Characterization of nutrient profiles from legume seeds

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

in

Plant Science

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Abstract

The overall goal of this research was to characterize components of legume seeds, and determine if specific seed components have potential functional properties for practical end-uses. The polymeric flavonoids proanthocyanidins (PA), also known as condensed tannins, accumulate in the seed coats of specific grain legume cultivars. In this study, the PA type found in pea seed coats (*Pisum sativum* L.) of 'Solido' was confirmed, and PA-enriched seed coat fractions and their flavan-*3*-ol subunits were investigated for antibacterial activity against food pathogens. Antimicrobial activity was influenced by the number of hydroxyl substitutions on the flavonoid aromatic B-ring. Our data suggest that at least part of the antimicrobial activity of flavan-*3*-ols and PAs involves their capacity to bind iron. Additionally, the fibre, protein and starch content of seeds from specific cultivars of the grain legumes pea (*Pisum sativum* L.), faba bean *(Vicia faba* L.*)*, and lentil (*Lens culinaris* L.), were determined to further understand the health-beneficial effects of legume seed consumption in the diet. The amount and type of fibre in the legume seed coats is discussed with specific relevance to the potential to ameliorate symptoms of type 2 diabetes.

Preface

In chapter 2 of this thesis, the extraction and processing procedures to obtain the crude proanthocyanidins extract were performed by Dr. Lihua Jin, University of Alberta. In Chapter 3 of this thesis, the pea seed coats fractions were prepared with the assistance of Dr. Lihua Jin. These fractions were provided to our collaborator Dr. Catherine Chan (human nutrition lab at Department of Agricultural, Food and Nutritional Science, University of Alberta) to test for their effects on amelioration of type 2 diabetes in a diabetic rat model. The performance of all other experiments and the data analysis in chapter 2 and chapter 3 are my original work, as well as the literature review in chapter 1.

Acknowledgement

I would like to thank Dr. Jocelyn Ozga for giving me the opportunity of being her graduate student. I could not finish this thesis without her supervision and support. She is not only my academic mentor but also a warm friend in my life. I also would like to thank Dr. Michael Gaenzle and Dr. Stephen Strelkov for serving as my committee members, and giving me valuable comments and directions on my research projects. I want to thank Dr. Lihua Jin for her suggestions with the flavonoids research; and Dr. Kelvin Lien for his advice on gas chromatography analysis. I also want to thank current and past lab members, the friendship will always be here. Moreover, I would like to thank the graduate students in AFNS who have helped me doing my experiments.

Last but not least, I would like to thank my family. My parents' love is the most important support during my study. Although they may not understand even one paragraph of my thesis, I cannot even write one word without them. I thank them for travelling thousand miles to help me taking care of my daughter, I cannot finish my work without their dedication. I want to thank my husband Zeyuan Li for his sacrifice and support for my graduate study. My daughter's birth on 2012 was the biggest change in my life. Every time when I was upset, her sweet smile was the best encouragement and made me optimistic.

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List of abbreviations

4CL 4-coumarate-CoA ligase

ANR Anthocyanidin reductase

ANS Anthocyanidin synthase

Ara Arabinose

C Catechin

C4H Cinnamate 4-hydroxylase

CHI Chalcone isomerase

CHS Chalcone synthase

CoA Coenzyme A

C-P Catechin-(4α→2)-phloroglucinol

DAD Photodiode array detector

DFR Dihydroflavonol 4-reductase

DW Dry weight

EC Epicatechin

EC-P Epicatechin-(4β→2)-phloroglucinol

EDTA Ethylenediaminetetraacetic acid

EGC-P Epigallocatechin-(4β→2)-phloroglucinol

EGC Epigallocatechin

F3H (or FHT) Flavanone 3-hydroxylase

F3'H Flavonoid 3' hydroxylase

F3'5'H Flavonoid 3'5' hydroxylase

FLS Flavanone synthase

FNS Flavone Synthase

Fuc Fucose

Gal Galactose

GC Gallocatechin

GC-P Gallocatechin-(4α→2)-phloroglucinol

Glc Glucose

GlcA Glucuronic acid

HPLC High performance liquid chromatography

LAR Leucoanthocyanidin reductase

LLE Liquid-liquid extraction

mDP Mean degree of polymerization

MIC Minimal inhibition concentration

nm Nano meter

PA Proanthocyanidin

PAL Phenylalanine ammonia-lyase

PPO Polyphenol oxidase

Rha Rhamnose

RP-HPLC Reversed phase HPLC

SPE Solid phase extraction

TFA Trifluoroacetic acid

λ wavelength

Xyl Xylose

Chapter 1 Literature review

Flavonoid classification

Flavonoids are plant secondary metabolites that serve a variety of functions. The flavonoid biosynthesis pathway produces various distinct groups of flavonoids that can accumulate in plant cells such as anthocyanins, proanthocyanidins (PAs), and flavonols. The basic flavonoid skeleton consists of three rings: two benzene rings (ring A and B) and one pyran ring (ring C). The structural differences of the flavonoid family of compounds are based on the saturation of the heteroatomic ring C; the attachment of the ring B at the carbon-2 or carbon-3 position in the ring C; the overall hydroxylation pattern, and the multiple substituents conjugated to the aromatic ring (monomers, oligomers or polymers) **(Figure 1.1)** (Grotewold, 1995).

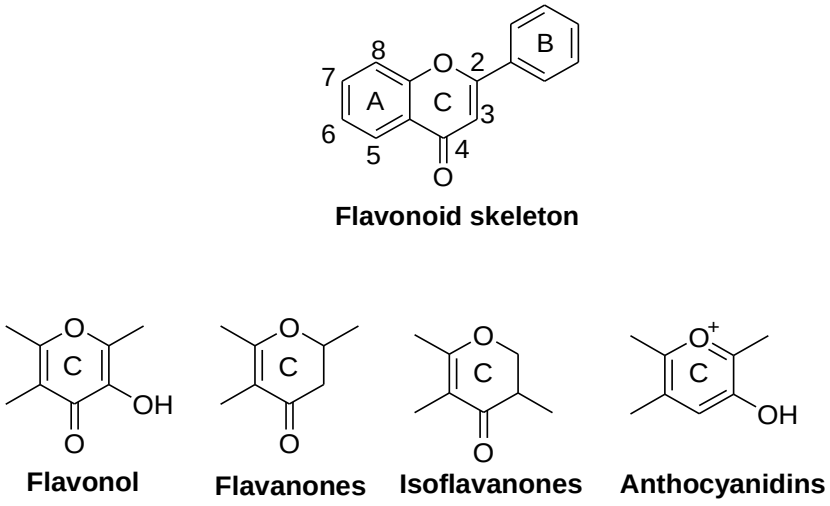


Figure 1.1 Basic flavonoid structure and several flavonoid subclasses as designated by ring C (Grotewold, 1995).

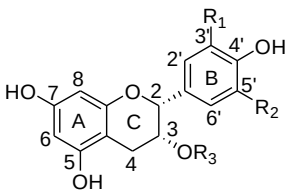
Flavonoids are involved in specific plant-pathogenic microbe interactions (phytoalexins, such as isoflavonoids in plants belonging to the Leguminosae family) (Koes 1994). Flavonoids also function as plant defense compounds to protect the plant against herbivore and microbial attack (Dixon et al., 2005; Ayres et al., 1997). Green tea flavanols (catechins) are well-known for their antimicrobial activity (Taylor et al., 2005). It has also been reported that anthocyanins from red wine are related to a lower risk of cardiovascular disease and cancer chemoprevention due to the anthocyanins' antioxidation and anticarcinogenesis activities (Hou, 2004). These potential properties make flavonoids a target for application in plant protection and for health-beneficial food ingredients.

Proanthocyanidins

The oligomeric or polymeric flavonoids proanthocyanidins (PAs), also known as condensed tannins, accumulate in the seed coats of specific cultivars of grain legumes including pea (*Pisum sativum* L.), lentil (*Lens culinaris* L.), and faba bean (*Vicia faba* L.) (Duenas et al., 2003; Duenas et al., 2004; Jin et al., 2012). PAs in legume seed coats can also be visually confirmed by the brown color which is a result of enzymatic browning of PAs caused by polyphenol oxidase (PPO). In the presence of oxygen, PPO catalyses the oxidation of diphenols (B ring of PAs) to semiquinones and quinones. Semiquinones and quinones are highly active and can react with phenols, amino acid and proteins from plant tissues to produce complex brown polymeric compounds (Grotewold E, 2005; Pourcel et al., 2007). PAs can function to protect plants against microbial pathogens. The proposed mechanisms of action for PAs antimicrobial activity include the effects of iron depletion, inhibition of cell-associated proteolysis and cell wall synthesis (Buzzini et al., 2007). PAs are hypothesized to act as digestion inhibitors or toxins to deter large herbivores (Ayres et al., 1997). PAs can also interact with the proteins in the mouth giving an astringent flavor; this could be the reason why PAs are unpopular for human consumption (Ariga et al., 1981).

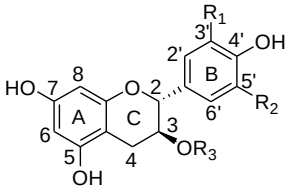
Moreover, PAs also have been reported to have human-health beneficial effects, such as antioxidant (Amarowicz et al., 2000) and radical scavenging activities (Gaulejac et al., 1999; Santos-Buelga et al., 2000). Since PAs have shown some interesting bioactivities, and some legume seed coats contain high PA levels, understanding the PA profile in legume seeds will aid in our understanding of health benefits of human consumption of these legume seeds.

PAs are oligomeric and polymeric (usually greater than 5 monomer units) flavan-*3*-ols. Two subclasses of flavan-*3*-ols, the 2,3-*cis*-flavan-*3*-ols and 2,3-*trans*-flavan-*3*-ols, make up the PA subunits found in plants (**Figure 1.2**). PAs are classified by the stereochemistry and hydroxylation patterns of the subunits; the linkage of subunits; the polymer length; and esterification of C-3 hydroxyl group with gallic acid or sugar units (Dixon, 2005).



2,3-*cis*-flavan-*3*-ols

R1=OH, R2=R3=H, (-)-epicatechin
R1=R2=OH, R3=H,(-)-epigallocatechin
R1=OH, R2=H, R3=Galloyl, (-)-epicatechin gallate
R1=R2=OH, R3=Galloyl, (-)-epigallocatechin gallate

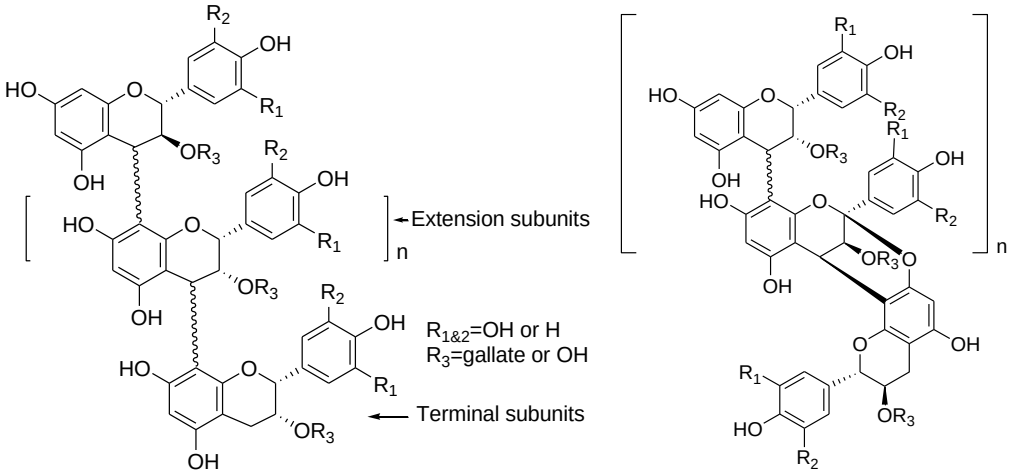


2,3-*trans*-flavan-*3*-ols

R1=OH, R2=R3=H, (+)-catechin
R1=R2=OH, R3=H, (+)-gallocatechin
R1=OH, R2=H, R3=Galloyl, (+)-catechin gallate
R1=R2=OH, R3=Galloyl, (+)-gallocatechin gallate

Figure 1.2 Chemical structures of common flavan-*3*-ols
(Adapted from Jin et al., 2012)

Two major classes of PAs are defined by the linkage of the extension units. If the C4 of one unit is linked to C6 or C8 of another unit, it is a B-type PA. If both C2 and C4 of one unit are linked to the C7 oxygen and C6 or C8 of another unit (double linkage), it is an A-type PA (Aron and Kennedy, 2008) (**Figure 1.3**).



B-type proanthocyanidins **A-type proanthocyanidins**

Figure 1.3 Structure of B-type and A-type proanthocyanidins.
(Adapted from Jin et al., 2012)

Flavonoid biosynthetic pathway

The flavonoid biosynthetic pathway is one of the most well-studied secondary metabolism pathways in plants. The flavonoid C6-C3-C6 skeleton is biosynthesized from phenylalanine (precursor that forms rings B and C) and three equivalents of malonyl-CoA (precursor that forms ring A) (**Figure 1.4**). In brief, the amino group of phenylalanine is removed by phenylalanine ammonia-lyase (PAL) to produce cinnamate. Then a hydroxyl group is added to the C-4 position of cinnamate by cinnamate 4-hydroxylase (C4H) to form 4-coumarate. Then a CoA is added to 4-coumarate by 4-coumarate-CoA ligase (4CL) to produce 4-coumaroyl-CoA. Malonyl-CoA is formed by adding a carboxyl group to acetyl CoA by acetyl-CoA carboxylase (ACCase). Condensation of three equivalents of malonyl-CoA and one equivalent of 4-Coumaroyl-CoA catalyzed by chalcone synthase (CHS) forms one chalcone molecule. Chalcone is stereospecifically isomerized by chalcone isomerase (CHI) to produce flavanone.

The biosynthesis of PAs from flavanone is described as follows. Flavanone 3-hydroxylase (FHT) adds a hydroxyl group to the C-3 position of flavanone to

form dihydroflavonol. Flavonoid 3' hydroxylase (F3'H) and flavonoid 3'5' hydroxylase (F3'5'H) catalyze the hydroxylation of B ring and result in the attachment of two hydroxyl groups, or three hydroxyl groups, respectively, forming flavan-*3,4*-diols. Flavan-*3,4*-diols are reduced to leucoanthocyanidins by dihydroflavonol 4-reductase (DFR).

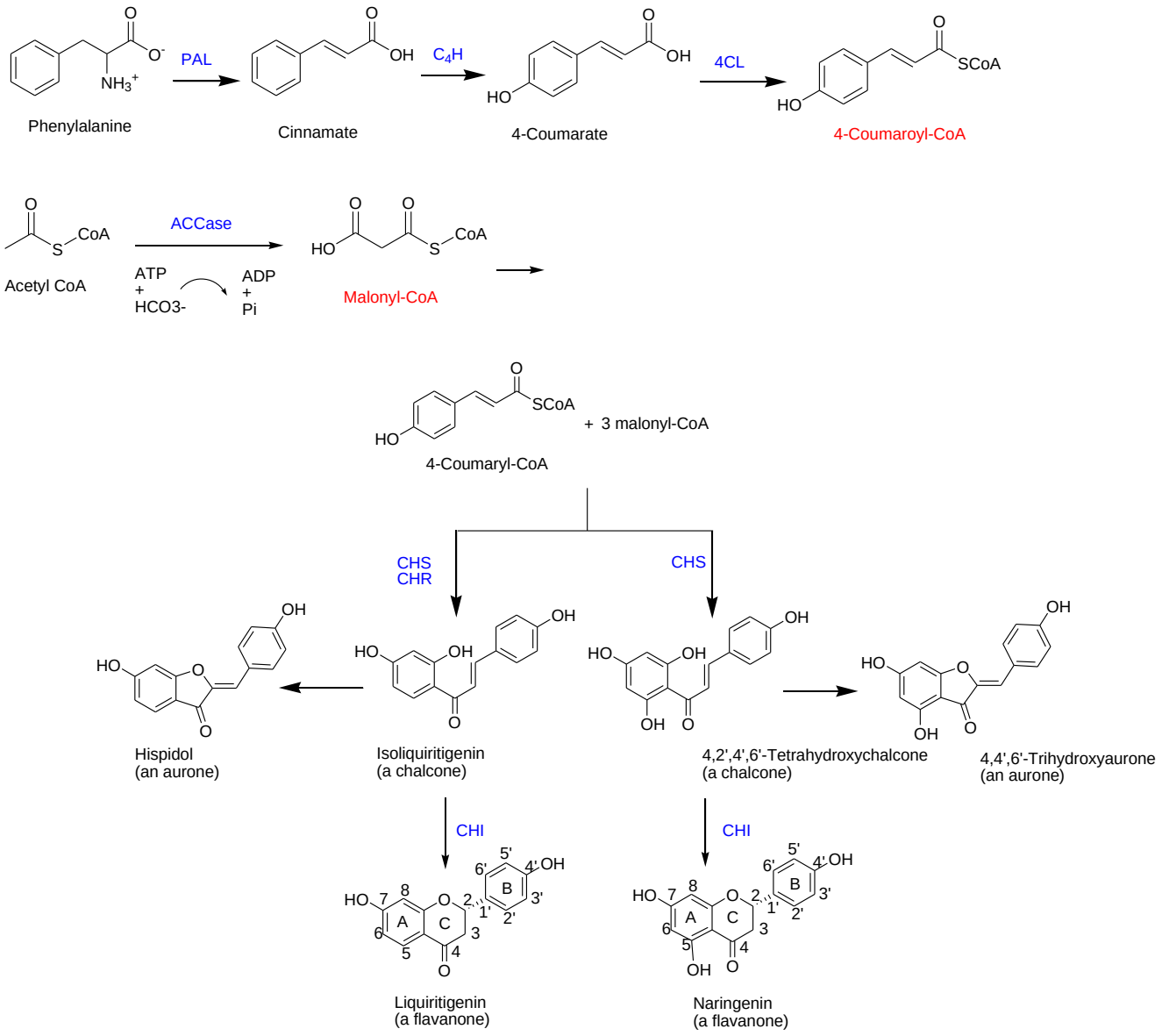


Figure 1.4 Formation of flavanone from phenylalanine (Adapted from Grotewold, 1995).

PAL, phenylalanine ammonia-lyase. **C4H,** cinnamate-4-hydroxylase. **4CL**, 4-coumaroyl-CoA-ligase. **ACCase**, acetyl-CoA carboxylase. **CHS**, chalcone synthase. **CHR**, chalcone reductase. **CHI**, chalcone isomerase.

Biosynthesis of proanthocyanidins

An important flavonoid biosynthesis branch pathway is the one leading to the PAs. This pathway is of significant interest because it can be a potential target for increasing the nutritional properties of food crops, including the legume seed crops.

Leucoanthocyanidin is converted to anthocyanidin by anthocyanidin synthase (ANS). There are two key enzymes, anthocyanidin reductase (ANR) and leucoanthocyanidin reductase (LAR), that regulate the chemical make up of PAs (Grotewold 2005). ANR converts anthocyanidin to 2,3-*cis*-flavan-*3*-ol, LAR generates 2,3-*trans*-flavan-*3*-ol from leucoanthocyanidin. Condensation of different flavan-*3*-ols forms the PAs oligomers and polymers (**Figure 1.5**).

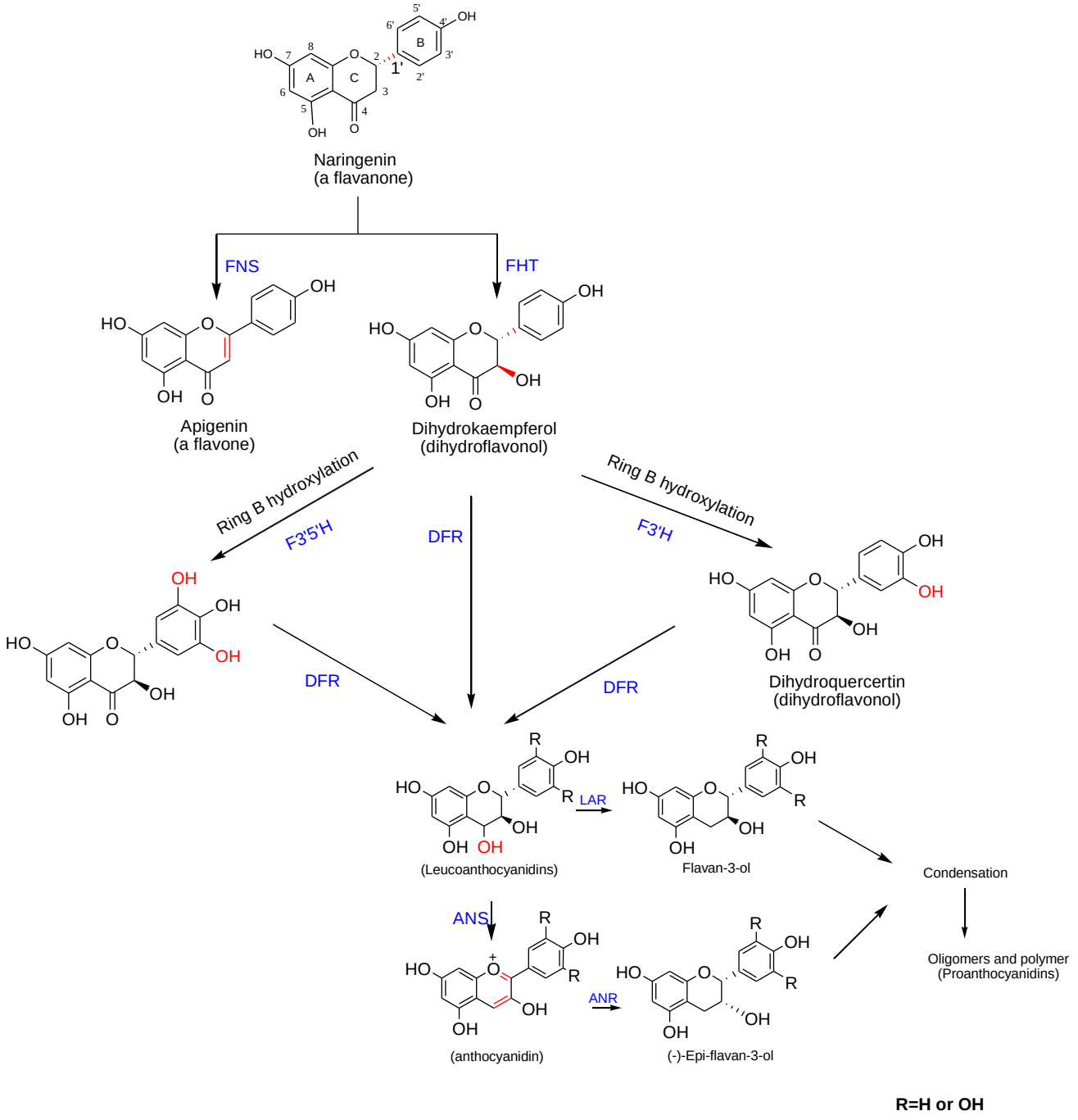


Figure 1.5 Formation of proanthocyanidins from flavanone

(Adapted from Grotewold, 1995).

FHT, flavanone 3-hydroxylase. **FNS**, flavone synthase. **DFR,** dihydroflavonol 4-reductase. A**NS**, anthocyanidin synthase. **LAR**, leucoanthocyanidin reductase. **ANR**, anthocyanidin reductase. **F3'H,** flavonoid 3' hydroxylase. **F3'5'H**, flavonoid 3'5'hydroxylase.

Proanthocyanidins chemical analysis

PAs are relatively unstable under sunlight, moisture and elevated temperature; moreover, they can be oxidized by polyphenol oxidase (PPO) (Pourcel et al., 2007). Therefore, methods for extraction, isolation, purification, identification and quantification of PAs from plant tissues need be carefully chosen.

