sinapic (89.9) acid	
Black bean	Gallic (41.1), protocatechuic (14.1), *p*-hydroxybenzoic (5.6), caffeic (17.5), syringic (17.1), *p*-coumaric (22.1), ferulic (170.1), and sinapic (50.5) acid	Ross et al., 2009
Kidney bean	Protocatechuic (64.4), *p*-hydroxybenzoic (21.3), *p*-coumaric (7.9), ferulic (138.0), and sinapic (73.4) acid	
Cowpea	Gallic (5.4) acid	Gutiérrez-Uribe et al., 2009
Chickpea	Gallic (82.8) and protocatechuic (110.9) acid	
Cow gram	Protocatechuic (172.6) acid	
Flower waist bean	Protocatechuic (128.4) acid	Wang et al., 2016
Hyacinth bean	Protocatechuic (111.8) acid	
Pearl bean	Protocatechuic (121.9) acid	
Oilseeds		
Sunflower	Gallic (11.2), protocatechuic (50.8), caffeic (25.5), *p*-coumaric (2.5), ferulic (16.9), and sinapic (15.6) acids	Pajak et al., 2014
Rapeseed meal (seven cultivars)	389-1050 µg sinapic acid equivalent /g DW (Main phenolic acids: sinapic, *p*-coumaric and *trans*-ferulic acid)	Naczk and Shahidi, 2009
Flaxseed	*p*-Coumaric acid glucoside (3800.0) and ferulic acid glucoside (4400.0)	Beejmohun et al., 2007

Moringa oleifera seed flour	Gallic (15.9) and *p*-coumaric (7.4) acids	Singh et al., 2013
Soy bean	Gallic (64.2), protocatechuic (238.8), vanillic (88.7), syringic (121.6), *p*-coumaric (101.7), and ferulic (87.1) acid	Min et al., 2013
Fruit seeds		
Blackberry seed meal	Protocatechuic (2.2), *p*-coumaric (0.2), gallic (3.4), and caffeic (2.1) acids	
Black raspberry seed meal	Protocatechuic (0.9), *p*-coumaric (2.4), gallic (2.4), and caffeic (1.5) acids	Ayoub et al., 2016
Blueberry seed meal	Protocatechuic (0.3), *p*-coumaric (0.2), gallic (3.5), and caffeic (0.2), syringic (0.9) acids; gallic hexoside (1.0)	

2.6.2 Flavonoids

Different bound flavonoids have been reported in the literature, as summarized in Table 2.4. Cereals are the primary natural sources of bound flavonoids. Das and Singh (2016) investigated bound flavonoids in maize and found 15.9 µg/g of kaempferol in pericarp portion and 28.8 µg/g of quercetin in germ parts, but bound flavonoids were not detected in the endosperm portion. In addition, three major flavonoids, namely catechin (16.28 µg/g), quercetin (47.2 µg/g) and kaempferol (30.4 µg/g) were identified in quinoa seeds (Tang et al., 2016). Other cereals such as rice, corn, wheat and barely were also excellent sources of bound flavonoids, showing 240, 760, 430 and 370 µg catechin equivalents/g of DW, respectively, however, their compositions were not provided (Min et al., 2016).

Aside from cereals, legumes are also valuable sources of bound flavonoids. For example, several bound flavonoids such as catechin (15.0–78.4 µg/g), epicatechin (0.5–7.9 µg/g), (+)-catechin-3-glucoside (0.0–122.0 µg/g) and luteolin 3-*O*-7-diglucoside (0.0–49.3 µg/g) were found in six lentil cultivars (Alshikh et al., 2015). In addition, cranberry beans contained bound flavonoids such as kaempferol (0.27–0.37 µg/g) in four different cultivars (Chen et al., 2015). Other legumes such as black bean, cow gram, hyacinth bean, pearl bean, red bean, red kidney bean and spring bay bean were also rich in bound flavonoids, with quercetin and catechin being the most prevalent flavonoids in the aforementioned legumes (Wang et al., 2016).

Oilseeds and other seeds are also abundant in bound flavonoids. For example, Moringa oleifera seed flour possessed three significant flavonoids, namely catechin (7490 µg/g), epicatechin (810 µg/g) and quercetin (18.7 µg/g) (Singh et al., 2016). Sunflower seeds contained quercetin,

kaempferol and apigenin at 1.5, 0.5 and 2.9 μg/g, respectively. In addition, epicatechin, isoquercitrin, and quercetin were found in soybean at high levels as shown in Table 2.4 (Wang et al., 2016). The concentration of bound flavonoids is mainly dependent on the food source and analytical methods employed in the hydrolysis and extraction procedures. In addition, fruit seeds were also a rich source of bound flavonoids. Ayoub et al. (2016) reported that bound flavonoids such as (+)-catechin, (-)-epicatechin, quercetin, epigallocatechin, myricetin, quercetin pentose, epicatechin gallate, kaempferol hexoside, quercetin-3-*O*-glucuronide were present in blackberry, black raspberry, blueberry seed meals, and their contents were 40–102, 7–116, 27–326, 40–136, 0–26, 0–330, 0–117, 0–68 and 0–1149 μg/100 g, respectively.

Table 2.4 Insoluble-bound flavonoids in cereals, legumes, and other seeds

Sources	Bound flavonoids (μg/g, DW)	Reference
Cereals		
Maize (pericarp)	Kaempferol (15.9)	Das and Singh, 2016
Maize (germ)	Quercetin (28.8)	
Quinoa seed	Catechin (16.28), quercetin (47.2), and kaempferol (30.4)	Tang et al., 2016
Dent corn (pericarp portion)	Quercetin (35.9)	Das and Singh, 2015
Flint corn (germ portion)	Quercetin (12.0)	
Rice	240 μg catechin equivalent/g DW	Min et al., 2012
Corn	760 μg catechin equivalent/g DW	
Wheat	430 μg catechin equivalent/g DW	
Barley	370 μg catechin equivalent/g DW	
Legumes		
Lentils (6 cultivars)	Catechin (15.0-78.4), epicatechin (0.5-7.9), (+)-catechin-3-glucoside (0.0-122.0), and luteolin 3′-7-diglucoside (0.0-49.3)	Alshikh et al., 2015
Lentils	Catechin (14.19-58.05), epicatechin (9.00-9.72)	Zhang et al., 2014
Cranberry beans (4 cultivars)	Kaempferol (0.2-0.3)	Chen et al., 2015
Mung bean	Quercetin (0.2) and kaempferol (0.1)	Pajak et al., 2014

Black bean	(+)-Catechin (109.7), epicatechin (93.8), rutin (93.8), isoquercitrin (462.3), and quercetin (86.6)	
Cow gram	Quercitrin (105.4)	
Hyacinth bean	Quercitrin (91.9)	
Pearl bean	(+)-Catechin (100.7)	Wang et al., 2016
Red bean	Rutin (89.5)	
Red kidney bean	Catechin (88.2) and isoquercitrin (97.1)	
Spring bay Bean	Catechin (158.8), rutin (124.2), isoquercitrin (85.7), and quercetin (86.8)	
Oilseeds		
Moringa oleifera seed flour	Catechin (7490.0), epicatechin (810.0), and quercetin (18.7)	Singh et al., 2013
Sunflower	Quercetin (1.5), kaempferol (0.5), and apigenin (2.9)	Pajak et al., 2014
Soybean	Epicatechin (95.2), isoquercitrin (396.0), and quercetin (101.0)	Wang et al., 2016
Fruit seeds		
Blackberry	(+)-Catechin (1.0), (-)-epicatechin (1.1), quercetin (2.0), quercetin pentose (3.3), epicatechin gallate (1.1), and quercetin-3-O-glucuronide (5.1)	
Black raspberry	(+)-Catechin (0.4), (-)-epicatechin (3.1), quercetin (3.2), epigallocatechin (1.3), epicatechin gallate (0.9), and quercetin-3-O-glucuronide (11.4)	Ayoub et al. 2016
Blueberry	(+)-Catechin (0.7), (-)-epicatechin (0.07), quercetin (0.2), epigallocatechin (0.4), myricetin (0.2), quercetin pentose (0.5), and kaempferol hexoside (0.6)	

2.7 Metabolism of soluble- and insoluble-bound phenolics in human digestive tract

Foods containing phenolic compounds undergo multi-enzyme reactions, following alteration of physical and chemical properties in the gastrointestinal tract, including the mouth, stomach, as well as small and large intestines (colon) after intake. Phenolic compounds can be released from the food matrix in the gastrointestinal tract by enzymes and pH conditions (Podsedek et al., 2014). The released free phenolics are absorbed in the small intestine, followed by conjugation with other compounds, leading to their introduction into the blood circulation system (Scalbert et al., 2009). However, only 5–10% of phenolics can be absorbed in the small intestine and the remaining 90–95% move directly to the colon (large intestine). Given the argument of Rodríguez-Roque et al. (2014) who stated that the phenolic bioavailability could be defined as the number of phenolic compounds that reach the blood circulation system so that they can exert bioactivity at the tissues and cells, a 5–10% absorption rate of phenolics indicates their low bioavailability. Conversely, insoluble-bound phenolics are not absorbed in the small intestine because they are bound to insoluble macromolecules such as cellulose, hemicellulose, structural protein, and pectin. Thus, they reach the colon, and they undergo fermentation by the colon microbiota and release the bound phenolics (Adom and Liu, 2002; Andreasen et al., 2001). In the colon, a variety of microorganisms, approximately 1000 different bacteria and around 1014 colony forming units (CFU), exist and take part in the fermentation of unabsorbed material as well as fungi, protozoa and archaea (Gibson and Roberfroid, 1995). The microorganisms, including *Bifidobacterium spp*. and *Lactobacillus spp*. secrete a variety of extracellular enzymes such as carbohydrases, proteases and other types of

enzymes, leading to the disruption of cell wall matrix or hydrolysis of covalent bonds of bound phenolics, followed by liberation of phenolics. The released phenolics render a myriad of health benefits that influence the fermentation environment of the colon by decreasing pH and preventing the growth of cancer-inducing microorganisms.

During fermentation in the colon, many different types of products are formed, however only a few of them can be absorbed. The primary compounds that can be absorbed in the colon include water and some salts and their sodium and potassium ions. In addition, vitamin K, vitamin B12, thiamin, riboflavin and short fatty acids can also be absorbed. However, the absorption of phenolics has not yet been well established. Thus additional research should be carried out on the incorporation of phenolics in the human colon to support the above bioactivities such as the inhibitory activity of colon cancer.

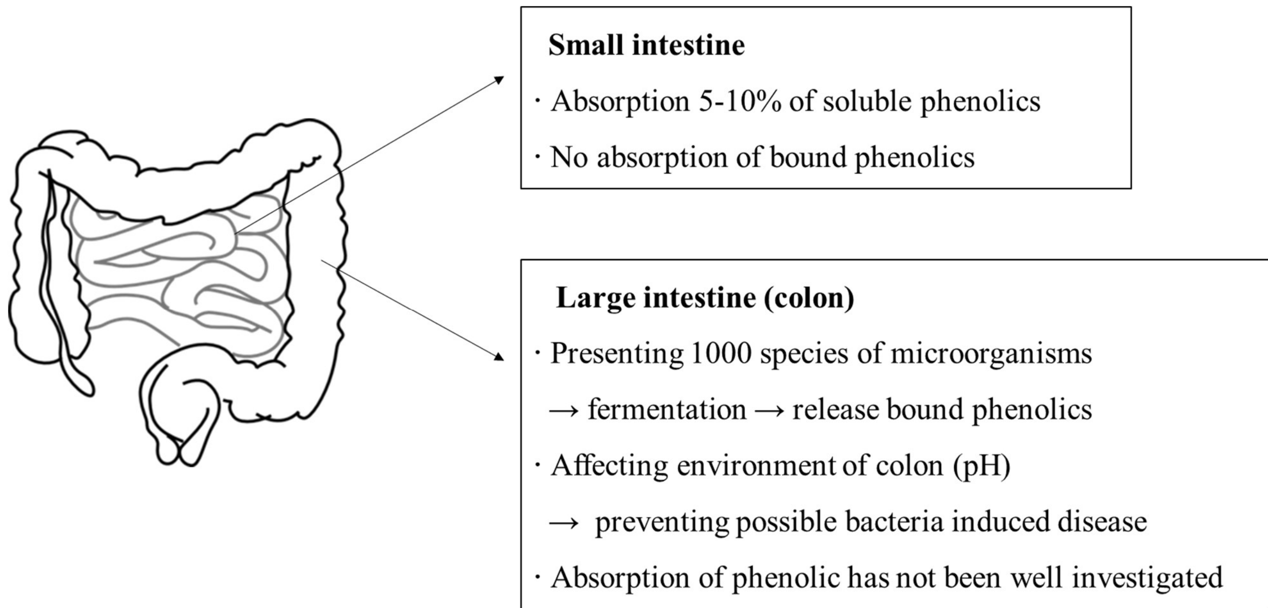


Figure 2.14 Absorption of soluble- and insoluble-bound phenolics in the digestive tract.

2.8 Bioactivities of phenolics

Phenolic Acids

In recent years, a variety of bioactivities of phenolic acids have been reported. First, gallic acid has shown remarkable anti-cancer activity in a number of studies (Inoue et al., 1995; Veluri et al., 2006; Kaur et al., 2009; Yeh et al., 2011). Gallic acid and methyl gallate extracted from *Givotia rottleriformis* reduced the growth of human epidermoid carcinoma (A431) skin cancer cells (Kamatham et al., 2015) and exhibited an inhibitory activity against hepatitis C virus (HCV), which is a major blood-borne pathogen and causes liver cirrhosis and hepatocellular carcinoma (HCC) that is an infection in primary human hepatocytes (Hsu et al., 2015; Morgan et al., 2013). In addition, methyl gallate possesses anti-bacterial and anti-viral properties (Choi et al., 2008; Kane et al., 1988; Kang et al., 2008). Ferulic acid, which has been used to relieve angina pectoris and hypertension in China (Hou et al., 2004), showed anti-cancer activity in cultured MIA PaCa-2 human pancreatic cancer cell (Fahrioğlu et al., 2016). Ferulic acid was also effective against diabetes, cardiovascular disease, neurodegenerative disease and cancer (Narasimhan et al., 2015; Eitsuka et al., 2014; Mancuso et al. 2014). Anti-cancer studies of ferulic acid were tested in different cell lines such as those of breast cancer (Eitsuka et al., 2014; Choi and Park, 2015) colon cancer (Janicke et al., 2011) and pancreatic cancer (Eitsuka et al., 2014). Feruloyl-L-arabinose was also effective when used on H1299 lung cancer cells (Fang et al., 2013).

Flavonoids

Flavonoids possess an effective antioxidant capacity due to the presence of functional groups such as hydroxyl groups, which render bioactivities such as anticancer, anti-inflammation and anti-virus as well as reducing cardiovascular diseases, type-2-diabetes and cholesterol. A variety of other bioactivities of bound flavonoids have also been demonstrated. Lin et al. (2000) studied the bioactivity of (+)-epigallocatechin 3-O-gallate extracted from roots of *Limonium sinense* and showed suppression of virus type-1 infection. Troxerutin, a derivative of rutin, showed a strong interaction and affinity for the major or minor groove of the DNA structure in cancer cells, leading to the potential for killing cancer cells by radiation-induced sensitizing, namely acting as a chemopreventive agent (Panat et al., 2016). Apigenin exhibited a radiation-induced sensitizing effect on lung carcinoma cells (Watanabe et al., 2007); rhamnetin and cirsiliol also showed the same effectiveness in tumor cells (Kang et al., 2013). However, flavonoids such as hesperidin, rutin, quercetin, and naringin did not support the secretion of inflammation factors such as NF-kB (Chtourou et al., 2016; Ahn et al., 2014; Hamad et al., 2015; Kamel et al., 2014). Apigenin also attenuated cisplatin-induced nephrotoxicity in human renal proximal tubular epithelial cells (Ju et al., 2015). Quercetin has been well documented as a great functional compound for the prevention of cancer, cardiovascular diseases and cognitive malfunction (Simkhada et al., 2009; Larson et al., 2012; Cherniack, 2012). Kaempferol revealed anticancer activity in human lung cancer cells and in *in vitro* cancer cell line systems (Leung et al., 2007; Bestwick et al., 2007). In addition, kaempferol suppressed other human cancer cell lines such as human cervical carcinoma (Hela), human stomach carcinoma (SGC-7901), human lung carcinoma (A549) and human breast carcinoma (MCF-7) by inducing nuclear condensation and mitochondria dysfunction (Liao et al., 2016).

2.9 Extraction of insoluble-bound phenolics

Insoluble-bound phenolics, as already mentioned, are covalently bound to the cell wall matrix via ester, ether and C-C bonds. Thus they should be hydrolyzed/liberated from the cell wall matrix in order to measure their contents. Acid and alkaline hydrolyses are the most common chemical methods used to extract the insoluble-bound phenolics, and recently, many other new techniques such as enzymatic hydrolysis and microwave assisted hydrolysis have been employed for better release of insoluble-bound phenolics from cell wall matrices.

2.9.1. Acid hydrolysis

Acid hydrolysis for the extraction of insoluble-bound phenolics from food materials has been widely employed by using 1–5% hydrochloric acid in water/methanol. The advantages of acid hydrolysis are its convenience and simple steps for the extraction. The extracted bound phenolics can be used directly for further experimentation after neutralization and filtration, unlike alkaline extraction which requires an additional extraction procedure using diethyl ether. However, phenolic compounds are unstable at low pH. Thus they may degrade during the extraction process or storage. For example, Sani et al. (2012) compared acidic and alkali hydrolysis for releasing of phenolics from germinated brown rice. Their results showed that some phenolics such as hydroxycinnamic, caffeic, syringic and protocatechuic acids were found in alkali hydrolysates, but were absent in acid hydrolysis products. Possible degradation or structural changes in the phenolics upon acid hydrolysis may be contemplated. According to the literature, flavonol extracts from *Opuntia ficus-indica* prickly fruit were partially degraded during acid hydrolysis (Moussa-Ayoub et al., 2011). In addition, Fazary and Ju (2007) argued that acid hydrolysis was not appropriate for hydrolyzing ester bonds of insoluble-bound phenolics, even though the treatment showed efficient

hydrolysis in breaking the glycosidic bonds. Thus, alkali hydrolysis has been commonly used for the release of insoluble-bound phenolics from the food matrix rather than acid hydrolysis.

2.9.2 Alkaline hydrolysis

The most common chemical method for extraction of insoluble-bound phenolics is alkaline hydrolysis that uses a wide range of concentrations of sodium hydroxide. This hydrolysis method has proven to be effective in hydrolyzing both ether and ester bonds that are rarely broken by acid hydrolysis (Acosta-Estrada et al., 2014). In addition, ordinarily alkaline hydrolysis is conducted at room temperature, leading to a low rate of loss of phenolics during the process than the acid hydrolysis method (Krygier et al., 1982). Alshikh et al. (2015) studied insoluble-bound phenolics in six lentil cultivars using alkali hydrolysis and found phenolic acids such as gallic acid, protocatechuic acid, *p*-coumaric acid and ferulic acid. The alkali hydrolysis was also employed for exploring the insoluble-bound phenolics in fruit seeds (Ayoub et al., 2016). In addition, alkali hydrolysis has been widely used for the release of insoluble-bound phenolics in many types of foods such as cereals, legumes and seeds (Chen et al., 2015; Verardo et al., 2015; Pajak et al., 2014; Ross et al., 2009; Gutiérrez-Uribe et al., 2010; Naczk and Shahidi. 1989). However, the disadvantage of this method is the more complicated procedure for the extraction in alkaline hydrolysis, as mentioned above, that requires further extraction steps after sodium hydroxide hydrolysis to isolate the liberated phenolics from food matrices.

2.9.3 Enzymatic hydrolysis

Aside from chemical methods such as acid and alkaline hydrolysis, enzymatic hydrolysis can serve as an efficient method for the extraction of insoluble-bound phenolics. In general, carbohydrate-hydrolyzing enzymes, including cellulase, hemicellulase, pectinase, amylase, and glucanase, are used to dismantle cell wall matrices that consist of cellulose, hemicellulose, pectin, and glucan (Landbo and Meyer, 2001). The cell wall matrix disintegrated by the enzymes would expose more surface to the solvent, facilitating extraction of the insoluble-bound phenolics. The advantage of enzymatic hydrolysis extraction is the minimization of the loss of phenolic compounds due to extreme (too low or too high) pH conditions during the extraction process. Xu et al. (2014) attempted the release of phenolic compounds from cell walls of muscadine grape (*Vitis rotundifolia Michx*) skins and seeds using cellulase, pectinase, and β-glucosidase. The enzymatic treatment shortened the extraction time and improved antioxidant capacity of the extracts compared to the typical solvent extraction. Tang et al. (2016) reported that enzymatic hydrolysis using pectinase, xylanase and feruloyl esterase released comparable amounts of bound phenolics to the soluble phenolics, but lower than acid and alkaline extraction, from quinoa seeds. Zheng et al. (2009) reported that carbohydrases such as cellulases, β-glucanase, and pectinase increased total phenolic contents of unripe apples by assisting the liberation of phenolics, however, some of the phenolics such as chlorogenic acid and phloridzin were significantly decreased during the enzymatic hydrolysis. Therefore, the enzymatic hydrolysis has both advantages and disadvantages.

2.10 Release of insoluble-bound phenolics from cell wall matrix

2.10.1 Non-thermal processing

2.10.1.1 Fermentation process in food

Fermentation refers to the microorganism-mediated bioconversion of sugar (or monosaccharide) into lactic acid, ethanol, and gas. Fermentation, as a traditional food processing method to extend the shelf-life of foods, has been suggested as a useful non-thermal food processing method for releasing insoluble-bound phenolics from the cell wall matrix of foods. During fermentation, microorganisms secrete a variety of extracellular enzymes such as carbohydrases, proteases, and lipases that break down the macromolecules into smaller entities so that they can use them as an energy source and as essential ingredients (Figure 2.15). The enzymes also include cell wall matrix disintegrating enzymes such as cellulase, hemicellulase, pectinase, amylase, and glucanase, leading to the liberation of insoluble-bound phenolics.

In the fermentation industry, bacteria and fungi are used for lactic acid fermentation, and yeast cells are employed in ethanol fermentation. Each of the microorganisms has different enzymes, leading to distinctive unique flavors and final products during fermentation, driven by different enzymes that are present in the medium. For instance, representative fungi in the lactic acid fermentation industry are the *Bacillus subtilis* and *Aspergillus oryzae*. Even though they are the same bacterial group of microorganisms, the enzymes that they secrete are apparently different. *Bacillus subtilis* releases amylase, cellulases, hydrolases, levansucrase, peptidase, proteases and xylanase, whereas *Aspergillus oryzae* releases acid protease, α-galactosidase, amylase, invertase, lignin peroxidase and tannase (Hur et al., 2014). Therefore, the fermentation process with different species of microorganisms can result in different enzymatic reactions, followed by differentiation in the liberation of phenolics from the cell wall matrix of food.

The main component of the cell wall matrix is cellulose. Thus cellulase plays a significant role in releasing the insoluble-bound phenolics. The hydrolysis process of cellulase can be divided into three steps. First, endoglucanase localizes at the low density of crystallinity of cellulose fibres,

releasing short oligosaccharides. Second, exoglucanase and cellobiohydrolase yield cellobiose units, a disaccharide with β-linkage, from the ends. Finally, the released cellobiose is hydrolyzed by β-glucosidase into glucose units (Sun and Cheng, 2002). In addition, the efficiency of cellulase was improved in the presence of phenolic acids such as ferulic acid and *p*-coumaric acid which act as a cofactor and assist their enzyme activity, enhancing their activity by up to 28.3 and 15.1%, respectively (Tian et al., 2013). Aside from cellulase, all cell wall matrix disintegrating enzymes such as cellulase, hemicellulase, pectinase, amylase, and glucanase play a major role in the degradation of cell walls, leading to the liberation of insoluble-bound phenolics.

