IMPROVEMENT OF THE PHYSICOCHEMICAL ATTRIBUTES AND ANTIOXIDANT PROFILES FROM PULSE SEEDS THROUGH GERMINATION

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Edible pulse seeds are good sources of food ingredients. Germination has been regarded as an effective process to further improve nutrient digestibility and accessibility of pulse seeds. Our aim was to observe the effect of germination on proximate composition, physicochemical attributes, and phenolic profiles of chickpea (*Cicer arietinum* L.), lentil (*Lens culinaris* Merr.), and yellow pea (*Pisum sativum* L.). In addition, mechanisms of how germination affects the antioxidant activity of phenolic compounds were proposed.

Chemical composition, thermal, pasting, and moisture adsorption properties of pulse flours were investigated over 6 days of germination. Protein contents increased by 3 percent points for all pulses over germination. However, lentil had the highest protein content. Lipid contents decreased over germination with chickpea having the greatest decline, from 8.00 to 5.90 g/100g (d.b.). Total starch decreased in lentil and yellow pea during germination. Thermal properties of pulse flours changed slightly, while pasting properties varied among pulses. The highest final viscosities for chickpea, lentil, and yellow pea flours were 1061, 981, and 1052 cP and were observed after 2, 1, and 0 days of germination, respectively. Moisture adsorption isotherms showed improved water adsorption capabilities after germination.

Soluble free (SFPs) and polar soluble bound phenolic compounds (PSBPs) were extracted from germinated pulse seeds. Their antioxidant activities were evaluated using both the *in vitro* system and stripped soybean oil (SSO)-in-water emulsions. Liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometry and size-exclusion chromatography with multiangle-light-scattering and refractive-index detection were employed to analyze the phenolic composition and molar mass, respectively. Antioxidant activities of SFPs increased in both *in vitro* and SSO-in-water emulsion system, however, much

lower than those of PSBPs based on SSO-in-water emulsion system. The effect of germination on PSBPs in SSO-in-water emulsion varied between pulses. By virtue of chemometric analysis, nine phenolic compounds were speculated as the pivotal phenolic compounds responsible for the antioxidant activity of PSBPs. In particular, the molar masses of PSBPs had a positive relationship with their antioxidant activity. Protective and co-antioxidative principles were proposed as reasons for the variability of antioxidant activities of PSBPs in oil-in-water emulsions with germination.

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DEDICATION

I dedicate this work to my wife and daughter:

To my wife, Zhao Jin, without your encourage and support. This work would never have been accomplished or even attempted.

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CHAPTER 1. GENERAL INTRODUCTION

1.1. Introduction

Pulses are edible seeds from plants in the legume family (*Fabaceae*), which includes chickpeas (*Cicer arietinum*), lentils (*Lens culinaris*), dry peas (*Pisum sativum*), and dry beans (*Phaseolus vulgaris*. Pulses have been a staple food in India, the Middle East, Medi, and South America for a thousand years (Sokhansanj and Patil, 2003). Currently, Canada and the USA are important exporters of pulses. Commercially available pulse forms in North America include whole and split seeds, while alternative pulse consumption form is as nutritional and functional food ingredients (Frohlich et al., 2014).

Pulses are high-value ingredients because they are gluten-free food ingredients that are high in protein, fiber, minerals, but are low in fat. Based on these characteristics, pulses have great potential to be the nutritional and functional ingredients for food systems, such as cracker, pasta, bread, cereal, chips, and baked goods (Schierhorn, 2018). Although fewer cases have been reported, pulse extracts are another possible form for extending the application of pulses. Pulses contain beneficial phytochemicals, in particular phenolic compounds, that have been shown to promote human health by playing a role in reducing the risk of chronic health conditions such as cardiovascular disease, diabetes, and certain types of cancer (Frohlich et al., 2014). Pulse extracts are, therefore, anticipated to be functional food ingredients (Girón-Calle et al., 2004; Amarowicz et al., 2009). In particular, pulse extracts are anticipated to be considered as natural antioxidants

instead of traditional artificial antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), *tert*-butylhydroquinone (TBHQ) (Xu et al., 2019b).

Application of pulses as food ingredients is mainly challenged by four continuous limitations. First, the presence of antinutritional factors that include phytate, enzyme inhibitors (trypsin inhibitors, chymo-trypsin inhibitors, and α-amylase inhibitors), polyphenolics (mainly tannins), lectins, and saponins (Pattterson et al., 2017); second, lower nutritional value to certain groups of consumers compared to cereal grains, (e.g. wheat, rice, and millet), due to the existence of resistant starch and dietary fiber (Bravo et al., 1998); third, relatively lower activity of bioactive chemicals and antioxidants in most of pulses is observed compared to cereals (Pellegrini et al., 2006); last, the undesired physicochemical properties of pulse flours, such as water holding capacity, pasting properties, and thermal properties, are required to be improved for pulse to be used as food ingredients (Benítez et al., 2013).

Pulses can be processed to improve their nutritional and functional attributes by one or more methods. These include dehulling and milling, thermal treatments, germination, extrusion, fermentation, and irradiation (Patterson et al., 2017). Amongst these, germination is an economical and effective method to improve the quality of pulse ingredients. Seeds germination starts from the imbibition of water at a moderate temperature. Gibberellins are formed in the embryo and diffused into aleurone to promote synthesis of hydrolytic enzymes in the aleurone, such as α-amylase, β-glucanase, and protease (Kermode, 1990). Polysaccharides and proteins are decomposed by these enzymes into low molecular weight nutrients, e.g. starch is broken down to

dextrin, maltose, and glucose, while proteins are broken down to polypeptides, peptides and amino acids (Kaur et al., 2015). These low-molecular nutrients are also the precursors of many other secondary metabolite nutrients, such as vitamins and phenolic compounds (Value and Nemzer, 2019). In addition, germination can decrease the antinutrients of pulse seeds as reported by many researchers (Wang et al., 1997; Vidal-Valverde et al., 2002; Sangronis and Machado, 2007; Pal et al., 2017). For instance, Pal et al. (2017) reported the reduction of trypsin inhibitor activity and phytic acids by 71.3% and 59.4%, respectively, after two days of lentil germination.

However, mixed reports for the effects of germination on physicochemical attributes and phenolic compounds are noted in the literature (Jayathilake et al., 2018). Increased pasting properties occurred flours from lentils that had been germinated for two days (Ghumman et al., 2016) and mung beans germinated for three days (Kaur et al., 2015) were reported. Conversely, decreased pasting properties was reported for flours from black bean that had been germinated for 5 days (Guajardo-Flores et al., 2017) as well as, in germinated kidney bean, cowpea, and pigeon pea (Owuamanam et al., 2014). Phenolic compounds and their antioxidative activities from germinated pulses have been widely studied by researchers, although with varied results (Yeo and Shahidi, 2015; Gan et al., 2017; Pattterson et al., 2017; Jayathilake et al., 2018). Gan et al. (2016) observed that the total phenolic content, ferric-reducing antioxidant power (FRAP), and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) increased after 5-day germination of 12 pulse cultivars. In contrast, Yeo et al. (2015) reported that soluble phenolics showed a declining trend in total flavonoids content, 2,2-diphenyl-1-picrylhydrazyl (DPPH)

radical and ABTS radical scavenging capabilities in 4-day germinated lentils. The contradictory results may be attributed to the different types of pulses used, the definition of phenolic compounds classes, and evaluation systems for antioxidative activity (e.g. *in vitro* assay vs in food systems).

In summary, germination is a promising process for improving the quality of pulse seeds, which is evidenced by decreases of antinutrients, and improvements in nutrients (Wang et al., 1997; Vidal-Valverde et al., 2002; Sangronis and Machado, 2007; Kaur et al., 2015; Pal et al., 2017). However, the attributes of both physicochemical and phenolics affected by the pulse germination process need clarification. The objectives of this research were, therefore, to better understand (1) how germination affects proximate composition and physicochemical attributes of pulses; (2) how the quantity and quality of phenolic compounds changed during pulse germination using evaluation systems suitable to the food industry; and (3) elucidate the mechanism(s) of how the germination changes the attributes of phenolics.

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CHAPTER 2. LITERATURE REVIEW

2.1. Pulse Classes Cultivated in North Dakota

Pulses are members of the subfamily *Faboideae* of the family *Fabaceae.* Three major pulses planted in North Dakota are chickpea, lentil, and dry pea. In 2018, the acreage of chickpea (*Cicer arietinum* L.), lentil (*Lens culinaris* Merr.), and dry pea (*Pisum sativum* L.) was 114,400, 185,000, and 375,000 in North Dakota, which was 18%, 24%, and 45% of total acreages for the United States, respectively (USDA, 2018). Chickpeas are divided into two types based on seed size, color, and the thickness and shape of the seed coat. Kabuli chickpeas have the larger seeds with thinner seed coats that range in color from white to pale cream, while Desi chickpeas have the smaller seeds with thick seed coats that range in color from light tan to solid black (USA Dry Pea & Lentil Council, 2019). Lentils are lens-shaped legumes with colors that include yellow, red-orange, green, brown, and black. Dry pea can be classified as garden peas (*Pisum sativum ssp. hortense*) and field peas (*Pisum sativum ssp. arvense*), which can be characterized as wrinkled seeds and smooth seeds, respectively. In this research, chickpea, lentil, and yellow pea referred in particular to Kabuli chickpea, green lentil, and yellow field pea, respectively.

2.2. Proximate Composition of Pulse seeds

2.2.1. Protein profiles of pulse seeds

Pulses are well-known sources of plant-based proteins, which are a potential alternative to animal proteins. The protein contents of pulse flours were well summarized by Foschia et al. (2017). In general, the protein content of pulses ranged from 17 to 35 g/100 g. In particular,

chickpea, lentil, and yellow pea have protein contents of 19-27 g/100 g, 23-31 g/100 g, and 14-31 g/100 g, respectively. The protein content from pulses is much higher than that from cereals (Foschia et al., 2017). The major portion of pulse proteins are storage proteins located in the cotyledon of pulse seeds, while the minor part of pulse proteins including enzymes, lectins, and enzyme inhibitors (Campos-Vega et al., 2010).

The Osborne classification system divides proteins into four classes: (i) globulins, soluble in salt-water; (ii) albumins, soluble in water; (iii) prolamins, soluble in ethanol-water solution; and (iv) glutelins, soluble in dilute acid or alkaline solution. In general, approximately 70% of the total protein in pulses are globulins, while only 10%-20% of total proteins are albumins (Hall et al., 2017), which results in the lower solubility of pulse proteins compared to animal proteins.

Proteins are composed of amino acids linked together by peptide bonds. In general, all pulse storage proteins are relatively low in sulfur-containing amino acids (methionine, cysteine) and tryptophan, but high in lysine, leucine, aspartic acid, glutamic acid, and arginine (Boye et al., 2010b). Therefore, enhancement of proteins rich in sulfur-containing amino acids and tryptophan by processing, such as germination or direct incorporation, would be good strategies to balance the amino acid profiles within a food.

2.2.2. Carbohydrate profiles of pulse seeds

Carbohydrates comprise approximately 60% - 70% of the dry matter of pulses (Value and Nemzer, 2019). Carbohydrates can be classified as starch (amylose, amylopectin, resistant

starch) and non-starch polysaccharides, as well as monosaccharides, disaccharides, and oligosaccharides.

Starch is the most abundant carbohydrates stored in the cotyledon of pulse seeds and constitutes between 37 to 52% of pulse dry weight (Hall et al., 2017). Generally, the legume starch granules are oval with sizes varying in width (5–55 μm) and length (5–70 μm) among the pulses (Hoover et al., 2010). Granule surfaces of pulse starches are commonly smooth compared to wheat. Starch was mainly composed of amylose, an essentially linear polysaccharide, and amylopectin, a highly branched polysaccharide. The amylose concentration of pulse seeds varies widely, from 24% to 88%, based on different types (Foschia et al., 2017). One example of variability is the huge difference in amylose contents between smooth and wrinkled peas, which are 33-48% and 60-88%, respectively (Ratnayake et al., 2002). Resistant starch is a special starch that cannot be completely digested by humans. Resistant starch is classified in three categories: (1) starch entrapped in an intact cell in pulses, which results in the physically inaccessible for digestion; (2) retrograded amylopectin and crystalline regions of native starch granules; and (3) retrograded amylose (Hoover and Zhou, 2003). The morphology and composition of the starches have a major impact on the physicochemical attributes of pulse flours (Horstmann et al., 2016).

Dietary fiber, similar to resistant starch, is the polysaccharide that cannot be completely broken down by human digestive enzymes, and includes hemicellulose, glucan, and pectin. (Hall et al., 2017). Due to the lower digestibility, dietary fiber is an effective transportive tool that can bring nutrients to the gastrointestinal system. Therefore, dietary fiber conjugated with

phytochemicals, such as phenolic compounds, are effective nutrients to be absorbed in the intestine, which favors the prevention of colon cancer (Bach Knudsen et al., 2017). Dietary fiber ranges from 3-20, 6-15, and 7-23 g/100 g, in pea, chickpea, and lentil, respectively (Hall et al., 2017).

The concentration of sugars including monosaccharides, disaccharides, and oligosaccharides are much lower in raw pulses when compared to starch and dietary fiber, and range from 5-12, 3-5, and 5-6 g/100 g, in raw pea, chickpea, and lentil, respectively (Hall et al., 2017). Some simple sugars, such as glucose, fructose, and sucrose, contribute to the sweetness of food ingredients. Consequently, sweetness is not the major function derived from raw pulse ingredients.

2.2.3. Lipid profiles of pulse seeds

In general, pulse seeds have a relatively low lipid content, which is typically below 3% (Hall et al., 2017). The exception is the lipid contents of chickpea and lupin, which are in the range of 2-7% and 5-15%, respectively. Lipid can be classified as non-polar lipids, such as triacylglycerol mainly presented in the germ, and polar lipid, such as phospholipids and glycolipids in the cell membrane (Shewry, 2010).

The major fatty acid composition of the lipids includes palmitic acid, stearic acid, oleic acid, linoleic acid, and linoleic acid. In particular, the fatty acid profile of chickpea, lentil, and yellow pea are characterized as 57% linoleic acid and 25% oleic acid, 48% linoleic acid and 21%

oleic acid, and 48% linoleic acid and 25% oleic acid, respectively (Gopala Krishna et al., 1997; Hall et al., 2017).

Lipids are the major source of flavor compounds such as aliphatic aldehyde, aliphatic alcohol, and carboxyl acids. Lipids also affect the functionality of pulse flours by conjugating with starch, known as the lipid-amylose complex (Shewry, 2010).

2.3. The Functionality of Pulse Flours

2.3.1. Water absorption capacity

Water is a major component of most food components, which affects the texture, taste, and shelf life of food. It is, therefore, appropriate to delve into the water sorption capability of food ingredients. Moisture content and water activity (a_w) are used for the evaluation of water characteristics in a food matrix. Although it usually is not a linear relationship, water activity and moisture content are related to each other with certain rules: foods with higher moisture content tends to have higher water activity. A plot of water content to water activity shows the relationship between moisture content and water activity, as is moisture sorption isotherm (MSI) (Al-Muhtaseb et al., 2002). Saturated salt slurry method has been extensively used for depicting MSI curve (Wolf et al., 1985) until Bell and Labuza (2000) developed a simplified, convenient, and inexpensive method, known as a_w measurement method.

Knowledge of MSI is of great importance for modeling, designing, and optimizing food processing units and procedures (Yang et al., 2015). In general, analysis of MSI demonstrated useful information for studying and controlling concentration and dehydration processes,

formulating food mixtures, analyzing moisture barrier properties, forecasting growth of microorganisms, and estimating the chemical and physical stability of foods (Damodaran et al., 2008). Generally, type I, II, III, IV, and V of MSI curves have been characterized (Deming et al., 1940), while only type I and II curves have special names, which are Langmuir and sigmoidal shapes, respectively. Most systems are known as type II, sigmoidal shape (Al-Muhtaseb et al., 2002). Ricardo et al. (2011) reviewed nine mathematics models developed for describing the MSI curves of food matrices, including Langmuir equation, Brunauer-Emmett-Teller (BET) equation, Halsey equation, Smith equation, Henderson Equation, Oswin equation, Iglesias-Chirife equation, Peleg model, and Guggenheim-Anderson-de Boer (GAB) equation, the most commonly used models in foods are BET and GAB.

An alternative method for interpreting MSI curves is to divide them conceptually into region I ($a_w < 0.2$), II ($0.2 < a_w < 0.85$), and III ($a_w > 0.85$). In region I, water molecules are tightly held by the food and have limited mobility; in region II, water activities increased rapidly with small increases in moisture, water molecules interact with the food but have some mobility; in region III, both moisture content and water activity are high and water molecules have high mobility. In addition to different moisture contents, foods in each region have different textural properties. The texture of foods from region I to III varied from dry, crisp (region I), chewy, moist (region II), and soft, juicy (region III) (Damodaran et al., 2008).

2.3.2. Thermal properties

Thermal treatment of food, such as cooking, baking, toasting, and even extrusion, is one of the most widely used operations in modern food processing. Thermal properties of food components and food ingredients are, therefore, of great importance in food research and food quality assurance (Farkas and Mohácsi-Farkas, 1996). Differential scanning calorimetry (DSC), which measures heat flow as a function of time and temperature, is widely employed for observing the thermal properties of proteins, starches, and lipids. For example, the detection of water loss, of protein denaturation, of starch crystallization, of thermophysical properties have been reported by many researchers (Semenova, 2007; Farah et al., 2018; Hădărugă et al., 2019).

The thermal properties of proteins are closely related to the denaturation of food protein, which means protein would lose its native conformation with heat processing. The denaturation temperature should be tested with excessive water due to the fact that proteins tend to be stable during heating if the water content is low. Denaturation of proteins is usually below 100 °C. The apparent enthalpy absorbed during protein denaturation is attributed to both endothermic and exothermic contributions, as are the net changes. Kaur et al. (2007) analyzed the thermal properties of 2.0 mg chickpea protein isolates in 10 μL phosphate buffer solution by DSC. The denaturation temperature ranged from 98.1 to 99.3 °C, with an apparent enthalpy ranging from 2.84 J/g to 5.83 J/g. It is worth mentioning that if the re-scanning of the apparent enthalpy showed significant different profiles from the original curves, it means the denaturation was irreversible (Farkas and Mohácsi-Farkas, 1996).

Thermal properties can be employed to study the carbohydrates, such as gelatinization of starch and phase transitions of polysaccharides. Gelatinization of pulse starch has been reviewed by Wani et al. (2016). Similar to the denaturation of protein, the gelatinization of starch needs excess water, otherwise only melting at high temperature can be observed. For example, the peak temperature of lentil starch was detected at the temperature range from 66 - 71 °C with the addition of excess water, while the apparent enthalpy ranged from 3.0 to 13.3 J/g. Some starches, such as amylose and cyclodextrin, had cavity structures which contribute to the ability to conjugate hydrophobic compounds (Hădărugă et al., 2019). The lipid-amylose complex is a representative example that demonstrate the interaction between starch and other matrices. An endothermic transition of 98 °C, much higher than that of native starch, 60-70 °C, has been observed during heating. This high temperature can be ascribed to the melting of amylose-lipid complexes (Shewry, 2010).

Thermal oxidative decomposition of edible oils examined by DSC can be used for predicting the melting and crystallization characteristics of oil stability. As reviewed by Farkas & Mohácsi-Farkas (1996), the melting temperatures of lipids from sunflower, linseed, olive, and grape oils are below 0 °C. However, the oil content is below 3% in most of the pulses. Therefore, lipid is not the major contributor to the thermal properties of pulse flours.