Plant samples for PAs analysis need to be immediately frozen and then are usually lyophilized in order to prevent/reduce PA degradation caused by enzymes. Liquid nitrogen is usually required during the sample grinding step to minimize the release of PPO to reduce PA degradation.

PAs are flavan-*3*-ol oligomers and polymers, therefore, polar solvent systems are applied in the extraction procedure of PAs from plant tissues. The most commonly used solvent systems include water, acetone and methanol, and aqueous acetone (66%) is widely used for PA extraction (Jin et al., 2012; Kennedy and Jones, 2001). The PA extraction efficiency can be enhanced by ultrasonication and vortexing (Grotewold, 2005). Liquid–liquid extractions (LLE) or solid phase extraction (SPE) are most commonly used techniques for initial purification of the crude PA extracts to remove chlorophylls, organic acids and sugars (Grotewold E 2005). LLE uses two immiscible solvents (e.g. water and ethyl acetate for PA purification) to separate compounds based on their solubilities. SPE, which usually follows LLE, uses a liquid mobile phase and a solid stationary phase to separate and purify the extracts based on the affinity of the substrate. In the case of PA purification, Toyopearl HW-40 resins, which are hydroxylated methacrylic polymers, are used as the size exclusion medium for the adsorption chromatography. Aqueous methanol (50%) containing 0.1% trifluoroacetic acid (TFA) is used to elute the impurities, including organic acids, flavan-*3*-ols, hydroxycinnamic acids and flavonols, from the size exclusion column. Then the PAs adsorbed to the size exclusion matrix are eluted by aqueous methanol (80%) containing 0.1% TFA (Kennedy 2003).

The characterization of PA composition can be achieved using the phloroglucinolysis method (Kennedy and Jones, 2001). In brief, the PA polymers are acid hydrolyzed and cleaved with nucleophile phloroglucinol, leading to the extension units phloroglucinol adducts and terminal flavan-*3*-ol units (**Figure 1.6**). High-performance-liquid chromatography (HPLC) (exclusively using a reversed-phase column), coupled with a diode-array detector (DAD) are commonly used for the identification of the PA phloroglucinolysis products (Merken and Beecher, 2000). The mean degree of polymerization (mDP) of the PAs can be calculated according to the method of Kennedy and Jones (2001). To calculate mDP, the sum of all subunits (in mole equivalents) is divided by the sum of only terminal units (in mole equivalents). Conversion yield is calculated as the sum of all subunits in mass (excluding the phloroglucinol moiety of the phloroglucinol adducts) divided by the weight mass of the starting PAs sample.

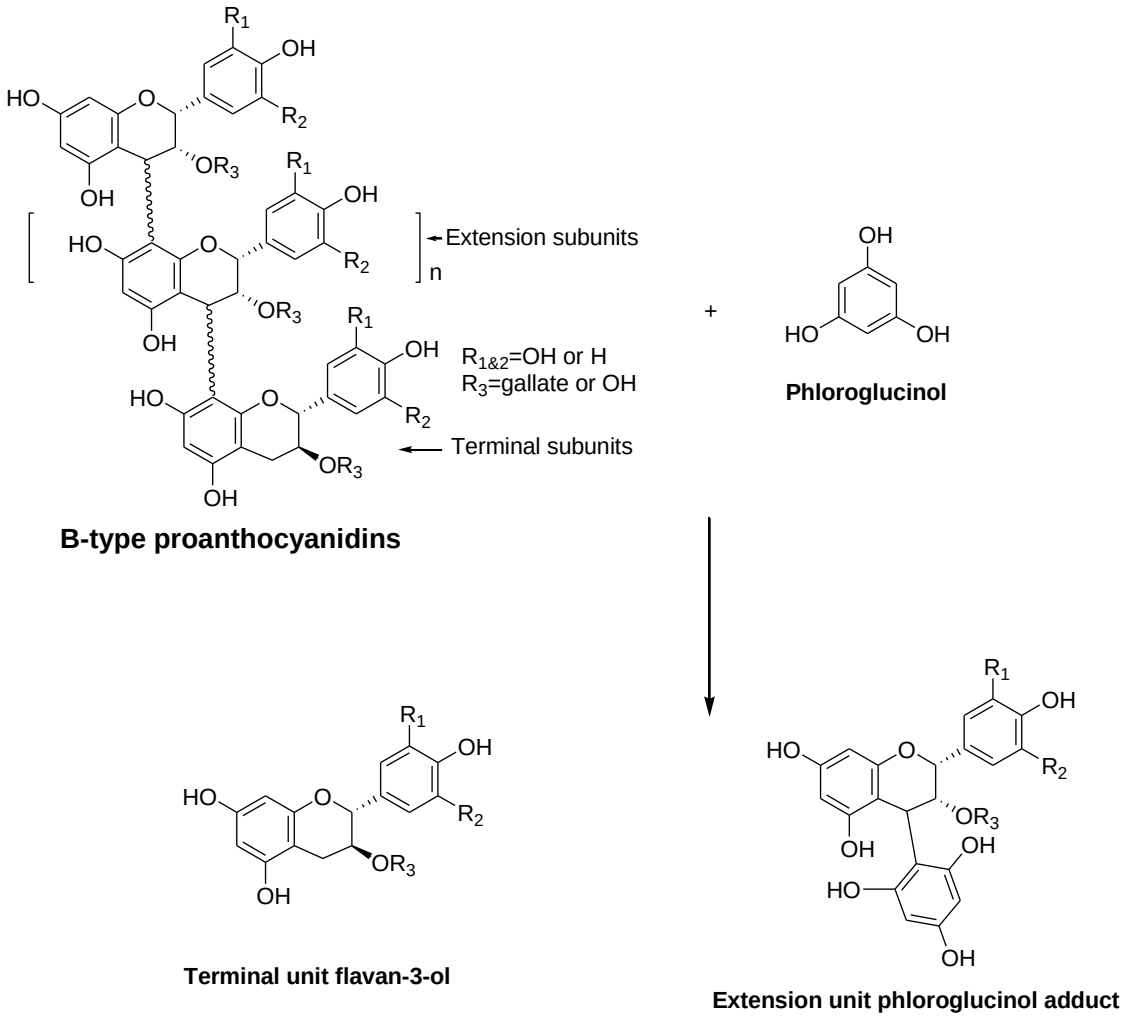


Figure 1.6 Phloroglucinolysis reaction leading to free terminal unit flavan-*3*-ol and extension unit phloroglucinol adduct (Jin et al., 2012).

Antimicrobial activity of flavonoids compounds

Antimicrobial agents are compounds that can inhibit the growth of microorganisms. Antimicrobial agents can be categorized according to the different microorganisms they can inhibit. For example, antibacterial agents can inhibit the growth of bacteria or kill the bacteria; antifungal agents are used to inhibit fungal growth; antiviral agents can inhibit virus growth, etc. There are many synthetic chemical compounds utilized as antimicrobial agents; however,

natural products are another important source of antimicrobial agents (Silver and Bostian, 1990).

Antibacterial agents are classified in three ways. Firstly, antibacterial agents can be bactericidal or bacteriostatic. Bactericidal antibacterial agents kill the bacteria; however bacteriostatic antibacterial agents only inhibit the bacteria growth. Secondly, antibacterial agents can be classified according to the target site of action. The five mains target sites are cell wall synthesis, protein synthesis, nucleic acid synthesis, metabolic pathways, and cell membrane function. Thirdly, antibacterial agents have diverse chemical structures originating from synthetic compounds or natural sources (Goering et al., 2012).

Bacteria are a large group of prokaryotic microorganisms. There are two different cell wall types in bacteria that differ in their ability to retain the crystal violet Gram stain, they are classified as Gram positive (stain-retaining) and Gram negative (non stain-retaining). A Gram-positive bacterium has a very thick layer of peptidoglycan in the cell wall, and it usually lacks the outer membrane; however, a Gram-negative bacterium cell wall typically has a thin peptidoglycan layer and an outer membrane that contains lipopolysaccharide (**Figure 1.7**) (Murray et al 2013).

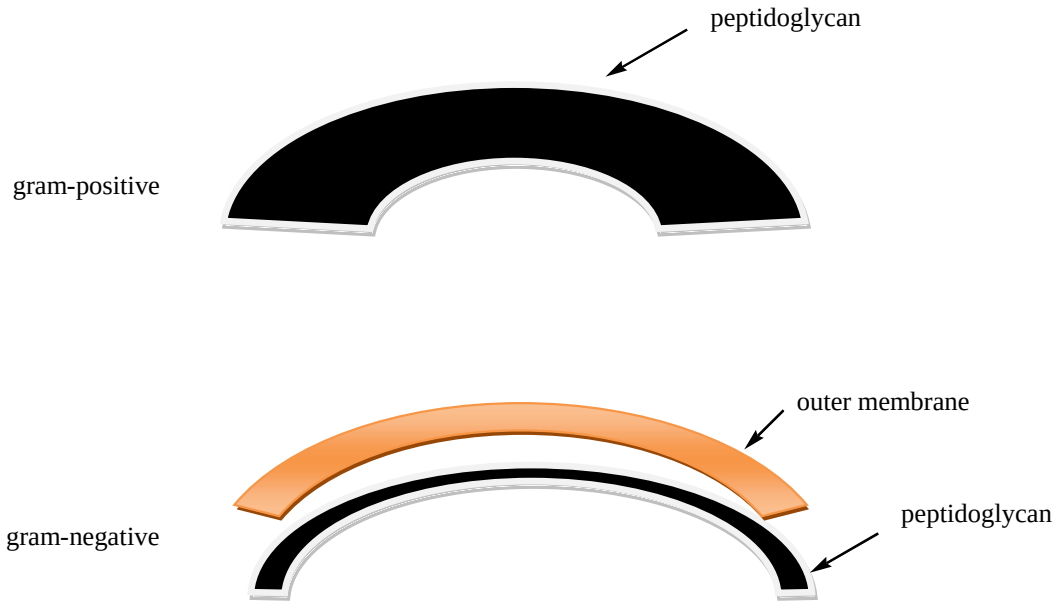
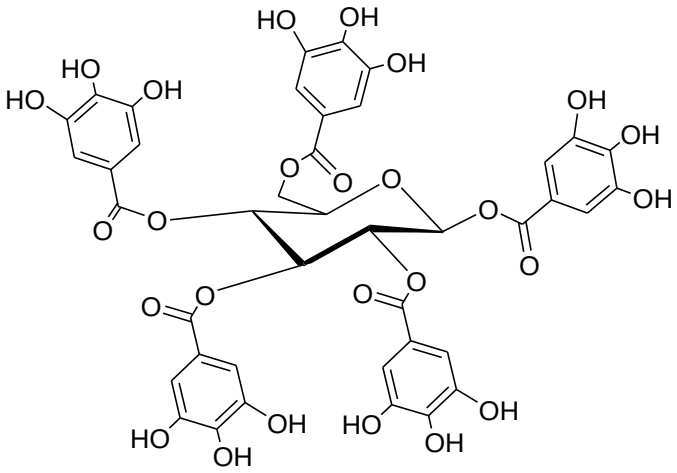


Figure 1.7 A simple comparison of Gram-positive and Gram-negative bacteria cell wall structure.

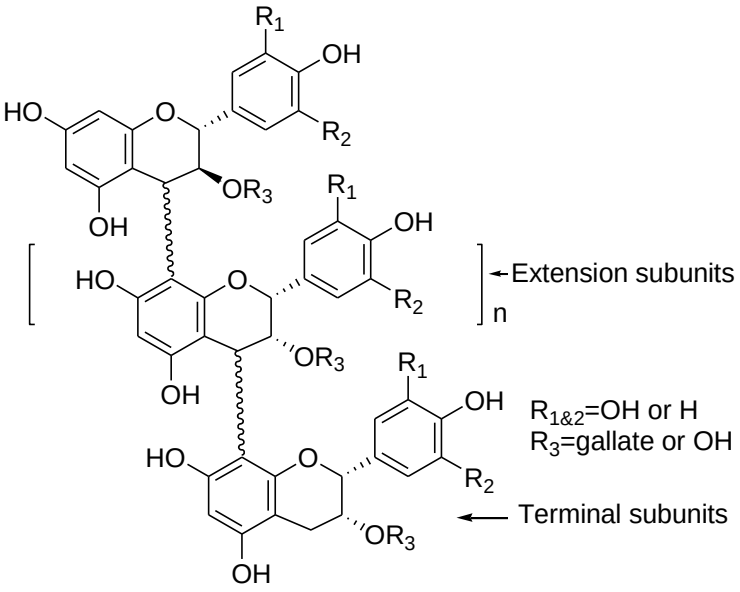
Flavonoids are a large group of compounds of secondary metabolites found in plant tissues including fruits, seeds and bark (Andersen and Markham, 2006). Many research groups have found that different subgroups of flavonoids have antibacterial, antifungal and antiviral activity (Cushnie and Lamb, 2005). For example, A-type PAs in cranberry fruit inhibit the urinary tract infection caused by *Escherichia coli* (Foo et al., 2000). Zang et al. (2013) also found A-type PAs from the herbaceous stems of *Ephedra sinica* (Ephedrae Herba in Traditional Chinese Medicine) have antimicrobial activity against the Gram-negative bacterial species *Pseudomonas aeruginosa*, and Gram-positive bacterial species methicillin-resistant *Staphylococcus aureus* and fungi *Canidia albicans*.

Antimicrobial activity of gallotannins, proanthocyanidins and flavan-3-ols

Tannins are polyphenolic compounds. There are two types of tannins according to structural differences: condensed tannins and hydrolysable tannins. Condensed tannins are polymers formed by condensation of flavans and do not have a sugar residue. Hydrolysable tannins are esters of phenolic acid and a sugar moiety (usually glucose). Esterifications with gallic acids produce gallotannins; esterifications with ellagic acids give ellagitannins (Serrano et al., 2009). **Figure 1.8** shows the structure of a hydrolysable tannin (penta-*O*-galloylglucose) and a condensed tannin (B-type PAs). Flavan-*3*-ols are the monomeric unit of PAs. Engels et al. (2009 and 2011) have studied the antimicrobial activities of gallotannins. Their work suggests that the antimicrobial mechanism of gallotannins involves iron chelation and likely inactivation of bacterial membrane-bound proteins.



Gallotannin (penta-O-galloyl-beta-D-glucose)



B-type proanthocyanidins

Figure 1.8 Gallotannin and proanthocyanidins structures. Gallotannins are esters formed by gallic acids with hydroxyl groups of a glucose core (Engels et al., 2009). Proanthocyanidins are polymeric flavan-3-ols (Jin et al., 2012).

The minimum inhibitory concentration (MIC) is generally used for the measurement of the activity of antimicrobial agents. MICs are defined as the

lowest concentration of the stock that will inhibit microorganism's growth after overnight incubation and are expressed in grams of dry matter of inhibitory substance per liter. The MIC is determined by using a critical dilution assay (Andrews, 2001). Engels et al. (2011) found epigallocatechin gallate had antimicrobial activity against *Staphylococcus aureus* (Gram positive) with a MIC of 0.2 g/L, *Bacillus cereus* (Gram positive) with a MIC of 0.1 g/L, and *Listeria monocytogenes* (Gram positive) with a MIC of 0.5 g/L. (-)-Epigallocatechin gallate from green tea (*Camellia sinensis*) extract possessed strong antibacterial activity against *Staphylococcus aureus* with a MIC of 0.073 g/L, and (-)-epicatechin had weaker bactericidal activity with a MIC of 0.183 g/L (Ikigai et al., 1993). Shinmamura et al. (2007) also reported that epigallocatechin gallate from tea (*Camellia sinensis*) had antibacterial activity against *Staphylococcus aureus* with a MIC of 0.05-0.1 g/L.

The presence of galloyl and gallic moieties on the position 3 of the catechin C-ring was found to enhance antibacterial activity. The mode of action involves the damage of membrane lipid bilayers (Ikigai et al 1993), disruption of the interaction of the cell membrane and membrane proteins (Engel et al 2011). Among the catechin-type compounds from green tea extracts, epigallocatechin gallate and epicatechin gallate exhibited the most antibacterial activity (Taylor et al., 2005). In green tea, (-)-gallocatechin-3-gallate had higher antimicrobial activity against *Bacillus cereus* (Gram positive) than (-)-gallocatechin (Friedman et al., 2006).

PAs isolated from fruit peels of *Zanthoxylum piperitum* are reported to suppress the antibiotic resistance of methicillin-resistant *Staphylococcus aureus* (Hatano et al., 2005). Epicatechin, epicatechin gallate and epigallocatechin gallate can inhibit the plasma coagulation caused by *Staphylococcus aureus* (Akiyama et al., 2001). Daglia (2012) have reviewed that flavan-*3*-ols from oolong tea and green tea inhibited *Vibrio cholera* (Gram negative), *Streptococcus mutans* (Gram positive), *Campilobacter jejuni* (Gram negative), *Clostridium perfringes* (Gram positive), *and Escherichia coli* (Gram negative). Flavan-*3*-ols are also reported to have iron chelating ability and inhibit the growth of invasive microorganisms by

iron-depletion (Aron and Kenndy, 2008). Structurally, the hydroxyl pattern of the B-ring of the flavan-*3*-ols affects the inhibition of microbial growth. In general, the gallocatechins, which have three hydroxyl groups on the B ring, are more inhibitory than the catechins which only have two hydroxyl groups (Scalbert, 1991). Cos et al. (2003) have summarized the mode of action for proanthocyanidins' antimicrobial activities. PAs not only induce the complexation of enzymes due to tannin-protein interaction, but they also inhibit microbial metabolism through inhibition of oxidative phosphorylation. Moreover, the tannin-metal ion binding ability contributes to the PAs antimicrobial activity because metal ions are required for the growth of most microorganisms.

Carbohydrates classification

Carbohydrates are aldehydes or ketones with various hydroxyl groups; they are the building blocks and the principle components of the plant cell wall (Buchanan et al., 2002). Carbohydrates are divided into four groups: monosaccharides, disaccharides, oligosaccharides, and polysaccharides. Monosaccharides are the simplest carbohydrates consisting of small molecules with three to nine carbon atoms. Disaccharides are formed by condensation of two monosaccharide molecules. Oligosaccharides are formed by the linkage of two of more monosaccharides by O-glycosidic bonds (**Figure 1.9**). Polysaccharides are long chain of multiple monosaccharides linked by glycosidic bonds (Berg et al., 2002).

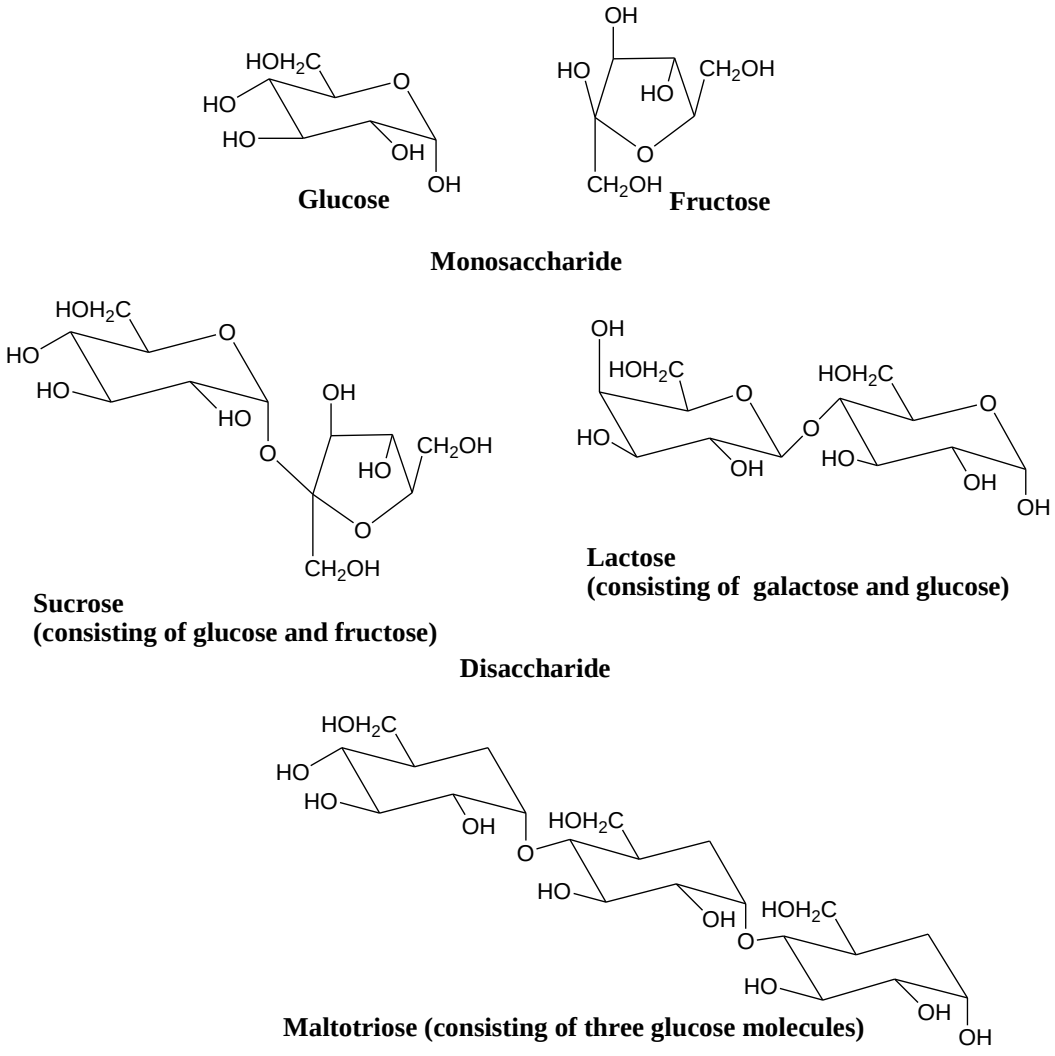


Figure 1.9 Examples of the structures of monosaccharides, disaccharides and trisaccharide

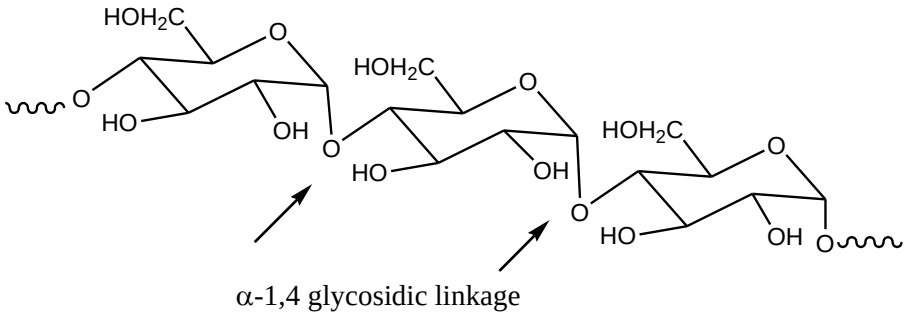
(Berg et al., 2002)**.**

Polysaccharides

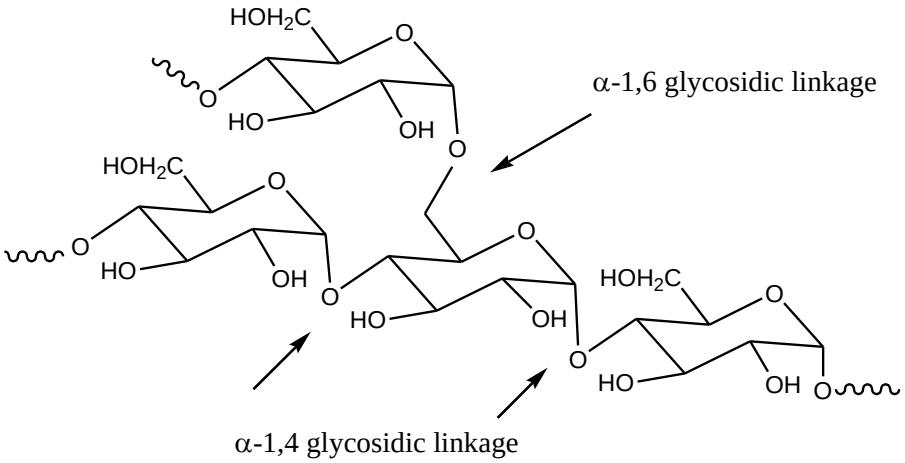
Polysaccharides are large polymeric sugars that are classified into two types in plants based on chemical structure and function: starches and non-starch polysaccharides (NSP).