Esterase also plays a significant role in the release of bound phenolics after the disintegration of cell wall matrix by cellulase. They hydrolyze ester bonds between phenolic acids and cell wall substances, leading to the release of phenolics (Figure 2.16). Some fungi and yeast cells such as *Lactobacillus lactis, Lactobacillus rhamnosus, Aspergillus niger, Cryptococcus flavus* and *Cryptococcus sp. S-2* release several types of esterases (Hur et al., 2014). Representative esterases for the liberation of bound phenolics are feruloyl esterases [E.C. 3.1.1.73], which are also known as ferulic acid esterases (FAE), cinnamoyl esterases and cinnamic acid hydrolases; these are subclasses of the carboxylic acid esterases (E.C. 3.1.1.1) (Williamson et al., 1998; Crepin et al., 2004). Tapin et al. (2006) used feruloyl esterase to release phenolic compounds from wheat and flax straws, and the enzyme efficiently increased the liberation of phenolics such as ferulic acid, coumaric acid, and vanillic acid, as well as vanillin.

Many studies have reported on the improvement of the antioxidant capacity of fermented foods and have suggested that the enhancement is due to the release of insoluble-bound phenolics by cell wall disintegrating enzymes. Kim et al. (2010) reported that fermented rice spent water, which is the byproduct of rice milling, improved the antioxidant capacity as reflected in the DPPH radical

scavenging ability and reducing power and suggested that the results might be due to the liberation of bound phenolics during fermentation. Dey and Kuhad (2014) reported the antioxidant capacity of four whole grain cereals, namely wheat, brown rice, maize and oat; they demonstrated that the antioxidant capacity of these cereals was enhanced by solid-state fermentation using several fungi such as Aspergillus oryzae NCIM 1212, Aspergillus awamori MTCC No. 548, Rhizopus oligosporus NCIM 1215 and Rhizopus oryzae RCK2012. They suggested that extracellular enzymes such as α-amylase, xylanase, β-glucosidase and esterases from fungi might play a significant role in releasing the insoluble-bound phenolics from cell wall matrices of cereals, leading to increased antioxidant capacity.

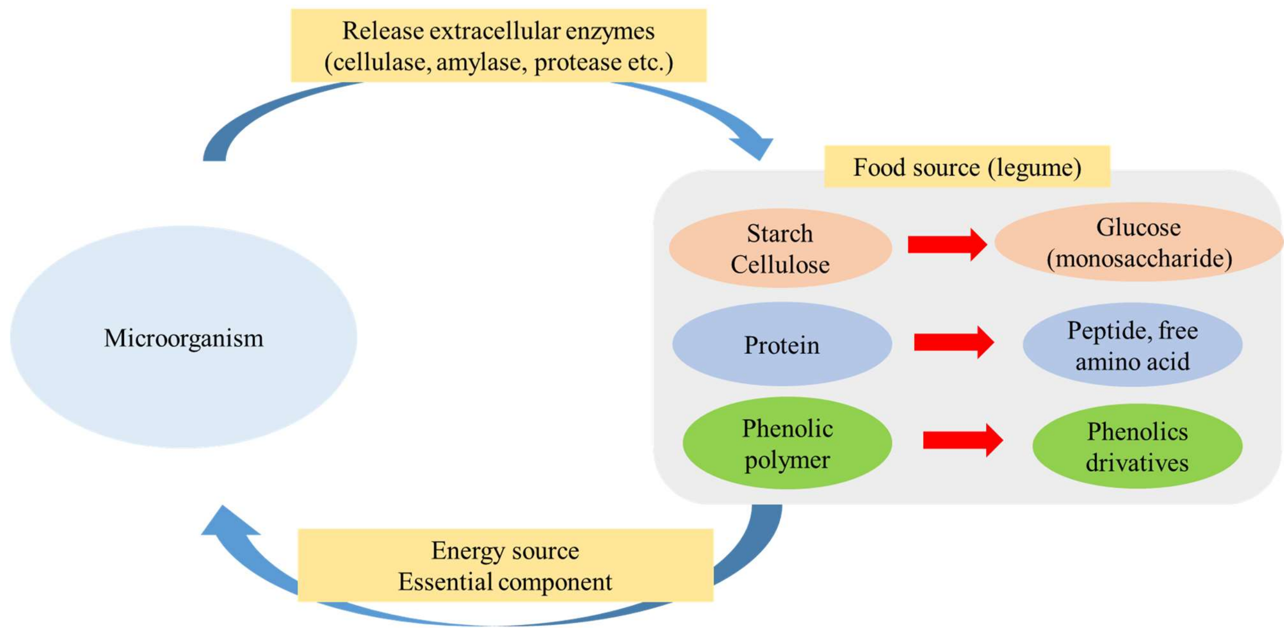


Figure 2.15 Hydrolysis of polymeric macromolecules by the extracellular enzymes released by microorganisms during fermentation.

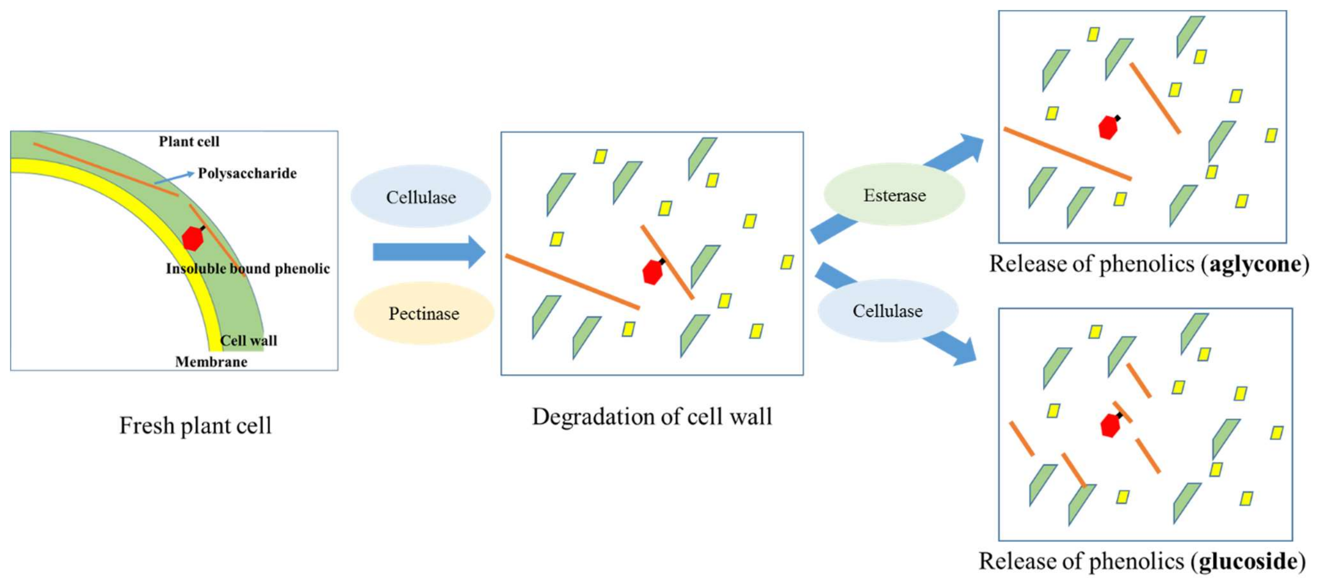


Figure 2.16 Release of bound phenolics from the cell wall matrix by enzymes.

2.10.1.2 Germination

Germination is the bioprocess that induces breaking of the dormancy of seeds by sprouting and growth. Germination is initiated with imbibition (hydration) that leads to swelling and breaking of the seed coat, followed by activation of cell metabolism. The increase in water intake allows an increase in the number of hydrated cells. The hydrated cell initiates aerobic respiration, mitochondrial repair, DNA repair, transcription and translation of new mRNAs at the same time, followed by DNA synthesis, cell division and seedling growth (Nonogaki et al., 2010). The activated cells release hydrolytic enzymes which hydrolyze macromolecules such as starch and protein into smaller molecules that are used for the metabolism and growth of the seeds, affecting the content of phenolic compounds and their formation (Raven et al., 2005).

Ti et al. (2014) showed that the amounts of insoluble-bound phenolics such as syringic acid, coumaric acid, and ferulic acid were increased from 1.7, 34.2 and 121.6 to 6.4, 117.9 and 343.8 μg/g DW, respectively, during 5 days of germination of brown rice. Yeo and Shahidi (2015) reported that the contents of insoluble-bound phenolics were enhanced during germination of lentils. The insoluble-bound phenolics of canary seeds were increased during 5 days of germination, especially ferulic acid which was the dominant bound phenolic and its content was increased from 98.20 to 313.07 μg/g DW (Verardo et al., 2015). Yang et al. (2001) argued that the enhancement of insoluble-bound phenolics during germination might be due to cell division (biosynthesis) of the sprouting or growth of seeds, which increased the total volume of cell wall part, followed by an increase of bound phenolics.

2.10.2. Thermal and hydrothermal processing

2.10.2.1. Roasting

Roasting is a traditional method that not only improves food flavor such as those of nuts and legumes but also serves as a useful pretreatment means for better oil release and oil extraction from oilseeds (Durmaz et al., 2011). During the roasting process, the heat used results in chemical reactions such as the Maillard reaction, leading to alteration of the chemical structures of phytochemicals present. In addition, the heat energy can also cause disruption of the cell wall matrix of foods, enhancing the release of bound phenolics (Dewanto et al., 2002; Nicoli et al., 1999; Veldsink et al., 1999). According to the literature, the roasting process affects the phenolics of nuts such as cashew nut, hazelnut, and peanut, leading to alteration of their antioxidant activities (Chandrasekara and Shahidi, 2011; Vinson and Cai, 2012). Nyembwe et al. (2015) reported that the contents of gallic acid and protocatechuic acid were increased from 34.8 and 78.0 to 81.0 and 123.0 mg/100 g DW of flour, respectively, upon roasting at 150 °C for 30 min, possibly due to the release of bound phenolics during the process. Chandrasekara and Shahidi (2011) reported that the protection factor by the Rancimat assay and total phenolic content of phenolic extracts from cashew nuts was increased by both the low and high-temperature roasting treatment and discussed that this improvement could be induced by the liberation of bound phenolics during the roasting process. However, the decrease of antioxidant potential of phenolics after roasting process was also shown in coffee beans such as Arabica and Robusta (Pokorna et al., 2015).

2.10.2.2 Extrusion Cooking

Extrusion cooking uses high temperature, pressure, and shear force to make individual unique products, leading to improvement of food quality such as digestibility of starch and protein as well as enhancement of food stability due to the inhibition of enzyme activity (Ragaee et al., 2014). The different characteristics of extrusion cooked foods are governed by the number of processing variables such as temperature, moisture content, pressure and processing time (Sarawong et al., 2014). For instance, breakfast cereals and corn curls are made under high temperature, low moisture, and high shear, while pasta is made under increased moisture content (about 40%) and low temperature, conferring specific texture and palatability. Meanwhile, extrusion processing, particularly under high thermal and pressure conditions, influence minor components such as phenolic compounds by disrupting cell wall matrix of foods, followed by the release of insoluble-bound phenolics. For example, extrusion cooking was found to liberate bound ferulic acid from cell walls of pigmented maize (Mora-Rochin et al., 2010). Gui and Ryu (2014) reported that the extrusion process enhanced total phenolic content in both the free and conjugated phenolic acids of ginsengs and this was thought to be due to the liberation of bound phenolics from the cell wall matrix. In support of these results, Ng et al. (1999) reported that extrusion cooking affects the cell wall matrix of onion waste, leading to depolymerization of pectins and hemicelluloses. Thus, extrusion cooking can serve as a useful means to release bound phenolics from the cell walls of foods. Meanwhile, some data indicated reduced antioxidant capacity upon extrusion processing. Altan et al. (2009) reported that extrusion processing resulted in 46–60% loss in total phenolic contents in barley compared to the corresponding raw material. The extrusion cooking reduced the antioxidant capacity of free and bound phenolics of green banana flour (Sarawong et al., 2014). Flavanones and flavones of sorghum (Sorghum bicolor L.) were significantly decreased (100%) during the extrusion cooking (Cardoso et al., 2015). Thus, the adverse effects indicate the need for

the development of alternative extrusion conditions such as minimizing shear and temperature stress to prevent loss of bioactive compounds (Killeit, 1994; Yajnik et al., 2010). In summary, extrusion cooking facilitates the liberation of bound phenolics from cell wall matrix via hydrolysis under high temperature and pressure. On the other hand, the high energy extrusion condition degrades the bioactive phenolic compounds with consequent loss of their antioxidant capacity.

Meanwhile, the baking process also affects the phenolic contents of staple foods. For example, insoluble-bound phenolics of rye whole meals were decreased upon baking and bread preparation from 1575 to 1472 μg/g dry matter; while the content of free phenolics such as ferulic acid was increased during the process due to the release of insoluble-bound phenolics (Hansen et al., 2002).

2.10.2.3. Hydrothermal or Boiling Process

The boiling process enhances the nutritional value, texture, and acceptability of legumes as well as degrading anti-nutritional factors such as tannins that reduce the digestibility of foods by interfering with the absorption of nutrients (Zdunczyk et al., 2003). The boiling process, which is a most common cooking method used as a hydrothermal treatment, involves heat energy, affecting the physicochemical properties of foods. The phenolics are also greatly influenced by the hydrothermal treatment that weakens the cell wall matrix and facilitates the liberation of bound phenolics (Chávez-Reyes et al., 2013), as well as causing a variety of chemical reactions with other compounds in which changes in the content and structures of phenolics occur.

Many studies on the boiling and hydrothermal treatment of foods have shown the increased content of phenolics and their antioxidant activity. The total phenolic contents of unripe and ripe plantains, eggplant and potato were increased after the boiling treatment (Ebun and Santosh, 2011; Chumyam

et al., 2013; Burgos et al., 2013). Bellail et al. (2012) investigated the effect of hydrothermal/boiling treatment on the changes of phenolic compounds of sweet potato and showed enhanced phenolic contents in some genotypes. Antioxidant capacity of potatoes was also improved after boiling as reflected in both ABTS and DPPH results (Burgos et al., 2013). Similar studies on cooked fruits and vegetables indicated enhancement of antioxidant capacity compared to their corresponding raw materials (Dewanto et al., 2002; Yamaguchi et al., 2001; Miglio et al., 2008).

On the contrary, some studies have revealed decreased phenolic contents after boiling treatment. Tsamo et al. (2015) reported that the boiling treatment reduced total phenolic content by about 34% in peel and most flavonols were decreased including kaempferol-3-O-rutinoside (61.3%) and rutin (59.8%), whereas ferulic acids were apparently increased (63.7%) in six plantain banana cultivars. Mazzeo et al. (2011) reported that chlorogenic acid, quercetin, and luteolin in carrots were degraded upon boiling (increase in kaempferol). Scaglioni et al. (2014) investigated the effect of boiling on changes of free and bound phenolics in rice and demonstrated that the contents of eight phenolic compounds, namely gallic, protocatechuic, chlorogenic, *p*-hydroxybenzoic, caffeic, syringic, *p*-coumaric and ferulic acids were reduced. López et al. (2013) reported that the boiling treatment decreased free phenolics such as protocatechuic acid, feruloyl aldaric acid, *p*-coumaric acid, proanthocyanidin dimer, hesperetin 7-neohesperidoside, naringenin 7-rutinoside and kaempferol 3-glucoside however, digestion and absorption rate at the intestinal level were improved and their neuroprotective and anticancer activities were maintained. Siah et al. (2014) studied the effect of boiling on the phenolic profiles and antioxidant capacity of faba bean. The results showed that boiling process decreased the total contents of phenolics and flavonoids as well as the antioxidant function as evaluated by the DPPH, TEAC, ORAC and FRAP assays. The

reduction of antioxidant capacity and phenolic content was thought to be due to the degradation of phenolics or binding with other insoluble-bound substances by the high heat energy upon boiling. Xiaoyun et al. (2009) reported 3-32% loss of ORAC value in eight potato cultivars after boiling; other studies also showed a reduction of polyphenolic content upon hydrothermal treatment (Aguilera et al., 2011; Siddhuraju et al., 2006; Turkmen et al., 2005). Hirawan et al. (2010) studied the changes in the phenolic content of pasta upon the boiling and reported a decreased level of ferulic acid in six different kinds of pasta. Thus, hydrothermal treatment reduces both the bound and soluble phenolics, possibly due to the chemical reaction with other compounds such as proteins and formation of irreversible covalent bonds that are not hydrolyzed in the extraction process (Ozdal et al., 2013).

CHAPTER 3

Materials and Methods

3.1 Materials

Four different varieties of lentils, namely CDC green land, CDC invincible, 3493-6, and maxim were provided by Professor Albert (Bert) Vandenberg of the Experimental Farm of the University of Saskatchewan, Saskatoon, Canada. Sodium chloride, methanol, ethanol, hexane, hydrochloric acid, acetic acid, sodium carbonate, diethyl ether, sodium hydroxide, ethyl acetate, acetone, mono- and dibasic potassium phosphates, potassium persulfate, acetonitrile, formic acid, sodium hydroxide, and trolox ((±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) were purchased from Fisher Scientific Co. (Nepean, ON, Canada). The compounds 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2′-azobis (2-methylpropionamidine) dihydrochloride (AAPH), hydrogen peroxide, 5,5-dimethyl-l-pyrroline-N-oxide (DMPO), 2,2-diphenyl-1-picrylhydrazyl (DPPH), trichloroacetic acid (TCA), aluminium chloride ($AlCl_3$), ethylenediaminetetraacetic acid (EDTA), potassium ferricyanide, sodium nitrite, quercetin, caffeic acid, gallic acid, sinapic acid, catechin, *p*-coumaric acid, epicatechin, ferrous sulfate, human LDL cholesterol, supercoiled plasmid pBR322 DNA, agarose, tris acetate, bromophenol blue, xylene cyanol, glycerol, *p*-nitrophenyl-α-D-glucopyranoside (PNPG), bovine serum albumin (BSA) and Folin Ciocalteu's phenol reagent were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). SYBR safe gel stain was obtained from Invitrogen Corporation (Carlsbad, CA, Canada).

3494-6

CDC invincible

CDC green land

Maxim

Figure 3.1 Four types of lentils used in the present study.

3.2 Methods

3.2.1 Sample preparation

3.2.1.1 Germination

Fifty grams of whole lentil (Invincible) were washed thoroughly with distilled water and then placed on aluminum plates and covered with a wet paper towel. The wetted paper towel was covered to prevent the evaporation of water. Aluminum foil was used to keep a dark environment, but allowing sufficient oxygen to enter as required for the respiration of lentils during germination through a small ventilation space (2cm diameter) and kept at 24.5±0.8 °C. The germination process was followed on days 1, 2, 3, and 4 (Figure 3.2). Water was supplied every 12 h to sustain adequate humidity. After germination, samples were dried in a convection oven at 55°C for 24h and then, stored at 4±1°C. Prior to the extraction process, defatting of germinated lentil was conducted using n-hexane. One gram of dried sample was ground to pass through a 2-mm mesh screen and then mixed with 15 mL of n-hexane; after removal of n-hexane this process was repeated two more times.

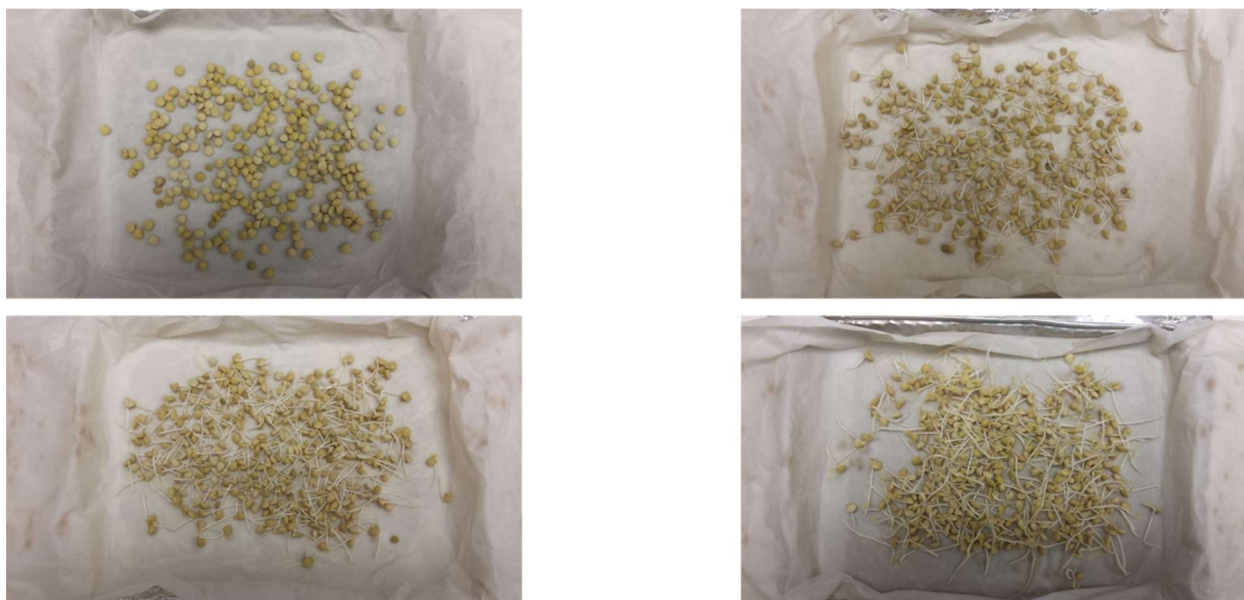


Figure 3.2 The growth of lentil seedlings during 4 days of germination (Invincible).

3.2.1.2 Boiling

Ten grams of each type of lentils were placed in 125 mL flask containing 50 mL boiling distilled water and then continuously boiled for 25 min on a hot plate. After 25 min, most of the distilled water was evaporated and subsequently cooled at ambient temperature. The boiled samples were then freeze-dried and analyzed for their antioxidant activity and phenolic composition. The significant differences between samples were established through one-way analysis of variance (ANOVA).

3.2.1.3 Dehulling

The dehulling process of lentils was followed as described by Chandrasekara and Shahidi (2011). Lentils were dehulled using a Seedburo hand grinder (Seedburo Equipment Co., Chicago, IL,

USA). The separated hulls and dehulled grains were isolated using air classification on a 757 South Dakota seed blower equipped with a column, which uses an air flow produced by a blower motor combination to separate hulls and dehulled grains (Seedburo Equipment Co., Des Plaines, IL, USA).

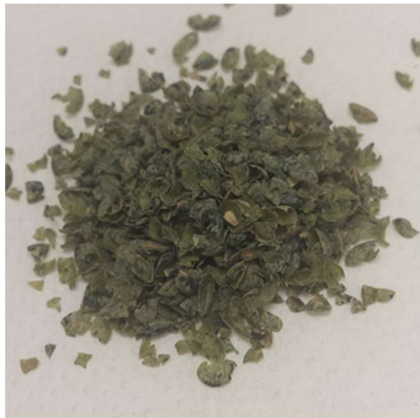


Figure 3.3 Hulls and dehulled grains of 3494-6.

3.2.2 Extraction of soluble- and insoluble-bound phenolics

Extraction of soluble phenolics (SPs) and insoluble-bound phenolics (IBPs) was carried out as described by Yeo and Shahidi (2017) with slight modification. One gram of hull was mixed with 10 mL of the extraction solvent (methanol/ water/ acetone, 1: 1: 1, v/v/v) and allowed to stand for

20 min at room temperature; this procedure was repeated two more times. Then, individual extracts were combined followed by the removal of the solvent using a rotary evaporator. The dried extracts were dissolved in 10 mL of methanol (HPLC grade) and subsequently stored at 5 °C until further analysis.

