

# **Biochemical Profiling of Phenolic Compounds in Lentil Seeds**

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In the Department of Plant Sciences  
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Saskatoon

By

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## ABSTRACT

Lentil (*Lens culinaris* Medikus) is an annual cool-season legume with a variety of seed coat colours. Seed coat colour is an important grading factor that affects the market value of lentils. In lentil, two independent loci gray ground colour (*Ggc*) and tan ground colour (*Tgc*) determine the four basic seed coat background colours; brown (*Ggc Tgc*), gray (*Ggc tgc*), tan (*ggc Tgc*) and green (*ggc tgc*). The zero tannin locus (*tan*) is epistatic to the *tgc* locus, producing clear seed coats. Lentil is a good source of protein, carbohydrates, dietary fiber components, minerals, vitamins, and secondary metabolites that include phenolic compounds. Phenolic compounds produce different pigments in plants and bring health benefits to humans. The overall objective of this study was to determine the relationship between seed coat colour and phenolic compounds in lentil.

In the first study, comparison of the phenolic profiles of four seed coat background colours in lentil (i.e., brown, gray, tan, and green) was performed using an optimized liquid chromatography-mass spectrometry (LC-MS) method. The results showed that for the levels of various phenolic compounds in lentil seeds varied with the seed coat colour. Specifically, seed coats of lentil genotypes carrying the homozygous recessive *tgc* allele (green and gray seed coats) had higher amounts of flavan-3-ols, proanthocyanidins, and some flavonols.

In the second study, a comparison was made between the phenolic profiles of lentil seed coats that do not express the *Tgc* phenotype (genotype *Tgc tan*) and those that express *Tgc* (genotype *Tgc Tan*). The LC-MS analysis detected several compounds

that were not influenced by *tan*, notably the phenolic acids, flavones, some flavonols, and some of dihydroflavonols. In contrast, myricetin, dihydromyricetin, and flavan-3-ols, and proanthocyanidin oligomers were detected only in *Ggc Tgc Tan* lines and therefore appear to be controlled by *tan*. The molecular analysis showed that *tan* is a basic-helix-loop-helix (bHLH) transcription factor that could interact with the regulatory genes in the phenylpropanoid pathway for the enzymes flavonoid-3',5'-hydroxylase (F3'5'H) and dihydroflavonol reductase (DFR).

The third study measured the effect of long term storage on specific changes in phenolic compounds in lentil seeds. Increases in phenolic acids and flavones occur in green lentil seeds during storage, possibly because of the breakdown of more complex species into smaller subunits. More interestingly, a significant decrease in 27 flavan-3-ols and proanthocyanidins also occurs. Polymerization of flavan-3-ols and proanthocyanidins and their conjugation to cellular constituents could reduce their extractability and produce dark pigments in long stored lentil seeds.

In conclusion, these studies determined that there is a relationship between phenolic compounds, specifically flavan-3-ols and proanthocyanidins, and seed coat colour genes *tgc* and *tan* in lentil. The findings of this study will help to develop future breeding strategies for lentil cultivars with aesthetic properties and nutritional benefits that appeal to consumers.

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## DEDICATION

*To my husband who supported me with his profound  
suggestions and unconditional love*

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## LIST OF ABBREVIATIONS

4CL	4-Coumaric acid:CoA ligase
ACN	Acetonitrile
ANR	Anthocyanidin reductase
ANS	Anthocyanidin synthase
bHLH	Basic-helix-loop-helix
C4H	Cinnamic acid 4-hydroxylase
CDC	Crop Development Center
CE	Condensing enzyme
CHI	Chalcone isomerase
CHS	Chalcone synthase
CoA	Coenzyme A
DFR	Dihydroflavonol reductase
ESI	Electrospray ionization
F3'5'H	Flavonoid-3',5'-hydroxylase
F3'H	Flavonoid-3'-hydroxylase
F3H	Flavanone-3-hydroxylase
FA	Formic acid
FLS	Flavonol synthase
FS	Flavone synthase
Ha	Hectare
HESI	Heated electrospray ionization
HPLC	High-performance liquid chromatograph
IFS	Isoflavone synthase
IS	Internal standard
LAR	Leucoanthocyanidin reductase

LC	Liquid chromatography
LDOX	Leucoanthocyanidin dioxygenase
LTS	Long-term storage
MARS	Mean area ratio per mg sample
MBW	MYB-bHLH-WD
MRM	Multiple reaction monitoring
MS	Mass spectrometry
Mt	Million tonnes
PAL	Phenylalanine ammonia lyase
PFP	Pentafluorophenyl
POD	Peroxidase
PPO	Polyphenol oxidase
RIL	Recombinant inbred line
RCF	Relative centrifugal force
ROS	Reactive oxygen species
SIM	Single ion monitoring
SIR	Single ion recording
SPG	Saskatchewan pulse growers
SR	Storage ratio
SRM	Selected reaction monitoring
STH	Sutherland
STS	Stilbene synthase
TF	Transcription factor
UPLC	Ultra performance liquid chromatography
WDR	WD-repeat



# CHAPTER 1 - INTRODUCTION

Lentil is an increasingly important pulse crop in the prairie regions of North America where it is grown in rotation with cereals and oilseeds. Canada, India, Australia, the USA and Turkey are the main producers of lentil and world production of lentil in 2013 was 4.95 Mt (FAOSTAT, 2015). Lentils are a good source of protein, carbohydrates, dietary fiber components, minerals, vitamins, and secondary metabolites that include phenolic compounds (Xu and Chang, 2009). Phenolic compounds (also known as polyphenols) exert adverse effects such as reducing iron bioavailability and growth-related enzymatic activity (Champ, 2002; Martin and Appel, 2010). However, several human health benefits have been linked with phenolic compounds such as anti-oxidant, anti-tumour, and anti-heart disease properties (Martin and Appel, 2010). Several subclasses of phenolic compounds are found in lentil seeds such as phenolic acids, stilbenes, and different types of flavonoids (Aguilera et al., 2010; Amarowicz et al., 2009; Amarowicz et al., 2010; Bartolomé et al., 1997; Dueñas et al., 2002; Dueñas et al., 2003; Escarpa et al., 2002; Takeoka et al., 2005; Tsopmo and Muir, 2010; Xu and Chang, 2009, 2010; Zhang et al., 2015; Zou et al., 2011).

Lentil seed coats exhibit a wide variation in colour and pattern. Two independent loci gray ground colour (*Ggc*) and tan ground colour (*Tgc*) determine the basic seed coat colours, brown, gray, tan and green (Vandenberg and Slinkard, 1990). The combined expression of the two alleles at both loci determines the four basic seed coat background colours. Phenolic compounds are natural pigments (Andersen and Jordheim, 2010), and coloured legumes contain high levels of phenolic compounds

(Elias et al., 1979; Kovinich et al., 2011; Segev et al., 2010; Trostyńska and Ciska, 2002). There is no information on how *Ggc* and *Tgc* alleles are related to the concentration of the phenolic compounds in lentil seeds.

There is a seed coat phenotype known as 'zero-tannin' which is characterized by thin seed coats, the result of the phenotypic expression of a single recessive gene (*tan*). Pleiotropic effects of the gene include green stems and white flower tissues. In contrast, most other lentil genotypes have reddish stems, purple/blue veins on flower petals, pink to blue floral tissue, as well as thicker and pigmented seed coats (Vaillancourt et al., 1986). The set of traits in zero-tannin phenotype is similar to Mendel's *A* gene in pea (Mendel, 1865), which is controlled by a basic-helix-loop-helix (bHLH) transcription factor in the phenylpropanoid pathway (Hellens et al., 2010). Zero-tannin lines should be a good tool for studying seed coat pigmentation in lentil as they naturally prevent formation of some pigments but not others.

The visual characteristics of lentils have an important role in their marketability, especially for the green market classes, which are distinguished by yellow cotyledons and green seed coats. Uniformly green seeds are valued more in the market place (Davey, 2007). However, green colour is not stable, and in response to environmental and temporal changes, seed coat colour may change over to yellow, yellow-brown, medium brown, and dark brown depending upon the storage situation and duration (Nozzolillo and Bezada, 1984). Darkening of the seed will decrease the grade of the sample and as a result the offered price will be reduced (Davey, 2007). Knowing the type of phenolic compounds associated with seed longevity during long-term storage (LTS) may provide strategies for use in breeding of lentil so that the commercial crop

can be stored for a longer time with less colour change in the seed coat. Longer term storage without seed coat colour change could preserve the market value of lentils.

This research aims to analyze the phenolic compound profiles of the seeds of a series of lentil genotypes with defined seed coat background colour based on genetic analysis. It includes an investigation of differences in the phenolic profile between tannin containing and zero-tannin phenotypes of lentil. It will also determine what changes occur in the phenolic profile of lentil seed coats during storage.

There were three hypotheses in this body of research,

1. Specific phenolic profiles are associated with the corresponding specific genetic combinations of the alleles of the *Ggc* and *Tgc* loci that determine the green, gray, tan, and brown seed coat phenotypes in lentil.
2. The *Ggc Tgc tan* and the *Ggc Tgc Tan* genotypes will have unique and detectable profiles of phenolic compounds, as the *tan* genotype blocks the expression of the *Tgc* seed coat genotypes in lentil seed coats.
3. Fresh and aged lentil samples will have specifically different phenolic profiles, as storage will cause both visual, and biochemical changes to lentil seed coats.

Globally, most lentils are consumed after removal of the seed coat by an abrasive dehulling process. Understanding the underlying genetics of seed coat biochemistry is an important step in determining the potential economic value of lentil seed coats that are a by-product of the dehulling process. The overall objective of this study was to define the relationship between seed coat colour and phenolic compounds in lentil.

The specific objectives of this study were as follows:

- 1) To determine if specific phenolic profiles of different lentil seed coat colours are characteristic of the four specific genetic combinations of alleles of the *Ggc* and *Tgc* loci;
- 2) To compare the phenolic profiles of lentil seed coats that do not express the *Tgc* gene (genotype *Tgc tan*) with those that express *Tgc* (genotype *Tgc Tan*);
- 3) To determine the effect of long-term storage on the specific changes in phenolic compounds in lentil seeds

# CHAPTER 2 - LITERATURE REVIEW

## 2.1. Lentil

### 2.1.1. Origin of Lentil and Production

Lentil (*Lens culinaris* Medikus) is a self-pollinating annual cool season grain legume, which originated in agricultural systems in the Near East. Domestication of lentil started around 7000-8000 BC, the same period in which emmer and einkorn wheat, barley, pea, chickpea, bitter vetch, and flax were domesticated (Erskine and Sarker, 2004). Lentil is consumed in many countries as a nutritious food, usually served in stews, soups, and its consumption is especially common in vegetarian diets (McVicar et al., 2010).

Lentil is cultivated in West and South Asia, Ethiopia, North Africa, southern Europe, South and North America, and in Oceania (Erskine and Sarker, 2004). The world production of lentil in 2013 was 4.95 Mt, of which Canada, India, and Turkey were the main producers (FAOSTAT, 2015). Canada is the major lentil producing and exporting country, whereas India is the major lentil importer.

Research on adaptation of lentil in Western Canada started in the 1970s and today lentil is an important pulse crop in this region (McVicar et al., 2010). Lentil is grown in prairie regions to enhance economic values for producers, vary crop rotations and increase the amount of nitrogen in the soil (McVicar et al., 2010). In 1970, approximately 600 ha of lentil were cultivated in Western Canada (McVicar et al., 2010). Lentil production is predicted to be more than 2 million ha in Canada in 2016.

### **2.1.2. Genetics of Lentil Seed Coat Colour and Pattern**

Lentil is a diploid ( $2n = 2X = 14$ ) plant with a genome size of 4000 Mbp (Arumuganathan and Earle, 1991). Two independent loci (*Ggc* and *Tgc*) determine the four basic seed coat background colours; brown (*Ggc Tgc*), gray (*Ggc tgc*), tan (*ggc Tgc*) and green (*ggc tgc*) (Vandenberg and Slinkard, 1990). A series of five alleles at the *Scp* locus (Ladizinsky, 1979) determine seed coat patterns. These include marbled-1, marbled-2, spotted, dotted, and absent (Vandenberg and Slinkard, 1990). The zero-tannin locus (*tan*) (Vaillancourt et al., 1986) is epistatic to the *tgc* locus, but not to the *Ggc* locus of lentil (Vandenberg and Slinkard, 1990). It is also assumed that genes for black seed coat are possibly epistatic to ground colour genes (Vandenberg and Slinkard, 1990). The underlying cotyledon colour can affect the perception of seed coat colour because many seed coat types are translucent to some extent. Cotyledon colour is controlled by a two-gene system. The expression of the gene *Yc* results in dominant (red cotyledon) and recessive (yellow cotyledon) phenotypes. A second gene, *i-yc*, inhibits the main cotyledon colour alleles and produces green cotyledon colour (Slinkard, 1978).

### **2.1.3. Market Characteristics of Lentil Seeds**

There are two major market classes of lentil, known as red and green. Red lentil represents the majority of lentil production worldwide. Red cotyledon lentils of various sizes are dehulled and marketed as “football” type (unsplit) or in split form. Green lentil types (typically yellow cotyledon), are marketed as whole seed of various sizes, and on the basis of seed coat colour (McVicar et al., 2010). Many minor market classes are produced in smaller quantities, including French green (yellow cotyledon, green seed coat, marbled seed coat pattern), Spanish brown (yellow cotyledon, gray seed coat with

dotted pattern), and Beluga (yellow cotyledon with black seed coat). Depending on the mass and diameter of seeds, lentils are classified in some markets as Chilean (seeds greater than 50 mg) and Persian or small-seeded (45 mg per seed or less) (McVicar et al., 2010).

Seed coat colour is an important grading factor that affects the market value of lentils, especially green lentil. According to the Canadian Grain Commission, green lentils exhibiting a good natural colour are graded as No. 1 (Canadian Grain Commission, 2014). An increase in seed coat colour variability, or darkening of the seed coat, will decrease the grade of the sample, thereby reducing the offered price (Davey, 2007). For No. 3 grade green lentil (severe discolouration, i.e., dark brown (Canadian Grain Commission, 2014)), the average price is approximately half that of No. 1 grade large green lentil (e.g., see <http://www.statpub.com/stat/prices/spotbid.html>(2015)). Therefore, long-term storage without seed coat colour change could serve as a strategy to preserve market value of lentil crops.

#### **2.1.4. Nutritional Value of Lentils**

Whole lentil seeds are a good source of protein, with an average protein content of 28.3% of total dry weight (Erskine et al., 2011). This valuable pulse crop has high concentrations of macro- and micronutrients (e.g., P, K, Ca, Fe, and Zn), vitamins (including niacin, vitamin A, and ascorbic acid), dietary fibre, carbohydrates, and essential amino acids such as lysine (Erskine et al., 2011). Lentil seeds are also a good source of secondary metabolites such as phenolic compounds (Xu and Chang, 2009). To date, the market value of lentil is not determined by nutritional profile. However, as the lentil industry evolves and the value of various seed components is determined,

there will be an increased demand for information about the nutritional value of those components in food processing applications.

## **2.2. Phenolic Compounds**

Humans require various types of nutritional compounds or natural products to maintain health. Carbohydrates, proteins, lipids and nucleic acids play a major role in primary nutrition and human health. Some secondary metabolites are also important for human health. Secondary metabolites include alkaloids, steroids, vitamins, hormones, and phenolic substances. Phenolic substances consist of a large group of metabolites that contain an aromatic ring with an attached OH group (Vermeris and Nicholson, 2006). There are different terms and types of classification for these compounds; phenolics, polyphenols, phenolic substances, and phenolic compounds. Various categorizing systems exist for polyphenols, largely based on number of carbon atoms in the molecule (Harborne and Simmonds, 1964).

### **2.2.1. Sub-classes of Phenolic Compounds**

Sub-classes of phenolic compounds detected in lentil seeds include phenolic acids (Amarowicz et al., 2009; Bartolomé et al., 1997; Xu and Chang, 2009; Zhang et al., 2015), flavan-3-ols and proanthocyanidins (Amarowicz et al., 2009; Amarowicz et al., 2010; Bartolomé et al., 1997; Dueñas et al., 2002; Dueñas et al., 2003; Escarpa et al., 2002; Zhang et al., 2015; Zou et al., 2011), anthocyanidins (Takeoka et al., 2005; Xu and Chang, 2010), flavonols (Aguilera et al., 2010; Amarowicz et al., 2009; Amarowicz et al., 2010; Dueñas et al., 2002; Escarpa et al., 2002; Tsopmo and Muir, 2010; Xu and Chang, 2009; Zhang et al., 2015; Zou et al., 2011), stilbenes (Dueñas et al., 2002), flavones (Amarowicz et al., 2009; Dueñas et al., 2002; Xu and Chang, 2009, 2010), and



flavanones (Aguilera et al., 2010). Descriptions for the detected phenolic compounds in lentils and some of the precursors are given in the following section.

### 2.2.1.1. Phenolic Acids

Phenolic acids include hydroxybenzoic acids and hydroxycinnamic acids.

#### 2.2.1.1.1. Hydroxybenzoic Acids

As shown in Figure 2.1, hydroxybenzoic acids consist of a C<sub>6</sub>-C<sub>1</sub> skeleton (Green, 2007). The alcohol group is substituted by a carboxyl group (-COOH) on a phenol ring (Vermeris and Nicholson, 2006). 4-Hydroxybenzoic acid, gallic acid, protocatechuic acid, and vanillic acid are all hydroxybenzoic acids occurring in lentil seeds (Amarowicz et al., 2009; Bartolomé et al., 1997; Xu and Chang, 2009; Zhang et al., 2015).

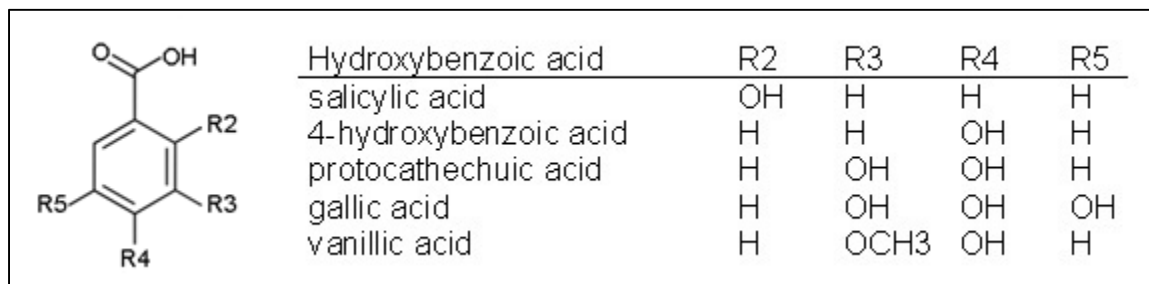


Figure 2.1. Hydroxybenzoic acids' structure and examples

#### 2.2.1.1.2. Hydroxycinnamic Acids

Figure 2.2 shows the basic structure of hydroxycinnamic acids with a common C<sub>6</sub>-C<sub>3</sub> skeleton (Green, 2007). Cinnamic acid, *p*-coumaric acid, and ferulic acid are some of the examples of hydroxycinnamic acids that have been detected in lentil seeds (Amarowicz et al., 2009; Bartolomé et al., 1997; Xu and Chang, 2009).

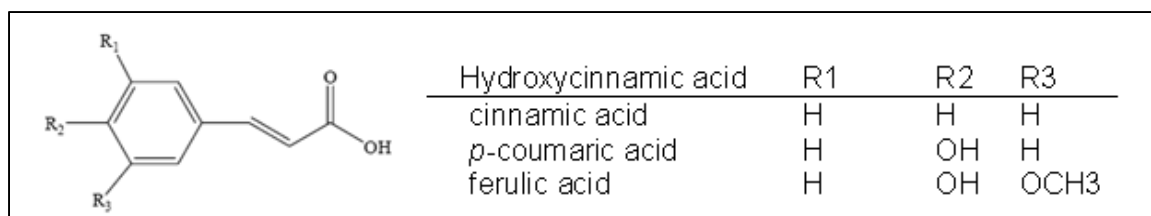


Figure 2.2. Hydroxycinnamic acids' structure and examples

### 2.2.1.2. Stilbenes

Stilbenes are characterized by a C<sub>6</sub>-C<sub>2</sub>-C<sub>6</sub> skeleton and contain 1, 2-diphenylethylene as a functional group (Vermeris and Nicholson, 2006). Resveratrol-3- $\beta$ -mono-*D*-glucoside is an example of a stilbene that can be found in lentil seeds (Figure 2.3) (Dueñas et al., 2002).

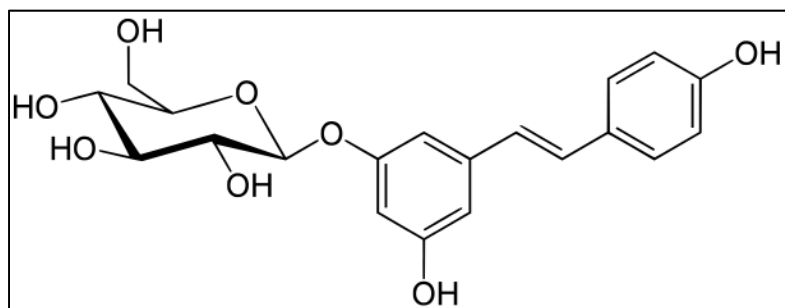


Figure 2.3. Structure of resveratrol-3- $\beta$ -mono-*D*-glucoside

### 2.2.1.3. Chalcones

Chalcones have a C<sub>6</sub>-C<sub>3</sub>-C<sub>6</sub> structure (Figure 2.4) with a linear unsaturated C<sub>3</sub>-chain which is connecting two rings (Vermeris and Nicholson, 2006). Because of their precursory role, chalcones are of great importance (Green, 2007). Chalcones can produce flavanones, which in turn serve as precursors for flavones and dihydroflavonols.

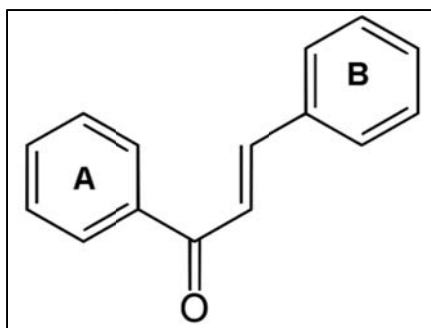


Figure 2.4. Basic structure of chalcones

#### 2.2.1.4. Flavonoids

Flavonoids represent a major sub-class of chemical within the C<sub>6</sub>-C<sub>3</sub>-C<sub>6</sub> group, including 60% of the total dietary phenolic compounds with an estimated number of 4000 (Green, 2007). They have an A-, B-, and C-ring with a six-member heterocycle (Figure 2.5). Flavonoids consist of flavanones, isoflavones, flavones, dihydroflavonols, flavonols, leucoanthocyanidins, anthocyanidins, flavan-3-ols and proanthocyanidins (Vermeris and Nicholson, 2006).

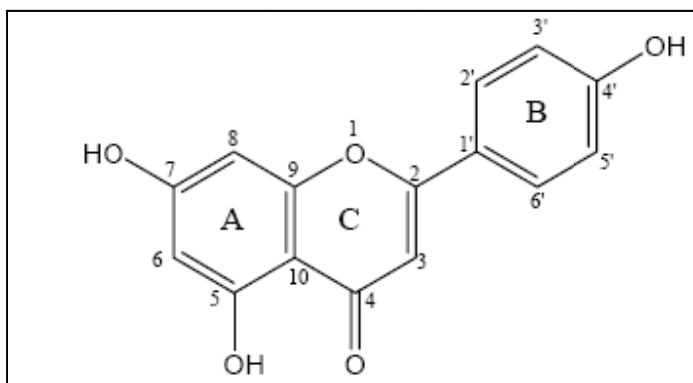


Figure 2.5. Basic structure of flavonoids

##### 2.2.1.4.1. Flavanones

The heterocycle of flavanones contain a ketone group without an unsaturated C-C bond (Figure 2.6). Naringenin, eriodictyol, and hesperetin are some examples of this group (Vermeris and Nicholson, 2006).

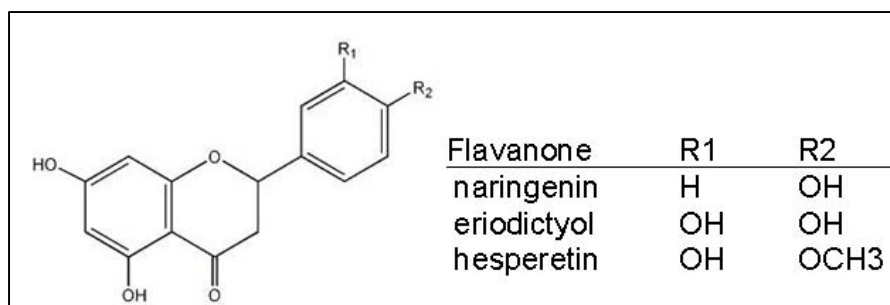


Figure 2.6. Flavanones structure with some examples

#### 2.2.1.4.2. Isoflavones

Isoflavones are structural isomers of flavanones that occur in sprouts and roots of pulses. In these compounds, the B-ring is attached to the C-ring at C-3 (Figure 2.7). The aglycones diadzein, genistein, glycitein and their glycosides are the most common plants' isoflavones (Green, 2007).

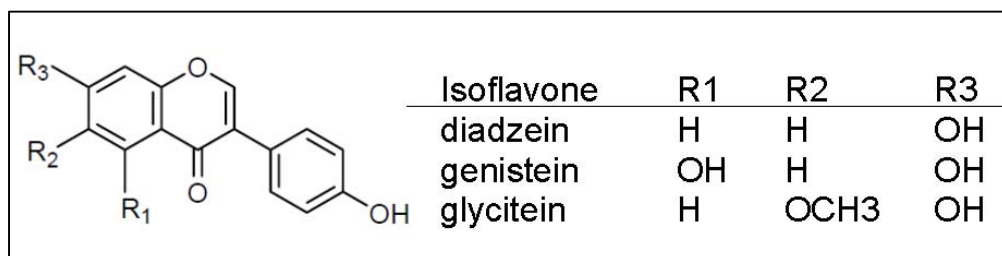


Figure 2.7. Structures of some common isoflavones

#### 2.2.1.4.3. Flavones

The structures of flavones are similar to flavanones, but they are unsaturated between C-2 and C-3 of C-ring (Figure 2.8). Apigenin and luteolin are two important examples of flavones that have been detected in lentil seeds (Amarowicz et al., 2009; Dueñas et al., 2002; Xu and Chang, 2009, 2010)

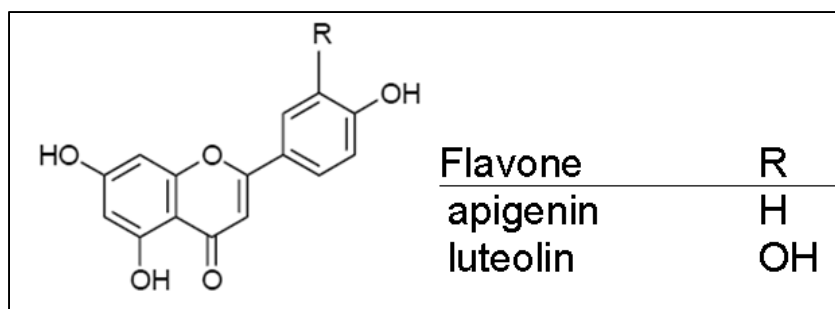


Figure 2.8. Structures and two examples of flavones

#### 2.2.1.4.4. Dihydroflavonols

Dihydroflavonols are also called flavanonols with a basic structure as represented in Figure 2.9. Dihydrokaempferol, dihydroquercetin, and dihydromyricetin are some examples of flavanonols (Vermeris and Nicholson, 2006).

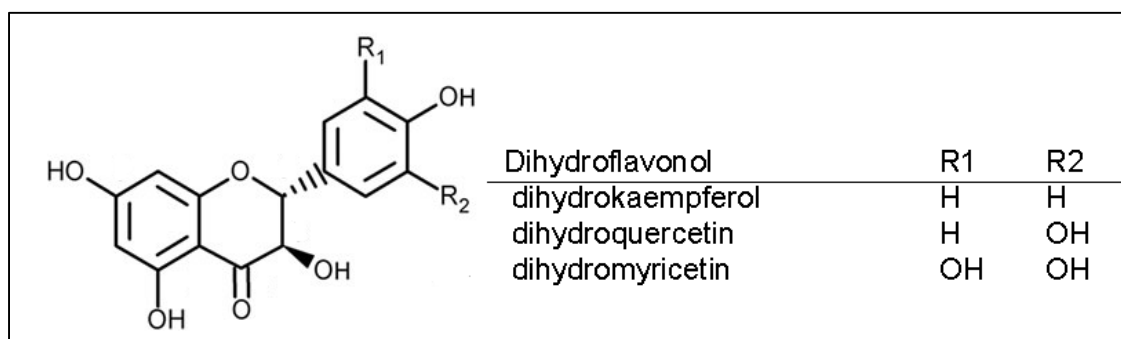


Figure 2.9. Structures of some common dihydroflavonols

#### 2.2.1.4.5. Flavonols

Flavonols are the most common plant flavonoids. The C-3 position in these substances tends to be hydroxylated or glycosylated (Green, 2007). Kaempferol, myricetin and quercetin are some examples of flavonols that have been found in lentil seeds (Figure 2.10) (Aguilera et al., 2010; Amarowicz et al., 2009; Amarowicz et al., 2010; Dueñas et al., 2002; Escarpa et al., 2002; Tsopmo and Muir, 2010; Xu and Chang, 2009; Zhang et al., 2015; Zou et al., 2011).

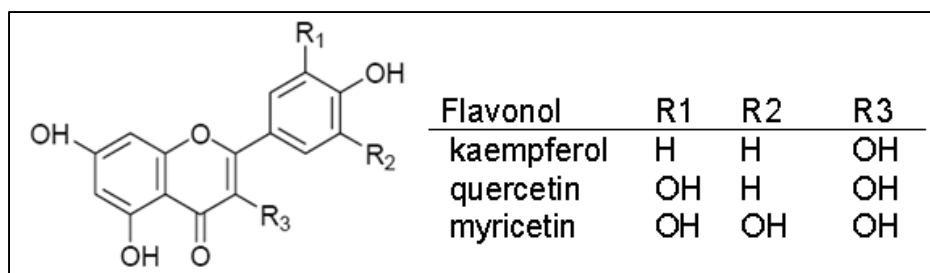


Figure 2.10. Structures of some flavonols

#### 2.2.1.4.6. Leucoanthocyanidins

Leucoanthocyanidins, also called flavan-3,4-cis-diols, are colourless substances that are made from flavanones (Vermeris and Nicholson, 2006). They are important precursors to flavan-3-ols and anthocyanidins. Leucocyanidin, leucodelphinidin, and leucopelargonidin are some examples of this group (Figure 2.11).

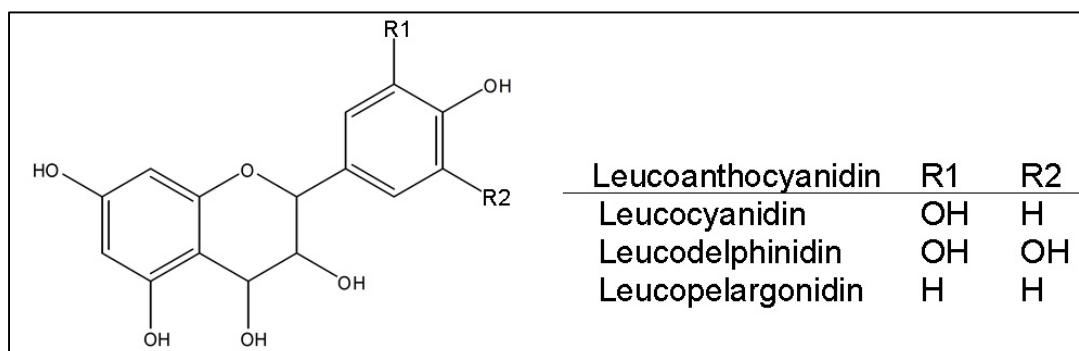


Figure 2.11. Structures of some common leucoanthocyanidins

#### 2.2.1.4.7. Anthocyanidins

The C-ring in anthocyanidins is a pyrilium cation (Figure 2.12) (Vermeris and Nicholson, 2006). Water-soluble glycosides of anthocyanidins are called anthocyanins; such as cyanidin-3-glucoside, delphinidin-3-glucoside, and malvidin-3-glucoside. Delphinidin 3-O-(2-O-β-D-glucopyranosyl-α-L-arabinopyranoside) has been detected in black lentil (Takeoka et al., 2005).