Starch

The storage polysaccharide starch is a polymer of glucose formed by α-1,4 and α-1,6 glycosidic linkages. There are two types of starch based on the glucose chain structure, amylose and amylopectin. Amylose consists of glucose residues connected by α-1,4 linkages with few or no α-1,6-glycosidic linkages. Amylose is referred to an unbranched starch molecule. Amylopectin, a highly branched starch molecule, consists of glucose units connected by α-1,4 linkages with additional α-1,6 linkages approximately every 30 glucose units (Berg et al., 2002) (**Figure 1.10**). Amylose is insoluble in water, and more resistant to enzymatic degradation as the amylose chains can pack tightly together, since it has few end-points for the enzymes to attach to. Amylopectin is soluble in water at temperature higher than 40 ^{0}C and is able to form an aqueous colloidal solution. The branched structure of amylopectin can be rapidly degraded by starch cleaving enzymes as the branched structure provides many end-points for the enzymes to attach (Green et al., 1975).



Amylose



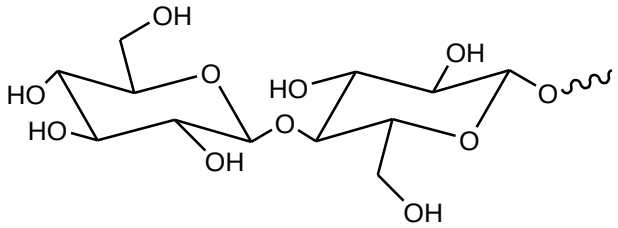
Amylopectin

Figure 1.10 Starch structures showing α-1,4 (amylose) and α–1,6 (amylopectin) glycosidic linkages (Berg et al., 2002).

Starches can also be classified into digestible starch and resistant starch (RS) based on their digestibility. Digestible starch is the starch absorbed in the small intestine of healthy individuals, and is hydrolysable by pancreatic amylase at 37 ^{0}C. RS is the sum of starch and its degradation products which are not absorbed in small intestine of healthy individuals (Leszczyński et al., 2004). RS is resistant to hydrolysis by pancreatic amylase in boiling water, but can be hydrolyzed by amyloglucosidase and pancreatic amylase in potassium hydroxide or dimethyl sulfoxide (Englyst, 1989). Amylose is an important type of resistant starch in the human diet.

Non-starch polysaccharides

The non-starch polysaccharides (NSP) make up the second class of plant polysaccharides. The NSP include cellulose, non-cellulosic polymers and pectin polysaccharides. These plant polysaccharides are made up of various monosaccharides linked by β-glycosidic linkages. NSP are the main components of plant cell walls (Englyst et al., 1994) (**Figure 1.11**).



β-1,4 glycosidic linkage

Figure 1.11 Non-starch polysaccharides (NSP) β-linkage (Berg et al., 2002).

There are several criteria for the classification of NSP. Based on reactions with water, NSP can be either soluble or insoluble (Englyst, 1989). Soluble NSP can form dispersions with water; whereas insoluble NSP do not. Based on extraction and isolation methodology, NSP consist of cellulose and hemicelluloses. Structurally, cellulose is an unbranched polymer of glucose joined by entirely β-1,4 linkages with an equatorial configuration, which allows very long straight chains (Kumar et al., 2012). Hemicellulose is a term for branched heteropolymers containing many different sugar monomers such as glucose, xylose, mannose, galactose, rhamnose and arabinose with a β−1,4 monomer linkage backbone (Scheller and Ulvskov, 2010) (**Figure 1.12**). Cellulose remains after a series of alkaline extractions of cell wall material; and hemicelluloses are solubilized by alkali (Neukom, 1976).

Based on the differences in solubility, NSPs that make up dietary fibre are classified into crude fibre (CF), neutral detergent fibre (NDF) and acid detergent fibre (ADF) (Degen et al., 2007; Englyst and Hudson, 1987; Englyst, 1989). CF is the residue that remains after acid and alkali extraction of plant material, and

includes variable portions of insoluble NSP. NDF consist of the insoluble NSP and lignin. Lignin is the second most abundant natural polymer which is only surpassed by cellulose. Lignin is not composed of sugar monomers, but consists of up to three phenyl propane monomers such as coumaryl alcohol, coniferyl alcohol, and syringyl alcohol (Freudenberg and Nash, 1968). ADF consist of a portion of insoluble NSP, mostly but not exclusively cellulose and lignin (Kumar et al., 2012). All of these classifications lack precision with respect to both chemical structures and biological functions, and the nutritional values are questionable. To avoid the vagueness in the classification, Bailey (1973) summarized the NSP into three groups: cellulose, non-cellulosic polymers, and pectic polysaccharides (**Figure 1.13**).

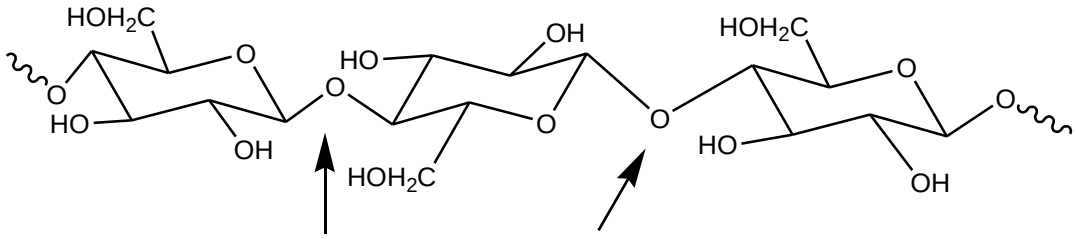
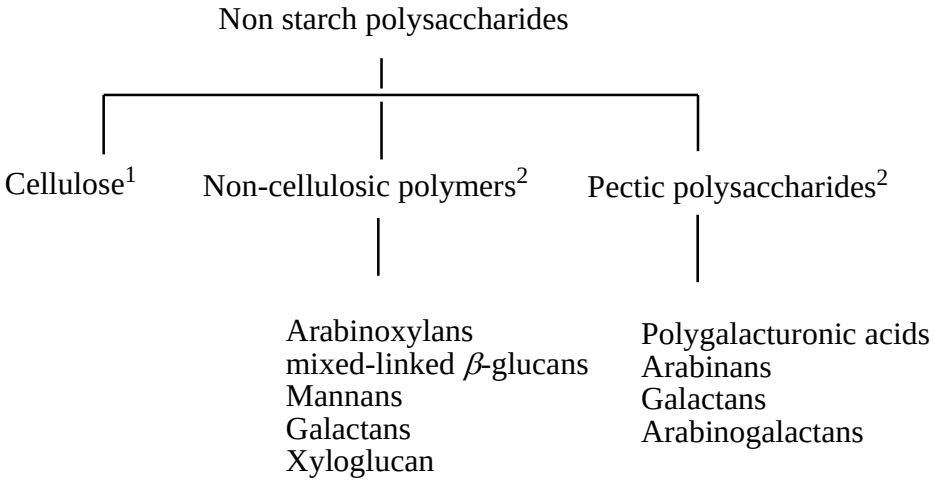


Figure 1.12 Cellulose structure showing β–1,4 glycosidic linkages



[1]Insoluble, [2]partially soluble in water

Figure 1.13 Classification of NSP (Choct, 1997)

Cellulose is the most abundant structural component of plant cell walls. Cellulose exists in the form of microfibrils in plant cell walls. Microfibrils are paracrystalline arrays of dozens of cellulose chains that are tightly hydrogen-bonded (Lavoine et al., 2012). Glycans are a class of polysaccharides that can hydrogen-bond to cellulose microfibrils and link them together to form a network; most cross-linking glycans are called by the common term 'hemicelluloses', which are defined as all materials extracted from the cell wall with molar concentrations of alkali, independent of structure ((Scheller and Ulvskov, 2010; Buchanan et al., 2002). Two major classes of cross-linking glycans in flowering plants are xyloglucans and glucuronoarabinoxylans. In the cell walls of dicots, the major cross-linking glycans are xyloglucans (Buchanan et al., 2002). Xyloglucans are linear chains of (1→4) β D–glucan with 1,6 linked xylose side chains; some of the xylosyl sites are substituted further with galactose, sometimes galactose is further substituted with fucose (Buchanan et al., 2002). Plants from the legume family (Fabaceae), which includes *Pisum sativum* (pea), contain the xyloglycan class known as fucogalacto-xyloglucans (Brennan and Harris, 2011).

In fucogalacto-xyloglucans, the α-D-xylosyl units are added to three contiguous glucosyl units of the backbone to form a heptasaccharide unit. On about one-half of these units, an α-L-Fuc-(1→2) β- D-Gal is added to the O-2 position of the xyl side group nearest the reducing end to form a monosaccharide unit (**Figure 1.14**) (Buchanan et al., 2002).

Dicots also contain glucuronoarabinoxylans in addition to the more abundant xyloglucans. Glucuronoarabinoxylans consist of the linear β-1,4-linked D-xylopyranosyl backbone, branched with arabinose and glucuronic acid (**Figure 1.15**) (Buchanan et al., 2002).

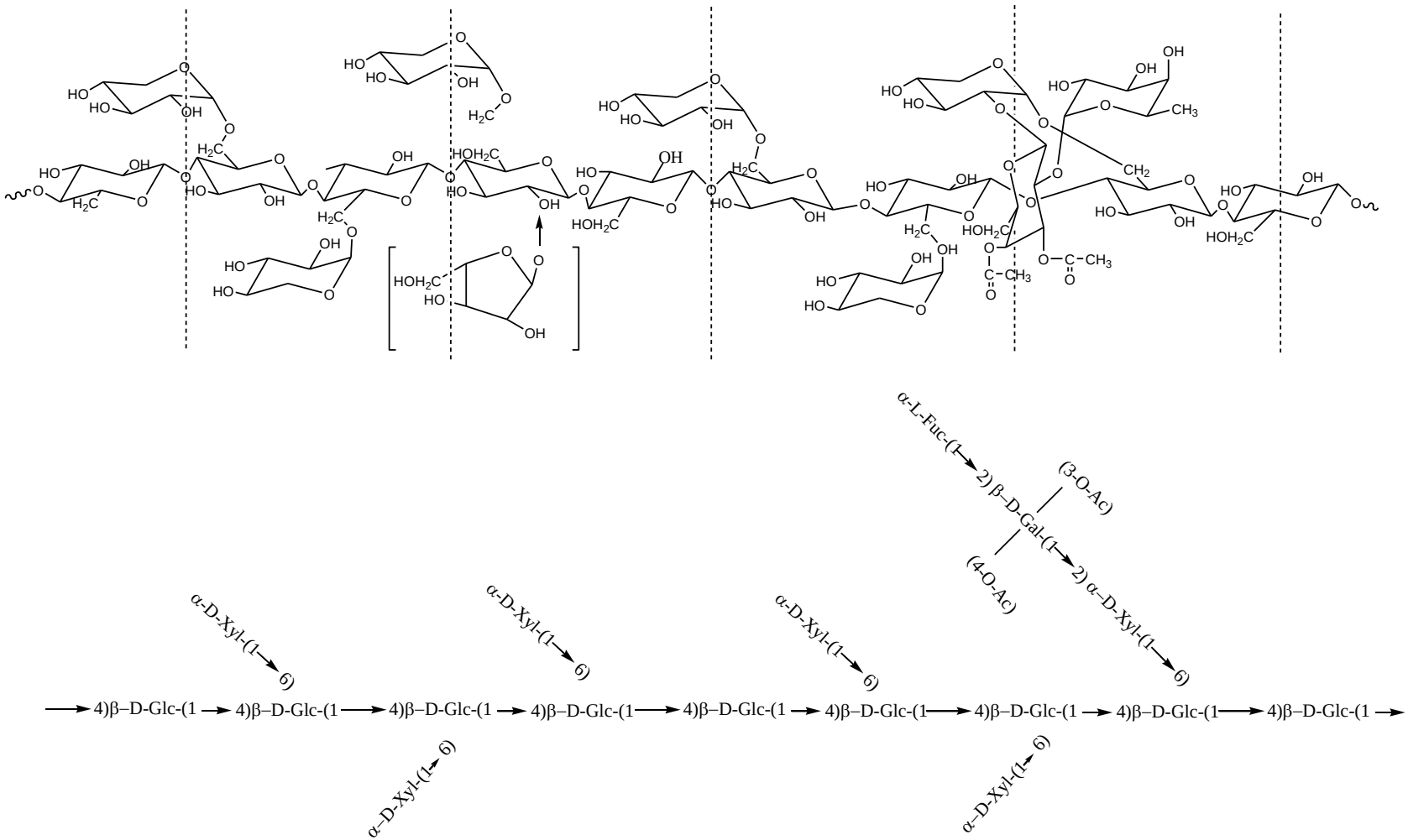


Figure 1.14 Structure of a fucogalacto-xyloglucan (Adapted from Buchanan et al., 2002).

Abbreviations: **Xyl-**Xylose**, Glc-**Glucose, **Fuc**-Fucose, **Gal**-Galactose

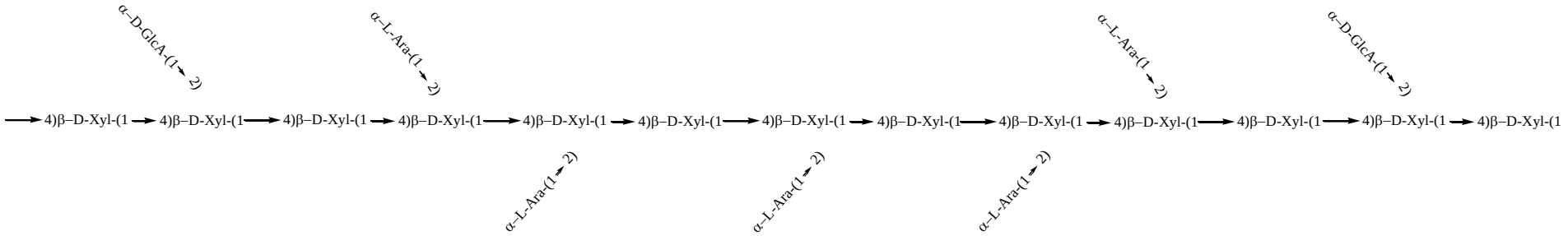


Figure 1.15 Structure of a glucuronoarabinoxylans.

(Adapted from Buchanan et al., 2002).

Abbreviations: **Xyl-**Xylose**, GlcA-**glucuronic acid, **Ara-**Arabinose

Pectins are a mix of polysaccharides rich in D-galacturonic acids. The most abundant pectic polysaccharide is homogalacturonan, which is a linear homopolymer of α-1,4-linked galacturonic acids (Mohnen, 2008). However, in grain legumes, the most common pectin is arabinogalactans type 1, which consist of a characteristic rhamnogalacturonan (in a 1:1 ratio) backbone. The side chains are substituted at the C-4 position of the rhamnose, and are mainly linear (1~4)-linked galactans and highly branched (l~5)-linked arabinans (Sinha et al., 2011; Cheetham et al., 1993) (**Figure 1.16**).

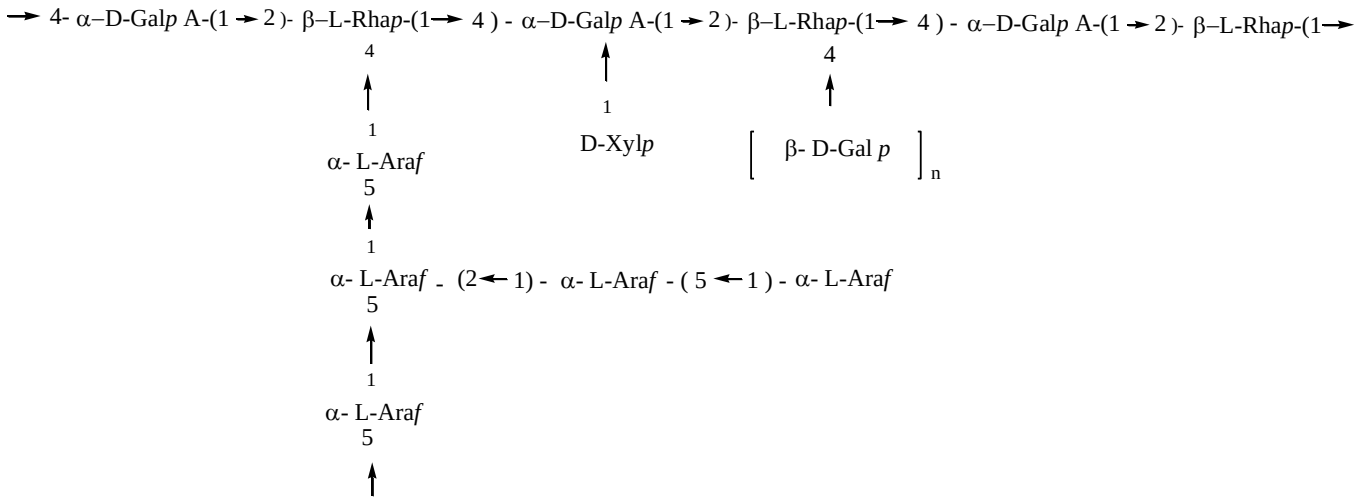


Figure 1.16 Structure of a pectin polymer from lupins
(Adapted from Cheetham et al., 1993).
Abbreviations: **Gal**-Galactose, **Ara**-Arabinose, **Rha**-Rhamnose
Gal A-galacturonic acids

Polysaccharides in human nutrition

Starches

Starch is an important energy source for humans. In the human diet, starch can be obtained from processed or unprocessed food such as bread, cereals, pulses, fruits and vegetables. Starch will be broken down to glucose after digestion in the human gastrointestinal tract, and glucose will be transported to tissues for energy use or storage. Starch found in storage organ plant tissues is produced and stored in organelles called amyloplasts. Amyloplasts are responsible for synthesis and storage of starch granules through polymerization of glucose in plants cells (BeMiller and Whistler, 2009). As mentioned previously, plants make both amylase and amylopectin starch molecules. Amylopectin, the branched starch chain, is the major component of most starch granules in crop plants. Starch granules grow by deposition of starch in layers. A starch granule is water insoluble and densely packed. The surface of starch granules is the first barrier for granule hydration, enzyme attack or chemical reaction (BeMiller and Whistler, 2009; Zobel, 1988). The amount of amylase to amylopectin, and the type of starch granule, will determine how quickly the starch can be enzymatically degraded.

Digestible starch can be easily broken down to glucose in the human small intestine, and resistant starch is not. It is reported that resistant starch is involved in decreasing glucose level in blood (Bornet et al., 1989; Shen et al., 2011). Resistant starch, compared to digestible starch, is not digested in the small intestine, but fermented by gut-colonising bacteria in the large intestine to produce short-chain fatty acids, acetic, propionic and butyric acids, which are responsible for reducing the levels of cholesterol, triglycerides, and urea in blood, as well as preventing the formation of gut cancer (Leszczyński, 2004).

Non-starch polysaccharides (NSP)

NSP have important benefits for human health, and a total NSP intake of 13 to 18 gram per day (approximately 50% from cereals, 40% from vegetables and 10% from fruit) is recommended for healthy people (Green, 2001). The beneficial effects of dietary NSP include the following: 1) Maintenance of colonic

absorption. For example, pectin and soy polysaccharides can increase colonic water absorption which is mediated by short chain fatty acids (SCFAs), as SCFAs stimulate the transportation of colonic fluid and electrolytes. Reduction in SCFAs in antibiotic-associated colitis may cause diarrhea; 2) Maintenance of gastrointestinal structure and health. For example, SCFAs produced from fermentable NSP can increase cell proliferation of colon epithelial cells, therefore increasing their absorptive capacity; 3) Fermented products of NSP can promote beneficial colonic bacterial growth and function; this is termed a prebiotic effect (Kumar et al., 2012). Another interesting health implication of NSP is their ability to lower the risk of developing diabetes mellitus. Soluble NSPs, especially mixed-link β-glucans can help to normalize blood glucose and insulin levels. This effect is proposed to be related to small intestinal viscosity and nutrient absorption, and systemic effects from colonic-derived SCFAs. NSP may also retard α-amylase action by capsuling starch and α-amylase, thus directly inhibiting the breakdown of starch to glucose (Kumar et al., 2012; Ou et al., 2001).

Determination of non-starch polysaccharides (NSPs) content

Since polysaccharides are classified by various methods, the determination of NSP from plant material is complex and diverse. In general, there are two types of measurements to determine the NSP from plant samples: the gravimetric method and chemical method (Englyst, 1989). The gravimetric method measures the dietary components gravimetrically which resist breakdown by starch and protein degrading enzymes. The Association of Official Analytical Chemists (AOAC) procedure is based on this method (Englyst, 1989). Crude fibre estimation is an example of the gravimetric method; however, it underestimates the total NSP because of the low recovery of cellulose, hemicelluloses and lignin. The chemical method, also called monomeric component analysis, quantifies the constituent individual sugars of the NSP by gas chromatography (GC), high-performance liquid chromatography (HPLC), or spectrophotometry; and determines the total NSP by summation (Sinha et al., 2011). Notably, monomeric component analysis yields lower values compared to gravimetric analysis because

of the exclusion of lignin and resistant starch during the chromatographic assay. Rapid technologies such as near infra-red reflectance (NIR) or transmission (NIT) spectroscopy are also applied to give fast, safe, and inexpensive analysis for dietary fibre (Blakeney and Flinn, 2005).

In the monomeric component technique using GC analysis, the NSP sugars are reduced to their alditols by alkaline sodium borohydride and then acetylated by acetic anhydride in the presence of methylimidazole as the catalyst (Sinha et al., 2011). In general, the procedure includes three steps. The first step is the hydrolysis and removal of starch (including resistant starch) by dispersing the sample in dimethyl sulphoxide (DMSO). The starch is then hydrolyzed by incubation with enzymes such as pancreatin and pullulanase. Pancreatin is an α-amylase enzyme which can hydrolyze the α-1,4 glycosidic bonds between glucose units in starch. Pullulanase (pullulan-6-glucanohydrolase) is an enzyme that can hydrolyze both α-1,6 and α-1,4 glycosidic starch bonds. The second step is the hydrolysis of the starch-free residue by 12 M sulphuric acid producing neutral sugar monomers. The third step is the derivatization of the monosaccharides to alditol acetates for GC analysis (Englyst, 1987; Englyst and Hudson, 1989).

Determination of starch content

Both total starch and resistant starch content can be measured based on enzymatic/spectrophotometric procedures. The total starch analysis procedure includes two steps for starch hydrolysis (Megazyme K-TSTA 07/11). Firstly, starch is partially hydrolyzed to maltodextrins by α-amylase which can catalyze the hydrolysis of α-1, 4-glycosidic linkages. Secondly, the starch dextrins are quantitatively hydrolyzed to D-glucose by amyloglucosidase which can cleave the terminal α-1, 4-glycosidic linkage and α-1, 6-glycosidic linkages. Then, D-glucose is oxidized to D-gluconate and hydrogen peroxide (H_2O_2) by glucose oxidase. H_2O_2 is quantitatively measured in a colourimetric reaction with *p*-

hydroxybenzoic acid producing a quinoneimine dye that can be monitored at λ 510 nm (**Figure 1.17**).