For the extraction of IBPs, 1g of the residue after extraction of SPs was blended with 15 mL of 2M NaOH, and the mixture vial was allowed to stand for 4h while stirring. The hydrolyzed samples were acidified with 4.63 mL of 6 M of HCl (< pH 2), and the liberated phenolics from the insoluble matrix of hulls were subsequently extracted with ethyl acetate five times. The solvent was then removed by using a rotary evaporator, followed by dissolution in 10 mL of methanol (HPLC grade) and storage at 5 °C until further analysis.

3.2.3 Antioxidant capacity

3.2.3.1 Total phenolic content (TPC)

Total phenolic contents of raw and processed lentils were determined as described by Singleton and Rossi (1965) with minor modification. An aliquot (125 µL) of each sample was mixed with 2 mL of distilled water and 63 µL of Folin-Ciocalteu reagent. Subsequently, the mixtures were allowed to react with 250 µL of 7% sodium carbonate and then, kept in the dark for 30 min at ambient temperature. The absorbance of the final products was read at 725 nm and results were expressed as mg gallic acid equivalents/g of dry weight (DW).

3.2.3.2 Total flavonoid content (TFC)

Total flavonoid content was measured as described by Kim et al. (2003) with slight modification. Briefly, 0.5 mL of lentil extract (20 mg/mL) was dissolved in 2 mL of distilled water, and then 0.15 mL of 5% $NaNO_2$ was added. After standing for 5 min, the samples were allowed to react with 0.15 mL of 10% $AlCl_3$ and 1 mL of 1 mol/L NaOH for 15 min in the dark. The absorbance was read at 510 nm, and total flavonoid content was expressed as mg catechin equivalents/g of DW.

3.2.3.3 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging ability using UV-visible spectrometry

DPPH radical scavenging ability of SPs and IBPs of geminated lentil was determined according to the procedure described by Brand-Williams et al. (1995) with slight modification. Briefly, 0.1 mM solution of DPPH in methanol (HPLC grade) was prepared and then allowed to stabilize for 12 h by standing with stirring at room temperature in the dark. To determine the DPPH radical scavenging ability of the samples, 1.9 mL of DPPH solution was mixed with 0.1 mL of sample, which was subsequently allowed to react for 30 min at room temperature in the dark. The absorbance was then read at 517 nm.

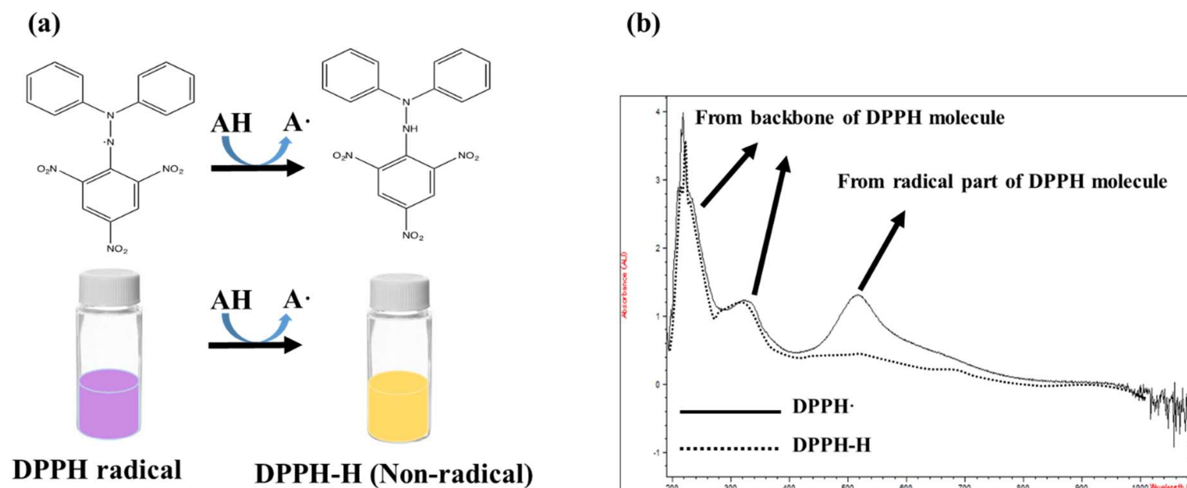


Figure 3.4 Changes in chemical structure, colour, and the absorbance of DPPH radicals after reduction with antioxidants.

3.2.3.4 2,2′-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation scavenging ability

ABTS radical cation was produced as described by Miller and Rice-Evans (1997) using ABTS and potassium persulfate. The ABTS radical cation (1 mL) so produced was diluted with 95% ethanol (90 mL) and adjusted to 0.070 ± 0.50 of absorbance at 750 nm. For the ABTS radical cation scavenging ability of sample, 100 uL of sample was mixed with 1.9 mL of the prepared ABTS solution and then allowed to react for 15 min in the dark room. The absorbance of the mixture was read at 750 nm.

3.2.3.5 Reducing power

The reducing power of raw and processed lentils was determined as described by Oyaizu (1986) with some modifications. The extracts (100 μL) were mixed with 0.5 mL of phosphate buffer solution (0.2 mol/L, pH 6.6) and 0.5 mL of potassium ferricyanide (1%, w/v). The mixture was incubated at 50 °C for 20 min, and then 0.5 mL of trichloroacetic acid (10%, w/v) was added to the mixture. Subsequently, the content was allowed to react with 0.5 mL of 0.1% (w/v) ferric chloride while standing for 5 min at room temperature. The absorbance was recorded at 700 nm and expressed as mg ascorbic acid equivalents (AAE)/g of DW.

3.2.3.6 DPPH radical scavenging ability using electron paramagnetic resonance (EPR) spectrometry

DPPH radical scavenging ability of SPs and IBPs of both raw and processed lentils was determined using a Brucker E-scan EPR spectrometer (Bruker Biospin Co., Billerica, MA, USA) according to the procedure described by Brand-Williams et al. (1995) with slight modification. The DPPH solution (0.2 mM, 1.9 mL) was mixed with 0.1 mL of sample and allowed to react at room temperature for 30 min in the dark. Subsequently, 1 mL of the resultant solution was injected via a syringe to the EPR spectrometer to read the changes of signals of DPPH radicals. The parameters selected for the analysis of signal were as follows: 1.86 G modulation amplitude, 5.02 X 102 receiver gain, 2.62 s sweep time100 G sweep width, 3495 G, 8 scans, 9.79 GHz microwave frequency, 1.86 G modulation, and 86.00 kHz modulation frequency. The signals of DPPH radicals were expressed as mg of trolox equivalents (TE)/g of DW.

3.2.3.7 Oxygen radical absorbance capacity (ORAC)

The ORAC assay was carried out using the method of Madhujith and Shahidi (2007) with slight modifications using a Fluostar Optima plate reader (BMG Labtech, Durham, NC). For the reaction, all chemicals and samples were diluted using a 75 mmol/L phosphate buffer (pH 7.0). Twenty microliters of test materials were placed on each plate well (Nepean, ON) and to which 200 µL of fluorescein (0.11 µM in PBS) were added. The mixture was pre-heated for 15 min at 37 °C in the built-in incubator. The equipment was programmed to inject 75 µL of AAPH (17.2 mg/mL in PBS) into each well before incubation. The changes of fluorescence were read every 210 s and expressed as the area under the fluorescein decay curve. The area was calculated with standard curve of trolox. Excitation and emission wavelengths were 485 and 520 nm, respectively.

3.2.4 Bioactivities of processed lentils

3.2.4.1 Cupric ion-induced human low-density lipoprotein (LDL) peroxidation

The inhibitory activity of human LDL peroxidation induced by cupric ion was evaluated according to the method described by Liyana-Pathirana and Shahidi (2015). In order to remove EDTA dissolved in LDL cholesterol, LDL was dialyzed in the 10 mM phosphate buffer (pH 7.4, 0.15 M NaCl) using dialysis at 4 °C under a nitrogen blanket in the dark for 12 h. For the observation of inhibitory activity against LDL oxidation, 0.8 mL of LDL cholesterol (0.04 mg LDL/mL) was mixed with 0.1 mL of soluble- and insoluble-bound phenolics in hulls and dehulled grains of lentils, subsequently pre-incubated at 37 °C for 15 min. After that, 0.1mL of cupric sulfate (50 µM) was added to the mixture and incubated at 37 °C for 11 h. The contents of yielded conjugated dienes (CD) from the oxidation of LDL cholesterol was read at 234 nm using a diode array spectrophotometer.

3.2.4.2 Inhibition of peroxyl and hydroxyl radical-induced supercoiled DNA strand scission

Inhibitory activity of phenolics from lentils against DNA (pBR 322 from *Escherichia coli RRI*) strand scission induced by peroxyl and hydroxyl radical was measured according to the method described by Chandrasekara and Shahidi (2011). First, DNA (50 μg/mL), which was dissolved in 10 mM phosphate buffer (PBS, pH 7.4), was mixed with PBS (2 μL), pBR 322 (50 μg/mL, 2 μL), 4 μL of 7 mM 2,2′-azobis (2-methylpropanimidamide dihydrochloride (AAPH), and phenolics extracted from lentils (0.1 mg/mL, 2 μL) to evaluate their inhibitory activity against peroxyl radicals induced oxidation. In addition, phenolics (6 mg/mL, 2 μL), PBS (2 μL), pBR 322 (50 μg/mL, 2 μL), $FeSO_4$ (0.5 mM, 2 μL), and H_2O_2 (0.5 mM, 2 μL) were mixed and incubated at 37 °C for 1 h for hydroxyl radical-induced oxidation. After the incubation, 1 μL of dye (0.25% bromophenol blue, 0.25% xylene cyanol, and 50% glycerol) was blended with the reaction mixture. Then, 10 μL of the resultant were loaded onto 0.7% agarose gel prepared in Tris−acetic acid−EDTA (TAE) buffer (40 mM Tri−-acetate containing 1 mM EDTA, pH 8.5), followed by the addition of SYBR safe (5 μL) into agarose gel solution (50 mL) as a gel stain. The analysis of electrophoresis was carried out using a model B1A horizontal mini gel electrophoresis system (Owl Separation Systems Inc., Portsmouth, NH, USA) and a model 300 V power supply (VWR International Inc., West Chester, PA) in TAE buffer. The reading of intensity (area %) of bands was conducted using the Chemi-Imager 4400 software (Cell Biosciences, Santa Clara, California, CA, USA).

3.2.4.3 α-Glucosidase inhibitory activity

α-Glucosidase inhibitory activity was determined according to Liu et al. (2011) with slight modification. The α-glucosidase (rat intestine) concentration used for this assay was 10 U/mL. Phenolics extracted were dissolved in 20 mM of sodium phosphate buffer (pH 6.8). In a test tube, 5 μL of α-glucosidase solution were mixed with 10 μL of phenolics and 620 μL of PBS, followed by pre-incubation at the oven for 20 min at 37 °C. The mixture was reacted with 10 μL of 10 mM *p*-nitrophenyl-α-D-glucopyranoside (PNPG) solution and incubated at 37 ºC for another 20 min. The reaction was terminated by adding 650 μL of 1 M Na_2CO_3, and absorbance was recorded at 410 nm.

3.2.5 HPLC-ESI-MS^n analysis

The HPLC analysis was carried out as described by Yeo and Shahidi (2017) with slight modification. The identification and quantification of phenolic compounds in SPs and IBPs of hulls were conducted using Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a G1311A quaternary pump, a G1379A degasser, and a G1329A ALS autosampler. Separation of phenolic compounds was carried out using SUPERLCOSIL LC-18 column (4.6 x 250 mm, 5 μm; Merck, Darmstadt, Germany), followed by detection using a diode array detector (DAD) at 280 and 330 nm. Analysis of data obtained was carried out by Chem Station software system (Agilent Technologies). The mobile phase was prepared with methanol/acetonitrile/formic acid (90:5:5; v/v/v) (eluent A) and 5% formic acid (eluent B) and gradient of elution was performed at 0 min, 20% A; 10 min, 30% B; 15 min, 40%B; 18 min, 45% B; 20 min, 50% B; 30 min, 70% B and 45 min, 80% B and the flow rate was 0.5 mL/min. HPLC-MS analysis was conducted with mass selective detector (MSD) ion trap system after ionization

by electrospray ionization (ESI) in the negative ion mode. The mass spectrometer detected ions in the range of *m/z* of 100 to 1000. The drying gas (N_2) temperature was fixed at 350 °C and nebulizer gas (N_2) pressure was 60 psi. The detected ions and fragment ions were used for the identification of phenolic compounds.

3.2.6 Statistical analysis

Statistical analysis was carried out among cultivars of lentils. All values yielded were the mean of three independent replicates, and then, the data was analyzed by one-way analysis of variance (ANOVA). The significant difference between individual groups was calculated by Tukey's HSD test using SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA). Significance was determined as a p-value of 0.05 or lower and expressed as a different letter.

Chapter 4

Effect of germination on the changes of antioxidant capacity of lentils

4.1 Introduction

Lentils have attracted much interest in recent years as an excellent source of dietary fibre, carbohydrates, protein, various vitamins, minerals, and several health-beneficial fatty acids (Adsule, 1996). Some epidemiological studies have reported their effects in lowering cholesterol and reducing colon cancer, heart diseases, and type-2-diabetes (Hsu et al., 2015; Morgan et al., 2013; Narasimhan et al., 2015; Eitsuka et al., 2014; Mancuso et al., 2014).

Germination is an efficient and economical bioprocess for improving the nutritional quality and functionality of different seeds, including legumes, and is well-studied in the plant physiology area. Reactive oxygen species (ROS) play a crucial role in seed development, dormancy breaking ability, and regulation of germination processes such as signaling molecules (Apel and Hirt, 2004). Meanwhile, phenolic compounds are responsible for controlling ROS that stimulates the production of phenolic compounds by activating enzymatic or nonenzymatic pathways (Świeca and Baraniak, 2014). This complex reaction process affects the composition and quantity of phenolic compounds, leading to changes in nutritional and functional quality of seeds.

The reason for alteration in the antioxidant activity of soluble phenolics (SPs) and insoluble-bound phenolics (IBPs) induced by germination has not been fully explored. For this reason, we tested the ratio of IBPs to SPs to see if it would provide a useful tool for better understanding and monitoring of changes in antioxidant activity by predicting the localization and molecular

transportation mechanism of phenolic compounds in plant cells during germination. Most phenolic compounds are synthesized in the intracellular space, especially in the endoplasmic reticulum, and are stored in vacuoles as SPs (Agati et al., 2012). On the other hand, IBPs are transported from the intracellular space and localized in cell walls by forming covalent bonds with insoluble macromolecules such as cellulose, arabinoglycan, and proteins or by self-polymerization. Therefore, changes in the ratio of these phenolics may provide a useful tool for predicting localization and transportation mechanisms of phenolic compounds and alteration of antioxidant activity upon processing, as exemplified for germination of lentils in this study. Thus, we monitored changes of antioxidant activity of SPs and IBPs procured from germinated lentils and used the ratio of IBPs to SPs to explain the results.

4.2 Results and Discussions

4.2.1 Total phenolic content (TPC)

The TPC of soluble- and insoluble-bound phenolics in germinated lentils was determined using Folin−Ciocalteu's assay, and results are shown in Table 4.1. The germination process led to the increase of TPC in both soluble phenolics (from 3.35 to 4.25 mg gallic acid equivalents (GAE)/g of defatted weight (DW)) and insoluble-bound phenolics (from 4.78 to 6.45 mg GAE/g of DW). In agreement with these findings, TPC of soluble phenolics isolated from germinated green lentils (*Lens esculenta*) showed an increase of TPC during 7 days (Cevallos-Casals and Cisneros-Zevallos, 2010). The sum of soluble phenolics and insoluble-bound phenolics, expressed as a total value, showed an increasing pattern from 8.13 to 10.69 mg/g, and this result might indicate a continuous synthesis of phenolic compounds during the germination process; longer germination time may lead to even better health beneficial effects, but this needs to be verified.

In the present study, a novel indicator, the ratio of insoluble-bound phenolics to soluble phenolics, is proposed as an efficient means to follow changes in the antioxidant activity of lentils during the germination process. The ratio of IBPs to SPs of lentil increased from 1.43 to 1.52. This result indicates slight alteration of phenolics from soluble to insoluble-bound phenolics during the germination process.

Table 4.1 Total phenolic content of soluble- and insoluble-bound phenolics of germinated lentils

Total phenolic content (GAE mg/g)				
Germination time (day)	Soluble	Bound	Total (soluble+bound)	Ratio (bound/soluble)
0	3.35 ± 0.02c	4.78 ± 0.03e	8.13 ± 0.05d	1.43 ± 0.00c
1	3.13 ± 0.04d	5.18 ± 0.08d	8.31 ± 0.10d	1.66 ± 0.03a
2	3.84 ± 0.06b	5.71 ± 0.01b	9.55 ± 0.06b	1.49 ± 0.02b
3	3.83 ± 0.06b	5.33 ± 0.11c	9.16 ± 0.07c	1.39 ± 0.05c
4	4.25 ± 0.06a	6.45 ± 0.08a	10.69 ± 0.14a	1.52 ± 0.00b

Values in each column having the same letter are not significantly different ($p > 0.05$)

4.2.2 Total flavonoid content (TFC)

The influence of germination on changes in TFC in SPs and IBPs is presented Table 4.2. TFC of SPs decreased from 2.49 to 1.96 mg catechin equivalents (CE)/g of DW during the 4 days of germination, whereas that of IBPs increased from 2.98 to 3.85 CE mg/g. The decrease of TFC in SPs may be related to the degradation of flavonoids by oxidants such as ROS, which is produced in cells, especially in the mitochondria, during electron transfer chain reaction (Nohl et al., 1998; Chance et al., 1979). ROS play a key role as signaling molecules and affect cell division and growth. In doing so, the concentration of ROS is controlled by phenolic compounds, which act as regulators in plant metabolism by scavenging ROS, resulting in degradation of phenolic compounds. On the other hand, a decrease of SPs can also be caused by their transport from intracellular space to cell wall and transformation from SPs into IBPs. This argument gains support from the findings of Strack et al. (1988) who reported that the increase in cell wall flavonoids during aging of Scots pine leaf paralleled a decrease in soluble flavonoids. They concluded that vacuolar efflux of these metabolites and deposition in the cell wall had occurred and that quercetin and kaempferol derivatives were also found in the cell wall of epidermal cells in lisianthus flowers petals (Markham et al., 2000). Phenylpropanoids, particularly hydroxycinnamic acid derivatives, contribute to cell wall formation through esterification with complex carbohydrates (McLusky et al., 1999). The phenolics synthesized at the endoplasmic reticulum were reported to be released in small vesicles that fuse in larger bodies and migrate to the cell wall, after fusion with the plasma membrane (Meyer et al., 2009).

The ratio of IBPs to SPs is also shown Table 4.2. This ratio was increased significantly from 1.19 to 1.96 ($p < 0.05$), indicating that the decrease of SPs is more likely due to the transport of flavonoids from intracellular to cell walls rather than degradation by ROS (Figure 4.1). This is because the decreased value (0.53) of SPs from 2.49 to 1.96 is comparable with the increased value in IBPs (0.87), and the difference (0.34) is similar to the increase of total value (0.35) from 5.47 to 5.82, which might be related to the synthesis of phenolic compounds during germination. Thus, both transport and synthesis of flavonoids may occur at the same time during germination. Further confirmation of this explanation requires a more detailed study using HPLC.

Table 4.2 Total flavonoid content of soluble- and insoluble-bound phenolics of germinated lentils

Total flavonoid contents (CE mg/g)				
Germination time (day)	Soluble	Bound	Total (soluble+bound)	Ratio (bound/soluble)
0	2.49 ± 0.03a	2.98 ± 0.18c	5.47 ± 0.21b	1.19 ± 0.06d
1	2.13 ± 0.05b	3.16 ± 0.01c	5.29 ± 0.05bc	1.49 ± 0.03c
2	2.17 ± 0.00b	3.56 ± 0.09b	5.73 ± 0.09a	1.64 ± 0.04b
3	2.01 ± 0.02c	3.16 ± 0.0c	5.17 ± 0.01c	1.58 ± 0.02b
4	1.96 ± 0.02c	3.85 ± 0.10a	5.82 ± 0.10a	1.96 ± 0.06a

Values in each column having the same letter are not significantly different ($p > 0.05$)

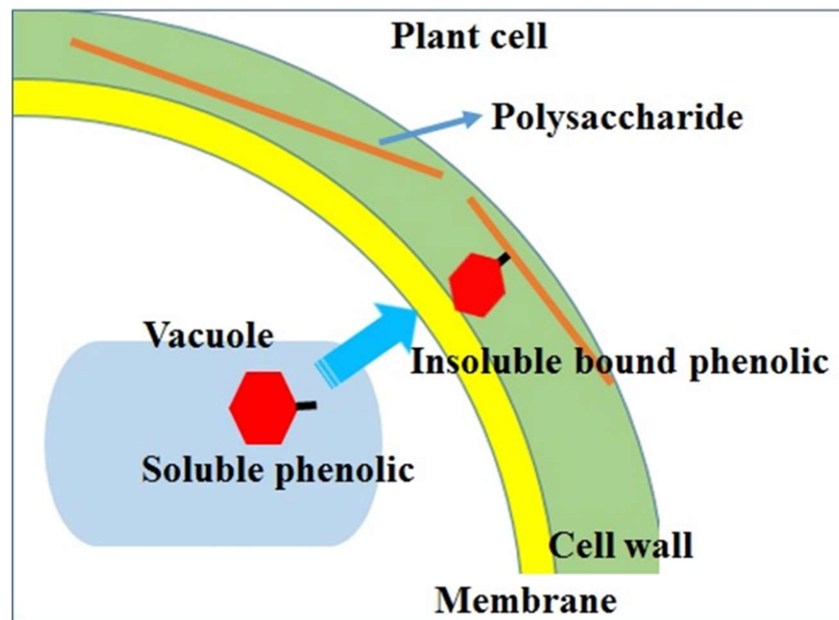


Figure 4.1 Possible pathway of transfer of phenolics from vacuole to the cell wall matrix (Yeo and Shahidi, 2015).

4.2.3 DPPH radical scavenging ability

The DPPH radical scavenging ability of SPs and IBPs in germinated lentils is shown in Table 4.3. The germination process decreased the DPPH radical scavenging ability of SPs from 61.3 to 53.5%, whereas an increase from 47.1 to 76.1% was found for IBPs. The continuous increase of total values induced by germination was from 111.5 to 122.8%, and this increment may be associated with the synthesis of phenolic compounds during the germination process. The ratio of IBPs to SPs significantly increased by approximately 2-fold from 0.78 to 1.42. This is most likely related to the enhancement of IBPs, possibly due to the transport of SPs into cell wall as discussed in the case of total flavonoids.

Table 4.3 DPPH radical scavenging abilities of soluble- and insoluble-bound phenolics of germinated lentils

DPPH radical scavenging ability (%)				
Germination time (day)	Soluble	Bound	Total (soluble + bound)	Ratio (bound/soluble)
0	61.3 ± 1.6a	47.7 ± 0.1e	109.0 ± 1.6d	0.78 ± 0.02e
1	55.0 ± 0.4c	53.2 ± 1.3d	108.2 ± 1.6d	0.97 ± 0.02d
2	58.4 ± 0.3b	65.8 ± 1.6b	124.2 ± 1.4b	1.13 ± 0.03c
3	53.1 ± 0.4d	62.9 ± 0.1c	116.1 ± 0.5c	1.19 ± 0.01b
4	53.5 ± 0.2d	76.1 ± 1.3a	129.7 ± 1.5a	1.42 ± 0.02a

Values in each column having the same letter are not significantly different ($p > 0.05$)

4.2.4 ABTS radical cation scavenging ability

The ABTS radical cation scavenging ability of SPs and IBPs of germinated lentils is shown in Table 4.4. Enhancement of ABTS radical cation scavenging ability of IBPs was observed in germinated lentils compared to the control (from 48.2 to 65.3%); a decreasing trend was found in SPs from 63.3 to 57.6% during 4 days of germination. A moderate increase in the total and ratio of IBPs to SPs was found in germinated lentils from 111.5 to 122.8% and from 0.76 to 1.13, respectively. The reason for this change may be the same as that discussed for TPC, TFC, and DPPH results.