Thermal properties of pulse flours are characterized by the thermal transitions of the combined constituents, mainly starch, protein, and lipid. The DSC data (Table 2.1) of chickpea, lentil, and pea flours indicates two peaks (Chung et al., 2008). The first peak occurred at a lower

temperature (60.1-82.0 °C), and was speculated to be related to the gelatinization of starch; The second peak occurred at higher temperatures (84.9-111.4 °C) and was thought to be related to the melting of amylose-lipid complex. Pea protein isolates showed two thermal transition peaks at temperatures of 67.15-68.15 °C and 85.07-92.90 °C, respectively (Shand et al., 2007). Therefore, absolute confirmation of the components responsible for the thermal transition of pulse flours by DSC alone is difficult.

Table 2.1. Thermal properties of both ungerminated and germinated pulse seeds

Pulse	Processing	Germination time (h)	Peak number	To (°C)	Tp (°C)	Tc (°C)	ΔH (J/g)	References
Pea	Flours	0	I	61.6-61.9	71.6-72.4	80.6-81.3	4.5-4.8	(Chung et al., 2008)
		0	II	85.2-89.7	93.2-95.9	102.1-103.6	2.2-6.4	
Lentil	Flours	0	I	67.3-68.4	75.6-76.1	82.0-82.0	3.0-3.2	
		0	II	84.9-85.0	92.2-94.8	102.4-102.6	5.0-5.1	
Chickpea	Flours	0	I	60.1-60.8	70.3-72.5	80.1-81.5	4.3-5.1	
		0	II	94.8-96.4	104.6-105.2	111.0-111.4	7.4-7.9	
Yellow pea	Flours	0	I	62.28	72.63	81.81	0.68	(Ma et al., 2017)
		0	II	81.89	86.15	89.91	0.02	
	Conventional cooked	0	I	44.65	57.63	70.05	0.58	
		72	I	44.38	56.61	70.31	0.72	
	Roasted	0	I	58.51	73.11	90.77	1.48	
		0	II	82.08	86.32	90.3	0.02	
		72	I	46.35	56.21	66.49	0.33	
		72	II	80.7	86.17	92.26	0.09	
Yellow pea	Flours	0	I	62	72.6	81.8	0.68	(Ma et al., 2017)
		0	II	81.9	86.2	89.9	0.02	
		72	I	60.2	71.9	80.9	0.9	
		72	II	80.9	87.2	91.9	0.03	
Pea	Protein isolates	0	I	N.A.	67.15-68.15	N.A.	0.095-0.146	(Shand et al., 2007)
		0	II	N.A.	85.07-92.90	N.A.	0.725-0.922	

*To, Tp, Tc, and ΔH represent the onset temperature, peak temperature, completion temperature, and enthalpy, respectively; N.A. denotes data not available; Germination time "0" represents raw pulses without germination.

2.3.3. Pasting properties

The pasting properties of pulse flours are mainly attributed to starch. Starch can be used as an adhesive, and for binding, clouding, dusting, film-forming, foam strengthening, gelling, glazing, moisture-retention, stabilizing, texturizing, and thickening applications (Damodaran et al., 2008). Physicochemical properties of starch, such as gelatinization, pasting, and retrogradation, are important properties related to film-forming, gelling, moisture-retention, stabilizing, texturizing, and thickening in the food industry.

Swelling, gelatinization, pasting, and retrogradation of starch are the typical physicochemical properties resulting from the configuration of starch with excess water (Shewry, 2010). These properties are influenced by the distribution of amylopectin short chains (DP 6-11) and the ratio of amylose to amylopectin (Hoover et al., 2010). For example, the high amylose content in pulse starches results in a higher pasting temperature, the absence of the peak viscosity, and an increased viscosity during retrogradation (Hoover et al., 2010).

Gelatinization temperature can be evaluated using the Rapid Visco-Analyzer (RVA) as well as by differential scanning calorimetry (DSC) (Huang et al., 2007). In addition to gelatinization temperature, the RVA viscogram has been widely used to demonstrate the temperature and viscosity of swelling, gelatinization, pasting, and retrogradation characteristics for starches (Hoover et al., 2010). Thus, the viscogram can be divided into four regions: (i) when an excessive amount of water incorporates into starch at room temperature, the slurry viscosity starts from zero. With the increase of temperature, the viscosity of the slurry increases due to

water uptake by the starch and swelling substantially. (ii) In the pasting region, swollen starch granules break under the shear and friction forces and lead to the leaching of amylose, which results in a decrease of slurry viscosity. Peak viscosity depends on the balance of both swelling and breaking of the starch granule. (iii) After peak viscosity, a shear thinning effect, corresponding to the viscosity decrease of starch, would be anticipated as the consequence of reorientation of soluble starch, as well as shear-induced destruction of the swollen granule. (iv) During cooling, because of the strengthening of hydrogen bonding and entanglement between starch chains, the viscosity of the slurry increases, referred to as setback (Shewry, 2010). These four regions translated into various viscosity parameters (Table 2.2) for various pulses.

Although starch is the major contributor to the pasting/thermal properties of flours, non-starch polysaccharides and proteins treated with heating also contribute to the gelling and pasting properties by way of swelling, denaturation, and unfolding (Henshaw et al., 1996; Kaur and Sandhu, 2010). This phenomenon is evidenced by the comparison of the peak viscosity, break down, setback, and final viscosity of lentil starches, which are approximately five times higher than those of lentil flours.

2.3.4. Phenolic compounds

Natural phenolic compounds, the ubiquitous secondary metabolites in plant foods, usually act as antioxidants to protect them from oxidation by combating oxidative stress derived from free radicals, reactive oxygen species (ROS), and prooxidants (Naczk and Shahidi, 2006; Brewer, 2011). The antioxidant properties of natural phenolic compounds in our diet have been

connected with their multifaceted functions in promoting human health (Rice-Evans, 2001; Robbins, 2003). Likewise, a well-reasoned hope to replace synthetic ones and effectively prevent food lipid oxidation has been placed on natural phenolic compounds extracted from plant foods. Pulse crops are one of the major plant foods, that provide significant calories and protein in the human diet. Recent intervention research performed on animals and humans indicate that pulse crops contain biologically active phenolic compounds that are related to the reduced risk of chronic diseases (Jayathilake et al., 2018).

Table 2.2. Pasting properties of both ungerminated and germinated pulse seeds

Sample	Pulse	Germination time (h)	Peak viscosity (cP)	Break down (cP)	Setback (cP)	Final viscosity (cP)	Pasting (°C)	References
Starch	Lentil	0	3925	1596	3004	6529	73.7	(Ghumman et al., 2016)
		48	3547	1448	2786	6433	73.2	
		96	3322	1359	2682	6304	73.2	
	Horse gram	0	6229	3066	1428	7657	80.5	
		48	6104	2889	1157	6954	79.7	
		96	6041	2789	757	6798	79.6	
Flour	Lentil	0	956	106	463	1419	74.2	
		48	1409	534	N.A.	1244	72.4	
		96	1027	529	N.A.	730	72.2	
	Horse gram	0	1603	511	N.A.	1660	81.7	
		48	1510	431	N.A.	1510	79.6	
		96	1605	1118	N.A.	684	79.2	
	Pea	0	1129-1371	93-172	576-706	1554-1870	69.5-69.7	(Chung et al., 2008)
	Lentil	0	1185-1359	140-239	605-662	1651-1781	70.0-70.1	
	Chickpea	0	755-1347	ND	320-610	1068-1938	69.171.8	
	Mung bean	0	2075	678	867	2264	N.A.	(Kaur et al., 2015)
		72	4601	1711	2277	5167	N.A.	
	Kidney bean	0	2676	1187	1479	2970	74.3	(Owuamanam et al., 2014)
		-	1181	557	677	1309	67	
	Cowpea	0	3052	1900	1640	3271	73	
		-	1512	922	1080	1670	68	
	Pigeon pea	0	2728	1408	494	3014	70	
		-	1026	675	933	1393	67.5	
	Black bean	0	4500	24	4884	9348	80	(Guajardo-Flores et al., 2017)
		120	1956	204	2364	4560	86	

*N.A. denoted data not available; Germination time "0" denote raw pulses without germination; "-" denote germination stopped when the length of the rootlets measured up to 2.54 cm

Over the past two decades, studies on various aspects of phenolic compounds have appeared in thousands of publications on the extraction and analysis of this category of compounds. However, the classification for crude phenolic compounds extracted from pulse

seeds is still limited. In general, crude phenolic compounds have been assigned on the basis of extraction methods, particularly their solubility or their partition ratio in the binary solvent system, yet these classification systems do not fully overlap. As reported by Xu et al. (2019), one way to classify phenolic compounds is based on the solubility of phenolic compounds in solvents (Table 2.3). For instance, phenolic compounds that dissolve in solvent (water/acetone/methanol) are named as free (Abdel-Aal et al., 2012; Xiang et al., 2017) or soluble phenolic compounds (Yeo and Shahidi, 2015), while residues which cannot be dissolved into such solvents are named as bound (Yeo and Shahidi, 2015; Xiang et al., 2017) or insoluble bound phenolic compounds (Krygier et al., 1982). Another way of classification relies on the partition ratio of phenolic compounds in solvents with different polarities, with water/acetone/methanol and diethyl ether/ethyl acetate (DE/EA) being the common combination. Phenolic compounds extracted by DE/EA from water are named as free phenolic compounds (Krygier et al., 1982; Chen et al., 2015a; Das and Singh, 2016), while the residues in the water phase are named as esterified (Krygier et al., 1982), conjugated (Chen et al., 2015a), or bound phenolic compounds (Yeo and Shahidi, 2015; Das and Singh, 2016; Xiang et al., 2017).

Two aforementioned classification methods have ignored a portion of phenolic compounds in pulse seeds that cannot be extracted by polar solvent systems. For instance, a whole group of natural phenolic lipids in pulse seeds have been excluded in the current crude phenolic compounds classifications, which is attributed to the defatting pretreatment among those most prevalent extraction methods (Krygier et al., 1982; Hung et al., 2011; Masisi et al.,

2016). This endows total phenolic compounds (TPC), a parameter frequently used to quantify the whole category, with two different meanings. The parameter of TPC either exclusively represents the total extractable phenolic compounds by polar solvents used (Ross et al., 2009; Xu and Chang, 2009; López et al., 2013), or includes both the extractable and non-extractable phenolic compounds (Abdel-Aal et al., 2012; Yeo and Shahidi, 2015; Xiang et al., 2017). Because of such differences in classification rather than others, contradictions are often observed in different studies.

Table 2.3. Nomenclature of phenolic compounds based on extraction methods in different seeds

Seeds	Water/organic solvent (DC 20-60)		Residues	References
	Organic solvent (DC 4-6)	Water phase		
Lentils	Soluble		Bound	(Yeo and Shahidi, 2015)
Edible seeds	Soluble		Bound	(Gan et al., 2017)
Mung bean	Soluble		Bound	(Gan et al., 2016b)
Edible beans	Solvent-soluble		Solvent-insoluble	(Gan et al., 2016a)
Beans	Free	Soluble conjugated	Insoluble bound	(Wang et al., 2016c)
Barley	Free	Esterified	Insoluble bound	(Dvořáková et al., 2008)
Dry beans	Total phenolic acid		NA	(Ross et al., 2009)
Dark beans	Phenolic compound		NA	(López et al., 2013)
Pinto and black beans	Total Phenolic Composition		NA	(Xu and Chang, 2009)

NA denotes not available.

Herein, we use a rational classification to completely define the phenolic compounds in pulse seeds by categorizing crude phenolic compounds into non-extractable (NEPs) and extractable phenolic compounds (EPs) groups (Figure 2.1). The EPs group can then be further classified as non-polar solvent extractable (NPSEPs) and polar solvent extractable (PSEPs) phenolic compounds, with the latter being further divided to soluble free (SFPs) and polar soluble bound phenolic compounds (PSBPs). As NPSEPs can be considered as non-polar soluble

bound phenolic compounds, NPSEPs and PSBPs together make up soluble bound phenolic compounds (SBPs). In principle, this annotation could answer central questions of the efficacy of natural antioxidants for each category.

2.3.4.1. Non-extractable phenolic compounds

Non-extractable phenolic compounds (NEPs), due to their insoluble nature in water or organic solvents, are also known as insoluble bound phenolic compounds. Insoluble moieties, to which the phenolic compounds are bound, such as cellulose, arabinoxylan, chitin, hemicellulose, and polysaccharide-protein complexes, make phenolic compounds non-extractable (Saura-Calixto, 2011; Quirós-Sauceda et al., 2014). NEPs exist in the cell wall of most pulse seeds (Naczk and Shahidi, 2004; Agati et al., 2012). NEPs play critical roles in the mechanical strength of cell walls, plant growth regulation, stress protection, and pathogen-resistant (Naczk and Shahidi, 2004).

The formation mechanism of NEPs has yet to be established. Agati et al. (2012) presented a hypotheises for the process. SFPs and SBPs are synthesized in plant cells, secreted, and are then escorted by vesicles to the cell wall. In the cell wall they link with hemicellulose, cellulose, arabinoxylan, and other insoluble moieties, through hydrogen bonds, hydrophobic interactions, and covalent bonds (Saura-Calixto, 2011). Alves et al. (2016) used α-amylase to hydrolyze NEPs of rice, which denoted that some insoluble bound moieties may contain 1-4 glucosidic linkage.

Phenolic compounds can also be entrapped by polysaccharides, which in turn, presents steric hindrance that prevents them from being extracted. As a result, phenolic compounds on NEPs have lower accessibility and availability (Quirós-Sauceda et al., 2014). Obviously, the antioxidant capability is limited by steric hindrance as well, which results in the increased endurance of antioxidant capability *in vivo*. The protective effect of non-starch polysaccharides on the attached phenolic compounds against enzymes in the mouth, stomach, and small intestine has recently been reported (Lovegrove et al., 2017). When NEPs come to the large intestine, non-starch polysaccharides can be digested by colonic microflora that releases phenolic compounds (Maurer et al., 2013) to protect against colon cancer.

In order to quantify the total phenolic compounds and characterize their properties, the attached phenolic compounds should be liberated. Alkaline hydrolysis followed by acidic hydrolysis is widely applied to liberate phenolic compounds from NEPs. Then, DE/EA is employed to extract phenolic compounds from the hydrolyzed solution (Çelik et al., 2013). Additionally, enzymatic hydrolysis and microwave-assisted hydrolysis have been utilized for the effective release of phenolic compounds on NEPs (Shahidi and Yeo, 2016).

2.3.4.2. Extractable phenolic compounds

2.3.4.2.1. Solvent extraction

A number of extractable phenolic compounds (EPs), especially PSEBs have been extensively reviewed (Robbins, 2003; Cheynier et al., 2013; Van Hung, 2014; Alves et al., 2016). Basically, extractable phenolic compounds must be extracted prior to any application.

Most of the extraction procedures are based on the solubility of phenolic compounds that are governed by their structure and polarity (Naczk and Shahidi, 2006). Dielectric constant (DC) is positively related to a solvent's polarity. Based on the principle that the more similar the DC, the higher inter-miscibility between two substances, NPSEPs, PSBPs, and SFPs can be separated, purified, and further investigated (Zhou and Elias, 2012; Cheynier et al., 2013).

A classical extraction procedure for phenolic compounds (Figure 2.1) has been detailed in numerous publications (Wang et al., 2014; Alves et al., 2016). Generally, NPSEPs are extracted by non-polar solvents with a process called defatting. Then, PSEPs are extracted from defatted seeds meal using water or water/polar solvents, such as methanol (Casazza et al., 2010), ethanol (Xu and Chang, 2007; Casazza et al., 2010), acetone (Bhat and Riar, 2017), methanol/water (7/3, v/v) (Xu and Chang, 2007), ethanol/water (63.5/100, v/v) (Bodoira et al., 2017), acetone/water (8/2, v/v) (Xu and Chang, 2007), or water/acetone/methanol (6/7/7, v/v/v). After that, SFPs are extracted from PSEPs using medium-polar solvent, mainly DE (Khoddami et al., 2013) or DE/EA (1/1, v/v) (Bodoira et al., 2017) for at least 3 times. In order to know the properties of phenolic compounds attached to the soluble moieties, PSBPs remaining in the water phase need to be hydrolyzed using sodium hydroxide (NaOH) and hydrochloric acid (HCl) so that attached phenolic compounds can be liberated. Using a medium-polar solvent (DE/DA), phenolic compounds from PSBPs can be extracted and can then be considered as soluble free. Following such procedure, tannins, flavonoids, phenolic compounds, and lignans are extracted from pulse seeds (Xu and Chang, 2007).

Nevertheless, it is difficult to extract the entire SFPs from PSEPs as medium polarity solvents for SFPs extraction can disperse into high polarity solvents at a certain ratio. In addition, small amounts of PSBPs and SFPs may disperse into medium-polar phase and polar phase, respectively (Chen et al., 2015a). Therefore, extraction with multiple times (at least 3) is encouraged to receive relatively pure SFPs from crude PSEPs.

2.3.4.2.2. Polar solvent extractable phenolic compounds (PSEPs)

2.3.4.2.3. Soluble free phenolic compounds (SFPs)

Pure phenolic compounds in their free form are synthesized in plant cells via two main pathways. The shikimate pathway directly provides phenylpropanoids such as the hydroxycinnamic acids (C6–C3) and coumarins (C6-C3), and the polyketide (acetate) pathway produces other simple phenolic compounds (Kozubek and Tyman, 1998). Most of the SFPs are derived from a combination of these two pathways. Chemically, SFPs have been classified into several groups such as phenolic acids (C6-C1 or C6-C3), coumarins (C6-C3), stilbenes (C6-C2-C6), flavonoids (C6-C3-C6), and polymers. Phenolic acids can be divided into hydroxybenzoic acids (C6–C1), e.g. gallic, *p*-hydroxybenzoic, vanillic, syringic, and ellagic acids, and hydroxycinnamic acids (C6-C3), e.g. *p*-coumaric, caffeic, ferulic, and sinapic acids (Amarowicz and Pegg, 2008). Coumarins (C6-C3) are present in different seeds (Shahidi and Yeo, 2016). Stilbenes (C6-C2-C6) exist mainly in the form of resveratrol (Song et al., 2010). Flavonoids (C6-C3-C6) can be further classified as flavones, flavanones, flavonols, flavanonols, isoflavones, flavanols, and anthocyanidins based on the substitution patterns at the C3 position of the basic

flavone backbone (Shahidi and Ambigaipalan, 2015). Owing to the polyhydroxy group (polar) and benzene ring (non-polar) structures, most of the pure phenolic compounds in the free form are soluble in polar and intermediate-polar solvents, such as water/acetone/methanol and DE/EA and can be categorized as soluble free phenolic compounds (SFPs). They are also the major constituents of both SBPs and NEPs.

2.3.4.2.4. Polar soluble bound phenolic compounds (PSBPs)

Polar soluble bound phenolic compounds (PSBPs) consist of a phenolic compound with a polar soluble moiety that cause phenolic compounds to have higher solubilities in polar solvents. As a result, PSBPs can be extracted by polar solvents such as water, methanol, and acetone, and purified by removing SFPs with DE/EA (Gan et al., 2016a). Herein, we mainly discuss carbohydrates as the dominant soluble moieties found in PSBPs from cereal and pulse seeds is presented.