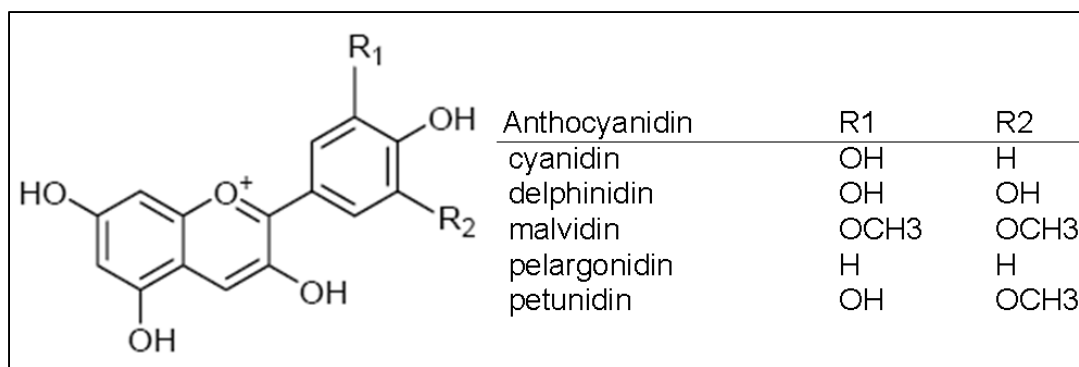


Figure 2.12. Structures of major anthocyanidins

#### 2.2.1.4.8. Flavan-3-ols

Flavan-3-ols have a completely saturated heterocycle (Figure 2.13). They contain a hydroxyl or galloyl group that is attached to C-3 which is a chiral center. *Trans* and *cis* diastereoisomers such as (+)-catechin and (-)-epicatechin are examples of flavan-3-ols. Flavan-3-ols can be found in lentil seeds in large amounts (Amarowicz et al., 2009; Amarowicz et al., 2010; Bartolomé et al., 1997; Dueñas et al., 2002; Dueñas et al., 2003; Escarpa et al., 2002; Zhang et al., 2015; Zou et al., 2011).

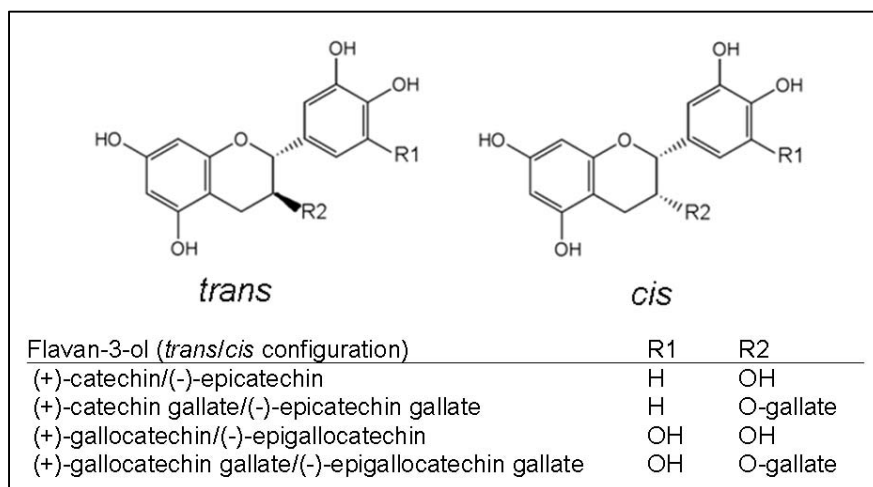


Figure 2.13. Structures of flavan-3-ols

#### 2.2.1.4.9. Proanthocyanidins

Proanthocyanidins, also referred to as condensed tannins, are oligomeric or polymeric tannins (beside hydrolysable and complex tannins) that consist of flavan-3-ol subunits. Polymers are formed by enzymatic activities and can form complexes of more than 50 flavan-3-ol units (Vermeris and Nicholson, 2006). Procyanidins, which are oligomers of (epi)catechin and prodelphinidins which are oligomers of epi(catechin) and epi(gallocatechin) are two major groups of proanthocyanidins that occur in lentil seeds in large amounts (Amarowicz et al., 2009; Amarowicz et al., 2010; Bartolomé et al., 1997; Dueñas et al., 2002; Dueñas et al., 2003; Escarpa et al., 2002; Zhang et al., 2015; Zou et al., 2011). Figure 2.14 shows an example of a procyanidin dimer.

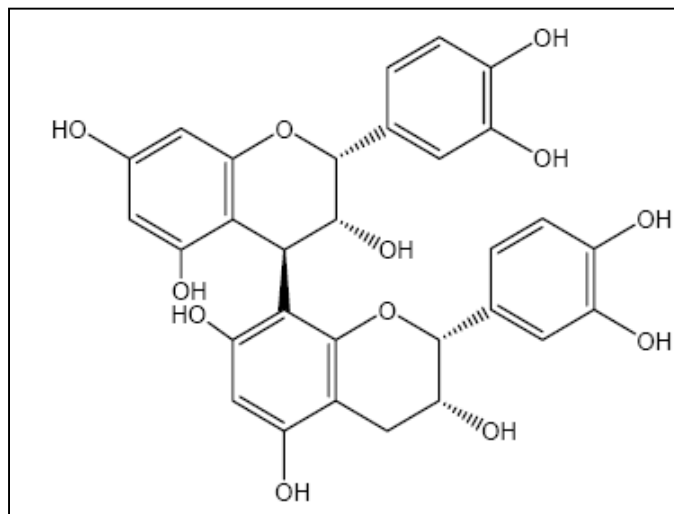


Figure 2.14. Structure of procyanidin B2 ((-)-epicatechin-(4β→8)-(-)-epicatechin)

#### 2.2.2. Phenolic Compounds in Plants

Phenolic compounds control different physiological processes of the plants including pigmentation and protection against biotic and abiotic stresses.



### **2.2.2.1. Phenolic Compounds as Pigments**

Phenolic compounds are part of the spectrum of pigmentation processes in plants. These processes include colours influenced by chlorophyll, carotenoids, betalains, and phenolic compounds. Chlorophylls are characterized by the green colour spectrum, while carotenoids give deep yellow to orange-red colours to plants. Phenolic compounds such as flavones and flavonols are white to pale yellow (Andersen and Jordheim, 2010). Proanthocyanins are colourless compounds, but will produce dark colours after oxidation (Appelhagen et al., 2011). Anthocyanidins are responsible for red, purple, and blue colours of flowers and fruits (Andersen and Jordheim, 2010).

### **2.2.2.2. Phenolic Compounds and Stresses**

Biotic and abiotic stresses can affect the production of phenolic compounds in plants. The amounts of vanillic and syringic acids in soybean (*Glycine max*) radicles increased under long-term and continuous cold/osmotic stress (Swigonska et al., 2014). Under sudden and short-term stress, however, the content of ferulic and *p*-coumaric acids increases. The action of these phenolic acids might be related to free radical scavenging potential and their antioxidant activity (Swigonska et al., 2014). Germination of *Vitis riparia* seeds under cold stress is accompanied by the increase in phenolic compounds such as gallic acid (Wróbel et al., 2005). Kaempferol derivatives, isoflavonoids and anthocyanins can protect plants against UV radiation (Solecka, 1997). Biotic stresses (i.e., fungi, bacteria, viruses, nematodes) can induce phenolic compounds such as stilbenes and flavonols. Plants with higher resistance to pathogens showed higher activity of both phenolic-related enzymes, such as phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO) and peroxidase (POD) and had higher

accumulation of phenolic compounds (Solecka, 1997). Flavonoids such as tannins can act as astringents that protect plants by deterring overgrazing by herbivores (Solecka, 1997).

### **2.2.3. Phenolic Compounds and Human Beings**

In the past, some phenolic substances were only thought of as anti-nutritional factors. For example, tannins can make complexes with iron and reduce bioavailability of iron-containing substances (Martin and Appel, 2010). Phenolics can interact with growth related proteins in humans and inhibit their enzymatic activity (Champ, 2002; Martin and Appel, 2010). More recently, phenolic compounds are considered as a “double-edged sword” (Martin and Appel, 2010). Scientists now pay attention to the health benefits of phenolics, for example their anti-oxidant, anti-tumour, and anti-heart disease properties (Martin and Appel, 2010).

#### **2.2.3.1. Anti-oxidant Properties of Phenolic Compounds**

Several classes of phenolic substances including phenolic acids have antioxidant activity. Phenolic acids donate a hydrogen atom to radicals and form a phenoxyl radical. This will terminate radical chain reactions and the formation of new radicals. Hydrophobic properties of benzene rings, the hydrogen donating potential of the hydroxyl group and the ability of phenolics to chelate metals will increase their anti-oxidant ability (Hon NG, 2011).

#### **2.2.3.2. Anti-tumour Properties**

Quercetin, *trans*-resveratrol, and flavan-3-ols from tea contain cancer preventative properties. Phenolic compounds in green tea transcriptionally activated a signaling cascade for the elimination of chemical carcinogens by detoxifying enzymes (Vermeris

and Nicholson, 2006). Treatment of colon carcinoma cell lines with phenolic acids such as gallic acid inhibited the growth of these cancer related cells (Hon NG, 2011).

### **2.2.3.3. Anti-heart Disease Properties**

The phenolics (+)-catechin, quercetin, and resveratrol showed protective effects against coronary heart disease, atherosclerosis, and heart attack. These properties may relate to the effect of reactive oxygen species (ROS) on low-density lipoproteins and on maturation of lesions in coronary veins (Vermeris and Nicholson, 2006).

### **2.2.4. Biosynthesis of Phenolic Compounds**

The general phenylpropanoid pathway generates substrates common to a number of phenylpropanoid substances such as phenolic acids, stilbenes, and flavonoids (Vermeris and Nicholson, 2006). However, the shikimate and acetate pathways might also act in the biosynthesis of some of the phenolic substances. The general phenylpropanoid pathway begins with deamination of phenylalanine, which is catalyzed by PAL, yielding in cinnamic acid (Figure 2.15). Cinnamic acid is hydroxylated by cinnamic acid 4-hydroxylase (C4H) to produce *p*-coumaric acid (Vermeris and Nicholson, 2006). Cinnamic acid and *p*-coumaric acid can be substrates for the other hydroxybenzoic and hydroxycinnamic acids (Green, 2007). *P*-coumaric acid is converted to *p*-coumaroyl Coenzyme A (*p*-coumaroyl CoA) by the enzyme 4-coumaric acid:CoA ligase (4CL) (Vermeris and Nicholson, 2006). *P*-coumaroyl CoA with three malonyl-CoAs will either produce stilbenes by the action of stilbene synthase (STS) or produce chalcone, which is catalyzed by chalcone synthase (CHS). Chalcone produces flavanones such as naringenin that is catalyzed by chalcone isomerase (CHI) (Vermeris and Nicholson, 2006). Flavones and isoflavones are produced from flavanones by

flavonoid-3'-hydroxylase (F3'H), flavone synthase (FS), and isoflavone synthase (IFS) (Reinprecht et al., 2013). Flavanone-3-hydroxylase (F3H) can convert flavanones to dihydroflavonols such as dihydrokaempferol. Other dihydroflavonols such as dihydroquercetin and dihydromyricetin can be produced from dihydrokaempferol by F3'H and flavonoid-3',5'-hydroxylase (F3'5'H) (Albert et al., 2014). Flavonols (e.g., kaempferol, quercetin, and myricetin) are produced from dihydroflavonols by the action of flavonol synthase (FLS) (Albert et al., 2014).

Dihydroflavonols are reduced to leucoanthocyanidins by dihydroflavonol reductase (DFR); for example leucocyanidin and leucodelphinidin are produced from dihydroquercetin and dihydromyricetin, respectively (Albert et al., 2014). Leucoanthocyanidin dioxygenase/anthocyanidin synthase (LDOX/ANS) dehydrates leucoanthocyanidins (Vermeris and Nicholson, 2006) and yields anthocyanidins such as cyanidin and delphinidin. Flavan-3-ols are produced via an enzymatic reduction; the *cis* configurations of flavan-3-ols (e.g., epicatechin, epigallocatechin) are made from anthocyanidins (e.g., cyanidin, delphinidin) by anthocyanidin reductase (ANR), while the *trans* configurations (e.g., catechin, gallic catechin) are produced by leucoanthocyanidin reductase (LAR) from leucoanthocyanidins (e.g., leucocyanidin, leucodelphinidin). In the next step, flavan-3-ols will be condensed to oligomers and polymers of proanthocyanidins by condensing enzyme (CE) in the vacuole (Koes et al., 2005). Beside these regulatory genes, a number of transcription factors (TFs) influence gene expression in this pathway. R2R3-MYB, WD-repeat (WDR), and basic-helix-loop-helix (bHLH) are the conserved TFs of the phenylpropanoid pathway in most plants. Together

they make an activation complex called MYB-bHLH-WD repeat complex (MBW) (Albert et al., 2014).

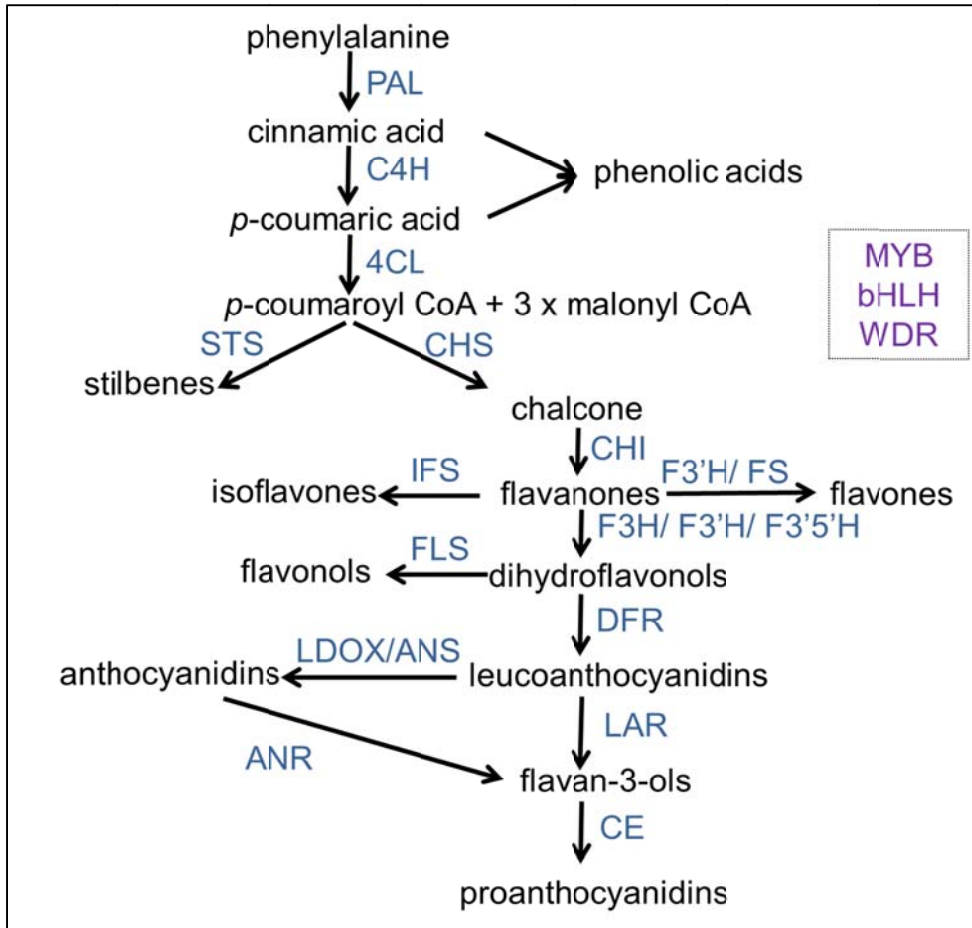


Figure 2.15. Phenylpropanoid pathway; Abbreviations: PAL, phenylalanine ammonia lyase; C4H, cinnamic acid 4-hydroxylase; 4CL, 4-coumaric acid:CoA ligase; STS, stilbene synthase; CHS, chalcone synthase; CHI, chalcone isomerase; IFS, isoflavone synthase; F3'H, flavonoid-3'-hydroxylase; FS, flavone synthase; F3H, flavanone-3-hydroxylase; F3'5'H, flavonoid-3',5'-hydroxylase; FLS, flavonol synthase; DFR, dihydroflavonol reductase; LDOX/ANS, leucoanthocyanidin dioxygenase /anthocyanidin synthase; LAR, leucoanthocyanidin reductase; ANR, anthocyanidin reductase; CE, condensing enzyme; WD-repeat, WDR; and basic-helix-loop-helix (bHLH). The information of the pathway originates from (Albert et al., 2014; Reinprecht et al., 2013; Vermeris and Nicholson, 2006; Winkel-Shirley, 2001; Koes et al., 2005).

## 2.3. Prologue to Chapter 3

The following research chapter describes a study to determine the phenolic profiles of lentil genotypes with four seed coat background colours, specifically green, gray, tan, and brown.

A series of optimization tests were conducted to achieve a fast separation with sufficient resolution for analysis of the phenolic profiles of lentil seeds. This series of related optimization tests was published in 2014 as a manuscript in J. Chromatogr. B and is provided in Appendix 1:

Mirali, M., Ambrose, S.J., Wood, S.A., Vandenberg, A., Purves, R.W., (2014). Development of a fast extraction method and optimization of liquid chromatography–mass spectrometry for the analysis of phenolic compounds in lentil seed coats. J. Chromatogr. B 969, 149–161.

<http://dx.doi.org/10.1016/j.jchromb.2014.08.007>

Copyright for use of this manuscript (# 1) in the thesis was obtained and is reported in Appendix 8 of the thesis.

Chapter 3 was submitted as a manuscript to the J. Nat. Prod. in June 2016 and is under review at this time.

# CHAPTER 3 - PROFILING THE PHENOLIC COMPOUNDS OF THE FOUR MAJOR SEED COAT TYPES IN LENTIL

## 3.1. Abstract

Phenolic compounds can provide antioxidant health benefits for humans, and foods such as lentil can be valuable dietary sources of different sub-classes of these secondary metabolites. This study used liquid chromatography-mass spectrometry (LC-MS) analyses to compare the phenolic profiles of lentil genotypes with four seed coat background colours (green, gray, tan, and brown) and two cotyledon colours (red and yellow) grown at two locations. The values of various phenolic compounds in lentil seeds varied with the different seed coat colours conferred by specific genotypes. Seed coats of lentil genotypes with the homozygous recessive *tgc* allele (green and gray seed coats) had higher values of flavan-3-ols, proanthocyanidins, and some flavonols.

## 3.2. Introduction

Lentil seed coats have a wide range of background colour and patterns. The seed coat ground colour of lentils is mainly determined by two independent genes: *Ggc* and *Tgc* (Vandenberg and Slinkard, 1990). The dominant and recessive combinations of two alleles at each locus determines the four basic seed coat ground colours known as brown (*Ggc Tgc*), gray (*Ggc tgc*), tan (*ggc Tgc*), and green (*ggc tgc*) (Vandenberg and Slinkard, 1990). Furthermore, a single gene controls the inheritance of red vs. yellow

cotyledon colour in lentil; the dominant *Yc* allele will produce a red cotyledon while the recessive *yc* allele produces a yellow cotyledon (Slinkard, 1978).

Information about the relationship between lentil seed coat colour genes and their associated phenolic compound profiles is unknown. Therefore, in this study the phenolic compound profile generated by liquid chromatography-mass spectrometry (LC-MS) for green, gray, tan, and brown seed coat ground colour phenotypes of lentil with either red or yellow cotyledons were compared. The objective was to determine if they are characteristic of specific genetic combinations of the alleles of the *Ggc* and *Tgc* loci.

### **3.3. Materials and Methods**

#### **3.3.1. Plant Material**

Seeds of a lentil recombinant inbred line (RIL) population LR-18 (Fedoruk et al., 2013) were obtained from fresh seed lots grown in 2013 at the Crop Development Centre (University of Saskatchewan) at the Sutherland (STH) and Saskatchewan Pulse Growers (SPG) farms near Saskatoon, Canada. The parents of LR-18 are CDC Robin (brown seed coat with red cotyledon) and 964a-46 (pale green seed coat with yellow cotyledon). The population segregated independently for both alleles of *Ggc*, *Tgc*, and *Yc* genes producing brown, gray, tan, and green seed coat colours and red and yellow cotyledon colours. For each seed coat colour combination, seed samples of a subset of eight RILs (four yellow and four red cotyledons) from the LR-18 population were randomly selected (Table 3.1). Seeds of three replications of each genotype grown at both locations were analyzed. Seeds were homogeneous for plumpness and diameter (Fedoruk et al., 2013) and had no evidence of seed coat pattern. Furthermore, the football fractions (entire decorticated seeds) of two RILs with green seed coat/yellow



cotyledon and two RILs with green seed coat/red cotyledon (obtained in three biological replicates) were compared.

Table 3.1. Genotypes and phenotypes of the lentil seed coat samples analyzed.

LR-18 RIL Group	Number of RILs	Genotype at <i>Ggc</i> locus	Genotype at <i>Tgc</i> locus	Seed Coat Colour Phenotype	Genotype at <i>Yc</i> locus	Cotyledon Colour Phenotype
1	4	<i>Ggc</i>	<i>Tgc</i>	Brown	<i>Yc</i>	red
2	4	<i>Ggc</i>	<i>Tgc</i>	Brown	<i>Yc</i>	yellow
3	4	<i>Ggc</i>	<i>Tgc</i>	Gray	<i>Yc</i>	red
4	4	<i>Ggc</i>	<i>Tgc</i>	Gray	<i>Yc</i>	yellow
5	4	<i>Ggc</i>	<i>Tgc</i>	Tan	<i>Yc</i>	red
6	4	<i>Ggc</i>	<i>Tgc</i>	Tan	<i>Yc</i>	yellow
7	4	<i>Ggc</i>	<i>Tgc</i>	Green	<i>Yc</i>	red
8	4	<i>Ggc</i>	<i>Tgc</i>	Green	<i>Yc</i>	yellow

### 3.3.2. HPLC-MS

In this experiment, previously optimized chromatographic conditions (Mirali et al., 2014) were applied using reversed phase HPLC on an Alliance 2695 (Waters, UK) with a 996 PDA UV/VIS detector coupled to a Quattro Ultima (Waters, UK) triple quadrupole MS equipped with an electrospray ionization (ESI) interface. The peak area of each analyte was integrated with Waters' MassLynx 4.1 software, and normalized to the peak area of a related internal standard (IS). All the flavan-3-ols and proanthocyanidins were normalized to the peak area of  $\pm$ -catechin-2,3,4-<sup>13</sup>C<sub>3</sub>. For the rests of phenolic compounds, the type of IS was based on the retention time (Rt). Therefore, the peak area of salicin/ 4-aminosalicylic acid, 3-hydroxy-4-methoxy-cinnamic acid, resveratrol-(4-hydroxyphenyl-<sup>13</sup>C<sub>6</sub>)/ 4-hydroxy-6-methylcoumarin were applied for the compounds with retention time ranges of 0-9, 9.1-13.6, and 13.7-30 min, respectively. The chromatographic column was a Core-shell Kinetex pentafluorophenyl (PFP), 100 × 2.1

mm id, 2.6  $\mu\text{m}$  particle size (Phenomenex, Torrance, CA).  $\text{H}_2\text{O}$ : formic acid (FA) (99:1, v/v) as solvent A and  $\text{H}_2\text{O}$ : acetonitrile (ACN): FA (9:90:1, v/v/v) as solvent B were used for mobile phases at a flow rate of 0.35 mL/min using a gradient detailed in Table 3.2. The column oven temperature was 40 °C and the injection volume was 2  $\mu\text{L}$ . Quantification of phenolic compounds was done using multiple reaction monitoring (MRM) and single ion recording (SIR) in negative or positive modes. For the MRM, several functions were defined with various time ranges in the mass spectrometry software, while for the SIR, one function with several transitions was used.

Table 3.2. Mobile phase gradient used in this experiment, where solvent A and B were  $\text{H}_2\text{O}$ :FA (99;1, v/v) and  $\text{H}_2\text{O}$ :ACN:FA (9:90:1, v/v/v), respectively.

Time (min)	A%	B%	Flow (mL/min)
0	99	1	0.35
1	99	1	0.35
21	59	41	0.35
24	40	60	0.35
24.1	20	80	0.35
26	20	80	0.35
26.1	99	1	0.35
30	99	1	0.35

To ensure good reproducibility of the LC-MS method, one of the samples was repeated at the start, middle, and the end of a run. In addition, a combination of standards plus reconstitution solvent (MeOH:  $\text{H}_2\text{O}$ , 10:90, (v/v)) was used as a quality control. Solvent blanks and method blanks (solvent plus IS) were also included to ensure that contamination was not present (Mirali et al., 2014).

### 3.3.3. Reagents and Standards

Since the optimization method was published (Mirali et al., 2014), some additional compounds incorporated into our analysis, in this study more than 70 compounds were included. The related information (including sub-class, retention time, molecular

ion, fragment ion, mode of action, and the company of origin) for the total phenolic compounds analyzed in the thesis is provided in Tables 3.3 and 3.4. Table 3.3 contains phenolic commercially available standards. However, catechin-3-glucoside and kaempferol dirutinoside were quantified using MRM based upon previous reports (Aguilera et al., 2010; Dueñas et al., 2003). Note that the type of sugar for kaempferol dirutinoside was determined based on previous literature, yet the exact bond location could not be confirmed. In addition, Table 3.4 presents the retention time and molecular ion in positive mode on several oligomers of proanthocyanidins that were detected in the lentil seed matrix but were not available commercially. For these oligomers that were analyzed based upon previous reports (Dueñas et al., 2003; Mirali et al., 2014), the order of C's (catechin or epicatechin) and G's (gallocatechin or epigallocatechin) given in Table 3.4 was arbitrary.

Table 3.3. Characteristics of the phenolic compounds including sub-class, retention time, optimum molecular and fragment ions in multiple reaction monitoring (MRM) and positive or negative mode

Compound	Sub-class	Retention time (min)	Mode	Molecular ion (m/z)	Fragment ion (m/z)	Company
Salicin	IS	3.5	Neg	285	123	Sigma
Protocatechuic acid	Phenolic acids	3.8	Neg	153	109	Sigma
4-Aminosalicylic acid	IS	3.9	Neg	152	108	Sigma
Vanillic acid-4- $\beta$ -D-glucoside	Phenolic acids	4.2	Neg	329	167	Sigma
(-)-Gallic acid	Flavan-3-ols	4.7	Neg	305	125	Sigma
4-Hydroxybenzoic acid	Phenolic acids	5	Neg	137	93	Sigma
Procyanidin B1	Proanthocyanidins	5.9	Neg	577	289	Extrasys
Cyanidin-3,5-di-O-glucoside	Anthocyanidins	6.1	Neg	609	447	Extrasys
(-)-Epigallocatechin	Flavan-3-ols	6.5	Neg	305	125	Extrasys
Catechin-3-glucoside*	Flavan-3-ols	6.5	Neg	451	137	
(+)-Catechin	Flavan-3-ols	6.6	Neg	289	203	Extrasys
$\pm$ -Catechin-2,3,4- $^{13}\text{C}_3$	IS	6.6	Neg	292	206	Sigma
Chlorogenic acid	Phenolic acids	6.9	Neg	353	191	Sigma
Delphinidin-3- $\beta$ -D-glucoside	Anthocyanidins	6.9	Neg	463	300	Santa
Procyanidin B2	Proanthocyanidins	7.1	Neg	577	289	Extrasys
(-)-Epicatechin	Flavan-3-ols	8	Neg	289	203	Extrasys
Dihydromyricetin	Dihydroflavonols	8.2	Neg	319	193	Sigma
Kaempferol dirutinoside*	Flavonols	8.6	Neg	901	755	
Cyanidin-3-O-rhamnoside	Anthocyanidins	8.7	Neg	431	285	Extrasys
Malvidin-3-O-galactoside	Anthocyanidins	8.7	Neg	491	313	Sigma
<i>trans-p</i> -Coumaric acid	Phenolic acids	8.7	Neg	163	119	Sigma
(-)-Epigallocatechin gallate	Flavan-3-ols	8.8	Neg	457	169	Extrasys
3-Hydroxy-4-methoxycinnamic acid	IS	9	Neg	193	134	Sigma
Quercetin-3,4'-di-O-glucoside	Flavonols	9.7	Neg	625	463	Extrasys
<i>trans</i> -Ferulic acid	Phenolic acids	9.9	Neg	193	134	Sigma
Dihydroquercetin	Dihydroflavonols	10.2	Neg	303	125	Sigma
Kaempferol-3-O-rhamnoside-7-O-rhamnoside	Flavonols	10.2	Neg	739	593	Sigma
Luteolin-3',7-di-O-glucoside	Flavones	10.5	Neg	609	285	Extrasys
Procyanidin A2	Proanthocyanidins	10.5	Neg	575	285	Extrasys
Resveratrol-3- $\beta$ -mono-D-glucoside	Stilbenes	10.5	Neg	389	227	Santa

Table 3.3. Continued

Compound	Sub-class	Retention time (min)	Mode	Molecular ion (m/z)	Fragment ion (m/z)	Company
(-)-Epicatechin gallate	Flavan-3-ols	10.6	Neg	441	169	Extrasyn
Myricetin-3-O-rhamnoside	Flavonols	10.8	Neg	463	316	Extrasyn
Quercetin-3-O-rutinoside	Flavonols	10.8	Neg	609	300	Extrasyn
Quercetin-3-O-galactoside	Flavonols	11	Neg	463	300	Extrasyn
Quercetin-3-O-glucoside	Flavonols	11.2	Neg	463	300	Extrasyn
Luteolin-7-O-glucoside	Flavones	11.6	Neg	447	285	Extrasyn
Kaempferol-3-O-rutinoside	Flavonols	11.8	Neg	593	285	Sigma
Dihydrokaempferol	Dihydroflavonols	12	Neg	287	125	Sigma
Kaempferol-3-O-glucoside	Flavonols	12.1	Neg	447	285	Extrasyn
Quercetin-3-O-rhamnoside	Flavonols	12.2	Neg	447	300	Extrasyn
Kaempferol-7-O-neohesperidoside	Flavonols	12.3	Neg	593	285	Extrasyn
Apigenin-7-O-glucoside	Flavones	12.8	Neg	431	268	Sigma
Quercetin-4'-O-glucoside	Flavonols	13	Neg	463	301	Sigma
Myricetin	Flavonols	13.3	Neg	317	151	Sigma
Luteolin-4'-O-glucoside	Flavones	13.4	Neg	447	285	Extrasyn
Resveratrol	Stilbenes	13.6	Neg	227	143	Sigma
Resveratrol-(4-hydroxyphenyl- <sup>13</sup> C <sub>6</sub> )	IS	13.6	Neg	233	149	Sigma
4-Hydroxy-6-methylcoumarin	IS	14	Neg	175	131	Sigma
Quercetin	Flavonols	15.5	Neg	301	151	Extrasyn
Luteolin	Flavones	15.9	Neg	285	133	Extrasyn
Naringenin	Flavanones	16.8	Neg	271	151	Extrasyn
Kaempferol	Flavonols	17.6	Neg	285	187	Sigma
Apigenin	Flavones	17.7	Neg	269	117	Extrasyn
Flavone	Flavones	20.2	Pos	223	77	Sigma
Flavanone	Flavanones	22	Pos	225	121	Sigma

IS is an abbreviation for internal standard, \* indicates a phenolic compound for which no authentic standard was available Procyanidins B1, B2, and A2 are epicatechin-(4 $\beta$ →8)-catechin, epicatechin-(4 $\beta$ →8)-epicatechin, and epicatechin-(2 $\beta$ →7,4 $\beta$ →8)-epicatechin, respectively.

Compounds were purchased from Sigma-Aldrich (Missouri, USA), Santa Cruz Biotechnology, Inc. (California, USA), and Extrasynthese (Genay, France).

A Core-shell Kinetex pentafluorophenyl (PFP) column was used with a flow rate of 0.35 mL/min. The solvent composition was A (H<sub>2</sub>O: FA (99:1, v/v)) and solvent B (H<sub>2</sub>O: ACN: FA (9:90:1, v/v/v)) with the gradients as shown in Table 3.2.