Table 4.4 ABTS radical cation scavenging ability (%) of soluble- and insoluble-bound phenolics of germinated lentils

Germination time (day)	Soluble	Bound	Total (soluble + bound)	Ratio (bound/soluble)
0	63.3 ± 0.6a	48.2 ± 0.7d	111.5 ± 1.2b	0.76 ± 0.00e
1	56.7 ± 1.7c	49.9 ± 1.1d	106.6 ± 2.2c	0.88 ± 0.03d
2	60.9 ± 1.2b	58.5 ± 1.9b	119.4 ± 3.1a	0.96 ± 0.01b
3	57.8 ± 0.1c	53.3 ± 1.2c	111.1 ± 1.3b	0.92 ± 0.02c
4	57.6 ± 0.3c	65.3 ± 0.5a	122.8 ± 0.7a	1.13 ± 0.01a

Values in each column having the same letter are not significantly different ($p > 0.05$)

4.3 Conclusion

A new indicator, the ratio of IBPs to SPs, was proposed in order to explain the changes in the formation of phenolic compounds in lentils during the germination process. In all measurements tested, this ratio showed increasing trends, allowing speculation such as transport of phenolic compounds from vacuole into cell wall followed transformation into insoluble bound phenolics by forming covalent bonds with insoluble macromolecules. Although only one type of processing was given in this study, this ratio can be applied to many other types of process-induced phenolic changes in food for the underlying reasons proposed.

Chapter 5

Effect of hydrothermal treatment on the changes of antioxidant capacities of lentils

5.1 Introduction

Lentils serve as a valuable dietary source of protein, carbohydrate, minerals, vitamins, and dietary fibre (Rochfort and Panozzo, 2007), thus providing essential nutrients to many populations around the world, especially the vegetarians. Recently, several studies on the antioxidant capacity of lentils have demonstrated their excellent radical scavenging potential in *in vitro* systems such as 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethyl benzothiazoline-6-sulfonic acid) (ABTS), and hydroxyl radicals as well as reducing power (Alshikh et al., 2015; Xu and Chang, 2008; Yeo and Shahidi, 2015; Zhang et al., 2015).

Phenolic compounds, based on their solubility in the extraction medium, can be divided into two groups, namely soluble phenolics that include both the free and esterified phenolics and insoluble-bound phenolics. Most of the soluble phenolics are localized in the vacuole of plant cells and are trapped by weak interaction with other compounds (Li et al., 2012), whereas insoluble-bound phenolics are localized in the cell wall matrices through covalent bonds. The mechanism of the formation of insoluble-bound phenolics has not yet been well discussed in the food science field (Yeo and Shahidi, 2016). However, other research areas such as biology have investigated their synthesis, transfer, and formation in a variety of plant-based foods at a cellular level. In the formation of insoluble-bound phenolics, the synthesized phenolic compounds in the intracellular organs, mainly endoplasmic reticulum, are released and transported to the cell wall matrices

through the vesicles transfer system, facilitating migration of phenolic compounds into the cell wall (Meyer et al., 2009). The transported phenolic compounds are bound to the insoluble macromolecules such as protein, cellulose, and pectin through covalent bonding, including ether, ester, and carbon-carbon bonds in the cell wall matrices in which they play a significant role in building cell wall matrices and protecting from outer predators such as pathogens and insects (Dai et al., 1996; Nicholson and Hammerschmidt, 1992).

The insoluble-bound phenolics are not absorbed in the small intestine, since they are covalently bound to the insoluble macromolecules, leading to their transfer to the large intestine (colon) where they are fermented by a number of microorganisms, followed by their liberation due to the action of cell wall disintegrating enzymes such as cellulase, amylase, and pectinase released by the microorganisms. The released phenolics act as health-promoting compounds by influencing the colon environment, such as pH, leading to the inhibition of the growth of harmful bacteria. Meanwhile, soluble phenolics can be absorbed in the digestive tract, and their effectiveness in the biological system depends on their bioavailability and bioaccessibility since the structure of phenolics is generally altered during absorption and transport in the blood stream. Thus, investigation on the bioavailability and bioaccessibility of phenolics would provide valuable information about the effectiveness of the phenolics in biological systems (Carbonell-Capella et al., 2014).

Boiling is the most common cooking method for improving the acceptability, texture, and nutritional value of legumes as well as eliminating anti-nutritional factors (Shahidi and Naczk, 2004). A number of studies have reported the changes in the content of phenolic compounds and antioxidant capacity of plant-based foods by heat treatment such as boiling. For example, boiling was found to reduce the content of phenolic compounds such as caffeic acid, gallic acid, apigenin-

7-*O*-glucoside, ferulic acid, syringic acid, isovitexin and phloridzin in fennel (Rawson et al., 2013). The boiling process also decreased the antioxidant capacity, total phenolic content and DPPH radical scavenging activity of green pea, yellow pea, chickpea and lentil (Xu and Chang, 2008) and attenuated the antioxidant capacity of faba beans (Siah et al., 2014). However, most of these studies have only reported changes in the soluble phenolics, with a few considering the insoluble-bound phenolics. Therefore, studies on the differences of antioxidant capacity and content of insoluble-bound phenolics of legumes are needed in order to fill the existing gap in the available knowledge in the field.

The aims of this study were to (1) monitor changes of antioxidant capacity of insoluble-bound phenolics in four lentil cultivars such as Greenland, 3494-6, Invincible, and Maxim during boiling and (2) determine the profiles of insoluble-bound phenolics and their contents using HPLC-ESI-MS/MS.

5.2 Results and Discussions

5.2.1 Total phenolic content (TPC)

Total phenolic content (TPC) and total flavonoid content (TFC) of SPs and IBPs of raw and boiled lentils are presented in Table 5.1 and the results are expressed as gallic acid equivalents (GAE) and catechin equivalents (CE), respectively. A slight increase (4.8–8.5%) was found in SPs upon the boiling process as compared to the corresponding raw lentil cultivars. Meanwhile, IBPs were significantly reduced in all cultivars ($p < 0.05$) during the hydrothermal treatment, showing a decrease of 40.9, 38.8, 29.6, and 33.1% of IBPs in Greenland, 3494-6, Invincible, and Maxim, respectively ($p < 0.05$). The reduced values of IBPs were approximately four times higher than the increased values in SPs. Thus the sum of SPs and IBPs expressed as a total in Table 5.1 decreased upon the boiling treatment.

A few studies have shown improvement of TPC in SPs during boiling treatment as was the case for chickpea and potato (Burgos et al., 2013; Xu and Chang, 2008). However, a majority of research on the hydrothermal treatment of various foods revealed a reduction of phenolic contents in both SPs and IBPs. Scaglioni et al. (2014) studied the effect of boiling on changes in SPs and IBPs of rice in which both of SPs and IBPs were significantly reduced during the hydrothermal treatment and the reduced phenolics were gallic, protocatechuic, chlorogenic, *p*-hydroxybenzoic, caffeic, syringic, *p*-coumaric, and ferulic acids as well as vanillin as determined by HPLC analysis. López et al. (2013) reported that SPs such as trans-ferulic acid, sinapyl aldaric acid, and procyanidin dimer in dark beans were decreased during boiling. Xu and Chang (2008) observed changes of TPC of SPs in green pea, yellow pea, and lentil upon boiling for a different time and

under pressure, showing loss of TPC in all the three legumes tested. The decreasing tendency of phenolics was also found in other foods such as vegetables. For instance, the loss of TPC (about 20%) in cauliflower was measured in two cultivars after boiling (Volden et al., 2009). In addition, loss of TPC was shown in fennel bulb, and phenolics such as caffeic acid, gallic acid, apigenin-7-*O*-glucoside, ferulic acid, syringic acid, isovitexin, and phloridzin were reduced during the boiling treatment (Rawson et al., 2013). According to the literature, while most studies have been conducted on SPs, only a few have been carried out on IBPs. However, a number of authors have argued that hydrothermal treatment weakens the cell wall matrix, which leads to the liberation of the insoluble-bound phenolics (Chuck-Hernandez et al., 2011; Chávez-Reyes et al., 2013; Mora-Rochin et al., 2010). Thus, the literature reports support our results about the reduction of IBPs upon boiling. Meanwhile, the ratio of insoluble-bound phenolics to soluble phenolics was found to serve as an indicator for changes of phenolic formation from bound to free phenolics during food processing (Yeo and Shahidi, 2015). This study demonstrated the decreased ratios of bound to free phenolics in all lentil cultivars, indicating that bound phenolics were changed into soluble/free phenolics. This might be due to the dissociation of a covalent bond of insoluble-bound phenolics by the thermal energy provided during the boiling process, leading to their liberation from seed matrices.

Table 5.1 Total phenolic content of raw and hydrothermally processed (boiled) lentils

Cultivar	Raw (GAE mg/g)				Boiled (GAE mg/g, loss %)			
	SPs	IBPs	Total (SPs+IBPs)	Ratio (IBPs/SPs)	SPs	IBPs	Total (SPs+IBPs)	Ratio (IBPs/SPs)
Greenland	3.63 ± 0.02b	3.12 ± 0.02c	6.75	0.86	3.83 ± 0.07 (+5.5)a	1.85 ± 0.02 (-40.9)d	5.67 (-16.0)	0.48 (-43.9)
3494-6	4.03 ± 0.02b	3.64 ± 0.07c	7.68	0.90	4.34 ± 0.14 (+7.5)a	2.23 ± 0.01 (-38.8)d	6.56 (-14.5)	0.51 (-43.1)
Invincible	3.91 ± 0.06b	3.37 ± 0.04c	7.28	0.86	4.10 ± 0.04 (+4.8)a	2.37 ± 0.04 (-29.6)d	6.47 (-11.1)	0.58 (-32.8)
Maxim	3.22 ± 0.07b	3.00 ± 0.05c	6.22	0.93	3.50 ± 0.02 (+8.5)a	2.00 ± 0.01 (-33.1)d	5.50 (-11.6)	0.57 (-38.3)

Values in each row having the same letter are not significantly different ($p > 0.05$).

5.2.2 Total flavonoid content (TFC)

Total flavonoid content (TFC) showed a similar pattern to TPC during the boiling process. In the SPs, Maxim showed 2.4% increase in TFC, whereas Greenland and Invincible were decreased about 5.1 and 8.4% compared to their corresponding raw counterparts (Table 5.2). Siah et al. (2014) investigated the effect of boiling on the soluble phenolics of five faba bean cultivars which demonstrated a reduction in all. In the boiling treatment of dark beans, flavonoids in SPs such as naringenin, quercetin, kaempferol, and their derivatives were also significantly reduced by the hydrothermal energy (López et al., 2013).

As noted for TPC, the boiling process rendered major changes in the IBPs. The hydrothermal treatment caused 39.6, 42.8, 26.1, and 27.9% loss of IBPs in Greenland, 3494-6, Invincible, and Maxim cultivars, respectively. The significant loss in IBPs led to a decrease in TFC (sum of SPs and IBPs) in all lentil cultivars, ranging from 9.8 to 20.2%. Thus, reduction in total flavonoids in the lentils upon hydrothermal treatment and the loss of IBPs might be due to the liberation of bound phenolics from cell wall matrices as was the case for TPC.

In the present study, we hypothesized that the SPs will be increased after boiling treatment due to the release from the insoluble-bound phenolics, which can occur due to the disintegration of cell wall matrix of food by the hydrothermal energy (Figure 5.1). However, the increase in SPs was less than the decrease in the amount of IBPs, thus overall phenolic/flavonoid contents were reduced upon boiling, which might indicate possible chemical reactions of the released phenolics with other molecules present in the food matrix; the possible chemical reactions taking place will be discussed in the antioxidant capacity section of this thesis.

Table 5.2 Total flavonoid content of raw and hydrothermally processed (boiled) lentils

Cultivar	Raw (CE mg/g)				Boiled (CE mg/g, loss %)			
	SPs	IBPs	Total (SPs+IBPs)	Ratio (IBPs/SPs)	SPs	IBPs	Total (SPs+IBPs)	Ratio (IBPs/SPs)
Greenland	2.95 ± 0.03a	1.91 ± 0.04c	4.86	0.65	2.80 ± 0.03 (-5.1)b	1.15 ± 0.02 (-39.6)d	3.96 (-18.7)	0.41 (-36.4)
3494-6	3.11 ± 0.03a	2.28 ± 0.02b	5.40	0.73	3.11 ± 0.03 (0.0)a	1.31 ± 0.02 (-42.8)c	4.42 (-18.1)	0.42 (-42.8)
Invincible	2.87 ± 0.05a	1.82 ± 0.03c	4.69	0.64	2.63 ± 0.03 (-8.5)b	1.35 ± 0.01 (-26.1)d	3.98 (-15.3)	0.51 (-19.2)
Maxim	2.30 ± 0.02b	1.55 ± 0.02c	3.85	0.67	2.36 ± 0.02 (+2.4)a	1.12 ± 0.02 (-27.9)d	3.47 (-9.8)	0.47 (-29.6)

Values in each row having the same letter are not significantly different ($p > 0.05$).

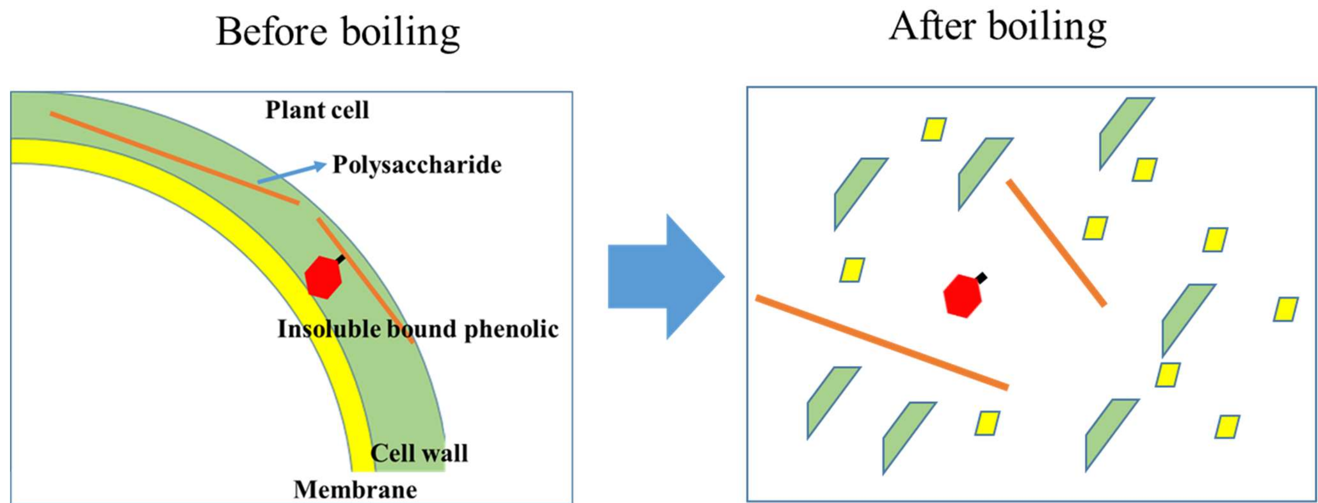


Figure 5.1 A possible pathway of the release of bound phenolics from cell wall matrix during hydrothermal processing.

5.2.3 Reducing power

The reducing power or electron donating ability of SPs and IBPs of the four lentil cultivars, namely Greenland, 3494-6, Invincible, and Maxim, are summarized in Table 5.3. The results are presented as mg ascorbic acid equivalents (AAE) per gram of dried weight (DW) of raw and processed lentils. A decrease of 8.0, 9.3, 49.1, and 11.3% in SPs was noted for Greenland, 3494-6, Invincible, and Maxim, respectively, upon boiling of raw lentil seeds. The Invincible cultivar however, showed the highest loss in reducing power (49.1%) among the tested lentil cultivars. Meanwhile, IBPs were significantly decreased in all lentil cultivars, ranging from 31.3 to 35.1% which are three times more than the loss in SPs. The loss of reducing power might be due to the reduced phenolic contents in both SPs and IBPs during the boiling process.

Table 5.3 Reducing power of raw and hydrothermally processed (boiled) lentils

Cultivar	Raw (AAE mg/g DW)				Boiled (AAE mg/g DW, loss %)			
	SPs	IBPs	Total (SPs+IBPs)	Ratio (IBPs/SPs)	SPs	IBPs	Total (SPs+IBPs)	Ratio (IBPs/SPs)
Greenland	8.78 ± 0.01a	7.69 ± 0.05b	16.47	0.88	8.08 ± 0.20 (-8.0)b	5.12 ± 0.28 (-33.5)c	13.19 (-19.9)	0.63 (-27.7)
3494-6	9.97 ± 0.06a	8.72 ± 0.03c	18.69	0.88	9.04 ± 0.11 (-9.3)b	5.75 ± 0.02 (-34.1)d	14.79 (-20.9)	0.64 (-27.3)
Invincible	9.75 ± 0.06a	8.55 ± 0.07b	18.30	0.88	4.96 ± 0.02 (-49.1)d	5.88 ± 0.03 (-31.1)c	10.84 (-40.8)	1.19 (+35.1)
Maxim	8.11 ± 0.18a	7.65 ± 0.05b	15.75	0.94	7.19 ± 0.06 (-11.3)c	4.96 ± 0.02 (-35.1)d	12.15 (-22.9)	0.69 (-26.8)

Values in each row having the same letter are not significantly different ($p > 0.05$).

5.2.4 DPPH radical scavenging capacity (DRSC) using EPR spectrometry

As the most common measure of radical scavenging capacity of natural products, DRSC has demonstrated much potential in predicting the electron or hydrogen atom donating capacity of functional food ingredients. In the present study, we employed DRSC to evaluate the radical scavenging capacity of SPs and IBPs of lentil cultivars as presented in Table 5.4. The boiling treatment increased DRSC of SPs in three lentil cultivars, namely Greenland, 3494-6, and Maxim, but that of Invincible cultivar decreased by 42%. The Invincible showed a different pattern compared to other cultivars, and the reason for this phenomenon has not been well explained. On the other hand, IBPs were significantly reduced by 18.9–39.4% in all lentil cultivars. Among the lentils used, Greenland cultivar exhibited the highest loss (39.4%) in DRSC, whereas 3494-6 showed 18.9% decrease which is lower than that of the other cultivars. All lentil cultivars used showed a reduction in their total DRSC during the hydrothermal treatment. However, 3494-2 lost only 2.5% of DRSC, exhibiting a relatively high hydrothermal stability compared to other cultivars.

Most prior work has so far been conducted on changes of soluble phenolics of natural products in which the boiling treatment primarily abated the DRSC. For example, Scaglioni et al. (2014) studied the effect of boiling on changes in SPs of rice and showed a decreasing tendency of DRSC during the hydrothermal process. The boiling process attenuated DRSC of SPs in fennel bulb by about 50% (Rawson et al., 2013). In addition, Xu and Chang (2008) observed changes in the DRSC of SPs in green pea, yellow pea, chickpea, and lentil during boiling for different times and pressure; they reported a loss of DRSC in all legumes tested. Zhang et al. (2014) reported that DRSCs of 4

lentil cultivars were decreased after boiling. Meanwhile, in some case, the boiling process enhanced DRSC of SPs in food. For instance, Burgos et al. (2013) reported that the hydrothermal treatment improved DRSC of SPs in purple-fleshed potatoes, possibly due to the release of phenolics from cell wall matrix, followed by conversion into soluble phenolics. However, the changes of the IBPs are rarely reported.

Table 5.4 DPPH radical scavenging ability of raw and hydrothermally processed (boiled) lentils

Cultivar	Raw (TE mg/g DW)				Boiled (TE mg/g DW, loss %)			
	SPs	IBPs	Total (SPs+IBPs)	Ratio (IBPs/SPs)	SPs	IBPs	Total (SPs+IBPs)	Ratio (IBPs/SPs)
Greenland	5.95 ± 0.23a	5.02 ± 0.06b	10.96	0.84	6.14 ± 0.08 (+3.3)a	3.04 ± 0.00 (-39.4)c	9.18 (-16.2)	0.49 (-41.4)
3494-6	6.67 ± 0.15b	4.74 ± 0.14c	11.41	0.71	7.28 ± 0.19 (+9.2)a	3.85 ± 0.09 (-18.9)d	11.13 (-2.5)	0.53 (-25.7)
Invincible	6.17 ± 0.27a	5.51 ± 0.00b	11.68	0.89	3.58 ± 0.06 (-42.0)c	3.56 ± 0.10 (-35.3)c	7.14 (-38.8)	1.00 (+11.4)
Maxim	5.22 ± 0.01b	5.24 ± 0.02b	10.46	1.00	5.65 ± 0.08 (+8.1)a	3.31 ± 0.15 (-36.8)c	8.96 (-14.4)	0.59 (-41.5)

Values in each row having the same letter are not significantly different ($p > 0.05$).

5.2.5 Oxygen radical absorption capacity (ORAC)

In a biological system, oxygen radicals such as superoxide and hydroxyl radicals exert a major effect on the oxidative stress that is induced by the imbalance between antioxidants and reactive oxygen species (ROS), which cause severe disorders in the normal cells, leading to fatal diseases such as cancer. The ORAC is an indirect *in vitro* method for measuring the potential of antioxidants to neutralize oxygen radicals by donating electrons. In the present study, ORAC values of SPs and IBPs derived from 4 lentil cultivars were evaluated and are presented in Table 5.5. In the SPs, the consistent pattern among lentil cultivars used was not found during the processing. Thus, Greenland showed increased ORAC (11.0%), while 3494-6, Invincible, and Maxim showed decreased values (6.5, 3.2, and 8.8%, respectively). In IBPs, three lentil cultivars showed reduced ORAC values, except 3494-2. The decrease ranged from 11.5 to 27.3%, and Invincible showed the highest reduction rate during the hydrothermal process. In short, a decreasing tendency in both of SPs and IBPs was found in ORAC values during the hydrothermal treatment. The loss of phenolics by the boiling process has also been reported in other foods. Xu et al. (2009) found that the boiling process reduced the ORAC of potato cultivars by approximately 3–32%. Siah et al. (2014) investigated the effect of boiling on the antioxidant capacity of faba bean in which the boiling process significantly reduced ORAC value of their SPs. Xu and Chang (2008) observed changes of ORAC of SPs in green pea, yellow pea, chickpea, and lentil during boiling treatment for different times and pressure showed a decreasing tendency of ORAC in the process. The loss of ORAC value was also observed in SPs of cauliflower by about 46% (Volden et al., 2009).

As mentioned earlier, we hypothesized that the SPs would be increased upon boiling due to the liberation of bound phenolics, which can occur by the disintegration of cell wall matrix of food by the hydrothermal energy. As we expected, IBPs showed an apparent decrease in contents as well

as antioxidant capacities during the hydrothermal processing, possibly due to the disintegration of cell wall matrices where the IBPs exist, thus facilitating the liberation of phenolics out of the cell wall matrices. However, SPs were not increased as much as the decreased amount of IBPs, thus overall phenolic/flavonoid contents were reduced after the boiling process, possibly due to the loss of soluble phenolics through chemical reactions with other molecules present in the food matrix under high energy treatment. The most probable mechanism is the interaction between phenolics and proteins during the hydrothermal treatment (Figure 5.2). O'Connell and Fox (1999) proposed that heat treatment can induce oxidation of phenolic compounds, which leads to formation of *o*-quinone and *o*-semiquinone; for example two hydroxyl groups of B-ring of flavonoids can donate hydrogen atom to the hydroxyl radicals, oxygen molecule, and transition metal, resulting in the formation of *o*-quinone by losing two hydrogen atoms or *o*-semiquinone by losing one hydrogen atom. The *o*-quinone so produced may react with thiol and amino groups of proteins via nucleophilic 1,4-Michael addition, which leads to the formation of irreversible covalent bond between the carbon atom of the aromatic ring of phenolics and nitrogen or sulfur atom of proteins (Le Bourvellec and Renard, 2012). Many different studies have reported on the interaction between phenolics and proteins such as interaction between soy protein and chlorogenic, caffeic, and gallic acids, flavones, apigenin, kaempferol, quercetin and myricetin (Rawel et al., 2002); β-lactoglobulin and sour cherry phenolics (Tantoush et al., 2011); and milk protein and caffeic acid (O'Connell and Fox, 1999) (Table 5.6). Thus, we may assume that IBPs are released from cell wall matrices during the hydrothermal treatment, but interestingly, the liberated phenolics were not observed in the SPs as much as the amount released, which might be due to the irreversible phenolic-protein or other possible interactions that cannot be released in the IBPs extraction procedure.