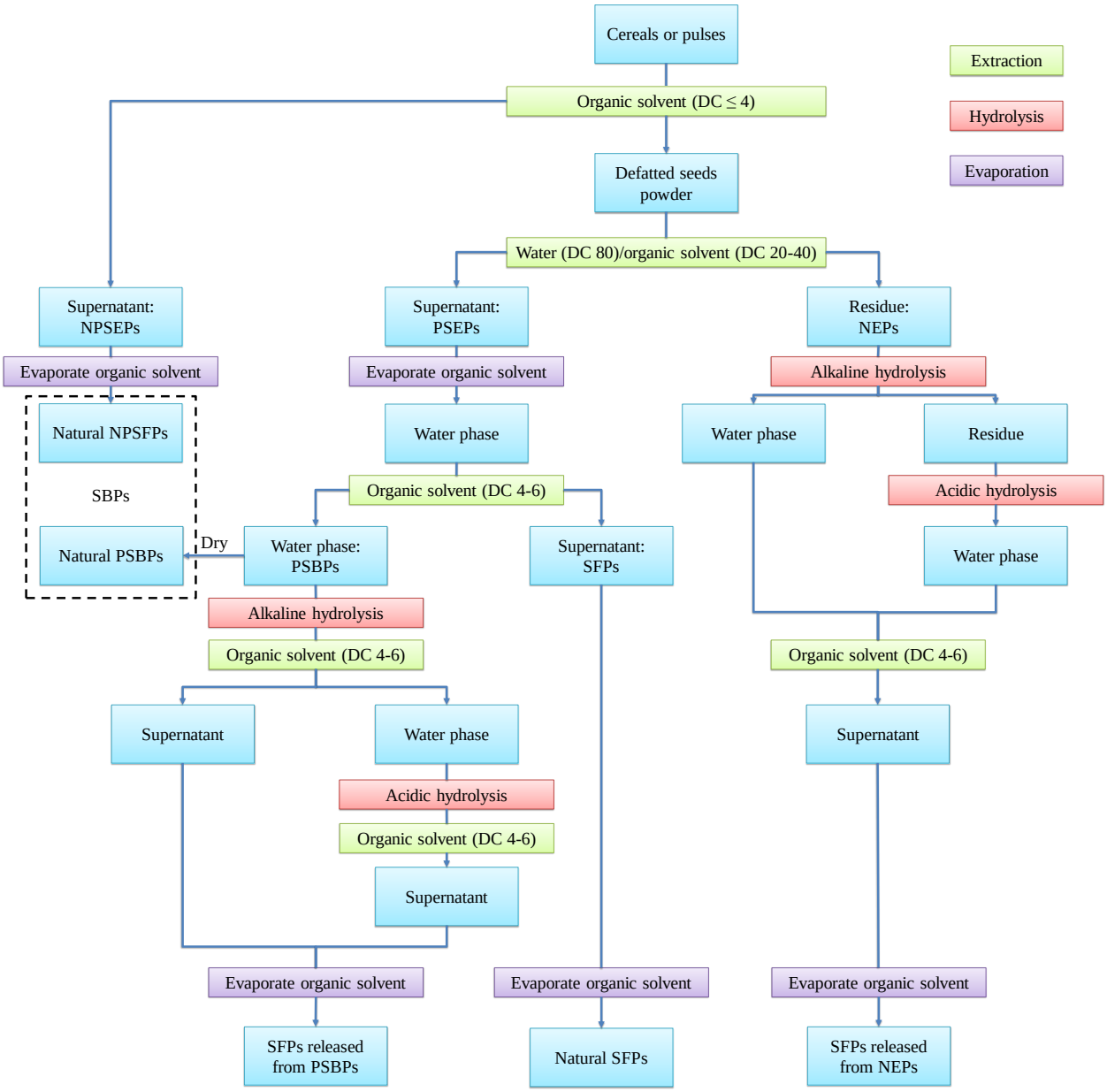

Figure 2.1. General extraction procedure of phenolic compounds.
The DC value of organic solvents (20 °C): methanol (DC 33), ethanol (DC 24.5), acetone (DC 20.7), ethyl acetate (DC 6.02), diethyl ether (DC 4.34), cyclohexane (DC 2.02), hexane (DC 1.88). DC, dielectric constant; NEPs, nonextractable phenolic compounds; NPSEPs non-polar solvent extractable phenolic compounds; PSEPs, polar solvent extractable phenolic compounds; SFPs, soluble free phenolic compounds; PSBPs, polar soluble bound phenolic compounds; SBPs, soluble bound phenolic compounds.

Phenolic compounds have been found to be conjugated with various soluble carbohydrates, including monosaccharides, disaccharides, oligosaccharides, and polysaccharides. Phenolic compounds bound to monosaccharides have been widely reported, such as quercetin-*O*-glucoside, apigenin-6-*C*-glucoside, and ferulic acid hexoside. (Gan et al., 2016b; Paucar-Menacho et al., 2017). Phenolic compounds can also be bound to disaccharides, such as quercetin-3-*O*-rutinoside and kaempferol-3-*O*-rutinoside (Zhang et al., 2015). Phenolic compounds bound to oligosaccharides have higher prebiotic activity, such as 3-*O*-(5-*O trans* feruloyl-*α*-*L*-arabinofuranosyl)-*D*-xylose and *O*-[5-*O*-(*trans*-feruloyl)-*α*-*L*-arabinofuranosyl]-(1→3)-*O*-*β*-*D*-xylopyranosyl-(1→4)-*D*-xylopyranose (Bento-Silva et al., 2018). Polysaccharides can be attached to phenolic compounds through hydrogen bonds, hydrophobic interactions, and covalent bonds, such as ester and ether bonds (Quirós-Sauceda et al., 2014; Dueñas et al., 2016). Rao and Muralikrishna (2004) reported that ferulic acids and coumaric acids conjugated with the soluble polysaccharides in rice, maize, wheat, and finger millet.

The bioavailability, chemical stability, and antioxidant activity of phenolic compounds can be impacted upon the conjugation with soluble carbohydrates. Soluble carbohydrates are polyhydroxy compounds, which can give rise to the hydrophilic property of the phenolic compounds attached, especially flavonoids (Tommasini et al., 2004; Xie et al., 2016). The majority of flavonoids, except for the subclass of catechin, are in the form of flavonoids-glycosides that result in higher bioavailability in the human body (Kumar and Pandey, 2013). Soluble carbohydrates, particularly polysaccharides, can improve the chemical stability of

phenolic compounds because of the steric hindrance that can protect phenolic compounds from other oxidants (Qiu et al., 2017; Fan et al., 2018). Soluble dietary fibers is an good example of a substance that trap and protect phenolic compounds via ester bonds, hydrogen bonds, or electrostatic bonds (Quirós-Sauceda et al., 2014). Meanwhile, polysaccharides can also keep the phenolic compounds away from part of the light. This is evidenced by a significant improvement in the photostability of phenolic compounds by encapsulating phenolic compounds with cyclodextrin (Kfoury et al. 2016). Soluble carbohydrates can also improve the antioxidant activity of phenolic compounds through a synergistic effect. Numerous studies have found that crude polysaccharides have excellent free radical scavenging capability (Sun et al., 2005, 2010; Zhao et al., 2012; Fan et al., 2014; Wu et al., 2014). It is possible that phenolic compounds conjugated on the polysaccharides are the main group of compounds responsible for the free radical scavenging (Wang et al., 2016). Thus, soluble polysaccharides synergistically donate hydrogen from their activated reducing ends to these oxidized phenolic compounds. Furthermore, PSBPs have been shown to exhibit higher antioxidant activity than hydrolyzed counterparts (Gan et al., 2016; Wang et al., 2015; Wang et al., 2016).

2.3.4.2.5. Non-polar solvent extractable phenolic compounds (NPSEPs)

Resorcinol lipids (Figure 2.2), also known as phenolic lipids, are the group of NPSEPs that have previously been precluded from the family of phenolic compounds in cereal and pulse seeds. This is because, the majority of this group in cereal and pulse seeds is removed during the initial defatting process employed before extracting and quantifying phenolic compounds. Thus,

there is a lack of information in the literature on this group of antioxidants in regard to their antioxidant activity. Resorcinol lipids are most abundant in outer layers of pulse seeds that makes up the dietary fiber. However, the amount of resorcinol lipids is relatively low in pulse seeds (~0.3 mg/kg d.w.) (Zarnowski and Kozubek, 1999; Landberg et al., 2007; Kulawinek et al., 2008).

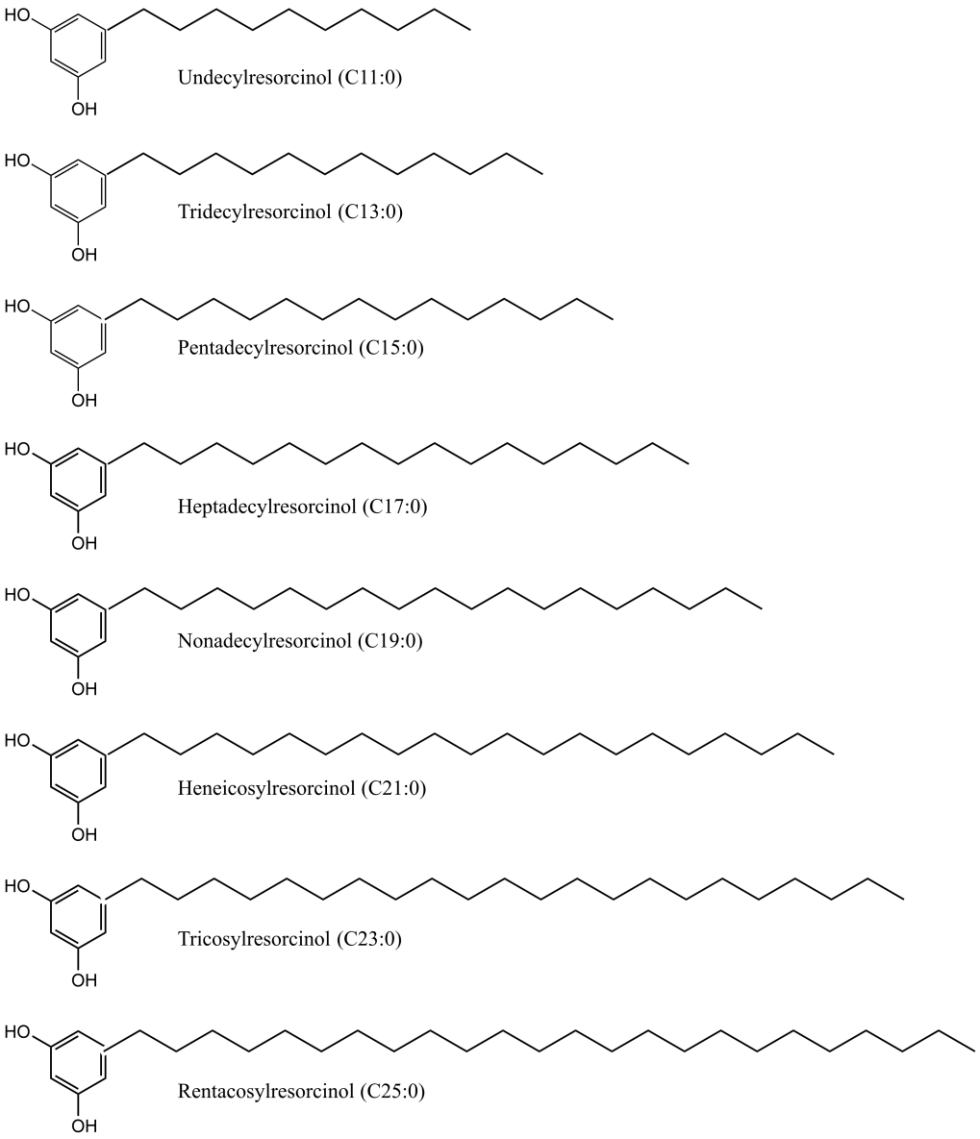

Figure 2.2. Chemical structures of common phenolic lipids (resorcinolic lipids) in cereal and pulse seeds.Effect of germination on proximate composition

2.4. Germination of Pulse Seeds

Germination is a process that improves the nutritive quality, reduce antinutritional effects, and soften pulses (López-Martínez et al., 2017). Basically, germination starts from seed hydration. Water is absorbed by dry seeds with concentration gradient and diffusion. In turn, the respiratory activity increases, while gibberellic acids are synthesized in the germ. With the increased mobility, which is attributed to water, gibberellic acids move from embryo to aleurone layer as a molecular signal. Enzymes such as carbohydrase, protease, and lipase are synthesized with the signal and secreted into the cotyledon. Starch, proteins, and lipids are degraded and used for respiration and synthesis of new cell constituents, such as phenolic compounds (Nelson et al., 2013; Sangsukiam and Duangmal, 2017). Pulse germination contributes to producing favorable changes in the proximate composition, physicochemical properties, and secondary metabolites, such as phenolic compounds, which turns it into an accessible and affordable ingredient choice for food technologists.

2.4.1. Effect of germination on proximate composition

2.4.1.1. Effect of germination on proteins

Enzymes are the functional proteins that accelerate the chemical reactions in pulse seeds. All kinds of enzymes, such as proteases, amylases, and lipases, are activated or generated during pulse germination via hormonal regulation, such as gibberellin and abscisic acid (Value and Nemzer, 2019). Enzymes are of great importance to all the substances changed during pulse germination, even proteins.

With the increased activity of protease, large molecular weight protein polymers are hydrolyzed into smaller molecules during pulse germination. For example, legumin and the largest subunit of vicilin (55 kDa) of lentil protein isolates were degraded after 3 days of germination (Bamdad et al., 2009). As a matter of course, the generation of new subunits ranged from 10 - 32 kDa. Nevertheless, certain proteins may be resistant to proteolysis. For example four original subunits of vicilin (35, 30, 12, 11 kDa) remain intact after five days of lentil germination (Bamdad et al., 2009).

Protein composition also changes during pulse germination. Fouad et al. (2015) germinated lentil for 6 days at room temperature. The free amino acids increased from 1.86 to 15.20 mg/g after 6 days of germination. In addition, the composition of amino acids made up of proteins changed. Total essential amino acids, including phenylalanine, valine, threonine, tryptophan, methionine, leucine, isoleucine, lysine, and histidine, increased from 36.9 to 38.88 g/100 g protein. In contrast, the non-essential amino acids and conditional essential amino acids decreased from 63.1 to 61.12 g/100 g protein. A similar trend was observed by Chilomer et al. (2010) during lupin germination that essential amino acids increased, while non-essential amino acids decreased.

Finally, the content of proteins tends to increase during pulse germination (Kavitha and Parimalavalli, 2014). However, proteins for proximate analysis are often quantified based on the measurement of nitrogen. This means all the nitrogen is counted as protein. As such, the variation in protein actually reflects the variation of nitrogen. Interestingly, many researchers

(Table 2.4) reported an increased protein content without any addition of nitrogen source during pulse germination, including mung bean, chickpea, pea, lentil, and yam bean (El-Adawy et al., 2003; Nwosu, 2013; Kavitha and Parimalavalli, 2014; Masood et al., 2014; Ghumman et al., 2016). A plausible explanation is that the consumption of carbohydrates into carbon dioxide results in the increased percentage of protein content (Hall et al., 2017).

2.4.1.2. Effect of germination on carbohydrates

Total carbohydrates content tends to decrease during pulse germination (Hall et al., 2017). The carbohydrate content of lentil, pea, mung bean, jack bean, and Africa yam bean decreased during germination (Table 2.4). Furthermore, subclass of carbohydrates, such as starch, sugar, and dietary fiber, were affected differently by germination.

As the hydrolytic enzymes are generated or activated, starches, one of the major matrix of the cotyledon, can be hydrolyzed into sugars, such as dextrin, maltose, and glucose. Starches in cowpea, jack bean, and mucuna decreased from 46.31, 26.74, 26.44 g/100 g to 26.83, 18.76, 22.25 g/100 g, respectively, after germination (Benítez et al., 2013). Amylose, a polysaccharide of starch, tend to decrease during germination. Ghumman et al. (2016) reported that apparent amylose content decreased from 19.5 - 22.1% to 10.8 - 21.3% and from 23.6 - 29.8% to 21.4 - 22.6% in lentil and horse gram, respectively, after 4 days of germination. Increased activity of α-amylase is responsible for the decreased content of amylose (Ghumman et al., 2016). Frias et al. (1998) also reported decreased ratio of amylose to amylopectin in lentil, from 26:74 to 24:76, after 6 days of germination further supporting amylose breakdown.

Resistant starch tends to decrease during pulse germination. Resistant starch decreased in cowpea, jack bean, and mucuna, from 79.1 mg/g, 47.4 mg/g, and 49.9 mg/g to 22.2 mg/g, 9.03 mg/g, and 30.1 mg/g, respectively, after 4 days of germination (Benítez et al., 2013). Hydrolytic enzymes affect intact cell and native starches. Resistant starch entrapped by the intact cell or located at the crystalline regions of native starch granules can be converted into digestible starch by hydrolytic enzymes.

Apparently, total sugars, including mono- and oligosaccharide, tend to increase due to the hydrolysis of starch and other polysaccharides. Following 3, 4, 5, and 6 days of lentil germination, total sugars increased from 5.01 g/100 g to 8.60 g/100 g, 10.42 g/100 g, 12.27 g/100 g, and 14.50 g/100 g, respectively (Fouad and Rehab, 2015). However, Pal et al. (2017) studied five cultivars of lentil, three of which had no reduction in total soluble sugar content after 2 days of germination. It can be inferred that a series of metabolic reactions occur differently among cultivars.