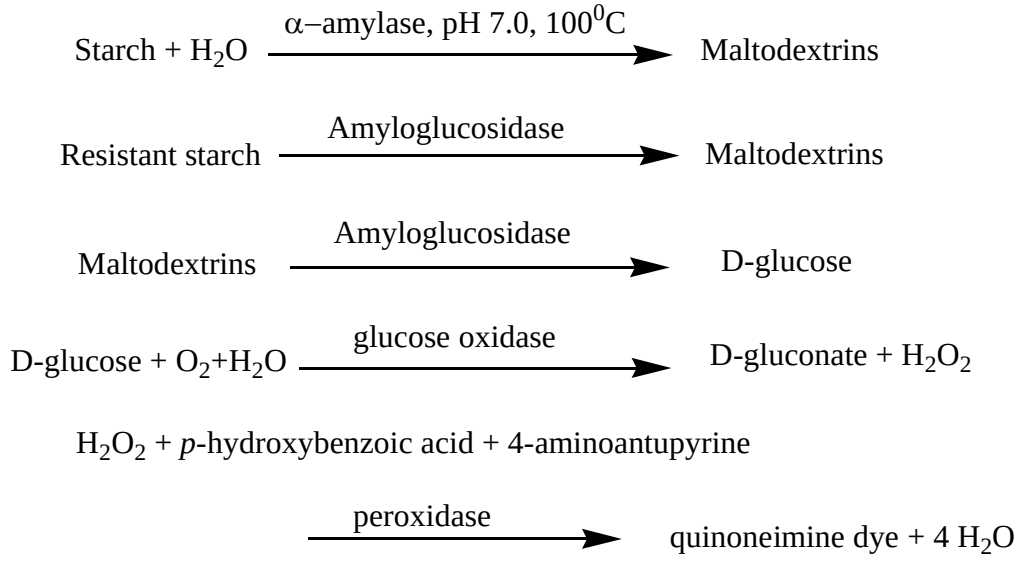


Figure 1.17 Total starch analysis principle.

For resistant starch analysis, firstly, the non-resistant starch is hydrolyzed to D-glucose by pancreatic α-amylase and amyloglucosidase. Secondly, the resistant starch is recovered as a pellet after centrifugation, and is quantitatively hydrolyzed to D-glucose and further processed for quantitation as described for total starch above (Megazyme RSTAR 11/02).

Determination of protein content

The nitrogen content can be determined by combustion using an automatic nitrogen analyzer. Total (crude) protein content can then be calculated by multiplying the sample nitrogen content with a conversion factor of 6.25 (method 968.06; AOAC, 2005). The nitrogen analyzer (TruSpec CN, model FP-428, Leco Instruments Ltd.) is an instrument that analyzes the carbon and nitrogen content of samples. One analysis cycle includes three phases: purge, combustion and analysis. In the purge phase, an encapsulated sample is purged of any atmospheric gases that have entered during sample loading. During the combustion phase, the

sample is dropped onto a hot surface (950 ^{0}C) and flushed with pure oxygen for very rapid combustion. The sample then undergoes oxidation. In the analysis phase, the gases from combustion are mixed with oxygen, and then carbon content is detected as carbon dioxide by a CO_2 infrared detector. By sweeping through hot copper, oxygen is removed, and NO_x is changed to N_2. The nitrogen content is detected by a thermal conductivity cell (Theory of operation, TruSpec CN, model FP-428, Leco Instruments Ltd., Mississauga, ON, Canada).

Legume seeds

Crop legumes belong to the *Fabaceae* (formerly *Leguminosae*) plant family. Crop legumes include some important and common food grains such as pea (*Pisum sativum* L.), lentils (*Lens culinaris* L.), and faba beans (*Vicia faba* L.). These crop legumes are important crops grown in western Canada with substantial economic significance. For human consumption, green peas are eaten as a vegetable (as fresh, canned, or frozen) and dried peas (also called field pea) can be made into flour or used in soups. These legume seeds are a rich source of protein, dietary fibre, and starch. For example, lentils are good source of dietary fiber, of which 93%-99.7% is insoluble fibre (Faris et al., 2013). More than that, legume seeds are also a good source of vitamins such as folate, thiamin (B1) and riboflavin (B2), and minerals such as iron (Crépon et al., 2010). In some cultivars, legume seed coats are rich sources of anthocyanidins (dry beans) and PAs (peas, faba beans, lentils) (Jin et al., 2012; Duenas et al., 2003). The high content of digestible protein and starch, as well as the presence of vitamins, minerals and flavonoids, makes legume seeds an excellent food source for humans and animals.

Thesis goal

The overall goal of this research was to characterize the components of legume seeds, and determine if specific seed components have potential functional properties for practical end-uses. To this end, one study (Chapter 2) investigated the antimicrobial activity of PA-enriched fractions derived from pea seed coats. A second study (chapter 3) was intended to specifically characterize

the fibre, protein and starch components of western Canadian grown grain legume seeds including pea, faba bean and lentils. Characterization of these seed components will aid in understanding their role in nutrition and potential amelioration of type 2 diabetes.

Chapter 2 Characterization and antimicrobial activity of proanthocyanidins from 'Solido' pea seed coats

Introduction

The seed coats of pea (*Pisum sativum L.*), are rich sources of proanthocyanidins (PAs) (Jin et al., 2012; Duenas et al., 2003). The PA-containing pea cultivars Acer, Rocket, and Solido contain PA subunits that mainly consist of the flavan-*3*-ols gallocatechin (GC) and epigallocatechin (EGC) that have tri-hydroxylated B-rings (**Figure 1.2**) (Jin, 2011; Jin et al., 2012). PAs (Scalbert, 1991) and their flavan-*3*-ol subunits (Ikigai et al., 1993; Taylor et al., 2005; Daglia, 2012) have antimicrobial properties. Since tri-hydroxylated flavan-*3*-ols (e.g. GC and EGC) have a higher antimicrobial potential compared to the di-hydroxylated forms (e.g. catechins, C and epicatechins, EC) (Scalbert 1991), we were interested in determining the antimicrobial activity of PAs made up of mainly GC and EGC derived from pea seed coats.

Staphylococcus is a genus of gram-positive bacteria; they are characterized by round shape (cocci) and can form grape-like clusters (Kloos and Bannerman, 1994). *Staphylococcus aureus* is an aerobic bacterium, and it is responsible for many infections (for example, boils as skin infection) and food poisoning (caused by toxins produced by *S. aureus in* contaminated foods) (Harris et al., 2002). *Staphylococcus pasteuri* is an aerobic bacterium and is recognized as the agent causing nosocomial infections and also considered as a blood derivatives contaminant (Savini et al., 2009). *Pediococcus* is a genus of gram-positive lactic acid bacteria that can grow in the absence of iron (Bryneel et al., 1989). *Pediococcus acidilactici* is an anaerobic bacterium usually found in fermented vegetables, dairy products and meat; it is a potential probiotic, having human and animal health beneficial effects (Klaenhammer, 1993).

In order to investigate the structure/function relationship of PAs and flavan-*3*-ols with antimicrobial activity, the flavan-*3*-ols C, EC, GC, EGC,

epigallocatechin gallate (EGCG), and PA extracts from 'Solido' pea seed coats were tested for antimicrobial activity against *S. aureus*, *S. pasteuri* and *P. acidilactici*. The two strains were selected to obtain strains that are sensitive (staphylococci) and resistant (pediococci) to epigallocatechin gallate and produce acid (Engels et al., 2011). Acid production was relevant because the turbidity of the stock solutions obscured the turbidity caused by bacterial growth, requiring the use of pH as an indicator of bacterial growth.

Materials and methods

Plant material

Pea seed coats (from *Pisum sativum* L. cv. Solido) were obtained from Mountain Meadows Food Processing Ltd. (Site 13, Box 45, RR 1, Legal, Alberta, Canada T0G 1L0). The seed coats were from pea seeds grown in 2009.

Chemicals

Organic solvents used for extraction, purification, and HPLC mobile phases (acetone, chloroform, methanol, acetonitrile) were purchased from Fisher Scientific (Ottawa, ON) and of HPLC grade. Ferric chloride hexahydrate was purchased from BDH chemicals (Toronto, ON). (+)-Catechin hydrate, (−)-epicatechin, (−)-gallocatechin, (−)-epigallocatechin, (−)-epigallocatechin gallate, phloroglucinol, trifluoroacetic acid (TFA), L-ascorbic acid, hydrochloric acid (36.5–38%), and Toyopearl resin (HW-40F) were purchased from Sigma (Oakville, ON). Grape skin and black currant leave PA phloroglucinolysis adducts were kindly provided by Dr. Lihua Jin, University of Alberta.

Extraction and purification of proanthocyanidins

The proanthocyanidins extraction procedure was adapted from Jin et al., (2012), and performed by Dr. Jin (University of Alberta) to obtain the "crude PA extract". The procedure was as follows. The seed coats were ground to a fine

powder in liquid nitrogen with a mortar and pestle. The ground powder was soaked in 80 mL of 66% aqueous acetone in a 250 mL Erlenmeyer flask. The flasks were immediately sparged with nitrogen to minimize oxygen content in the flask and capped with glass stoppers. Subsequently, the flasks were placed on a shaker at 100 rpm for 24 hours at 4 ℃ in the dark. Extracts were then filtered through 5.5 cm Whatman #1 filter paper using a Buchner funnel under moderate vacuum. The residue was washed with 66 % aqueous acetone, the rinse liquid was pooled, and the acetone was removed under vacuum using a Speed-Vac concentrator (AES 2000, Savant, NY, USA). The remaining aqueous extract was partitioned 4 times with chloroform (3:1,v/v) to remove lipophilic compounds and flavan-*3*-ol monomers using a separatory funnel. The remaining aqueous extract was dried to a powder using a freeze-drier. The final powder was defined as the "crude PA extract".

The "crude PA extracts" were further purified by adsorption chromatography using Toyopearl resin (HW 40-F, Supelco, 1.5 ×12 cm polypropylene column, ~10-14 ml bed volume) preconditioned with 50% (v/v) aqueous methanol containing 0.1 % (v/v) trifluoroacetic acid (TFA). The extract powder was dissolved in a minimum amount of 50% v/v aqueous methanol with 0.1% v/v TFA, and then loaded onto the Toyopearl column (1g extract powder per 100 mL resin). The column was washed with 5 bed volumes of 50% v/v aqueous methanol with 0.1% v/v TFA. The column was then eluted with 4 bed volumes of 80% aqueous methanol with 0.1% v/v TFA. The methanol was removed from the aqueous methanol extracts with a Speed-Vac concentrator, and the remaining extract was dried down to a powder using a freeze-drier. The final powder was defined as the "PA-enriched extract" (**Figure 2.1**).

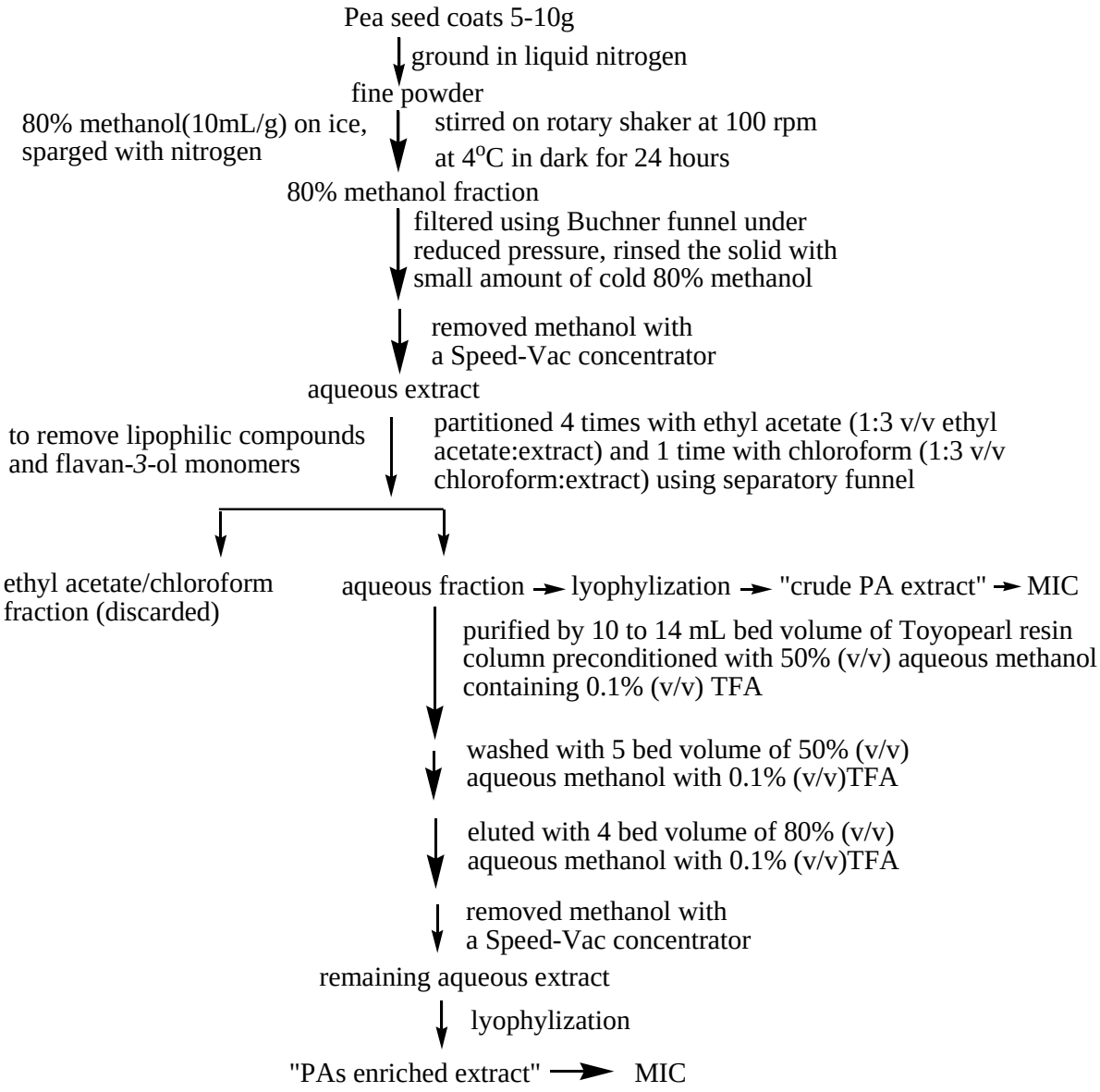


Figure 2.1 Extraction and purification of PAs from 'Solido' seed coats

(Jin et al., 2012)

Phloroglucinolysis and HPLC quantification of proanthocyanidin subunits

The phloroglucinolysis reaction of PA-enriched extract powder was performed in acidic conditions to cleave the PAs into their constitutive subunits, followed by derivatization with excess phloroglucinol (phloroglucinolysis) according to Jin et al. (2012). Approximately 5 mg of the lyophilized PA-enriched extract was dissolved in a 1 mL 0.1 N methanolic hydrochloric acid solution containing 100 g L^{-1} phloroglucinol and 10 g L^{-1} ascorbic acid. This reaction solution was incubated in a 50 ℃ water bath for 20 minutes, then a 200 µL aliquot of the solution was added to 1 mL of 40 mM sodium acetate solution to quench the reaction. A 20 µL aliquot of the diluted reaction solution was subjected to HPLC-DAD analysis to monitor free-flavan-*3*-ols (released from PA terminal subunits) and flavan-*3*-ol phloroglucinol adduct (from extension PA subunits) production.

A 20 µL aliquot of the diluted reaction mixture was injected onto two Chromolith RP-18e columns (4.6×100 mm) connected in series, protected by a guard column (Chromolith RP-18e, 4.6×10 mm), stabilized at 30 ℃, using an SHIMAZU Prominence HPLC system equipped with a SHIMAZU SPD M20A diode array detector (DAD). The samples were eluted at 3 mL/min using a linear gradient with 1% (v/v) aqueous acetic acid (solvent A) and acetonitrile with 1% acetic acid (v/v; solvent B) as follows: isocratic at 3% B from 0 to 4 min, 3% to 18% B by 14 min, and 80% B from 14 to 18 min. Free PA (terminal) and phloroglucinol-conjugated PA (extension) subunits were monitored at 280 nm. Solido seed coat PA terminal subunits were identified by comparison of RP-HPLC-DAD retention times and absorbance spectra with commercially available flavan-*3*-ol standards. Solido seed coats phloroglucinol-PA adducts were identified by comparison of RP-HPLC retention times to PA-phloroglucinol derivatization products from grape skin (epigallocatechin-(4β→2)-phloroglucinol, EGC-P) and black currant leaf (gallocatechin-(4α→2)-phloroglucinol, GC-P) PA reaction products that have been previously characterized (Kennedy and Jones, 2001; Tits et al., 1992). The mean degree of polymerization and conversion yield were calculated according to the method of Kennedy and Jones (2001).

Strains and culture conditions

S. aureus FUA 2082, *S. pasteuri* FUA 2077 and *P. acidilactici* FUA 3072 were kindly provided by Dr. Michael Gänzle (University of Alberta). *S. aureus* and *S. pasteuri* were incubated overnight in Luria-Bertani broth (LB) (Difco, Becton, Dickinson & Co., Sparks, MD) at 37^{o}C, *P. acidilactici* was incubated overnight in de Man, Rogosa, Sharpe (MRS) broth (Difco, Becton, Dickinson & Co., Sparks, MD) under anaerobic condition (BBL GasPak System, Becton, Dickinson & Co) at 37^{o}C. Stock cultures were stored at -80 ^{o}C in 50% glycerol.

MIC assays of proanthocyanidin fractions and flavan-*3*-ols

The antimicrobial activity as minimum inhibitory concentration (MIC) of extracts was investigated following Engels et al. (2009). MICs are defined as the lowest concentration of the extract that will inhibit the growth of a microorganism after overnight incubation and were expressed in grams of dry matter (dm) of the test substance per liter of bacterial solution. The MIC is determined by using a critical dilution assay. The stock solutions of each sample were prepared by dissolving the compounds or samples of interest into 100% methanol. Serial 2-fold dilutions of the crude PA extract, PA-enriched extract or flavan-*3*-ol standards stock solution were prepared in 96 well microtiter plates with LB broth or MRS broth. In brief, using a multi-channel pipette, 100 µL of sterile medium was added to each well of the microtiter plate. Sterile medium (50 µL) was added into wells as the negative control. The stock solution (100 µL) was added into wells as the highest inhibitory concentration. Two-fold serial dilutions were made by serially pipetting 100 µL of a higher concentration into 100 µL of sterile media. Methanol was evaporated from the microtiter plate solutions by placing the microtiter plates under a laminar flow hood for 2 hours. After methanol removal, the overnight bacterial culture was diluted by taking 1 mL overnight bacterial culture to 10 mL of medium, then 50 µL of diluted overnight bacterial culture was added to the microtiter wells. Subsequently, the microtiter plate was incubated overnight at 37^{o}C. After overnight incubation, 20 µL of bromocresol green aqueous solution (0.04% w/v, 0.04 g bromocresol green dissolved in 100 mL distilled water) was added to wells to measure pH changes in the bacterial

solution. The change in pH was measured by the addition of bromocresol green solution because bacterial growth as measured by turbidity was obscured with the precipitation of medium with the PA stock solutions. The MIC was determined as the lowest concentration of the compound or extract of interest that can inhibit the acidification of the bacterial medium. All serial dilution experiments were performed in triplicate.

Results and Discussion

Phloroglucinolysis and HPLC quantification of proanthocyanidin subunits from 'Solido' seed coats

Phloroglucinolysis is an acid-catalyzed cleavage reaction followed by phloroglucinol derivatization. In brief, the PAs polymers are cleaved under acidic condition in the presence of the nucleophile phloroglucinol. Under these reaction conditions, the flavan-*3*-ol extension units form phloroglucinol adducts, and the terminal flavan-*3*-ol units do not. The subunits can then be characterized by HPLC-DAD, and the PAs composition and polymer mean degree of polymerization (mDP) can be calculated according to Kennedy and John (2001).

Grape skin and black currant leaf PA standards (kindly provided by Dr. Lihua Jin, Jin et al., 2012) were subjected to phloroglucinolysis followed by RP-HPLC-DAD analysis, epigallocatechin-(4β→2)-phloroglucinol (EGC-P) from grape skin and gallocatechin-(4β→2)-phloroglucinol (GC-P) from black currant leaves were used as the reference compounds. A HPLC retention time comparison of references compounds and 'Solido' seed coat phloroglucinolysis reaction products confirmed the identity of the following compounds: GC-P: gallocatechin- (4α→2)-phloroglucinol; EGC-P: epigallocatechin- (4β→2)-phloroglucinol; GC: gallocatechin; EGC: epigallocatechin; EC: epicatechin (**Table 2.1**). The PA flavan-*3*-ols extension subunits were gallocatechin and epigallocatechin. The PA flavan-*3*-ols terminal subunits consisted mainly of gallocatechin, epigallocatechin, and with minor amounts of epicatechin. The PA

extension units and terminal units are assumed to be linked in a B-type configuration (**Figure 1.3**) because the interflavonoid bonds were easily cleaved within 20 minutes of acid-hydrolysis. The cleavage of the double linkage in the A-type PAs requires more reaction time and/or stronger acid condition. The mDP of the PA polymers was calculated at 2 subunits in length (**Table 2.1**).

Table 2.1 Summary of pea ('Solido') seed coat PA subunit composition following phloroglucinolysis and RP-HPLC-DAD analysis.

Compound	**Retention time(min)**	**Molar percentage**
'Solido' GC-P	1.97	30.0 ± 1.15[a]
Black currant leaf GC-P	1.80	
'Solido' EGC-P	2.36	23.4 ± 0.22
Grape skin EGC-P	2.20	
'Solido' GC	3.13	20.5 ± 0.33
GC standard	3.13	
'Solido' EGC	8.19	25.3 ± 1.38
EGC standard	8.21	
'Solido' EC	11.24	8.0 ± 0.03
EC standard	11.21	
mDP		***2.1 ± 0.1***
Conversion yield [b]		***70.4 ± 0.04%***

[a] Molar % ± SE (n=3)
[b] Yield of PAs extract calculated (%)

Abbreviations: GC-P: gallocatechin- (4α→2)-phloroglucinol; EGC-P: epigallocatechin- (4β→2)-phloroglucinol; GC: gallocatechin; EGC: epigallocatechin; EC: epicatechin.

The 'Solido' seed coat PAs subunits and terminal flavan-3-ols monomers were consistent with those identified by Jin (2011) in this cultivar. However, the mDP of the PA extract was only 2 in this study, compared to a mDP of 5 reported

by Jin (2011) for 'Solido'. The mDP of 2 subunits is indicative of the presence of flavan-*3*-ol monomers in the PA-enriched extract. The difference between the two studies could be due to different column chromatography dynamics and solvent systems. In Jin (2011), the column bed volume was 4 mL, whereas the column bed volume used in this purification procedure was 10 to 14 mL. The larger bed volume used in this study may have increased the retention time of the flavan-*3*-ol monomers on the column (due to increased flow resistance) resulting in some monomers eluting with the PA polymer fraction. The solvents used for purification in this study also varied from those used by Jin (2011). Jin (2011) used 66% aqueous acetone with 0.1% v/v TFA for the final wash of the Toyopearl column. In this study, 80% methanol with 0.1% v/v TFA was used for the column final wash. Acetone has better solubility for PAs-containing material compared to methanol (Kennedy, 2003), and as a result, 66% aqueous acetone with 0.1% v/v TFA might result in a higher efficiency for PAs extraction. Even though the mDP was low in the PA-purified fraction, this PA extract was enriched in PA polymers compared to the initial crude PA extract.

Antibacterial activity of pea PA-extracts and flavan-*3*-ols monomers

The crude PA and PA-enriched extracts, along with the flavan-*3*-ols standards C, EC, GC, EGC, and EGCG exhibited different antimicrobial activities against the aerobic bacteria *S. aureus*, *S. pasteuri*, and the anaerobic bacteria *P. acidilactici*. In this study, the MIC of EGCG on *S. aureus* was 0.05 g/L, and supplementation of iron increased the MIC to 0.10 g/L. These results are in agreement with the EGCG antimicrobial data reported by Engels et al. (2011) (**Table 2.2**). Additionally, the MIC of EGCG on *S. pasteuri* was 0.07 g/L, and supplementation with iron decreased the inhibitory activity with a MIC of 0.23 g/L. *P. acidilactici* was found to be resistant to the presence of EGCG (MIC > 1.7 g/L) in this study.