Table 3.4. Characteristics of the proanthocyanidins including, retention time, and optimum molecular ion wave length in single ion recording (SIR) and positive mode

Composition	Sub-class	Retention time (min)	Mode	Molecular Ion (m/z)
GGC_I	Proanthocyanidins	4.7	Pos	899.7
GC_I	Proanthocyanidins	4.9	Pos	595.6
GGGC_I	Proanthocyanidins	5	Pos	1204.8
GGG	Proanthocyanidins	5.3	Pos	915.6
GGC_II	Proanthocyanidins	5.4	Pos	899.7
GCC_I	Proanthocyanidins	5.5	Pos	883.7
CC-gallate	Proanthocyanidins	5.5	Pos	731.7
GGGC_II	Proanthocyanidins	5.6	Pos	1204.8
GGCC_I	Proanthocyanidins	5.6	Pos	1187.8
GGC_III	Proanthocyanidins	5.7	Pos	899.7
GGGCC_I	Proanthocyanidins	5.7	Pos	1492.2
GC_II	Proanthocyanidins	5.9	Pos	595.6
GGCC_II	Proanthocyanidins	6.2	Pos	1187.8
GCCC_I	Proanthocyanidins	6.3	Pos	1171.8
GGGC_III	Proanthocyanidins	6.3	Pos	1204.8
GCC_II	Proanthocyanidins	6.4	Pos	883.7
GCC_III	Proanthocyanidins	6.5	Pos	883.7
±-Catechin-2,3,4- <sup>13</sup> C <sub>3</sub>	IS	6.6	Pos	294.2
GGGCC_II	Proanthocyanidins	6.9	Pos	1492.2
GCCCC_I	Proanthocyanidins	6.9	Pos	1460.1
CCCC_I	Proanthocyanidins	7.3	Pos	1155.9
GCCC_II	Proanthocyanidins	7.3	Pos	1171.8
CCCCC	Proanthocyanidins	7.9	Pos	1444.1
GCCCC_II	Proanthocyanidins	7.9	Pos	1460.1
CCCC_II	Proanthocyanidins	8.5	Pos	1155.9

IS, C, and G stand for internal standard, catechin/epicatechin, and gallocatechin/epigallocatechin, respectively.

The chromatographic conditions were the same as those described in Table 3.2.

### 3.3.4. Sample Preparation

Phenolic compounds were extracted from lentil seeds as described previously (Mirali et al., 2014) with some minor modifications.. Briefly, for each biological replicate 1 mL of the extraction solvent (acetone: water (70:30 v/v)) was added to a ~250 mg freeze-dried lentil sample in a micro centrifuge tube. Thereafter, two ¼ inch ceramic sphere beads were added to the micro centrifuge tube, and samples were crushed to a fine paste with a Fast Prep®FP120 (Qbiogene, Inc., Canada) for a maximum of seven consecutive times of 45 s each at a speed setting of 4.0. Samples were shaken for 1 h on a rocking platform at a speed of 1400 rpm. The tubes were centrifuged twice (12,000 rpm for 5 min each with the maximum relative centrifugal force rcf of 15300) and 100 µL aliquot of the supernatant was dried with a Speed Vac (LABCONCO, Kansas City, USA). Dried samples were then reconstituted in 100 µL methanol: water (10:90, v/v) solution.

### 3.3.5. Data Analyses

The analysis of lentil seeds in this research is based upon relative quantification (i.e., area ratio of a phenolic compound to an internal standard). Relative quantification is commonly used for comparative analyses, especially in metabolomics applications (Lei et al., 2011), whenever a quantitative measure of the *relative* amount but not the *absolute* amount is required, as is the case here in examining *changes* in phenolic compounds. Thus, with relative quantification, the analyte signal intensity is normalized to that of an internal standard by dividing the integrated area of each phenolic compound to the integrated area of a related IS and reported as area ratio for a given analyte. The area ratio was described per mg of dry weight of each seed sample variety

and the average of three replicates was reported as mean area ratio per mg sample (MARS).

Data of this chapter were analyzed with a linear mixed model using the lmerTest package (Kuznetsova et al., 2016) in R (v. 3.2.4) (R Core Team, 2016). For the best model fit, location was considered as a random effect, whereas seed coat colour, cotyledon colour, and their interaction were considered as fixed effects. Appendices 2-4 show the P-values from mixed model ANOVA F-test for the response variables. Location showed significant effect on resveratrol-3- $\beta$ -mono-D-glucoside, catechin, galocatechin, procyanidin B1, GGC\_I, GGC\_II, GGG, GGCCs, GGGCs, GCCCC\_I and GGGCCs. Therefore, these compounds were analyzed separately for SPG (Appendix 2) and STH (Appendix 3) locations. For the rest of phenolic compounds, data from two locations were combined (Appendix 4).

### **3.4. Results**

The retention time and the optimized molecular and fragment ions of the standards and the potential compounds from different sub-classes of phenolic compounds are provided in Tables 3.3 and 3.4. In these studies, several phenolic compounds were not detected, which included 4-hydroxybenzoic acid, chlorogenic acid, and *trans*-ferulic acid (phenolic acids sub-class), resveratrol aglycone (stilbenes sub-class), naringenin and flavanone aglycones (flavanones sub-class), apigenin, flavone, and luteolin-3',7-di-O-glucoside (flavones sub-class), kaempferol-3-O-glucoside, kaempferol-7-O-neohesperidoside, quercetin, quercetin-3-O-glucoside, quercetin-3-O-galactoside, quercetin-4'-O-glucoside, quercetin-3,4'-di-O-glucoside (flavonols sub-class),



epigallocatechin gallate and epicatechin gallate (flavan-3-ols sub-class), procyanidin A<sub>2</sub> and B<sub>2</sub> (proanthocyanidin sub-class), and the anthocyanidins.

Appendices 2-4 show the *P*-values from the ANOVA F-test for the effect of cotyledon and seed coat colour on MARS of phenolic compounds in lentil seeds from SPG, STH or the combination of these two locations. Resveratrol-3- $\beta$ -mono-*D*-glucoside and procyanidin B1 at STH location (Appendix 3) and vanillic acid-4- $\beta$ -*D*-glucoside and kaempferol dirutinoside in combination at two locations (Appendix 4) were not significantly different among seed coat colours, cotyledon colours or their interactions. Significant differences among MARS were observed for the remaining phenolic compounds in case of seed coat colours, cotyledon colours or their interactions.

Figures 3.1.a-c show mean comparisons for the main effect of seed coat colour at SPG, STH or the combination of both locations. The levels of procyanidin B1 (at SPG location; Figure 3.1.a) and GGGCC\_I (at STH location; Figure 3.1.b), were similar in lentils with gray and green seed coat colour, and greater than those apparent in seeds with brown and tan seed coat colour.. Similar phenomena were apparent for GGGC\_I, GCCCC\_I, and GGGCC\_II (at both SPG and STH locations) and quercetin-3-*O*-rhamnoside, myricetin-3-*O*-rhamnoside, and GCCCC\_II (Figure 3.1.c). Green seed coat lentils contained the highest levels of flavan-3-ols including catechin (at both SPG and STH locations; Figures 3.1.a-b), gallic catechin (at STH location; Figure 3.1.b), and catechin-3-glucoside (Figure 3.1.c). In contrast, resveratrol-3- $\beta$ -mono-*D*-glucoside (SPG location) was similar between tan and green seed coat samples; both of these had higher levels than lentils with brown and gray seed coats.

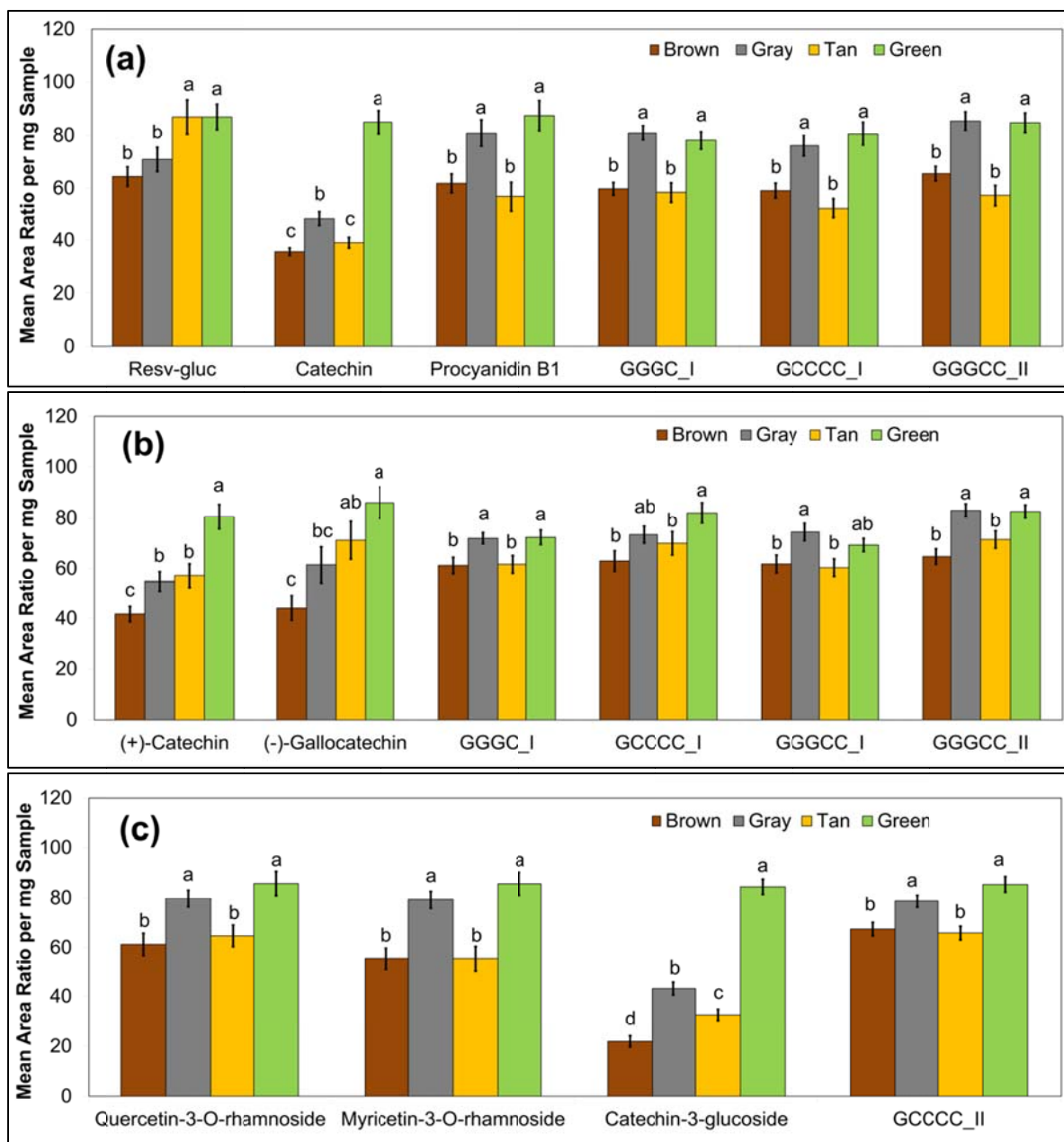


Figure 3.1. Effect of four genetically distinct lentil seed coat colours (brown, gray, tan, and green) on mean area ratio per mg sample of phenolic compounds at (a) SPG location, (b) STH location, and (c) combination of two locations. Error bars are the standard errors of two cotyledon colours (3.1.a,b) and two locations (3.1.c) in three replicates. Resv-gluc, C and G stand for resveratrol-3- $\beta$ -mono-D-glucoside, catechin/epicatechin, and gallocatechin/epigallocatechin, respectively. Means with different letters for each phenolic compound within each panel were significantly different ( $P \leq 0.05$ ).

Figures 3.2.a-c compare the MARS values of phenolic compounds with respect to the interaction of seed coat and cotyledon colours. For gallocatechin (SPG location), green

seed coats with either red and yellow cotyledon had significantly higher levels as compared to brown, gray, and tan seed coats (Figure 3.2.a). The levels of GGC\_I, GGC\_II, GGG, GGCCs, GGGC\_II, GGGC\_III, GGGCC\_I were greater in gray and green seed coats than brown and tan seed coats, especially for yellow cotyledon containing seeds from the SPG location. A similar trend is observed for luteolin-4'-O-glucoside, CC-gallate, CCCCs, CCCCC, GCs, GCCs, GGC\_III, and GCCCs (Figure 3.2.c). For kaempferol-3-O-robinoside-7-O-rhamnoside, lentils with a green seed coat and red cotyledon had higher levels relative to all other genotypes.

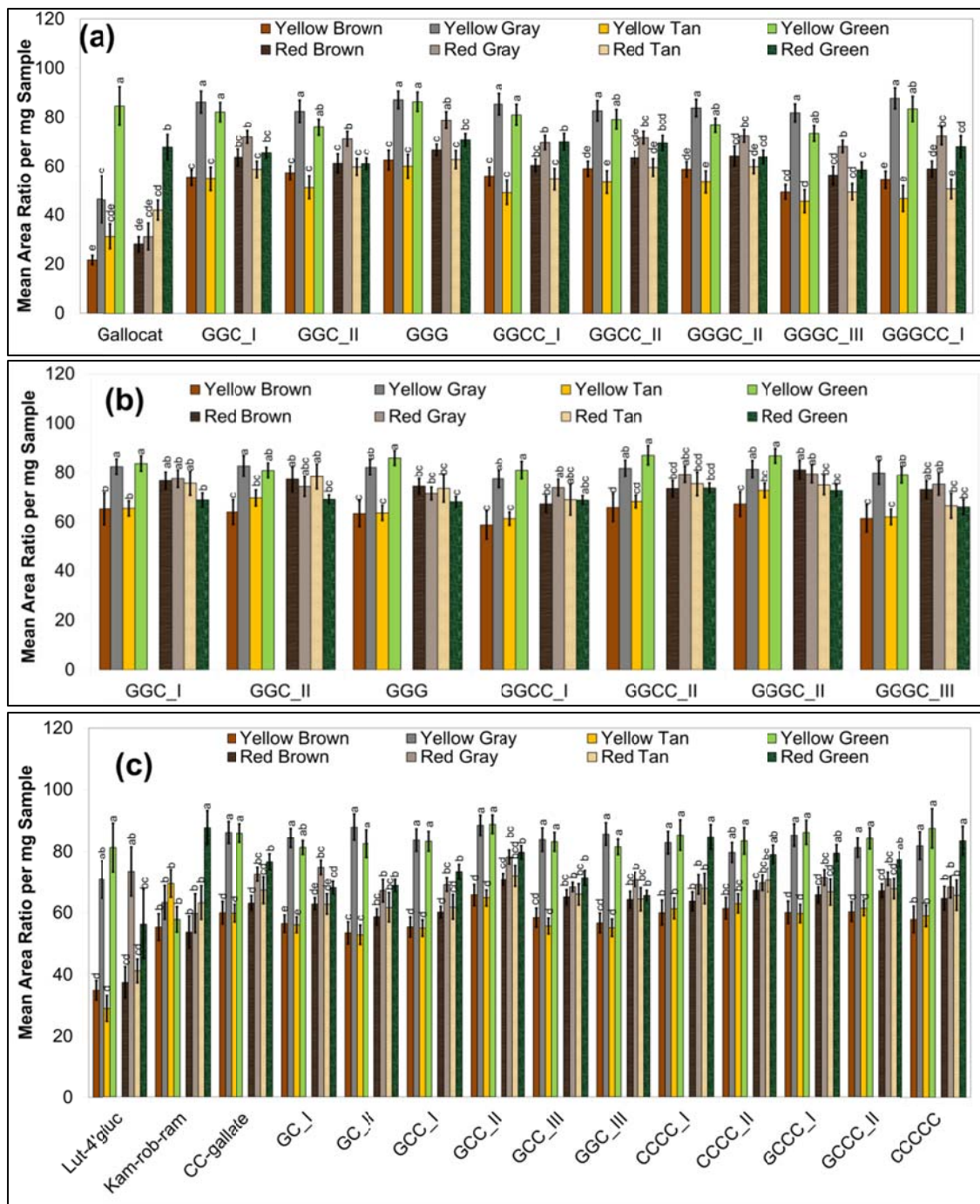


Figure 3.2. Interaction of cotyledon colour (red, yellow) and seed coat colour (brown, gray, tan, and green) on mean area ratio per mg sample of phenolic compounds at (a) SPG location, (b) STH location, and (c) combination of two locations. Error bars are the standard errors for three replicates (3.2.a and 3.2.b) or two locations with three replicates (3.2.c). Gallocat, lut-4'gluc, kam-rob-ram, C, and G stand for gallic acid, luteolin-4'-O-glucoside, kaempferol-3-O-robinoside-7-O-rhamnoside, catechin/epicatechin and gallic acid/epigallocatechin, respectively. Means with different letters for each phenolic compound within each panel were significantly different ( $P \leq 0.05$ ).

To confirm if cotyledon colour influences the phenolic profile, dehulled seeds of two genotypes with the green seed coat/red cotyledon combination were compared with two green seed coat/yellow cotyledon genotypes (Figure 3.3). No proanthocyanidin oligomers were detected in any of the football fractions. Among the phenolic compounds detected in the cotyledons, the levels of kaempferol dirutinoside, catechin, and catechin-3-glucoside in red and yellow cotyledon colours were not significantly different. The levels of vanillic acid-4- $\beta$ -D-glucoside and kaempferol-3-O-robinoside-7-O-rhamnoside were higher in the red cotyledons than for the yellow cotyledons.

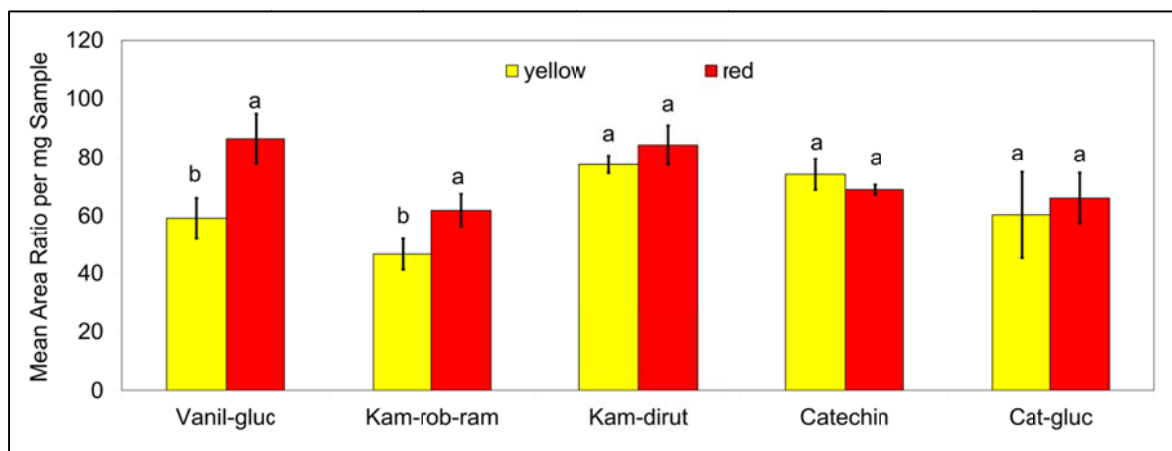


Figure 3.3. Mean area ratio of different phenolic compounds per mg of red and yellow cotyledons. Error bars are the standard errors of two genotypes with three replicates. Vanil-gluc, kam-rob-ram, kam-dirut, and cat-gluc stand for vanillic acid-4- $\beta$ -D-glucoside, kaempferol-3-O-robinoside-7-O-rhamnoside, kaempferol dirutinoside, and catechin-3-glucoside, respectively. Means with different letters for each phenolic compound were significantly different ( $P \leq 0.05$ ).

### 3.5. Discussion

LC-MS was implemented to compare the profiles of several phenolic compounds in lentil seeds. These levels were compared for lentils with brown, gray, tan, and green seed coats containing with red or yellow cotyledons that were grown at two locations. Similar trends were observed for luteolin-4'-O-glucoside (flavone sub-class), myricetin-

3-O-rhamnoside and quercetin-3-O-rhamnoside (flavonol sub-class), and proanthocyanidin dimers, trimers, tetramers, and pentamers. Specifically, the levels were higher for gray (*Ggc tgc*) and green (*ggc tgc*) lentil seed coats than in brown (*Ggc Tgc*) and tan (*ggc Tgc*) seed coats. Gray and green seed coats have the recessive *tgc* in common, whereas brown and tan seed coats both have the dominant allele (*Tgc*). This indicates that the production of some phenolic compounds, specifically the proanthocyanidins, is controlled by the *Tgc* seed coat colour gene. QTLs for tannins are located at the seed coat pattern gene *Z* (zonal) and seed coat colour *V* gene (violet factor) of common bean (*Phaseolus vulgaris*) (Caldas and Blair, 2009). *Z* and *V* genes of common bean map close to the phenylpropanoid pathway genes of 4-coumarate:CoA ligase (*4CL1*) and flavonoid-3',5'-hydroxylase (*F3'5'H*), respectively (Reinprecht et al., 2013). The *T* locus of soybean (*Glycine max*), which produces brown (*iRT*) vs. gray (*iRt*) seed coats, is associated with flavonoid-3'-hydroxylase (*F3'H*) (Toda et al., 2002). Anthocyanidins accumulate in the black (*iRT*) seed coat of soybean, but are undetectable in the brown (*iRt*) seed coat. The genes that affect anthocyanidin production are up-regulated in black seeded soybean (Kovinich et al., 2011). Proanthocyanidins are basically colourless, but changes by polyphenol oxidase (PPO) could oxidize them and cause a change from yellow to brown (Lepiniec et al., 2006; Marles et al., 2003). This might be similar to the origin of tan and brown colours in lentil. The lower levels of phenolic compounds in tan and brown lentil seed genotypes could be due to oxidative events. Therefore, *Tgc* might be linked to, or associated with, an oxidizing enzyme such as PPO, while *tgc* could be a recessive form that results in less oxidation.

Although the types of phenolic compounds are consistent for different seed coat colours, the MARS value is affected by location and genotype. The random effect of location shows significant effect on some phenolic compounds (resveratrol-3- $\beta$ -mono-*D*-glucoside, catechin, gallic acid and several of the proanthocyanidins), while the rest were not affected by location (including vanillic acid-4- $\beta$ -*D*-glucoside, luteolin-4'-*O*-glucoside, catechin-3-glucoside, flavonols, and some proanthocyanidins). Within each location, and specifically SPG, the MARS values for gray and green seed coats were similar and higher than for tan and brown seed coats. The interaction of seed coat and cotyledon colour shows higher MARS values for green and gray seed coat colours, specifically for yellow cotyledon containing seeds cultivated at the SPG location. Vaillancourt et al. (1986) report a significant genotype  $\times$  location interaction for total tannin content in lentil seed coat; however, the ranking of lentil lines was similar for different locations.

The main effect of seed coat colour or its interaction with cotyledon colour on the levels of catechin-3-glucoside, catechin, and gallic acid indicate the highest values for the green seed coat. Catechin and its glycone were detected in both red and yellow dehulled samples. There could be an interaction from cotyledons for these that affect the values of the analyzed flavan-3-ols in whole seed.

Most of the phenolic compounds detected in the whole seed were not detected in the cotyledons, which is in agreement with (Dueñas et al., 2002). Kaempferol dirutinoside, catechin, and catechin-3-glucoside were not significantly different between red and yellow cotyledons. However, red cotyledons had higher levels for vanillic acid-4- $\beta$ -*D*-glucoside and kaempferol-3-*O*-rhamnoside-7-*O*-rhamnoside compared to yellow

cotyledons. The red cotyledon is controlled by a dominant allele of *Yc*, while the homozygous recessive form (*yc*) will produce the yellow cotyledon (Slinkard, 1978). Cotyledon colour in lentil is associated with the amount of carotenoids (Thomas, 2016). Investigation using a wide range of samples could help determine if compounds within the phenolic acid and flavonol sub-classes could affect cotyledon colour in lentil.

Higher antioxidant activity in lentil might be related to the presence of phenolic acids, flavonols, flavan-3-ols, and proanthocyanidins (Fратиanni et al., 2014; Xu and Chang, 2010; Zou et al., 2011).

As part of the diet, lentils might contribute to the control of blood glucose levels and obesity because they contain flavonols (Zhang et al., 2015). Considering the greater amounts of flavan-3-ols, proanthocyanidin oligomers, and some flavonols found in green and gray seed coats, these types might possess greater antioxidative and health promoting properties.

### **3.6. Conclusion**

The levels of various phenolic compounds in lentil seeds varied with the different seed coat colours conferred by specific genotypes. Specifically, seed coats of lentil genotypes with the homozygous recessive *tgc* allele (green and gray seed coats) had higher amounts of flavan-3-ols, proanthocyanidins, and some flavonols. This suggests lentils with green and gray seed coats might be more promising as health-promoting foods.

### **3.7. Prologue to Chapter 4**

The aim of the following study was to develop a better understanding of the phenolic compound profiles of seed coats in the zero-tannin (*tan*) compared with normal (*Tan*)



genotypes of lentil. This work was submitted as a manuscript to PLOS One in May 2016. The manuscript and this chapter included complementary molecular results that were carried out by Dr. Kirstin Bett, Rob Stonehouse, and Rui Song.

# CHAPTER 4 - BIOCHEMISTRY AND GENETICS OF ZERO-TANNIN LENTILS

## 4.1. Abstract

The zero-tannin trait in lentil is controlled by a single recessive gene (*tan*) that results in a phenotype that is characterized by green stems, white flowers, and thin, transparent or translucent seed coats. In this study, one of the major groups of plant pigments, phenolic compounds, was compared among zero-tannin and normal phenotypes and genotypes of lentil. Biochemical data were obtained by liquid chromatography-mass spectrometry (LC-MS). Genomic sequencing was used to identify a candidate gene for the *tan* locus. Phenolic compound profiling revealed that myricetin, dihydromyricetin, flavan-3-ols, and proanthocyanidins were only detected in normal lentil phenotypes, but not in zero-tannin types. The molecular analysis showed that the *tan* gene encodes a bHLH transcription factor, homologous to the *A* gene in pea. The results of this study suggest that *tan* as a bHLH transcription factor interacts with the regulatory genes in the biochemical pathway of phenolic compounds starting from flavonoid-3',5'-hydroxylase (*F3'5'H*) and dihydroflavonol reductase (*DFR*).

## 4.2. Introduction

Phenolics are associated with health benefits including antioxidant activity and protection against diseases such as cardiovascular disorders, cancer, HIV, and diabetes (Calderón-Montaña et al., 2011; Delgado-Vargas et al., 2000; Manach et al., 2004; Spilioti et al., 2014; Xiao et al., 2014). Physical removal of the seed coat of lentils

leads to improved iron bioavailability (DellaValle et al., 2013), likely due to the removal of phenolic compounds, implying that these compounds interfere with iron nutrition.

The phenylpropanoid pathway plays an important role in the biosynthesis of different groups of phenolic compounds (Vermeris and Nicholson, 2006). The enzymes and related genes for branches of the pathway have been defined extensively in model plants (Grignon-Dubois and Rezzonico, 2012; Kaushik et al., 2015; Welcha et al., 2008; Winkel-Shirley, 2001). Among the numerous phenotypic traits controlled by this pathway, pigmentation has been well characterized in several plant species. Generally, variability in black, purple, red, pink, brown, and yellow colouration in many tissues is the result of different combinations of the end products of this pathway (Andersen and Jordheim, 2010). A number of transcription factors (TFs) and modifying enzymes that influence gene expression have been identified in this pathway. The conserved TFs R2R3-MYB, WD-repeat (WDR), and basic-helix-loop-helix (bHLH) form an activation complex called the MYB-bHLH-WD (MBW) repeat complex (Albert et al., 2014) that controls the phenylpropanoid pathway in most plants.

The lentil market class known as 'zero-tannin' is determined by expression of a single recessive gene, *tan* (Vaillancourt et al., 1986). Homozygous recessive *tan* is epistatic to *Tgc*, but not to *Ggc* (Vandenberg and Slinkard, 1990). In *tan* genotypes, the expression of the dominant *Ggc* produces a gray translucent seed coat, while the recessive *ggc* results in a transparent seed coat. The colour of seed coats in *tan* genotypes does not change during storage (Matus et al., 1993) or cooking, and imbibition occurs more rapidly. The thinner seed coat results in faster cooking, easier dehulling, and rounder seed shape. These characteristics are desirable for processors and consumers,

creating opportunities for breeding lentils with higher value. Zero-tannin seeds also imbibe water more quickly leading to imbibitional injury at the time of germination (Matus et al., 1993; Smýkal et al., 2014), which is a negative agronomic characteristic that can be overcome using modified agronomic techniques.

The *tan* gene also influences pigmentation of stems and flowers. Wild type lentil plants have reddish stems, purple veins on floral tissues, and thicker, pigmented seed coats (Vaillancourt et al., 1986). The *tan* phenotype is characterized by green stems and white flowers. This set of traits is similar to Mendel's *A* gene in pea (*Pisum sativum*) (Mendel, 1865) which encodes a bHLH TF that has a regulatory function with pleiotropic effects (Hellens et al., 2010). The absence of pigmentation in pea is the result of a mutation in this bHLH with mis-spliced mRNA caused by a premature stop codon (Hellens et al., 2010). The striking similarities between the two sets of phenotypes suggest that the lentil homologue of the pea *A* gene could be the same as the lentil *tan* gene.

The objective of this study was to compare the phenolic compound profiles obtained by LC-MS of seed coats in the zero-tannin (*tan*) and normal (*Tan*) genotypes of lentil along with the corresponding genotypic data. This information will help determine what exactly *tan* is and which segments of the phenylpropanoid pathway this gene influences.

## **4.3. Materials and Methods**

### **4.3.1. Plant Material**

The lentil recombinant inbred line (RIL) population LR-30, which consists of 138 lines, was derived from a cross between the brown seed coat cultivar CDC Robin (genotype *Ggc Tgc Tan*) and a zero-tannin plant from the breeding line 2670b (genotype *Ggc Tgc tan*). Both genotypes are homozygous for *Tgc* and the RILs of this population have

either normal brown or gray zero-tannin seed coats based on segregation of the dominant or recessive alleles at the *Tan* locus. Seed coats of RILs were phenotyped visually and classified as brown opaque (*Ggc Tgc Tan*) or zero-tannin gray translucent (*Ggc Tgc tan*).

In a preliminary test, two subsets of 10 RILs of each phenotype were randomly selected for analysis of the phenolic profile of the lentil seeds. Whole seeds of these 20 RILs were obtained from three biological replicates that were grown in a randomized complete block design in the field in 2013 at Saskatoon, SK, Canada.

In a second preliminary test, one available gray seed coat normal genotype, CDC Maxim (*Ggc tgc Tan*), was decorticated and seed coats were separated from cotyledons and embryos (Mirali et al., 2014). All three seed fractions were similarly analyzed with three technical replicates.

Based on the preliminary analyses, one representative RIL from the *Ggc Tgc Tan* genotype group (LR-30-76) and one representative from the *Ggc Tgc tan* genotype group (LR-30-98) were compared with seed coats of a *ggc tan* genotype (CDC Gold) (Figure 4.1). CDC Gold has a transparent seed coat which allowing its cotyledon colour to be easily observed. Seeds of CDC Gold were also produced in the field in 2013 at Saskatoon.



Figure 4.1. Scanned images of lentil seeds with normal brown (*Ggc Tgc Tan*), zero-tannin gray (*Ggc tan*), and zero-tannin transparent (*ggc tan*) seed coats

The seeds of all three genotypes were decorticated to obtain the seed coat fractions that were analyzed using three technical replicates.

#### 4.3.2. HPLC-MS

The HPLC-MS method was described previously (Mirali et al., 2014). The HPLC hardware was an Agilent 1290 UPLC (with G4212 DAD detector) coupled to a Thermo Finnigan TSQ Quantum Ultra (Thermo Fisher Scientific Inc., UK) triple quadrupole MS equipped with a heated electrospray ionization (HESI) interface. The chromatographic column, mobile phases, flow rate, and the gradient were the same as described in §3.3.2. Relative quantification was determined for phenolic compounds, using selected reaction monitoring (SRM), and for the proanthocyanidins, using single ion monitoring (SIM). Peak areas of each analyte were integrated with Thermo Xcalibur 2.1 software and values are reported as the area ratio per mg weight as described in §3.3.5.

### **4.3.3. Sample Preparation**

Sample preparation was conducted similar to that described under §3.3.4 of this thesis. For each replicate of whole lentil seeds, 1000 µL of the extraction solvent was added to 250 mg of freeze-dried sample in micro-centrifuge tubes. When separate seed fractions (cotyledons, embryos, and seed coats) were analyzed, the extraction solvent and the freeze-dried samples were reduced to 250 µL and 50 mg, respectively.

### **4.3.4. Statistical Analysis**

Analysis of variance and means comparisons of area ratio per mg of sample were done using R software (v. 3.2.4) (R Core Team, 2016). Duncan's multiple range test was used for comparing the means of area ratio per mg samples (95% confidence level).