Dietary fiber increased during pulse germination. Dietary fiber is the major component of cotyledon cell wall, which can be hydrolyzed and result in the release of starch and protein. In contrast, new cell walls are synthesized for protecting new cells associated with sprouts (Martín-Cabrejas et al., 2003). Comprehensively, dietary fiber tends to be increased during pulse germination. The increased total dietary fiber has been observed in mucuna, pea, Jack bean, cowpea, chickpea, and lentil (Table 2.4). Martín-Cabrejas et al. (2003) further reported that some insoluble dietary fibers and soluble dietary fibers increased during pulse germination, such as

Table 2.4. Effect of germination on proximate composition of pulse seeds

	Germination time (h)	Ash %	Lipid %	Protein %	Total carbohydrate %	Carbohydrate %			References
						Starch	Fiber	Sugar	
Lentil	0	2.67	-	24.69	-	-	-	-	(Ghumman et al., 2016)
	48	2.52	-	25.74	-	-	-	-	
	96	2.50	-	27.14	-	-	-	-	
Horse gram	0	3.74	-	23.64	-	-	-	-	
	48	3.54	-	24.86	-	-	-	-	
	96	3.09	-	25.21	-	-	-	-	
Jack bean	0	2.76	-	23.30	-	24.70	23.38	0.92	(Akpapunam and Sefa-Dedeh, 1997)
	72	3.15	-	20.00	-	18.60	40.08	1.58	
Winged bean	0	-	19.90	-	-	-	-	-	(King and Puwastien, 1987)
	24	-	19.40	-	-	-	-	-	
	48	-	19.40	-	-	-	-	-	
	72	-	19.60	-	-	-	-	-	
	96	-	19.30	-	-	-	-	-	
	120	-	19.10	-	-	-	-	-	
Mung bean	0	3.67	1.79	23.50	-	-	-	-	(Masood et al., 2014)
	24	3.64	1.62	25.53	-	-	-	-	
	48	3.25	1.56	26.77	-	-	-	-	
	72	3.81	1.51	27.17	-	-	-	-	
	96	3.81	1.43	27.80	-	-	-	-	
	120	3.82	1.32	30.43	-	-	-	-	
Chickpea	0	1.83	5.80	17.80	-	-	-	-	
	24	1.72	5.73	18.43	-	-	-	-	
	72	2.95	5.09	21.60	-	-	-	-	
	96	2.84	4.93	22.77	-	-	-	-	
	120	3.52	4.62	23.37	-	-	-	-	

Table 2.4. Effect of germination on proximate composition of pulse seeds (continued)

	Germination time (h)	Ash %	Lipid %	Protein %	Total carbohydrate %	Carbohydrate %			References
						Starch	Fiber	Sugar	
Mung bean	0	4.50	1.75	26.40	61.20	-	-	-	(El-Adawy et al., 2003)
	72	5.53	1.28	24.54	59.73	-	-	-	
	120	7.54	1.15	22.52	57.94	-	-	-	
Pea	0	3.93	2.40	34.70	54.72	-	-	-	
	72	5.97	1.77	32.60	52.51	-	-	-	
	120	7.51	1.65	30.73	50.31	-	-	-	
Lentil	0	4.16	1.15	31.41	56.53	-	-	-	
	72	5.87	1.09	29.80	54.50	-	-	-	
	120	6.97	0.93	28.37	53.85	-	-	-	
African Yam Bean	0	3.23	1.64	23.24	58.05	-	-	-	(Nwosu, 2013)
	24	3.23	1.63	22.63	58.04	-	-	-	
	48	3.21	1.61	22.58	57.84	-	-	-	
	72	3.20	1.61	23.81	57.53	-	-	-	
	96	3.19	1.59	23.81	57.53	-	-	-	
Lentil	0	2.77	2.20	25.63	48.70	-	21.70	5.08	(Fouad and Rehab, 2015)
	72	3.10	1.32	27.51	46.27	-	22.30	8.60	
	96	3.19	1.24	27.90	44.56	-	23.61	10.42	
	120	3.25	1.15	28.41	43.33	-	24.29	12.27	
	144	3.35	0.90	28.86	41.69	-	25.40	14.50	
Chickpea	0	3.12	4.08	20.37	51.76	-	11.25	-	(Uppal and Bains, 2012)
	36	3.79	3.43	23.40	49.35	-	12.68	-	
	48	3.84	3.34	23.58	49.01	-	13.12	-	
	60	3.85	3.12	23.62	48.77	-	13.19	-	

Table 2.4. Effect of germination on proximate composition of pulse seeds (continued)

	Germination time (h)	Ash %	Lipid %	Protein %	Total carbohydrate %	Carbohydrate %			References
						Starch	Fiber	Sugar	
Cowpea	0	3.47	1.50	22.53	59.13	-	3.78	-	(Uppal and Bains, 2012)
	16	3.55	1.41	24.28	56.54	-	4.54	-	
	20	3.61	1.36	24.51	56.34	-	4.62	-	
	24	3.72	1.34	24.94	55.80	-	4.70	-	
Cowpea	0	-	-	-	-	46.31	31.00	-	(Benítez et al., 2013)
	96	-	-	-	-	26.83	35.00	-	
Jack bean	0	-	-	-	-	26.74	42.50	-	
	96	-	-	-	-	18.76	41.00	-	
Mucuna	0	-	-	-	-	26.44	41.00	-	
	96	-	-	-	-	22.25	42.00	-	
Pea	0	-	-	25.50	-	-	15.30	-	(Martín-Cabrejas et al., 2003)
	48	-	-	24.60	-	-	15.50	-	
	96	-	-	24.00	-	-	24.40	-	
	144	-	-	25.80	-	-	27.00	-	

*Germination time “0” denote raw pulses without germination; “-” denote data not available

cellulose and β-glucan, respectively. The ratio of insoluble dietary fiber to soluble dietary fiber in pea decreased from 1.73 to 0.96 after 6 days of dark germination. The increased soluble dietary fiber percentage denoted that germination improved the availability of dietary fiber of pulse seeds.

2.4.1.3. Effect of germination on lipids

During germination, lipids tend to decrease due to the increased activity of lipases. Free fatty acids hydrolyzed from lipids contribute to the catabolic and metabolic activities . In addition, the decomposed products from lipids play an important part in the synthesis of constituents for young seedlings (Devi et al., 2015). Chickpea, lentil, cowpea, African yam bean, pea, and mung bean had a significant reduction in lipid content compared to its non-germinated counterparts (Table 2.4).

2.4.2. Effect of germination on the quantity of phenolic compounds in pulse seeds

Germination is an efficient and economical means for improving the antioxidant contents of pulse seeds (Yeo and Shahidi, 2015). During germination, three states of phenolic acids are mutually transformed (Wu et al., 2013). As observed in cereal grains, the content of many phenolic acids decreased in the earlier stage of germination, including steeping, while the content of total phenolic acids increased in the later stages of germination (Hübner and Arendt, 2013). It can be concluded that the germinated seeds have more total phenolics than that of ungerminated seeds (Hübner and Arendt, 2013).

In addition, many studies have shown that reactive oxygen species (ROS) play a pivotal role in seed germination as signaling molecules. Gomes and Garcia (2013) use ROS theory to

explain the germination procedure. ROS are derived from the reduction of oxygen, which gives rise to superoxide, hydrogen peroxide, and hydroxyl radicals, as well as, singlet oxygen (Carocho and Ferreira, 2013). After quiescent seeds imbibing water, ROS intentionally generated from electron transport chain in the mitochondria, electron transfer of photosynthesis in the chloroplast, lipid catabolism in glyoxysome, and other biosynthetic pathways (Bailly, 2004).

However, excess ROS can cause damage to DNA (Buetler et al., 2004), carbohydrate, protein, and lipids (Carocho and Ferreira, 2013). The damage of ROS to DNA promote a signal that seeds need to produce more enzymes to protect themselves against ROS. Three different kinds of enzymes are then synthesized (Gill and Tuteja, 2010) which include (i) enzymes for directly scavenging radicals, such as catalase, superoxide dismutase, and glutathione peroxidase; (ii) enzymes for synthesizing phenolic compounds, such as 3-dehydroquinate synthase, 3-dehydroquinate dehydratase, shikimate dehydrogenase, chalcone synthase, and chalcone isomerase; and (iii) enzymes for transformation of phenolic compounds, such as acyltransferase, methyltransferase, and glycosyltransferase.

As a result, biopolymer moieties and phenolic parts in both extractable phenolic compounds and non-extractable phenolic compounds would be dramatically changed during pulse seed germination. In general, TPC of pulse seeds increased during germination, such as chickpea, red lentil, mung bean, and kidney bean (Mamilla and Mishra, 2017).

2.4.3. Effect of pulse seeds germination on the antioxidant activity of phenolic compounds in preventing lipid oxidation

The antioxidant activity, rather than content of phenolic compounds, is the key factor contributing to making pulse extracts a source of alternative antioxidants.

2.4.3.1. Principle of lipid oxidation

The principles of lipid oxidation can be explained as free radicals repeatedly attacking unsaturated lipids and converting them into small molecules of aldehydes, ketones, and acids, some of which have off-flavor in foods and beverages. One lipid oxidation mechanism includes initiation, propagation, and termination stages (Shahidi and Ambigaipalan, 2015). In the initiation step, ultra-violet light, metal ions, or singlet oxygen, attack lipids to generate a lipid radical (L•). The L• can also be formed with the abstraction of hydrogen from lipid molecules by high energy radicals from the second oxidation step. The second step is propagation: oxygen reacts with L• to form a lipid hydroperoxide radical (LOO•), which can further generate lipid hydroperoxides (LOOH) and L•. Then LOOH can react with metal ions to produce lipid alkyl radicals (LO•) and LOO•. Procedures above results in the accumulation of L•, LO•, and LOO•. The accumulated radicals from the propagation step can be terminated by self-interactions to form stable aliphatic alcohol, aldehydes, and other reactive products, which impart off-flavors and odors to fats, oils, and lipid-containing foods (Shahidi and Ambigaipalan, 2015). In general, free radicals and metal ions are the two key factors responsible for lipid oxidation.

2.4.3.2. Prevention of lipid oxidation

Existence of singlet oxygen, metal ions, pro-oxidative enzymes (lipoxygenases), free radical oxidation reactions, and autoxidation chain reaction are responsible for lipid oxidation (Carocho and Ferreira, 2013). Antioxidants are effective additives to scavenge these factors during initiation and propagation steps.

Most antioxidants function to scavenge free radicals. Antioxidant compounds, which have a lower reduction potential than lipid radicals, can donate hydrogen atoms to inactive LOO•, LO•, and L• and produce lipid derivatives and antioxidant radicals, which can further impede the propagation reaction (Chaiyasit et al., 2007). For example, ascorbate has a lower reduction potential ($E^{0'}$) = 282 mV than that of LOO• ($E^{0'}$ = 1000 mV) and thus can react as an antioxidant. In addition, native antioxidants can regenerate antioxidant radicals, which is also called redox recycling, makes antioxidants much more effective (Panya, 2012).

The inactivation of metal cations is another scheme to prevent lipid oxidization. Flavonoids and other phenolic compounds exhibit both metal chelating and radical scavenging activities (Panya, 2012). Industrially, citric acid, phosphoric acid and ethylenediaminetetraacetic (EDTA) are widely used as metal chelators (Chaiyasit et al., 2007).

2.4.3.3. Effect of germination on the antioxidant activity of pulse extracts

Radical scavenging activity assays, such as DPPH• and ABTS•, have been commonly used due to their efficiency and accuracy (Moon and Shibamoto, 2009). Metal chelating assays focus on metal cations such as FRAP (ferric reducing antioxidant power) and total oxidant potential using Cu (II) as a prooxidant (Huang et al., 2005). There are a variety of methods for

the determination of lipid oxidation products, such as conjugated diene, FOX (ferrous oxidation-xylenol), FTC (ferric thiocyanate), GSHPx (glutathione peroxidase), TEAC assay (Trolox equiv. antioxidant capacity), TRAP (total radical-trapping antioxidant parameter), heme degradation of peroxides and the electron paramagnetic resonance (Polak et al., 2015).

Germination has been reported to improve the in vitro antioxidant activities of phenolic compounds. After 2 and 3 days of germination, chickpea had an increased TRAP and TEAC, which increased from 16 to 55%, and from 12 to 23%, respectively (Fernandez-Orozco et al., 2009). In addition, López-Amorós et al. (2006) reported a significant increase antioxidant activity of peas, beans, and lentils with respect to IC50 (scavenge 50% of free radical ABTS) after 4 days of germination.

2.4.4. Germinated pulse seeds as food ingredients

Germinated pulse seeds have been used as food ingredients for many years, based on the general belief that they impart significant nutritional, flavor, and textural benefits over their unsprouted or sound grain counterparts. As an example, Captain James Cook used germinated seeds to combat scurvy in sea voyages. This highlights the enigmatic understanding of the true benefits of germinated seeds. Although researchers have unveiled the mask of germinated pulse with advanced technologies as discussed above, there is still many details that need to be uncovered. For example, variation of moisture, carbohydrate, protein, lipid, and their physicochemical properties are well reported, however, the effect of germination on thermal properties, moisture sorption isotherm, and pasting properties of pulse flours have not been well described (Foschia et al., 2017). In addition, it is well reported that the quantities of phenolic

compounds in pulse seeds increased during germination. However, it is still unknown if the antioxidant activities of these phenolic compounds are strong enough to be utilized as natural antioxidants in preventing lipid oxidation. Further research is expected to be performed to answer these questions.

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CHAPTER 3. EFFECT OF GERMINATION ON THE CHEMICAL COMPOSITION, THERMAL, PASTING, AND MOISTURE SORPTION PROPERTIES OF FLOURS FROM CHICKPEA, LENTIL, AND YELLOW PEA*

3.1. Abstract

Chemical composition, thermal, pasting, and moisture adsorption properties of flours from chickpea, lentil, and yellow pea were investigated over a 6-day germination. Protein content increased for pulses over germination while lentil had the highest protein content that increased from 30.65 to 33.60 g/100 g dry basis (d.b.). Two amino acids, lysine and leucine, increased significantly during germination of chickpea and lentil, respectively. Lipid content in pulse flours decreased over germination with chickpea having the greatest decline, i.e. from 8.00 to 5.90 g/100 g (d.b.). Total starch decreased in lentil and yellow pea flours during germination. Thermal properties of pulse flours changed slightly, while pasting properties varied among pulses. The highest final viscosities for chickpea, lentil, and yellow pea flours were 1061, 981, and 1052 cP and were observed after 2, 1, and 0 days of germination, respectively. Moisture adsorption isotherms showed improved water adsorption capability after germination.

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3.2. Introduction

Currently, there has been an increasing market demand on gluten-free products as gluten can cause autoimmune, i.e. immune-mediated health problem to certain people (Sapone et al., 2012). In the United States, gluten-free food made up 2.8 % and 6.5 % of all food sales in 2013 and 2015, respectively. In 2015, about 4.63 billion U.S. dollars in retail sales were reported (Gao et al., 2018). The market is projected to be valued at 7.59 billion U.S. dollars by 2020 (Statista, 2017).

To meet the gluten-free market demand, the ideal scenario would be to select raw materials that can be inexpensively produced and have an outstanding nutrition value. Pulse crops, including chickpea (*Cicer aretinium* L.), yellow pea (*Pisum sativum* L.), and lentil (*Lens culinaris* Merr.), are gluten-free. Pulse flours are high in proteins, carbohydrates, vitamins, and minerals. For example, the protein content in pulse flours (17–40 g/100 g on the dry basis (d.b.)) is much higher than in cereal flours (3–7 g/100 g d.b.) (Ghumman et al., 2016). Lysine content, one of the essential amino acids, in pulse seeds is much higher than in cereals (Damodaran et al., 2008). In addition, pulse flours have higher dietary fiber than wheat flours and have been used for producing food with a lower glycemic index (Fujiwara et al., 2017). Furthermore, the consumption of pulse flours has been associated with improved human health, owing mainly to the presence of bioactive components such as polyphenols (Xu et al., 2018c).

Three continuous limitations for the use of pulse as food ingredients have been reported. First, the presence of antinutritional factors such as trypsin inhibitors (Savelkoul et al., 1992); second, lower nutritional value due to the existence of resistance starch and dietary fiber (Li, Oh,

Lee, Baik, & Chung, 2017); and third, relatively lower bioactive chemicals and antioxidants (Jan et al., 2018). Germination of pulse has been employed to modify macronutrient structure (Jan et al., 2018), improve the digestibility (Tharanathan and Mahadevamma, 2003), generate new compounds with higher bioactivities (Xu et al., 2018b; c), mitigate beany flavors (Xu et al., 2019a), increase nutritional values, and improve the amino acid profiles (Guajardo-Flores et al., 2017). Therefore, germinated pulse seems to be a potential functional food ingredient rather than a raw pulse crops.

As is well known, physicochemical characteristics are critical for the application of food ingredients. It is important to understand the impact of germination on the physical property and chemical composition changes of pulse crops before they can be incorporated into foods as food ingredients. Thermal properties reflect the gelatinization temperature and enthalpy of pulse flours. Commonly, differential scanning calorimetry (DSC) is employed to investigate the gelatinization along with the thermal transition. Higher pasting properties is ideal for thickening food or gel formation, otherwise, lower pasting property would be preferred. The knowledge of moisture sorption isotherm is essential to determine product stability, and needed for design of storage, packaging, and drying systems for extension of shelf life.

The composition changes of pulse crops during germination have been investigated to a certain degree. However, the impact of germination on the physical properties of pulse flours has not been fully explored. Therefore, the objective of the present study was to determine the effect of germination on the physicochemical composition changes of chickpea, lentil, and yellow pea. Variability of their thermal and pasting properties, and moisture sorption isotherms following

germination were also evaluated. The findings provide important information that could be used to formulate gluten-free foods with germinated pulse flours.

3.3. Materials and Methods

3.3.1. Chemicals

Chickpea (*Cicer aretinium* L.), lentil (*Lens culinaris* Merr.), and yellow pea (*Pisum sativum* L.) from the 2017 crop year were gifted from JM Grain (Garrison, ND, USA), Viterra Inc (Grand Forks, ND, USA), and AGT Food and Ingredients (Minot, ND, USA). The manufacturers have noted that the samples were a mixture of different cultivars. Total starch assay kit K-TSTA-50A was purchased from Megazyme (Megazyme Inc., Chicago, IL). The amino acid assay kit AccQ•Fluor was purchased from (Waters, Milford, MA, USA). All other chemicals were purchased from VWR International (West Chester, PA, USA).

3.3.2. Germination of pulse seeds

Each pulse seed from three different sources was mixed thoroughly before germination. The procedure of pulse germination was adapted from our previous research without any modification (Xu et al., 2018c). Briefly, 500 g of seeds were soaked in 2000 mL of 0.07% sodium hypochlorite for 30 min. Then, these seeds were washed with distilled water until reaching neutral pH. Afterward, seeds were soaked with 2000 mL of distilled water for 5.5 h, shaking every 30 min. The hydrated seeds were stored on a covered wet laboratory paper in germination trays, which were in contact with the circulating moist air to maintain the relative humidity and moisture of seeds at 99% and 50%, respectively. Seeds were germinated at 25 °C

in the dark for 6 days. Germination was performed in duplicate at the same time with two separate containers.

The sprouted seeds with 0, 1, 2, 3, 4, 5, and 6 days of germination were collected and frozen in the refrigerator at -63 °C overnight. Frozen pulse seeds were freeze-dried in a freeze dryer (Virtis, GPFD 24D×48, Gardner, NY). The temperature of the condenser was set at -50 °C, while initial temperature of chamber was -10 °C. After the pressure of the chamber was stablized at 10 millitorr, the chamber temperature was increased to 25 °C so that ice could sublimate faster. After 3 days in the freeze-drier, pulses seeds were stored at -4 °C in a refrigerator.

3.3.3. Preparation of pulse flours

Freeze-dried pulse seeds were ground using either a cyclone mill or disc mill for comparison. A Udy cyclone mill (MODEL 3010-030, UDY Corp., Fort Collins, CO) with a 0.5 mm screen was used to create the pulse flours. A disc mill (Perten, Laboratory Mill 3600, Springfield, IL) with a fine disc was used to mill raw and germinated pulse samples into flours. The distance between the stationary and the rotating disc was set as 1 (the minimum gap). Three hundred grams of freeze-dried pulses were milled by the cyclone mill and disc mill for 7 min and 5 min, respectively.

3.3.4. Chemical composition analysis

Moisture and ash contents were determined using AACC Approved Methods 44-15.02 and 08-01.01, respectively. Total dietary fiber (TDF) including soluble dietary fiber (SDF) and insoluble dietary fiber (IDF) were tested using an automated Ankom TDF Dietary Fiber Analyzer (Ankom Technology Corp., Macedon, NY). Crude protein content was measured by

nitrogen combustion method using a LECO FP428 nitrogen analyzer (LECO Corporation, St Joseph, MN, USA). A nitrogen conversion factor of 6.25 was used to calculate crude protein content. A total starch assay kit K-TSTA-50A (Megazyme International) was used to determine the starch content following the AACC Approved Method 76-13.01. Crude lipid was estimated using a Dionex™ ASE™ 350 Accelerated Solvent Extractor (Thermo Fisher Scientific, Sunnyvale, CA) with 22 mL stainless-steel extraction cells.

Amylose content in pulse flours was measured by the method adapted from Sandhu & Singh (2007). Briefly, 10 mg of pulse flours were thoroughly mixed with 10 mL of NaOH (0.5 N). The dispersed sample was diluted with distilled water in a 50 mL volumetric flask and 10 mL subsequently transferred into a 50 mL volumetric flask. Five mL of 0.1 N HCl was added followed by 0.5 mL 2% iodine solution. The volume was brought up to 50 mL with distilled water and allowed to stand for 2 min before the absorbance was measured at 625 nm.