Table 5.5 Oxygen radical absorbance capacity (ORAC) of raw and hydrothermally processed (boiled) lentils

Cultivar	Raw (TE mg/g DW)				Boiled (TE mg/g DW, loss %)			
	SPs	IBPs	Total (SPs+IBPs)	Ratio (IBPs/SPs)	SPs	IBPs	Total (SPs+IBPs)	Ratio (IBPs/SPs)
Greenland	15.68 ± 0.76a	16.04 ± 0.28b	31.71	1.02	17.41 ± 0.72 (+11.0)a	12.07 ± 0.11 (-24.7)b	29.48 (-7.0)	0.69 (-32.2)
3494-6	14.98 ± 0.65a	13.55 ± 0.42b	28.54	0.90	14.01 ± 0.68 (-6.5)a	14.61 ± 1.27 (+7.8)b	28.63 (+0.3)	1.04 (+15.3)
Invincible	16.60 ± 0.91a	21.44 ± 5.06a	38.04	1.29	16.06 ± 1.10 (-3.2)a	15.58 ± 0.30 (-27.3)a	31.64 (-16.8)	0.97 (-24.9)
Maxim	14.94 ± 0.10b	16.44 ± 0.55a	31.38	1.10	13.63 ± 0.26 (-8.8)b	14.55 ± 0.45 (-11.5)b	28.18 (-10.2)	1.07 (-3.0)

Values in each row having the same letter are not significantly different ($p > 0.05$).

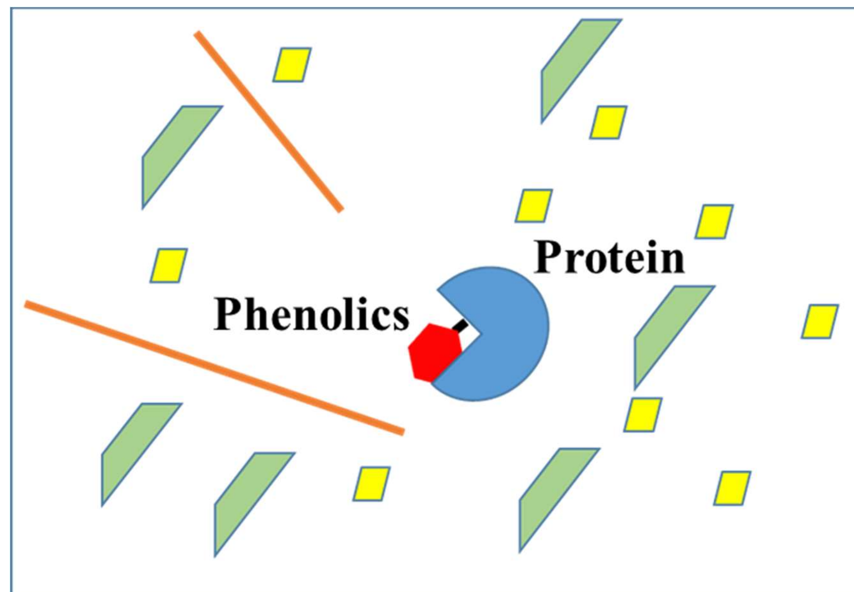


Figure 5.2 Formation of irreversible covalent bond between phenolic compound and protein.

Table 5.6 Formation of irreversible covalent bond between phenolics and proteins

Protein	Phenolic compounds	References
Soy protein	Chlorogenic-, caffeic-, gallic acid, flavones, apigenin, kaempferol, quercetin and myricetin	Rawel et al. (2002)
Fish myofibrillar protein	Caffeic acid, catechin, ferulic acid and tannic acid	Prodpran et al. (2012)
β-Lactoglobulin	Sour cherry phenolics (anthocyanins)	Tantoush et al. (2011)
Milk protein	Caffeic acid	O'Connell and Fox (1999)

5.2.6 HPLC-ESI-MS^n analysis

The HPLC-ESI-MS^n analysis was carried out in order to identify and quantify the individual IBPs upon boiling. A representative HPLC chromatogram of IBPs of lentils, SB-2, is shown in Fig. 5.3. In the HPLC-MS analysis, 10 compounds were positively identified from the four lentil cultivars along with another 7 compounds that were tentatively identified. As shown in the chromatogram, catechin was the dominant molecule among the detected compounds, accounting for approximately 70% of total IBPs in the raw lentils.

The seven tentatively identified insoluble-bound phenolics in four tested lentil cultivars are presented in Table 5.7. Procyanidin dimer B2 was tentatively identified according to its fragment ions and the literature data; one fragment ion was *m/z* at 289 (epicatechin) and deducting 289 from 577 (*m/z* of precursor ion) is 288, indicating that the compound has two catechin molecules and losing two hydrogen atom for the covalent bond, presumably leading to procyanidin dimer B2. From the identification analysis, catechin and catechin derivatives were the overwhelming insoluble-bound phenolics in the four lentil cultivars tested.

The quantification data for IBPs in raw and processed lentils are presented in Table 5.8. The data reflect changes in the antioxidant capacity of IBPs during hydrothermal treatment. As shown in the chromatogram earlier, catechin was the dominant phenolic compound in IBPs and was also the major compound that had its concentration decreased in IBPs, meaning that the reduction of antioxidant capacity in IBPs might be due to the loss of catechin. All lentil cultivars showed loss of catechin, ranging from 59 to 70% upon boiling. In addition, other compounds detected were

also decreased during the hydrothermal process. The HPLC analysis clearly shows the reduction of IBPs during the boiling process in all lentil cultivars tested.

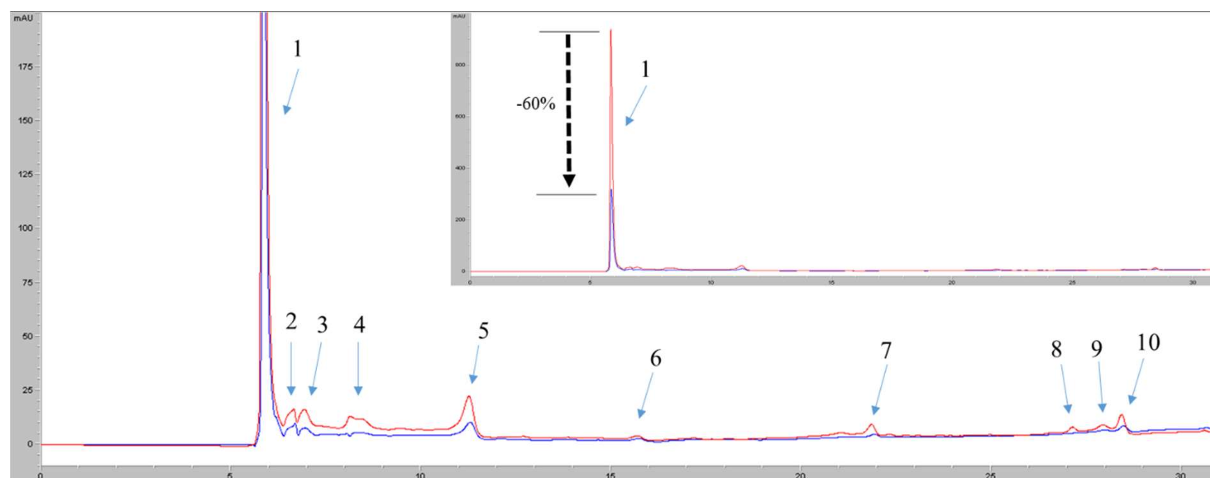


Figure 5.3 HPLC chromatogram of insoluble-bound phenolics of Greenland (raw: red, boiled: blue)(Yeo and Shahidi, 2017).

Table 5.7 Individual insoluble-bound phenolic compounds identified in raw and hydrothermally processed (boiled) lentils

Peak	Tentative assignment	$[M-H]^-$ (*m/z*)	RT(min)	Fragment ions
1	Catechin	289	5.8	287, 167, 137, 194, 247, 269
2	Protocatuic acid derivative	316	6.6	249, 181, 113
3	Catechin	289	6.9	245, 205, 179
4	Procyanidin dimer B2	577	8.1	425 407 289 451
5	Epicatechin	289	11.2	245, 205, 179
6	Epicatechin	289	15.6	245, 205, 179
7	Carboxylated quercetin	345	21.8	125, 301, 327, 243, 219

Table 5.8 The contents of insoluble-bound phenolics in raw and hydrothermally processed (boiled) lentils (mg/g of DW)

Peak	Tentative assignment	Greenland		3494-6		Invincible		Maxim	
		Raw	Boiled	Raw	Boiled	Raw	Boiled	Raw	Boiled
1	Catechin	2.07±0.01	0.83±0.01 (-60%)	2.17±0.11	0.74±0.00 (-66%)	1.09±0.01	0.32±0.01 (-70%)	2.16±0.02	0.89±0.02 (-59%)
2	Protocatechuic acid derivative	0.34±0.01	0.24±0.00 (-28%)	0.40±0.02	0.25±0.01 (-39%)	0.28±0.01	0.16±0.00 (-42%)	0.52±0.00	0.26±0.01 (-49%)
3	Catechin	0.13±0.00	0.08±0.00 (-35%)	0.17±0.04	0.08±0.00 (-55%)	0.06±0.00	ND (-100%)	0.14±0.01	0.08±0.00 (-46%)
4	Procyanidin dimer B2	0.11±0.00	ND (-100%)	0.09±0.00	ND (-100%)	0.06±0.00	ND (-100%)	0.10±0.00	0.06±0.00 (-42%)
5	Epicatechin	0.26±0.00	0.14±0.00 (-48%)	0.25±0.00	0.10±0.00 (-60%)	0.15±0.00	ND (-100%)	0.29±0.00	0.08±0.00 (-71%)
6	Epicatechin	0.06±0.00	ND (-100%)	ND	ND	ND	ND	0.06±0.00	ND (-100%)
7	Carboxylated quercetin	0.10±0.00	0.05±0.00 (-53%)	ND	0.05±0.00	0.10±0.01	0.05±0.00 (-51%)	0.09±0.00	0.05±0.00 (-47%)

ND: Not detected

5.3 Conclusion

Effect of boiling/hydrothermal treatment on the changes in antioxidant potential of insoluble-bound phenolics of lentil cultivars was investigated. The boiling treatment significantly reduced insoluble-bound phenolics in lentil cultivars as shown in the HPLC-ESI-MS^n analysis and antioxidant capacity determinations ($p < 0.05$). The total amount of phenolics, i.e., the sum of soluble and insoluble-bound fractions, was also accordingly decreased, possibly due to the formation of the irreversible covalent bond between liberated phenolics and proteins due to the hydrothermal energy. For better understanding of the loss of phenolics, further research on the formation of irreversible covalent bonds of phenolic compounds with proteins is required; other possible chemical reactions such as degradation and binding with other molecules should also be considered.

Chapter 6

Further exploration of the interaction of released phenolics with proteins in a model system

6.1 Introduction

In the previous chapter, the boiling treatment was shown to significantly reduce the content of insoluble-bound phenolics in lentil cultivars as shown in the HPLC-ESI-MS^n analysis and antioxidant capacity determinations. However, there was loss of phenolics, which may possibly be due to the formation of the irreversible covalent bonds between liberated phenolics and proteins resulting from the hydrothermal energy. Thus, further studies are needed in order to confirm the formation of irreversible covalent bonds of phenolic compounds with proteins.

Therefor, the aim of this supplementary experiment was to explore the reason for the loss of phenolics upon hydrothermal treatment, thus their degradation and binding with proteins were investigated in some preliminary experiments.

6.2 Methods

6.2.1 Interaction of bovine serum albumin (BSA) with phenolic compounds under hydrothermal treatment

The interaction of BSA with standard phenolics such as catechin, quercetin, coumaric acid, and caffeic acid was investigated under hydrothermal treatment conditions. Briefly, 5 mL of 0.1% (w/v)

solution of BSA were mixed with 1 mL of 2 mM phenolic solution. The mixture was subsequently heated in a convection oven at 125°C for 60 min to induce the reaction of BSA with phenolics. The content of the remaining phenolics after the reaction was measured using HPLC-ESI-TOF/MS.

6.2.2 Measuring the loss of free phenolics using HPLC-ESI-TOF/MS

HPLC analysis was carried out as described by Yeo and Shahidi (2017) with slight modification. Quantification of the remaining phenolics after the reaction was carried out using an Agilent 6230 TOF-HPLC/MS system (Agilent Technologies, Palo Alto, CA) equipped with a 1260 quaternary pump, a 1260 degasser, and a 1260 DAD (diode array detector). Separation of phenolic compounds was achieved using a SUPERLCOSIL LC-18 column (4.6 x 250 mm, 5 µm; Merck, Darmstadt, Germany), followed by detection using DAD at 280 and 330 nm. Analysis of data obtained was carried out by using Chem Station software system (Agilent Technologies). The mobile phase was prepared with methanol/acetonitrile/formic acid (90:5:5; v/v/v) (eluent A) and 5% formic acid (eluent B) and gradient of elution was performed at 0 min, 20% A; 10 min, 30% B; 15 min, 40%B; 18 min, 45% B; 20 min, 50% B; 30 min, 70% B and 45 min, 80% B and the flow rate was 0.5 mL/min. HPLC-MS analysis was conducted with mass selective detector (MSD) TOF system after ionization by electrospray ionization (ESI) in the negative ion mode. The mass spectrometer detected ions in the *m/z* range of 100 to 1000. The drying gas (N_2) temperature was fixed at 350 °C and nebulizer gas (N_2) pressure was 60 psi.

6.3. Results

6.3.1 Confirmation of phenolic-protein interaction under hydrothermal treatment

The interaction of phenolics with protein (BSA) upon hydrothermal treatment was tested, and the results are summarized in Table 6.1. Catechin, quercetin, coumaric acid, caffeic acid were selected to test the complexation and loss of free phenolics due to possible structural changes by chemical reactions using HPLC-ESI-TOF/MS. The results were expressed as the remaining content (%) of phenolics tested. The intact catechin and quercetin were significantly decreased by 77.1 and 58.3% upon hydrothermal treatment for 60 min, whereas the loss of coumaric acid and caffeic acid during the treatment was insignificant, indicating that no chemical interaction had occurred between BSA and these phenolic acids. The structural alterations of catechin and quercetin may possibly result from the interaction with BSA as well as other possible chemical reactions such as self-oxidation. Therefore, it is unclear whether the interaction with BSA leads to structural alteration of these flavonoids or not as these limited data indicate. Thus, further studies are required to reach a reasonable conclusion about their loss upon hydrothermal treatment.

Table 6.1 Remaining intact phenolics (%) after hydrothermal treatment at 125 °C in the presence of BSA using HPLC-ESI-TOF-MS

Heating time (min)	Catechin	Quercetin	Coumaric acid	Caffeic acid
0	100.0 ± 0.0a	100.0 ± 0.0a	100.0 ± 0.0a	100.0 ± 0.0a
30	99.8 ± 0.2b	61.3 ± 0.0b	95.8 ± 0.5b	99.5 ± 0.1b
60	77.1 ± 0.3c	58.3 ± 0.1c	100.0 ± 0.1a	99.2 ± 0.1b

Values in each column having the same letter are not significantly different ($p > 0.05$).

6.3.2 Confirmation of structural changes of phenolics under hydrothermal treatment

The effect of hydrothermal treatment on the stability of phenolics such as catechin, quercetin, coumaric acid and caffeic acid without BSA was carried out in order to confirm their possible structural changes (Table 6.2). The changes of intact phenolics after hydrothermal treatment were monitored using HPLC-ESI-TOF/MS. As the results indicate, the intact catechin and quercetin were significantly decreased upon hydrothermal treatment even in the absence of BSA, whereas coumaric and caffeic acids did not display any decreasing trend. Thus, structural changes of catechin and quercetin had occurred during the hydrothermal treatment, followed by alteration of their molecular structures.

Overall, the loss of catechin and quercetin had occurred in both the presence and absence of BSA upon hydrothermal treatment. The result so obtained indicate that structural changes of catechin and quercetin had taken place prior to any interaction with BSA. According to O'Connell and Fox (1999), the *o*-quinone formed by losing two hydrogen atoms in the B-ring of flavonoids reacts with thiol and amino groups of proteins via nucleophilic 1,4-Michael addition and this leads to the formation of irreversible covalent bonds. Therefore, oxidation of catechin, as evidenced in the present study, is interpreted as the initiation step, which yields *o*-quinone at the B-ring of flavonoids, followed by the formation of an irreversible covalent bond with BSA (protein) and is responsible for the decrease in the content of flavonoids upon hydrothermal treatment.

In addition to this supplementary data, the release of insoluble-bound phenolics from the lentil matrix (SB-2) and subsequent conversion into soluble phenolics was tested under hydrothermal treatment (data is not shown). The procedure for proving the liberation of bound phenolics was started with removing of the soluble phenolics from the lentil matrix, which facilitates the

identification of the liberated bound phenolics that are converted from bound to soluble phenolics. Subsequently, the remaining lentil matrix containing only bound phenolics was subjected to hydrothermal treatment to induce the release of bound phenolics. The released bound phenolics were extracted using appropriate medium and analyzed by HPLC-ESI-TOF-/MS. According to the result, the conversion of several phenolics from bound phenolics into soluble phenolics was confirmed; namely, catechin, quercetin, *p*-coumaric acid, syringic acid. This proves the liberation of bound phenolics from the lentil matrix as these limited data indicate. Thus, this preliminary experiment provides valuable information on the liberation of bound phenolics from the lentil matrix under hydrothermal treatment.

Table 6.2 Remaining intact phenolics (%) after hydrothermal treatment at 125 °C in the absence of BSA using HPLC-ESI-TOF/MS

Heating time (min)	Catechin	Quercetin	Coumaric acid	Caffeic acid
0	100.0 ± 0.0a	100.0 ± 0.0a	100.0 ± 0.0a	100.0 ± 0.0a
30	85.5 ± 1.4b	80.0 ± 0.7b	99.9 ± 0.8a	98.4 ± 0.4b
60	68.2 ± 0.2c	67.3 ± 0.2c	99.4 ± 0.1a	99.4 ± 1.3ab

Values in each column having the same letter are not significantly different ($p > 0.05$).

Chapter 7

Effect of dehulling on the changes of antioxidant capacities and bioactivities of lentils

7.1 Introduction

Lentils are valuable dietary sources of proteins, carbohydrates, minerals, vitamins, and dietary fibre (Rochfort, 2007). Moreover, they contain a number of health-promoting compounds such as phenolics, which are responsible for the effective bioactivities such as anticancer and anti-hepatitis C virus (HCV), as well as reducing diabetes and cardiovascular disease (Hsu et al., 2015; Morgan et al., 2013; Narasimhan et al., 2015; Eitsuka et al., 2014; Mancuso et al., 2014).

Legumes mainly consist of hull/seed-coat and dehulled grain part that includes endosperm, hypocotyl, and radicle. The hull portion is composed of several types of plant cells such as epidermis, hypodermis, chlorenchyma, palisade, parenchyma, and endothelium cells. These cells possess phenolics-containing organs such as vacuole and cell wall, thereby having high contents of phenolics in both soluble- and insoluble-bound forms. On the other hand, dehulled grain part is composed of endosperm, hypocotyl, and radicle, which are mostly composed of nutrient-storage cells that mainly contain proteins, starch granules and lipids, leading to a relatively low content of phenolics than the hulls (Figure 7.1).

Insoluble-bound phenolics are abundant in cereals, legumes, and other seeds, such as oilseeds and nuts. They are responsible for the rigid structure of the cell wall by connecting macromolecules such as cellulose, pectin, and structural protein to each other. The insoluble-bound phenolics are

covalently associated with the macromolecules through ether, ester, and covalent bonds. Cereals, legumes, and oilseeds contain high contents of insoluble-bound phenolics, 4~168 mg GAE/100g of insoluble-bound phenolics accounting for 20-60% of their total phenolics. Meanwhile fruits and vegetables contain a relatively low concentration of insoluble-bound phenolics of 3~29 mg GAE/100g, accounting for 7-38 % of total phenolics present (Nayak et al., 2015; Sun et al., 2002; Wolfe et al., 2008; Miller et al., 2000; Wang et al., 1996; Wu et al., 2006; Koponen et al., 2007).

Antioxidant potentials of lentil cultivars such as free radical scavenging ability, metal chelation, and oxygen radical absorbance capacity (ORAC) have been well established by many studies all over the world. However, bioactivities such as inhibitory activity against oxidation of LDL cholesterol and DNA strand breakage of lentils have not been well studied, especially for the insoluble-bound phenolics. Therefore, the aim of this study was to evaluate antioxidant potential and bioactivities of hulls and dehulled grains of four lentil cultivars such Greenland, 3494-6, Invincible, and Maxim.

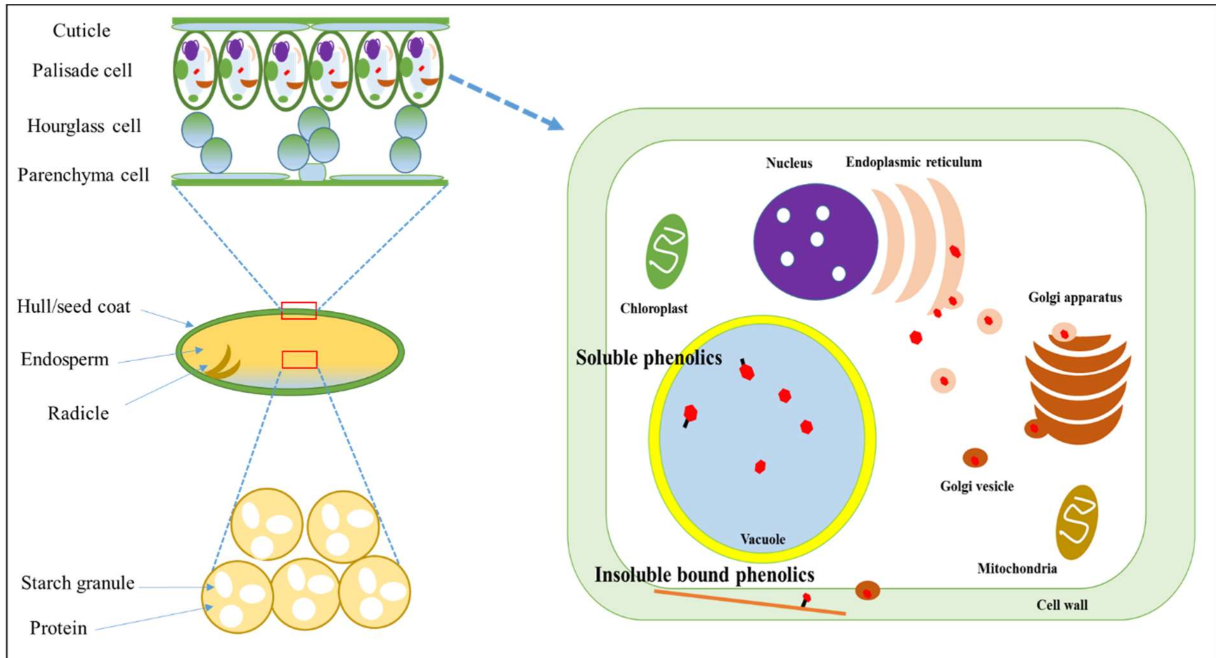


Figure 7.1 The microstructure of lentils and localization of soluble- and insoluble-bound phenolics in cells.