### **4.3.5. Molecular Markers**

To initially test if the lentil homologue of the *A*-gene of pea segregated with *tan*, the nucleotide sequence for the pea gene [GU132941] was used to tBLASTx an in-house collection of 3' transcript sequences of lentil from which SNPs had been identified (Sharpe et al., 2013). A number of sequences from various lentil lines matched the pea sequence. An alignment of these sequences, using BioEdit alignment software (Hall, 1999), revealed a SNP (LcC01900p336) located 52 nucleotides downstream of the STOP codon. A KASP assay (LGC Genomics, Hoddesdon, UK) was designed to assay genotypes at this SNP. The allele specific primers were A1=GAAGGTGACCAAGTTCATGCTGACAAAATCACGTGATGTTGTGACTC and A2=GAAGGTCGGAGTCAACGGATTGACAAAATCACGTGATGTTGTGACTT. The conserved primer was C1=AAGCCAATGTGTACCAATGATGTATCATT. DNA was extracted from a single individual of each LR-30 RIL using a modified CTAB extraction

(Doyle and Doyle, 1990). The reaction volume was 10  $\mu$ L and consisted of 50 ng/ $\mu$ L DNA, 2X KASP Reaction Mix and 0.17  $\mu$ M KASP Assay Mix (allele-specific primers, A1 and A2, and common primer, C1). PCR amplification was carried out in a StepOnePlus<sup>™</sup> Real-Time PCR System (Applied Biosystems, California, US) and fluorescence was analyzed using StepOne Software version 2.1 (Applied Biosystems, California, US).

To identify the putative causal mutation in the gene, the pea sequence was compared to a preliminary assembly of the lentil genome (CDC Redberry v0.3) using tBLASTx to identify the full lentil homologue. Nested primers were designed to span the introns across the full gene and to amplify fragments from several *Tan* and *tan* lines. Amplified fragments were electrophoresed on a 1% agarose gel, excised bands were purified with a Qiagen gel extraction kit (cat.no. 28706), and the resulting DNA was sequenced using the Sanger method. Sequences were aligned to the reference genome (CDC Redberry v0.3) and SNPs identified using BioEdit alignment software.

A KASP assay, LcZT-Exon6p343,

A1 = GAAGGTGACCAAGTTCATGCTGCCCGATGATATTCGGATCGGA,

A2 = GAAGGTCGGAGTCAACGGATTGCCCGATGATATTCGGATCGGT,

C = GGCCAACAAATGAAAATCTGAGTCCAAT) was designed for a candidate SNP and used to survey a panel of 96 lentil genotypes representative of a wide range of seed coat colours and patterns and the zero-tannin cultivars Cedar, Shasta, CDC Zt-4 and CDC Gold.



## 4.4. Results

### 4.4.1. Biochemical Analysis

In a preliminary experiment, the analysis of variance showed no significant differences between the RILs within either of the same phenotypic groups of normal brown opaque seed coats (genotype *Ggc Tgc Tan*) or gray translucent zero-tannin seed coats (genotype *Ggc tan*) for most of the analyzed phenolic compounds (Appendix 5). In a second preliminary experiment, the phenolic profiles of the three seed fractions (cotyledon, seed coat, and embryo) of CDC Maxim (gray seed coat) were significantly different (Figure 4.2.a,b). Vanillic acid-4- $\beta$ -*D*-glucoside, luteolin, kaempferol glycones and aglycone, and flavan-3-ols (including catechin, gallocatechin, and catechin-3-glucoside) were detected in all three seed fractions. Resveratrol-3- $\beta$ -mono-*D*-glucoside, luteolin-4'-*O*-glucoside, quercetins, myricetins, and oligomers of proanthocyanidins (i.e., dimers, trimers, tetramers, and pentamers) were detected only in the seed coat fraction.

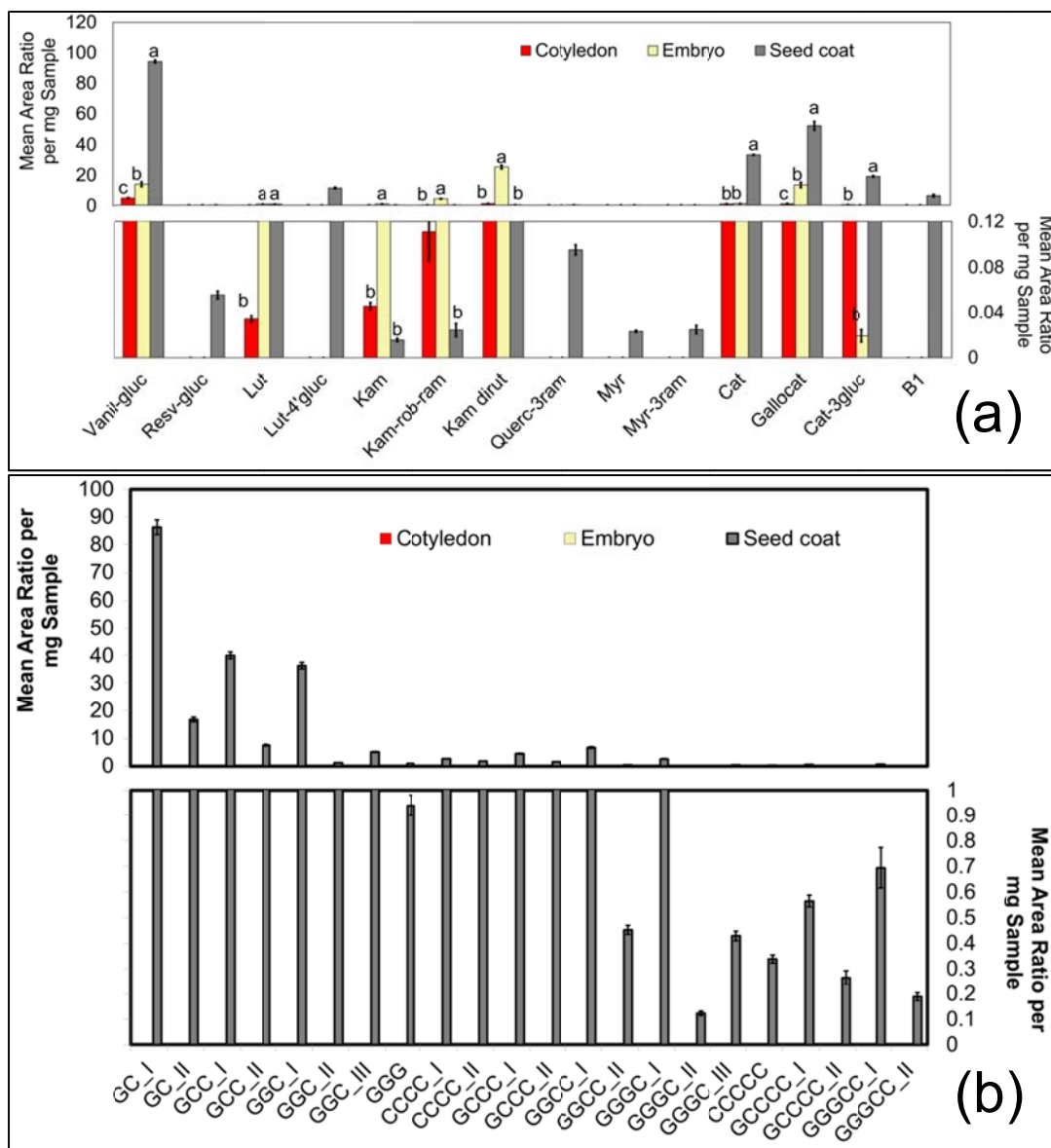


Figure 4.2. Mean area ratio per mg sample obtained for different sub-classes of phenolic compounds in a) SRM mode and b) SIM mode in cotyledon, embryo, and seed coat fractions of CDC Maxim lentil with genotype *Ggc Tgc Tan*; Vanil-gluc, resv-gluc, lut, lut-4'gluc, kam, kam-rob-ram, kam-dirut, querc-3ram, myr, myr-3ram, cat, gallocat, B1, C and G indicate vanillic acid-4- $\beta$ -D-glucoside, resveratrol-3- $\beta$ -mono-D-glucoside, luteolin, luteolin-4'-O-glucoside, kaempferol, kaempferol-3-O-robinoside-7-O-rhamnoside, kaempferol dirutinoside, quercetin-3-O-rhamnoside, myricetin, myricetin-3-O-rhamnoside, catechin, gallo catechin, procyanidin B1, catechin/epicatechin and gallo catechin/epigallo catechin. Means with different letters for each phenolic compound were significantly different ( $P \leq 0.05$ ).

Based on the results of the two preliminary tests, a deeper investigation of the phenolic profile for seed coats that were normal brown opaque (*Ggc Tgc Tan*), gray translucent zero-tannin (*Ggc tan*), and transparent zero-tannin (*ggc tan*) was conducted. Among the phenolic acids, *trans-p*-coumaric acid, protocatechuic acid, and vanillic acid-4- $\beta$ -D-glucoside were detected in all three phenotypes (Figure 4.3.a). Resveratrol-3- $\beta$ -mono-D-glucoside and flavones, including apigenin-7-O-glucoside and luteolin aglycone and glycones, were found in all three seed coat types. Among the dihydroflavonols, dihydrokaempferol was found in all three seed coat phenotypes, while dihydroquercetin was predominantly found in brown opaque and to a lesser amount in the gray translucent seed coats. Dihydromyricetin was detected only in the brown opaque phenotype. Kaempferol glycones occurred in all three seed coat phenotypes. Quercetin-3-O-rutinoside was detected at a low level in gray phenotypes, whereas quercetin-3-O-rhamnoside was at a very low level in transparent zero-tannin. Myricetin-3-O-rhamnoside, however, was found in the brown opaque phenotype, but absent in other phenotypes. Similarly, flavan-3-ols including catechin, epicatechin, gallocatechin, epigallocatechin, and catechin-3-glucoside were detected in brown opaque seed coats. Similar results were observed for proanthocyanidin dimers, trimers, tetramers, and pentamers (Figures 4.3.a, b).

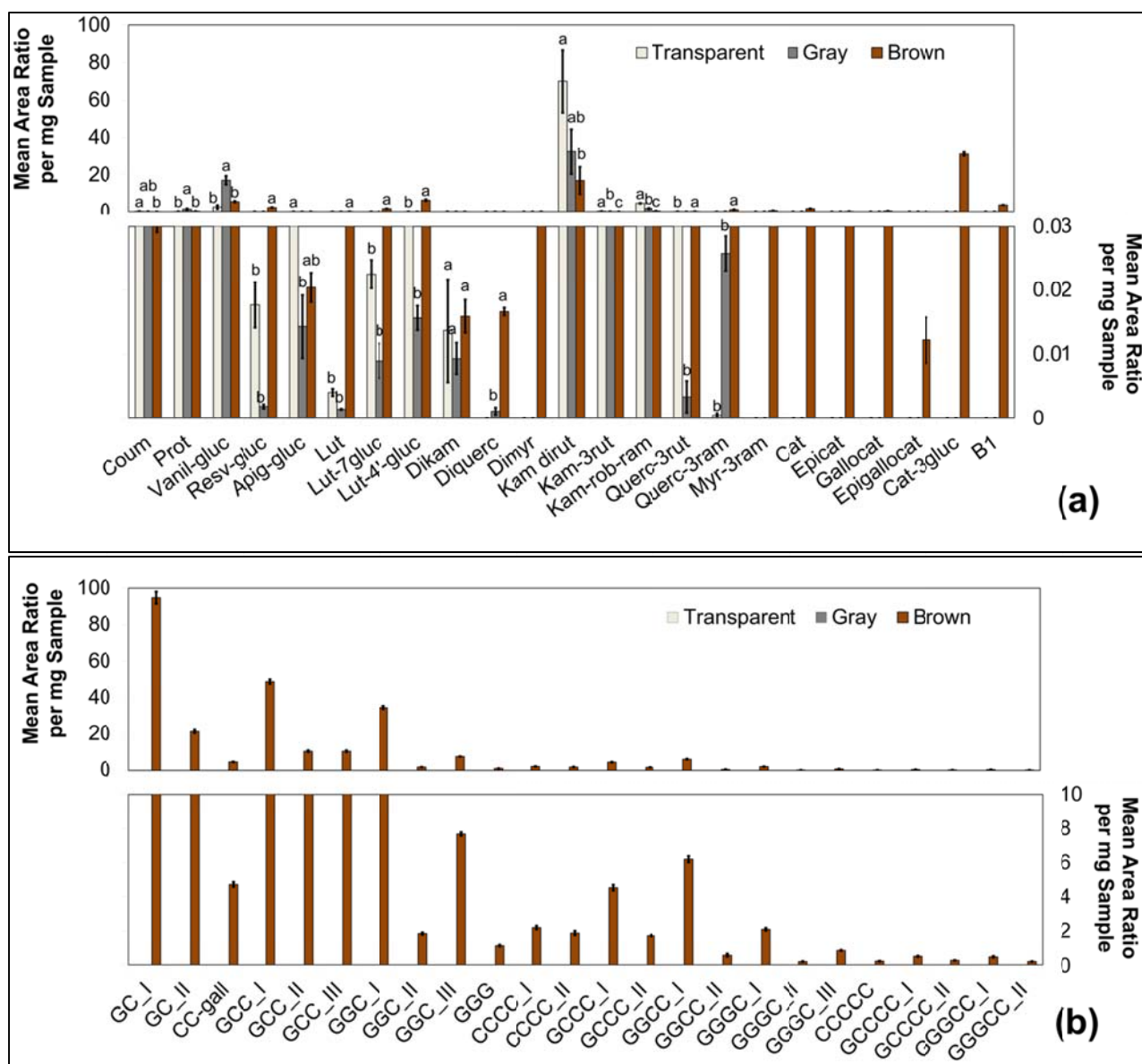


Figure 4.3. Mean area ratio per mg sample obtained for different sub-classes of phenolic compounds in a) SRM mode and b) SIM mode for transparent, gray translucent, and brown opaque lentil seed coats; Coum, prot, vanil-gluc, resv-gluc, apig-gluc, lut, lut-7gluc, lut-4'-gluc, dikam, diquerc, dimyr, kam-3rut, kam-rob-ram, querc-3rut, querc-3ram, cat, epicat, gallocat, epigallocat, cat-3gluc, B1, C, G, and CC-gall stand for trans-*p*-coumaric acid, protocatechuic acid, vanillic acid-4- $\beta$ -*D*-glucoside, resveratrol-3- $\beta$ -mono-*D*-glucoside, apigenin-7-*O*-glucoside, luteolin, luteolin-7-*O*-glucoside, luteolin-4'-*O*-glucoside, dihydrokaempferol, dihydroquercetin, dihydromyricetin, Kaempferol-3-*O*-rutinoside, kaempferol-3-*O*-rabinoside-7-*O*-rhamnoside, quercetin-3-*O*-rutinoside, quercetin-3-*O*-rhamnoside, catechin, epicatechin, gallo catechin, epigallo catechin, catechin-3-glucoside, procyanidin B1, catechin/epicatechin, gallo catechin/epigallo catechin, and CC-gallate, respectively. Means with different letters for each phenolic compound were significantly different ( $P \leq 0.05$ ).

#### 4.4.2. Molecular Analysis

The SNP marker LcC01900p336 was identified in a contig that was homologous to the 3' end of the *A* gene of pea. It was polymorphic between the parents of LR-30 and it co-segregated with the seed coat phenotype in the segregating RILs. However, when tested on a panel of 96 lines, the genotyping results were not correlated with the phenotypes (data not shown), suggesting it is not the causative mutation and is simply genetically linked in the LR-30 population. Sequencing the exonic regions of this gene in multiple *tan* and *Tan* lines revealed a common SNP in all three *tan* lines that was not apparent in the *Tan* lines (Figure 4.4). The gene consisted of seven exons and this SNP, at position 343 in exon 6, introduces a premature STOP codon, which would result in a truncated protein and a non-functioning enzyme. Note that the mutation in pea that causes the white flower character is caused by a SNP in the splice site at the end of Exon 6, and approximately 165 bp after this deletion. The KASP assay LcZT-Exon6p343, designed to test for this SNP, consistently identified all *tan* lines and demonstrated that none of the *Tan* lines have the variant allele in the mapping population LR-30 and in the diversity panel.

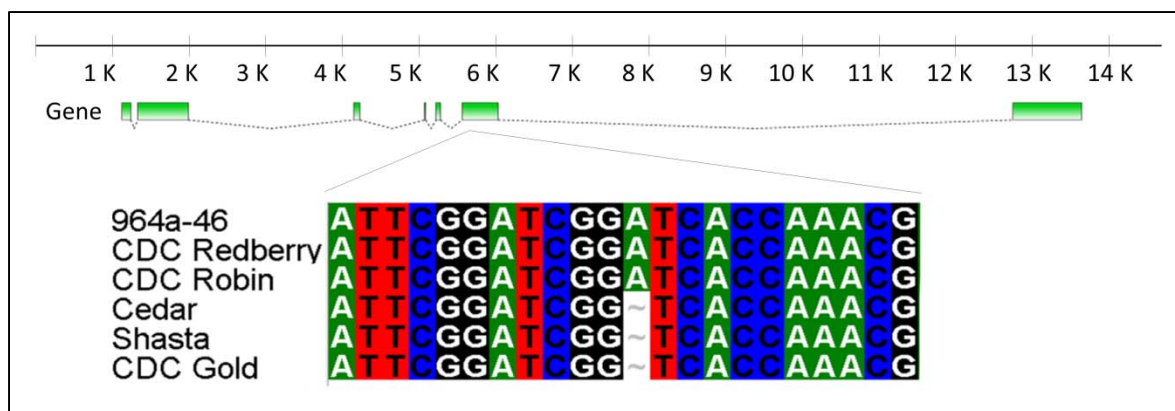


Figure 4.4. Structure of *LcubHLH* highlighting the region of the variant related to *tan*. The *tan* lines (Cedar, Shasta and CDC Gold) all have a deletion relative to the *Tan* lines (964a-46, CDC Redberry, and CDC Robin). The gene structure and the sequence alignment were obtained by FancyGene (Rambaldi and Ciccarelli, 2009) tool and BioEdit (Hall, 1999) alignment software, respectively.

## 4.5. Discussion

Phenolic compounds are produced through the actions of numerous regulatory genes and TFs in the phenylpropanoid pathway. These compounds fulfill different roles including seed pigmentation for plants, and health benefits for humans who eat the seeds. A combination of biochemical and genetic approaches was used to investigate whether a restriction in the phenylpropanoid pathway is responsible for the lack of seed coat pigmentation in zero-tannin (*tan*) lentil phenotypes. To accomplish this, seed coats from brown opaque (*Ggc Tgc Tan*), gray translucent zero-tannin (*Ggc tan*) were compared transparent zero-tannin (*ggc tan*) phenotypes.

The most obvious differences between the *Tan* and *tan* genotypes were the presence of dihydromyricetin, myricetin-3-*O*-rhamnoside, flavan-3-ols, and proanthocyanidin oligomers in the brown lines, and the absence of these in the zero-tannin phenotypes (Figures 4.3.a, b). Phenolic compound profile results were superimposed on structural steps of the pathway (Grignon-Dubois and Rezzonico, 2012; Kaushik et al., 2015; Welcha et al., 2008; Winkel-Shirley, 2001) and is presented as a putative biochemical

pathway in Figure 4.5. Dihydromyricetin can be produced by F3'5'H from dihydroquercetin and/or dihydrokaempferol (Figure 4.5). Thereafter, myricetin, galocatechin/epigallocatechin (from flavan-3-ols) and several proanthocyanidins are produced from dihydromyricetin at the next steps. None of these phenolic compounds were detected in the zero-tannin phenotypes. This shows that the phenylpropanoid pathway in these phenotypes is being blocked at the point where *F3'5'H* acts. Catechin/epicatechin requires dihydroquercetin as a precursor; therefore, the phenylpropanoid pathway should also be blocked at the location of *DFR* activity. In *Brassica carinata* seeds, dihydrokaempferol, dihydroquercetin and trace amounts of dihydromyricetin accumulate in yellow-seeded (i.e. transparent seed coat) phenotypes, while proanthocyanidins occurred in brown-seeded phenotypes (Marles et al., 2003). The level of mRNA for *flavanone-3-hydroxylase (F3H)* and *flavonoid-3'-hydroxylase (F3'H)* was similar between dark and transparent seed coats of *B. rapa*. However, the amounts of mRNA for *DFR*, *anthocyanidin synthase (ANS)*, and *anthocyanidin reductase (ANR)* were similar in transparent seed coat phenotypes (Li et al., 2012). *Arabidopsis thaliana tt3* mutant seeds have transparent seed coats, and visible anthocyanidins or proanthocyanidins are absent in the *tt3* mutant, because it lacks *DFR* mRNA (Shirley et al., 1995). Strong down-regulation of *ANR* and *ANS* lead to reduced amounts of proanthocyanidins and anthocyanidins and a transparent seed coat in *Medicago truncatula* (Li et al., 2016).

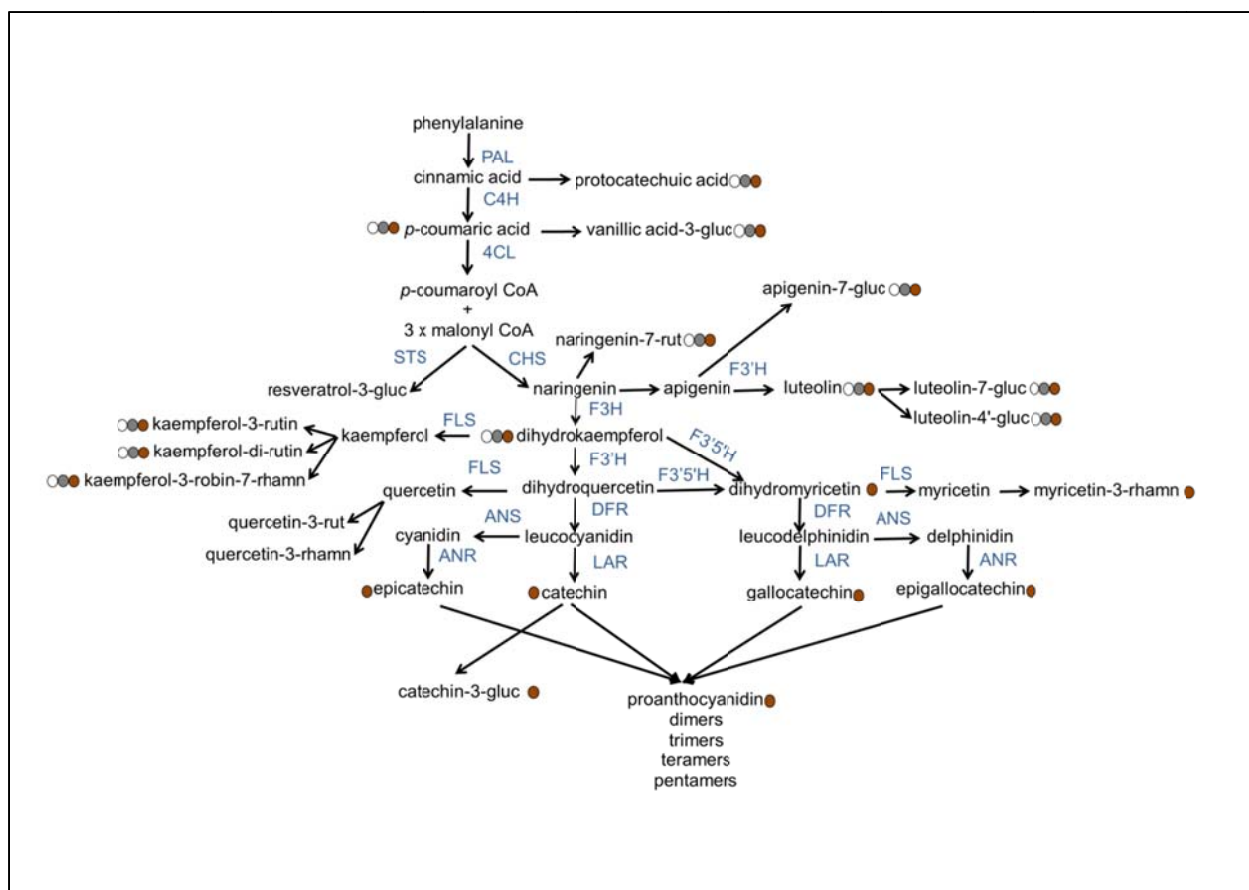


Figure 4.5. Putative biochemical pathway of phenolic compounds in lentil seed coats; The figure also depicts whether or not the final product of different branches of phenylpropanoid were observed in *Tan* and/or *tan* genotypes. Abbreviations: PAL, phenylalanine ammonia lyase; C4H, cinnamic acid 4-hydroxylase; 4CL, 4-coumaric acid:CoA ligase; STS, stilbene synthase; CHS, chalcone synthase; F3H, flavanone-3-hydroxylase; F3'H, flavonoid-3'-hydroxylase; F3'5'H, flavonoid-3',5'-hydroxylase; FLS, flavonol synthase; DFR, dihydroflavonol reductase; ANS, anthocyanidin synthase; LAR, leucoanthocyanidin reductase; ANR, anthocyanidin reductase; gluc, glucoside; rut, rutinoside; robin, robinoside; rhamn, rhamnoside. Circles filled with different colours illustrate the seed coat colours of transparent, gray-translucent and brown. The information of the pathway originates from (Grignon-Dubois and Rezzonico, 2012; Kaushik et al., 2015; Welcha et al., 2008; Winkel-Shirley, 2001).

The molecular analyses confirmed that *tan* most likely encodes for a bHLH, orthologous to the *A*-gene in pea. The LcZT-Exon6p343 allele found in *tan* lentil genotypes, introduces a premature STOP codon that prevents the expression of a full copy of bHLH. As *tan* is epistatic to *Tgc*, the *tan* ground colour is not observed in *Ggc Tgc tan* or



*ggc Tgc tan* genotypes. The gene *MtTT8* in *M. truncatula* (which is a homologous bHLH) controls proanthocyanidin- and anthocyanidin-related genes such as *ANR* and *ANS* (Li et al., 2016). A large insertion mutation in *BrTT8* results in transparent phenotype in *B. rapa*. This bHLH controls the expression of *ANS* and *ANR* (Li et al., 2012). The allele *tt3* (*DFR*) in *A. thaliana* seeds with transparent seed coat is controlled by a group of TFs including bHLHs such as TT8 (Appelhagen et al., 2011). TT2 (R2R3-MYB protein) and TTG1 (WDR protein) control *DFR*, and thus also interact to control the phenylpropanoid pathway genes. A ternary MBW complex has been proposed for controlling the downstream sections of the phenylpropanoid pathway (Appelhagen et al., 2011). Therefore *tan*, as the bHLH component of this MBW complex, interacts with the regulatory genes in the phenylpropanoid pathway for *F3'5'H* and *DFR*, as well as downstream processes.

All the analyzed phenolic acids were found in all three lentil seed coat types (Figure 3a). Among the flavonols, kaempferols were found in all three seed coat phenotypes tested. The aglycone and glycones of kaempferol were detected in the embryo, cotyledon, and seed coat fractions (Figure 4.2.a). However, the remaining flavonols, including quercetins and myricetins, were detected in the seed coat, but not other fractions (Figure 4.2.a). The analysis did not detect dihydroquercetin in the transparent phenotype, however quercetin glycones were found in all three lentil seed coat colours. This suggests that dihydroquercetin should be present in the transparent seed coat. Since the signal intensity of the dihydroquercetin peak in brown seed coat extracts was observable but weak, it appears this compound is in low abundance.

The phenylpropanoid pathway affects characteristics of plants besides pigmentation, including protection of the plant against stresses. Although seed coat phenolics can provide a good barrier against pathogens, newly germinated seeds also need to be protected by chemical defense materials such as phenolic compounds (Gallery et al., 2010). Dueñas et al. (2002) reported that catechin and various phenolic acids were present in lentil cotyledons (Dueñas et al., 2002) but they did not report flavonoids in cotyledons of lentils. My study detected a diversity of phenolic compounds, including phenolic acids, flavones, flavonols, and different flavan-3-ols in the cotyledon and embryo fractions. All or some of these compounds could play a role in the protection of the embryo and cotyledons.

Zero-tannin lentils do not change seed coat colour during storage (Matus et al., 1993), which could be due to the lack of flavan-3-ols and proanthocyanidins. However, phenolic compounds improve seed establishment (Randhir and Shetty, 2003), and as a result seeds harbouring lower levels could be more prone to damage caused by rapid water imbibition during germination (Matus et al., 1993; Smýkal et al., 2014). Zero-tannin lentils are more susceptible to soil- and seed-borne diseases, a problem that is circumvented by application of seed-applied fungicides (Matus and Slinkard, 1993).

Flavan-3-ols such as catechin and gallic acid show anti-inflammatory and anti-oxidative activity and have been associated with the reduction of some cardiovascular diseases (Gonza' Lez et al., 2011; Toh et al., 2013) and proanthocyanidins are the major antioxidants entering the colon (Manach et al., 2004). These compounds reduce cholesterol (Toh et al., 2013), inhibit the growth of breast cancer cells (Ramljak et al., 2005), and protect the prostate (Lei et al., 2014). Zero-tannin lentils cannot provide the

health benefits associated with flavan-3-ols and proanthocyanidins. However, some health advantages could be associated with zero-tannin phenotypes. Phenolic acids, flavones, and flavonols showed anti-oxidative (Calderón-Montaña et al., 2011; Delgado-Vargas et al., 2000; Spilioti et al., 2014 ), anti-cardiovascular disease (Calderón-Montaña et al., 2011), anti-cancer (Calderón-Montaña et al., 2011; Manach et al., 2004; Spilioti et al., 2014; Xiao et al., 2014), anti-diabetic (Calderón-Montaña et al., 2011; Xiao et al., 2014), and anti-HIV (Calderón-Montaña et al., 2011; Xiao et al., 2014) effects. They also increase the bioavailability of iron (Hart et al., 2015), and represent better candidates for iron biofortification programs in lentil (Tako et al., 2014).

## 4.6. Conclusion

Using lentil RILs, the biochemical seed coat phenotype determined by the gene *Tgc* in RILs was found to be altered by the epistasis resulting from the expression of *tan*. The LC-MS analysis detected several phenolic compounds that were not influenced by *tan*, notably the phenolic acids, flavones, some flavonols, and some of dihydroflavonols. Myricetin, dihydromyricetin, and all the analyzed flavan-3-ols, and proanthocyanidin oligomers were detected only in *Ggc Tgc Tan* lines and therefore can be controlled by *tan*. Molecular analysis showed that *tan* is a bHLH transcription factor, and is the same as the *A* gene in pea. In other plant species, this transcription factor interacts with the regulatory genes in the phenylpropanoid pathway, including those controlling the expression of *F3'5'H* and *DFR* and downstream steps. This new knowledge of the underlying basis of the genotypes and phenotypes of zero-tannin lentil seed coats will be useful in designing breeding strategies for the development of lentil cultivars with improved nutritional profiles.

## 4.6. Prologue to Chapter 5

The goal of the following research chapter was to compare phenolic compound profiles of fresh lentils with those stored for long periods. This chapter was published in Eur. Food Res. Technol. in June 2016.

Mirali, M., Purves, R.W., Vandenberg, A. (2016). Phenolic profiling of green lentil (*Lens culinaris* Medic.) seeds subjected to long-term storage. Eur. Food Res. Technol. DOI 10.1007/s00217-016-2713-1

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# **CHAPTER 5 - PHENOLIC PROFILING OF GREEN LENTIL SEEDS SUBJECTED TO LONG-TERM STORAGE**

## **5.1. Abstract**

Lentils have several desirable properties that make them a healthy and nutritious food option. The visual characteristics of the seed coat are important factors that determine the marketability and, ultimately, the sale price of whole lentils. However, the seed coat colour is not stable and green lentil in particular changes colour over time. While total phenolic content significantly affects darkening of lentil seeds, this study investigated the effect of specific phenolic compounds in the seed darkening process in detail. The phenolic compound profiles of six green lentil cultivars were analysed using liquid chromatography-mass spectrometry. To maximize the potential amount of change, the oldest seeds available were compared with fresh seeds. Some increases in amounts were noted for some phenolic acids and flavones. The most notable result was a decrease in the amount of all 27 flavan-3-ols and proanthocyanidin oligomers. Polymerization of these oligomers (the major phenolic compounds in green lentil seed coat tissue) results in their cross-linking with the cell wall. The consequence will be seed darkening and reduction in the extractability of these oligomers.

## **5.2. Introduction**

The range of background colours and patterns of lentil seed coats determines the market classes for whole lentil seeds. Approximately 25% of the lentil crop in Canada,

the world's major producer, is marketed as "green lentil", which typically has a pale green seed coat covering a yellow cotyledon (SPG, 2015).



Figure 5.1. Change in the seed coat colour of CDC Improve lentil from green (left-2014) to dark brown (right-2006) after eight years of storage.

The green seed coat colour in lentil is not stable and changes over time to yellow, yellow-brown, medium brown, and dark brown depending upon the storage conditions and duration (Nozzolillo and Bezada, 1984). Figure 5.1 shows how the seed coat colour of CDC Improve darkened after 8 years of storage. Seed coat colour is an important grading factor that affects the market value of lentils. According to the Canadian Grain Commission, green lentils with good natural colour are graded as No. 1 lentils (Canadian Grain Commission, 2014). An increase in seed coat colour variability, or darkening of the seed coat, will decrease the grade of the sample thereby reducing the offered price (Davey, 2007). For No. 3 grade green lentil (severe discolouration, i.e., dark brown (Canadian Grain Commission, 2014)), the average price is approximately half that of No. 1 grade large green lentil (e.g., see <http://www.statpub.com/stat/prices/spotbid.html>(2015)). A better understanding of the

biochemicals involved in seed coat darkening will inform breeding strategies aimed at overcoming this process, thereby preserving economic value.