Non-starch carbohydrate was calculated by the (Equation 3.1). The unit of all the values was g/100 g on dry basis.

$$\text{Non-starch carbohydrate} = (100 - \text{moisture} - \text{ash} - \text{protein} - \text{starch} - \text{lipid}) \quad \text{(Equation 3.1)}$$

3.3.5. Amino acid analysis

Pulse protein isolates were extracted from pulse flours for the amino acid analysis. The pulse protein isolates, including chickpea protein isolate, lentil protein isolate, and yellow pea protein isolate, were extracted using a alkaline extraction-isoelectric precipitation method adapted from Lan et al. (2018). Briefly, germinated pulse flour (70.0 g) was dispersed in double distilled water at a ratio of 1:15 (w/v) and pH of solution was adjusted to 9.5. Solutions were

magnetic stirred for 1 h at 25 °C followed by centrifugation at 6,000 rpm (Beckman J2–HS, Beckman Coulter Inc., Indianapolis, IN, USA) for 20 min. Supernatant was collected and filtered using Whatman grade 1 paper. The pH of supernatant was adjusted to their isoelectric points pH 4.5 (Boye et al., 2010a), to precipitate proteins and then centrifuged at 6,000 rpm for 10 min. Pellets were collected and re-suspended in water, followed by a pH adjustment to 7.0 using 1.0 M NaOH. The protein isolate was obtained by freeze drying the pellet for 48 h with a same lyophilizer used to obtain dried pulses and stored in a refrigerator at -20 °C until needed.

The method of amino acid analysis was adapted from Kabelová et al. (2009). Ten mL of 6 N HCl was added into tubes with 50 mg of pulse protein isolates and 0.2 mL of 25 mM α-aminobutyric acid (AABA). Three droplets of phenol was added to prevent the halogenation of tyrosine, while nitrogen purge was required to prevent the oxidation of amino acids. Then, tubes were sealed tightly and set in the oven for 22 hours at 105 °C for the protein hydrolysis. The hydrolyzed solution were cooled and diluted to 50 mL with distilled water. After filtering through a 0.2 μm polytetrafluoroethylene (PTFE) disposable membrane filter, 1 mL of the solution was freeze dried and then dissolved with 1 mL of citric acid buffer (pH 2.2) labeled as prepared sample. The amino acid calibration standards (0, 0.5, 1, 5, 10, and 25 μg/L) were prepared with 0.1 mM of AABA as an internal standard. Ten μl of the calibration standard or prepared sample was added into 70 μl of AccQ•Fluor Borate Buffer in a vial. After 15 seconds of vortex, 20 μl of reconstituted AccQ•Fluor Reagent was added into the tube, and mixed immediately for several seconds with Vortex. The content was then transferred to an autosampler vial, which was heated in a water bath at 55 °C for 10 minutes.

An Agilent 1200 HPLC system with a diode-array detector (DAD) (Agilent, Milford, Massachusetts, USA) was employed for amino acids analysis. AccQ•Tag amino acid column (Nova-Pak C18, 4 μm, 150 × 3.9 mm) from Waters was used. The column was thermostatic at 55 °C. After 5 µl of each sample injected into the column, a gradient mobile phase was used. The mobile phase consisted of eluent A (prepared from Waters AccQ•Tag Eluent A concentrate, by adding 100 ml of concentrate to 1 L of Milli-Q water and mixing) and eluent B (acetonitrile, HPLC grade). The best gradient separation program was in Table 3.1:

Table 3.1. The parameters of gradient eluent program

Time (min)	Eluent A (%)	Eluent B (%)	Flow (mL/min)
0	99.9	0.1	0.4
0.5	99	1	0.4
18	95	5	0.4
19	91	9	0.4
29.5	83	17	0.4
36	0	100	0.4
39	99.9	0.1	0.4
43	99.9	0.1	0.4

3.3.6. Lipoxygenase activity

Lipoxygenase (LOX) was analyzed following the method of Poudel et al with some modifications (Poudel et al., 2017). Pulse flours (1.0 g) and 5 mL of phosphate buffer (0.2 mol/L, pH 6.5) were mixed, and then extracted for 2 h using an ultrasound containing an ice bath. Tubes were shaken by hand each half hour. The mixture was centrifuged at 4,000 × g for 10 min at 4 °C. The supernatant was used as the crude enzyme extract for further analysis. For the substrate solution, linoleic acid (L-1376, Sigma-Aldrich) (140 μL) and Tween 20 (140 μL) were mixed and then emulsified into 8 mL of 0.2 mol/L phosphate buffer (pH 6.5). The solution was clarified

using 1.1 mL of 0.5 N NaOH and the volume brought to 50 mL with phosphate buffer (pH 6.5). The stock solutions were diluted (1:40, v/v) with 0.2 mol/L sodium borate buffer (pH 9.0) before use. The substrate solution was flushed with nitrogen to prevent any oxidation. Enzymatic activity was measured at room temperature (25 °C) at 234 nm (VWR 6300 Double Beam UV–Vis Spectrophotometer, VWR, Palo Alto, CA, USA). The quartz cuvette containing 1.25 mL of the substrate and 20 μL of the extract was rapidly mixed for 5 s, and the change in absorbance was recorded for 5 min with a time interval of 5 seconds. If the rate of absorbance increase was larger than 0.05/min, the extract was diluted with 0.2 mol/L sodium borate buffer (pH 9.0). If there was no or very little change in absorbance, the addition of extract was changed to 100 μL. The units of LOX activity were U/g, where U was defined as the numeric increase in absorbance per minute.

3.3.7. Particle size analysis

The particle size of flours was measured by laser light scattering using a Malvern Mastersizer 3000 instrument with an automated Aero S dry powder disperser (Malvern Instruments Ltd, Worcestershire, UK). The particle sizes of flours are reported as d_{43} and d_{50}, which defines the volume-weighted diameter and the diameter at 50% cumulative volume, respectively.

3.3.8. Determination of thermal properties

Differential scanning calorimeter (DSC) Q2000 (TA Instruments, New Castle, Del, USA) was employed to test the thermal properties of pulse flours. The procedure followed White et al. (1990). Approximately 3.5 mg (d.b.) of pulse flours was weighed accurately into an aluminum

pan, followed by the addition of 8 μL of distilled water (70% moisture content). This ratio of pulse flours to water provided enough water to achieve one symmetrical DSC gelatinization peak. The pan was hermetically sealed and allowed to sit for 2 hrs. The instrument was calibrated for temperature and enthalpy measurement with indium, and an empty pan was used as a reference. Samples were heated from 30 to 120 °C at a rate of 10 °C/min and cooled from 120 to 40 °C at a rate of 25 °C/min. Thermal transitions were characterized by computing onset temperature (T_o), peak temperatures (T_p), the completion of the transition temperature (T_c), and melting enthalpy (ΔH) using TA Universal Analysis software.

3.3.9. Determination of pasting properties

Pasting properties of pulse flours were determined with a Rapid Visco Analyzer (RVA 4500, Perten Instruments, Springfield, IL) using AACC Approved Methods 76-21.01. Briefly, pulse flours (3.0 g, d.b.) was added to 25 mL deionized water in an RVA canister. Slurries were held at 50 °C for 1 min before heating from 50 to 95 °C at a rate of 12 °C/min and held at 95 °C for 2.5 min. The slurry was then cooled at a rate of 12 °C /min to 50 °C and held for 12 min.

3.3.10. Moisture adsorption isotherms

A fully automated Aqualab vapor sorption analyzer (VSA, Decagon Devices Inc., Pullman, WA) was used to generate moisture adsorption isotherms of pulse flours at 25 °C following the dynamic vapor sorption method as described by Syamaladevi et al. (2016). The VSA is capable of generating equilibrium relative humidity conditions between 20% and 85% that corresponds to water activities of 0.20–0.85. During the test, approximate 2.0 g of pulse flours inside the VSA were exposed to 20% humidity until a constant sample mass was achieved.

Once the sample reached equilibrium, the corresponding water activity and water contents were recorded. The VSA then incrementally set another level of relative humidity to bring the water content of the sample to a different equilibration value. A 0.1 a_w of resolution was selected for the water sorption curve. The equilibrium water content value at each water activity step was calculated from the weight change data. The Guggenheim–Anderson–de Boer (GAB) isotherm model (Equation 3.2) was used to describe the dry basis moisture content (m) as a function of water activity (a_w).

$$m = \frac{CKm_0a_w}{(1-Ka_w)(1-Ka_w+CKa_w)} \quad \text{(Equation 3.2)}$$

Where *C* and *K* are constants and m_0 is described as the monolayer moisture content on the dry basis.

3.3.11. Statistical analysis

The experimental treatments were arranged in a completely randomized design (CRD) with two replicates. All measurements were performed with duplicate samples, and values were expressed as means ± SD of duplicates from each of two independent experiments. The data were statistically analyzed using statistical software, SAS version 9.4 (SAS Institute Inc. Cary, NC). One-way analysis of variance (ANOVA) was conducted, and a significant difference was defined at $p < 0.05$ by Tukey's test.

3.4. Results and Discussion

3.4.1. Milling process selection

Particle size is a predictor of flours quality, thus, we compared the impact of two widely used laboratory milling equipment, cyclone mill and disc mill, on the pasting properties and

particle sizes of raw chickpea, lentil, and yellow pea flours (Kaiser et al., 2019). Pasting properties of raw chickpea, lentil, and yellow pea flours differed significantly between the two milling systems (Table 3.2). In particular, the flours processed by the cyclone mill had much higher peak, breakdown, and final viscosities than flours from the disc mill. The pasting temperature of cyclone milled pulse flours was significantly lower. Nonetheless, the setback viscosity of pulse flours remained the same irrelevant of the milling equipment except that of yellow pea.

Table 3.2. Effect of milling on pasting properties and particle size of chickpea, lentil, and yellow pea flour

	Chickpea		Lentil		Yellow pea	
	Disc	Cycl	Disc	Cycl	Disc	Cycl
Peak viscosity (cP)	357±6 a	773±9 b	483±27 a	687±20 b	353±7 a	1089±115 b
Breakdown (cP)	14±1 a	29±7 b	12±1 a	38±4 b	21±3 a	37±1 b
Final visc (cP)	592±50 a	1009±11 b	789±37 a	956±14 b	679±28 a	1656±115 b
Setback (cP)	256±48 a	266±10 a	318±12 a	307±1 a	347±18 a	605±22 b
Peak time (min)	6.9±0.1 a	7.0±0.0 a	7.0±0.0 b	5.3±0.0 a	7.0±0.0 b	5.5±0.1 a
Pasting temp. (°C)	82.9±0.7 b	79.5±0.5 a	79.5±0.6 b	76.3±0.6 a	84.4±0.7 b	77.0±0.6 a
d43 (μm)	375±27 b	108±29 a	459±2 b	76±6 a	473±24 b	62±4 a
d50 (μm)	342±18 b	23±1 a	446±1 b	28±1 a	460±18 b	25±0 a

*Cycl and Disc denotes cyclone mill and disc mill, respectively. Breakdown is presented as the peak viscosity – hot paste. Data points represent mean ± standard deviation of two independent experiments. Different letters indicate statistically significant different results between two milling systems ($p < 0.05$).

With respect to the particle size, all three pulse flours processed by the disc mill showed bimodal distributions, with the maximum weight percent of particles appearing at ~ 500 μm (Figure 3.1). Conversely, uniformed particle sizes with monomodal distributions were observed in the pulse flours prepared by cyclone mill, with an exception of lentil flours which showed a

small shoulder in the high particle size end. The smaller d_{43} and d_{50} observed the cyclone milled flours supports the finer flours it produced (Table 3.2). In the cyclone mill, samples remain in the grinding chamber until impact-shattering and abrasion make them small enough to flow out the screen with the air current. The particle size of cyclone milled flours relies on the sieve size of the screen. In disc mill, the particle size of samples is generally bigger as it is reduced by the friction between one stationary and one rotating disc and is determined by the disc type and distance between the discs. For instance, Assefa et al. (2018) highlighted that Tef [*Eragrostis tef* (Zucc.) Trotter] flours processed by disc mill exhibited a broad particle size distribution ranging from 90 μm to 710 μm. The differences in pasting properties of the flours from the two different mills might be attributed to the courser particles that prevented the water from penetrating into the starch granules, thus resulting in the lower pasting viscosities than the finer ones. Therefore, it is worth mentioning that the particle size of flours should be reported when comparing the pasting properties of the same variety of sample. Based on the results of pasting properties and particle sizes, cyclone mill was employed for the preparation of germinated pulse flours.

3.4.2. Impact of germination on the chemical composition of pulse flours

Germination is a complex green bioprocess in which pulse seeds physically recover from maturation drying, resume a sustained metabolic intensity, and complete essential cellular events. Thus, the alteration of macronutrients, such as proteins, lipids, and carbohydrate, is anticipated as they serve as energy sources during seed germination. Therefore, the content of macronutrients in germinated chickpea, lentil, and yellow pea was determined (Table 3.3).

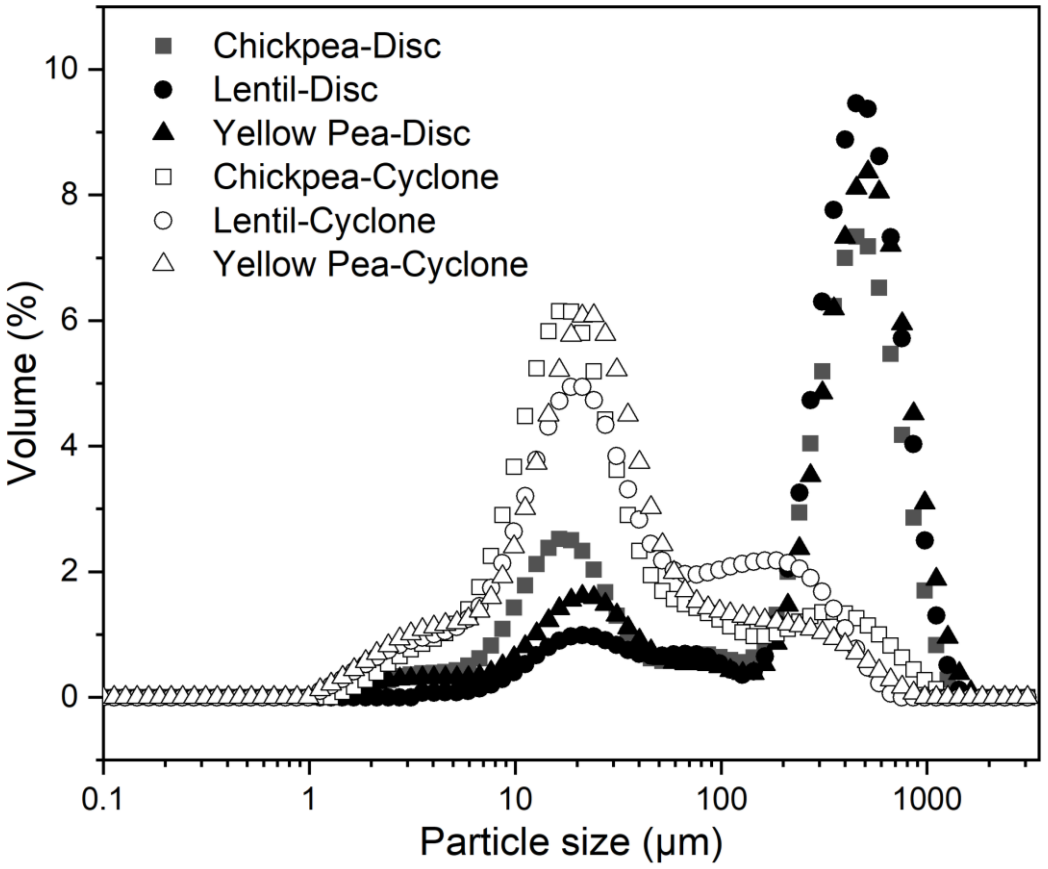

Figure 3.1. Particle size distribution of chickpea, lentil, and yellow pea flours processed by disc mill and cyclone mill

3.4.2.1. Effect of germination on ash content

Ash is considered as a low commercial value constituent (Delcour & Hoseney, 2010). The ash content of raw chickpea, lentil, and yellow pea was 3.13 g/100 g, 2.57 g/100 g, and 2.63 g/100 g, respectively (Table 3.3). Raw chickpea had the highest ash content among the three crops. During germination, the ash content of chickpea, lentil, and yellow pea changed slightly among the range of 3.10–3.33 g/100 g, 2.36–2.64 g/100 g, and 2.63–2.83 g/100 g, respectively, with no significant difference being observed among the samples (Table 3.3).

Table 3.3. Effect of germination on macronutrients of chickpea, lentil, and yellow pea flour*

	Germination time (days)	Ash (g/100g)	Protein (g/100g)	Lipid (g/100g)	Starch			Non-starch carbohydrate (g/100g)
					Total starch (g/100g)	Apparent amylose content (g/100g)	Apparent amylose/total starch	
Chickpea	0	3.13±0.06 a	24.36±0.64 a	8.00±0.00 f	40.28±1.28 a	13.70±0.22 a	34.01±0.53 a	19.55±0.51 bc
	1	3.10±0.04 a	24.65±0.25 ab	7.59±0.02 e	40.55±0.22 a	13.41±0.19 a	33.08±0.28 a	20.22±0.37 c
	2	3.21±0.00 a	25.65±0.20 bc	7.19±0.06 d	40.70±0.12 a	16.03±0.39 b	39.38±1.07 b	18.87±0.02 bc
	3	3.20±0.02 a	25.64±0.06 bc	6.88±0.14 cd	40.99±0.86 a	16.32±0.06 b	39.81±0.68 b	19.69±0.92 bc
	4	3.19±0.14 a	26.06±0.13 cd	6.66±0.03 bc	40.55±0.41 a	15.62±0.36 b	38.51±0.50 b	20.45±0.57 c
	5	3.33±0.15 a	26.77±0.05 de	6.39±0.15 b	40.47±0.35 a	16.27±0.35 b	40.20±0.53 b	17.68±0.69 ab
	6	3.26±0.15 a	27.75±0.01 e	5.90±0.02 a	40.57±0.35 a	16.63±0.49 b	41.01±1.56 b	15.64±0.46 a
Lentil	0	2.57±0.06 ab	30.65±0.48 a	1.72±0.02 e	41.02±2.04 d	14.06±0.32 b	32.61±1.06 a	20.32±0.87 b
	1	2.36±0.02 a	30.82±0.13 ab	1.62±0.01 d	42.36±0.17 d	16.33±0.13 c	38.55±0.46 c	19.22±0.12 b
	2	2.37±0.02 ab	30.88±0.16 ab	1.57±0.01 d	41.83±0.60 cd	16.02±0.19 c	38.30±1.00 bc	19.93±0.80 b
	3	2.45±0.03 ab	31.66±0.03 b	1.46±0.02 c	39.51±0.90 bc	15.72±0.07 c	39.80±1.09 c	20.6±0.66 b
	4	2.59±0.06 ab	33.13±0.19 c	1.34±0.03 b	41.32±0.83 cd	13.34±0.07 b	32.30±0.81 a	12.74±1.34 a
	5	2.52±0.00 ab	33.03±0.10 c	1.29±0.01 b	38.51±0.71 b	12.24±0.14 a	31.78±0.94 a	15.43±0.37 a
	6	2.64±0.16 b	33.60±0.00 c	1.16±0.01 a	34.96±0.57 a	12.17±0.53 a	34.80±0.95 ab	19.19±0.52 b
Yellow pea	0	2.63±0.00 a	25.83±0.16 a	1.87±0.03 e	43.81±0.29 c	12.04±0.37 a	27.50±1.04 a	19.39±0.64 a
	1	2.74±0.07 a	26.03±0.12 a	1.76±0.02 d	43.59±0.42 c	16.47±1.20 bc	37.79±3.12 b	20.65±0.25 ab
	2	2.70±0.07 a	26.00±0.67 a	1.72±0.02 d	43.69±0.06 c	18.36±0.81 c	42.03±1.91 b	23.49±0.68 bc
	3	2.67±0.09 a	25.68±0.44 a	1.68±0.01 cd	42.91±0.46 bc	17.92±0.21 bc	41.77±0.94 b	24.92±0.86 c
	4	2.71±0.06 a	27.8±0.47 b	1.63±0.01 bc	39.86±0.93 a	15.61±0.56 b	39.17±0.50 b	21.57±1.76 abc
	5	2.83±0.07 a	27.74±0.08 b	1.57±0.01 b	41.44±0.64 ab	15.48±0.39 b	37.37±1.51 b	18.14±1.12 a
	6	2.73±0.02 a	28.01±0.02 b	1.43±0.04 a	40.94±0.38 a	16.04±0.49 bc	39.18±1.56 b	18.42±0.07 a

*All the data were calculated on the dry weight basis. Data points represent mean ± standard deviation of two independent experiments. Different letters indicate statistically significant differences intraspecies ($p < 0.05$)

3.4.2.2. Effect of germination on protein content

The protein content of raw pulse flours followed the order of lentil (30.65 g/100 g) > yellow pea (25.83 g/100 g) > chickpea (24.36 g/100 g). A small increase of crude protein was observed at the beginning of germination. Crude protein of chickpea, lentil, and yellow pea increased significantly after germination for 3, 4, and 5 days, respectively (p<0.05). By the end of the 6-day germination, crude protein content increased by 3.39 g/100 g, 2.95 g/100 g, and 2.81 g/100 g in chickpea, lentil, and yellow pea, respectively (Table 3.3). The increase of crude protein content during pulse germination has been observed by other researchers. Masood et al. (2014) reported the crude protein of chickpea increased from 17.80 g/100 g to 23.37 g/100 g after a 120-hour germination. Fouad and Rehab (2015) reported the crude protein of lentil increased from 25.63 g/100 g to 28.86 g/100 g after 6 days of germination. The increased crude protein content during germination had been explained by (i) the synthesis of enzymes by germinating seed; (ii) a compositional change following the degradation of other constituents, and (iii) the synthesis of newly-formed protein that occurs during germination. The increased protein is plausible if germination were performed with the supplement of a nitrogen source. However, many germination experiments were performed with pure water (Masood et al., 2014; Fouad and Rehab, 2015), which means negligible nitrogen source was available. In addition, it is well known that crude protein is estimated indirectly on the basis of nitrogen content. According to the mass balance calculation, crude protein content tends to be constant if there is no net gain or loss of nitrogen. In this sense, an increase in protein is surprising for seeds germinated with pure water. Since the crude protein content is calculated and presented as a percentage of dry

weight, our interpretation is that the loss of dry weight, such as metabolic loss and shoot snap, occurred during germination and processing. Therefore, crude protein divided by the decreased total dry weight resulted in the increase of relative crude protein percentage rather than the absolute protein content.