7.2 Results and Discussions

7.2.1 Total phenolic content (TPC)

Total phenolic content (TPC) was evaluated using Folin Ciocalteu's reagent for the prediction of the phenolic contents in lentil cultivars such as Greenland, 3494-6, Invincible, and Maxim and the results were expressed as mg gallic acid equivalents (GAE)/g of DW (Table 7.1). In hulls, the range of TPC in SPs was 31.5-33.6 mg GAE/g and the corresponding IBPs were 19.7-24.3 mg GAE/g, showing a higher TPC of SPs than IBPs. On the other hand, the dehulled grains of lentils showed significantly lower TPC than the corresponding hulls of lentils; the soluble phenolics of dehulled grains was 1.6 mg GAE/g of DW in four lentil cultivars and 1.0-1.2 mg GAE in the insoluble-bound phenolics. The results reveal the biased distribution of phenolics in lentils; in other words, both SPs and IBPs are more abundant in hulls than the corresponding dehulled grains of lentils. The biased distribution of phenolics toward the hull portion of this pulse has been well documented in other literature. For example, Das and Singh (2016) reported that maize possesses *p*-hydroxybenzoic, vanillic, syringic, caffeic, *p*-coumaric, ferulic, and isoferulic acid and their contents were 499, 1788, 83, 0, 3, 3247, and 842 µg/g in hulls, respectively, while only 0-27 µg/g was found in the endosperm. This supports the biased distribution of phenolics toward the hull portion than the dehulled part in pulses. Some studies have shown a lower TPC in whole lentils than their hulls. For example, Alshikh et al. (2015) investigated TPC in whole seeds of six lentil cultivars and the range of TPC in SPs was 3.6-27.0 and 1.2-17.5 mg GAE/g DW in IBPs. Moreover, Xu and Chang (2007) reported that the range of TPCs in lentils was 4.9–9.6 mg GAE/g, which is

lower than the TPC of hulls in the present study. These studies also support the biased distribution of phenolics toward hulls than their corresponding dehulled grains.

Meanwhile, the ratio that indicates the proportion of IBPs to SPs is summarized in Table 7.1. In our previous report, this new concept was suggested as an efficient indicator for the prediction of formation of phenolics, whether the phenolics are soluble- or insoluble-bound form in foods (Yeo and Shahidi, 2015). The range of the ratio was from 0.6 to 0.8 in four lentil cultivars in both hulls and dehulled grains, which indicates that a high percentage of phenolics is present in the soluble form as compared to the insoluble-bound form in both hulls and dehulled grains of lentils.

Table 7.1 Total phenolic content of SPs and IBPs in hulls and dehulled grains of lentils (Gallic acid equivalents, mg/g DW)

Cultivar	Hull				Dehulled			
	SPs	IBPs	Total	Ratio (IBPs/SPs)	SPs	IBPs	Total	Ratio (IBPs/SPs)
Greenland	32.8 ± 0.3b	19.7 ± 0.2d	52.5	0.6	1.6 ± 0.0a	1.1 ± 0.0b	2.7	0.7
3494-6	33.6 ± 0.1a	23.1 ± 0.2b	56.7	0.7	1.6 ± 0.0a	1.0 ± 0.0a	2.6	0.6
Invincible	32.5 ± 0.3b	24.3 ± 0.1a	56.8	0.8	1.6 ± 0.0a	1.2 ± 0.0b	2.8	0.7
Maxim	31.5 ± 0.1c	22.4 ± 0.2c	53.8	0.7	1.6 ± 0.1a	1.0 ± 0.0a	2.6	0.6

Values in each column having the same letter are not significantly different ($p > 0.05$).

7.2.2 Total flavonoid content (TFC)

Total flavonoid content of soluble- and insoluble-bound phenolics of four lentil cultivars showed a similar tendency to the total phenolic content (Table 7.2). Thus, TFC was in the range of 1.1-1.2 mg CE/g DW in the soluble phenolics and 0.7-0.9 mg CE/g in the insoluble-bound phenolics of dehulled lentils, whereas hulls showed a higher amount of total flavonoids, 7.2-8.4 mg CE/g in the soluble phenolics and 11.0-14.7 mg CE/g in the insoluble-bound phenolics. Thus, flavonoids were also dominant in the hulls compared to the corresponding dehulled grains of lentils.

Overall, the occurrence of phenolics was dominated in hulls as can be seen in the total phenolic content and total flavonoids. The reason for the concentration of phenolics in the hulls might be due to their physical structure and the difference in composition of cells in order to protect the seeds from the environment. As described in Figure 6.1, the structure of legumes can be largely divided into hulls and dehulled grains which include the endosperm and the radicle. The endosperm, which accounts for the most weight of legumes, is a nutrition-storage organ in which plants save energy sources such as starch, proteins, and lipids; thus, the endosperm cell mainly consists of starch granules and proteins, not the phenolic-containing organs such as vacuoles. On the other hand, hulls are composed of different types of cells such as palisade, hourglass, and parenchyma cells. These cells have phenolic-containing organs such as vacuoles, the organ where most soluble phenolics are localized. Therefore, the different composition of cells in hulls and dehulled grains can be a primary reason for the biased distribution of phenolics.

Table 7.2 Total flavonoid content (TFC) of SPs and IBPs in hulls and dehulled grains of lentils (Catechin equivalent, mg/g DW)

Cultivar	Hull				Dehulled			
	SPs	IBPs	Total	Ratio (IBPs/SPs)	SPs	IBPs	To tal	Ratio (IBPs/SPs)
Greenland	7.4 ± 0.5bc	11.0 ± 0.0d	18.4	1.5	1.1 ± 0.1a	0.8 ± 0.1a	2.5	0.8
3494-6	7.9 ± 0.1ab	13.0 ± 0.3b	20.9	1.6	1.2 ± 0.1a	0.9 ± 0.1a	2.8	0.7
Invincible	8.4 ± 0.1a	14.7 ± 0.1a	23.1	1.7	1.1 ± 0.1a	0.8 ± 0.1ab	2.8	0.8
Maxim	7.2 ± 0.0c	12.7 ± 0.1b	19.9	1.7	1.2 ± 0.1a	0.7 ± 0.1b	2.9	0.6

Values in each column having the same letter are not significantly different ($p > 0.05$).

7.2.3 DPPH radical scavenging capacity (DRSC) using EPR spectrometry

Antioxidant potentials such as DPPH radical scavenging ability, reducing power, and hydroxyl radical scavenging ability of dehulled lentils were determined and the results are presented in Table 7.3. First, DPPH radical scavenging ability was conducted to determine the hydrogen atom or electron donating ability of phenolics. According to the results, soluble phenolics from dehulled lentils did not show DPPH radical scavenging ability. This might be due to the low concentration of phenolics in dehulled lentils as already mentioned in the section for TPC and TFC to scavenge DPPH radicals. The insoluble-bound phenolics also showed a low DPPH radical scavenging ability. These results contrast with previous findings of the DPPH radical scavenging ability of the hulls of lentils in both soluble- and insoluble-bound phenolics. Hulls showed 97.4-134.7 mg trolox equivalent/g of DW in the soluble phenolics and 26.3-71.9 mg trolox equivalent/g of DW for the insoluble-bound phenolics. Thus, hulls showed a better DPPH radical scavenging ability than the corresponding dehulled grains of lentils. Chandrasekara et al. (2012) reported that the hulls of millets displayed a more effective DPPH radical scavenging ability than the corresponding dehulled grains of five tested cultivars, which agrees with the result of the present study. Shahidi et al. (2006) compared the DPPH radical scavenging ability of hulls and whole sesame seeds and the results revealed a three times higher DPPH radical scavenging ability of hulls than the whole seeds of black sesame. In addition, a better DPPH radical scavenging ability of the hulls compared to the whole grains was found in two faba beans (Boudjou et al., 2013), which also agrees with the results of the present study.

Table 7.3 DPPH radical scavenging ability of SPs and IBPs in hulls and dehulled grains of lentils (trolox equivalent, mg/g DW)

Cultivar	Hull				Dehulled			
	SPs	IBPs	Total	Ratio (IBPs/SPs)	SPs	IBPs	Total	Ratio (IBPs/SPs)
Greenland	104.6 ± 9.0b	26.3 ± 8.7c	130.8	0.3	0.0 ± 0.8a	0.0 ± 0.4b	0.0	N/A
3494-6	134.7 ± 2.8a	79.9 ± 3.3a	214.6	0.6	0.0 ± 0.1a	2.1 ± 0.4a	2.1	N/A
Invincible	103.1 ± 3.0b	45.5 ± 1.4b	148.7	0.4	0.0 ± 0.1a	2.1 ± 0.1a	2.1	N/A
Maxim	97.4 ± 4.9b	71.9 ± 4.8a	169.3	0.7	0.6 ± 1.7a	0.0 ± 0.1b	0.6	N/A

Values in each column having the same letter are not significantly different ($p > 0.05$).

7.2.4 Reducing power

The reducing power is defined as a capacity to donate one electron to molecules, which can contribute to the breaking radical chain reaction of lipid oxidation. Thus, the reducing power of phenolics was determined to evaluate electron donating ability of lentils, and the results are summarized in Table 7.4. As shown, soluble- and insoluble-bound phenolics of dehulled lentils hardly showed any reducing power; in other words, 1.0-1.2 mg AAE/g DW in soluble phenolics and 0.5-0.9 mg AAE/g DW in insoluble-bound phenolics were found in dehulled grains of lentils. On the other hand, hulls showed better reducing power by exhibiting 60.6-70.4 mg AAE/g for soluble phenolics and 44.6-54.5 mg AAE/g for insoluble-bound phenolics, which clearly reveals the uneven distribution of phenolics in lentils as already discussed in the TPC and TFC section.

Table 7.4 Reducing power of SPs and IBPs in hulls and dehulled grains of lentils (ascorbic acid equivalent, mg/g DW)

Cultivar	Hull				Dehulled			
	SPs	IBPs	Total	Ratio (IBPs/SPs)	SPs	IBPs	Total	Ratio (IBPs/SPs)
Greenland	61.3± 0.3c	44.6 ± 0.4d	106.0	0.8	1.2 ± 0.0b	0.9 ± 0.1a	2.1	0.8
3494-6	70.4 ± 1.8a	51.3 ± 0.7b	121.7	0.5	1.2 ± 0.0ab	0.6 ± 0.1bc	1.8	0.5
Invincible	67.4 ± 1.0b	54.5 ± 0.9a	121.9	0.5	1.2 ± 0.0a	0.6 ± 0.0b	1.9	0.5
Maxim	60.6 ± 0.9c	47.7 ± 0.8c	108.3	0.4	1.0 ± 0.0c	0.5 ± 0.0c	1.5	0.4

Values in each column having the same letter are not significantly different ($p > 0.05$).

7.2.5 Hydroxyl radical scavenging ability

Another antioxidant potential measured was the hydroxyl radical scavenging ability. Hydroxyl radicals are commonly found in biological systems, as they are naturally produced during Fenton's reaction in the mitochondria. The generation of hydroxyl radicals performs a key function in the cell life, for instance, they are involved in the metabolism of cells by acting as a signaling molecule. However, they could also have a negative effect on the body cells once they are produced excessively, which can cause an imbalance between free radicals and antioxidants, also known as oxidative stress. Thus, providing appropriate concentrations of antioxidants such as phenolics through the diet can reduce oxidative stress in the body cells. In this study, we determined the hydroxyl radical scavenging ability of phenolics extracted from dehulled lentils and compared the results with those from the hull portion conducted in the previous study (Table 7.5). In the results, unlike the DPPH radical scavenging ability and reducing power, dehulled lentils showed a comparable hydroxyl radical scavenging ability to the corresponding hulls; in other words, the hydroxyl radical scavenging ability of soluble- and insoluble-bound phenolics in the dehulled seeds were 7.1-10.1 and 6.1-12.3 mg CE/g of DW, which is lower than the hull fraction that exhibited 14.5-19.8 and 17.4-20.4 mg CE/g of DW, as was reported in the previous study. However, they displayed a better activity than the other antioxidant capacity assays employed. Chandrasekara et al. (2012) reported that the hydroxyl radical scavenging abilities of millets were 7.7-28.4 in the dehulled section of the grains and 39.1-196.0 µmol ferulic acid equivalents/g defatted meal in hull parts in six cultivars, indicating a better hydroxyl radical scavenging ability of the hull part than the corresponding dehulled grains. In all measurements of antioxidant potentials, the hulls displayed better antioxidant capacity than the corresponding dehulled grains

of lentils, which might be related to the different distribution of phenolics in the hulls and the dehulled grains of lentils, as exhibited in their TPC and TFC.

Table 7.5 Hydroxyl radical scavenging activity of SPs and IBPs in hulls and dehulled grains of lentils (catechin equivalent, mg/g DW)

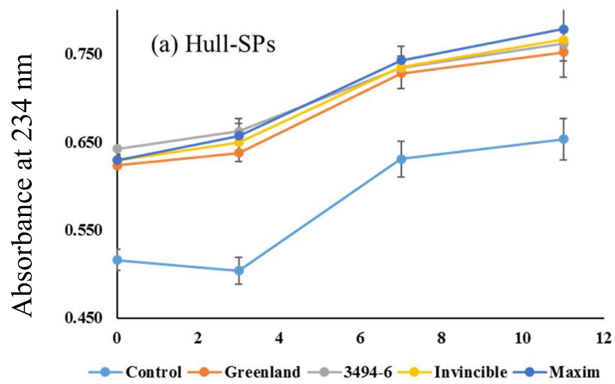
Cultivar	Hull				Dehulled			
	SPs	IBPs	Total	Ratio (IBPs/SPs)	SPs	IBPs	Total	Ratio (IBPs/SPs)
Greenland	19.5 ± 0.7a	17.4 ± 0.5c	36.9	0.9	8.4 ± 0.5a	6.1 ± 0.9c	14.5	0.7
3494-6	19.8 ± 1.4a	19.0 ± 0.3b	38.7	1.0	10.1 ± 0.6a	7.9 ± 0.4b	18.0	0.8
Invincible	14.5 ± 2.6b	20.2 ± 0.3a	34.7	1.4	8.5 ± 3.3a	8.7 ± 0.4b	17.2	1.0
Maxim	14.6 ± 0.1b	20.0 ± 0.2a	34.6	1.4	7.1 ± 0.1a	12.3 ± 0.2a	19.4	1.7

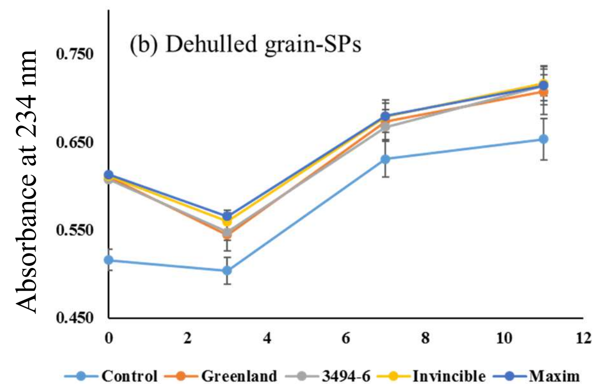
Values in each column having the same letter are not significantly different ($p > 0.05$).

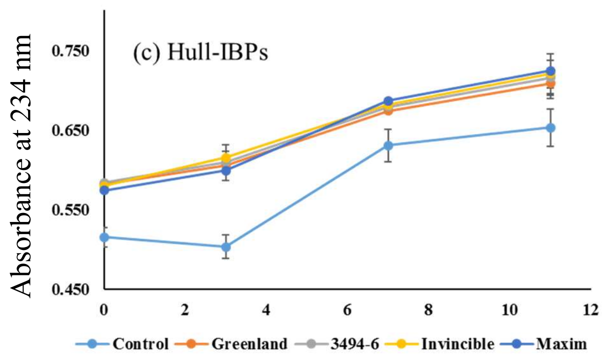
7.2.6 Inhibitory activity of soluble- and insoluble-bound phenolics in hulls and dehulled grains of lentils against oxidation of LDL cholesterol

LDL cholesterol performs a key function in transporting triacylglycerols and cholesterol to body cells by acting as a shuttle, thus serving as an essential compound for the life and growth of cells. Nevertheless, they are known as a "bad cholesterol" and are considered as a harmful factor in causing cardiovascular disease, especially once they are oxidized in the blood stream and are accumulated at the arterial wall, leading to the narrowing of blood vessels and finally blocking the blood stream. Therefore, preventing the oxidation of LDL cholesterol is a key to reducing possible vascular diseases. In the present study, the LDL cholesterol oxidation inhibitory activity of soluble- and insoluble-bound phenolics in hulls and dehulled lentils was determined as summarized in Figure 7.2. According to the results, the dehulled grains of lentils in both soluble- and insoluble-bound phenolic fractions showed a decrease in absorbance at 234nm after 3h of incubation, which was not the case in the hulls. The decrease of absorbance might be due to the loss of phenolics since absorbance at 234 nm is partially derived from phenolics, as can be seen in the absorbance at 0 h of incubation in Figure 7.2. Therefore, the oxidation of LDL cholesterol is calculated after 3h of incubation. Absorbance at 234 nm of LDL cholesterol without phenolics (control) was increased from 0.504 (3h) to 0.653 (11h), with an increase of 0.149 in absorbance, which indicates the formation of oxidation products such as conjugated dienes during the oxidation of LDL cholesterol. The LDL cholesterol consists of an outer surface, which is mainly composed of phospholipids and lipoproteins, and an inner space packed with cholesterol, cholesterol-esters, and triacylglycerols. Therefore, the formation of conjugated dienes is mainly caused by the oxidation of phospholipids and triacylglycerols. In the soluble phenolics of hulls, the absorbances in the

Greenland, 3494-6, Invincible, and Maxim cultivars was increased by 0.115, 0.099, 0.117, and 0.121, respectively, during the 11h of incubation, which corresponds to 23, 22, 21, and 18% of inhibition against the oxidation of LDL cholesterol compared to the control (an increase of 0.149). In the same way, the insoluble-bound phenolics in the hulls of lentils also attenuated the oxidation of LDL particles by 16-32% during LDL oxidation. On the other hand, the dehulled grains of lentils did not suppress oxidation of LDL cholesterol. This result is probably related to the different content of phenolics between the hulls and the dehulled grains of lentils as was already discussed in the TPC and TFC section. Chandrasekara and Shahidi (2011) studied the inhibitory activities of hulls and dehulled grains of millets against oxidation of LDL cholesterol and reported a better inhibitory activity of hulls than the corresponding dehulled grains, which agrees with the results of the present study.







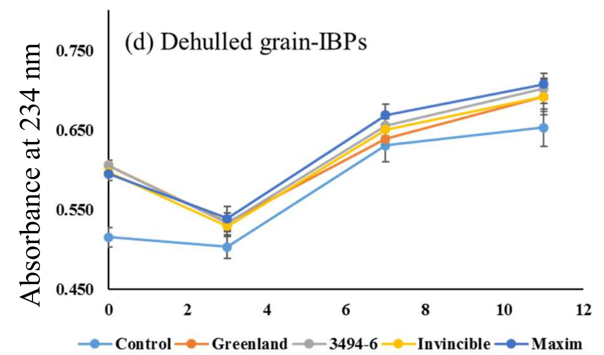


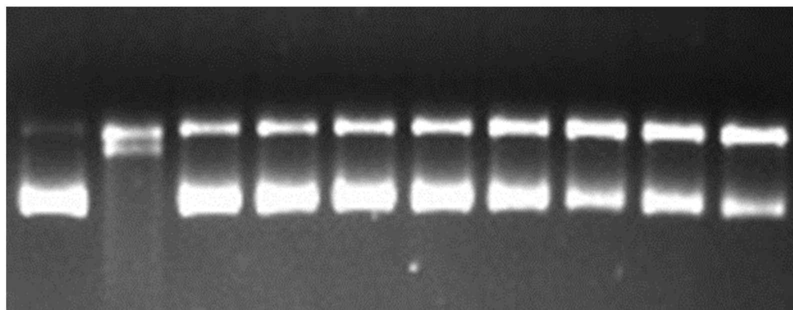
Figure 7.2 LDL oxidation inhibitory activity of hulls and dehulled grains of lentils.

7.2.7 DNA scission inhibitory activity of hulls and the dehulled grains of lentils

The oxidation of DNA is one mechanism responsible for the mutation of cells, which causes the dysfunction of cells and subsequently leads to serious adult disease such as cancer. Thus, preventing the oxidation of DNA is very important for maintaining the health of the cells. In the present study, we investigated the inhibitory activity of phenolics extracted from the hulls and the dehulled grains of lentils against the oxidation of the DNA; the results are summarized in Table 7.6. Figure 7.3 shows one example which clearly displays the relatively strong inhibitory activity of hulls compared to the corresponding dehulled grains of lentils. In both hydroxyl radical- and peroxyl radical-induced oxidation, hulls showed a stronger inhibitory activity than the dehulled grains; the hulls having a 41-54% inhibitory activity in the soluble phenolics and 58-65% of inhibition in the insoluble-bound phenolics for the hydroxyl radical-induce oxidation. On the other hand, the dehulled grain fractions showed a somewhat lower inhibitory activity against DNA oxidation than the corresponding hulls by displaying 20-44% inhibition in the soluble phenolics and 32-52% in the insoluble-bound phenolics. In the peroxyl radical-induced oxidation, similar results were observed with a better inhibitory activity in hulls than in the dehulled grains. Chandrasekara and Shahidi (2011) studied the inhibitory activity of the hulls and the dehulled grains of millets against oxidation of DNA and reported a better inhibitory activity of the hull fraction than the corresponding dehulled grains, which agrees with the results of the present study.

In intracellular spaces, reactive oxygen species (ROS) are simultaneously produced at the mitochondria through Fenton's reaction, and they act as a signaling molecule in which they perform essential works for the metabolism of the cells in the body. Meanwhile, a high

concentration of the ROS in the body cells can cause oxidative stress, further leading to the oxidation of DNA strand. Thus, with respect to the prevention of oxidation of DNA strands, the intake of additional antioxidants through the diet might be helpful in maintaining the balance between ROS and antioxidants in the body cells. Therefore, lentils can serve as a suitable diet source for the prevention of oxidation of DNA.



1 2 3 4 5 6 7 8 9 10

Figure 7.3 The DNA scission inhibitory activity of insoluble-bound phenolics of hulls and dehulled grains of lentils at the hydroxyl radical oxidation system (1, blank; 2, control; 3, Greenland hull; 4, 3494 hull; 5, Invincible hull; 6, Maxim hull; 7, Greenland dehulled; 8, 3494 dehulled; 9, Invincible dehulled; and 10, Maxim dehulled.