Different pigments such as chlorophylls and phenolic compounds form the variety of pigments occurring in plants. While chlorophylls are characterized by a green colour, phenolic compounds such as flavan-3-ols, proanthocyanidins, flavones, and flavonols, are colourless, white, and pale yellow (Andersen and Jordheim, 2010). Anthocyanidins make intense colours from orange and red to blue (Andersen and Jordheim, 2010). Greener lentil seeds have higher amounts of chlorophyll a and b (Davey, 2007). Chlorophyll breaks down with age and is converted to colourless compounds in chloroplasts. These colourless compounds will change to differently structured compounds that are finally stored in vacuoles (Christ and Hörtensteiner, 2014; Hörtensteiner, 2006). Seed coat darkening is a phenomenon that can significantly affect the greenness of the seed coat and phenolic compounds play an important role. The effect of storage on the total amount of phenolics (as detected by spectrophotometric methods) or sub-classes of phenolic compounds (applying chromatographic methods) has been studied in various plant tissues (Appendix 6). Spectrophotometric analysis of total phenolics and total proanthocyanidins in lentil seed reveals a decrease is possible during storage (Nozzolillo and Bezada, 1984; Pirhayati et al., 2011). To date no studies have examined the impact of storage on the profiles of phenolic compounds in lentil seed coats.

The objective of the current study was to use an optimized LC-MS method to compare phenolic compound profiles of fresh green seeds with those that have experienced long-term storage (LTS).

## 5.3. Materials and Methods

### 5.3.1. Plant Material

A preliminary study analyzed seeds of the recombinant inbred line (RIL) LR-18-183 from the LR-18 (CDC Robin × 964a-46) (Fedoruk et al., 2013) population harvested in 2009 and 2014. Seeds were obtained from the Crop Development Centre, University of Saskatchewan, Saskatoon, Canada. Breeder seeds of six green lentil cultivars (Table 5.1) were also obtained from the same source. Fresh seeds of these cultivars were harvested in 2014 and, for comparison, older seeds (harvested in 2000-2007) were obtained from the long-term storage facility. The storage facility was a routine type that is commonly used for seed storage; as a result, the temperature and humidity were not controlled and subject to seasonal fluctuations. Both fresh and LTS seeds were analyzed four months after harvest of the 2014 seeds in three replicates for each cultivar as described below.

Table 5.1. Genotype names and storage duration of green lentils used in this experiment

Sample number	Variety	Year
1	CDC Imigreen	2007
2		2014
3	CDC Impower	2007
4		2014
5	CDC Greenland	2006
6		2014
7	CDC Improve	2006
8		2014
9	CDC Meteor	2005
10		2014
11	CDC Plato	2000
12		2014



### 5.3.2. HPLC-MS

Previously optimized chromatographic conditions (Mirali et al., 2014) were applied using an Agilent 1100 (Agilent, Germany) high-performance liquid chromatograph (HPLC) with G1315 PDA UV/VIS detector coupled to a Thermo Finnigan TSQ Quantum Ultra (Thermo Fisher Scientific Inc., UK) triple quadrupole MS equipped with a heated electrospray ionization (HESI) interface. Peak areas were obtained with Thermo Xcalibur 2.1 software. The chromatographic column, mobile phases, flow rate, and the gradient were the same as what described in §3.3.2.

Relative quantification of phenolic compounds was done using SRM in positive or negative mode and SIM in positive mode. The values are reported as the area ratio per mg weight as described in §3.3.5. Also, to ensure that the targeted method was not missing any significant changes in the phenolic compound profiles, full scan LC-MS spectra ( $m/z$  140-1500) with UV-VIS detection (250-600 nm) were also acquired in both positive and negative modes (Figure 5.2).

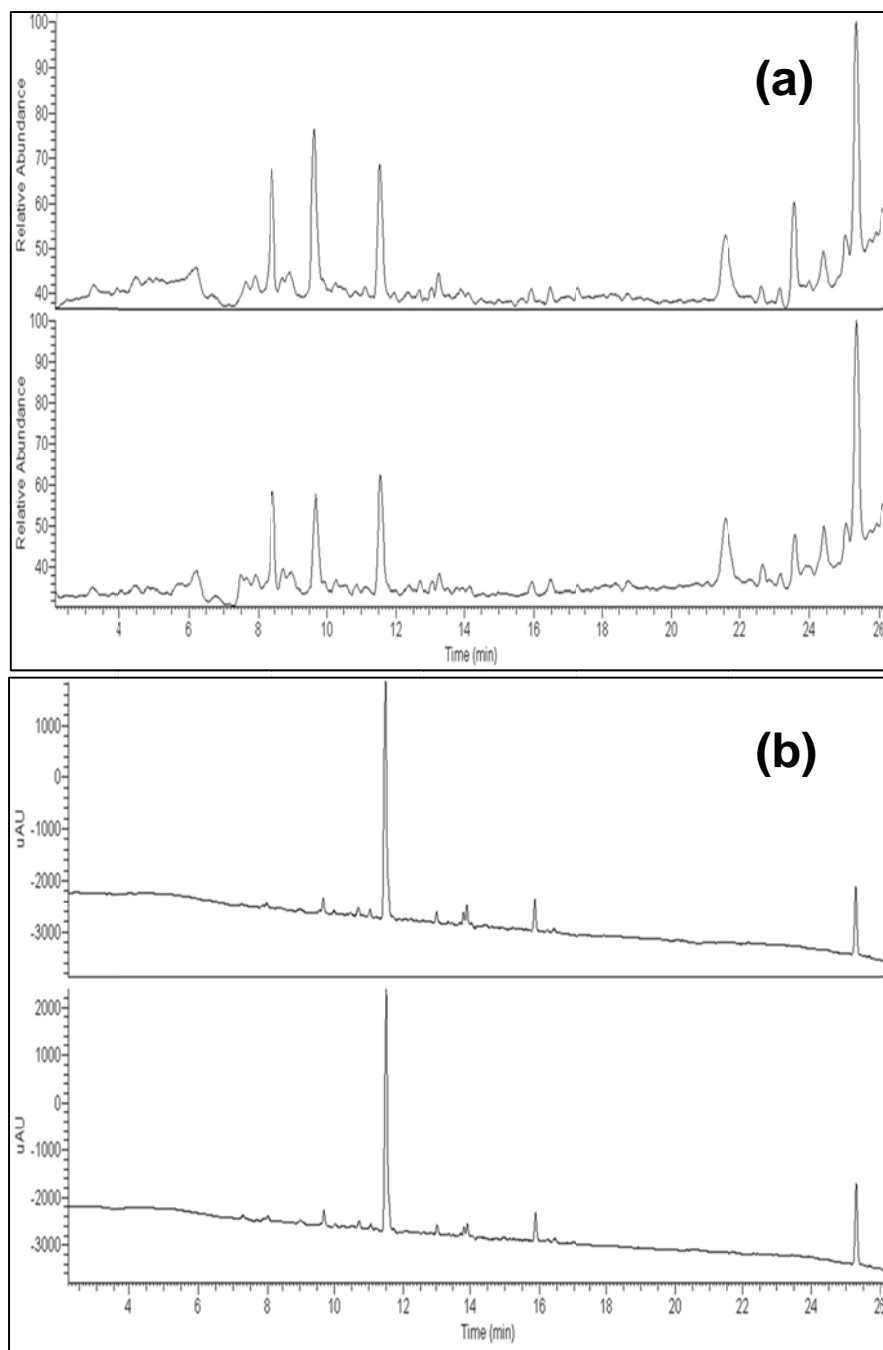


Figure 5.2. (a) Chromatograms obtained using mass spectrometry and total ion current detection, and (b) Chromatograms obtained using UV-VIS detection (250-600 nm) using seeds of CDC Improve lentil (fresh are top trace, LTS are lower trace in both (a) and (b)).

### **5.3.3. Sample Preparation**

Sample preparation was conducted similar to that described under §3.3.4 with minor modifications. Samples were pulverized with a Mini-Beadbeater-16 (BioSpec Products Inc., OK, USA) for 5.5 min. In addition, samples were shaken for 1 h on a Thermomixer (Eppendorf, Germany) at a speed of 1400 rpm at room temperature. Thereafter, an aliquot of the final supernatant was diluted 10 times with Milli-Q water and transferred to glass vials for analysis.

### **5.3.4. Statistical Analysis**

A comparison among means was done using R software (v. 3.2.4).(R Core Team, 2016). The experimental design was based on completely randomized design.

## **5.4. Results**

Samples of LR-18-183 lentils used in the preliminary test were harvested in 2009 and 2014 and compared using LC-MS/MS (Mirali et al., 2014) to investigate changes in the phenolic profile that occurred during storage (Appendix 7). The study was used to assess whether the changes in the levels of phenolic compounds were sufficient to warrant a more rigorous test of the rest of the population. Minimal differences between the samples were apparent over a period of 5 years. Note that during the course of the year, the daily average temperature varies between -14 C and +19 C in Saskatoon (Government-of-Canada, 2016). Thus, a plausible explanation for the minimal change is the influence of the very low temperatures (below 0 °C) experienced during winter storage in Saskatoon (6 months); that is, during the winter months little to no change is expected to occur. Because the available RILs that were less than 5 years old showed

only small changes, it was therefore decided to investigate further using older seed of several cultivars with green seed coats. This was done in order to maximize the amount of time for change to occur, which in turn, should help to simplify data interpretation. Consequently, the oldest breeder seed available was used and the storage durations varied from 7 to 14 years (Table 5.1).

Signal responses were found for all phenolic compounds analyzed using SRM and SIM methods and in both fresh and LTS seeds for all six genotypes including phenolic acids, stilbenes, flavones, flavonols, and flavan-3-ols. Moreover, dimers, trimers, tetramers, and pentamers of proanthocyanidins were observed in lentil samples.

Figures 5.3.a-f show mean area ratio of phenolic compounds per mg of fresh and old samples of CDC Imigreen, CDC Impower, CDC Greenland, CDC Improve, CDC Meteor, and CDC Plato genotypes. Vanillic acid-4- $\beta$ -D-glucoside (phenolic acids sub-class) and luteolins (flavones sub-class) were elevated in LTS seeds compared to fresh seeds of most of the genotypes. Mean area ratio of the flavonols sub-class per mg of different genotypes remained mostly unchanged between fresh and LTS samples; though kaempferol-3-O-rubinoside-7-O-rhamnoside and kaempferol dirutinoside showed lower values in some of the long-term stored genotypes (e.g., CDC Impower, CDC Greenland, and CDC Plato), while quercetin-3-O-rhamnoside showed higher values (e.g., CDC Meteor and CDC Plato). A significant reduction after storage in mean area ratio of flavan-3-ols (including catechin, gallic catechin, and catechin-3-glucoside) per mg of all six genotypes observed. A similar reduction was noticed in the 24 detected proanthocyanidins (including dimers, trimers, tetramers, and pentamers of catechin/epicatechin and gallic catechin/epigallocatechin) for all the genotypes.

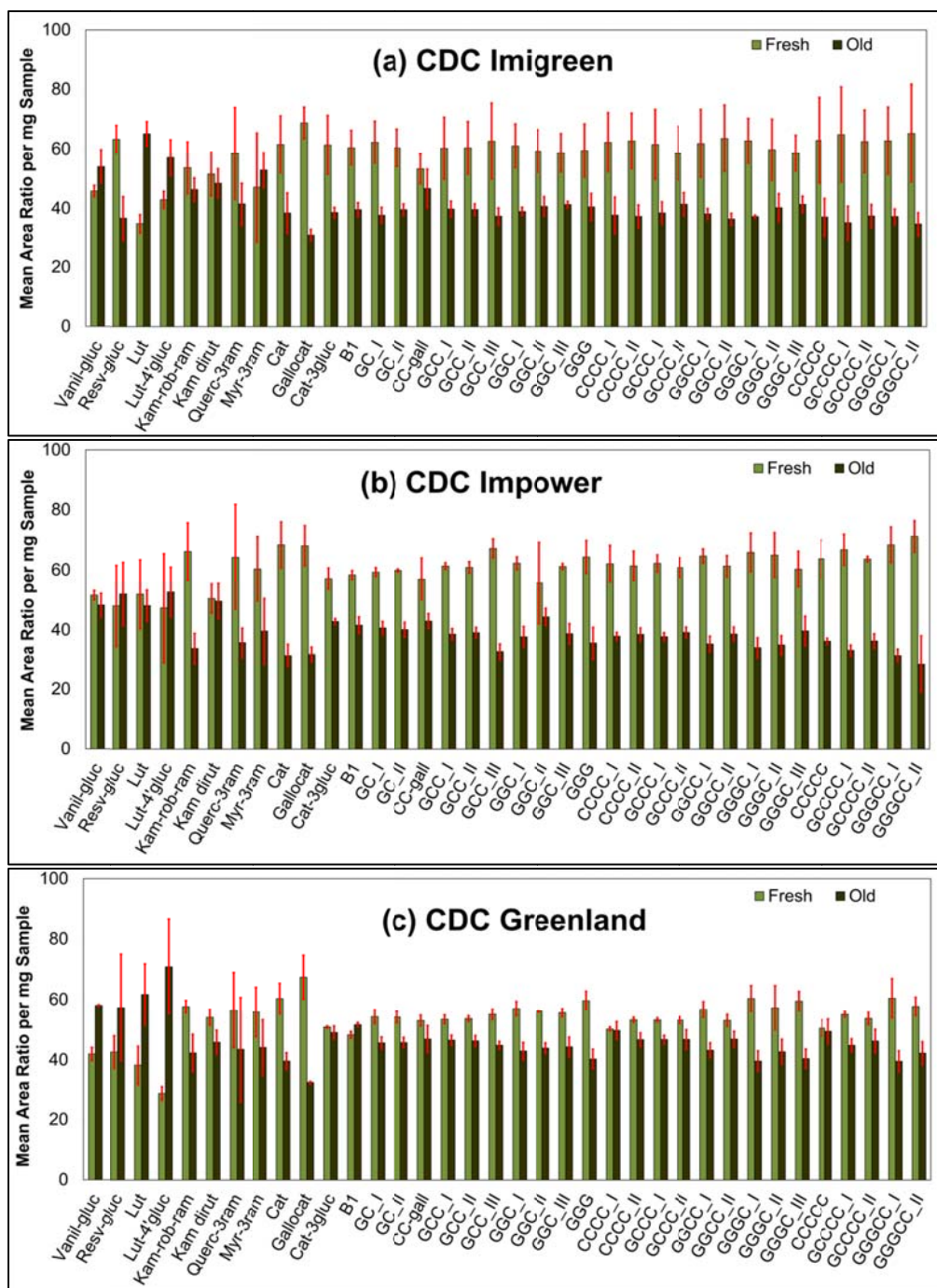


Figure 5.3. Mean area ratio per mg of fresh and old samples in (a) CDC Imigreen, (b) CDC Impower (c) CDC Greenland, (d) CDC Improve, (e) CDC Meteor, and (f) CDC Plato varieties. Error bars are standard errors of three replicates. vanil-gluc, resv-gluc, lut, lut-4'gluc, kam-rob-ram, querc-3ram, myr-3ram, cat, gallocat, epigallocat, cat-3gluc, B1, C, G, and CC-gall stand for vanillic acid-4- $\beta$ -D-glucoside, resveratrol-3- $\beta$ -mono-D-glucoside, luteolin, luteolin-4'-O-glucoside, kaempferol-3-O-robinoside-7-O-rhamnoside, quercetin-3-O-rhamnoside, catechin, gallo catechin, catechin-3-glucoside, procyanidin B1, catechin/epicatechin, gallo catechin/epigallo catechin, and CC-gallate, respectively.

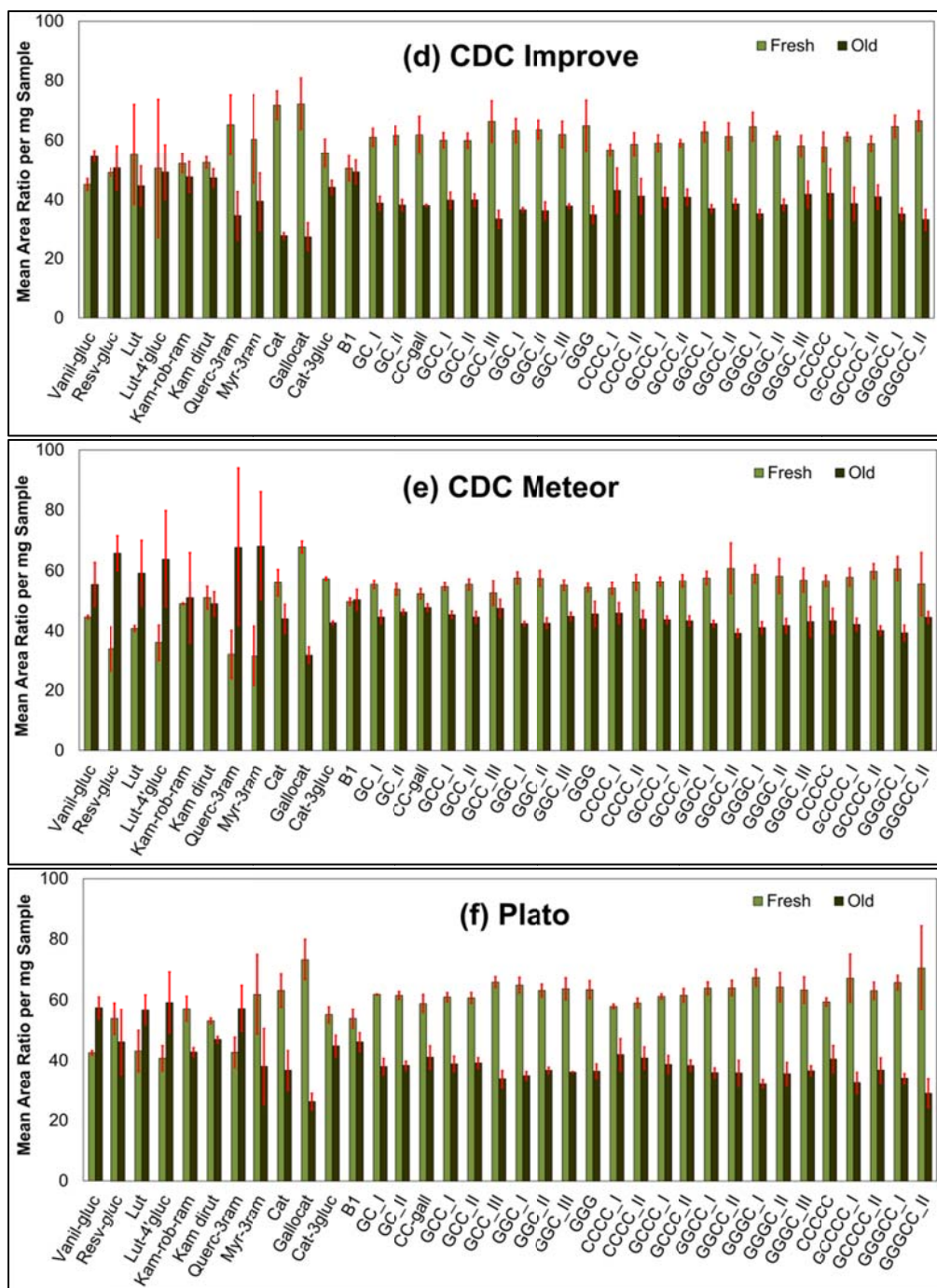


Figure 5.3. Continued

Full scan analyses of the fresh and LTS lentil seeds did not show the addition or omission of any major peaks in the chromatograms obtained using total ion current plots or UV-VIS detection (e.g., see traces for fresh and LTS samples of CDC Improve, Figures 5.2.a and b). As a result, the possibility of significant changes occurring in the

phenolic compounds profile from species that were not targeted by the SRM and SIM methods was ruled out.

## 5.5. Discussion

Marketability of green lentil depends largely on a stable green colour of the seed coats, and storage can dramatically affect the greenness and marketability of this crop. Phenolic compounds are involved with seed darkening (Aaby et al., 2007; Beninger et al., 2005; Cakmak et al., 2010; Carbone et al., 2011; Howard et al., 2010; Mareuardt et al., 1978; Martín-Cabrejas et al., 1997; Nasar-Abbas et al., 2009; Nozzolillo and Bezada, 1984; Srisuma et al., 1989; Zhou et al., 2010). Using LC-MS, the phenolic profiles of fresh seeds and the oldest available breeder seed samples were compared (to maximize the aging time). The type of phenolic compounds observed in the samples is consistent with previous analyses of lentil seeds (Aguilera et al., 2010; Bartolomé et al., 1997; Dueñas et al., 2002; Dueñas et al., 2003; López-Amorós et al., 2006; Zou et al., 2011).

Storage increased the amount of vanillic acid-4- $\beta$ -D-glucoside, luteolin, and luteolin-4'-O-glucoside. Srisuma et al. (2007) and Aaby et al. (1989) reported the rise of phenolic acids, such as ferulic, sinapic, and ellagic acids, in other plant materials. These increases could be related to the degradation of more complex phenolic compounds such as those with several sugar conjugates (Rothwell et al., 2015).

Although some increases (e.g., quercetin-3-O-rhamnoside) and decreases (e.g., kaempferol-3-O-robinoside-7-O-rhamnoside and kaempferol dirutinoside) observed in flavonols when comparing fresh and LTS samples, these compounds, on average, remained essentially unchanged during LTS conditions. This is similar to reports for

pinto bean, for which the levels of analyzed flavonols in non-aged and aged seeds was not significantly different (Beninger et al., 2005). The pattern for the changes in mean area ratio per mg sample indicated that flavonols with a largest number of sugars decrease slightly whereas those with only one sugar increase slightly. These data support the hypothesis that more complex phenolic compounds (e.g., those with a larger number of sugars) may be breaking down to produce more compounds with a smaller number of sugars.

The LC-MS analysis of the lentil samples showed a significantly declining trend for all 27 flavan-3-ols and oligomers of proanthocyanidins after LTS. Some of the variations in the degree of change among the different genotypes may have to do other variables (e.g., environmental factors), but since all six genotypes showed the same decreasing trend after storage, it is extremely likely that these changes are attributed to storage effects. In other plant materials, flavan-3-ols and oligomers of proanthocyanidins analyzed separately by chromatography (Carbone et al., 2011; Howard et al., 2010; Zhou et al., 2010) and/or combined with MS methods (Aaby et al., 2007) as well as total proanthocyanidins analyzed by spectrophotometric methods (Beninger et al., 2005; Mareuardt et al., 1978; Nasar-Abbas et al., 2009; Nozzolillo and Bezada, 1984) also declined after storage.

Green lentil seed coats darken over time during storage to dark brown, and this results in leakage of different soluble materials into the imbibition medium (Nozzolillo and Bezada, 1984). Nevertheless, phenolic compounds were not found in large quantities among the leaked materials. The authors proposed polymerization of low molecular weight flavan-3-ols and oligomers of proanthocyanidins as the possible reason



(Nozzolillo and Bezada, 1984). Different models have been introduced for the biosynthesis of proanthocyanidins, including conversion of flavan-3-ols to quinone methides or their protonated carbocation, which results in polymerization into colourless proanthocyanidins (He et al., 2008). Proanthocyanidins can be produced either through the endoplasmic reticulum or via plastids such as chloroplasts (Zhao, 2015). Re-differentiation of chloroplasts, occurs under stress or ageing processes (Kaewubon et al., 2015), and causes swelling of chloroplasts and formation of tannosomes, which are structures that contain thylakoids and tannins (Brillouet, 2015). This could explain why the green colour is replaced by a darker colour. Proanthocyanidins produced in the endoplasmic reticulum and tannosomes from chloroplasts are mostly stored in vacuoles with a minor percentage in cell walls (Toivonen and Brummell, 2008). Proanthocyanidins have oxidizable OH groups (OH groups that are adjacent to each other), which make them good substrates for oxidative enzymes such as polyphenol oxidase (PPO) and peroxidase (POD) (Madinez and Whitaker, 1995). Note that PPO is located in plastids, whereas POD occurs in plastids, mitochondria, and cytosol (Toivonen and Brummell, 2008). Stress or ageing could increase reactive oxygen species (ROS) in the stored plant materials. ROS could react with cell membrane lipids and cause breakdown of membranes and decompartmentalization of organelles (Lattanzio, 2003). This will intermix oxidative enzymes with their potential substrates, i.e., proanthocyanidins (Toivonen and Brummell, 2008), a process that will produce short-lived highly reactive intermediates, such as semiquinones and quinones (Pourcel et al., 2006; Toivonen and Brummell, 2008). As a result, some non-enzymatic reactions with other phenolic compounds in particular, but also with proteins and polysaccharides

in the cell wall, could be expected to occur (Pourcel et al., 2006). Cross-linking of proanthocyanidins with other phenolic compounds and especially with the cell wall has been proposed as the source of the brownish compounds that give the seed coat colour (Pourcel et al., 2005; Zhao, 2015). Some quinone products have been observed *in vitro* (Guyot et al., 1996; Tanaka et al., 2002), but an LC-UV-MS analysis did not provide any evidence for this in the lentil samples. A comparison of chromatograms obtained using total ion current and UV-VIS detection of fresh and LTS samples of green lentil seeds did not show significant differences. The possibility remains that lower abundance species were also affected because not all peaks were large enough to be separated from the baseline signal in these chromatograms. More likely, numerous possible new quinone species could form from ROS, but these species are highly reactive and could react with several different phenolic compounds thereby producing many possible products. Thus the overall signal would be diluted into many small signals that would become indistinguishable from the baseline. This is in contrast to *in vitro* experiments in which only a small number of compounds were present and therefore specific favored pathways could produce identifiable peaks.

Proanthocyanidins make up the largest proportion of phenolic compounds in lentil seed coat tissue (Dueñas et al., 2002). The bursting of vacuoles caused by cell death might transport proanthocyanidins from the vacuole to the cell wall (Hörtensteiner, 2006). Pang et al. (2007) suggest that the proanthocyanidins are initially soluble when they are in vacuoles and then become insolubilized after attaching to the cell wall. Proanthocyanidins are good H-donors and can easily make hydrogen-bonds (Cheynier et al., 2013). Having several reactive sites, oligomers and polymers of

proanthocyanidins can be encapsulated within the gel structure of cell wall polysaccharides. Proanthocyanidins and cell wall polysaccharides bind through H-bonding and hydrophobic interactions (Renard et al., 2001). All of these will result in stronger binding of proanthocyanidins with cell wall materials and make them extremely hard to extract (Hanlin et al., 2010), which is the reason for the significant reduction in their storage ratio in the lentil samples.

## **5.6. Conclusion**

Overall, this work addresses the fact that LTS darkens the green colour in lentil seeds; this reduces marketability and affects the economic value of green lentil, which is based on the visual characteristics of the seed coat. Increases in phenolic acids and flavones occur in green lentil seeds after storage, possibly because of the breakdown of more complex phenolic species into smaller subunits. A significant decrease in 27 flavan-3-ols and proanthocyanidins also occurs. During storage, enzymatic and non-enzymatic reactions will polymerize proanthocyanidins and result in cross-linking of these major phenolic compounds with cell wall materials. This will produce dark pigments and reduce their extractability. The findings of this study help to narrow down the genes of interest responsible for lentil seed darkening. Enzymatic analysis could be used as a more explicit indicator of how phenolic compounds in lentil seed coats change over time.

# CHAPTER 6 - GENERAL DISCUSSION

## 6.1. Discussion

Lentil has a diverse range of seed coat colours including clear, green, tan, gray, brown, and black. Seed coat colour is an important grading factor that affects the market value of lentils. In lentil, two independent loci (*Ggc* and *Tgc*) determine the four basic seed coat background colours; brown (*Ggc Tgc*), gray (*Ggc tgc*), tan (*ggc Tgc*) and green (*ggc tgc*). The zero-tannin locus (*tan*) is epistatic to the *tgc* locus, resulting in clear seed coats. Lentil is a good source of macronutrients and secondary metabolites including phenolic compounds. Phenolic compounds are responsible for different colours in plants from yellow and red to blue. Phenolic compounds have been associated with human health benefits such as anti-oxidant, anti-tumour, and anti-heart disease properties. The purpose of this research was to analyze the phenolic compound profiles of the seed coats of a series of lentil genotypes with defined seed coat background colour based on genetic analysis. It included an investigation of differences in the phenolic profile between tannin containing and zero-tannin phenotypes of lentil. It also determined what changes occur in the phenolic profile of lentil seed coats during storage.

The first objective of this research was to determine if specific phenolic profiles of different lentil seed coat colours are characteristic of the four specific genetic combinations of alleles for the *Ggc* and *Tgc* loci. The results showed that the levels of various phenolic compounds in lentil seeds varied among the green, gray, tan, and brown seed coat colours. Specifically, seed coats of lentil genotypes with the homozygous recessive *tgc* allele (green and gray seed coats) had higher amounts of flavan-3-ols, proanthocyanidins, and some flavonols. This indicated that the production

of some phenolic compounds in lentil, specifically the flavan-3-ols and proanthocyanidins, is controlled by the *Tgc* seed coat colour gene.

The phenolic profiles of lentil seed coats that do not express the *Tgc* gene (genotype *Tgc tan*) were compared with those that express *Tgc* (genotype *Tgc Tan*). The LC-MS analysis detected several compounds that were not influenced by *tan*, notably the phenolic acids, flavones, some flavonols, and some dihydroflavonols. Myricetin, dihydromyricetin, and all the analyzed flavan-3-ols, and proanthocyanidin oligomers were detected only in *Ggc Tgc Tan* lines and therefore can be controlled by *tan*.

Molecular analysis revealed that *tan* is a bHLH transcription factor, and is the same as the *A* gene in pea. The LcZT-Exon6p343 allele found in *tan* lentil genotypes, introduces a premature STOP codon that prevents the expression of a full copy of bHLH. As *tan* is epistatic to *Tgc*, the tan ground colour is not observed in *Ggc Tgc tan* or *ggc Tgc tan* genotypes. This transcription factor interacts with the regulatory genes in the phenylpropanoid pathway starting at the point *F3'5'H* and *DFR* act.

The last objective of this research was to determine the effect of LTS on the specific changes in phenolic compounds in lentil seeds. Increases in phenolic acids and flavones occur in green lentil seeds after storage. In addition, a significant decrease in 27 flavan-3-ols and proanthocyanidins also occurs, possibly because of the polymerization of these sub-classes during storage.

Two major market classes determine lentil value. The green market class has green seed coat colour and is consumed as whole seed, while the red market class has gray, tan or brown seed coat and is consumed as dehulled football or split. There are some niche market classes such as French green, Spanish brown, Beluga, and zero-tannin.

Results of this research add more value for consuming whole green lentil seeds specifically with green and gray seed coat colours. Flavonols and procyanidin trimers showed the most antioxidant capacity in lentil seed coat (Dueñas et al., 2006), whereas in whole lentil seeds, the tannin fraction (i.e., proanthocyanidins) showed higher antiradical activity and higher reducing power (Amarowicz et al., 2009; Amarowicz et al., 2010).

Typically, seed coats of dehulled lentils are fed to livestock. Since the seed coats are a good source of phenolic compounds, more profit could be obtained from lentil seed coats. Considering each ton of lentil produces 80-110 Kg seed coats (Dueñas et al., 2002), and 9-22 % seed coat-methanol-water extract can be yielded out of it (i.e., 7-24 Kg extract) (Ronzio et al., 1998); Out of this amount of extract, 700-2400 g phenolic compounds may be obtained (Ronzio et al., 1998). Therefore, lentil seed coat extract can be a potential plant-based antioxidant in producing supplements. Also, protecting ground meat against oxidation by using lentil seed coat flour (Der, 2010) is an innovative idea. More uses for this source of natural antioxidant could be developed for lentil seed coats in biofortifying foods and nutraceutical purposes.

Zero-tannin lentils and specifically their transparent phenotype may increase the bioavailability of iron. While normal lentil seed coats decreased the iron uptake in Caco-2 cells experiment, gray zero-tannin extracts with up to 50 µg/mL extract concentration increased the iron uptake (Hart et al., 2016. Unpublished data). Interestingly, the iron uptake for zero-tannin transparent lentil showed an increase of eight times higher concentration (400 µg/mL) (Hart et al., 2016. Unpublished data); this could be due to the lack of phenolic compounds such as myricetin in zero-tannin lentils (Hart et al., 2015).