3.4.2.3. Effect of germination on amino acids profiles

Proteins are made up of 20 amino acids, 9 of which are essential amino acids, such as histidine (His), phenylalanine (Phe), valine (Val), threonine (Thr), methionine (Met), leucine (Leu), isoleucine (Ile), lysine (Lys), histidine (His), and tryptophan (Try), while 11 of which are non-essential amino acids, such as alanine (Ala), arginine (Arg), asparagine (Asn), aspartic acid (Asp), glutamic acid (Glu), glutamine (Gln), glycine (Gly), proline (Pro), serine (Ser), cysteine (Cys), and tyrosine (Tyr). Some of the non-essential amino acids are conditional essential amino acid, such as cysteine (Cys).

Sum of essential amino acids were not significantly different ($p < 0.05$) in raw chickpea, lentil and yellow pea; however, some individual essential amino acids are significantly different in pulses (Table 3.4). The sum of cysteine and lysine are high in raw lentil and yellow pea, i.e., 7.45 ± 0.60 and 6.55 ± 0.50 g/100 g, respectively, while low in chickpea (3.52 ± 0.75 g/100 g). Cereal seeds are low in lysine compared to meat amino acids and pulse seeds are reported to have higher lysine than cereal seeds (Damodaran et al., 2008). Therefore, raw lentil and yellow pea should be a good lysine supplement. Lower methionine is the major shortage of pulse protein compared to the animal proteins. Methionine is high in raw chickpea, which is 0.64 ± 0.07 g/100 g, while lentil and yellow pea are low in methionine (Damodaran et al., 2008), which is 0.10 ±

0.00 and 0.19 ± 0.01 g/100 g protein, respectively. Methionine is a limiting amino acids in chickpea as reported by Khan et al. (1995). However, proteins of cereals, such as rice, wheat, barley, and maize are rich in methionine (Damodaran et al., 2008). Consequently, combination of cereal and pulse protein is a good strategy to improve their amino acid quality.

As shown in Table 3.4, essential amino acids did not significantly change during germination. The sum of cysteine and lysine increased significantly ($p < 0.05$) in chickpea after germination from 3.52 ± 0.75 to 7.74 ± 0.47 g/100 g, while histidine decreased from 6.03 ± 0.19 to 4.06 ± 0.18 g/100 g after germination. As pulse has a low cysteine content in pulse protein (Hall et al., 2017), lysine is the major amino acids that likely increased during chickpea germination. Lysine increased from 7.1 to 7.8 g/100 g after 24 hours of mung bean germination (Wongsiri et al., 2015). Leu increased significantly at 5 days of lentil germination, from 7.23 ± 0.02 to 8.28 ± 0.14 g/100 g. Kuo et al. (2004) germinated lentil for 2, 4, and 6 days and observed a consistent increase in leucine content.

Sum of non-essential amino acids in raw lentil is significantly ($p < 0.05$) higher than that in raw chickpea and yellow pea, which were 55.02 ± 0.43, 59.44 ± 0.41, and 59.37 ± 0.75 g/100 g, respectively. The content of glutamic acid and glutamine contributed to this difference. Sum of glutamic acid and glutamine were high in chickpea and yellow pea, 16.04 ± 0.27 g/100 g and 16.67 ± 0.39 g/100 g, respectively, while low in lentil, i.e., 14.67 ± 0.2 g/100 g. As glutamic acid

Table 3.4. Effect of pulse germination on the amino acids profile

	CP0	CP1	CP3	CP5	LE0	LE1	LE3	LE5	YP0	YP1	YP3	YP5
His	6.03 ± 0.19 a	5.82 ± 0.09 ab	5.67 ± 0.00 abc	4.06 ± 0.18 e	4.67 ± 0.11 bcde	4.02 ± 0.23 ef	4.90 ± 0.11 abcde	4.67 ± 0.69 bcde	5.43 ± 0.61 abcd	4.48 ± 0.16 de	4.50 ± 0.19 cde	2.84 ± 0.02 f
Thr	3.16 ± 0.19 a	3.25 ± 0.28 a	3.14 ± 0.14 a	3.15 ± 0.06 a	3.43 ± 0 a	3.65 ± 0.30 a	3.58 ± 0.03 a	3.60 ± 0.01 a	3.40 ± 0.11 a	3.43 ± 0.07 a	3.39 ± 0.00 a	3.39 ± 0.02 a
Cys+lys	3.52 ± 0.75 e	5.84 ± 0.45 cd	6.73 ± 0.22 bc	7.74 ± 0.47 ab	7.45 ± 0.60 abc	6.78 ± 0.17 abc	6.15 ± 0.49 bc	7.30 ± 0.60 abc	6.55 ± 0.50 bc	8.65 ± 0.6 a	7.43 ± 0.21 abc	4.16 ± 0.28 de
Met	0.64 ± 0.07 a	0.43 ± 0.03 bc	0.53 ± 0.06 ab	0.50 ± 0.01 ab	0.10 ± 0.00 e	0.18 ± 0.03 de	0.16 ± 0.01 e	0.09 ± 0.04 e	0.19 ± 0.01 de	0.16 ± 0.04 e	0.1 ± 0.03 e	0.33 ± 0.01 cd
Val	3.93 ± 0.04 b	3.91 ± 0.18 b	3.93 ± 0.07 b	4.06 ± 0.01 b	4.36 ± 0.01 ab	4.65 ± 0.38 a	4.64 ± 0.01 a	4.78 ± 0.09 a	4.41 ± 0.04 ab	4.47 ± 0.01 ab	4.46 ± 0.2 ab	4.66 ± 0.08 a
Ile	3.28 ± 0.04 bc	3.05 ± 0.09 c	3.26 ± 0.11 bc	3.50 ± 0.03 abc	3.33 ± 0.01 abc	3.66 ± 0.32 ab	3.62 ± 0.01 ab	3.83 ± 0.07 a	3.52 ± 0.05 abc	3.57 ± 0.03 ab	3.53 ± 0.25 abc	3.69 ± 0.04 ab
Leu	7.50 ± 0.11 abc	7.08 ± 0.22 c	7.27 ± 0.16 bc	7.82 ± 0.08 abc	7.23 ± 0.02 c	7.90 ± 0.64 abc	7.86 ± 0.02 abc	8.28 ± 0.14 a	7.77 ± 0.09 abc	7.97 ± 0.07 abc	7.89 ± 0.49 abc	8.26 ± 0.03 ab
Phe	5.31 ± 0.60 a	5.12 ± 0.18 ab	5.12 ± 0.00 ab	5.35 ± 0.29 a	4.32 ± 0.05 b	4.77 ± 0.25 ab	4.79 ± 0.06 ab	5.09 ± 0.01 ab	4.53 ± 0.10 ab	4.65 ± 0.01 ab	4.58 ± 0.28 ab	4.87 ± 0.01 ab
Essential*	33.36 ± 0.10 bc	34.5 ± 0.43 abc	35.66 ± 0.76 abc	36.18 ± 1.13 ab	34.88 ± 0.4 abc	35.61 ± 1.98 abc	35.7 ± 0.36 abc	37.64 ± 0.27 a	35.79 ± 1.51 abc	37.38 ± 0.29 a	35.88 ± 1.28 abc	32.19 ± 0.35 c
Ser	6.08 ± 0.19 ab	5.78 ± 0.20 ab	5.54 ± 1.02 ab	4.99 ± 0.48 b	5.85 ± 0.17 ab	6.36 ± 0.25 ab	6.32 ± 0.06 ab	6.50 ± 0.00 a	5.79 ± 0.15 ab	6.07 ± 0.02 ab	6.10 ± 0.06 ab	5.99 ± 0.12 ab
Arg	7.90 ± 0.16 a	7.78 ± 0.56 a	5.44 ± 1.10 b	7.52 ± 0.28 a	6.43 ± 0.36 ab	6.86 ± 0.24 ab	6.55 ± 0.04 ab	6.88 ± 0.10 ab	7.14 ± 0.15 a	7.69 ± 0.19 a	7.25 ± 0.37 a	7.47 ± 0.01 a
Gly	5.93 ± 0.39 ab	6.01 ± 0.46 ab	5.35 ± 0.45 ab	5.00 ± 0.48 b	5.73 ± 0.36 ab	6.32 ± 0.48 a	5.90 ± 0.02 ab	5.82 ± 0.14 ab	6.39 ± 0.15 a	6.16 ± 0.10 ab	6.18 ± 0.28 ab	5.87 ± 0.07 ab
Asp+asn	11.93 ± 0.22 bcd	11.76 ± 0.37 cd	12.86 ± 0.17 ab	13.21 ± 0.49 a	11.02 ± 0.25 d	11.24 ± 0.28 cd	13.29 ± 0.15 a	12.14 ± 0.23 bc	11.73 ± 0.04 cd	9.56 ± 0.13 e	11.82 ± 0.04 cd	12.19 ± 0.03 bc
Glu+gln	16.04 ± 0.27 abc	15.70 ± 0.06 abcd	16.47 ± 0.57 a	16.36 ± 0.25 ab	14.67 ± 0.2 cdef	14.36 ± 0.47 def	15.06 ± 0.21 bcde	13.55 ± 0.30 f	16.67 ± 0.39 a	13.75 ± 0.64 ef	15.32 ± 0.13 abcd	14.90 ± 0.23 cdef
Ala	5.35 ± 0.07 a	5.55 ± 0.62 a	5.56 ± 0.09 a	5.39 ± 0.08 a	5.13 ± 0.04 a	4.97 ± 0.44 a	4.88 ± 0.04 a	5.02 ± 0.33 a	5.05 ± 0.08 a	5.17 ± 0.13 a	4.81 ± 0.15 a	4.92 ± 0.05 a
Pro	4.10 ± 0.02 ab	4.12 ± 0.18 ab	4.09 ± 0.12 ab	4.11 ± 0.04 ab	3.91 ± 0 b	4.13 ± 0.29 ab	4.29 ± 0.02 ab	4.40 ± 0.10 a	4.14 ± 0.02 ab	4.21 ± 0.05 ab	4.27 ± 0.11 ab	4.38 ± 0.05 ab
Tyr	2.11 ± 0.25 cd	1.97 ± 0.06 d	1.97 ± 0.09 d	1.94 ± 0.08 d	2.27 ± 0.03 bcd	2.43 ± 0.11 abc	2.40 ± 0.05 abc	2.53 ± 0.00 ab	2.47 ± 0.02 abc	2.62 ± 0.07 ab	2.45 ± 0.08 abc	2.70 ± 0.03 a
Non-essential*	59.44 ± 0.41 a	58.67 ± 1.12 ab	57.28 ± 1.96 ab	58.50 ± 0.54 ab	55.02 ± 0.43 b	56.68 ± 2.07 ab	58.69 ± 0.35 ab	56.82 ± 1.00 ab	59.37 ± 0.75 a	55.23 ± 0.74 b	58.2 ± 0.56 ab	58.41 ± 0.22 ab

*All the units is g/100 g protein. Essential amino acids including histidine (His), phenylalanine (Phe), valine (Val), threonine (Thr), methionine (Met), leucine (Leu), isoleucine (Ile), lysine (Lys), histidine (His), and a conditional amino acid, cysteine (Cys), however, not including tryptophan. Non-essential amino acids including alanine (Ala), arginine (Arg), asparagine (Asn), aspartic acid (Asp), glutamic acid (Glu), glutamine (Gln), glycine (Gly), proline (Pro), serine (Ser), and tyrosine (Tyr), however, not including Cys.Sum of non-essential amino acids were not significantly different ($p < 0.05$) after the germination of chickpea, lentil, and yellow pea, except aspartic acids.

is well known contribute to umami flavor (Kabelova et al., 2008), raw chickpea and yellow pea protein isolates tend to have more umami flavor than raw lentil protein isolates.

During acid hydrolysis, asparagine are converted to aspartic acid. Therefore, the content aspartic acids is actually the sum of aspartic acids and asparagine. The sum of aspartic acids and asparagine increased in chickpea, lentil, and yellow pea, from 11.93 ± 0.22, 11.02 ± 0.25, and 11.73 ± 0.04 g/100 g to 13.21 ± 0.49, 12.14 ± 0.23, and 12.19 ± 0.03 g/100 g, respectively. Germination is a good process for improving aspartic acid, which has a salty-umami taste (Kabelova et al., 2008). The increased Aspartic acids have also been observed in germinated winged bean (King and Puwastien, 1987).

3.4.2.4. Effect of germination on starch and non-starch carbohydrate

Starch contents in raw chickpea, lentil, and yellow pea were 40.28 g/100 g, 41.02 g/100 g, and 43.81 g/100 g, respectively. Over 6 days of germination, starch decreased 6.06 g/100 g and 2.87 g/100 g in lentil and yellow pea, respectively. Although the percentage of reduction varied due to different species and germination conditions, these decreasing trends are consistent with previous reports in lentil and peas. Starch in lentils decreased 3.8 g/100 g after one day of germination (Ghavidel and Prakash, 2007), while starch content in peas decreased 5.16 g/100 g with 6 days of germination (Urbano et al., 2005). As is well known, a whole spectrum of hydrolytic enzymes, including α-amylase, glucosidase, and dextranase are generated from the aleurone layer of pulse seeds, and β-amylase existing in the cotyledon, is activated during pulse seeds germination (Olaerts et al., 2016). These hydrolytic enzymes are responsible for the

conversion of starch into oligosaccharides or monosaccharides, resulting in the reduction of starch.

Interestingly, the total starch of chickpea did not change significantly ($p > 0.05$) over the course of the 6-day germination. Kaczmarska et al. (2017) reported a similar observation that no significant change in the starch content of lupin flour from germinated lupins at temperature (~25 °C) and humidity (~90%) controlled conditions. It is no doubt that the total starch is hydrolyzed by enzymes. Similar to crude protein, no significant change of starch percentage could be explained by the dry matter loss due to the leaching of seed constituents during steeping, such as dust and soluble phenolic compounds (known as pigments), or metabolic loss due to respiration (Sumathi et al., 1995).

Amylose content of raw chickpea, lentil, and yellow pea was 13.70, 14.06, and 12.04 g/100 g, respectively, while the ratio of apparent amylose/total starch was 34.01, 32.61, and 27.50, respectively. Similar to Hoover et al. (2010), results fell within the expected range of 30.4–35.0, 23.5–32.3, and 24.0–49.0 for chickpea, lentil, and pea (smooth), respectively. The ratio of apparent amylose/total starch increased by 20.6%, 22.0%, and 42.5% in chickpeas germinated for 6 days, lentils germinated for 3 days, and yellow pea germinated for 6 days, respectively. One speculation for the increase of amylose is that debranched amylopectin can be converted into amylose, while amylopectin can only be degraded (Li et al., 2017).

Non-starch carbohydrate in raw chickpea, lentil, and yellow pea was 19.55 g/100 g, 20.32 g/100 g, and 19.39 g/100 g, respectively. Non-starch carbohydrate of chickpea and lentil decreased after 5 and 4 days of germination, respectively, while that of yellow pea gradually

increased during 3 days of germination and then decreased. As a result, change of non-starch carbohydrate varied due to species of pulse seeds. In order to know how much dietary fibers (DF) accounts for non-starch carbohydrate, we measured DF content in germinated pulse seeds. The total dietary fiber (TDF) in chickpea after 2 days of germination was 14.9 g/100 g (78.9% of non-carbohydrate starch), which consisted of 11.3 and 3.6 g/100 g of insoluble (IDF) and soluble dietary fiber (SDF), respectively. The TDF in lentil after 1 day of germination was 9.9 g/100 g (51.5% of non-carbohydrate starch), which included 7.1 and 2.8 g/100 g of IDF and SDF, respectively. The TDF in lentil after 1 day of germination was 17.3 g/100 g (89.2% of non-carbohydrate starch), with 13.9 and 3.4 g/100 g being IDF and SDF, respectively. Our findings indicated that dietary fiber is the major contributor to the non-starch carbohydrate content in germinated pulse seeds and it varied depending on the type of seed.

3.4.2.5. Effect of germination on lipid content

Crude lipids in pulses is mainly comprised of triacylglycerol and phospholipids (Hall et al., 2017). Crude lipids of raw chickpea, lentil, and yellow pea were 8.00 g/100 g, 1.72 g/100 g, and 1.87 g/100 g, respectively. A significant decrease ($p < 0.05$) in lipid contents was observed during germination. Lipids decreased 2.10 g/100 g, 0.56 g/100 g, and 0.44 g/100 g after 6 days of germination. Similar trends of lipid reduction were reported by many researchers in pulse seeds germination studies. For instance, lipids content of Guar (*Cyamopsis tetragonoloba* L.) and Faba bean (*Vicia faba* L.) decreased from 0.90 g/100 g to 0.75 g/100 g and from 1.31 g/100 g to 0.99 g/100 g, respectively, after 3 days of germination (Khalil, 2001). Rumiyati et al. (2012) also reported a 70% reduction in lipid content in germinated lupin after 9 days of germination.

The majority of the crude lipid is distributed in the germ of pulse seeds (Kosson, Czuchajowska, & Pomeranz, 1994; Delcour & Hoseney, 2010). It is generally accepted that triacylglycerol is hydrolyzed by enzymes to release free fatty acids during germination. These free fatty acids will undergo β-oxidation, which takes place in both cytosol and mitochondria to produce essential energy to support seeds growth. As a result, a decrease of crude fat content is anticipated over the course of germination (Cornejo et al., 2015).