Table 7.6 DNA scission inhibitory activity of phenolics in hulls and dehulled grains of lentils

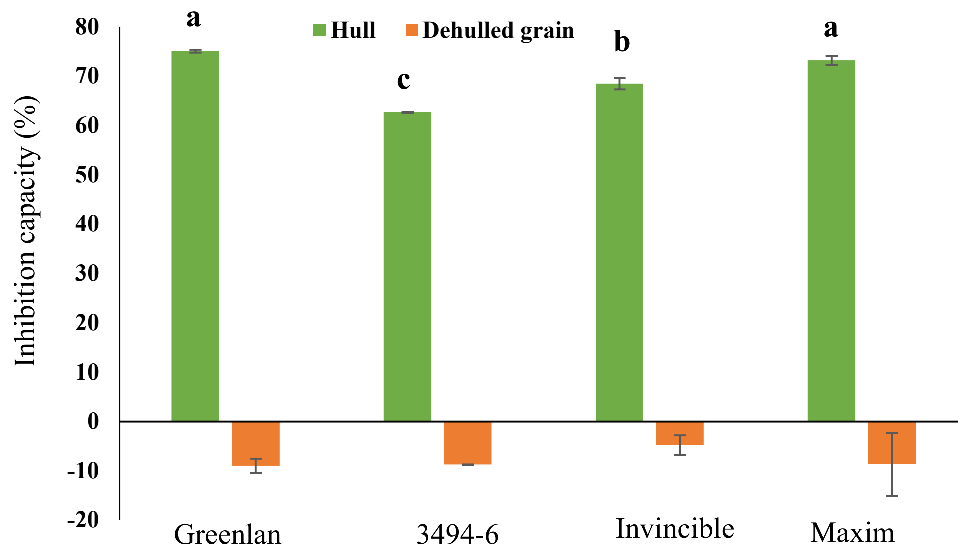
Cultivar	*Hydroxyl radical-induced oxidation (inhibition %)*			
	Hull		Dehulled grain	
	SPs	IBPs	SPs	IBPs
Greenland	43 ± 8a	65 ± 4a	31 ± 4b	52 ± 14a
3494-6	45 ± 9a	65 ± 7a	30 ± 2bc	45 ± 15a
Invincible	41 ± 13a	58 ± 6a	44 ± 5a	48 ± 11a
Maxim	54 ± 2a	63 ± 6a	20 ± 2c	30 ± 5a
Cultivar	***Peroxyl radical induced oxidation (inhibition %))***			
	Hull		Dehulled grain	
	SPs	IBPs	SPs	IBPs
Greenland	97 ± 1a	97 ± 8a	67 ± 2a	24 ± 1a
3494-6	99 ± 4a	98 ± 6a	60 ± 1ab	18 ± 3a
Invincible	97 ± 0a	95 ± 4a	59 ± 3ab	19 ± 8a
Maxim	97 ± 1a	95 ± 4a	53 ± 6b	10 ± 8a

Values in each column having the same letter are not significantly different ($p > 0.05$).

7.2.8 α-Glucosidase inhibitory activity of hulls and dehulled grains of lentils

The occurrence of type-2-diabetes has dramatically increased in recent years globally. It is well-known that type-2-diabetes starts with high blood sugar. A high concentration of blood sugar increases the release of insulin to absorb the excessive sugar molecules present in the blood stream. However, chronic high blood sugar causes insulin resistance, thus subsequently cells do not respond to insulin by inactivating the insulin-receptor at the surface of the cells. This further leads to type-2-diabetes, causing serious complications such as heart disease, stroke, and diabetic retinopathy. Thus, controlling sugar content in blood stream is key to the prevention of type-2-diabetes. Meanwhile, α-glucosidase is involved in the hydrolysis of polysaccharides into simple sugars, facilitating the supplement of energy sources to the body cells. However, α-glucosidase has a negative effect on the patients who are suffering from diabetes, since the enzyme yields more sugar and increases sugar content in the blood stream of patients. Therefore, inhibition of the activity of α-glucosidase is one of the effective treatments for both reducing complications of the patients who are suffering from type-2-diabetes and preventing the initiation of type-2-diabetes. The α-glucosidase inhibitory activities of phenolics in hulls and dehulled grains of lentils were measured and the results are shown in Figure 7.4. According to the results, soluble phenolics in hulls showed a better α-glucosidase inhibitory activity than the corresponding dehulled grains; soluble phenolics in hulls reduced 62–75% activity while dehulled grains did nothing. Similar results were found in the insoluble-bound phenolics; hulls of lentil cultivars attenuated α-glucosidase activity by approximately 45-80%, whereas dehulled grains did not generally show any α-glucosidase inhibitory activity, except for Maxim (23%). Overall, hulls in both soluble- and insoluble-phenolics showed a higher α-glucosidase inhibitory activity than the corresponding

dehulled grains, which might be due to the difference in localization of phenolics in the structure of lentils.



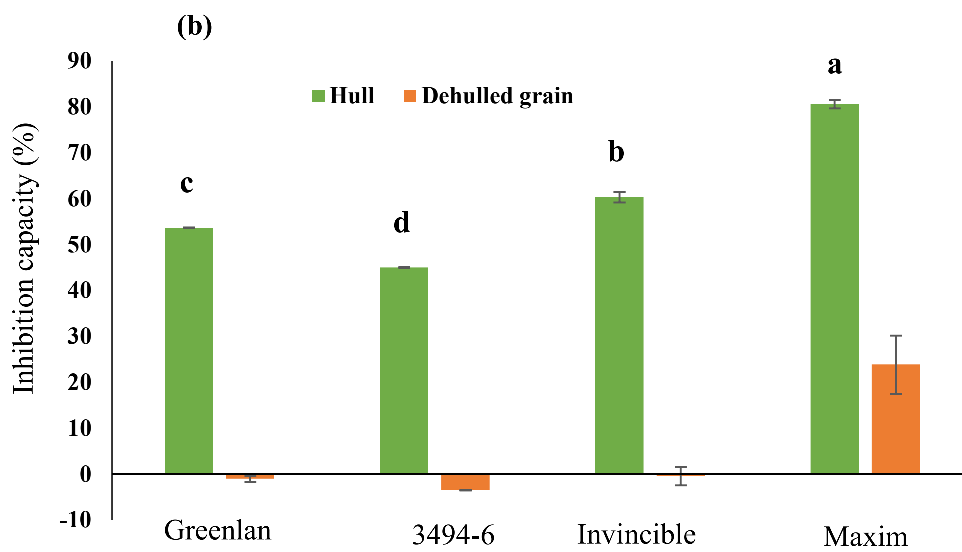


Figure 7.4 α-Glucosidase inhibitory activity of soluble (a) and insoluble-bound phenolics in lentil cultivars.

7.3 Conclusion

The antioxidant potentials of soluble- and insoluble-bound phenolics from hulls and dehulled grains of lentils as well as their bioactivities indicated that hulls in both soluble- and insoluble-phenolics showed higher antioxidant activity and bioactivity than the corresponding dehulled grains of lentils. This observation might be related to the different distribution of phenolics in hulls and dehulled grains of lentils.

Chapter 8

Identification and quantification of soluble- and insoluble-bound phenolics in lentil hulls

8.1 Introduction

Lentils are abundant in macro- and micronutrients such as proteins, carbohydrates, minerals, vitamins, and dietary fibre and provide essential components in the human diet (Rochfort, 2007). In recent years, many studies on the antioxidant potential of lentils have been conducted, revealing their efficient free radical scavenging abilities (Amarowicz et al., 2009; Alshikh et al., 2015; Yeo and Shahidi, 2015).

The hulls of legumes, which are the outermost part of legumes, not only protect the inside constituents such as endosperm and cotyledon, but are also responsible for physical defense against outer enemies such as bacteria, insects, and animal predators (Auger et al. 2010). Phenolic compounds in the hulls such as phenolic acids, flavonoids, and tannins are heavily involved in the development of seed longevity and their dormancy breaking by acting as signaling molecules (Auger et al. 2010). Apart from such pivotal functions in plant biology, they are also well-known as useful bioactive compounds in the human body by providing inhibitory activities against cancer, inflammation, and cardiovascular diseases (Shahidi and Yeo, 2016).

With respect to the structure of hulls, they consist of many different cells, including epidermis, hypodermis, chlorenchyma, palisade, and parenchyma cells (Shahidi and Yeo, 2016), and such cells possess phenolic-containing organs, namely vacuole and cell wall matrix. The vacuole stores

a large amount of water along with various organic compounds, including phenolics. Meanwhile, cell wall matrix is composed of insoluble macromolecules, such as cellulose, hemicellulose, pectin, and structural protein that contribute to the rigid structure of plants in which phenolics are present in different chemical interaction with neighboring constituents. For instance, soluble phenolics are trapped in the vacuolar membrane, while insoluble-bound phenolics are localized at the cell wall matrix by yielding covalent bonds such as ether, ester, and carbon-carbon bonds. Thus, a number of phenolics are present in the hulls in the soluble and insoluble-bound forms.

Many different phenolics in lentil hulls of different cultivars have been reported, including catechins, procyanidins, quercetin, myricetin, luteolin, apigenin as well as dimer, trimer, and tetramer proanthocyanidins (Dueñas et al., 2003; Mirali et al., 2014; Dueñas, 2002). Up until now, most of the research has been conducted on the soluble phenolics, whereas insoluble-bound phenolics and their antioxidant capacity have rarely been investigated. Thus, the aim of this study was to investigate the phenolic profiles and their antioxidant capacity of both soluble and insoluble-bound phenolics present in hulls of four lentil cultivars such as Greenland, 3494-6, Invincible, and Maxim.

8.2 Results and Discussions

8.2.1. Identification of soluble phenolics in hulls of lentils using HPLC-ESI-MS^n

Soluble phenolics in hulls of lentil cultivars were tentatively identified using HPLC-ESI-MS^n by comparing retention times and wavelength-absorption of standard compounds, as well as by consideration of fragment ions by mass analysis. HPLC chromatogram was recorded by using a diode array detector (DAD) and the results are presented in Figure 8.1. In order to detect a wide range of compounds, two wavelengths were set up at the same time by adjusting the analysis conditions, namely 280 nm for detecting phenolic acids and proanthocyanidins as well as 330 nm for flavonoids in which they showed a higher resolution than at 280 nm. In the chromatogram, SPs revealed more peaks than IBPs in all tested lentil cultivars. For identification of phenolics, the precursor ions and fragment ions yielded from precursor ions were mainly used as described in Figure 7.2.

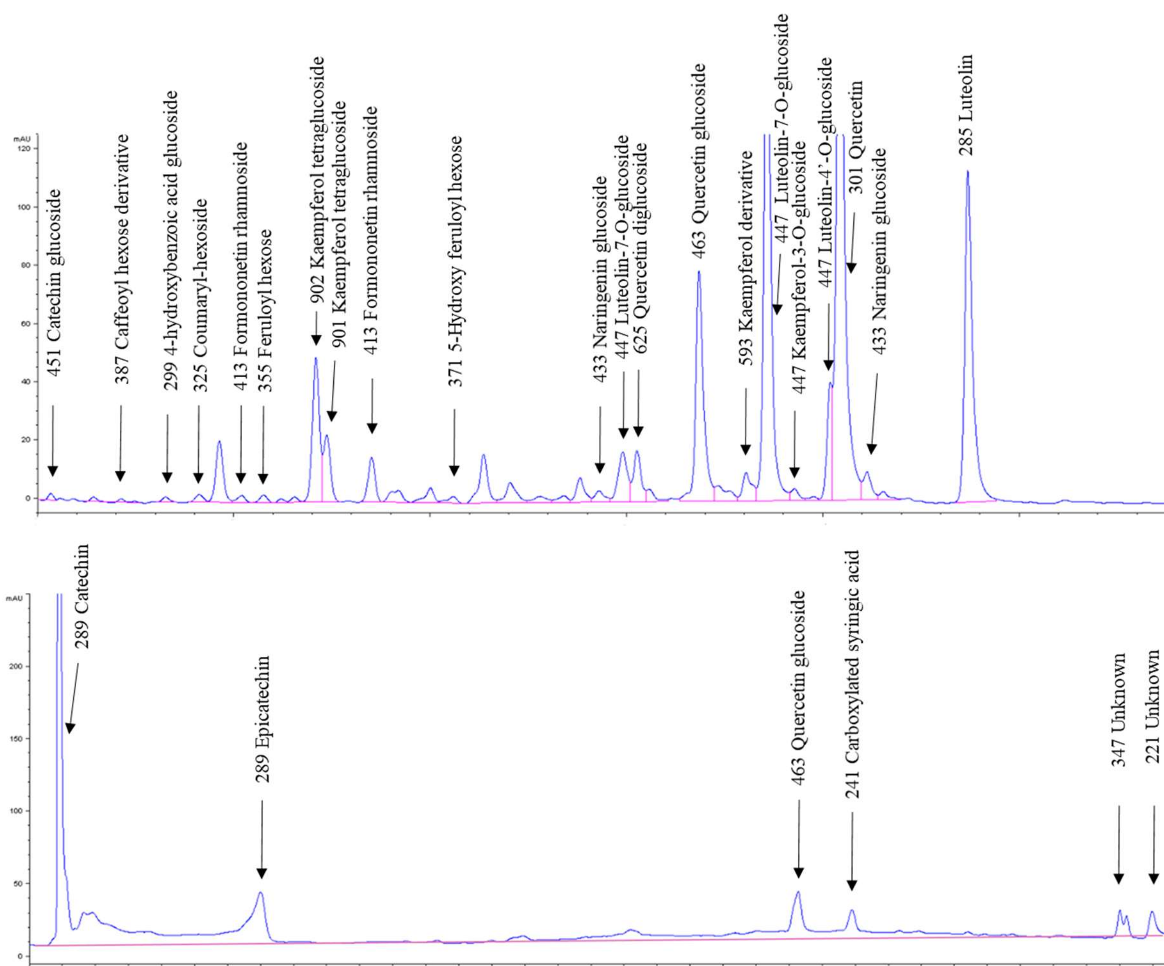


Figure 8.1 Representative HPLC chromatogram of soluble (SPs) and insoluble-bound phenolics (IBPs) of lentil hulls. (a) SPs of 3494-6 at 330nm; (b) IBPs of Greenland at 280nm (the numbers mean their deprotonated ion $[M\text{-}H]^-$).

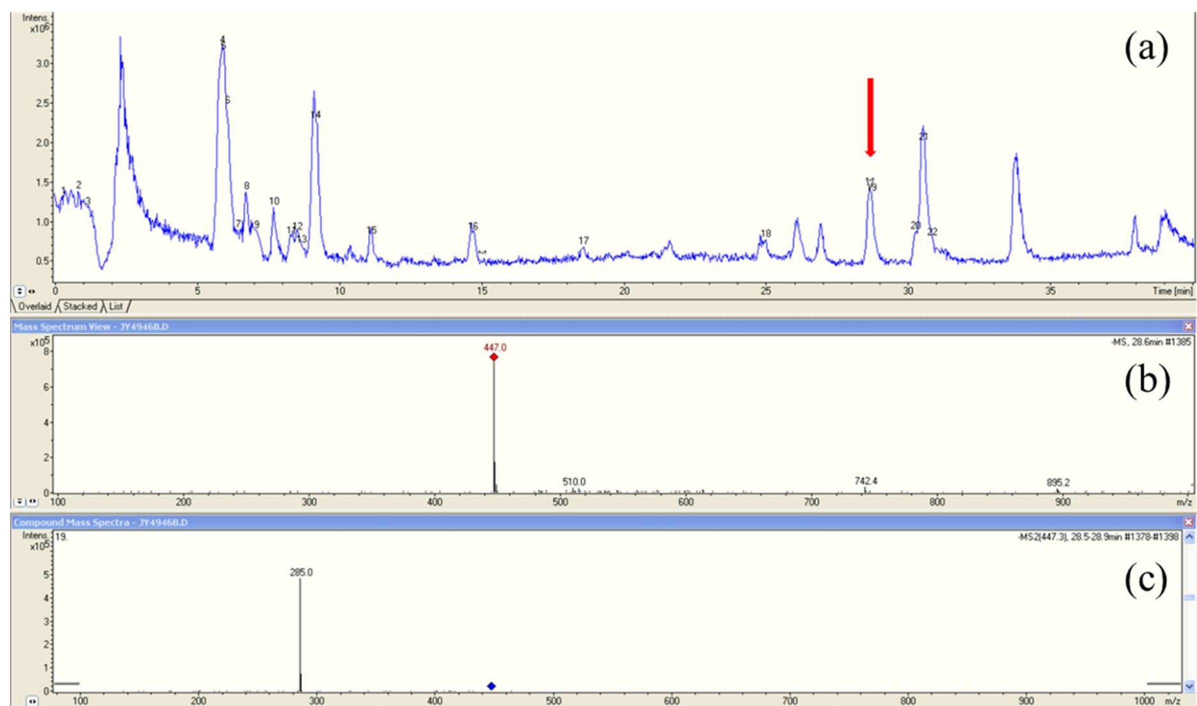


Figure 8.2 Representative mass spectra of kaempferol-3-O-glucoside (*m/z* at 447). (a) Mass spectra of SPs from 3494-6 hull; (b) The deprotonated ion $[M-H]^-$ of kaempferol-3-O-glucoside; (c) MS^2 spectra (fragment ions) of kaempferol-3-O-glucoside.

8.2.1.1 Phenolic acids and their derivatives

Phenolic acids are the secondary metabolites of plant-based foods in that they perform essential functions, such as regulation of growth by acting as signaling molecules and for protection from outer predators and adverse conditions (Cheynier et al., 2013). Meanwhile, they also act as effective health-promoting agents in the biological system by rendering anti-cancer effect and inhibitory activity against hepatitis C virus (HCV) (Hsu et al., 2015). We tentatively identified several phenolic acids, namely caffeoyl hexose derivative, coumaryl-hexoside, feruloyl hexose, 5-hydroxyferuloyl hexose, and 4-hydroxybenzoic acid glucoside in the SPs of hulls of the four tested lentil cultivars (Table 8.1). First, a deprotonated molecule $[M-H]^-$ at *m/z* 387 was detected and tentatively identified as a caffeoyl hexose derivative based on the presence of a fragment ion at *m/z* 341. Chandrasekara and Shahidi (2011) suggested a deprotonated ion at *m/z* 341 as being a caffeoyl hexose in the negative mode, which leads to the tentative suggestion that it was a caffeoyl hexose derivative. Coumaryl-hexoside was identified by comparison with the relevant literature; precursor ion $[M-H]^-$ at *m/z* 325 and fragment ions such as that at 163, which corresponds to *p*-coumaric acid, and 119 which perfectly matches with coumaryl-hexoside as reported by Ibrahim et al. (2015). The MS spectrum exhibited a deprotonated molecule $[M-H]^-$ at *m/z* 355 and the peak was suggested as a feruloyl hexose; the fragment ion at *m/z* 193 indicates ferulic acid and the MS^2 fragmentation indicates $[M-H-162]^-$ ions, which means loss of a hexose, leading to this identification. In addition, 5-hydroxyferuloyl hexose was also identified in MS analysis with the deprotonated molecule $[M-H]^-$ at *m/z* 371, and this identification was made by considering MS data, UV spectrum, and the literature data (Chandrasekara and Shahidi, 2011).

8.2.1.2 Prodelphinidins and proanthocyanidins

Prodelphinidin dimer (GC-C) was found in MS data along with its *m/z* signal at 593 and fragment ions including those at *m/z* 289, 407, and 425. This identification was based on the relevant literature in which the same precursor ion and fragment ions were found and suggested as a prodelphinidin dimer (GC-C) (Amarowicz, 2009). In addition, two proanthocyanidins, namely procyanidin dimer B_2 and procyanidin trimer (3C) C_1 were also tentatively identified; the precursor ion with *m/z* at 577 corresponds to the dimer of catechin that is found in fragment ions, suggested as a procyanidin dimer B_2 (Chandrasekara and Shahidi, 2011). In the same way, a deprotonated ion at *m/z* 865 along with fragment ion at 289 in MS data signifies the presence of a procyanidin trimer (3C) C_1, which is consistent with the findings of Mirali et al. (2014).

Table 8.1 List of phenolic acids, prodelphinidins, and proanthocyanidins identified from soluble phenolics of lentil hulls

Tentative assignment	Wavelength (nm)	RT (min)	$[M-H]^-$ (*m/z*)	Fragment ions
Phenolic acids				
Caffeoyl hexose derivative	280	6.2	387.1	341
Coumaryl-hexoside	330	14.1	325.0	163, 119
Feruloyl hexose	330	15.7	355.0	193, 134
5-Hydroxy feruloyl hexose	330	21.5	371.0	ND
4-hydroxybenzoic acid 4-O-glucoside	330	13.2	299.0	137
Prodelphinidin				
Prodelphinidin dimer (GC-C)	280	7.0	593.1	425, 407, 289
Proanthocyanidin				
Procyanidin trimer (3C) C1	280	7.9	865.2	695, 847, 739, 731, 577, 287
Procyanidin dimer B2	280	8.4	577.1	425, 407, 289, 451
Procyanidin dimer B2	330	10.7	577.1	425, 407, 289, 451

8.2.1.3 Flavonoids

A number of compounds belonging to different classes of flavonoids, such as flavonol, flavan-3-ol, flavone, and isoflavone and their derivatives were found in SPs of hull fractions of the four lentil cultivars tested as given in Table 8.2. The representative flavonoids such as catechin, myricetin, quercetin, and luteolin were identified using authentic standards and by comparison of their retention times and MS data. In the present study, three compounds having the same deprotonated molecule $[M-H]^-$ at *m/z* 433, but with different retention times were found. The first compound displayed a fragment ion at *m/z* 301 in the MS data, which corresponds to quercetin. Chandrasekara and Shahidi (2011) found the same MS data with a precursor ion at *m/z* 433 and a fragment ion at *m/z* 301, along with MS^2 fragmentation of $[M-H-132]^-$, which indicates loss of pentose in the fragmentation, suggested as a quercetin-*O*-pentoside. The second and third compounds yielded a different fragment ion at *m/z* 271, which corresponds to naringenin. The MS^2 fragmentation of this peak indicated $[M-H-162]^-$ which means loss of a glucose molecule, leading to the tentative identification of naringenin glucoside. A precursor at *m/z* 451 along with fragment ions at *m/z* 137, 271, and 289 (catechin) was observed in the MS data which showed MS^2 fragmentation of $[M-H-162]^-$ due to the loss of one glucose molecule, tentatively identified as catechin glucoside, in agreement with studies reported by Amarowicz (2009; 2010). Five peaks displaying the same deprotonated ion $[M-H]^-$ at *m/z* 447 were found in the present study. Of those, three showed a fragment ion at *m/z* 285 corresponding to kaempferol or luteolin alone with the loss of a glucose molecule in the fragmentation pattern, which was tentatively suggested as kaempferol or luteolin glucoside. Mirali et al. (2014) detected the same three deprotonated ions that indicated a precursor ion $[M+H]^+$ at *m/z* 449 with fragment ion $[M+H-Glc]^+$ at *m/z* 287 in the

positive mode in the lentil hulls, which corresponds to $[M-H]^-$ at *m/z* 447 and 285 in the negative mode. The report proposed luteolin-7-O-glucoside, kaempferol-3-O-glucoside, and luteolin-4'-O-glucoside, respectively; therefore, the three peaks in the present study were tentatively identified in the same way. Meanwhile, two other peaks exhibited different fragment ions at 235, 401, and 161 compared to three earlier compounds. However, the pattern of fragmentation was similar to kaempferol hexoside as reported by Chandrasekara and Shahidi (2011) in which they found a precursor ion at *m/z* 447 with fragment ion at 401, identified as kaempferol hexoside. The last 447 was proposed as a kaempherol derivative, considering fragment ion $[M-H]^-$ at *m/z* 285. A deprotonated molecule $[M-H]^-$ at *m/z* 901 was found, which had a fragment ion at *m/z* 755, corresponding to kaempferol triglucoside. Zou et al. (2011) observed two precursor ions at *m/z* 901 and 755 in lentils and identified them as kaempferol tetraglucoside and kaempferol triglucoside, respectively; thus, this compound at *m/z* at 901 was suggested to be kaempferol tetraglucoside. A similar compound at *m/z* 902 was also detected along with the same fragment ion and tentatively proposed as being the same molecule; the slight difference in mass might be due to the surrounding ions in the ionization process. A precursor ion at *m/z* 319 $[M-H]^-$ in the MS spectra was found along with fragment ions at *m/z* 301, 283, and 203. The fragment ion at *m/z* 301 indicates quercetin and the fragmentation of $[M-H-18]^-$ indicates loss of a water molecule, thus identified as a quercetin hydrate. Quercetin glucoside and quercetin diglucoside were also found in the present study with similar fragment ions. The MS spectrum showed a signal at *m/z* 463, and the fragment ion in the MS/MS was at *m/z* 301 specific for quercetin; loss of a glucose molecule in the fragmentation $[M-H-162]^-$ was also noted, thus suggesting it to be quercetin glucoside. Mirali et al. (2014) found the same precursor ion and fragment ions in lentils and similarly proposed it as a quercetin glucoside. The quercetin diglucoside, having a deprotonated molecule

$[M\text{-}H]^-$ at 625 was also tentatively identified using the fragment ion at *m/z* 463 that was already suggested as a quercetin glucoside. The loss of 162 amu in the fragmentation explains the presence of a second glucose molecule, hence a quercetin diglucoside being proposed. A possible fragmentation pathway of quercetin diglucoside is suggested as detailed in Figure 7.3. This identification procedure is consistent with that reported by Amarowicz (2009; 2010). A peak showing a deprotonated ion $[M\text{-}H]^-$ at 593 was also detected and was suggested as a kaempferol derivative due to a fragment ion at *m/z* 285.