Increasing the iron uptake will make zero-tannin lentils more suitable candidates for iron biofortification (Tako et al., 2014), as they should have less inhibitors of iron uptake. The seeds of zero-tannin lentils are rounder or plumper compared to normal lentil genotypes. This might be due to alteration of the seed coat structure caused by lack of proanthocyanidins. Seed plumpness may also increase dehulling efficiency by increasing dehulling recovery after decortication because of seed coat structural change (Fedoruk, 2013). Plumpness of dehulled lentils may also change the perceived colour of the product. Plumper seeds appear to be richer in cotyledon colour and they may also cook faster, which will increase consumer appeal. Zero-tannin lentils also do not darken during storage. All of these characteristics make zero-tannin lentil a good candidate for diversification of breeding programs and development of potential new markets for lentil. The flavan-3-ols and proanthocyanidins are controlled by *tgc*, while *tan*, a bHLH transcription factor, can affect their production. The major reason for changes in seed coat colour during long-term storage is the polymerization of these two sub-classes of phenolic compounds, which will reduce their extractability and probably their bioavailability. Optimizing storage condition of lentil seeds might decrease the benefit loss after storage. It may also be possible to develop genotypes that exhibit reduced rate of polymerization by investigating the factors that influence the activity and concentration of key enzymes. Findings of these studies will be helpful to design lentil cultivars with improved nutritional profiles and better marketability.

## **6.2. Future work**

Our optimized LC-MS method analyzed several compounds from various sub-classes of phenolics. 49 Phenolic compounds were detected including phenolic acids, stilbenes,

flavones, flavonols, flavan-3-ols, and proanthocyanidins from 73 analyzed chemicals. These types of optimization tests should be extended and more phenolic standards should be added for building a larger database. Such a database provides an opportunity for a more specific profiling of phenolic compounds for wider range of lentils, which can also be extended to seed coats of other pulse crops.

In this set of studies, phenolic compounds were analyzed based on relative quantification. The major goal was comparing the phenolic profiles among lentils with different seed coat colours, which makes the relative quantification an appropriate option. However, it will be necessary in the future to analyze the phenolic compounds based on absolute quantification in order to estimate the exact amount of phenolic compounds that can be extracted from lentil seed coats of different colours.

Future studies could focus on the quantification of the phenolic compounds in the major seed coat types. The seed coat represents approximately 10 % of the weight of dry seeds. Since 80% of Canadian lentil production will at some point be dehulled, the seed coat fraction of the total production represents several hundred thousand tonnes of biological materials that contain phenolic compounds. A deeper understanding of the specific amounts of the compounds, and their potential antioxidant activity would provide a baseline for determining the potential economic value of the lentil seed coat fraction.

Our optimized analysis method had a short 30-min running time using HPLC equipment. It would be worth modifying the optimized method for shorter analysis duration by using UPLC equipment. In Chapters 3 and 4, we were forced to analyze sub-set of RIL populations because of limitations of the total duration analysis. Using UPLC, analysis



of all the RILs of a population might become much easier. Generating such data would facilitate genetic-mapping analysis. The extraction method was optimized based on the available equipment in the lab. Although this method is fast and reproducible, it will not be suitable for analyzing large genetic populations. The advantage of using automated extraction methods should be considered for phenolic profiling of such large sample sizes.

Acquiring UV/VIS-detection and full scan chromatograms on the lentil samples, some intense peaks were noticed that we were not able to identify with the applied mass spectrometer. It will be necessary to identify such unknown compounds using accurate mass instrumentation. Using such mass spectrometers, elemental composition will be determined as the error in the  $m/z$  assignment is much less compared with the triple quadrupole. This means that fewer molecular formula will have a specific  $m/z$  range which might lead to identification of the unknown samples.

In these studies, a specific biochemical relationship between the *Ggc/ggc* gene (the other important locus for lentil seed coat background colour besides the *Tgc/tgc*) and the presence or absence of phenolic compounds were not found. For these analyses only 73 phenolic compounds were used. More than 8000 phenolic compounds have been described in nature, and pigmentation (gray vs non-gray) may also be related to presence of other pigments, for example carotenoids and betalains. For future research, the presence or absence of other phenolic compounds besides carotenoids and betalains should be investigated in different seed coat colours of lentil. This might provide evidence of associations between *Ggc/ggc* and other pigments.

Legumes have a shared synteny, including the genes that are related to seed coat colour. Bioinformatics research works on lentil has started and *Lens culinaris* Genome v1.2 was released recently (June 2016). However, more bioinformatics research is needed and in the meantime, querying the lentil genome through a blast in phenylpropanoid genes in gene sequences from other legumes such as alfalfa and soybean might be helpful for identifying genes associated with gray colour.

In Chapter 3, a limited number of yellow and red cotyledons were compared with each other and found some differences between these two cotyledon colours. Red and yellow colours of lentil cotyledons are basically associated with carotenoids. It would be interesting to continue these comparisons among cotyledons of a RIL population in order to see if there are associations between these colours and any of sub-classes of phenolic compounds.

Biochemical clues about some of the specific phenolic compounds that are controlled by the *tgc* gene were provided. Some molecular analyses could be used to develop a more explicit indication of the exact regulatory genes in the phenylpropanoid pathway that are related to *tgc*.

In Chapter 5, seed coats of fresh lentils with green seed coat colour were compared with those of green lentils stored for long periods. A future storage experiment could be designed to include all four seed coat colours. This might provide more detailed understanding of changes in phenolic compound profile after storage. It would be necessary to analyze fresh seeds (for example, from the LR-18 population with four seed coat background colours) and then compare them to samples stored for long periods of 7-14 years to determine how phenolic compounds profiles might change. In

addition, the effect of temperature and packaging techniques on phenolic profile could be investigated.

In Chapter 5, increases in phenolic acids and flavones observed in green lentil seeds after storage. Breakdown of more complex species into smaller subunits proposed as the possible reason. Acid or alkaline hydrolysis on the extracted samples before the LC-MS analysis should be applied to confirm this hypothesis.

These studies were focused on the biochemical phenotyping and genetic influences of background seed coat colour in the absence of seed coat pattern. A separate group of genes that confer seed coat pattern, such as marbled, spotted, dotted, or combinations are available as true breeding lentil genotypes. The black seed coat phenotype could also be subjected to a deeper analysis.

These set of studies detected phenolic compounds in raw lentil seeds. Changes in phenolic compound profiles after cooking lentils with different seed coat colour could provide deeper knowledge of potential nutritional effects of phenolic compounds. In addition, the effect of different methods of cooking can be investigated on the phenolic profiles of lentils.

Determining of phenolic profiles of a specific lentil seed coat colour does not mean that all the detected phenolics can be available after digestion. Some studies could be designed on the bioavailability of phenolics after digestion *in vitro* or *in vivo* to gain insight into potential dietary influence of specific phenolics.

Iron chelation could be a concern because of the adverse effects of some of the phenolic compounds, for example myricetin. Interestingly, some other phenolics such as kaempferol have been detected that increase the bioavailability of iron. Studies on the

effect of different types of phenolics on bioavailability of iron are ongoing in some collaborating laboratories such as the USDA at Ithaca, NY. It will be of great interest to design breeding programs to increase the amount of phenolic compounds with improving effects and to reduce the amount of specific phenolic compounds with inhibitor effects on iron bioavailability.

In collaboration with USDA, the iron bioavailability in normal, zero-tannin gray, and zero-tannin transparent lentils were compared using the caco-2 test. The results were promising and showed the highest iron bioavailability for zero-tannin transparent lentils. It will be interesting to compare lentil seeds with different seed coat background colour and pattern by this test. It would be possible to investigate all the phenolic compounds that have been detected in these sets of analyses and to determine the complete set of inhibitors and promoters of iron uptake in lentil samples.

Higher amounts of flavan-3-ols, proanthocyanidins, and flavonols in green and gray seed coats suggests these lentil seed coats might possess greater antioxidative properties, and might be more promising as health-promoting foods. While only 20 % of lentils are consumed as whole seeds, the results of these experiments might lead to improve the knowledge of consumers and might increase the marketability of whole lentils seeds especially with green and gray seed coat colours.

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# APPENDICES

## Appendix 1. Manuscript # 1

### Development of a fast extraction method and optimization of liquid chromatography - mass spectrometry for the analysis of phenolic compounds in lentil seed coats

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#### Highlights

- A systematic optimization for LC-MS analysis of polyphenols in lentil seed coats.
- Acid extraction of polyphenols resulted in the breakdown of polymeric compounds.
- Polyphenols were extracted efficiently in 1 hr with acetone: H<sub>2</sub>O (70: 30, v/v).
- A core-shell Kinetex PFP column gave better isomer separation than C18 columns.

- We developed a 30 min LC-MS method that showed differences between three genotypes.

### Abstract

A systematic set of optimization experiments was conducted to design an efficient extraction and analysis protocol for screening six different sub-classes of phenolic compounds in the seed coat of various lentil (*Lens culinaris* Medik.) genotypes. Different compounds from anthocyanidins, flavan-3-ols, proanthocyanidins, flavanones, flavones, and flavonols sub-classes were first optimized for use as standards for liquid chromatography mass spectrometry (LC-MS) with UV-VIS detection. The effect of maceration duration, reconstitution solvent, and extraction solvent were investigated using lentil genotype CDC Maxim. Chromatographic conditions were optimized by examining column separation efficiencies, organic composition, and solvent gradient. The results showed that a 1 h maceration step was sufficient and that non-acidified solvents were more appropriate; a 70:30 acetone: water (v/v) solvent was ultimately selected. Using a Kinetex PFP column, the organic concentration, gradient, and flow rate were optimized to maximize the resolution of phenolic compounds in a short 30-min analysis time. The optimized method was applied to three lentil genotypes with different phenolic compound profiles to provide information of value to breeding programs.

### Key words

Polyphenols; *Lens culinaris*; Extraction; Core-Shell Column; HPLC condition optimization

### Introduction

Phenolic compounds (also known as polyphenols) make up a large group of secondary

metabolites characterized by the presence of an -OH group and an aromatic ring (Vermeris and Nicholson, 2006). The basic C6-C3-C6 (A-, C-, and B-ring) structure (Figure I) is typically observed, with different phenolic sub-classes being dependent upon further hydroxylation, methylation, or other modifications (Abad-García et al., 2009). In human nutrition, phenolic compounds act as a “double-edged sword”, exerting both adverse effects and health benefits (Martin and Appel, 2010). There are several publications regarding health benefits of phenolic compounds, for example in protection against cancer (Martin and Appel, 2010; Thomasset et al., 2006) and cardiovascular diseases (Martin and Appel, 2010). Conversely, adverse effects described in the literature include inhibition of non-haem iron (Hooper and Frazier, 2012; Lynch, 1997) and induction of pro-oxidative stress and H<sub>2</sub>O<sub>2</sub> production when polyphenols are present in high amounts (Lambert et al., 2007; Oikawa et al., 2003). These contrasting effects highlight the importance of analyzing these compounds in food resources.

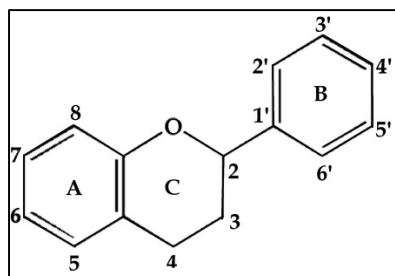


Figure I. C6-C3-C6 structure in phenolic compounds.

Lentil (*Lens culinaris* Medik.) is a good source of protein, carbohydrates, dietary fiber components, minerals, vitamins, and secondary metabolites that include phenolic compounds (Xu and Chang, 2009). Simple phenolic compounds (Tsopmo and Muir, 2010), phenolic acids (Amarowicz et al., 2009; Bartolomé et al., 1997; López-Amorós et



al., 2006; Xu and Chang, 2009), flavan3-ols and proanthocyanidins (Amarowicz et al., 2009; Amarowicz et al., 2010; Bartolomé et al., 1997; Dueñas et al., 2002; Dueñas et al., 2003; Escarpa et al., 2002; Zou et al., 2011), anthocyanidins (Takeoka et al., 2005; Xu and Chang, 2010), flavonols (Aguilera et al., 2010; Amarowicz et al., 2009; Amarowicz et al., 2010; Dueñas et al., 2002; Escarpa et al., 2002; Tsopmo and Muir, 2010; Xu and Chang, 2009; Zou et al., 2011), stilbenes (Dueñas et al., 2002), flavones (Amarowicz et al., 2009; Dueñas et al., 2002; Xu and Chang, 2009, 2010), and flavanones (Aguilera et al., 2010) are the major sub-classes of phenolic compounds found in lentil seeds. Phenolic compounds are much more diverse in the lentil seed coat than in the cotyledon and mostly consist of oligomers and polymers of proanthocyanidins (Dueñas et al., 2002). Quantifying phenolic compound concentrations in lentils is needed to assess the potential for long-term development of breeding crops with improved quantity and quality of such compounds. Any suitable analytical method must be able to simultaneously quantify different sub-classes of phenolic compounds and do so as efficiently as possible due to the need to analyze large numbers of samples.

Several methods are used to analyze phenolic compounds. Liquid chromatography-mass spectrometry (LC-MS) methods are well-suited because of their selectivity and sensitivity. When coupled with ultraviolet detection (LC-UV-MS), these methods offer a fast solution to determine unknown phenolic compounds (Abad-García et al., 2009). A critical step in these methods is sample preparation. Although several methods have been reported for extraction of phenolic compounds in lentil seeds, no systematic comparisons among these methods have been made to determine the differences

among them or relative advantages or disadvantages of each. Extraction time is another important parameter as long extraction times (for example (Bartolomé et al., 1997; Dueñas et al., 2002; Takeoka et al., 2005)) make analytical methods very time consuming and not readily applicable to the analysis of phenolic compounds in a large number of lentil genotypes. If extraction efficiencies are similar, the method that can be accomplished in the shortest time would be the preferred approach.

Previous experiments involving lentil seeds have used chromatographic gradients to separate phenolic compounds in a time range of 70 to 120 min (Aguilera et al., 2010; Bartolomé et al., 1997; Dueñas et al., 2002; Zou et al., 2011). These separations were typically done using columns with large particles (e.g., 5  $\mu\text{m}$ ); in particular, C18 columns are commonly used (Aguilera et al., 2010; Amarowicz et al., 2009; Amarowicz et al., 2010; Bartolomé et al., 1997; Dueñas et al., 2002; Dueñas et al., 2003; López-Amorós et al., 2006; Takeoka et al., 2005; Tsopmo and Muir, 2010; Xu and Chang, 2010; Zou et al., 2011). However, the implementation of newer column technologies (e.g., core-shell) with smaller particle sizes (e.g., 2.6  $\mu\text{m}$ ) can improve the separation efficiency and thereby allow for a shorter analysis time (Lesellier, 2012). As the column is a critical parameter in LC-MS experiments, an investigation of the performance of core-shell columns for the analysis with phenolic compounds in lentil is warranted; to our knowledge, no such work has been previously reported.

In addition, optimizing the gradient and the organic modifier (Biesaga et al., 2007) can contribute to improvements in LC-MS methods. We employed H<sub>2</sub>O: acetonitrile (ACN): formic acid (FA) to affect separation, while making changes to the amount of acid and organic solvent (separately) to improve the resolution of peaks.

We optimized phenolic compound extraction for speed and efficiency by examining both solvent type and extraction duration for several sub-classes of phenolic compounds in lentil seed coats. We also examined three different types of columns, the amount of organic modifier, and the gradient of the organic phase for their ability to achieve fast separation and sufficient resolution of peaks for different types of isomeric phenolic compounds.

Ultimately, the optimized method was applied to three different seed coat colours of lentil to demonstrate similarities and differences in the phenolic compound profiles. The results indicate that the time efficient procedure we developed can be successfully applied to the extraction and analysis of phenolic compounds for phytochemical screening of various lentil genotypes in breeding programs.

## Materials and methods

### Plant material

Seeds from three lentil genotypes (CDC Maxim, 946a-46, Indianhead) were obtained from the Crop Development Centre at the University of Saskatchewan (Saskatoon, Canada). The seeds were decorticated using an abrasive mill (SATAKE Engineering Co. Ltd No# 554046 Japan) and the seed coats separated using sieves and a column blower. To analyze the lentil seed coat, ~50 mg (for each replicate) was weighed into micro centrifuge tubes. The tubes were covered, put in a -80 °C freezer for 1 h, and then freeze-dried overnight. A ¼ inch ceramic sphere bead was added to each tube and the seed coats pulverized using Fast Prep®FP120 (Qbiogene, Inc., Canada) two consecutive times for 10 s at a speed setting of 4.0.

## HPLC-MS

Experiments were conducted using an HP1100 series (Agilent, Germany) reversed phase high performance liquid chromatograph (HPLC) with UV-VIS detection coupled to a Quattro LC (Waters, UK) triple quadrupole mass spectrometer (MS) equipped with an electrospray ionization (ESI) interface. Waters' MassLynx 4.1 software was used for integration of peak areas. Unless otherwise specified, the chromatographic column was a Genesis C18 and the mobile phases used were H<sub>2</sub>O: FA (95:5, v/v) as solvent A and H<sub>2</sub>O: ACN: FA (5:90:5, v/v/v) as solvent B. The column oven temperature was 25 °C and the injection volume was 2 µL. The mobile phase was delivered at a flow rate of 0.2 mL/min and the gradient used is given in Table I.

Table I. Typical gradient of organic solvent used in this study; solvent A and B were H<sub>2</sub>O: FA (95:5, v/v) and H<sub>2</sub>O: ACN: FA (5:90:5, v/v/v), respectively.

Time (min)	Solvent A (%)	Solvent B (%)	Flow rate (mL/min)
0	99	1	0.2
2	99	1	0.2
10	95	5	0.2
20	90	10	0.2
30	80	20	0.2
40	60	40	0.2
50	20	80	0.2
55	20	80	0.2
55.05	99	1	0.2
60	99	1	0.2

## Reagents and standards

Fourteen phenolic compounds (based upon the literature and their commercial availability) were considered, including sub-classes of anthocyanidins, flavan-3-ols, proanthocyanidins, flavanones, flavones, and flavonols (Table II). Internal standards (ISs) were compounds that were not detectable in preliminary seed coat studies but had

similar structures to the analytes of interest so should respond in an analogous way and thereby provide a reference with respect to extraction efficiency and ionization variability due to differences in the sample matrix. Malvidin-3-*O*-galactoside, (-)-gallo catechin, flavone, 3-hydroxy-4-methoxy-cinnamic acid, 4-aminosalicylic acid, 4-hydroxy-6-methylcoumarin, salicin, and procyanidin C1(epicatechin-(4 $\beta$ →8)-epicatechin-(4 $\beta$ →8)-epicatechin) were purchased from Sigma-Aldrich (Missouri, USA), whereas (+)-catechin, naringenin, luteolin, luteolin-4'-*O*-glucoside, luteolin-7-*O*-glucoside, kaempferol-3-*O*-glucoside, myricetin-3-*O*-rhamnoside, quercetin-3-*O*-glucopyranoside, quercetin-3-*O*-galactoside, and procyanidin B1 (epicatechin-(4 $\beta$ →8)-catechin) were purchased from Extrasynthese (Genay, France). A series of experiments using flow injection analysis were used to confirm the *m/z* of the parent ion (full MS scan); determine the *m/z* value(s) of the most intense fragment ion(s) (product ion scan); and to optimize the MS conditions, namely the cone voltage (CV) and collision energy (CE), for quantification using multiple reaction monitoring (MRM); all were done using ESI in positive mode. Note that MRM is a very sensitive, selective technique in which the first quadrupole is used to select the desired molecular ion, the second as a collision cell to fragment the ion, and the third to monitor an intense fragment ion.

Table II. Characteristics of the phenolic compounds including sub-class, optimum Molecular and Fragment ions, Cone voltage (CV), Collision energy (CE), and UV-VIS wavelength; IS, C, and G stand for internal standard, epi(catechin), and epi (gallocatechin), respectively. A Genesis C18 column was used with a flow rate of 0.2 mL/min. Solvent composition was A (H<sub>2</sub>O: FA (95:5, v/v)) and solvent B (H<sub>2</sub>O: ACN: FA (5:90:5, v/v/v)) with the gradients as described in §2.2.

Compound	Sub-class	Retention time (min)	Molecular weight (g/mol)	Molecular ion (m/z)	Fragment ion (m/z)	Cone voltage (V)	Collision energy (eV)	Wave length (nm)
GG	proanthocyanidins	2.4		610.7	306.7	37	25	274
GGC	proanthocyanidins	2.8		898.7	290.7	35	25	276
GC	proanthocyanidins	3.6		594.7	290.7	43	25	276
(-)-gallocatechin	flavan-3-ols	3.7	306.3	307.1	138.8	30	20	274
4-aminosalicylic acid	IS	3.9	153.1	154	118.6	48	14	234, 300
GCC	proanthocyanidins	4		882.7	290.7	35	25	276
CC	proanthocyanidins	7.6		579.3	126.8	36	32	280
Salicin	IS	8	286.3	287.4	106.8	8	14	270
procyanidin B1	proanthocyanidins	8.9	578.5	579.3	126.8	36	32	280
(epi) catechin-glucoside	flavan-3-ols	9.5		452.7	290.7	40	20	280
(+)-catechin	flavan-3-ols	10	290.3	291.2	138.8	27	18	280
CCC	proanthocyanidins	20.3		867.4	579	38	15	278
procyanidin C1	proanthocyanidins	22	866.8	867.4	579	38	15	280
3-hydroxy-4-methoxy-cinnamic acid	IS	25.9	194.2	195.2	116.9	17	10	268, 326
malvidin-3- <i>o</i> -galactoside	anthocyanidins	29	528.9	493.4	331	40	23	278, 528
myricetin-3- <i>o</i> -rhamnoside	flavonols	29.7	464.4	465.4	319.1	20	13	264, 358
quercetin-3- <i>o</i> -galactoside	flavonols	30.2	464.4	465.2	303.2	28	15	265, 358
quercetin-3- <i>o</i> -glucopyranoside	flavonols	30.4	464.4	465.2	303.2	21	14	265, 358
luteolin-7- <i>o</i> -glucoside	flavones	30.9	448.4	449.3	287.3	28	24	268, 348
kaempferol-3- <i>o</i> -glucoside	flavonols	33.7	448.4	449.3	287.3	25	15	266, 348
luteolin-4'- <i>o</i> -glucoside	flavones	34.7	448.4	449.3	287.3	36	25	268, 334
4-hydroxy-6-methylcoumarin	IS	36.2	176.2	176.9	134.9	41	18	268, 334
Naringenin	flavanones	37.6	272.3	273	152.8	34	22	290
Luteolin	flavones	38.5	286.2	287.1	152.8	60	33	267, 358
Flavone	flavones	46.4	222.2	223.1	76.8	60	40	254,298

With optimum MRM conditions, initial LC conditions were applied using the C18 column allowing the retention time and UV-VIS wavelength to be determined for each analyte.

Furthermore, many proanthocyanidin oligomers (e.g., procyanidin dimers (CC),

prodelphinidin dimers (GG and GC), procyanidin trimers (CCC), and prodelphinidin trimers (GGC and GCC)) as well as (epi) catechin glucoside were not commercially available but are major components in the lentil seed coat. The procyanidin oligomers contain monomers of epi(catechin) while prodelphinidin oligomers contain monomers of epi(galocatechin) and/or epi(catechin). MRM conditions were optimized considering a range of CV and CE and the transitions (molecular ion > fragment ion) determined in previous papers (Dueñas et al., 2003), with Rt values then determined (Table II). Because standards were not available, the identification of which oligomer was present could not be confirmed; therefore, they are assigned only as dimers, trimers, and so on. Similarly, for the glycosylated form of (epi) catechin, the exact bond location and type of sugar could not be determined. Note that ions with the same transitions were monitored in one channel with the Rt being used to differentiate these compounds. Using one channel decreased the number of transitions and promoted chromatogram accuracy by increasing the number of points per peak. Thus, the 579.3 > 126.8 transition was used for procyanidin B1 and CC, 867.4 > 579 for procyanidin C1 and CCC, 465.2 > 303.2 for quercetin-3-O-glucopyranoside and quercetin-3-O-galactoside, and 449.3 > 287.3 for luteolin-7-O-glucoside, kaempferol-3-O-glucoside, and luteolin-4'-O-glucoside.

#### Optimizing extraction method

All extraction optimizing tests were conducted using CDC Maxim lentils.

#### Duration of extraction method

The extraction method was modified from (Dueñas et al., 2002). The seed coat powder (~50 mg) was macerated with 750 µL of a solution containing 0.1% HCl in methanol (MeOH): water (80:20, v/v) three times for a) 3 x 24 h on platform, b) 2 h + 24 h + 2 h on

platform, c) 1 h on platform + 1 h in ultrasonic bath + 1 h on platform, and d) 3 x 1 h in ultrasonic bath. The rate of shaking on the rocking platform was 1400 rpm, and 80 kHz frequency/100% power was applied in the ultrasonic bath. After each extraction, the tubes were centrifuged twice (12,000 rpm for 5 min each with the maximum relative centrifugal force (rcf) of 15300) and as much of the supernatant as possible was removed while keeping this amount the same among samples. Then 100  $\mu$ L of each extract were combined and dried down with a Speed Vac (LABCONCO, Kansas City, USA). Samples were then re-dissolved in a reconstitution solvent of 300  $\mu$ L methanol: water (5:95, v/v). The chromatographic conditions used were as described in §2.2.

An additional experiment was designed to determine how the intensity of compounds changed by increasing the number of extraction steps. Phenolic compounds of lentil seed coat were extracted four times with the extract of each step analyzed separately. The areas under each peak in the chromatograms were integrated and normalized from 1 to 100 with MS Excel to determine the percent of the total recovered in each step.

#### Reconstitution solvent

The high organic percentage of the extraction solvent will adversely affect chromatography. Consequently, the samples were dried down and reconstituted to better match the starting mobile phase. Three reconstitution solvents were compared, namely MeOH: H<sub>2</sub>O (5:95, v/v), MeOH: H<sub>2</sub>O (10:90, v/v), and acetic acid: MeOH: H<sub>2</sub>O (0.5:50:49.5, v/v/v).

#### Extraction solvent

The effect of different extraction solvents on the concentration of phenolic compounds was investigated. Based on previously published protocols (Aguilera et al., 2011;



Bartolomé et al., 1997; Pang et al., 2007), five solvents were considered, namely methanol (with 0.1% HCl): water (80:20, v/v), acetone: water: acetic acid (70:29.5:0.5, v/v/v), acetone: water (70:30 v/v), methanol: water (70:30, v/v), and methanol: water: acetic acid (80:19.5:0.5, v/v/v).

#### Optimizing chromatographic conditions

This part of the experiment was conducted using the mixture of standards described in § 2.3.

#### Selection of chromatographic column

Three different columns were compared: Core-shell Kinetex pentafluorophenyl (PFP), 100 × 2.1 mm id, 2.6 µm particle size (Phenomenex, Torrance, CA); Core-shell Kinetex C18, 100 × 2.1 mm id, 2.6 µm particle size (Phenomenex, Torrance, CA); and Genesis C18, 100 × 2.1 mm id, 4 µm particle size (Grace Vydac, Illinois, US).

#### Organic percentage and gradient

The effect of the acid concentration in the mobile phase was investigated using 0.5, 1, 2, and 5% formic acid in Solvents A and B. Furthermore, the optimum gradient was tested by changing the slope of the linear gradient from 5 to 50 min and therefore the composition of the organic solvent in the isocratic gradient from 50 to 55 min, as shown in Table III.

Table III. Different gradients of organic solvents that were used for optimizing chromatographic conditions

Time (min)	Solvent B (%)			
0	1	1	1	1
5	1	1	1	1
50	30	50	70	90
55	30	50	70	90
55.05	1	1	1	1
60	1	1	1	1

Results obtained using the different chromatographic conditions were compared based on the

$$\text{capacity factor } (K') = \frac{\text{compound } Rt - \text{Void volume } Rt}{\text{Void volume } Rt} \quad \text{Eq. 1}$$

and the

$$\text{resolution} = 2 * \frac{(Rt \text{ b} - Rt \text{ a})}{(\text{Peak width b} + \text{Peak width a})} \quad \text{Eq. 2}$$

where compound “b” elutes later than compound “a”.

Method applicability for comparing different genotypes of lentil

Chromatograms for three different genotypes (964a-46, Indianhead, and CDC Maxim) with different seed coat colours (green, black, and grey, respectively) were compared to see how applicable the optimized method was for analyzing contrasting seed coat phenotypes.

LC-MS reproducibility

To ensure good reproducibility of the LC-MS method, one of the samples was repeated at the start, middle, and the end of a run. In addition, a combination of 18 standards plus reconstitution solvent (MeOH: H<sub>2</sub>O, 5:95, (v/v)) was used as a quality control. Solvent blanks and method blanks (solvent plus IS) were also included to ensure that contamination was not present.

Statistical analysis

Data exploration and analysis of variance was done using R software (v. 2.15.3) (R Core Team, 2013). Compounds resulting in low chromatogram peak areas near the background noise were omitted due to reliability concerns. Data were transferred to an Excel® spreadsheet and final results were recorded as the average of replicates ±

standard deviation. The experimental design was based upon randomized complete blocks.

## Results and discussion

### Optimizing compounds for use as standards in an LC-MS method

The MS allows for quantification of expected phenolic compounds, and UV-VIS detection will help to identify unexpected phenolic compounds. Because phenolic compounds absorb UV-VIS radiation between 250 and 600 nm, we can look for peaks in that UV-VIS range that are not associated with a corresponding MS peak and investigate further. For this study, quantification of expected phenolic compounds was done with a triple quadrupole mass spectrometer using MRM as described in §2.2. For all phenolic standards, the optimum molecular ions were protonated  $[M+H]^+$  (Table II) except for malvidin-3-O-galactoside, which is already a cation in solution (purchased as a chloride salt). As a result malvidin-3-O-galactoside does not require a proton to ionize and therefore produces an  $M^+$  ion.

With the collision gas on, the molecular ion collides with neutral gas molecules causing fragmentation and enabling the optimum ions to be identified for use in MRM analyses. The major fragment ion in (MS/MS spectra of) malvidin-3-O-galactoside, luteolin-4'-O-glucoside, luteolin-7-O-glucoside, kaempferol-3-O-glucoside, quercetin-3-O-galactoside, quercetin-3-O-glucopyranoside, myricetin-3-O-rhamnoside, and (epi) catechin-glucoside is related to sugar loss (162 for glucose/galactose and 146 for rhamnose). The fragment ion for flavan-3-ols at  $m/z$  138.8 is the result of C-ring cleavage at 1/3 bond (Figure I). In proanthocyanidins, fragment ions at  $m/z$  291,  $m/z$  307, and  $m/z$  579 are related to the

breaking of bonds between their monomers (flavan-3-ols). The fragment ion at  $m/z$  127 could be due to the rupture of the C-ring at the 2/4 bond. In flavanones and flavones, fragment ion  $m/z$  153 is the result of C-ring cleavage at the 1/3 bond (Abad-García et al., 2009). With the optimum molecular and fragment ions identified, MRM was carried out to optimize CV and CE (Table II).

Different standard solutions were run on a Genesis C18 column using the default conditions (§2.2). More polar compounds (e.g., proanthocyanidins and flavan-3-ols) eluted at the start of the run; later eluting compounds were mostly flavones (Table II). Besides compound polarity, the type of functional group and the nature and position of conjugate can also affect the elution order (Abad-García et al., 2009). Glycosylated luteolins came out sooner than their aglycones (38.5 min). On the other hand, luteolin-7-*O*-glycoside ( $R_t$  = 30.9 min) eluted before luteolin-4'-*O*-glycoside ( $R_t$  = 34.7 min). Quercetin-3-*O*-galactoside eluted a bit earlier than quercetin-3-*O*-glucopyranoside and the prodelphinidins in Table II eluted sooner than the procyanidins. The UV spectra contain major absorption peaks for prodelphinidins and procyanidins at 274-276 and 278-280 nm, respectively (Table II). For both flavones and flavonols, two major peaks were observed at 254-268 and 298-358 nm. The UV-VIS spectrum for anthocyanidin (malvidin-3-*O*-galactoside) was very specific, with two major peaks observed at 278 and 528 nm (Table II).

#### Optimization of extraction method

##### Extraction duration

Maceration times that were used previously for lentil seeds ranged from ~12 to 36 h (Bartolomé et al., 1997; Dueñas et al., 2002; Takeoka et al., 2005). Figure II shows the

effect of maceration duration on different phenolic compounds in CDC Maxim lentil seed coats. In this figure, C and G represent (epi) catechin and (epi) gallocatechin, respectively. Thus, CC and CCC are procyanidin dimers and trimers consisting of two and three (epi) catechins, respectively; GC and GG represent prodelphinidin dimers with one and two (epi) gallocatechin(s), respectively; and GCC and GGC are prodelphinidin trimers that have, respectively, one and two (epi) gallocatechins beside two or one (epi) catechins. Note that both stereoisomers of catechin and epicatechin have the same molecular weight; this is also true for both gallocatechin and epigallocatechin. Areas of the LC-MS/MS peaks for each phenolic compound were divided by the sample dry weight (in mg) and values for 3, 28, and 72 h total extraction durations were compared to determine any effect attributable to lentil seed coat maceration time. No significant differences are evident for most of the phenolic compounds (Figure II). Previous work shows the duration of extraction to have only a minor effect on recovery of isoflavones from soy protein (Griffith and Collison, 2001). For profiling phenolic compounds in a large number of genotypes, a fast maceration step is advantageous. Based on our results, a maceration time of 1 h ( $\times 3$ ) was determined to be sufficient and time efficient.