Our recent research revealed that aromatic compounds in chickpea, lentil, and yellow pea flours increased drastically after 1 day of germination (Xu et al., 2019a). Some of those distinctive fishy, green, and beany flavors associated compounds are derived from lipid oxidation. Hence, we proposed another possible mechanism that concomitantly attributes to lipid reduction during germination, i.e., the accelerated oxidation of lipids initiated by lipoxygenase (LOX). The activity of LOX increased significantly ($p<0.05$) after 1 day of germination (Figure 3.2), from 7.6 to 31.3 U/g, 3.2 to 21.5 U/g, and 9.5 to 24.8 U/g in chickpea, lentil, and yellow pea, respectively. There was no significant change of LOX activity in lentil and yellow pea afterward, while it kept rising to a peak of 45.7 U/g after 2 days of germination in chickpea. The highest LOX activity was also observed in chickpea, which corresponds to the greatest reduction (26.2%) of crude lipid content after 6 days of germination.

3.4.3. Thermal properties of germinated pulse flours

Thermal property of cereal and grains is one of the critical parameters for food manufacturers in engineering design involving thermal processing (Subramanian and Viswanathan, 2003). Thermal properties of cereal flours are moisture-dependent and the

endothermic transition of DSC thermogram reflects flours gelatinization with the addition of excess water (Chung & Liu, 2012). DSC was employed to examine the gelatinization temperatures and enthalpy changes (ΔH) of germinated pulse flours (Table 3.5**).** All tested pulse flours showed a single endothermic transition from 50 to 100 °C, which reflects gelatinization. Thermal properties of lentil flours were significantly different from that of chickpea and yellow pea (p<0.05). In particular, T_o, T_p, and T_c of raw lentil flour were 62.50 °C, 67.94 °C, and 81.92 °C, respectively. Thermal properties T_o and T_p of raw chickpea and yellow pea flours were not significantly different (p<0.05), but were higher than that of raw lentil flours. Nevertheless, raw chickpea and yellow pea flours had T_c that were significantly different (p<0.05), which were 86.80 °C and 82.13 °C, respectively. It is well known that thermal transition temperatures of cereal flours are influenced by amylose and protein contents, distribution of amylopectin branch chains, lipid complexed amylose chains, and other factors (Asmeda et al., 2016). The differences among the thermal properties of raw chickpea, lentil, and yellow pea flours may be attributed to the different crystalline structure of starches as well. Content of crystalline structure positively affects melting enthalpy (Tester and Morrison, 1990; Cooke and Gidley, 1992). Lentil may have lower content of crystallites resulting in a lower onset temperature and lower entropy.

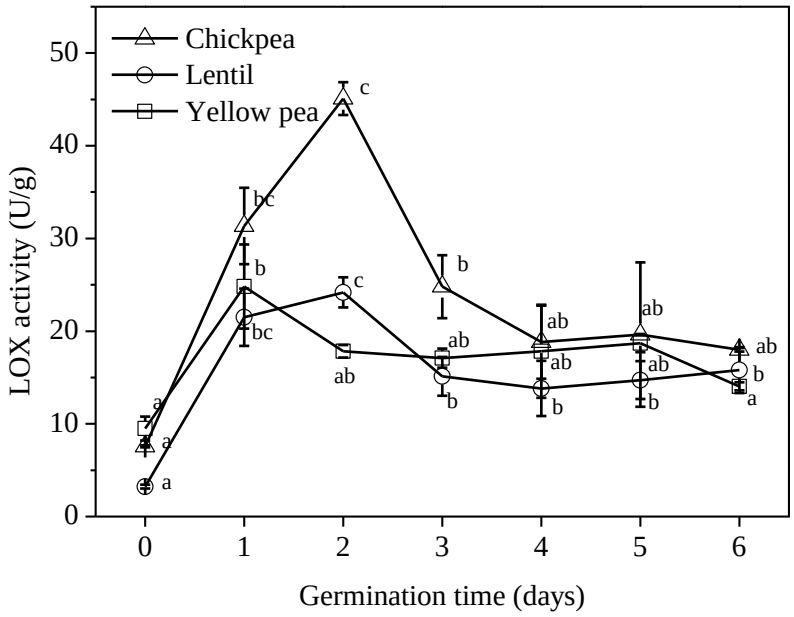

Figure 3.2. Effect of germination time on LOX activity in chickpea, lentil, and yellow pea flours. Activity (U) was defined as the numeric increase in absorbance at 234 nm per minute of the reaction. Data points represent mean ± standard deviation of two independent experiments. Different letters indicate statistically significant different results intraspecies ($p < 0.05$)

Germinated pulse flours exhibited similar thermal properties as raw flours, irrespective of germination time, while only T_p and T_c of both chickpea and yellow pea showed a slight increase over the course of germination (Table 3.5). These results were somewhat similar to the results of Ma et al. (2018), who reported that thermal properties of yellow pea (*Pisum sativum* L.) with 3 days of germination were not significantly different from that of the raw yellow pea. The thermal properties indicated that the hydrogen bond of starch in germinated pulse can be broken down at a temperature similar to that of raw pulse flours.

ΔH can be used to predict the energy required to break down the intermolecular hydrogen bonds of starch granules (Hoover et al., 2010). The ΔH of raw chickpea, lentil, and yellow pea

flours were 5.81, 1.95, and 3.15 J/g, respectively. The ΔH of chickpea and yellow pea decreased after 4 and 3 days of germination, respectively, suggesting the reduction of energy required to convert chemical composition of pulse flours from an ordered to disordered form. During germination, the partial hydrolysis of starch by the activated enzymes diminish the intermolecular hydrogen bonds of starch, making them easily detached during heating. However, the ΔH of lentil did not change significantly during germination. This might be due to the starch structure and composition differences.

Table 3.5. Effect of germination on thermal properties of chickpea, lentil, and yellow pea flour

Species	Germination time	To	Tp	Tc	ΔH
	Days		°C		J/g
Chickpea	0	66.35±0.06 b	70.48±0.44 a	86.80±0.16 a	5.81±0.01 c
	1	65.92±0.37 b	70.40±0.71 a	87.50±0.24 a	5.89±0.01 c
	2	65.71±0.06 b	71.57±0.07 ab	88.05±1.20 a	5.31±0.04 bc
	3	66.09±0.24 b	70.71±0.19 a	88.27±0.64 a	4.83±0.02 ab
	4	65.95±2.14 b	73.10±0.17 c	90.25±0.04 b	4.63±0.26 a
	5	66.52±0.93 b	73.13±0.28 c	89.99±0.23 b	4.79±0.23 a
	6	66.88±1.00 b	72.28±0.18 bc	88.66±0.78 a	4.38±0.21 a
Lentil	0	62.50±0.04 a	67.94±1.15 a	81.92±0.66 a	1.95±0.08 a
	1	61.81±0.04 a	66.64±1.13 a	80.71±0.16 a	2.15±0.08 a
	2	61.30±1.15 a	66.15±0.37 a	80.67±0.20 a	2.14±0.06 a
	3	62.85±0.33 a	66.65±0.74 a	80.63±0.08 a	2.12±0.24 a
	4	62.60±0.18 a	66.90±0.42 a	81.48±0.23 a	1.84±0.10 a
	5	63.37±0.45 a	68.44±1.95 a	80.98±0.20 a	1.82±0.12 a
	6	62.83±1.69 a	68.36±0.27 a	81.74±0.71 a	1.83±0.01 a
Yellow pea	0	65.76±0.14 b	69.87±0.66 a	82.13±0.03 a	3.15±0.07 d
	1	66.02±0.49 b	70.61±0.08 a	81.93±0.45 a	3.32±0.06 d
	2	65.85±0.41 b	70.3±0.11 a	82.41±0.52 a	2.88±0.00 c
	3	66.11±0.71 b	70.70±0.04 a	82.34±0.23 a	2.62±0.10 b
	4	66.17±0.03 b	70.70±0.07 a	81.49±0.71 a	2.10±0.05 a
	5	66.96±0.45 b	71.31±0.17 a	84.30±0.63 b	1.97±0.05 a
	6	67.10±1.60 b	72.38±1.38 b	82.99±0.05 ab	1.90±0.09 a

Data points represent mean ± standard deviation of two independent experiments. Different lower-case letters indicate statistically significant differences intraspecies ($p < 0.05$). To, Tp, Tc, and ΔH represents the onset temperature, peak temperature, completion temperature, and enthalpy, respectively.

3.4.4. Pasting properties of germinated pulse flours

Pasting properties of flours, the core factor determining its application in food products, provides information about the changes in the behavior of flour paste viscosity with change in temperature. We, therefore, examined the impact of germination on the pasting properties of the pulse flours using RVA (Figures 3.3 – 3.5).

When an excessive amount of water was introduced to pulse flours at room temperature, the slurry viscosity of chickpea (Figure 3.3), lentil (Figure 3.4), and yellow pea (Figure 3.5) flours was close to zero. With the increase of temperature, the viscosity of the slurry increased due to the starch taking up water and swelling substantially. Raw chickpea, lentil, and yellow pea flours start to paste at the temperatures of 79.5, 76.3, and 77.0 °C, respectively (Table 3.6). This discrepancy in pasting properties was consistent with the results of thermal properties, i.e., lentil flours had a lower range of gelatinization temperature than that of chickpea and yellow pea flours. Interestingly, the temperature of gelatinization tested by RVA is different from that of DSC. Chung et al. (2008) also reported that the pasting temperature of pulse flours tested by DSC was lower than that tested by RVA. The reason may be caused by the differential pressure during measurement. Flours tested by DSC were set in a sealed pan that keeps accumulate pressure in the closed system; while the sample tested through RVA was achieved in an open system avoiding pressure build up. Pasting temperatures of all three pulse flours decreased gradually upon germination. This might be due to the enzymatic degradation of an cotyledon cell wall in pulse seeds facilitating starch and protein to contact and absorb water. As is well known, protein and starch are wrapped by the cell wall in the cotyledon. When the cell wall decompose,

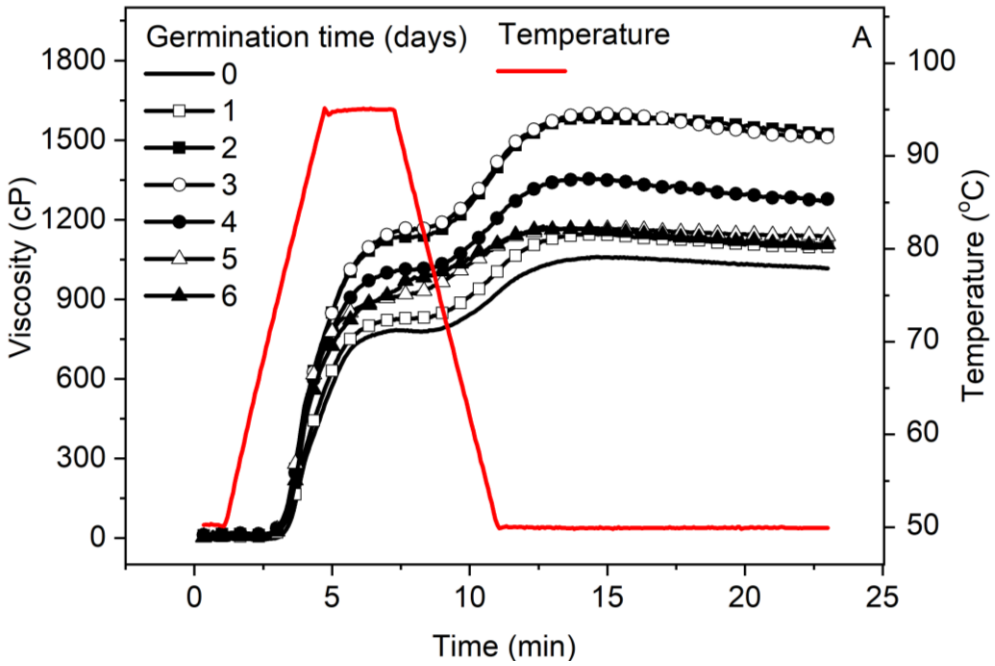

Figure 3.3. The effect of germination on viscosity profile of chickpea flours. Curves were plotted based on the average of replicates (n=2).

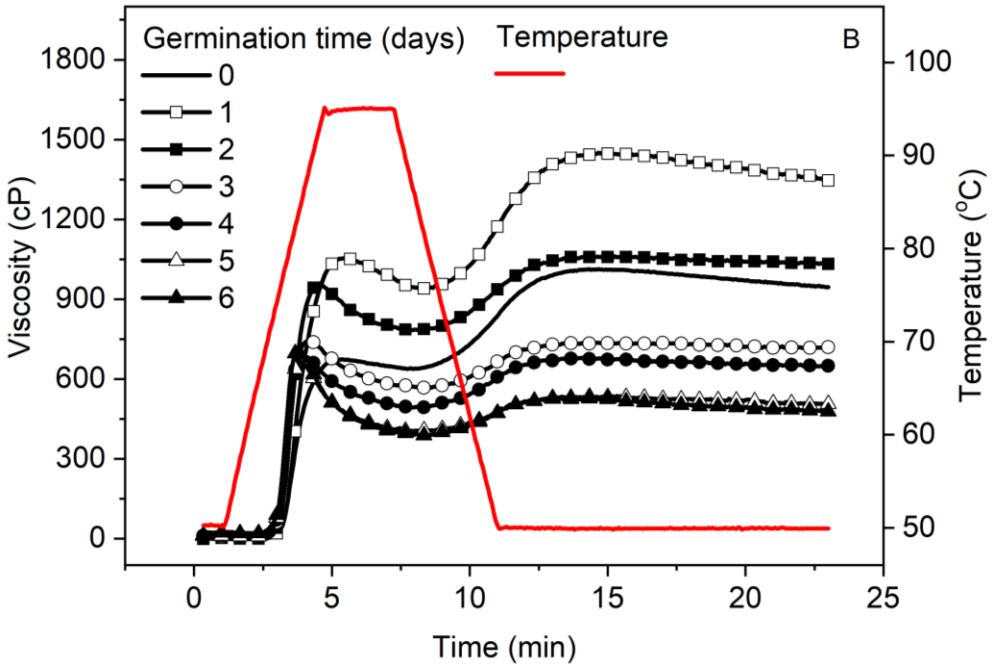

Figure 3.4. The effect of germination on viscosity profile of lentil flours. Curves were plotted based on the average of replicates (n=2).

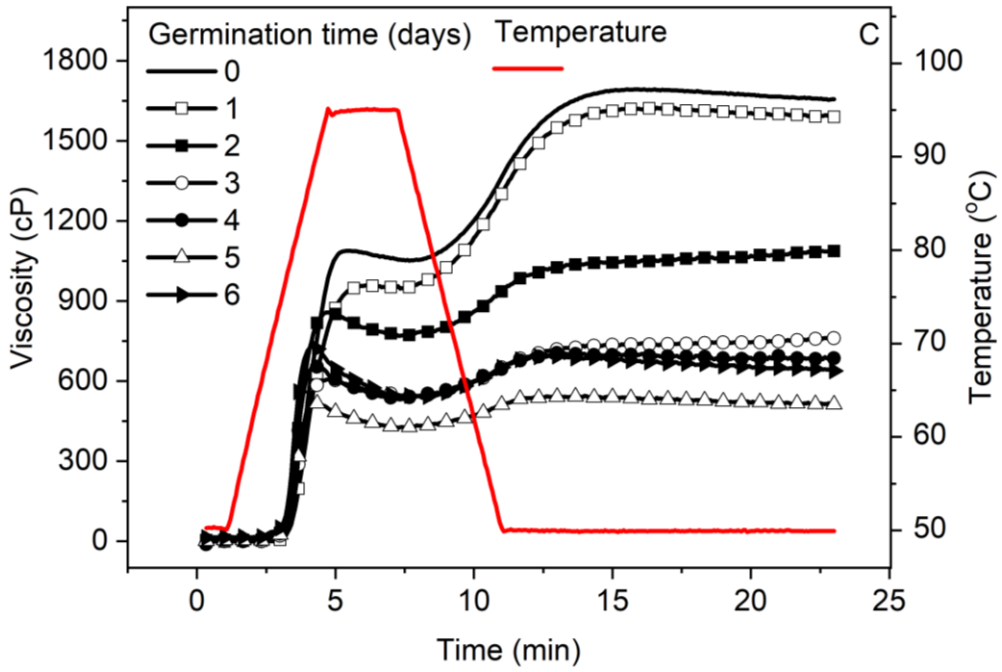

Figure 3.5. The effect of germination on viscosity profile of yellow pea flours. Curves were plotted based on the average of replicates (n=2).

water is more accessible to the starch and protein. In addition, following their exposure to the hydrolases, the compact structure of starch and protein can be loosened, which results in a larger surface area to water (Shewry, 2010).

In the pasting region, swollen starch granules break to release amylose, which results in a decrease of slurry viscosity. Peak viscosity depends on the balance of both swelling and breaking of the starch granule. The highest peak viscosity of yellow pea was observed in the raw pulse flours than its counterparts (Figure 3.5). Most of the starch in raw pulse seeds are native. With germination, the activation of enzymes such as amylase can degrade native starch, bringing about a lowered viscosity of paste. Differently, the highest peak viscosities of chickpea and lentil were observed at 3 days and 2 days of germination, respectively. The comparably lower peak viscosity of raw chickpea and lentil flours may be caused by the protective effect of their

cotyledon cell wall so that starch granule swelled gradually. Nevertheless, with excessive germination time, native starch granules were degraded by amylase activated during germination. Consequently, peak viscosity decreased in all three pulses with continuous germination. Such trend was reported by Ghumman et al. (2016), where peak viscosity of lentil starch increased after 2 days of germination and decreased after 4 days of germination, while peak viscosity decreased in horsegram following 4 days of germination.

After peak viscosity, a shear thinning effect, corresponding to the viscosity reduction of starch, would be anticipated as the consequence of reorientation of soluble starch, as well as shear-induced destruction of the swollen granule (Delcour & Hoseney, 2010). However, this does not apply to raw chickpea, lentil, and yellow pea flours (Li et al., 2019). Chung et al. (2008) reported that raw chickpea, lentil, and yellow pea had little shear thinning effect. Resistant starch, which is less soluble, is responsible for this observation (Delcour & Hoseney, 2010). Following the process of germination, shear thinning was observed in lentil (Figure 3.4) and yellow pea (Figure 3.5). It is plausible due to the partial modification of resistant starch in lentil and yellow pea. In this sense, germination could potentially increase the digestibility of lentil and yellow pea.

During cooling, because of the strengthening of hydrogen bonding and entanglement between starch chains, the viscosity of the slurry increases, referred to as setback. Amylopectin retrogradation and amylose crystallization are involved in setback (Delcour & Hoseney, 2010). The increased setback viscosity from raw pulse to lentil (Figure 3.4) and yellow pea (Figure 3.5) germinated for one day, and chickpea (Figure 3.3) germinated for two days was observed (Table

3.6). However, the setback viscosity decreased with continuing germination. It is reasonable that more amylose and amylopectin were hydrolyzed by enzymes, which resulted in less entanglement between starch chains. Acevedo et al. (2017) reported decreased setback viscosity

Table 3.6. Effect of germination on RVA attributes of chickpea, lentil, and yellow pea flours.