In this study, the presence of isoflavones was also noted as reflected in a precursor ion $[M\text{-}H]^-$ at *m/z* 387 with fragment ions at 267 and 249. According to Jin et al. (2012), formononetin has *m/z* at 267, thus this compound was tentatively proposed as being a formononetin derivative.

Table 8.2 List of flavonoids identified from soluble phenolics of lentil hulls

Tentative assignment	Wavelength (nm)	RT (min)	$[M-H]^-$ (*m/z*)	Fragment ions
Flavonoids				
Catechin	280	5.7	289.0	
Myricetin	280	6.7	317.1	271 161 109
Quercetin-O-pentoside	280	9.0	433.0	301 169 415
Catechin glucoside	280	9.3	451.0	137 271 289
Kaempferol glucoside	330	10.5	447.1	152 315 271 177 207 297
Kaempferol tetraglucoside	330	17.0	902.2	755
Kaempferol tetraglucoside	330	17.3	901.2	755
Kaempferol hexoside	330	21.0	447.1	235 401 161
Quercetin hydrate	330	22.1	319.0	301 283 203
Naringenin glucoside	330	24.2	433.0	271
Luteolin-7-O-glucoside	330	24.8	447.0	285
Quercetin diglucoside	330	26.0	625.0	463
Quercetin glucoside	330	20.0	463.0	301
Kaempferol derivative	330	28.5	593.1	285
Luteolin-7-O-glucoside	330	29.0	447.1	285
Kaempferol-3-O-glucoside	330	30.5	447.0	285
Luteolin-4'-O-glucoside	330	30.6	447.1	285
Quercetin	330	30.8	301.0	301
Naringenin glucoside	330	31.2	433.0	271
Luteolin	330	33.8	285.0	ND
Kaempferol derivative	280	40.0	447.1	148 417 347 431 285
Isoflavone				
Formononetin rhamnoside	330	15.6	413.1	267 368
Formononetin rhamnoside	330	19.6	413.1	267 249
Formononetin rhamnoside	330	20.0	413.1	267 249

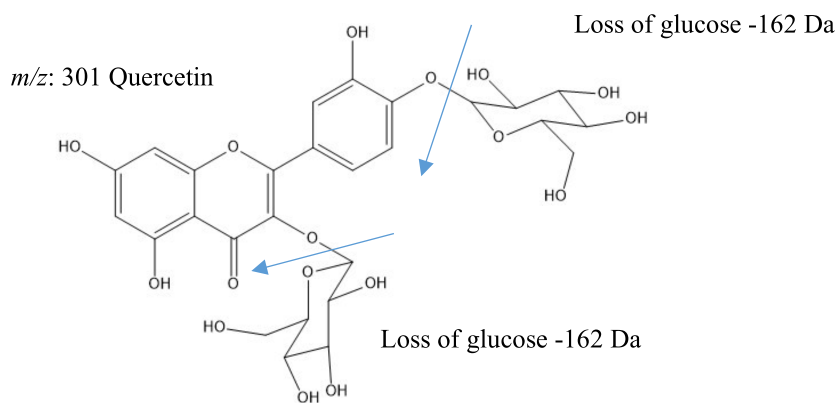


Figure 8.3 Proposed fragmentation pathway for the peak at *m/z* $[M-H]^-$ 625.0 (Quercetin diglucoside).

8.2.2 Identification of insoluble-bound phenolics in hulls of lentils using HPLC-ESI-MS^n

Up until now, a number of studies on the identification of phenolics in many plant-based foods have been carried out. However, most of the research has so far been focused on soluble phenolics and only a few studies have reported the insoluble-bound phenolics. Thus, the determination of phenolic profiles of insoluble-bound phenolics in lentil hulls would fill the existing gap in the available knowledge in this field. In addition, in the human digestive tract, the insoluble-bound phenolics in foods reach directly the large intestine without absorption in the small intestine. In the large intestine/colon, the insoluble-bound phenolics undergo fermentation and are subsequently released from the insoluble cell wall macromolecules. The released bound phenolics render their heath-beneficial effects such as inhibitory activity against the growth of disease-inducing bacteria by reducing the pH in the large intestine, among others (Shahidi and Yeo, 2016). Thus, the insoluble-bound phenolics have great potential as health-promoting agents. In the present study, a total of 15 insoluble-bound phenolics belonging to the phenolic acid and flavonoid classes were tentatively identified, as shown in Table 8.3.

8.2.2.1 Phenolic acids

Two phenolic acids, which are probably present in the cell wall matrix of a plant cell, were found in the hulls of the four tested lentil cultivars (Table 7.3**)**. First, a deprotonated precursor ion $[M-H]^-$ at *m/z* 249 was found with its fragment ion at *m/z* 153 that corresponds to the protocatechuic acid as reported by Chandrasekara and Shahidi (2011), thus a protocatechuic acid derivative is proposed. Another phenolic acid detected in the IBPs was carboxylated syringic acid. The

compound showed a precursor ion $[M-H]^-$ at *m/z* 241 along with a fragment ion at *m/z* 197, corresponding to syringic acid; in addition, loss of 44 amu in the fragmentation in MS/MS indicates its substitution with a carboxyl group (Leenheer et al., 2001), leading to the tentative identification of the molecule as a carboxylated syringic acid.

8.2.2.2 Flavonoids

Flavonoids that are present in the insoluble-bound form were also found in the hulls of the four lentil cultivars studied in this work. The identification of the representative flavonoids such as catechin, epicatechin, quercetin, and kaempferol was carried out by comparing the retention times of standard compounds and their MS/MS data, as well as their maximum absorption wavelength. Several hydrated flavonoids, which bind one or more water molecules, were detected in the present study, including catechin dihydrate, catechin hydrate, and apigenin hydrate. Catechin dihydrate showed a deprotonated ion $[M-H]^-$ at *m/z* 325 with the fragment ion at *m/z* 289 that corresponds to catechin. The loss of 36 amu indicates two water molecules $[M-H-18-18]^-$, thus it was suggested to be a catechin dihydrate. The identification of catechin hydrate and apigenin hydrate was also conducted in a similar manner. A deprotonated ion $[M-H]^-$ at *m/z* 653 was detected and tentatively identified as carboxylated kaempferol diglucoside. The MS/MS data provided fragment ions at *m/z* 447 [M-H-162-44] and 285 [M-H-162-162-44], which implies the substitution of two glucose molecules and one carboxyl group (the loss of amu 44), thus tentatively identified as carboxylated kaempferol diglucoside. To the best of our knowledge, this molecule is identified for the first time in any lentil cultivars. A precursor ion at *m/z* 557 $[M-H]^-$ was also detected and tentatively proposed as a dihydrokaempferol dimer as supported by its fragmentation pattern. The fragment

ion at *m/z* 287 corresponds to hydrokaempferol and the binding of two hydrokaempferol along with the release of an oxygen atom (amu 16) and a hydrogen atom (amu 1) in the formation of an ester bond $[2M-H+17]^-$ matched with dihydrokaempferol.

Table 8.3 List of phenolic compounds identified from insoluble-bound phenolics of lentil hulls

Tentative assignment	Wavelength (nm)	RT (min)	$[M-H]^-$ (*m/z*)	Fragment ions
Phenolic acids				
Protocatechuic acid derivative	280	6.8	249	113, 181, 153
Carboxylated syringic acid	280	29.8	241	197
Flavonoids				
Catechin	280	5.7	289	287, 167, 137, 194, 247, 269
Catechin dihydrate	280	8.1	325	289
Epicatechin	280	11.2	289	245, 205, 179
Catechin hydrate	280	15.8	325	289
Luteolin-7-O-glucoside	280	24.5	447	285
Quercetin glucoside	280	27.0	463	301
Luteolin-7-O-glucoside	280	28.6	447	285
Quercetin	280	30.1	301	ND
Kaempferol	280	33.2	285	ND
Apigenin hydrate	280	40.4	287	269, 241, 155
Carboxylated kaempferol diglucoside	280	49.1	653	447, 285, 417
Dihydrokaempferol hydrate	280	54.8	305	155, 261, 134, 233
Dihydrokaempferol dimer	280	57.5	557	287, 269

8.2.3 Quantification of soluble and insoluble-bound phenolics in lentil hulls

The contents of soluble and insoluble-bound phenolics in the hulls of four lentil cultivars were determined by HPLC analysis and the results are summarized in Tables 8.4 and 8.5. The quantification of phenolics was made by using the corresponding standard compounds and expressed as their equivalent concentrations. Meanwhile, the contents of phenolic derivatives were calculated using their corresponding aglycones, which may affect the result by either overestimation or underestimation. For example, the contents of caffeoyl hexose derivative, coumaryl-hexoside, feruloyl hexose, and 4-hydroxybenzoic acid glucoside were reported using standard curves of caffeic, coumaric, ferulic, and hydroxybenzoic acids, respectively; the same process was applied to other phenolics including proanthocyanidins, prodelphinidin, and flavonoids.

8.2.3.1 Soluble phenolics

In the HPLC analysis of soluble phenolics, the total content of phenolics, which is the sum of all compounds quantified, in Greenland, 3494-6, Invincible, and Maxim cultivars was 59.2, 40.9, 31.4, and 26.7 mg/g DW, respectively. Overall, all lentil cultivars showed a high content of flavonoids such as myricetin, quercetin, and kaempferol, as well as their derivatives. Flavonoids have proven to display strong bioactivities such as anticancer, anti-inflammation, and anti-virus effects as well as being effective in reducing cholesterol, cardiovascular disease and type-2-diabetes (Shahidi and Yeo, 2016). In particular, myricetin that is found in the present study showed effective inhibitory activity against colon carcinogenesis (Harnly et al., 2006; Nirmala and Ramanathan, 2011).

Quercetin has been reported to be an excellent bioactive compound for the inhibition of cancer, cardiovascular disease, and cognitive malfunction (Simkhada et al., 2009; Larson et., 2012; Cherniack, 2012). In addition, kaempferol serves as a strong anticancer agent in human lung cancer cell and other cell lines (Leung et al., 2007; Bestwick et al., 2007). For these reasons, lentils are thought to provide an excellent functional food with a rich supply of health beneficial phenolic compounds.

Table 8.4 Quantification of soluble phenolics in lentil hulls (mg/g)

Tentative assignment	$[M-H]^-$ (*m/z*)	Greenland	3494-6	Invincible	Maxim
Phenolic acids					
Caffeoyl hexose derivative	387.1	13.40 ± 7.77	9.77 ± 1.01	9.60 ± 6.01	9.69 ± 4.14
Coumaryl-hexoside	325.0		0.13 ± 0.02	0.13 ± 0.02	
Feruloyl hexose	355.0	0.01 ± 0.01	0.02 ± 0.00	0.001± 0.01	0.01 ± 0.00
5-Hydroxy feruloyl hexose	371.0		0.01 ± 0.00	0.002 ± 0.01	
4-hydroxybenzoic acid 4-O-glucoside	299.0	0.01 ± 0.00	0.004 ± 0.00	0.004 ± 0.002	
Prodelphinidin					
Prodelphinidin dimer (GC-C)	593.1	5.26 ± 3.41	3.34 ± 1.29	1.45 ± 1.18	5.80 ± 3.06
Proanthocyanidin					
Procyanidin trimer (3C) C1	865.2	2.51 ± 0.93	1.57 ± 0.32	1.51 ± 0.23	2.59 ± 0.48
Procyanidin dimer B2	577.1	4.34 ± 0.42	1.85 ± 0.52	1.63 ± 0.58	3.24 ± 0.98
Procyanidin dimer B2	577.1				0.15 ± 0.00
Flavonoids					
Catechin	289.0			2.41 ± 0.96	
Myricetin	317.1	20.80 ± 7.13	10.98 ± 1.67	10.99 ± 2.62	
Quercetin-O-pentoside	433.0	3.27 ± 2.99	2.12 ± 0.61		
Catechin glucoside	451.0	6.59 ± 1.43		2.23 ± 0.33	2.79 ± 0.28
Kaempferol glucoside	447.1			0.04 ± 0.02	0.06 ± 0.04
Kaempferol tetraglucoside	902.2		0.55 ± 0.47	0.19 ± 0.15	0.28 ± 0.23
Kaempferol tetraglucoside	901.2	0.20 ± 0.01	0.26 ± 0.21	0.07 ± 0.06	0.14 ± 0.11
Kaempferol hexoside	447.1	0.11 ± 0.09			0.17 ± 0.13
Quercetin hydrate	319.0	0.26 ± 0.04			0.12 ± 0.09
Naringenin glucoside	433.0		0.12 ± 0.05	0.09 ± 0.02	
Luteolin-7-O-glucoside	447.0		0.28 ± 0.02	0.07 ± 0.01	
Quercetin diglucoside	625.0	0.41 ± 0.08	0.43 ± 0.31	0.16 ± 0.09	0.23 ± 0.17
Qercetin glucoside	463.0		1.55 ± 1.29	0.11 ± 0.05	0.27 ± 0.20
Kaempferol derivative	593.1	0.17 ± 0.02	0.18 ± 0.14	0.05 ± 0.03	
Luteolin-7-O-glucoside	447.1	0.24 ± 0.03	2.08 ± 1.82	0.07 ± 0.05	0.08 ± 0.06
Kaempferol-3-O-glucoside	447.0	0.07 ± 0.01	0.09 ± 0.07	0.04 ± 0.02	0.14 ± 0.11
Luteolin-4'-O-glucoside	447.1		0.37 ± 0.04	0.005 ± 0.01	0.08 ± 0.02
Quercetin	301.0	0.31 ± 0.01	3.30 ± 2.80	0.10 ± 0.04	0.15 ± 0.08
Naringenin glucoside	433.0	0.16 ± 0.02	0.20 ± 0.13	0.07 ± 0.01	0.12 ± 0.06
Luteolin	285.0	0.12 ± 0.00	1.44 ± 0.03	0.02 ± 0.00	0.10 ± 0.03
Kaempferol derivative	447.1		0.06 ± 0.02		0.06 ± 0.04
Isoflavone					
Formononetin rhamnoside	413.1	0.11 ± 0.02	0.03 ± 0.01	0.05 ± 0.04	0.13 ± 0.02
Formononetin rhamnoside	413.1	0.70 ± 0.08	0.18 ± 0.01	0.35 ± 0.07	0.03 ± 0.02
Formononetin rhamnoside	413.1	0.09 ± 0.05	0.01 ± 0.01	0.06 ± 0.06	0.17 ± 0.00
Total		**59.25**	**40.92**	**31.49**	**26.74**

8.2.3.2 Insoluble-bound phenolics

The total content of insoluble-bound phenolics, which is the sum of all compounds quantified in the HPLC analysis, of Greenland, 3494-6, Invincible, and Maxim was 8.1, 10.3, 6.7, and 14.1 mg/g DW, respectively, is provided in Table 8.5. Catechin was the predominant phenolic present in the insoluble-bound form in all lentil cultivars tested in this work and might contribute most to the antioxidant capacity as reflected in DPPH radical scavenging ability, reducing power, and hydroxyl radical scavenging ability of the tested extracts.

Catechin is well-known as a health-promoting compound, which is heavily involved in human gene expression. It induces the formation of PTGS2 (aka COX-2 for cyclooxygenase-2), which is a dioxygenase and involved in the synthesis of prostaglandin that is an essential molecule in the human body (Noé et al., 2004; http://ctdbase.org/). In addition, catechin also provides effective bioactivities in reducing diabetes, cardiovascular disease, and cancer (Clarke, 2013). For instance, catechin inhibited the growth of colon cancer cells (Baek et al., 2004), and showed antimicrobial activity against *Helicobacter pylori* and *Staphylococcus aureus and Vibrio cholera* (Toda et al. 1990; Shin et al. 2005). In addition, catechin serves as a strong neuroprotective agent (Katergaris et al., 2015). Therefore, insoluble-bound phenolics of lentils, which can be released during food processing, such as boiling, steaming, and fermentation, can be considered as valuable bioactive compounds that contribute to human health promotion.

Table 8.5 Quantification of insoluble-bound phenolics in lentil hulls (mg/g)

Tentative assignment	$[M-H]^-$ (*m/z*)	RT	Greenland	3494-6	Invincible	Maxim
Phenolic acids						
Protocatechuic acid derivative	249	6.8	1.58 ± 0.06			1.94 ± 1.47
Carboxylated syringic acid	241	29.8	0.62 ± 0.01			
Flavonoids						
Catechin	289	5.7	3.77 ± 0.21	6.48 ± 4.60	4.72 ± 2.75	9.13 ± 7.18
Catechin hydrate	325	8.1		0.24 ± 0.21	0.23 ± 0.08	0.20 ± 0.08
Epicatechin	289	11.2	0.62 ± 0.06	0.42 ± 0.31	0.44 ± 0.00	0.27 ± 0.14
Catechin hydrate	325	15.8		0.14 ± 0.05	0.15 ± 0.01	
Luteolin-7-O-glucoside	447	24.5		0.25 ± 0.00		
Quercetin glucoside	463	27.0	1.10 ± 0.02	0.59 ± 0.12		
Luteolin-7-O-glucoside	447	28.6		1.01 ± 1.15	0.12 ± 0.01	0.14 ± 0.05
Quercetin	301	30.1		0.92 ± 0.65		
Kaempferol	285	33.2		0.26 ± 0.20		
Apigenin hydrate	287	40.4			0.79 ± 0.11	2.22 ± 1.61
Carboxylated kaempferol diglucoside	653	49.1	0.06 ± 0.02			
Dihydrokaempferol hydrate	305	54.8	0.13 ± .06		0.26 ± 0.09	0.17 ± 0.02
Dihydrokaempferol dimer	557	57.5	0.23 ± 0.00			0.11 ± 0.01
Total			**8.11**	**10.34**	**6.71**	**14.18**

8.3 Conclusion

In the present study, the identification, quantification, and antioxidant potential of soluble- and insoluble-bound phenolics in hulls of four lentil cultivars were reported. Through HPLC-MS analysis, a relatively diverse spectrum of phenolics was found in the soluble phenolics compared to the insoluble-bound phenolics, leading to a higher antioxidant capacity such as DPPH radical scavenging ability and reducing power of soluble phenolics compared to the latter. Up until now, only a few studies have been conducted on the insoluble-bound phenolics of plant-based foods, especially on the identification of the bound phenolics using HPLC-MS/MS. Thus, this study provided valuable information that fills the existing gap in the available literature in this and related research areas.

Chapter 9

Summary and future research

9.1 Summary

The effect of food processing operations such as germination, hydrothermal treatment (boiling), and dehulling on the antioxidant capacities and bioactivities of lentil cultivars was evaluated. In the germination process, a novel indicator, the ratio of insoluble-bound (IBPs) to soluble phenolics (SPs), was proposed as an efficient means to follow changes in the antioxidant activity of lentils during the germination process. This indicator may also be employed to monitor other process-induced changes in the antioxidant capacity of food phenolics in lentils and other foods. The antioxidant activity of SPs, IBPs, and total value, SPs + IBPs, of germinated lentils were assessed during a 4-day period. Total phenolic content (TPC), total flavonoid content (TFC), DPPH radical, and ABTS radical cation scavenging abilities were used to record antioxidant capacities. An increase in IBPs was found in TPC, TFC, DPPH, and ABTS radical cation scavenging ability, whereas SPs revealed a declining trend in TFC, DPPH, and ABTS, except for TPC during the 4 days of germination. Longer germination time may further improve health beneficial effects of lentils. The ratio of IBPs to SPs increased using most of the methods employed, and this may be due to the alteration of phenolic compounds from soluble to insoluble-bound form during germination. This ratio can possibly be used as a new means for monitoring process-induced changes in the antioxidant capacity of foods.

In addition, the changes in the insoluble-bound phenolics of lentil cultivars upon hydrothermal (boiling) processing were monitored using HPLC-ESI-MS^n analysis and by following different

antioxidant capacity measurements. The hydrothermal energy disintegrated or loosened the cell wall matrix, hence the content of insoluble-bound phenolics decreased as shown by HPLC analysis and measurement of phenolics and antioxidant capacity during the boiling process, indicating their possible release from cell wall matrix. However, the released bound phenolics did not remain as soluble/free phenolics as their increase was less than the decrease in the content of insoluble-bound phenolics, indicating loss of phenolic compounds. This loss was speculated to be due to the formation of irreversible covalent bonds to other molecules such as protein, starch, and cellulose, which are not affected by the alkali hydrolysis procedure used in this work. Preliminary experiments indicated that interaction of oxidized flavonoids with proteins was possibly responsible for this observation. Thus, structural changes of bound phenolics and possible alteration of chemical bonding during boiling may be contemplated.

In the present study, we also studied the antioxidant potential of soluble and insoluble-bound phenolics derived from hulls and dehulled lentils, as well as their bioactivities such as inhibitory activity against oxidation of LDL cholesterol and DNA strand breakage. The α-glucosidase inhibitory activities were also assessed. Results so obtained indicated better antioxidant potential and bioactivities of hulls compared to the corresponding dehulled grains in all measurements, indicating that the hull fraction possesses a higher content of phenolics in both the soluble and insoluble-bound forms. These results might be directly connected to the localization of phenolics in the lentil structure.

Finally, phenolic profiles and their quantities in both soluble and insoluble-bound fractions in hulls of the four lentil cultivars were studied. Through HPLC-MS analysis, a relatively diverse spectrum of phenolics was found in the soluble phenolics compared to the insoluble-bound phenolics. Up until now, only a few studies have been conducted on the insoluble-bound phenolics of plant-based

foods, especially on the identification of the bound phenolics using HPLC-MS/MS. Thus, this study provided valuable information that fills the existing gap in the available literature in this and related fields.

9.2 Future research

Future research should be conducted in several associated areas in order to shed further light in this field for lentils and possibly other legumes.

1. The health promoting effects of phenolics in lentils can be determined using cell line and rat test.
2. Changes of phenolic profiles and their contents during the germination process needs to be further examined.
3. The exact details about the interaction of oxidized flavonoids in seeds of lentils and other legumes needs to be further studied.

Publications Included in Thesis

1. **JuDong Yeo,** Fereidoon Shahidi. (2017). Effect of hydrothermal processing on changes of insoluble-bound phenolics of lentils. *Journal of Functional Foods*, 38, 716-722.

   Contribution of author: Planning, designing and executing of experiments, data analysis, manuscript writing was carried out by first author.

2. Fereidoon Shahidi and JuDong Yeo. (2016). A review- Insoluble-bound phenolics in foods. *Molecules*. 21, 1-22.

   Contribution role: Co-Author – writing the draft of the manuscript.

3. JuDong Yeo, Fereidoon Shahidi. (2015). Letter- Critical evaluation of changes in the ratio of insoluble bound to soluble phenolics on antioxidant activity of lentils during germination. *Journal of Agricultural and Food Chemi*stry, 63, 379−381.

   Contribution of author: Planning, designing and executing of experiments, data analysis, manuscript writing was carried out by first author.

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