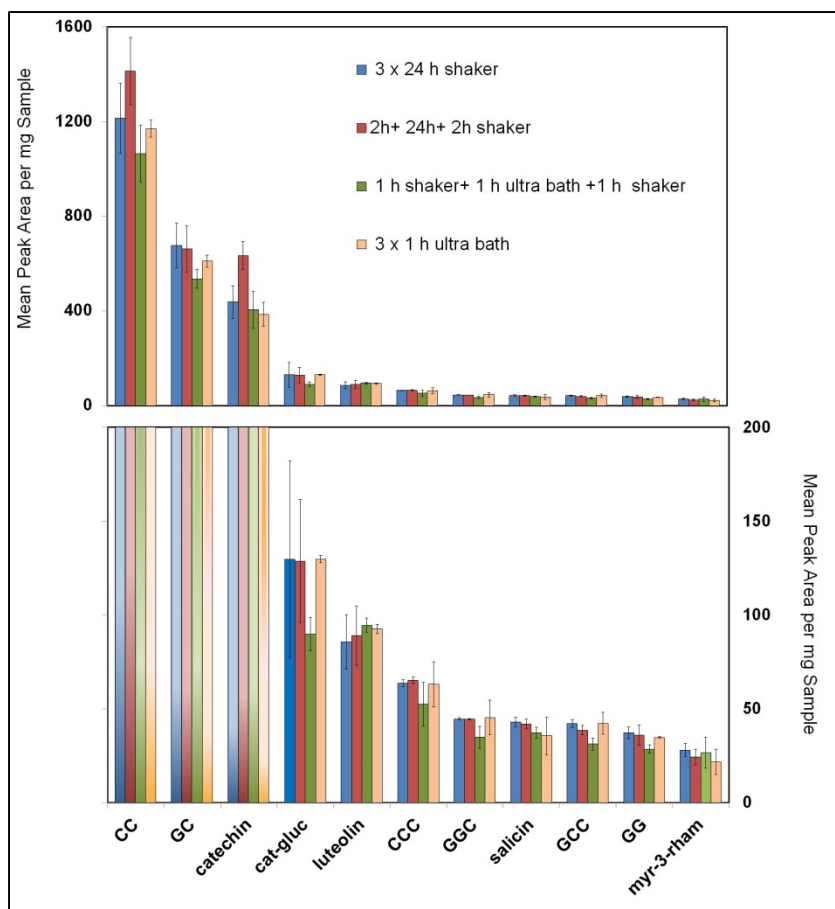


Figure II. Effect of maceration time on the mean area per mg sample of phenolic compounds; Maceration treatments were 3×24 h on platform, 2 h + 24 h + 2 h on platform, 1 h on platform + 1 h ultrasonic bath + 1 h platform, and 3×1 h in ultrasonic bath. C, G, cat-gluc, and myr-3-rham represent (epi) catechin, (epi) gallicocatechin, (epi) catechin-glucoside and myricetin-3-O-rhamnoside, respectively. A Genesis C18 column with a flow rate of 0.2 mL/min was used with solvents and gradients as described in §2.2.

### Optimizing the number of extractions

Figure III shows the normalized area of the lentil seed coat phenolic compounds in the first to fourth extraction steps. Compounds were extracted four times for 1 h using the ultrasonic bath and/or platform shaker. With respect to the ISs, the first extraction removed more than 80% and virtually none remained at the fourth extraction. The same trend was observed for flavonols (kaempferol-3-O-glucoside and myricetin-3-O-rhamnoside) and flavones (luteolin-4'-O-glucoside). Different results were obtained for

oligomers of proanthocyanidins (B1, CC, GC, C1, CCC, GCC, and GGC) and, more specifically, their monomers (catechin and gallic acid), as a significant amount of the monomers, dimers, and trimers were extracted in the fourth step. We believe this is due to the acid causing the proanthocyanidin polymers to slowly breakdown, as these polymers are found in large quantities in the lentil seed coat (Dueñas et al., 2003). Further evidence for this hypothesis is given in §3.2.3 and 3.2.4. We decided to use three extractions to ensure good recovery. Note that during this experiment (and the previous one), the differences between application of an ultra-sonic bath and a shaker platform on the extraction were not significant for the phenolic compounds analyzed. However, as heating of the water (and hence samples) was observed when using the ultrasonic bath, we decided to continue the experiments using only the platform shaker.

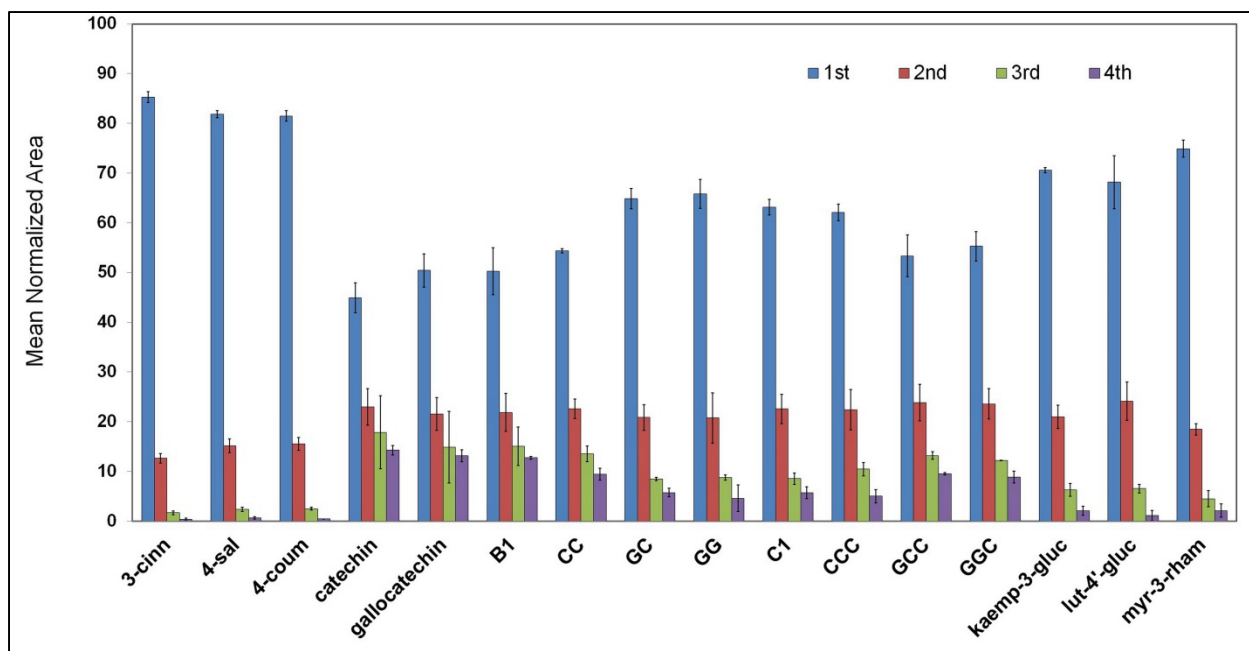


Figure III. Effect of the number of extractions using ultrasonic/platform/ultrasonic/platform on normalized area of different phenolic compounds; error bars are the standard deviation of three replicates. 3-Cinn, 4-sal, 4-coum, C, G, kaemp-3-gluc, lut-4'-gluc, and myr-3-rham represent 3-hydroxy-4-methoxy-cinnamic acid, 4-aminosalicylic acid, 4-hydroxy-6-methylcoumarin, (epi) catechin, (epi) gallo catechin, kaempferol-3-O-glucoside, luteolin-4'-O-glucoside, and myricetin-3-O-rhamnoside, respectively. A Genesis C18 column was used with a flow rate of 0.2 mL/min and solvents and gradients as described in §2.2.

#### Reconstitution solvent

Figure IV shows the effect of different reconstitution solvents on the peak areas of phenolic compounds. The left side of the plot shows peak areas obtained from reconstituting extracts from lentil seed coats, and the right side shows peaks areas from reconstituting chemical standards. The reconstitution solvents in both cases were MeOH: H<sub>2</sub>O (5:95, v/v), MeOH: H<sub>2</sub>O (10:90, v/v), and acetic acid: MeOH: H<sub>2</sub>O (0.5:50:49.5, v/v/v). Overall, the use of different reconstitution solvents for the chemical standards resulted in minimal differences in the peak areas obtained. The largest change was in the mean area of 3-hydroxy-4-methoxy-cinnamic acid, which increased by 17% when the acid-containing reconstitution solvent was applied. A high amount of



ethanol was required for stock preparation of this standard, suggesting that the higher amount of organic solvent improves solubility. However, significant changes were evident for the lentil seed coat extracts. Note that the peak areas of the IS, 3-hydroxy-4-methoxy-cinnamic acid showed the same pattern for both the chemical standard solution and the lentil seed coat extract solution. Similarly, Peak areas for luteolin were similar for the various solvents with respect to lentil seed coat extracts and chemical standards. However, catechin and gallic acid had significantly higher peak areas in the lentil seed coat extract when reconstituted with acetic acid: MeOH: H<sub>2</sub>O (0.5:50:49.5, v/v/v); this is in contrast to the similar mean areas of both of these flavan-3-ols in the chemical standards data for all three reconstitution solvents considered. Notably, the area of the dimer proanthocyanidin B1 in the lentil seed coat extract decreased upon reconstitution in acetic acid: MeOH: H<sub>2</sub>O (0.5:50:49.5, v/v/v) but was the same for the three different reconstitution solvents when the chemical standard was used. These data suggest that the acid may contribute to the breakdown of this dimer (epicatechin-(4 $\beta$ →8)-catechin) into monomers (and therefore other oligomers extracted from the seed coat would break down after reconstitution as well). Because the data suggest that the presence of acid in either the reconstitution or extraction solvent (§3.2.1) can cause breakdown of the polymers, investigation of other extraction solvents (with and without acid) would be worthwhile to determine a more suitable extraction solvent. Peak areas obtained using 5 or 10% organic reconstitution solvent were comparable and either of these two could be applied. We chose 10% for the remainder of this study as it did not have an adverse effect on the chromatography.

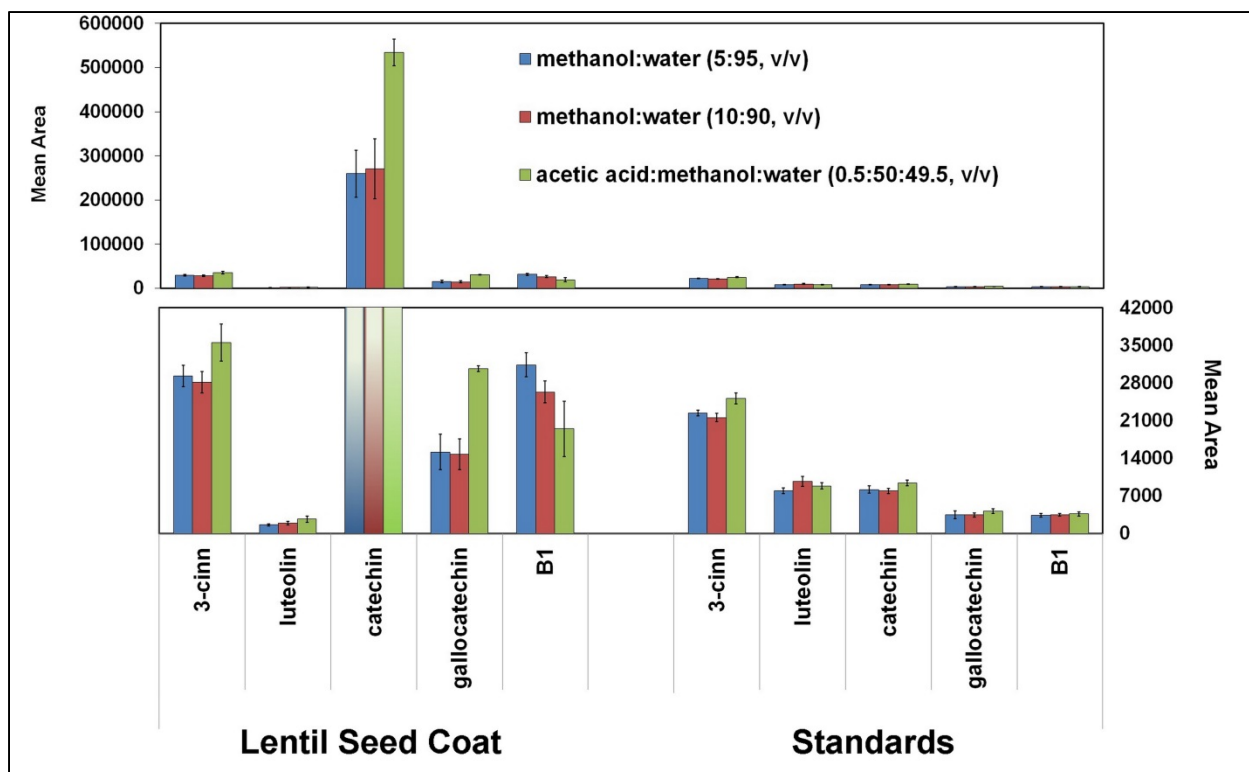


Figure IV. Effect of the reconstitution solvent on the peak areas of phenolic compounds obtained from solutions prepared from lentil seed coat extractions (left) and chemical standards (right). 3-Cinn represents 3-hydroxy-4-methoxy-cinnamic acid. Reconstitution solvents were MeOH: H<sub>2</sub>O (5:95, v/v), MeOH: H<sub>2</sub>O (10:90, v/v), and acetic acid: MeOH: H<sub>2</sub>O (0.5:50:49.5, v/v/v). A Genesis C18 column with a flow rate of 0.2 mL/min was used, with solvents and gradients as described in §2.2.

### Extraction solvent

Several solvents have been used for extraction of phenolic compounds from lentil seeds, including methanol (Tsopmo and Muir, 2010), acidified methanol (Dueñas et al., 2002; Escarpa et al., 2002; López-Amorós et al., 2006; Takeoka et al., 2005), acetone (Amarowicz et al., 2009; Amarowicz et al., 2010; Bartolomé et al., 1997), and acidified acetone (Xu and Chang, 2010; Zou et al., 2011), yet a thorough experiment to compare their effectiveness in extracting different sub-classes of phenolic compounds has not been reported. Based on these previous studies, five different extraction solvents

comprised of acetone, methanol, and acidified forms with acetic acid and/or hydrochloric acid were considered. The peak areas per sample weight were obtained for a range of phenolic compounds (Figure V). Areas were similar for all solvents except methanol/HCl: water. Use of acidified methanol with HCl increased the area/weight for catechin and gallic catechin. As this solvent increased the extraction of flavan-3-ols, it would be expected to also improve extraction of proanthocyanidins, which are oligomers and polymers of flavan-3-ols. However, it led to decreased area/weight of (epi) catechin-glucoside and dimers and trimers of (epi) catechin (CC and CCC). A similar trend was observed for prodelphinidin dimers and trimers, which have (epi)catechin and (epi)gallic catechin (GC, GGC, and GCC) building blocks. Xu and Chang (Xu and Chang, 2007) found that both acetone and acidified acetone extracted more total condensed tannins than other solvents, but separate analyses of monomers and/or oligomers were not discussed. As described earlier, a large amount of proanthocyanidin oligomers and polymers exist in lentil seed (Dueñas et al., 2003) and our goal is to accurately reflect the relative amounts of these phenolic compounds in our measurements. Because the presence of acid appears to cause some breakdown of these species (and also the sugar containing (epi) catechin) without providing any apparent benefits, acidified solvents were not considered further. As the area/weight of the other extraction solvents were similar, acetone: H<sub>2</sub>O (70: 30, v/v) was chosen because it improved the rate at which the samples could be dried down.

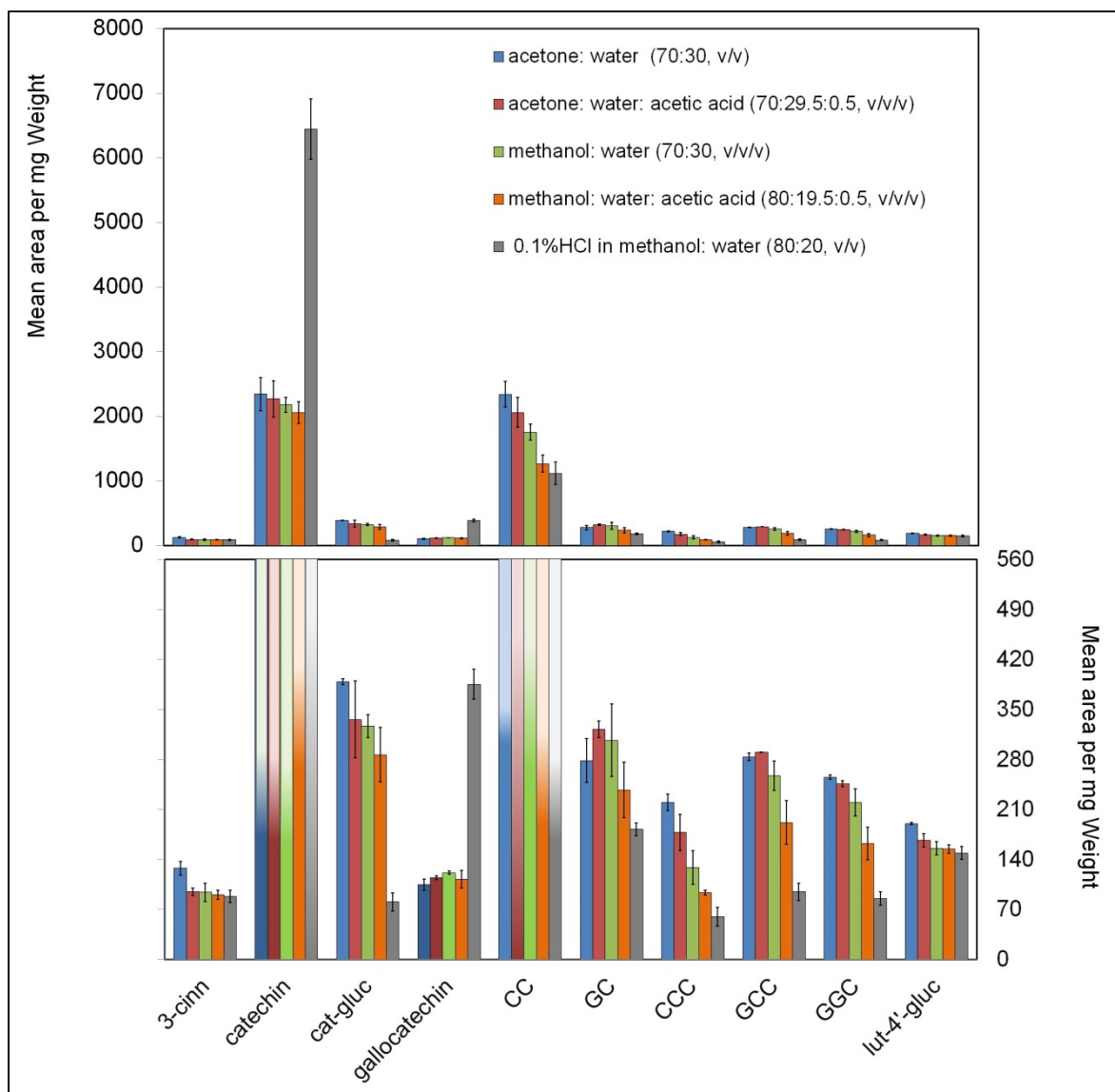


Figure V. Effect of extraction solvent on area/ mg weight of different phenolic compounds; error bars reflect the standard deviation of three replicates. 3-Cinn, cat-gluc, C, G, and lut-4'-gluc represent 3-hydroxy-4-methoxy-cinnamic acid, (epi) catechin-glucoside, (epi) catechin, (epi) gallo catechin, and luteolin-4'-O-glucoside, respectively. A Genesis C18 column with a flow rate of 0.2 mL/min was used, with solvents and gradients as described in §2.2.

Optimization of chromatographic conditions

Type of column

Different types of C18 fully porous columns have been used for analyzing phenolic compounds in lentil, including Zorbax Stablebond C18 (Xu and Chang, 2010; Zou et al., 2011), Nova Pak C18 (Aguilera et al., 2010; Amarowicz et al., 2009; Bartolomé et al., 1997; Dueñas et al., 2002; Dueñas et al., 2003), Nucleosil C18 (Escarpa et al., 2002), and Symmetry C18 (Tsopmo and Muir, 2010) columns. With the exception of the Nova Pak C18 column (4  $\mu\text{m}$ ), all employ a 5  $\mu\text{m}$  particle size. Although superficially porous (core-shell) columns are comparable to Ultra-HPLC columns and superior to HPLC in terms of speed and efficiency (Guillarme et al., 2010), we could find no report of core-shell column use with C18 or other stationary phases (e.g., PFP) for analyzing lentil seed coats. Here, core-shell columns (Kinetex C18 and PFP) with a 2.6  $\mu\text{m}$  particle size were compared with a fully porous column with a 4  $\mu\text{m}$  particle size (Genesis C18). Unlike conventional C18 columns, the Kinetex PFP has a different selectivity because it contains a PFP phase that provides aromatic and polar selectivity by incorporating fluorine atoms on the phenyl ring. Isoflavones in rat serum were resolved better using a core-shell PFP column than a C18 column; the fluorinated phenyl ring on the stationary phase of the PFP column appears to be more hydrophilic than the C18 chain and, as a result, has greater interaction with hydrophobic isoflavones (Gavina et al., 2013).

Using the gradient with a maximum of 50% solvent B, as described in Table III, the performance of the three columns was tested; the results are summarized in Table IV. The earliest eluting compound (gallic acid) came out near to the void volume in both the Genesis and Kinetex C18 columns, whereas gallic acid was retained longer in the Kinetex PFP column (Table IV). Overall, the two core-shell columns (Kinetex C18 and PFP) retained phenolic compounds longer than the conventional C18 column

(Genesis C18). Peak widths of the core-shell columns were similar and significantly narrower than for the Genesis C18. In addition to the smaller pore size, the inner core of the core-shell columns is solid fused silica; because analytes cannot penetrate, the diffusion path is shorter and results in sharper peaks and better resolution compared with fully porous particles with similar diameters (Guillarme et al., 2010). For isomeric compounds (compounds with the same  $m/z$ ), separation of these compounds by LC is critical. An example of isomeric compounds is kaempferol-3-*O*-glucoside and luteolin-4'-*O*-glucoside. For the different isomeric compounds present in our mixture, the best resolution was achieved with the Kinetex PFP column. Figure VI shows chromatograms of the traces for the transition  $449.3 > 287.3$  (luteoin-7-*O*-glucoside, luteoin-4'-*O*-glucoside, and kaempferol-3-*O*-glucoside). The compounds eluted in the same order in all three columns (confirmed with individual standards in separate runs); however, all three compounds are baseline resolved using the Kinetex PFP (Figure VI.a) but the peaks for luteoin-4'-*O*-glucoside and kaempferol-3-*O*-glucoside overlap using the C18 columns (Figures VI.b and VI.c). The change in selectivity of the fluorinated phenyl ring on the stationary phase of the PFP column enabled this separation. Consequently, the Kinetex PFP column was used for the remainder of the study.

Table IV. Effect of column type on the capacity factor (K'), peak width, and resolution for Genesis C18, Kinetex C18 and Kinetex PFP columns

phenolic compound	K'			peak width (sec)			Resolution		
	Genesis C18	kinetex C18	kinetex PFP	Genesis C18	kinetex C18	kinetex PFP	Genesis C18	kinetex C18	kinetex PFP
Gallocatechin	1.2	2	3	20.2	14.2	10	11.7	28.6	22.5
B1	3.9	7.5	6.3	24.6	15.6	13.8	2.1	1.9	14.4
Catechin	4.6	7.9	8.4	36.8	16.4	10.7	15.8	29.3	27.1
malvidin-3-O-galactoside	12.5	15.8	14.2	17.9	15.5	10.6	1.1	6	13
myricetin-3-O-rhamnoside	12.7	16.9	15.7	17.3	13.2	8	1.3	4.7	4.1
quercetin-3-O-galactoside	13	17.6	16.1	22.8	8.8	7.9	1.5	2.2	3.3
quercetin-3-O-glucoside	13.4	17.8	16.4	22.5	9	8.1	0.6	0.8	7.8
luteolin-7-O-glucoside	13.5	17.9	17.2	22.6	9.8	7.7	5	16.1	7.3
kaempferol-3-O-glucoside	14.8	19.7	17.9	24.7	7.7	8.5	1.6	1.4	16.3
luteolin-4'-O-glucoside	15.1	19.9	19.7	20.7	9.7	9.8	4.4	24.3	11.4
4-hydroxy-6-methylcoumarin	16.4	23.6	21	32	14.1	8.9	3.1	2.8	22.4
Luteolin	17.3	24.1	23.6	26.3	13.7	9.5	1	6.9	10.9
Naringenin	17.6	25.3	24.9	27.9	13.4	10.1	27	54.9	45.1
Flavone	25	35.3	30.6	24.7	14.6	10.5			

Solvent A was H<sub>2</sub>O: FA (95:5, v/v) and solvent B was H<sub>2</sub>O: ACN: FA (5:90:5, v/v/v) with the gradient utilized 50% B maximum

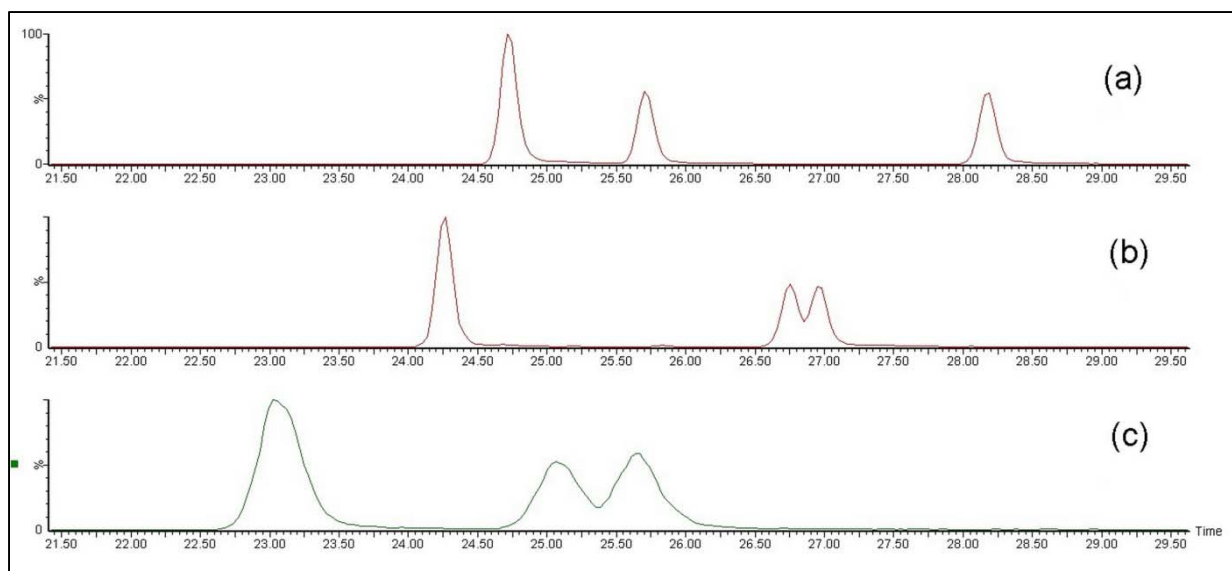


Figure VI. Chromatograms monitoring  $m/z$  449.3>287.3 with a) Kinetex PFP columns, b) Kinetex C18, and c) Genesis C18; peaks are luteolin-7-*O*-glucoside, kaempferol-3-*O*-glucoside, and luteolin-4'-*O*-glucoside (from left to right). Solvent A was H<sub>2</sub>O: FA (95: 5, v/v), solvent B was H<sub>2</sub>O: ACN: FA (5:90:5, v/v/v), and the flow rate was 0.2 mL/min. A linear gradient from 5 to 50% B from 5 to 50 min and an isocratic gradient of 50% B from 50.1 to 55 min were used, as shown in Table III.

#### Mobile phase gradient and composition

The next step involved optimizing the chromatographic conditions for analyzing lentil seed coats. Mobile phases consisting of mixtures of H<sub>2</sub>O: ACN:acetic acid (Aguilera et al., 2010; Amarowicz et al., 2009; Amarowicz et al., 2010; Dueñas et al., 2002; López-Amorós et al., 2006), H<sub>2</sub>O: ACN: trifluoroacetic acid (TFA) (Tsopmo and Muir, 2010), or H<sub>2</sub>O: MeOH: FA (Takeoka et al., 2005) have been used for the chromatographic analysis of phenolic substances in lentil. ACN was employed in this study as the organic solvent since it showed better separation and had a shorter chromatographic run time for tomato flavonoids compared with methanol (Biesaga et al., 2009). FA was selected as the acid since it can achieve low pH values (2-3) without causing electrospray ionization suppression issues inherent with TFA (Kuhlmann et al., 1995). To optimize the amount of FA in the mobile phase, four different percentages of formic acid in



solvent A (H<sub>2</sub>O: FA (v/v)) and solvent B (H<sub>2</sub>O: ACN: FA (v/v/v)) were applied to determine the effect on the *K'* and selectivity of phenolic compounds: A (99.5:0.5), B (9.5:90:0.5), pH=2.33; A (99:1), B (9:90:1), pH=2.17; A (98:2), B (8:90:2), pH=2.02; and A (95:5), B (5:95:5), pH=1.82. Increasing the percentage of formic acid reduced the retention of phenolic compounds (Figure VII), with the effect being more dramatic for the highly hydrophilic compounds (i.e., flavan-3-ols and proanthocyanidins); with 5% formic acid, gallicocatechin eluted near the void volume. Adequate resolution was maintained at lower FA concentrations as the selectivity of critical pairs was virtually identical for different percentages of formic acid (data not shown). As a result, 0.5 and 1% FA were both suitable options to maintain good retention of the polar analytes. We selected H<sub>2</sub>O: FA (99:1, v/v) as the peak shapes for some compounds (e.g., malvidin-3-O-galactoside) were marginally better in 1% FA.

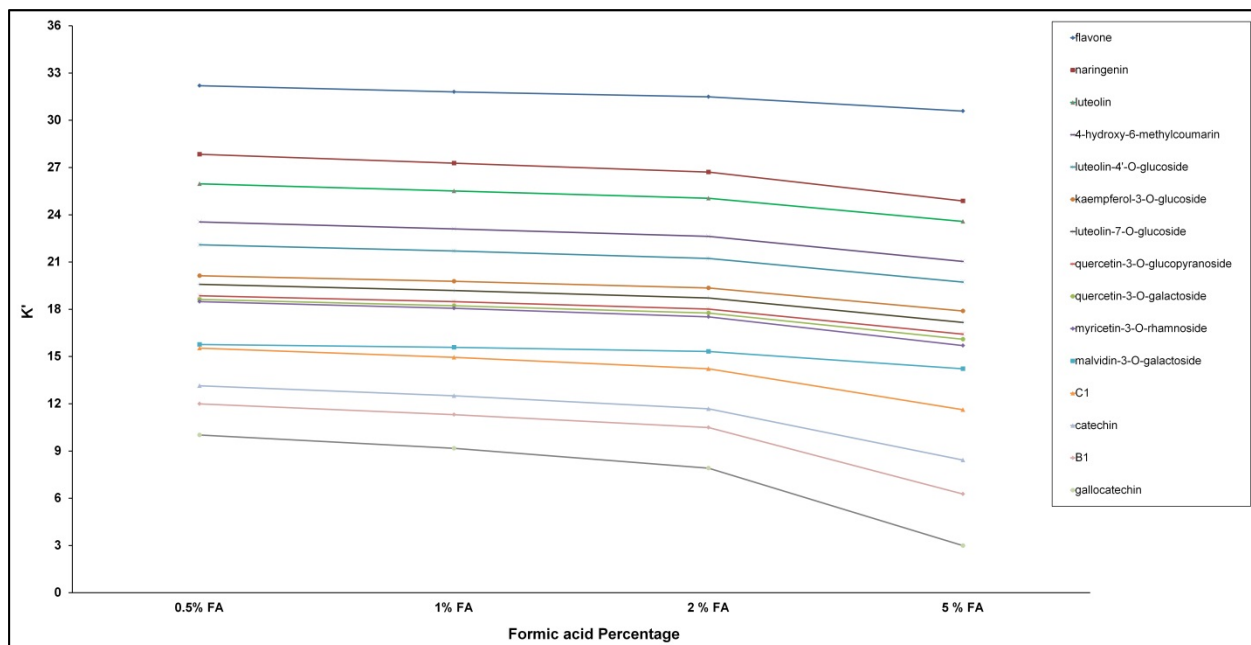


Figure VII. Effect of formic acid concentration on *K'* of phenolic standards; Solvent A (H<sub>2</sub>O: FA (v/v)) was 99.5:0.5 (pH=2.33), 99:1 (pH=2.17), 98:2 (pH=2.02), or 95:5 (pH=1.82), as shown on x-axis. A Kinetex PFP column with a flow rate of 0.2 mL/min was used. A linear gradient from 5 to 50% B from 5 to 50 min and an isocratic gradient of 50% B from 50.1 to 55 min were used, as shown in Table III.