EGCG is the most studied flavan-*3*-ol-type flavonoid compound, and it was used as a reference compound for the MICs assays in this study. *S. aureus* and *S. pasteuri* were both inhibited by EGCG. The mode of antibacterial action of

EGCG involves membrane damaging activity as shown by 5,6-carboxyfluorescein released from the intra liposomal space in *S. aureus* (Ikigai et al., 1993); and membrane protein binding ability as shown by EGCG covalently binding to sulfhydryl groups of membrane proteins at the C2 and C6 position of the B-ring, and inducing membrane protein intermolecular cross-linking (Chen et al., 2011). Furthermore, EGCG is more inhibitory in the absence of iron than in the presence of iron. This is due to the iron binding ability of EGCG (Khokhar et al., 2003). Hydroxyl groups from the B-ring and the galloyl groups of EGCG can bind with iron (Khokhar et al., 2003). *P. acidilactici* does not require iron for growth. Consistently, *P. acidilactici* was resistant to the presence of EGCG in MIC assays (Engels et al., 2011). These data support that at least one antibacterial mode of action of ECGC involves iron binding.

Table 2.2 MICs of flavan-*3*-ols monomers and PAs enriched extract

	S. aureus MIC(g/L)	*S. pasteuri* MIC(g/L)	*P. acidilactici* MIC(g/L)
Epigallocatechin gallate W/Fe^{3+}	0.10±0[a]	0.23±0.03	>1.7
W/O Fe^{3+}	0.05±0	0.07±0.03	>1.7

	S. aureus MIC(g/L)	*S. pasteuri* MIC(g/L)	*P. acidilactici* MIC(g/L)
Catechin W/ Fe^{3+}	3.37±0	4.49±1.94	3.37±0
W/O Fe^{3+}	2.81±0.97	3.37±0	6.73±0
Epicatechin W/ Fe^{3+}	3.42±0	6.83±0	3.42±0
W/O Fe^{3+}	5.53±1.91	6.83±0	>6.63
Gallocatechin W/ Fe^{3+}	>0.17	>0.17	>0.17
W/O Fe^{3+}	>0.17	0.08±0	>1.0
epigallocatechin W/ Fe^{3+}	>0.56	0.56±0	>0.28
W/O Fe^{3+}	0.23±0.08	0.07±0	>1.1

	S. aureus MIC(g/L)	*S. pasteuri* MIC(g/L)	*P. acidilactici* MIC(g/L)
Crude PA W/Fe^{3+}	0.16±0.07	0.38±0	1.91±0
W/O Fe^{3+}	0.10±0.03	0.10±0	1.91±0
PA enriched W/Fe^{3+}	0.42±0	0.56±0.24	>0.83
W/O Fe^{3+}	0.28±0.12	0.21±0	>0.83

[a] g/L+SD (n=3). [b] W=with. [c] W/O=without

The flavan-*3*-ols C and EC have two hydroxyl groups on the B ring (4', 5'-hydroxylated), and they differ in their stereochemistry by the configuration of two chiral centers on carbons 2 and 3 of C-ring (2, 3-*cis* and 2, 3-*trans* flavan-*3*-ols, respectively) (**Figure 1.2**). GC and EGC have three hydroxyl groups on the B ring (3', 4', 5'-hydroxylated), and also differ in their stereochemistry by the configuration of carbon 2 and 3 of C-ring (2, 3-*cis* and 2, 3-*trans* flavan-*3*-ols, respectively).

The MIC data of the flavan-*3*-ols showed that in general GC and EGC were much more inhibitory to *S. aureus* and *S. pasteuri* growth than C and EC (**Table 2.2**). The MIC of C on *S. aureus* was 2.81 g/L, and supplementation with iron did not markedly change the inhibitory activity (MIC of 3.37 g/L, similar to that obtained for *S. pasteuri*). The MIC of EC on *S. aureus* was 5.53 g/L, and supplementation with iron also did not markedly change the inhibition activity (MIC of 3.42 g/L). *S. pasteuri* was resistant to the presence of EC (MIC of 6.83 g/L). However, *S. aureus* and *S. pasteuri* growth was markedly inhibited by the presence of GC and EGC (MICs ranged from 0.07 to 0.23 g/L), and supplementation with iron decreased the inhibition activity of GC and EGC on both these bacterial species. *P. acidilactici* growth was resistant to the presence of all the flavan-*3*-ols at the concentrations used in this study (**Table 2.2**). In conclusion, the increase of one hydroxyl group in the flavan-*3*-ol B-ring resulted in increased antimicrobial activity, likely due to enhanced iron binding abilities (Scalbert, 1991). The stereochemistry configuration on carbons 2 and 3 of the flavan-*3*-ol C-ring did not appear to affect the antimicrobial activity tested in these assays.

The crude PA and PA-enriched extracts both showed antimicrobial activity against *S. aureus* and *S. pasteuri* (**Table 2.2**). However, *P. acidilactici* was resistant to the presence of the PA extracts. The crude PA extract contained PA polymers, flavan-*3*-ols, and other small phenolic compounds. The PA-enriched extract was mainly PA polymers along with a small amount of flavan-*3*-ols monomers. The PA-enriched extract was less inhibitory than the crude PA extract in the MIC assays for *S. aureus* and *S. pasteuri*, possibly due to the presence of phenolic acids in this extract. All the MICs values are higher in the presence of iron than in the absence of iron, this would suggest that the antimicrobial mode of action is related to PA-iron binding capability. PAs are reported to have ability to complex metals through their *ortho*-diphenol groups (Buzzuni et al., 2007), and bacterial growth can be severely inhibited by iron depletion. These results generally agree with the literature that specific PAs and flavan-*3*-ols possess

antimicrobial activity (Buzzuni et al., 2007; Aron and Kenndy, 2008; Serrano et al., 2009).

In conclusion, this study confirmed ‘Solido’ seed coat PA composition and investigated the antimicrobial activity of PA-containing seed coat extracts against the gram-positive bacteria *S. aureus*, *S. pasteuri* and *P. acidilactici*. ‘Solido’ seed coats contain B-type proanthocyanidins and the composition units are mainly gallocatechin and epigallocatechin. An increasing number of hydroxyl groups on the aromatic B-ring could enhance the antimicrobial activity of the flavan-*3*-ols. The antimicrobial mechanism of flavan-*3*-ols and PAs appears to involve their iron binding capacity.

Chapter 3 Protein, starch and fibre components of pea (*Pisum sativum* L), faba bean (*Vicia faba* L) and lentil (*Lens culinaris* L) seeds grown in western Canada

Introduction

The embryos of pea, faba bean, and lentil seeds are rich sources of protein and starch (Dahl et al., 2012; Faris et al., 2013; Crépon et al., 2010). Their seed coats are also a rich source of fibre which is mainly made up of complex carbohydrates of the cell walls (Reyes-Moreno and Paredes-López, 1993). Data obtained using a rat model suggested that the carbohydrate components (fibre) of pea seed coats can ameliorate the symptoms of type 2 diabetes (Whitlock et al., 2012). Therefore, we were interested in analyzing the polysaccharide (fibre) components of legume seed coats as well as obtaining a base level of the other main seed nutrients. In this study, the protein, starch and fibre components (total NSP and soluble NPS) of faba bean ('Fatima' and 'Snowbird'), lentil ('CDC LeMay', 'CDC Plato', and 'CDC Redberry') and pea ('Courier', 'Solido', and 'Canstar') seeds grown in western Canada have been investigated to determine their specific nutritional values. The specific fibre components of pea seed coat fractions used to further understand the effects of pea seed coat fibre on the amelioration of type 2 diabetes symptoms in the rat model system were also characterized. The data from this study can be used to further understand the health/nutritionally- beneficial components of pea, faba bean and lentil seeds for human or animal consumption and for amelioration of chronic diseases such as type 2 diabetes.

Materials and Methods

Plant Materials

Seeds of lentil (*Lens culinaris* L., cvs. CDC LeMay, CDC Plato, and CDC Redberry), faba bean (*Vicia faba* L., cvs. Fatima and Snowbird), and pea (*Pisum*

Sativum L., cvs. Solido, Courier, Canstar) were produced in Alberta, Canada in 2008, except for Canstar, which was grown in Alberta, Canada in 2011.

Chemicals

Megazyme total starch kit (K-TSTA) and Megazyme resistant starch kit (K-RSTAR) were purchased from Megazyme Cedarlane (Burlington, ON, Canada). Organic solvents (ethanol, glacial acetic acid, dichloromethane, acetic anhydride, and acetone) were of HPLC grade and purchased from Fisher Scientific (Ottawa, ON). Sulfuric acid (95-98%), food grade hydrogen chloride (36.5-38%), ammonia, sodium acetate, monobasic sodium phosphate monohydrate, sodium borohydride were purchased from Fisher Scientific (Ottawa, ON). 2-deoxy-D-glucose, 1-methylimidazole, pancreatin (P-7545) and pullulanase (P-5420) were purchased from Sigma-Aldrich (Oakville, Ontario).

Cooked and hydrolyzed seed coats preparation

For cooked seed coat samples, 'Canstar' seed coats (146 g) and 'Solido' seed coats (193 g) were heated in boiling water (1 L) for 30 minutes. The seed coats and the water fraction remaining after the heating were lyophilized using a freeze dryer (Virtis Ultra 35L Freeze Dryer, Stone Ridge, New York, United States) for 7 days. For hydrolyzed seed coats sample, 'Canstar' seed coats (146 g) and 'Solido' seed coats (193 g) were incubated in a acidic solution (1 L, 2N made by 660 mL ethanol, 330 mL food grade HCl, and water in a 2 L volumetric flask) at 100 ^{0}C for 1.5 hour, then NaOH (54 g) was added to partially neutralize the acidic solution. The seed coats and solution remaining were then lyophilized using Virtis Ultra 35L Freeze Dryer for 7 days. The seed coat samples were stored in a freezer (-80 ^{0}C) before use.

Determination of protein content

Whole seeds were dehulled manually to seed coats and embryos. Mature embryos (20 g) and seed coats (10 g) per cultivar were ground using a Retsch, ZM 200 (PA, USA) mill equipped with a 0.5 mm screen to produce finely ground samples. The ground samples were then lyophilized using a freeze dryer (Virtis Ultra 35L Freeze Dryer, Stone Ridge, New York, United States) for 7 days. The

total (crude) protein content of the legume seed coats and embryos was calculated by multiplying the nitrogen content with a conversion factor of 6.25 (method 968.06; AOAC, 2005). The nitrogen content of each sample (100mg) was determined with a nitrogen analyzer (model FP-428, Leco Instruments Ltd., Mississauga, ON, Canada). Caffeine (150 mg) and EDTA (Ethylenediaminetetraacetic acid) (100 mg) were analyzed as the standards for calibration.

Determination of total and resistant starch content

Whole seeds of lentil ('CDC LeMay', 'CDC Plato', and 'CDC Redberry'), faba bean ('Fatima' and 'Snowbird'), and pea ('Solido', 'Courier', and 'Canstar') were ground using a Retsch, ZM 200 (PA, USA) mill equipped with a 0.5 mm screen to produce finely ground samples. The unprocessed ground samples were lyophilized using a freeze dryer (Virtis Ultra 35L Freeze Dryer, Stone Ridge, New York, United States) for 7 days. For the cooked treatment, the ground powder was boiled in water for 30 minutes, then lyophilisation was performed as described above.

The total starch assay was based on the Megazyme total starch assay procedure AA/AMG 11/01. In brief, a ground and lyophilized sample (50 mg) was placed into a Corning culture test tube (20 mL) (Sigma-Aldrich, Oakville, Ontario). Aqueous ethanol (0.2 mL 80 % v/v), then DMSO (2 mL) were added immediately to the tube, followed by vortexing and incubation in a boiling water bath for 5 minutes. Thermostable α-amylase (3 mL; 300 units) was added immediately to the tube after removal from the water bath. The reaction solution was vortexed then incubated in the boiling water bath for another 6 minutes, with vortexing at every other 2 minutes. The reaction solution was cooled to about 50 ^{o}C, then sodium acetate buffer (4 mL; 200 mM, pH 4.5) and amyloglucosidase (0.5 mL) were added. The solution was vortexed and incubated at 50 ^{o}C for 30 minutes, with vortexing at every 15 minutes. Subsequently, a 1 mL aliquot of the reaction solution was transferred to a 2 mL microfuge tube. For whole seeds

samples, the whole content was diluted to 34.7 mL by adding 25 mL double distilled water, then a 1 mL aliquot from the diluted solution was transferred to a 2 mL microfuge tube. This was repeated to give a technical replicates. The microfuge tubes were centrifuged at 3511 *g* for 10 minutes. A 50 μL aliquot from each tube was transferred into a 2 mL glass test tube, and GOPOD (glucose oxidase/peroxidise) (1.5 mL) solution was added. The solution was mixed well by inverting, then incubated in a 50 ^{o}C water bath for 20 minutes. After incubation, a 350 μL aliquot from each tube was loaded into a 96-well UV plate and the absorbance was read at 510 nm with a spectrophotometer (SpectraMax Multi-Mode Microplate Reader, Sunnyvale, California, United States).

The resistant starch assay was based on the Megazyme resistant starch assay procedure (Megazyme RSTAR 11/02). This procedure includes two major steps. The first step is the hydrolysis and solubilisation of non-resistant starch. A ground and lyophilized sample (80~100 mg) was placed into a Corning culture test tube (20 mL). Pancreatic α-amylase (4.0 mL; 10 mg/mL) containing amyloglucosidase (3 U/mL) was added to the tube. The solution was vortexed and incubated at 37^{o}C with continuous shaking at 200 strokes/min (setting of 100 on the water bath is equivalent to 200 strokes/min, which means 100 forward and 100 reverse) for exactly 16 hours. The sample was removed from the water bath and 99% ethanol (4.0 mL) was added with vigorous stirring on a vortex mixer, then the solution was centrifuged at 1,500 *g* for 10 minutes using an ultracentrifuge (Beckman L8-70M**,** Mississauga, Ontario, Canada). After centrifugation, the supernatant was carefully decanted and the pellet was re-suspended in 50% aqueous ethanol (2 mL) with vigorous stirring using a vortex mixer. An additional 50% aqueous ethanol (6 mL) was then added to the solution and mixed by vortexing. The solution was centrifuged at 1,500 *g* for 10 minutes. The supernatant was then discarded, and this suspension and centrifugation step was repeated. Finally the supernatant was carefully decanted and the tube was inverted onto an absorbent paper to drain the excess liquid. The second step of the procedure is the measurement of resistant starch. A magnetic stirrer bar (5 x 15 mm) and aqueous KOH (2 mL; 2 M) were added to the tube containing the starch pellet, and the tubes contents were stirred

for 20 minutes in an ice water bath over a magnetic stirrer. Then sodium acetate buffer (8 mL; 1.2 M, pH 3.8) was added to the solution with continuous stirring using the magnetic stirrer, followed by amyloglucosidase (0.1 mL; 3300 U/ml). The reaction solution was mixed well and incubated in a water bath at 50^{o}C for 30 minutes with occasional vortexing. After the incubation, the solution was centrifuged at 1,500 *g* for 10 minutes and the final solution volume in each tube was 10.3 mL. A 1 mL aliquot from the sample solution was transferred into a microfuge tube. This was repeated to give a technical replicates. The microfuge tubes were centrifuged at 3511 *g* for 10 minutes. A 50 µL aliquot from each microfuge tube was transferred into 2 mL glass test tubes and GOPOD solution (1.5 mL) was added and mixed well by inverting. The solution was incubated in 50 ^{o}C water bath for 20 minutes. After incubation, a 350 µL aliquot from each tube was loaded into a 96-well UV plate and the absorbance was read at 510 nm with a spectrophotometer (SpectraMax Multi-Mode Microplate Reader, Sunnyvale, California, United States).

Determination of non starch polysaccharides content

Mature air-dried whole seeds were separated into seed coats and embryos. Embryos (20 g) and seed coats (10 g) per cultivar were ground using a Retsch, ZM 200 (PA, USA) mill equipped with a 0.5 mm screen to produce finely ground samples. The ground samples were lyophilized with a freeze dryer (Virtis Ultra 35L Freeze Dryer, Stone Ridge, New York, United States) for 7 days. NSP components analysis of embryos and seed coats were performed according to the method described in Englyst and Hudson (1987) and Englyst (1989).

Measurement of total and insoluble non starch polysaccharides

Briefly, the procedure can be summarized as three major steps. The first step is the hydrolysis and removal of starch. Seed coat sample (45 to 50 mg) was added to a glass test tube (16 x 125 mm). DMSO (dimethyl sulphoxide) (0.25 mL) was added to the tube, after vortexing, the solution was heated at 100^{o}C in a water

bath for 1 hour. The tubes were immediately transferred to another water bath heated to 42 ^{o}C, then sodium acetate buffer (1 mL; 0.1M, pH 5.2), aqueous pancreatin solution (100 μL; 25 mg/mL) and aqueous pullulanase solution (50 μL; made by 30 μL enzyme and 2.97 mL distilled water) were added to the tube, vortexed and incubated for 16 hours at 42 ^{o}C. The second step is the acid hydrolysis of the starch-free residue with sulphuric acid. Total NSP and insoluble NSP were measured in independent samples. For total NSP samples (two technical replicates), 95 % ethanol (6 mL) was added to the tube, vortexed and incubated 1 hour at room temperature. For insoluble NSP samples (two technical replicates), phosphate buffer (6 mL; 0.2 M, pH 7.0) was added to the tube, vortexed and heated for 1 hour in 100^{o}C water bath. Then the solution was centrifuged at 1914 *g* for 20 minutes using an ultracentrifuge (Beckman L8-70M, Mississauga, Ontario, Canada), and the supernatant was aspirated off. Subsequently, 85 % ethanol (5 mL) was added to the solution for total NSP measurement, and phosphate buffer (5 mL; 0.2 M, pH 7.0) was added to the solution for insoluble NSP measurement. After vortexing, the tube was centrifuged at 1914 *g* for 20 minutes and the supernatant was aspirated off. The tube was washed one more time use 85% ethanol (5 ml) and the residue was dried in a 65^{o}C water bath. The dried starch-free residue was dispersed in H_2SO_4 (0.5 mL; 12 M) and incubated in a 35^{o}C water bath for 1 hour. Then distilled water (5.5 mL) was added into the solution, vortexed and heated in 100^{o}C water bath for 2 hours. The solution was then cooled to room temperature, and aqueous myo-inositol (0.1 mL; 20 mg/mL) was added to the tube as the internal standard. The solution was vortexed and centrifuged at 2000 *g* for 5 minutes. A 1 mL aliquot of the hydrolysate was transferred to another 15 mL glass tube. The third step is the preparation of alditol acetates as constituent sugars for GC analysis. Ammonia solution (0.2 mL; 12 M) was added to the hydrolysate and the mixture was vortexed, then freshly prepared sodium borohydride solution (0.1 mL; 100 mg sodium borohydride per mL of 3 M ammonia solution) was added and the solution was incubated for 1 hour in a 40^{o}C water bath. Then glacial acetic acid (0.1 mL) was added to the solution. After vortexing, the acidified solution (0.2

mL) was transferred to another 15 mL glass tube, and 1-methylimidazole (0.3 mL) was added to the solution and mixed. Subsequently, acetic anhydride (2 mL) was added to the solution and vortexed continuously for 10 minutes, then distilled water (5 mL) was added to the solution to decompose excess acetic anhydride and aid in phase separation. When the solution was cooled to room temperature, dichloromethane (4 ml) was added and mixed for 15 seconds. After centrifugation at 700 *g* for 5 min, the top layer was aspirated off and distilled water (5 mL) was added. The solution was centrifuged at 700 *g* for 5 minutes, the top layer was aspirated off, and the bottom layer was dried with a 50 ^{o}C evaporator to obtain the residue. Dichloromethane (1mL) was added to dissolve the residue and the solution was transferred into screw cap GC vials for GC analysis (**Figure 3.1**).

A 0.5 μL aliquot of the derivatized sample was injected onto a DB-17 fused silica capillary column (0.25 mm i.d. x 30m; J&W Scientific, Folsom, CA) in Varian 3400 GC with cool-on-column injector. Helium was used as the carrier gas at the rate of 1.5 mL/min. The injector temperature was increased from 60 ^{o}C to 270 ^{o}C at the rate of 150 ^{o}C/min and maintained for 20 minutes. Oven temperature was raised from 50 ^{o}C to 190 ^{o}C at the rate of 30 ^{o}C/min, and maintained for 3 minutes; then increased to 270 ^{o}C at the rate of 5 ^{o}C/min, and maintained for 5 minutes. The flame ionization detector (FID) temperature was set at 270 ^{o}C. Peak area integration for carbohydrate analyses were according to a Shimadzu Ezchrom Data System (Shimadzu Scientific Instruments Inc., Columbia, MD). The difference of total NSP and insoluble NSP values was the soluble NSP value.

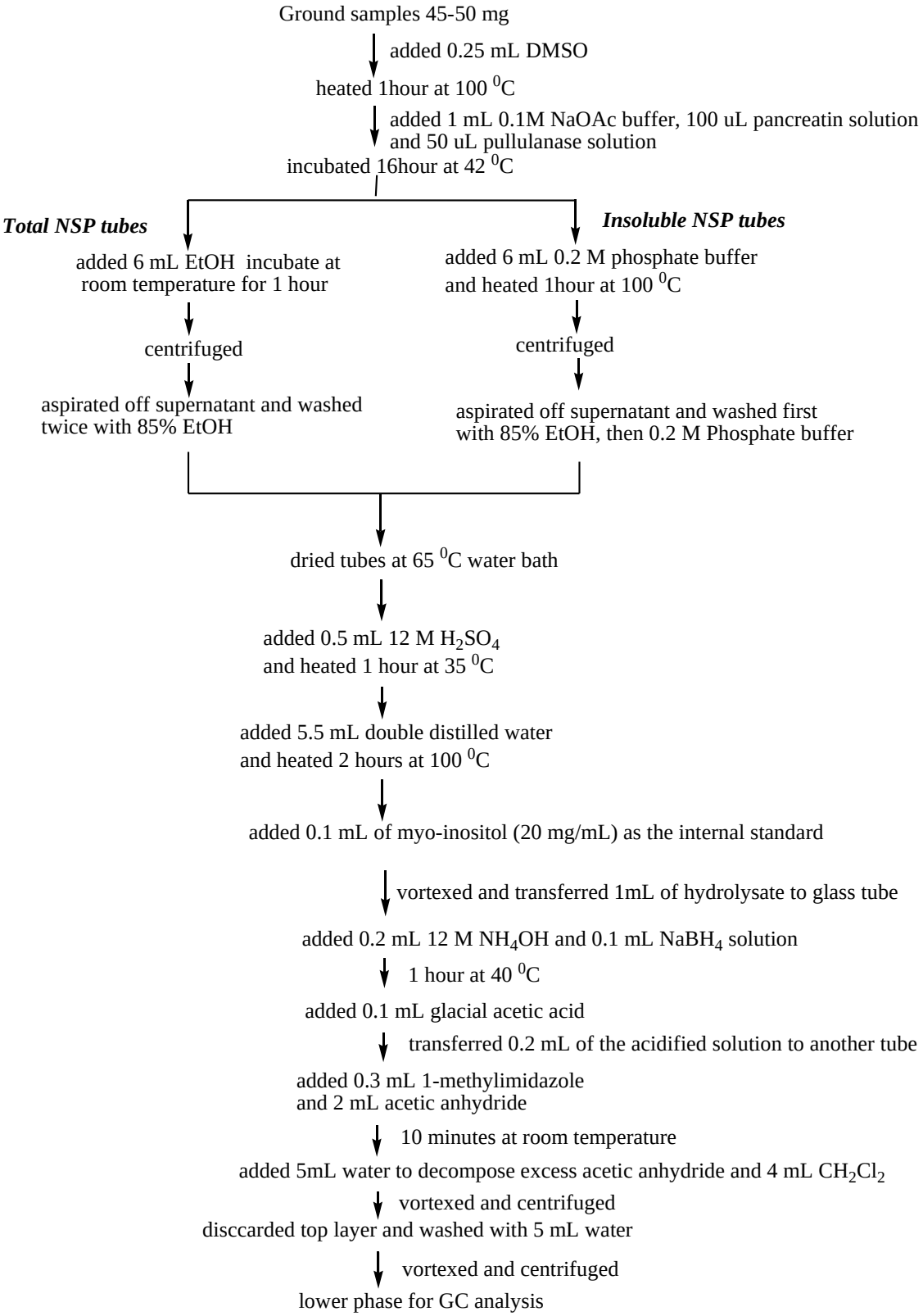


Figure 3.1 Analysis procedure for non-starch polysaccharides (NSP)

Results and discussion

Protein, starch and fibre components in seeds of different cultivars of lentil, faba bean and pea.

Protein

The embryos of crop legume seeds contain the majority of the seed protein compared to the seed coat. In our study, the protein content of embryos ranged from 28 to 29% in lentil, 34 to 35% in faba bean, and 25 to 26% in pea (**Table 3.1**). Protein content in the seed coats ranged from 11% to 12% in lentil, 7 to 10% in pea, and 7 to 8% in faba bean (**Table 3.1**). The western Canadian grown grain legume embryo protein content was consistent in general with reported values for the whole seed, as the seed is approximately 90% embryo (26% for whole lentil seeds, Faris et al., 2013; 25 to 37% for whole faba bean seeds, Crépon et al., 2010; 21 to 33 % for whole pea seeds, Dahl et al., 2012).

Starch

Among these grain legume raw whole seeds, total starch content ranged from 34 to 42% and resistant starch ranged from 7 to 13%. Specifically, the total starch content was 36 to 38% for lentil, 34 to 35% for faba bean, and 36 to 42% for pea (**Table 3.1**). Resistant starch values were 10 to 13% for lentil, 9 to 10% for faba bean, and 7 to 12% for pea (**Table 3.1**). The legume grain seed total and resistant starch values in this study are consistent to those found by Chung et al. (2008), who reported a total starch content for raw pea seed of 44.6 to 49.4%, and 46.0 to 47.1% for raw lentil seed; resistant starch content for raw pea seed as 10.1 to 14.7%, and 14.4 to 14.9% for raw lentil seeds; and by Crépon et al. (2010), who reported 41.2 to 42.7% of total starch content for raw faba bean seeds.

The cooking treatment (boiling in water for 30 minutes) increased the total seed starch content by 1 to 6%, and decreased the resistant starch content by 4 to 9% (on a dry weight basis) in pea, lentil and faba bean **(Table 3.1)**. Lentil cooked whole seeds had the highest total starch, and faba bean cooked whole seed had the

lowest total starch. Lentil raw whole seed contained the highest resistant starch, and there was no significant change in resistant starch level among lentil, faba bean and pea cooked whole seed **(Table 3.1)**. According to the literature, raw lentil seed flour contains a mean value of 16.1% resistant starch; cooked lentil seeds flour contains a mean value of 7.6% resistant starch (García-Alonso et al., 1998). Costa et al (2006) reported that cooking treatment decreased the resistant starch value in lentil and pea seeds. Pea and lentil seeds were first soaked in water for 16 hours and then freeze-dried in Costa's sample preparation method. Raw pea seed flour contains 2.5% resistant starch, cooked pea seed flour contains 1.9% resistant starch; raw lentil seed flour contains 3.3% resistant starch, cooked lentil seed flour contains 2.5% resistant starch (Costa et al., 2006). The lentil resistant starch values were lower than that reported by Garc á-Alonso et al. (1998), and this is likely because soaking allows seeds to imbibe water into their cells, and the seeds swell as they hydrate, which makes starch become more digestible (Carmona-Garcia R et al., 2007)

Our data also indicated that the cooking treatment decreased the resistant starch content for all cultivars. This is likely because in the presence of water and high temperature, gelatinization occurs when the starches absorb water and begin to swell, and the starch granules unwind, making the starch molecules more accessible for enzymatic degradation and more digestible (García-Alonso et al., 1998). In our data, the resistant starch content in the lentil cultivars were lower than what was found by Garc á-Alonso et al. (1998) and higher than that found by Costa et al. (2006); resistant starch content in pea cultivars were higher than that found by Costa et al. (2006). The cultivars, processing methods and analysis methods can affect starch content results. In conclusion, our data matched the previous reports, which showed that the cooking treatment decreased resistant starch amount.

Table 3.1 Protein, total starch and resistant starch profiles in seeds of different cultivars of lentil, faba bean and pea.

Cultivar	Protein[a] (%)	Total starch[b] (%)	Resistant starch[b] (%)
Lentil	***Seed coat/embryo***	***Raw/cooked***	***Raw/cooked***
CDC LeMay	11.44±0.06[c]/29.26±0.03	36.84±1.44/41.14±0.85	13.35±0.11/3.98±0.02
CDC Plato	12.38±0.20/28.34±0.05	37.93±0.97/44.49±0.22	12.89±0.27/3.63±0.04
CDC Redberry	11.48±0.06/29.28±0.07	36.31±1.15/42.35±0.3	9.89±0.31/3.79±0.00
Faba bean	***Seed coat/embryo***	***Raw/cooked***	***Raw/cooked***
Fatima	7.03±0.04/35.3±0.14	33.79±0.17/35.67±0.63	9.02±0.47/2.74±0.13
Snowbird	8.38±0.53/34.01±0.07	35.55±0.05/37.27±0.72	10.03±0.44/3.44±0.06
Pea	***Seed coat/embryo***	***Raw/cooked***	***Raw/cooked***
Solido	7.96±0.21/25.76±0.09	37.75±0.35/38.90±1.19	7.26±0.42/3.30±0.16
Courier	9.93±0.10/25.64+0.18	36.41±0.65/38.80±0.60	9.95±0.09/4.14±0.15
Canstar	6.65±0.05/24.62+0.25	42.36±0.43/44.48±0.29	11.58±0.38/3.77±0.01

[a] Protein content was determined in the seed coats and embryos of samples directly after grinding (raw).

[b] The total starch and resistant starch content was determined in raw or cooked whole seed samples.

[C] Data are means ± SE, n=3.

Fibre

The seed coats of crop legume seeds contain the majority of the fibre content of the seed. In lentil embryos, the total, insoluble and soluble fibre content ranged from 6 to 8%, 5 to 7%, and 1 to 3%, respectively, with 'LeMay' having the highest embryo soluble NPS content (**Table 3.2**). In lentil seed coats, the total, insoluble and soluble fibre content ranged from 39 to 54%, 40 to 47%, and 0 to 7%, respectively, with 'Plato' having the highest seed coat soluble NSP content (**Table 3.2**). For lentil whole seeds, the total, insoluble and soluble fibre ranged from 9 to 12%, 8 to 10% and 1 to 3% (**Table 3.3**). Tosh et al. (2010) reported that lentil whole seeds contained 18 to 20% total fibre, 11 to 17% insoluble fibre, and

2 to 7% soluble fibre using a gravimetric method. Kubicka et al. (2003) characterized the seed coat components of lentil (grown in Poland) with a total fibre of 83.4%, insoluble fibre of 72.0%, and soluble fibre of 11.5% as measured with a gravimetric method (Asp et al., 1983). In our study, the lentil seed coat fibre content in the cultivars studied were lower than that reported by Kubicka (2003) which included lignin (not included as a fibre component in our analysis), but the ratios of total, insoluble and soluble fibre content were general consistent with the above reported values.

In faba bean embryos, the total, insoluble and soluble fibre content ranged from 6 to 7%, 5%, and 1 to 2%, respectively; in faba bean seed coats, these values were 61 to 72%, 59 to 67%, and 2 to 5%, respectively (**Table 3.2**). For faba bean whole seeds, the total, insoluble and soluble fibre ranged from 14%, 12% and 1 to 2%, respectively (**Table 3.3**). The fibre values in this study were lower those reported by Giczewska et al. (2003), who reported that faba bean whole seeds grown in Poland contained 20 to 26.8% total fibre and 3 to 4% soluble fibre, using a fibre analysis method similar to that used in this study.

In pea embryos, the total, insoluble and soluble fibre content ranged from 7 to 8%, 4 to 5%, and 2 to 3%, respectively; in pea seed coats, these values were 50 to 68%, 43 to 65%, and 3 to 8% respectively (**Table 3.2**). For pea whole seeds, the total, insoluble and soluble fibre ranged from 11 to 13 %, 9 to 10% and 2 to 4% (**Table 3.3**). Dahl et al. (2012) summarized whole pea seed fibre data from seven published studies and reported pea seeds consist of 14 to 26% total dietary fibre, 10 to 15% insoluble fibre, and 2 to 9% soluble fibre. Daveby et al. (1993) reported fibre content values for pea embryos where the total dietary fibre includes lignin and uronic acid (which was not included as fibre components in our analysis). Their value for insoluble fibre content was 1.9 to 2.3%, the soluble fibre content was 1.2 to 1.3%, and the total fibre content was 5.5 to 6.8%. In pea seed coats, the total fibre content was 57.1 to 71.3%. In general, the data from our study on pea embryo and seed coat fibre contents were similar to those reported by Daveby et al. (1993).

Among the different legume species studied, pea embryos in general contained the highest soluble fibre (2 to 3%) (**Table 3.2**). The soluble fibre content of seed coats varied within cultivar among the legume grain seeds studied, with the lentil cultivar CDC Plato and the pea cultivar Courier having the highest values (**Table 3.2**). For whole seeds, pea seeds contained the highest soluble fibre (**Table 3.3**)

Table 3.2 Total fibre, insoluble fibre and soluble fibre content of seed coats and embryos from the seeds of various cultivars of lentil, faba bean and pea.

Species	Cultivar	Tissue	Total fibre (%)[a]	Insoluble fibre (%)[a]	Soluble fibre (%)[a]
Lentil					
	CDC LeMay	Seed coat	44.9 ±1.4[b]	39.8 ±0.6	5.0 ±1.0
		Embryo	8.2 ±0.4	5.8 ±0.9	2.4 ±0.6
	CDC Plato	Seed coat	53.7 ±2.2	46.6 ±2.3	7.1 ±1.7
		Embryo	7.6 ±0.2	6.4 ±0.5	1.2 ±0.6
	CDC Redberry	Seed coat	38.5 ±2.1	39.4 ±5.4	ND[c]
		Embryo	6.4 ±0.5	4.9 ±0.0	1.5 ±0.5
Faba bean					
	Fatima	Seed coat	60.6 ±0.2	59.1 ±1.9	1.5 ±1.9
		Embryo	6.6 ±0.1	4.8 ±0.4	1.8 ±0.4
	Snowbird	Seed coat	71.6 ±2.5	67.4 ±1.4	4.3 ±1.8
		Embryo	6.2 ±0.1	5.1 ±0.2	1.1 ±0.4
Pea					
	Solido	Seed coat	60.1 ±1.0	55.5 ±1.6	4.6 ±1.3
		Embryo	7.6 ±0.5	4.7 ±0.9	2.9 ±0.7
	Canstar	Seed coat	68.1 ±2.0	65.3 ±0.2	2.9 ±2.1
		Embryo	7.3 ±0.9	5.3 ±0.5	2.1 ±0.4
	Courier	Seed coat	50.3 ±0.5	42.8 ±1.7	7.6 ±1.3
		Embryo	7.9 ±0	5.3 ±0.4	2.6 ±0.4

[a] % = mg/100 mg dry weight of sample

[b] Data are means ± SE, n=3.

[c] ND=not detected

[d] Experimental error. Soluble fibre value is calculated as total fibre value subtracts insoluble fibre value.

Table 3.3 Total fibre, insoluble fibre and soluble fibre content of whole seeds of various cultivars of lentil, faba bean and pea.

Species	Cultivar	Tissue	Total fibre (%) [a]	Insoluble fibre (%)	Soluble fibre (%)
Lentil					
	CDC LeMay	Whole seed	11.7 ±0.5[b]	9.0 ±0.9	2.7 ±0.6
	CDC Plato	Whole seed	11.6 ±0.3	9.9 ±0.3	1.7 ±0.6
	CDC Redberry	Whole seed	9.1 ±0.4	7.9 ±0.5	1.2 ±0.8
Faba bean					
	Fatima	Whole seed	13.7 ±0.1	11.9 ±0.5	1.7 ±0.6
	Snowbird	Whole seed	14.0 ±0.3	12.5 ±0.1	1.5 ±0.3
Pea					
	Solido	Whole seed	13.0 ±0.5	9.9 ±0.7	3.1 ±0.5
	Canstar	Whole seed	11.3 ±0.8	8.6 ±0.4	2.7 ±0.7
	Courier	Whole seed	12.7±0.1	9.6 ±0.6	3.2 ±0.5

[a] mg/100 mg dry weight of sample

[b] Data are means ± SE, n=3.

Fibre components of embryos and seed coats of different cultivars of pea, faba bean and lentil.

Pea

In the embryos of the pea cultivars studied, the main components of the total fibre fraction were arabinose (46 to 51%) and glucose (33 to 38%) (calculated as the percent of a specific monosaccharide within the total monosaccharide detected by GC analysis of the alditol acetates derivatized from polysaccharide hydrolysates) (**Table 3.4**). The less abundant components of the total fibre fraction were galactose (5.9 to 7.2%), xylose (3.7 to 4.3%), rhamnose (2.0 to 2.2%), ribose (0.9 to 1.2%), fucose (0.5 to 0.6%) and mannose (0.5 to 0.6%).

The main components of the soluble fibre fraction of the pea embryos also consisted of arabinose (52 to 65%) and glucose (12 to 25%). The less abundant components of the soluble fibre fraction were galactose (9.2 to 13.2%), xylose (2.9 to 6.1%), ribose (2.5 to 3.5%), rhamnose (1.9 to 2.5%), mannose (0.2% to 0.7%) and fucose (0.1 to 0.6%) (**Table 3.4**).

Table 3.4 Fibre components of pea embryos by GC analysis

Embryo cultivars	NSP		Rhamnose (%)[a]	Ribose (%)	Fucose (%)	Arabinose (%)	Xylose (%)	Mannose (%)	Glucose (%)	Galactose (%)	total (%)
Courier	Total	% (mg/100mg dwt)	0.17±0.03[b]	0.08±0.00	0.04±0.00	3.92±0.03	0.30±0.01	0.04±0.00	3.00±0.05	0.47±0.01	8.01±0.05
		% (sugar/total sugar)	2.1±0.4	0.9±0	0.5±0	48.9±0.5	3.7±0.1	0.5±0	37.4±0.6	5.9±0.1	
	Insoluble	% (mg/100mg dwt)	0.11±0.02	0.01±0.00	0.04±0.00	2.45±0.20	0.21±0.01	0.03±0.01	2.30±0.31	0.24±0.01	5.40±0.42
		% (sugar/total sugar)	2.1±0.3	0.3±0.1	0.7±0.1	45.6±3.1	4.0±0.1	0.6±0.1	42.5±3.0	4.4±0.1	
	Soluble	% (mg/100mg dwt)	0.06±0.01	0.06±0.00	0.00±0.01	1.47±0.23	0.09±0.02	0.01±0.01	0.69±0.30	0.23±0.01	2.62±0.38
		% (sugar/total sugar)	2.4±0.9	2.5±0.4	0.1±0.3	57.3±9.1	3.2±0.2	0.4±0.2	25.0±10.4	9.2±0.9	
Canstar	Total	% (mg/100mg dwt)	0.17±0.04	0.09±0.01	0.04±0.01	3.84±0.47	0.29±0.02	0.04±0.00	2.48±0.31	0.53±0.04	7.48±0.87
		% (sugar/total sugar)	2.2±0.3	1.2±0.1	0.6±0.0	51.2±1.2	4.0±0.3	0.6±0.0	33.1±1.2	7.2±0.3	
	insoluble	% (mg/100mg dwt)	0.12±0.01	0.02±0.00	0.03±0.01	2.79±0.41	0.24±0.03	0.04±0.00	1.86±0.03	0.26±0.02	5.37±0.50
		% (sugar/total sugar)	2.3±0.2	0.4±0.1	0.6±0.1	51.4±3.1	4.4±0.2	0.7±0.1	35.2±3.1	4.9±0.1	
	Soluble	% (mg/100mg dwt)	0.05±0.04	0.07±0.01	0.01±0.00	1.04±0.06	0.05±0.01	0.00±0.01	0.62±0.30	0.27±0.02	2.11±0.41
		% (sugar/total sugar)	1.9±1.4	3.5±0.6	0.4±0.3	51.8±6.2	2.9±1.1	0.2±0.3	26.1±7.9	13.2±1.4	
Solido	Total	% (mg/100mg dwt)	0.16±0.02	0.08±0.01	0.05±0.00	3.61±0.52	0.34±0.05	0.05±0.01	2.96±0.48	0.51±0.03	7.75±0.46
		% (sugar/total sugar)	2.0±0.2	1.0±0.1	0.6±0.0	46.4±5.0	4.3±0.4	0.6±0.1	38.4±6.0	6.6±0.3	
	Insoluble	% (mg/100mg dwt)	0.17±0.05	0.02±0.00	0.03±0.01	2.05±0.28	0.19±0.02	0.04±0.01	2.13±0.59	0.20±0.02	4.83±0.90
		% (sugar/total sugar)	3.8±1.5	0.3±0	0.6±0.1	43.2±2.1	4.1±0.3	0.7±0.2	42.9±4.1	4.3±0.3	
	Soluble	% (mg/100mg dwt)	0[c]	0.06±0.01	0.01±0.00	1.56±0.24	0.14±0.02	0.01±0.01	1.76±1.01	0.31±0.02	2.92±0.67
		% (sugar/total sugar)	0[c]	2.6±0.9	0.6±0.2	65.4±27.4	6.1±2.6	0.4±0.3	12.1±36.8	12.4±3.9	

[a] % = mg/100 mg dry weight of sample
[b] Data are means ± SE, n=3
[c] Experimental error. Soluble fibre value is calculated as total fibre value subtracts insoluble fibre value

Among the seed coats of the pea cultivars studied, the main components of the total fibre fraction were glucose (71 to 74%) and xylose (15 to 20%). The less abundant components of the total fibre fraction were arabinose (5.9 to 6.6%), galactose (1.6 to 2.1%), rhamnose (0.6 to 1.2%), mannose (0.3 to 0.4%), fucose (0.4%) and ribose (0.1%) (**Table 3.5**).

The main components of the soluble fibre fraction of the pea seed coats also consisted of glucose (30 to 45%), arabinose (20 to 33%), xylose (19 to 20%), galactose (8 to 13%), rhamnose (3.2 to 4.8%), fucose (1 to 2%), mannose (0.5 to 1.9%), and ribose (0.1 to 0.7%) (**Table 3.5**).

The composition of the dietary fiber varies in the seed coat (termed outer fiber of the seed by food scientists) and in the embryo (termed inner fiber of the seed by food scientists) (Brooks et al., 2008). The greatest difference between seed coat fibre and embryo fibre is the amount of cellulosic and non-cellulosic polysaccharide present. For example, the fibre from seed coats of various lupin species (belongs to legume family) contains large quantities of cellulose ranging from 35 to 56% (Brillouet and Riochet, 1983), and lower amounts of hemicelluloses and pectins (Weightman et al. 1994). The fibre components of pea and faba bean embryos contain about 55% pectic substances, about 9% cellulose, and 6% to 12% non-starchy non-cellulosic glucans (a polymer of glucose) (Brillouet and Carre, 1983).

As discussed in the literature review, the major cross-linking glycans in dicot cell walls are xyloglucans (Buchanan et al., 2002). Xyloglucans are linear chains of glucose with xylose side chains, and sometimes the xylosyl sites are substituted further with galactose, then some galactose is further substituted with fucose (Buchanan et al., 2002). The legume family contains the xyloglycan class known as fucogalacto-xyloglucans which are made up of mainly linear chains of glucose, some xylose and very minor amounts of galactose from the side chains (Brennan and Harris 2011) (**Figure 1.14**). Dicots also contain glucuronoarabinoxylans in addition to the more abundant xyloglucans (Buchanan et al., 2002). Glucuronoarabinoxylans are composed of linear chains of xylose with arabinose and glucuronic acids as the branching side chains (**Figure 1.15**).

In our study, the main components of total fibre in the pea embryo (arabinose 46 to 51% and glucose 33 to 38%) varied from that in the pea seed coat (glucose 71 to 74% and xylose 15 to 20%) (**Table 3.4 and 3.5).** These data indicate that the most abundant component of the pea seed coat total fibre was cellulose (made up of linear chains of glucose). Since cellulose is a water-insoluble polysaccharide, the insoluble fibre component is also mainly made up of cellulose (35 to 43% glucose in pea embryos, 71 to 80% in pea seed coats) (**Table 3.4 and 3.5**). The occurrence of fucose, galactose, and xylose is indicative of the presence of the microfibril cross-linking polysaccharide fucogalacto-xyloglucan. The presence of arabinose (5.9 to 6.6%) in total fibre of pea seed coats suggests the presence of glucuronoarabinoxylans and/or pectins (Buchanan et al., 2002). Rhamnose is also a constituent of pectins. Mannose was also present at very low levels, indicating the presence of glucomannans, galactoglucomannans, or galactomannans in interlocking microfibrils in the cell wall (Buchanan et al., 2002).

The pea embryo consists of polysaccharides having various degrees of solubility, including hemicelluloses, pectins and celluloses (Dahl et al., 2012). In our study, the high amount of arabinose in total fibre indicates the presence of pectins as a main component in embryo fraction. The glucose was likely from the backbone of xyloglucans. Very small amount of rhamnose, ribose, and mannose were present (**Table 3.4**).

The main component of the soluble fibre of the pea seed coats is arabinose (20 to 33%) (**Table 3.5**), likely from glucuronoarabinoxylans or a small amount of pectin. Soluble fibre glucose (30 to 45%) (**Table 3.5**) is likely from xyloglucans and a small amount of cellulose. Galactose, xylose and the small amount of fucose indicated the presence of fucogalacto-xyloglucans in this fraction. Rhamnose, ribose and mannose were in very small amounts in the seed coat soluble fibre fraction.

In pea embryos, the soluble fibre component arabinose was the main component likely from pectins and/or glucuronoarabinoxylans. Rhamnose also was likely from pectins. Galactose, xylose and small amount of fucose indicated

the presence of fucogalacto-xyloglucans. Ribose and mannose were in very small amounts in the seed embryo soluble fibre fraction (**Table 3.4**).