In separate runs, the slope of the linear gradient (5-50 min) was changed such that the endpoint (and subsequent isocratic gradient from 50-55 min) changed from 30 to 90% solvent B. ACN is a common organic modifier for extraction of phenolic compounds and its use has resulted in better separation than that achieved with methanol (Biesaga et al., 2007). Adjusting the maximum % of the organic solvent affected the retention (Figure VIII.a) and separation of critical isomers (Figure VIII.b). Specifically, a higher percentage of organic solvent for the endpoint of the gradient reduced the analysis time but at the expense of decreasing the resolution of critical pairs (Figure VIII.b). In the run that used 30% ACN as the endpoint of the linear gradient, the late eluting compound (flavone) came out after 60 min (Figure VIII.a). Therefore, a modified concentration of the mobile phase was introduced. The same gradient was used for the first 44 min and then the amount of ACN increased from 26.7% at 44 min (the value of B at that time in the gradient) to 50% by 50 min. Addition of this step resulted in elution of flavone from the Kinetex PFP column before 55 min while not affecting the critical pairs of phenolic compounds (which eluted before 44 min).

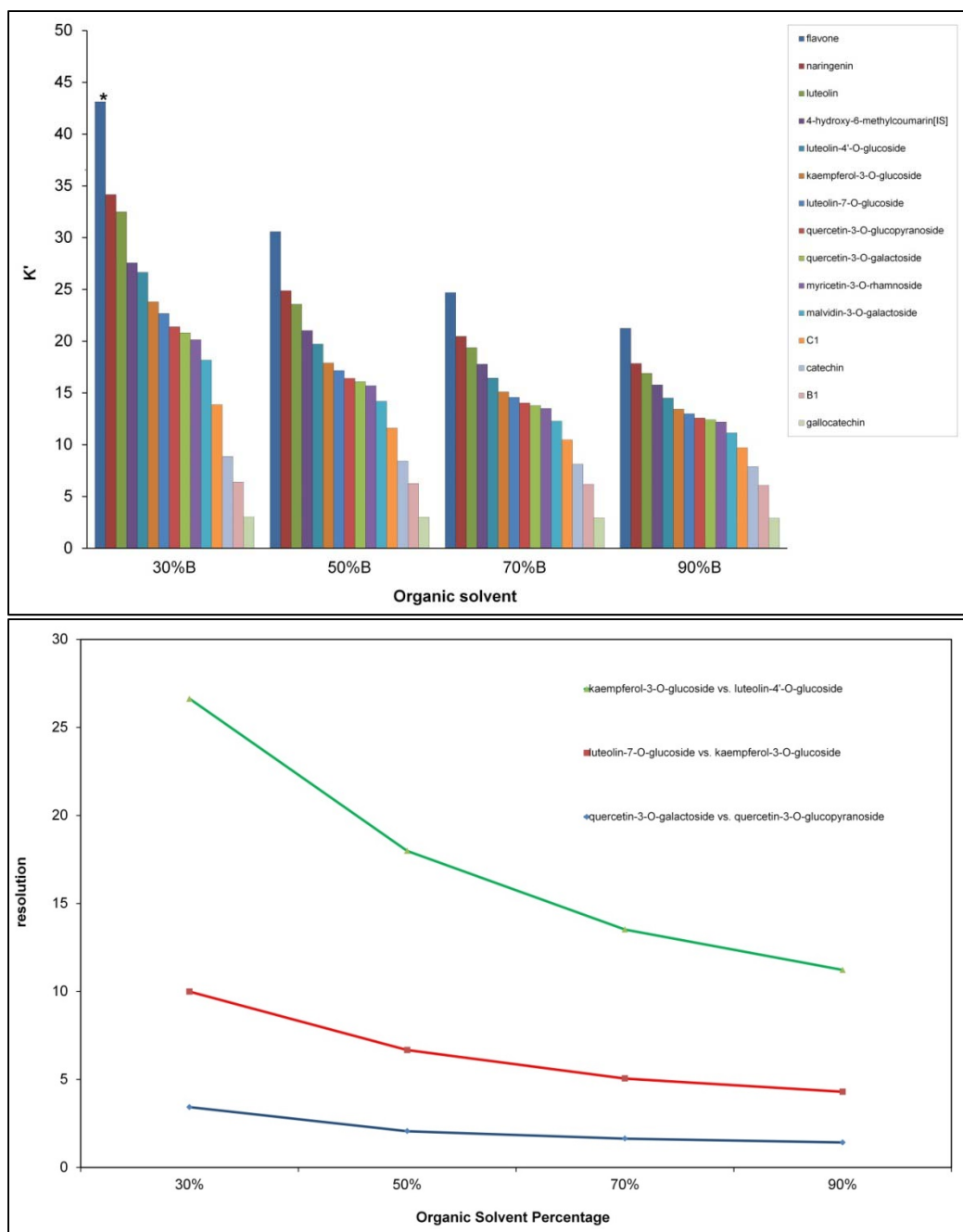


Figure VIII. a) Effect of organic solvent percentage (30, 50, 70, and 90% B) on capacity factor of phenolic compounds (\* minimum  $K'$  for flavone if it came out at 60 min). b) Effect of acetonitrile concentration on  $K'$  of phenolic standards. For both (a) and (b), a Kinetex PFP column, 5% FA, and a flow rate of 0.2 mL/min were used.

Effect of flow rate. As a final step in the method development, the flow rate was optimized. To minimize the analysis time, the flow rate was increased to the maximum that could be reliably achieved without the concern of over-pressuring the column. Our

final flow rate of 0.4 mL/min was double the flow rate used in previous optimization stages, and enabled the gradient interval to be decreased by half without any degradation in separation. Figure IX shows the final gradient for the optimized chromatographic conditions using phenolic compound chemical standards.

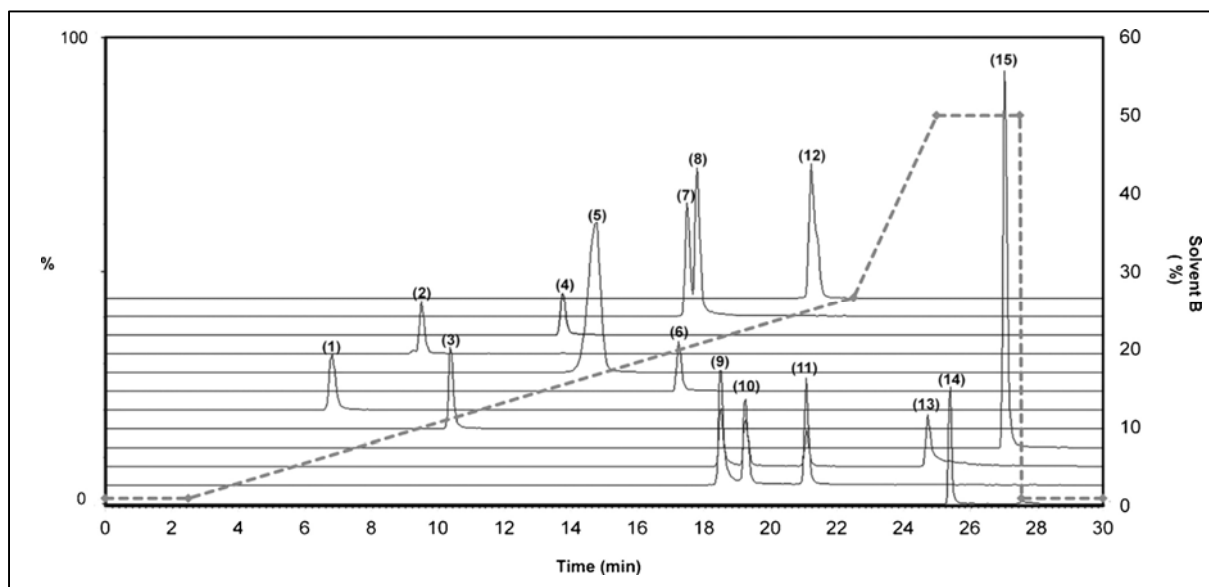


Figure IX. Chromatogram of phenolic standards applying the optimized method: (1) galocatechin, (2) B1, (3) catechin, (4) C1, (5) Malvidin-3-O-galactoside, (6) myricetin-3-O-rhamnoside, (7) quercetin-3-O-galactoside, (8) quercetin-3-O-glucopyranoside, (9) luteolin-7-O-glucoside, (10) kaempferol-3-O-glucoside, (11) luteolin-4'-O-glucoside, (12) 4-hydroxy-6-methylcoumarin[IS], (13) luteolin, (14) naringenin, and (15) flavone. Chromatograms of each transition were overlaid. A Kinetex PFP column was used with a flow rate of 0.4 mL/min. Solvent composition was solvent A (H<sub>2</sub>O: FA (99:1, v/v)) and solvent B (H<sub>2</sub>O: ACN: FA (9:90:1, v/v/v)), with the following gradient: 0 min (1% B), 2.5 min (1% B), 22.5 min (26.7% B), 25.0 min (50% B), 27.5 min (50% B), 27.55 min (1% B), 30 min (1% B).

Applying optimized method to the analysis of different lentil genotypes

Within the mass spectrometry software, three functions were defined with time ranges of 0-10.5, 8-16.5, and 16-30 min. Each function contained 6-7 transitions, which made it efficient enough to sample at least eight points per chromatographic peak. The optimized extraction and chromatographic methods were applied to three genotypes of

lentil with different seed coat colours. Figure X shows the total ion current (TIC) chromatograms of a) black (Indian head), b) green (964a-46), and c) grey (CDC Maxim) lentil seeds; peak intensities were normalized to the most intense peak in the black lentil. The seed coat of lentil is enriched with proanthocyanidin oligomers and polymers (Dueñas et al., 2003), which can be observed clearly in the TIC chromatograms of all genotypes (Figure X.a,b,c). Peaks for gallocatechin (peak 1), catechin (peak 8), (epi) catechin-glucoside (peak 6), GC (peak 3), B1 (peak 5), CC (peak 7), GGC (peak 2), GCC (peak 4), and myricetin-3-O-rhamnoside (peak 9) are very similar for the black, green and grey seed coat genotypes. The major differences are related to the flavones luteolin-4'-O-glucoside (peak 10) and luteolin (peak 12), which are much more concentrated in the black seed coat genotype compared with the green and grey seed coat genotypes.

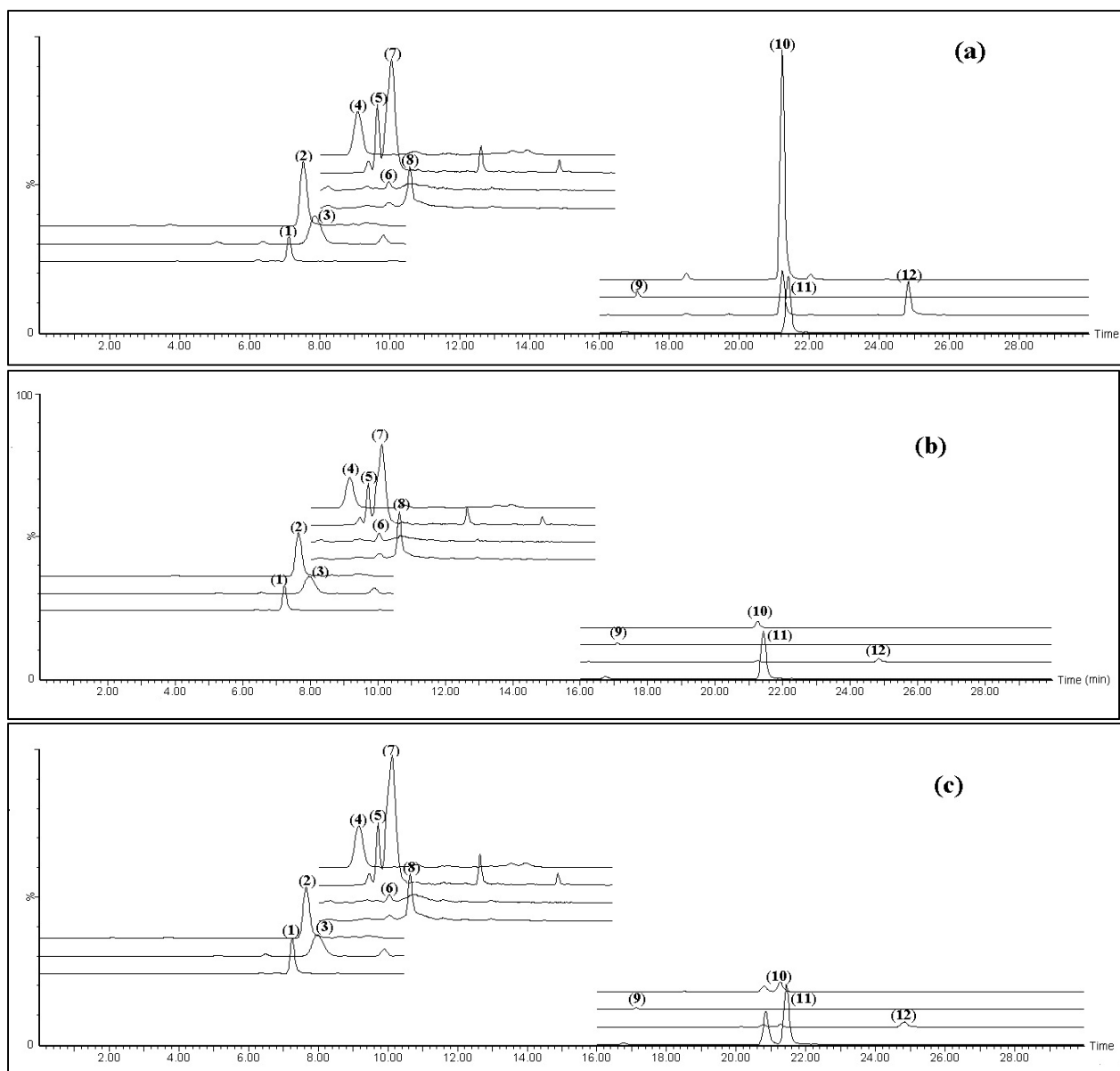


Figure X. Chromatograms of phenolic compounds in the seed coats of a) Indian head, b) 964a-46, and c) CDC Maxim lentil obtained using the optimized method: (1) gallic catechin, (2) GGC, (3) GC, (4) GCC, (5) B1, (6) (epi) catechin-glucoside, (7) CC, (8) catechin, (9) myricetin-3-O-rhamnoside, (10) luteolin-4'-O-glucoside, (11) 4-hydroxy-6-methylcoumarin, and (12) luteolin.

## Conclusions

A systematic approach was used to develop and optimize an LC-MS method for the analysis of phenolic compound composition in lentil seed coats. The optimum MS conditions (CV and CE), molecular and fragment ions,  $R_t$ , and UV-VIS wavelength were

determined for 18 different phenolic standards using LC-MS with UV-VIS detection. The use of long duration maceration was found to be unnecessary for extracting phenolic compounds from lentil seed coats; 1 h maceration times provided similar results to those obtained using longer times. Three extraction steps gave good recovery and were used in this study; however, only one extraction would be required to compare levels among the different genotypes if  $^{13}\text{C}$  or  $^2\text{D}$ -labelled internal standards for each analyte were added to the extraction solvent. Alternatively, only one labelled standard or a suitable analog would be required for each compound sub-class if the extraction efficiencies are similar within a sub-class; we are currently investigating this approach.

The use of acidified reconstitution or extraction solvents resulted in a breakdown of the polymeric phenolic compounds into smaller subunits. When comparing genotypes, the composition of the phenolic compounds should ideally not be altered by the analytical method. As a result, a non-acidified extraction solvent composed of acetone:  $\text{H}_2\text{O}$  (70:30, v/v) and a non-acidified reconstitution solvent of MeOH:  $\text{H}_2\text{O}$  (10:90, v/v) were chosen for extraction and reconstitution of phenolic compounds, respectively.

Both core-shell columns retained early eluting compounds longer than the Genesis C18 column, with the Kinetex PFP column having the longest retention time. The isomeric compounds were best resolved using the Kinetex PFP column. Higher percentages of formic acid in the mobile phase (>1%) should be avoided as this results in polar phenolic compounds eluting near the void volume. The concentration of the organic solvent was also optimized to maximize the resolution of critical isomers. Through this process and further optimization of the flow rate, a 30 min LC-MS method was developed.

The LC-MS method was successfully applied to the analysis of three lentil genotypes (black, green and grey seed coat). The intensities of oligomeric flavan-3-ols were very similar for all three genotypes, while luteolin and its glycosylated form were more concentrated in the black seed coat genotype. This illustrates that the developed method could be successfully applied to phenolic compound profiling of various lentil genotypes and this improved phenotyping capability used to develop genotyping systems for breeding strategies with objectives related to altering or improving the spectrum of phenolic compounds in lentils.

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Appendix 2. P-values from mixed model ANOVA F-test for the effect of cotyledon colour and seed coat colour on phenolic compounds in SPG location, where numbers represent the mean square of area ratio per mg sample.

	Df	Resveratrol-3-β-mono-D-glucoside	(+)-Catechin	(-)-Gallocatechin	Procyanidin B1	GGC_I	GGC_II	GGG	GGCC_I	GGCC_II	GGGC_I	GGGC_II	GGGC_III	GCCCC_I	GGGCC_I	GGGCC_II
Seed Coat Colour	3	0.183 **	0.059 ***	0.031 * ***	0.084 ***	<.001 ***	0.003 **	0.015 *	0.006 **	0.018 *	0.06 ***	0.003 **	0.002 **	0.057 ***	0.018 *	0.288 ***
Cotyledon Colour	1	0.55	0.06	0.38	0.13	0.08	0.21	0.12	0.15	0.35	0.23	0.22	0.09	0.32	0.09	0.64
Seed coat * Cotyledon	3	0.183 **	0.059 ***	0.031 * ***	0.084 ***	<.001 ***	0.003 **	0.015 *	0.006 **	0.018 *	0.06 ***	0.003 **	0.002 **	0.057 ***	0.018 *	0.288 ***

\*, \*\*, \*\*\*, denote significance at the 0.05, 0.01, and 0.001 probability levels, respectively.  
C and G stand for catechin/epicatechin and gallocatechin/epigallocatechin, respectively.

Appendix 3. P-values from mixed model ANOVA F-test for the effect of cotyledon colour and seed coat colour on phenolic compounds in STH location, where numbers represent the mean square of area ratio per mg sample.

	Df	Resveratrol-3-β-mono-D-glucoside	(+)-Catechin	(-)-Galocatechin	Procyanidin B1	GGC_I	GGC_II	GGG	GGCC_I	GGCC_II	GGGC_I	GGGC_II	GGGC_III	GCCCC_I	GGGCC_I	GGGCC_II
Seed Coat Colour	3	0.751	0.75	0.86	0.497	0.003	0.004	<.001	0.031	0.018	0.058	0.002	0.023	0.334	0.108	0.127
Cotyledon Colour	1	0.283	<.001	<.001	0.289	0.053	0.257	0.019	0.002	<.001	0.005	0.112	0.009	0.011	0.007	<.001
Seed coat * Cotyledon	3	0.052	<.001	<.001	0.057	0.091	0.091	0.44	1	0.35	0.99	0.96	0.88	0.25	0.6	0.76
			***	***		**	**	***	*	*	**	**	*	*	**	***

\*, \*\*, \*\*\*, denote significance at the 0.05, 0.01, and 0.001 probability levels, respectively.

C and G stand for catechin/epicatechin and galocatechin/epigallocatechin, respectively.

Appendix 4. P-values from mixed model ANOVA F-test for the effect of cotyledon colour and seed coat colour on phenolic compounds in SPG and STH locations, where numbers represent the mean square of area ratio per mg sample.

	Df	Vanillic acid-4- $\beta$ -D-glucoside	Luteolin-4'-O-glucoside	Kaempferol-3-O-robinoside-7-O-rhamnoside	Kaempferol dirutinoid	Myricetin-3-O-rhamnoside	Quercetin-3-O-rhamnoside	Catechin-3-glucoside	CC-gallate	GC_I	GC_II	GCC_I	GCC_II	GCC_III	GGC_III	CCCC_I	CCCC_II	GCCC_I	GCCC_II	CCCCC	GCCCC_II
Seed Coat Colour	3	0.242	<.001 ***	0.004 **	0.539	<.001 ***	<.001 ***	<.001 ***	<.001 ***	<.001 ***	<.001 ***	<.001 ***	<.001 ***	<.001 ***	<.001 ***	<.001 ***	<.001 ***	<.001 ***	<.001 ***	<.001 ***	<.001 ***
Cotyledon Colour	1	0.24	0.69	0.2	0.4	0.23	0.59	0.72	0.18	0.22	0.06	0.15	0.37	0.22	0.1	0.74	0.94	0.42	0.63	0.78	0.69
Seed coat * Cotyledon	3	0.212	0.039 *	0.001 **	0.651	0.117	0.502	0.136	0.002 **	<.001 ***	<.001 ***	<.001 ***	<.001 ***	<.001 ***	<.001 ***	0.028 *	0.014 *	0.001 **	0.001 **	0.03 *	0.097

\*, \*\*, \*\*\* denote significance at the 0.05, 0.01, and 0.001 probability levels, respectively.

C and G stand for catechin/epicatechin and galocatechin/epigallocatechin, respectively.

Appendix 5 (a). Analysis of variance for 10 brown normal *Ggc Tgc Tan* lines in three replicates.

Residual	Genotypes	Df
20	9	
0.01667	0.01348	Vanillic acid-4- $\beta$ -D-glucoside
1.171	10.536 ***	Kaempferol-3-O-robinoside-7-O-rhamnoside
1642.2	511.1	Kaempferol dirutinose
0.000667	0.0023704 **	Resveratrol-3- $\beta$ -mono-D-glucoside
0.001667	0.003704	Myricetin-3-O-rhamnoside
0.0437	0.383 ***	Luteolin-4'-O-glucoside
0.004333	0.002815	Quercetin-3-O-rhamnoside
0.01	0.01707	Catechin
0.011	0.02607	Gallocatechin
0.008	0.006519	Procyanidin B1
6.758	9.752	Catechin-3-glucoside
0.06016	0.05715	C-C-gallate
0.877	0.7052	GC_I
0.171	2.768 ***	GC_II
90.28	67.1	GCC_I
5.822	4.005	GCC_II
0.01123	0.01831	GCC_III
2.697	1.745	GGC_I
1.253	1.027	GGC_II
59.96	44.74	GGC_III
0.1732	0.1305	GGG
4.681	10.008	CCCC_I
4.223	8.333	CCCC_II
3.486	4.071	GCCC_II
30.13	15.51	GGCC_I
2.824	0.864	GGCC_II
1.528	1.03	GGGC_I
0.6985	0.4389	GGGC_II
4.778	4.673	GGGC_III
0.6791	1.4968	CCCCC
1.877	2.557	GCCCC_I
1.178	2.061	GCCCC_II
1.739	0.4771	GGGCC_I
0.6072	0.3398	GGGCC_II

\*\* and \*\*\* denote significance at the 0.01 and 0.001 probability levels, respectively.

Appendix 5 (b). Analysis of variance for 10 gray zero-tannin *Ggc Tgc tan* lines in three replicates.

	Df	
Genotypes	9	0.01496
Residual	20	0.016
		Vanillic acid-4- $\beta$ -D-glucoside
	33.6	33.47
		Kaempferol-3-O-robinoside-7-O-rhamnoside
	15443	46725
		Kaempferol dirutinoside
	0.000333	0.000333
		Luteolin-4'-O-glucoside



Appendix 6. Effect of storage of different plant materials on storage ratio for samples analyzed by chromatographic (thin layer chromatography, HPLC, or LC-MS) and spectrophotometric (Prussian blue, Folin-Ciocalteu's Phenol Reagent, or vanillin assay) methods

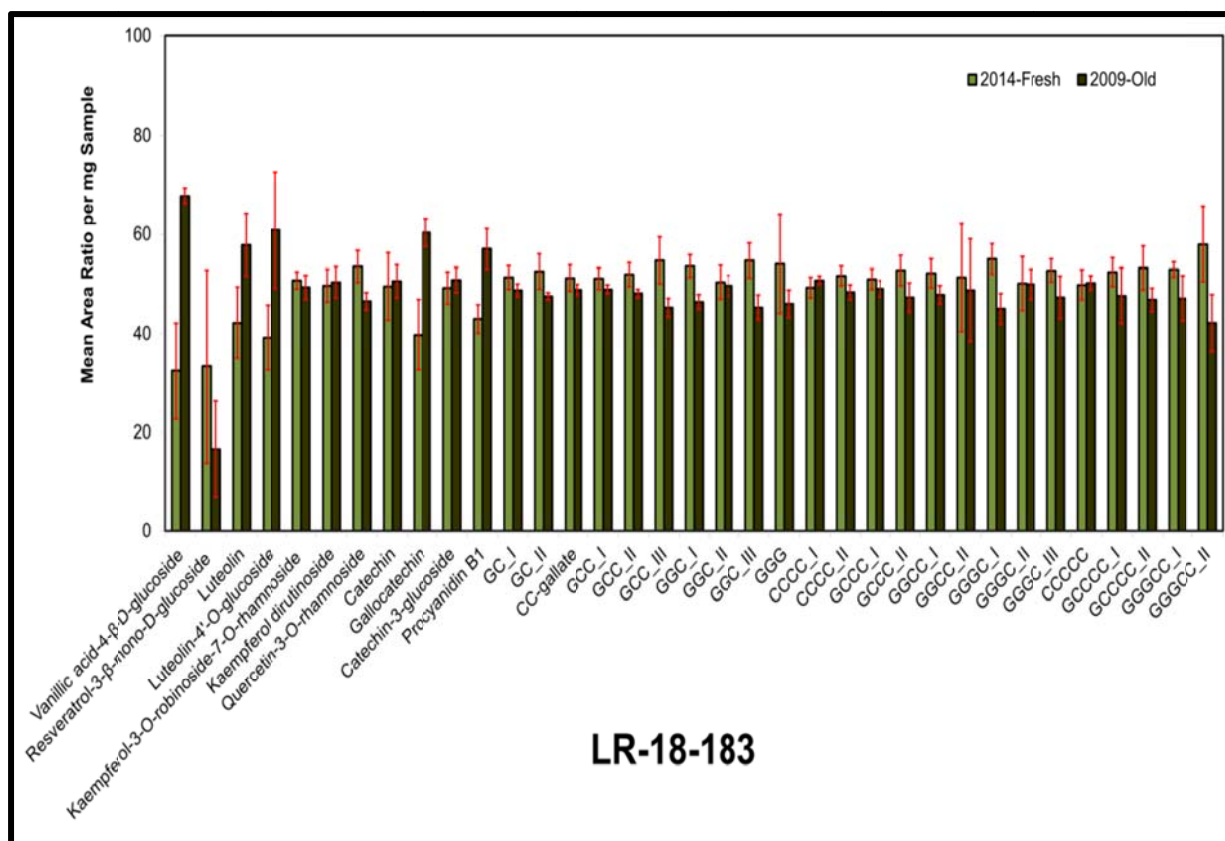
Sub-class	Compound	Plant	Mean Storage Ratio $\pm$ SD	Analytical Method	Reference
Anthocyanidins	Cyanidin-3-O-glucoside	Soybean	0.9	Chromatographical	(Zhou et al., 2010)
Anthocyanidins	Delphinidin-3-O-glucoside	Soybean	0.8	Chromatographical	(Zhou et al., 2010)
Flavonols	Kaempferol	Pinto bean	0.7 $\pm$ 0.1	Chromatographical	(Beninger et al., 2005)
Flavonols	Kaempferol-3-O-acetylglucoside	Pinto bean	1.3 $\pm$ 0.1	Chromatographical	(Beninger et al., 2005)
Flavonols	Kaempferol-3-O-glucoside	Pinto bean	1.6 $\pm$ 0.9	Chromatographical	(Beninger et al., 2005)
Flavonols	Kaempferol-3-O-glucosylxylose	Pinto bean	0.9 $\pm$ 0.4	Chromatographical	(Beninger et al., 2005)
Phenolic acids	Ellagic acid	Strawberry	4.2 $\pm$ 2.5	Chromatographical	(Aaby et al., 2007)
Phenolic acids	Ferulic acid	Dry bean	3.1 $\pm$ 2.3	Chromatographical	(Srisuma et al., 1989)
Phenolic acids	Sinapic acid	Dry bean	1.5 $\pm$ 0.7	Chromatographical	(Srisuma et al., 1989)
Flavan-3-ols	(-)-Epicatechin	Apple	0.8 $\pm$ 0.2	Chromatographical	(Carbone et al., 2011)
Flavan-3-ols	(-)-Epicatechin	Soybean	0.4	Chromatographical	(Zhou et al., 2010)
Flavan-3-ols	(+)-Catechin	Apple	1 $\pm$ 0.4	Chromatographical	(Carbone et al., 2011)
Flavan-3-ols	(+)-Catechin	Strawberry	0.3 $\pm$ 0.1	Chromatographical	(Aaby et al., 2007)
Proanthocyanidins	Procyanidin dimers	Blueberry	0.5 $\pm$ 0.2	Chromatographical	(Howard et al., 2010)
Proanthocyanidins	Procyanidin trimers	Blueberry	0.3 $\pm$ 0.2	Chromatographical	(Howard et al., 2010)
Proanthocyanidins	Procyanidin tetramers	Blueberry	0.3 $\pm$ 0.1	Chromatographical	(Howard et al., 2010)
Proanthocyanidins	Procyanidin pentamers	Blueberry	0.3 $\pm$ 0.2	Chromatographical	(Howard et al., 2010)
Proanthocyanidins	Procyanidin hexamers	Blueberry	0.3 $\pm$ 0.1	Chromatographical	(Howard et al., 2010)
Proanthocyanidins	Procyanidin heptamers	Blueberry	0.3 $\pm$ 0.1	Chromatographical	(Howard et al., 2010)
Proanthocyanidins	Procyanidin octamers	Blueberry	0.3	Chromatographical	(Howard et al., 2010)

Appendix 6. Continued

Sub-class	Compound	Plant	Mean Storage Ratio $\pm$ SD	Analytical Method	Reference
Total	Total non-tannins	Dry bean	0.8	Spectrophotometrical	(Martín-Cabrejas et al., 1997)
Total	Total non-tannins	Faba bean	0.8	Spectrophotometrical	(Nasar-Abbas et al., 2009)
Total	Total free phenolics	Faba bean	0.9	Spectrophotometrical	(Nasar-Abbas et al., 2009)
Total	Total phenolics	Alfalfa	1.4	Spectrophotometrical	(Cakmak et al., 2010)
Total	Total phenolics	Dry bean	0.9	Spectrophotometrical	(Martín-Cabrejas et al., 1997)
Total	Total phenolics	Lentil	0.24	Spectrophotometrical	(Pirhayati et al., 2011)
Total	Total phenolics	Pinto bean	0.2	Spectrophotometrical	(Pirhayati et al., 2011)
Total	Total proanthocyanidins	Lentil	0.75	Spectrophotometrical	(Nozzolillo and Bezada, 1984)
Total	Total proanthocyanidins	Faba bean	0.2	Spectrophotometrical	(Nasar-Abbas et al., 2009)
Total	Total proanthocyanidins	Faba bean	0.9	Spectrophotometrical	(Mareuardt et al., 1978)
Total	Total proanthocyanidins	Pinto bean	0.7	Spectrophotometrical	(Beninger et al., 2005)
Total	Total tannins	Dry bean	7 $\pm$ 3.1	Spectrophotometrical	(Martín-Cabrejas et al., 1997)
Total	Total tannins	Faba bean	0.9	Spectrophotometrical	(Nasar-Abbas et al., 2009)

Storage ratio values were mostly obtained from the Phenol-Explorer web database (Rothwell et al., 2013) or calculated according to the following equation;

$$SR = \frac{\text{Quantity of phenolic compounds in stored plant material}}{\text{Quantity of phenolic compounds in fresh plant material}}$$



Appendix 7. Mean area ratio per mg of sample in LR-18-183 inbred line from 2009 and 2014. Error bars are standard errors of three replicates. C and G represent catechin/epicatechin and galocatechin/epigallocatechin.

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