Table 3.5 Fibre components of pea seed coats by GC analysis

Seed coat cultivars	NSP		Rhamnose (%)[a]	Ribose (%)	Fucose (%)	Arabinose (%)	Xylose (%)	Mannose (%)	Glucose (%)	Galactose (%)	total (%)
Courier	Total	% (mg/100mg dwt)	0.63 ±0.03[b]	0.06 ±0.01	0.20 ±0.01	3.41 ±0.17	7.49 ±0.42	0.21 ±0.01	38.26 ±0.89	1.08 ±0.03	51.34 ±0.56
		% (sugar/total sugar)	1.2 ±0.1	0.1 ±0.0	0.4 ±0.0	6.6 ±0.04	14.6 ±0.9	0.4 ±0.0	74.5 ±1.1	2.1 ±0.1	
	Insoluble	% (mg/100mg dwt)	0.37 ±0.03	0.02 ±0.00	0.11 ±0.01	1.81 ±0.10	5.89 ±0.19	0.16 ±0.01	34.77 ±1.40	0.50 ±0.03	43.61 ±1.69
		% (sugar/total sugar)	0.8 ±0.1	0.0 ±0.0	0.2 ±0.0	4.1 ±0.1	13.5 ±0.3	0.4 ±0.0	79.7 ±0.1	1.1 ±0.0	
	Soluble	% (mg/100mg dwt)	0.26 ±0.02	0.05 ±0.01	0.10 ±0.01	1.60 ±0.27	1.61 ±0.55	0.05 ±0.01	3.49 ±0.58	0.58 ±0.00	7.73 ±1.37
		% (sugar/total sugar)	3.7 ±0.7	0.7 ±0.2	1.3 ±0.3	20.8 ±0.3	19.7 ±3.2	0.7 ±0.1	45.3 ±0.9	7.9 ±1.3	
Canstar	Total	% (mg/100mg dwt)	0.33 ±0.01	0.02 ±0.00	0.22 ±0.00	3.53 ±0.09	11.98 ±0.33	0.15 ±0.01	41.55 ±1.37	0.95 ±0.01	58.72 ±1.97
		% (sugar/total sugar)	0.6 ±0.0	0.0 ±0.0	0.4 ±0.0	6.0 ±0.0	20.4 ±0.3	0.3 ±0.0	70.7 ±0.3	1.6 ±0.0	
	Insoluble	% (mg/100mg dwt)	0.19 ±0.01	0.01 ±0.00	0.16 ±0.00	2.21 ±0.06	10.96 ±0.29	0.12 ±0.00	34.84 ±3.49	0.59 ±0.03	49.07 ±3.17
		% (sugar/total sugar)	0.4 ±0.0	0.0 ±0.0	0.3 ±0.0	4.5 ±0.4	22.6 ±2.1	0.2 ±0.0	70.7 ±2.7	1.2 ±0.1	
	Soluble	% (mg/100mg dwt)	0.14 ±0.02	0.01 ±0.00	0.06 ±0.00	1.32 ±0.11	1.02 ±0.45	0.03 ±0.01	6.71 ±4.69	0.36 ±0.02	9.65 ±6.75
		% (sugar/total sugar)	3.2 ±2.0	0.1 ±0.0	1.6 ±1.1	33.3 ±21.9	19.7 ±9.2	0.5 ±0.2	31.6 ±40.8	10.1 ±7.1	
Solido	Total	% (mg/100mg dwt)	0.57 ±0.03	0.04 ±0.01	0.24 ±0.01	3.58 ±0.20	10.00 ±0.39	0.28 ±0.01	44.11 ±0.55	1.31 ±0.04	60.14 ±0.97
		% (sugar/total sugar)	0.9 ±0.0	0.1 ±0.0	0.4 ±0.0	5.9 ±0.2	16.7 ±0.5	0.4 ±0.0	73.4 ±0.8	2.1 ±0.0	
	Insoluble	% (mg/100mg dwt)	0.38 ±0.01	0.01 ±0.01	0.17 ±0.00	2.44 ±0.17	9.07 ±0.43	0.21 ±0.01	42.47 ±1.45	0.79 ±0.05	55.55 ±1.62
		% (sugar/total sugar)	0.7 ±0.0	0.0 ±0.0	0.3 ±0.0	4.4 ±0.4	16.3 ±0.4	0.4 ±0.0	76.4 ±0.8	1.4 ±0.1	
	Soluble	% (mg/100mg dwt)	0.19 ±0.02	0.03 ±0.00	0.07 ±0.01	1.14 ±0.11	0.93 ±0.39	0.07 ±0.02	1.65 ±1.00	0.52 ±0.03	4.59 ±1.32
		% (sugar/total sugar)	4.8 ±1.4	0.7 ±0.1	1.7 ±0.6	29.3 ±8.7	18.8 ±3.2	1.9 ±0.8	29.7 ±11.9	13.2 ±3.6	

[a] % = mg/100 mg dry weight of sample
[b] Data are means ± SE, n=3

In general, our data with respect of pea seed fibre composition were similar to the data reported by Daveby et al. (1993) for pea embryos and seed coats (see **Table 3.6** for comparison). The only noted differences were that our pea embryo fibre glucose content was about 10% higher and galactose content was 4% lower than that reported by Daveby et al. (1993). Different pea cultivars and the place of origin could affect the exact sugar components that make up the fibre of the embryos.

Table 3.6 Comparison of data for the composition sugar components of pea seed fibre

Pea		Fucose	Arabinose	Xylose	Glucose	Galactose
Embryo	Daveby et al 1993[a]		43%-45%[b]	5.4%-7.9%	23%-25%	9.4%-11.6%
	Current study[c]		46%-49%	3.7%-4.3%	33%-38%	5.8%-7.1%
Seed coats	Daveby et al 1993	0.4%-0.5%		13.5%-16.8%	74.9%-75.8%	2.5%-2.7%
	Current study	0.3%-0.4%		15%-20%	70%-75%	1.6%-2.2%

[a] Pea cultivars 'Timo' 'Vreta' 'Capella' grown in Ultuna, Sweden
[b] Calculated as the amount of constituent sugar divided by the sum of total sugars
[c] Pea cultivars 'Solido' 'Canstar' 'Courier' grown in Alberta, Canada

Faba bean

In the embryos of the faba bean cultivars studied, the main components of the total fibre fraction were glucose (49 to 50%) and arabinose (35 to 38%). The less abundant components of the total fibre fraction were galactose (5.1 to 5.4%), xylose (4.3 to 4.6%), rhamnose (1.4 to 2.1%), ribose (1.1 to 1.3%), fucose (0.6 to 0.7%), and mannose (0.6%) (**Table 3.7**).

In the faba bean embryo soluble fibre fraction, the main components were arabinose (63 to 70%) and glucose (4 to 33%) (**Table 3.7**). The less abundant components of the soluble fibre fraction were galactose (13 to 17%), xylose (4.5 to 5.3%), ribose (4.2 to 2.7%), rhamnose (2.9 to 3.4%), mannose (1.2 to 1.8%), and fucose (0 to 0.3%) (**Table 3.7**).

Table 3.7 Fibre components of faba bean seed embryos by GC analysis.

Embryo cultivar	NSP		Rhamnose (%)[a]	Ribose (%)	Fucose (%)	Arabinose (%)	Xylose (%)	Mannose (%)	Glucose (%)	Galactose (%)	total (%)
Fatima	Total	% (mg/100mg dwt)	0.09 ±0.02[b]	0.08 ±0.01	0.04 ±0.00	2.49 ±0.20	0.28 ±0.02	0.04 ±0.00	3.22 ±0.31	0.33 ±0.00	6.59 ±0.07
		% (sugar/total sugar)	1.4 ±0.3	1.1 ±0.1	0.6 ±0.0	37.9 ±3.4	4.3 ±0.4	0.6 ±0.0	48.9 ±4.1	5.1 ±0.1	
	Insoluble	% (mg/100mg dwt)	0.06 ±0.01	0.01 ±0.00	0.04 ±0.00	1.45 ±0.17	0.20 ±0.01	0.02 ±0.00	2.89 ±0.26	0.14 ±0.01	4.82 ±0.38
		% (sugar/total sugar)	1.2 ±0.1	0.2 ±0.0	0.8 ±0.0	30.0 ±2.3	4.2 ±0.2	0.4 ±0.0	60.1 ±2.7	3.0 ±0.1	
	Soluble	% (mg/100mg dwt)	0.03 ±0.02	0.06 ±0.02	0.01 ±0.00	1.04 ±0.23	0.08 ±0.02	0.02 ±0.01	0.33 ±0.28	0.19 ±0.05	1.77 ±0.46
		% (sugar/total sugar)	2.9 ±2.0	4.2 ±1.2	0.3 ±0.1	70.2 ±22.3	5.3 ±1.5	1.2 ±0.4	33.4 ±11.0	12.6 ±3.6	
Snowbird	Total	% (mg/100mg dwt)	0.13 ±0.04	0.08 ±0.00	0.04 ±0.00	2.20 ±0.08	0.28 ±0.01	0.04 ±0.00	3.10 ±0.16	0.34 ±0.01	6.22 ±0.12
		% (sugar/total sugar)	2.1 ±0.6	1.3 ±0.1	0.7 ±0.0	35.4 ±1.9	4.6 ±0.2	0.6 ±0.0	49.9 ±1.6	5.4 ±0.2	
	Insoluble	% (mg/100mg dwt)	0.08 ±0.01	0.01 ±0.00	0.04 ±0.00	1.60 ±0.07	0.23 ±0.02	0.02 ±0.00	2.92 ±0.15	0.17 ±0.00	5.09 ±0.23
		% (sugar/total sugar)	1.5 ±0.1	0.3 ±0.0	0.9 ±0.0	31.6 ±0.7	4.6 ±0.3	0.4 ±0.0	57.4 ±0.8	3.4 ±0.2	
	Soluble	% (mg/100mg dwt)	0.06 ±0.06	0.06 ±0.03	0.00 ±0.01	0.59 ±0.77	0.05 ±0.11	0.02 ±0.01	0.19 ±1.16	0.17 ±0.12	1.13 ±2.26
		% (sugar/total sugar)	3.4 ±2.4	6.7 ±1.9	0	62.8 ±17.0	4.5 ±0.7	1.8 ±0.3	3.8 ±20.6	17.3 ±4.2	

[a] % = mg/100 mg dry weight of sample [b] Data are means ± SE, n=3

Among the seed coats of the faba bean cultivars, the main components of the total fibre fraction were glucose (77 to 81%) and xylose (15 to 18%). The less abundant components of the total fibre fraction were galactose (1.5 to 1.6%), arabinose (1.7 to 2.1%), fucose (0.2%), rhamnose (0.6%), mannose (0.2%), and ribose (0.01%) (**Table 3.8**).

In the faba bean seed coat soluble fibre fraction, the main components were glucose (58 to 86%) and xylose (14 to 16%). The less abundant components of the total fibre fraction were galactose (10 to 19%), arabinose (11 to 13%), rhamnose (3.6 to 7.4%), fucose (0.8 to 1.5%), ribose (0 to 0.7%), and mannose (0.5 to 1.0%) (**Table 3.8**).

The main components of total fibre in the faba bean embryo glucose (49 to 50%) and arabinose (35 to 38%) varied from that in the faba bean seed coat glucose (77 to 81%) and xylose (15 to 18%) (**Table 3.7 and 3.8).** These data indicate that the most abundant component of the faba bean seed coat total fibre was cellulose. The insoluble fibre component is also mainly made up of cellulose (57 to 60% glucose in faba bean embryos, 79 to 82% in faba bean seed coats) (**Table 3.7 and 3.8**). The occurrence of fucose, galactose, and xylose is indicative of the presence of the microfibril cross-linking polysaccharide fucogalacto-xyloglucan. The presence of arabinose (1.7 to 2.1%) in the total fibre of faba bean seed coats suggests the presence of small amount of glucuronoarabinoxylans and/or pectins (Buchanan et al., 2002). Very small amounts of rhamnose, ribose, and mannose were present (**Table 3.8**).

The faba bean embryo consists of high amount of arabinose (35 to 38%) in total fibre, which indicates the presence of pectins as a main component in embryo fraction. The glucose was likely from the backbone of xyloglucans. Fucogalacto-xyloglucan was likely present, and small amounts of rhamnose, ribose, and mannose also were present **(Table 3.7)**.

The main components of the soluble fibre of the faba bean seed coats were glucose (58 to 86%) and xylose (14 to 16%) (**Table 3.8**), likely from xyloglucans and a small amount of cellulose. Arabinose (11 to 13%) was likely from pectins and glucuronoarabinoxylans. Galactose, xylose and small amount of fucose

indicated the presence of fucogalacto-xyloglucans in this fraction. Rhamnose was likely indicative of the presence of small amount of pectins. Ribose and mannose were in very small amounts in the seed coat soluble fibre fraction (**Table 3.8**).

In faba bean embryos, the soluble fibre component arabinose (63 to 70%) was the main component likely from pectins and/or glucuronoarabinoxylans. Glucose (4 to 33%) was likely from the backbone of xyloglucans and a small amount of cellulose. Rhamnose is also a constituent of pectins. Galactose, xylose and small amount of fucose indicate the presence of fucogalacto-xyloglucans. Ribose and mannose were found in very small amounts in the seed embryo soluble fibre fraction (**Table 3.7**).

Table 3.8 Fibre components of faba bean seed coats by GC analysis.

Seed coat cultivars	NSP		Rhamnose (%)[a]	Ribose (%)	Fucose (%)	Arabinose (%)	Xylose (%)	Mannose (%)	Glucose (%)	Galactose (%)	total (%)
Fatima	Total	% (mg/100mg dwt)	0.33 ±0.03[b]	0.02 ±0.01	0.11 ±0.02	0.97 ±0.08	8.53 ±0.36	0.10 ±0.01	45.35 ±3.81	0.89 ±0.07	56.30 ±4.36
		% (sugar/total sugar)	0.6 ±0.0	0.0 ±0.0	0.2 ±0.0	1.7 ±0.1	15.2 ±0.6	0.2 ±0.0	80.5 ±0.6	1.6 ±0.0	
	Insoluble	% (mg/100mg dwt)	0.17 ±0.03	0.01 ±0.00	0.07 ±0.01	0.62 ±0.05	8.31 ±0.73	0.08 ±0.01	45.20 ±5.11	0.50 ±0.03	54.96 ±5.95
		% (sugar/total sugar)	0.3 ±0.0	0.0 ±0.0	0.1 ±0.0	1.1 ±0.0	15.2 ±0.4	0.1 ±0.0	82.1 ±0.5	0.9 ±0.1	
	Soluble	% (mg/100mg dwt)	0.16 ±0.06	0.01 ±0.01	0.05 ±0.03	0.34 ±0.12	0.22 ±1.00	0.02 ±0.01	0.15 ±7.84	0.39 ±0.09	1.35 ±9.14
		% (sugar/total sugar)	7.4 ±5.9	0.7 ±0.7	1.5 ±1.3	11.4 ±11.3	15.7 ±4.8	0.5 ±0.4	86.3 ±6.2	18.4 ±15.2	
Snowbird	Total	% (mg/100mg dwt)	0.41 ±0.04	0.01 ±0.00	0.11 ±0.01	1.54 ±0.07	13.04 ±0.80	0.12 ±0.00	55.35 ±1.64	1.09 ±0.03	71.65 ±2.53
		% (sugar/total sugar)	0.6 ±0.0	0.0 ±0.0	0.2 ±0.0	2.1 ±0.0	18.2 ±0.5	0.2 ±0.0	77.3 ±0.5	1.5 ±0.1	
	Insoluble	% (mg/100mg dwt)	0.18 ±0.03	0.02 ±0.01	0.06 ±0.01	0.74 ±0.08	11.09 ±0.99	0.06 ±0.01	48.20 ±4.99	0.53 ±0.07	60.87 ±6.13
		% (sugar/total sugar)	0.3 ±0.0	0.0 ±0.0	0.1 ±0.0	1.2 ±0.1	18.3 ±0.3	0.1 ±0.0	79.1 ±0.4	0.9 ±0.1	
	Soluble	% (mg/100mg dwt)	0.22 ±0.05	0	0.05 ±0.02	0.77 ±0.13	1.95 ±1.52	0.06 ±0.01	7.15 ±5.30	0.57 ±0.05	10.77 ±6.93
		% (sugar/total sugar)	3.6 ±1.3	0	0.8 ±0.3	12.6 ±4.4	14.0 ±13.0	1.0 ±0.3	58.0 ±15.2	10.0 ±3.8	

[a] % = mg/100 mg dry weight of sample
[b] Data are means ± SE, n=3

Lentil

In the embryos of the lentil cultivars studied, the main components of the total fibre fraction were glucose (53 to 57%) and arabinose (32 to 34%). The less abundant components of the total fibre fraction were ribose (1.4 to 14%), galactose (4 to 5%), xylose (3.5 to 4.0%), rhamnose (0.7 to 1.3%), mannose (0.6 to 0.8%), and fucose (0.4 to 0.5%) (**Table 3.9**).

The soluble lentil embryo fibre fraction had a similar component profile to the total fibre fraction, where glucose (15 to 44%) and arabinose (32 to 36%) were the main components. The less abundant components of the total fibre fraction were galactose (6.5 to 26%), ribose (5 to 14%), xylose (4.6 to 6.3%), mannose (1.8 to 6.9%), rhamnose (0 to 4.3%), and fucose (0.4 to 0.7%) (**Table 3.9**).

Table 3.9 Fibre components of lentil seed embryos by GC analysis.

Embryo cultivars	NSP		Rhamnose (%)[a]	Ribose (%)	Fucose (%)	Arabinose (%)	Xylose (%)	Mannose (%)	Glucose (%)	Galactose (%)	total (%)
LeMay	Total	% (mg/100mg dwt)	0.08 ±0.02[b]	0.11 ±0.00	0.04 ±0.00	2.60 ±0.04	0.30 ±0.01	0.06 ±0.00	4.71 ±0.35	0.33 ±0.01	8.23 ±0.43
		% (sugar/total sugar)	0.9 ±0.2	1.4 ±0.1	0.4 ±0.0	31.8 ±1.3	3.7 ±0.1	0.8 ±0.0	57.1 ±1.3	4.0 ±0.1	
	Insoluble	% (mg/100mg dwt)	0.04 ±0.00	0.01 ±0.00	0.03 ±0.00	1.77 ±0.15	0.20 ±0.02	0.03 ±0.01	3.55 ±0.70	0.18 ±0.03	5.80 ±0.91
		% (sugar/total sugar)	0.6 ±0.0	0.3 ±0.1	0.4 ±0.0	31.4 ±2.7	3.5 ±0.2	0.4 ±0.1	60.3 ±3.0	3.1 ±0.0	
	Soluble	% (mg/100mg dwt)	0.04 ±0.02	0.10 ±0.00	0.01 ±0.00	0.83 ±0.13	0.10 ±0.02	0.04 ±0.00	1.16 ±0.43	0.15 ±0.02	2.43 ±0.58
		% (sugar/total sugar)	1.8 ±0.9	4.7 ±1.2	0.4 ±0.0	36.2 ±5.0	4.6 ±1.0	1.8 ±0.5	43.9 ±8.6	6.5 ±1.0	
Plato	Total	% (mg/100mg dwt)	0.05 ±0.00	0.10 ±0.02	0.03 ±0.00	2.59 ±0.20	0.27 ±0.03	0.06 ±0.00	4.17 ±0.08	0.38 ±0.01	7.65 ±0.21
		% (sugar/total sugar)	0.7 ±0.0	1.3 ±0.2	0.4 ±0.0	33.8 ±1.7	3.5 ±0.3	0.8 ±0.0	54.7 ±2.2	4.9 ±0.1	
	Insoluble	% (mg/100mg dwt)	0.04 ±0.00	0.01 ±0.00	0.03 ±0.00	2.21 ±0.26	0.22 ±0.03	0.02 ±0.00	3.68 ±0.32	0.22 ±0.01	6.44 ±0.52
		% (sugar/total sugar)	0.7 ±0.0	0.2 ±0.0	0.4 ±0.0	34.2 ±2.3	3.5 ±0.3	0.3 ±0.0	57.2 ±2.6	3.5 ±0.1	
	Soluble	% (mg/100mg dwt)	0.01 ±0.00	0.08 ±0.02	0.01 ±0.00	0.38 ±0.20	0.05 ±0.02	0.04 ±0.00	0.49 ±0.35	0.15 ±0.02	1.21 ±0.59
		% (sugar/total sugar)	0.6 ±0.1	13.8 ±7.5	0.7 ±0.3	31.9 ±2.2	5.4 ±1.7	6.9 ±4.3	15.1 ±29.8	25.6 ±15.0	
Redberry	Total	% (mg/100mg dwt)	0.08 ±0.01	0.08 ±0.00	0.03 ±0.00	2.16 ±0.08	0.25 ±0.02	0.04 ±0.01	3.43 ±0.41	0.30 ±0.02	6.37 ±0.51
		% (sugar/total sugar)	1.3 ±0.2	1.3 ±0.1	0.5 ±0.1	34.2 ±1.6	4.0 ±0.1	0.6 ±0.1	53.4 ±2.1	4.7 ±0.2	
	Insoluble	% (mg/100mg dwt)	0.08 ±0.02	0.01 ±0.00	0.03 ±0.00	1.71 ±0.08	0.17 ±0.02	0.02 ±0.00	2.76 ±0.09	0.16 ±0.00	4.92 ±0.02
		% (sugar/total sugar)	1.5 ±0.4	0.2 ±0.0	0.5 ±0.0	34.7 ±1.5	3.4 ±0.3	0.3 ±0.0	56.1 ±2.1	3.3 ±0.1	
	Soluble	% (mg/100mg dwt)	0.00 ±0.02	0.07 ±0.00	0.01 ±0.00	0.46 ±0.15	0.08 ±0.03	0.02 ±0.00	0.67 ±0.42	0.13 ±0.01	1.45 ±0.52
		% (sugar/total sugar)	0	6.2 ±1.8	0.7 ±0.4	35.6 ±13.6	6.3 ±2.6	1.8 ±0.6	37.4 ±22.0	11.0 ±2.5	

[a] % = mg/100 mg dry weight of sample
[b] Data are means ± SE, n=3

Among the seed coats of the lentil cultivars, the main components of the total fibre fraction were glucose (61 to 65%), and xylose (21 to 25%). The less abundant components of the total fibre fraction were arabinose (8 to 10%), galactose (2.2 to 2.8%), rhamnose (0.6 to 1.0%), ribose (0.1%), and mannose (0.3 to 0.4%) and fucose (0.2%) (**Table 3.10**).

In the lentil seed coat soluble fibre fraction, the main components were arabinose (21 to 49%) and glucose (7.6 to 26%). The less abundant components of the total fibre fraction were xylose (16 to 25%), galactose (7 to 13%), rhamnose (1.6 to 3.3%), mannose (0.8 to 1.5%), ribose (0.3 to 0.8%) and fucose (0.3 to 0.7%) (**Table 3.10**).

The main components of total fibre in the lentil embryo (glucose 53 to 57% and arabinose 32 to 34%) varied from that in the lentil seed coat (glucose 61 to 65% and xylose 21 to 25%) (**Table 3.9 and 3.10).** These data indicate that the most abundant component of the lentil seed coat total fibre was cellulose (made up of linear chains of glucose). The insoluble fibre component is also mainly made up of cellulose (56 to 60% glucose in lentil embryos, 67 to 72% in lentil seed coats) (**Table 3.9 and 3.10**). The occurrence of fucose, galactose, and xylose in lentil seed coat total fibre is indicative of the presence of the microfibril cross-linking polysaccharide fucogalacto-xyloglucan. The presence of arabinose (8 to 10%) in the total fibre of lentil seed coats suggests the presence of glucuronoarabinoxylans and/or pectins. Rhamnose was also a constituent of pectins. Mannose was also present at very low levels indicating the presence of glucomannans, galactoglucomannans, or galactomannans for interlocking microfibrils in the cell wall (Buchanan et al., 2002) (**Table 3.10**).

The lentil embryo consists of a high amount of arabinose (32 to 36%) in total fibre, this indicates the presence of pectins as a main component in embryo fraction. The glucose (15 to 44%) was likely from backbone of xyloglucans. Very small amounts of rhamnose, ribose, and mannose were present (**Table 3.9**).

The main component of the soluble fibre of the lentil seed coats is arabinose (21 to 49%) (**Table 3.10**), likely from glucuronoarabinoxylans or a small amount of pectin. Soluble fibre glucose (7.6 to 26%) (**Table 3.10**) is likely from

xyloglucans and a small amount of cellulose. Galactose, xylose and a small amount of fucose indicate the presence of fucogalacto-xyloglucans in this fraction. Rhamnose, ribose and mannose were found in very small amounts in the seed coat soluble fibre fraction.

In lentil embryos soluble fibre components, arabinose (32 to 36%) was the main component likely from pectins and/or glucuronoarabinoxylans. Rhamnose was likely from pectins. Glucose (15 to 44%) was likely from the xyloglucans backbone. Galactose, xylose and small amount of fucose indicate the presence of fucogalacto-xyloglucans. Ribose and mannose were found in very small amounts in the seed embryo soluble fibre fraction (**Table 3.9**).

Table 3.10 Fibre components of lentil seed coats by GC analysis.

Seed coat cultivars	NSP		Rhamnose (%)[a]	Ribose (%)	Fucose (%)	Arabinose (%)	Xylose (%)	Mannose (%)	Glucose (%)	Galactose (%)	total (%)
LeMay	Total	% (mg/100mg dwt)	0.34 ±0.00[b]	0.05 ±0.00	0.09 ±0.00	4.50 ±0.32	9.37 ±0.67	0.17 ±0.00	29.17 ±0.54	1.17 ±0.02	44.86 ±1.40
		% (sugar/total sugar)	0.7 ±0.0	0.1 ±0.0	0.2 ±0.0	10.0 ±0.4	20.8 ±0.9	0.4 ±0.0	65.1 ±1.2	2.6 ±0.0	
	Insoluble	% (mg/100mg dwt)	0.18 ±0.00	0.02 ±0.00	0.05 ±0.00	2.15 ±0.10	8.09 ±0.27	0.10 ±0.00	28.64 ±0.25	0.57 ±0.01	39.81 ±0.63
		% (sugar/total sugar)	0.5 ±0.0	0.0 ±0.0	0.1 ±0.0	5.4 ±0.2	20.3 ±0.3	0.3 ±0.0	71.9 ±0.5	1.4 ±0.0	
	Soluble	% (mg/100mg dwt)	0.15 ±0.00	0.03 ±0.00	0.03 ±0.00	2.34 ±0.24	1.27 ±0.41	0.07 ±0.00	0.53 ±0.50	0.60 ±0.02	5.04 ±0.95
		% (sugar/total sugar)	3.3 ±0.7	0.8 ±0.2	0.7 ±0.1	49.0 ±7.3	24.3 ±4.2	1.5 ±0.3	7.6 ±10.3	12.9 ±6.6	
Plato	Total	% (mg/100mg dwt)	0.33 ±0.03	0.05 ±0.00	0.09 ±0.01	4.42 ±0.27	13.35 ±0.81	0.16 ±0.00	33.83 ±1.21	1.49 ±0.22	53.73 ±2.21
		% (sugar/total sugar)	0.6 ±0.0	0.1 ±0.0	0.2 ±0.0	8.2 ±0.3	24.8 ±0.9	0.3 ±0.0	63.0 ±0.9	2.8 ±0.4	
	Insoluble	% (mg/100mg dwt)	0.18 ±0.01	0.02 ±0.00	0.06 ±0.00	2.24 ±0.19	11.59 ±0.95	0.10 ±0.00	31.74 ±1.06	0.72 ±0.18	46.63 ±2.29
		% (sugar/total sugar)	0.4 ±0.0	0.0 ±0.0	0.1 ±0.0	4.8 ±0.2	24.8 ±1.0	0.2 ±0.0	68.2 ±1.3	1.5 ±0.3	
	Soluble	% (mg/100mg dwt)	0.16 ±0.03	0.03 ±0.00	0.03 ±0.00	2.18 ±0.16	1.77 ±0.42	0.06 ±0.01	2.09 ±0.95	0.77 ±0.23	7.09 ±1.70
		% (sugar/total sugar)	2.3 ±0.2	0.5 ±0.1	0.5 ±0.1	34.3 ±7.3	24.8 ±1.0	1.0 ±0.2	25.6 ±7.9	10.9 ±1.5	
Redberry	Total	% (mg/100mg dwt)	0.38 ±0.08	0.04 ±0.01	0.06 ±0.01	3.65 ±0.34	9.79 ±0.98	0.14 ±0.01	23.60 ±0.84	0.86 ±0.02	38.51 ±2.06
		% (sugar/total sugar)	1.0 ±0.2	0.1 ±0.0	0.2 ±0.0	9.5 ±0.4	25.3 ±1.3	0.4 ±0.0	61.4 ±1.7	2.2 ±0.1	
	Insoluble	% (mg/100mg dwt)	0.22 ±0.03	0.01 ±0.00	0.05 ±0.01	2.39 ±0.30	9.74 ±1.28	0.12 ±0.01	26.35 ±3.68	0.52 ±0.06	39.41 ±5.38
		% (sugar/total sugar)	0.6 ±0.0	0.0 ±0.0	0.1 ±0.0	6.1 ±0.1	24.7 ±0.2	0.3 ±0.0	66.8 ±0.3	1.3 ±0.0	
	Soluble	% (mg/100mg dwt)	0.16 ±0.06	0.02 ±0.01	0.01 ±0.00	1.26 ±0.19	0.05 ±0.53	0.02 ±0.02	0	0.33 ±0.07	0
		% (sugar/total sugar)	1.6 ±n/a[c]	0.3 ±n/a	0.3 ±n/a	21.3 ±n/a	15.7 ±n/a	0.8 ±n/a	0[d]	6.6 ±n/a	

[a] % = mg/100 mg dry weight of sample
[b] Data are means ± SE, n=3
[c] Only one replicate data was acceptable
[d] experimental error

Protein, starch and fibre components in the seed coat fractions of the pea cultivars 'Solido' and 'Canstar'.

The seed coats of the pea cultivars 'Solido' and 'Canstar' contained approximately 6% to 8% protein and 0.2% to 0.6 % starch (**Table 3.11**). The protein content in 'Solido' seed coats was slightly higher (1%) than that in 'Canstar' seed coats. The cooking treatment and the acid-hydrolysis treatment had minimal effects on the seed coat protein and starch content in both cultivars (**Table 3.11**).

Table 3.11 Protein and total starch components of raw, cooked and hydrolyzed pea seed coats of 'Solido' and 'Canstar'

Pea seed coat	Protein (%)[a]	Total starch (%)
'Solido' raw	7.96±0.21[b]	0.46±0.04
'Solido' cooked[c]	8.36±0.09	0.36±0.04
'Solido' hydrolyzed[d]	8.22±0.17	0.16±0.03
'Canstar' raw	6.65±0.05	0.16±0.01
'Canstar' cooked	6.91±0.03	0.59±0.02
'Canstar' hydrolyzed	5.86±0.07	0.16±0.01

[a] % = mg/100 mg dry weight of sample

[b] Data are means ± SE, n=3

[c] placed in boiling water for 30 minutes

[d] incubated in 2N HCl solution at 100 ^{0}C for 1.5 hour, then partially neutralized by NaOH solution.

There was no change in total fibre content with cooking treatment in 'Canstar' and 'Solido' (**Tables 3.12, 3.13**). Heating the seed coat fractions to 100^{o}C for 30 min in water does not cleave the linkages between the fibre components. However, hydration of the fibre components is possible. The acid-hydrolysis treatment decreased the total fibre content of 'Canstar' by about 20%,

and hydrolyzed all the soluble fibre (**Table 3.12**). For 'Solido' seed coat fractions, the hydrolysis treatment decreased total fibre by about 30%, and also hydrolyzed all the soluble fibre **(Table 3.13**).

For 'Canstar' seed coat total fibre components, the acid-hydrolysis treatment decreased glucose from 42 to 34 mg/100mg dry weight (dwt), xylose from 12 to 2.7 mg/100mg dwt, arabinose from 3.5 to 0.5 mg/100mg dwt, rhamnose from 0.3 to 0.2 mg/100mg dwt, fucose from 0.2 to 0.03 mg/100mg dwt, and galactose from 0.9 to 0.2 mg/100mg dwt. Mannose content did not change with the hydrolysis treatment (**Table 3.12**).

For 'Solido' seed coats total fibre components, the acid-hydrolysis treatment decreased glucose from 44 to 30 mg/100mg dwt, xylose from 10 to 2 mg/100mg dwt, arabinose from 3.6 to 0.4 mg/100mg dwt, rhamnose from 0.6 to 0.1 mg/100mg dwt, fucose from 0.2 to 0.02 mg/100mg dwt, galactose from 1.3 to 0.2 mg/100mg dwt, and mannose from 0.3 to 0.1 mg/100mg dwt **(Table 3.13**).

The acid hydrolysis treatment can cleave the side-chain components of the complex fibres, liberating xylose, arabinose, and fucose from fucogalacto-xyloglucans and arabinoxyloglucans (leading to lower values of these sugars in the fibre fraction after hydrolysis). The glucose component also decreased in both cultivars with acid-hydrolysis. This is likely due to the cleavage of the glucose backbone of the fucogalacto-xyloglucans and arabinoxyloglucans.

Table 3.12 Sugar components of raw, cooked and hydrolyzed seed coats of 'Canstar' by GC analysis

Canstar' seed coat Treatment	NSP		Rhamnose (%)[a]	Ribose (%)	Fucose (%)	Arabinose (%)	Xylose (%)	Mannose (%)	Glucose (%)	Galactose (%)	total (%)
Raw	Total	% (mg/100mg dwt)	0.33 ±0.01[b]	0.02 ±0.00	0.22 ±0.00	3.53 ±0.09	11.98 ±0.33	0.15 ±0.01	41.55 ±1.37	0.95 ±0.01	58.72 ±1.97
		% (sugar/total sugar)	0.6 ±0.0	0.0 ±0.0	0.4 ±0.0	6.0 ±0.0	20.4 ±0.3	0.3 ±0.0	70.7 ±0.3	1.6 ±0.0	
	Insoluble	% (mg/100mg dwt)	0.19 ±0.01	0.01 ±0.00	0.16 ±0.00	2.21 ±0.06	10.96 ±0.29	0.12 ±0.00	34.84 ±3.49	0.59 ±0.03	49.07 ±3.17
		% (sugar/total sugar)	0.4 ±0.0	0.0 ±0.0	0.3 ±0.0	4.5 ±0.4	22.6 ±2.1	0.2 ±0.0	70.7 ±2.7	1.2 ±0.1	
	Soluble	% (mg/100mg dwt)	0.14 ±0.02	0.01 ±0.00	0.06 ±0.00	1.32 ±0.11	1.02 ±0.45	0.03 ±0.01	6.71 ±4.69	0.36 ±0.02	9.65 ±6.75
		% (sugar/total sugar)	3.2 ±2.0	0.1 ±0.0	1.6 ±1.1	33.3 ±21.9	19.7 ±9.2	0.5 ±0.2	31.6 ±40.8	10.1 ±7.1	
Cooked	Total	% (mg/100mg dwt)	0.55 ±0.04	0.12 ±0.02	0.16 ±0.08	3.37 ±0.12	13.35 ±0.32	0.22 ±0.03	48.73 ±1.06	1.13 ±0.04	67.13 ±1.13
		% (sugar/total sugar)	0.8 ±0.1	0.2 ±0.0	0.2 ±0.1	5.0 ±0.2	19.8 ±0.6	0.3 ±0.0	72.0 ±0.7	1.7 ±0.1	
	Insoluble	% (mg/100mg dwt)	0.29 ±0.03	0.05 ±0.00	0.14 ±0.01	1.66 ±0.09	11.61 ±0.52	0.18 ±0.01	43.60 ±1.59	0.54 ±0.02	58.07 ±2.27
		% (sugar/total sugar)	0.5 ±0.0	0.1 ±0.0	0.2 ±0.0	2.9 ±0.1	20.0 ±0.1	0.3 ±0.0	75.1 ±0.2	0.9 ±0.0	
	Soluble	% (mg/100mg dwt)	0.26 ±0.06	0.06 ±0.02	0.03 ±0.07	1.71 ±0.20	1.74 ±0.80	0.05 ±0.02	5.13 ±0.92	0.59 ±0.04	9.56 ±1.94
		% (sugar/total sugar)	2.6 ±0.2	0.7 ±0.2	0.5 ±0.7	18.7 ±2.0	16.3 ±5.0	0.5 ±0.2	54.2 ±2.0	6.5 ±1.0	
Hydrolyzed	Total	% (mg/100mg dwt)	0.15 ±0.01	0.10 ±0.00	0.03 ±0.00	0.54 ±0.03	2.66 ±0.17	0.13 ±0.01	33.68 ±0.31	0.24 ±0.01	37.54 ±0.49
		% (sugar/total sugar)	0.4 ±0.0	0.3 ±0.0	0.1 ±0.0	1.4 ±0.1	7.1 ±0.4	0.4 ±0.0	89.7 ±0.5	0.6 ±0.0	
	Insoluble	% (mg/100mg dwt)	0.05 ±0.00	0.06 ±0.01	0.02 ±0.01	0.45 ±0.01	2.36 ±0.04	0.11 ±0.00	36.76 ±0.46	0.16 ±0.01	39.95 ±0.50
		% (sugar/total sugar)	0.1 ±0.0	0.1 ±0.0	0.1 ±0.0	1.1 ±0.0	5.9 ±0.1	0.3 ±0.0	92.0 ±0.1	0.4 ±0.0	
	Soluble	% (mg/100mg dwt)	0	0	0	0	0	0	0	0	0
		% (sugar/total sugar)	0	0	0	0	0	0	0	0	0

[a] % = mg/100 mg dry weight of sample
[b] Data are means ± SE, n=3

Table 3.13 Sugar components of raw, cooked and hydrolyzed seed coats of 'Solido' by GC analysis

Solido' seed coat Treatment	NSP		Rhamnose (%)[a]	Ribose (%)	Fucose (%)	Arabinose (%)	Xylose (%)	Mannose (%)	Glucose (%)	Galactose (%)	total (%)
Raw	Total	% (mg/100mg dwt)	0.57 ±0.03[b]	0.04 ±0.01	0.24 ±0.01	3.58 ±0.20	10.00 ±0.39	0.28 ±0.01	44.11 ±0.55	1.31 ±0.04	60.14 ±0.97
		% (sugar/total sugar)	0.9 ±0.0	0.1 ±0.0	0.4 ±0.0	5.9 ±0.2	16.7 ±0.5	0.4 ±0.0	73.4 ±0.8	2.1 ±0.0	
	Insoluble	% (mg/100mg dwt)	0.38 ±0.01	0.01 ±0.01	0.17 ±0.00	2.44 ±0.17	9.07 ±0.43	0.21 ±0.01	42.47 ±1.45	0.79 ±0.05	55.55 ±1.62
		% (sugar/total sugar)	0.7 ±0.0	0.0 ±0.0	0.3 ±0.0	4.4 ±0.4	16.3 ±0.4	0.4 ±0.0	76.4 ±0.8	1.4 ±0.1	
	Soluble	% (mg/100mg dwt)	0.19 ±0.02	0.03 ±0.00	0.07 ±0.01	1.14 ±0.11	0.93 ±0.39	0.07 ±0.02	1.65 ±1.00	0.52 ±0.03	4.59 ±1.32
		% (sugar/total sugar)	4.8 ±1.4	0.7 ±0.1	1.7 ±0.6	29.3 ±8.7	18.8 ±3.2	1.9 ±0.8	29.7 ±11.9	13.2 ±3.6	
Cooked	Total	% (mg/100mg dwt)	0.51 ±0.04	0.12 ±0.01	0.17 ±0.01	2.12 ±0.10	8.91 ±0.51	0.19 ±0.01	42.22 ±1.30	1.07 ±0.01	55.30 ±1.92
		% (sugar/total sugar)	0.9 ±0.0	0.2 ±0.0	0.3 ±0.0	3.8 ±0.1	16.1 ±0.4	0.4 ±0.0	76.4 ±0.5	1.9 ±0.1	
	Insoluble	% (mg/100mg dwt)	0.23 ±0.02	0.07 ±0.01	0.10 ±0.01	1.21 ±0.01	7.52 ±0.15	0.16 ±0.01	39.04 ±1.13	0.47 ±0.02	48.80 ±1.05
		% (sugar/total sugar)	0.5 ±0.0	0.1 ±0.0	0.2 ±0.0	2.5 ±0.1	15.4 ±0.6	0.3 ±0.0	80.0 ±0.9	1.0 ±0.0	
	Soluble	% (mg/100mg dwt)	0.27 ±0.02	0.05 ±0.00	0.07 ±0.00	0.91 ±0.10	1.39 ±0.66	0.03 ±0.00	3.18 ±0.56	0.59 ±0.02	6.50 ±0.93
		% (sugar/total sugar)	4.4 ±0.6	0.8 ±0.1	1.2 ±0.2	14.5 ±2.1	19.9 ±6.9	0.5 ±0.1	49.2 ±6.9	9.6 ±1.7	
Hydrolyzed	Total	% (mg/100mg dwt)	0.14 ±0.01	0.09 ±0.01	0.02 ±0.00	0.38 ±0.01	2.01 ±0.04	0.13 ±0.01	29.45 ±0.20	0.24 ±0.01	32.47 ±0.26
		% (sugar/total sugar)	0.4 ±0.0	0.3 ±0.0	0.1 ±0.0	1.2 ±0.0	6.2 ±0.1	0.4 ±0.0	90.7 ±0.2	0.7 ±0.0	
	Insoluble	% (mg/100mg dwt)	0.04 ±0.00	0.04 ±0.00	0.02 ±0.00	0.29 ±0.01	1.64 ±0.09	0.12 ±0.01	32.43 ±0.37	0.14 ±0.00	34.70 ±0.42
		% (sugar/total sugar)	0.1 ±0.0	0.1 ±0.0	0.0 ±0.0	0.8 ±0.0	4.7 ±0.2	0.3 ±0.0	93.5 ±0.3	0.4 ±0.0	
	Soluble	% (mg/100mg dwt)	0	0	0	0	0	0	0	0	0
		% (sugar/total sugar)	0	0	0	0	0	0	0	0	0

[a] % = mg/100 mg dry weight of sample
[b] Data are means ± SE, n=3

The seed coat fractions of ‘Canstar’ had higher soluble NSP levels than those of ‘Solido’. The cooking treatment did not significantly change soluble NSPs of ‘Solido’ or ‘Canstar’ seed coats (**Tables 3.12, 3.13**), but likely hydrated the fibre components. The hydrolysis treatment completely removed the soluble NSP from the seed coat fibre fraction (**Tables 3.12, 3.13**). These characterized pea seed coat fractions can now be used to test if insoluble, soluble and/or hydrated pea seed coat fibre has health-beneficial effects when consumed. Soluble NSPs have been shown to help normalize blood glucose and insulin levels (Kumar et al., 2012). The ability of soluble NSPs to ameliorate symptoms of diabetes mellitus has been attributed to their effects on small intestinal viscosity and nutrient absorption, and systemic effects from colonic-derived short chain fatty acids produced from fermentable NSP (Kumar et al., 2012; Ou et al., 2001). The characterization of the grain legume seed fibre components and pea seed coat fractions in this study will aid nutritional studies aimed at determining if legume seed fibre with higher soluble NSP levels (like ‘Canstar’ seed coats) or specifically processed seed coat fractions have health-related benefits on consumption.

Chapter 4 Summary and conclusion

Summary of thesis work

This thesis aims to investigate the bioactive component proanthocyanidins (PAs) with antimicrobial activity against food pathogens; and the nutrient profile of legume seeds, including fibre, starch, and protein.

PAs were isolated from pea seed coat (from *Pisum sativum* L., cv. Solido) by extraction, solvent partition and size exclusive chromatography. The PA-enriched extract underwent acid-catalyzed cleavage and the cleaved products were derivatized with phloroglucinol to their subsequent flavan-*3*-ol phloroglucinol adducts and the free flavan-*3*-ols terminal subunits. The phloroglucinolysis products were identified using HPLC-DAD by comparing their retention times with the standard flavan-*3*-ols' retention times. The PA subunit composition, average degree of polymerization, and the conversion yield of PA-enriched extract were determined using HPLC-DAD analysis method. The antimicrobial activity of flavan-*3*-ol monomers and PA-enriched extract were tested against food pathogens including three Gram-positive bacteria by MIC assays. The structure-antimicrobial activity relationship was analyzed by comparison of MIC values of flavan-*3*-ols with two or three hydroxyl groups on the flavonoid B-ring. The antimicrobial mode of action for flavan-*3*-ols and PA-enriched extract were analyzed by comparison of MIC values in the condition of with or without iron during bacterial growth.

Different cultivars of mature legume seeds including pea, faba bean, and lentil were studied for their nutrients composition in whole seeds, embryos and seed coats respectively. The total starch and resistant starch of raw and cooked legume whole seeds were determined using Megazyme methods. The protein content in legume seed embryos and seed coats were determined by multiplying a conversion factor of 6.25 with the nitrogen content analyzed by a nitrogen analyzer. The non starch polysaccharides (fibre) in legume seed embryos and seed coats were first cleaved in acidic condition, and the cleaved subunits were

derivatized to alditol acetates as the constituent sugars and determined by GC analysis.

In order to investigate the health benefits of pea seed coats on the amelioration of type 2 diabetes, the seed coats of two pea cultivars (contain or do not contain PAs) were prepared as cooked and acid-hydrolyzed samples. The reason to prepare cooked samples of legume seeds is that they are consumed in this form in the human diet. The reason to prepare acid-hydrolyzed samples is that the PAs from pea seed coats can be acid-hydrolyzed to anthocyanidins. Anthocyanins are glycosides of anthocyanidins which have health benefits such as antioxidant, anti-carcinogenic, and antidiabetic activities (Zafra-Stone et al., 2007). We would like to investigate the health benefits with respects to proanthocyanidins/anthocyanins and/or fibre from legumes seeds. The pea seed coat fraction preparations and components analyse were my original work. The pea seed fractions were provided to our collaborator Dr. Catherine Chan (human nutrition lab at Department of Agricultural, Food and Nutritional Science, University of Alberta) to test for their effects on amelioration of type 2 diabetes in a diabetic rat model.

General conclusions

Overall, the thesis work showed that legume seeds are a good source of proanthocyanidins, starch, protein and fibre for the human diet.

1. Proanthocyanidin in legume seed coat and their antimicrobial activity.

From the study of proanthocyanidins from legume seed coats (Chapter 2), we confirmed that the PA content in cultivar ‘Solido’ seed coats consisted mainly of gallocatechin and epigallocatechin flavan-*3*-ol subunits. These flavan-*3*-ols and pea seed PAs showed antimicrobial activity against *Staphylococcus aureus* and *Staphylococcus pasteuri*. The antimicrobial activity was enhanced with increased hydroxyl substitution on the flavonoid B-ring. The flavan-*3*-ol stereochemistry of

the *cis*-2,3 and *trans*-2,3 configuration was not associated with antimicrobial activity. The antimicrobial mode of action for PAs and their flavan-*3*-ol subunits appeared to involve iron-binding. The hydroxyl groups of flavan-*3*-ols and PAs can bind with iron which is an element that is required for most aerobic bacterial growth. These results show that PAs in pea seeds coats can act as an antibacterial agent. The antibacterial activity of flavan-*3*-ol monomers was higher than that of pea seed coats PA extracts (flavan-*3*-ol oligomers/polymers). The iron chelating capacity of PAs extracted from Sea buckthorne was reported to decrease with increasing PA polymer length (Arimboor and Arumughan, 2012). This is likely due to increasing steric hindrance of hydroxyl groups with increasing PA polymer size.

2. Fibre, protein and starch content in legume seeds

In Chapter 3, we studied the protein, starch and fibre content in the seeds of grain legumes including pea (*Pisum sativum L.*), faba bean (*Vicia faba* L.), and lentil (*Lens culinaris L.*) grown in western Canada. Legume seeds are a rich source of protein, starch and fibre. Cooked legume whole seeds contained less resistant starch than raw seeds. Resistant starch has health beneficial effects involved in ameliorating blood glucose since it is not digested in human small intestine (Bornet et al., 1989; Shen et al., 2011). Legume seed coats are also a major source of dietary fibre. We found that the composition of fibre differed among the legume seed crops of pea, faba bean and lentil, as well as between cultivars within each crop. The health benefits of fibre include amelioration type 2 diabetes, mainly from soluble non-starch polysaccharides (Kumar et al., 2012; Ou et al., 2001). These data on protein, starch and fibre content among pea, faba bean and lentil (and within specific cultivars of each crop) grown in western Canada can be used to target specific uses. For example, faba bean seeds have higher protein content, lentil and pea seeds have higher starch content. For soluble fibre, pea seed cultivars have the highest amount compared to lentil and faba bean seeds.

Overall, the high content of protein and starch content in seed embryos, and the high fibre content in seed coats indicate that these legume grain seeds are a good food source for the human diet. These food attributes along with the presence of bioactive PAs in the seed coats of specific legume cultivars, give legume seeds great potential for enhancing human health.

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