Sample	Germination time (day)	Peak viscosity	Hot paste viscosity	Breakdown	Final Viscosity	Setback	Peak Time (min)	Pasting Temp (°C)
				(cP)				
Chickpea	0	773±9 a	743±1 a	30±8 a	1009±11 a	266±10 ab	7.0±0.0 a	79.5±0.5 b
	1	805±22 ab	769±22 ab	36±0 a	1075±33 a	306±11 bc	7.0±0.0 a	79.3±0.2 b
	2	1103±29 d	1036±27 d	67±2 c	1518±8 c	482±19 d	7.0±0.0 a	77.0±0.6 a
	3	1135±10 d	1061±9 d	75±1 c	1500±16 c	439±7 d	7.0±0.0 a	76.8±0.0 a
	4	976±35 c	927±33 c	49±1 b	1246±45 b	320±12 c	7.0±0.0 a	77.0±0.5 a
	5	878±38 bc	847±38 bc	31±0 a	1107±45 a	260±6 ab	7.0±0.0 a	77.0±0.5 a
	6	933±20 c	880±18 c	53±1 b	1110±2 a	230±16 a	6.9±0.1 a	77.9±0.4 ab
Lentil	0	687±20 a	649±16 c	12±1 a	956±14 d	307±1 c	5.3±0.0 e	76.3±0.6 c
	1	1096±59 c	981±58 e	115±1 b	1416±99 e	435±41 d	5.4±0.0 e	76.3±0.6 c
	2	942±19 b	773±13 d	169±6 c	1024±13 d	251±0 c	4.5±0.0 d	74.2±0.0 b
	3	763±23 a	576±13 bc	187±10 cd	739±28 c	164±15 b	4.2±0.0 c	74.3±0.0 b
	4	690±4 a	490±3 ab	200±6 d	643±9 bc	153±6 ab	4.0±0.0 b	73.4±0.1 ab
	5	655±10 a	398±8 a	257±1 e	498±11 ab	100±3 ab	3.9±0.0 ab	72.7±0.0 a
	6	726±25 a	396±13 a	330±13 f	483±6 a	87±6 a	3.8±0.0 a	72.6±0.0 a
Yellow pea	0	1089±115 d	1052±93 d	37±3 ab	1656±115 c	605±22 d	5.5±0.1 b	77.0±0.6 ab
	1	924±50 cd	915±46 cd	9±4 a	1537±74 c	622±28 d	6.3±0.2 c	78.3±0.1 b
	2	830±39 bc	745±35 bc	85±4 c	1044±61 b	300±26 c	4.8±0.1 a	76.7±0.1 a
	3	617±15 ab	543±6 ab	74±9 bc	765±7 ab	222±1 bc	4.7±0.0 a	77.1±0.7 ab
	4	677±31 ab	555±33 ab	123±2 cd	722±52 a	167±18 ab	4.3±0.1 a	76.7±0.0 a
	5	575±83 a	468±61 a	107±22 c	580±95 a	112±35 a	4.4±0.0 a	77.4±0.1 ab
	6	736±11 ab	566±40 ab	171±29 d	683±63 a	117±23 a	4.3±0.2 a	76.3±0.6 a

*Breakdown value is presented as the peak viscosity – hot paste. Data points represent mean ± standard deviation of two independent experiments. Different letters indicate statistically significant different results intraspecies ($p < 0.05$).

3.4.5. Moisture sorption isotherms of germinated pulse flours

Moisture sorption behavior of germinated pulse flours is a predominant factor to adequately select the drying and storage conditions for themselves and the processed products.

We therefore experimentally obtained the water adsorption isotherm of germinated pulse flours over a wide range of water activities (a_w 0.20–0.85).

Moisture adsorption isotherms constructed for chickpea, lentil, and yellow pea flours exhibited a sigmoidal shape isotherm, a sigmoid curve generally observed in complex foods containing proteins and polysaccharides (Figures 3.6 – 3.8). Raw yellow pea flours had higher water adsorption capability than raw chickpea and lentil flours. Germination improved all water adsorption ability of chickpea, lentil, and yellow pea flours, despite the variation tendency among them. In particular, the water adsorption ability of chickpea flours increased gradually during 6 days of germination. The sharp increase of lentil flours occurred from day 2 to day 4, while that of yellow pea flours was from day 4 to day 6. Such sigmoidal shape arises partly because of the structural changes of macronutrients during germination. Vertucci and Leopold (1987) also found that germinated soybean had higher water adsorption capability. Generally, weak bindings are formed between water and ionic and hydrogen-bonding groups in raw pulse flours due to the chemical compositions (Kermode, 1990). Thus, increased water adsorption isotherm implied that more ionic interaction and hydrogen bonding occurred between constituents in pulse flours and water molecules. Starch in the germinated pulse flours tended to transform from their semi-crystalline form into amorphous form, while proteins in cotyledon were loosened so that larger surface area was available to interact with water molecules.

Experimental adsorption data of germinated pulse flours was modeled by means of GAB (Eq. 3.2) and found to be satisfactory in the current study because $R^2 > 0.999$ for all adsorption isotherms. GAB monolayer moisture values (m_o) were similar among the three raw pulse flours

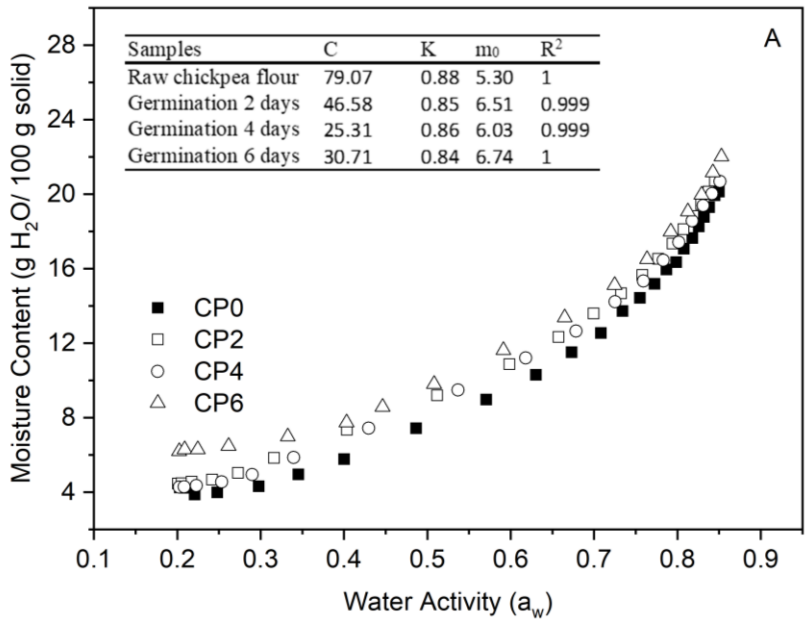

Figure 3.6. The effects of germination on moisture adsorption isotherms of chickpea flours. The calculated Guggenheim-Anderson-de Boer model parameters of the flours are included in the table insert (m_o is the monolayer moisture value, K is a multilayer factor, C is the surface heat constant, and R^2 is the goodness of fit).

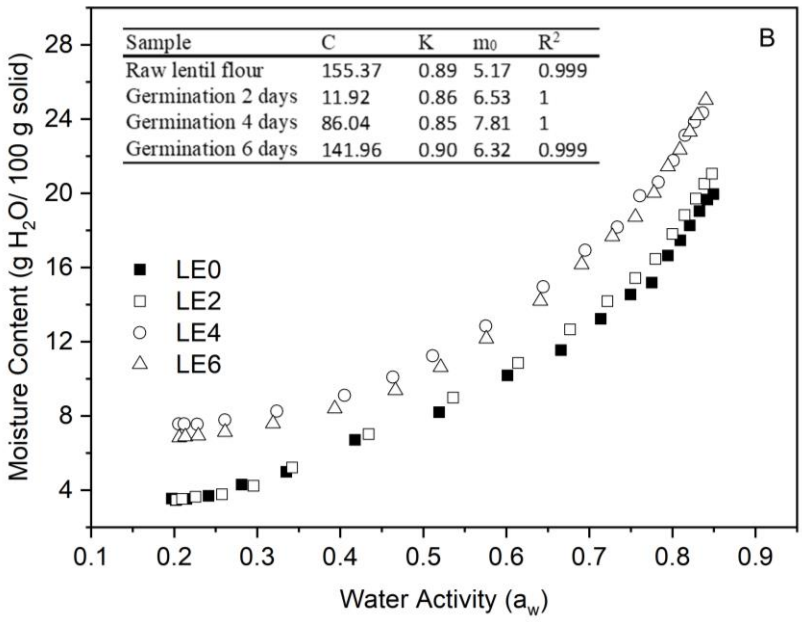

Figure 3.7. The effects of germination on moisture adsorption isotherms of lentil flours. The calculated Guggenheim-Anderson-de Boer model parameters of the flours are included in the table insert (m_o is the monolayer moisture value, K is a multilayer factor, C is the surface heat constant, and R^2 is the goodness of fit).

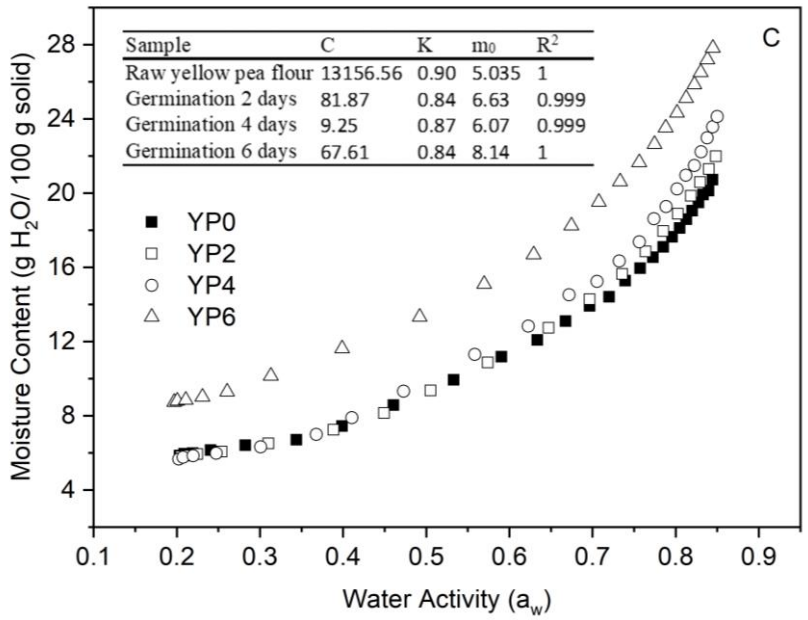

Figure 3.8. The effects of germination on moisture adsorption isotherms of yellow pea flours. The calculated Guggenheim-Anderson-de Boer model parameters of the flours are included in the table insert (m_o is the monolayer moisture value, K is a multilayer factor, C is the surface heat constant, and R^2 is the goodness of fit).

at 25 °C (~5.2 g H_2O/100 g solid), again indicating that raw pulse flours expose few adsorption sites to the water molecule. The values of GAB model parameters, particularly m_o, increased with the extension of germination. For instance, m_o of chickpea and yellow pea flours presented the lowest and highest increment after 6 days germination, reaching 6.73 and 8.14 H_2O/100 g solid, respectively. The increased m_o implicated the greater hygroscopicity of the flours after germination. This may be ascribed to an improvement in a total adsorption ability of germinated pulse flours, which again reflected physical and chemical modifications by germination

3.5. Conclusion

Variations in chemical composition in chickpea, lentil, and yellow pea flours were observed over the course of a 6-day germination. The physicochemical properties and thus the

food applications will be dictated by the change of chemical composition. Short-term germination led to a higher pasting property and lower water adsorption capacity of germinated pulse flours than long-term germination. Taking into account that germination balanced the amino acids profiles of pulse protein isolates, germination of pulse seeds as functional ingredients provides a solution to design healthy foods such as weaning foods and baked goods for people suffering from celiac disease.

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CHAPTER 4. PULSE SEED GERMINATION AFFECT ANTIOXIDANT ACTIVITY OF PHENOLIC COMPOUNDS*

4.1. Abstract

The purpose of this study was to investigate the antioxidant activity of phenolic compounds extracted from germinated pulse seeds including chickpeas, lentils, and yellow peas. Phenolic compounds in soluble free and polar soluble bound form were extracted at different germination time and total phenolic content was examined by Folin Ciocalteu's reaction. After diluting all the extracts into 187 μg gallic acid equivalence/mL, the antioxidant activity of phenolic compounds was investigated in both *in vitro* and stripped soybean oil-in-water emulsions based on the formation of lipid hydroperoxides, hexanal, and hexanol. The results suggested that total phenolic contents of pulses increased following pulse germination. Germination influenced the antioxidant activity of phenolic compounds in both *in vitro* assay and emulsion systems. Polar soluble bound phenolic compounds showed higher antioxidative ability in emulsion system with the order of chickpea > yellow pea > lentil.

4.2. Introduction

Pulse grains are a dry edible variety of beans, peas, and lentils that have been consumed as staple foods in many regions throughout the world for centuries. Pulses are rich in plant-based

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proteins, carbohydrates, dietary fibers, and minerals (Vaz Patto et al., 2015). In addition to nutritional properties, a great deal of attention has been devoted to the phenolic compounds in pulses due to their health benefits associated with prevention of chronic diseases, such as diabetes, chronic inflammation, and cardiovascular disease (Wang et al., 2015a). Numerous *in vitro* assays also demonstrated that phenolic compounds extracted from pulses possess superior antioxidant activity (Bartolomé et al., 1997; Fernandez-Orozco et al., 2009; Cevallos-Casals and Cisneros-Zevallos, 2010; Shahidi and Yeo, 2016). However, the application of pulse phenolic compounds as natural antioxidants to prevent food/oil oxidation has yet to be investigated. It is widely accepted that significant antioxidant activity of food is related to high total phenolic content (TPC). Unlike fruits, which have been considered as excellent source of phenolics (>500 mg gallic acid equivalence (GAE)/100 g), most of pulse crops can only be categorized as having a low to medium TPC (~100 mg GAE/ 100 g) (Vasco et al., 2008). In addition, phenolic compounds vary in concentration amongst different pulse species and varieties. Padhi et al reported TPC of 14 commercial Canadian pulse cultivars and found green lentil had the highest TPC at about 6 times higher than in pea and chickpea (Padhi et al., 2016). Consequently, it is of great interest to effectively boost the TPC and antioxidant activity of pulses as functional ingredients.

Germination is an efficient and economical means for improving antioxidant contents of pulse seeds as observed in the germination of barley amaranth, quinoa, buckwheat, wheat and oat (Mäkinen and Arendt, 2015). Solvent extractable phenolic compounds can be classified as non-polar solvent extractable phenolic compounds and polar solvent extractable phenolic compounds

(PSEPs), while PSEPs can be further classified into soluble free (SFPs) and polar soluble bound phenolic compounds (PSBPs) (Figure 2.1). SFPs mainly exist as phenolic acids and flavonoids; PSBPs are recognized as esterified phenolics. In addition to PSEPs, non-extractable phenolic compounds (NEPs) are typically in the cell wall combined with cellulose, lignin, and arabinoglycan (Cheynier et al., 2013). During germination, three types of phenolics are mutually transformed. For instance, both PSEPs and NEPs were reported to increase in germinated chickpea (Tarzi et al., 2012) and yellow pea (Yeo and Shahidi, 2015). However, the decrease of PSEPs in lentil was also reported after germination for 2 days (López-Amorós et al., 2006), while Yeo and Shahidi (Yeo and Shahidi, 2015) reported that both PSEPs and NEPs increased consistently during germination. Such inconsistency elucidated that change of phenolic compound form and composition during germination are complicated, which makes it difficult to estimate the most effective extract antioxidant composition over the course of germination using *in vitro* assay.

In this study, oil-in-water emulsions prepared using stripped soybean oil (SSO), free of endogenous antioxidants, were employed as the model food system to evaluate antioxidant activity of phenolics extracted from chickpea (*Cicer aretinium* L.), yellow pea (*Pisum sativum* L.) and lentil (*Lens culinaris* Merr.) at varying stage of germination process. The objectives of this study were therefore to i) assess the impact of germination time on TPC; ii) investigate the impact of germination time on the antioxidant activity *in vitro* and in an emulsion system; and iii) compare the antioxidant activity between SFPs and PSBPs at different germination time.

4.3. Materials and Methods

4.3.1. Chemicals and reagents

Chickpea (*Cicer aretinium* L.), lentil (*Lens culinaris* Merr.) and yellow pea (*Pisum sativum* L.) were gifted from JM Grain (Garrison, ND, USA), Viterra Inc (Grand Forks, ND, USA), and AGT Food and Ingredients (Minot, ND, USA). The manufactures have noted that chickpea, lentil, and yellow pea were a mixture of different cultivars obtained from growers. Commercial soybean oil was purchased locally. Hydroxylamine hydrochloride, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and ferrozine were purchased from Acros Organics (Morris Plains, NJ, USA); 2, 2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH), fluorescein sodium salt, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), iron(II) sulfate heptahydrate ($Fe_2SO_4.7H_2O$), 2,2-diphenyl-1-picryhydrazyl (DPPH), ammonium thiocyanate, hexanal, hexanol, gallic acid, Folin Ciocalteu's phenol reagent were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were purchased from VWR International. (West Chester, PA, USA).

4.3.2. Germination of pulse seeds

The method of pulse germination followed a modified version used by Fernandez-Orozco and coworkers (Fernandez-Orozco et al., 2009). Briefly, 500 g of seeds were soaked in 2000 mL of 0.07% sodium hypochlorite solution for 30 min. The seeds were then washed with distilled water until reaching neutral pH and subsequently soaked in 2000 mL of distilled water for 5.5 h with shaking every 30 min. The hydrated seeds were stored on and covered by wet laboratory paper in germination trays, which were in contact with circulating moist air to maintain the

relative humidity and the moisture of seeds at 99% and 50%, respectively. Seeds were germinated at 25 °C in the dark for 6 days. The sprouted seeds at 2, 4, or 6 days into the germination process were collected, freeze-dried, and ground in an IKA M20 mill (Staufen, Germany) fitted with a 0.5 mm sieve. The ground flour was placed in plastic bags and stored in darkness under vacuum conditions in a desiccator at 4 °C until further analysis. Seed germination was performed in duplicate.

4.3.3. Extraction of phenolic compounds

The extraction of phenolic compounds from germinated pulses were followed as described by Chandrasekara and Shahidi with modification (Chandrasekara and Shahidi, 2010). Twelve grams of ground sample was defatted with 60 mL of n-hexane by shaking for 1 h under nitrogen, and then centrifuged at 2,000×*g* for 5 min and supernatants collected. The defatting was repeated and the two defatted meal samples were combined. The defatted meal was mixed with 60 mL of acetone/H_2O (7/3, v/v), purged with N_2, and then shaken for 1 h with shaker. After centrifugation of the resulting slurry for 5 min at 2,000×*g*, the supernatant was collected. The extraction was repeated and supernatants were combined. The crude phenolic solution was then prepared by evaporating the acetone from the supernatants at 40 °C with rotary evaporation (RE 111 Buchi, Flawil, Switzerland), and the pH of the solution was adjusted to 2~3.

Soluble free phenolic compounds (SFPs) were extracted from the crude solution by partitioning 3 times with ethyl ether/ethyl acetate (1/1, v/v). The supernatants were combined and the solvent removed with rotary evaporation. Remaining solvent traces were removed with

nitrogen purge. The dried samples were redissolved using 15 mL methanol, the solution transferred into storage vials and purged with nitrogen. The vials were stored at 4 °C.

PSBPs were collected by evaporating any remaining organic solvent from the rest of the crude phenolic solution. The remnant was transferred to vials using 50 mL water as a solvent and stored at 4 °C.

4.3.4. Determination of total phenolic content (TPC)

The TPC of each extract was determined using the method described by Alshikh and Shahidi (Alshikh et al., 2015) with modification. The mixture of deionized water (3 mL), the phenolic extract (150 μL), and Folin-Ciocalteu's reagent solution (250 μL) were vortexed and incubated for 5 min at room temperature, followed by the addition of 20% sodium carbonate (750 μL) to each tube. The solutions were stored in dark at ambient temperature for 1 h. The absorbance was measured at 765 nm (VWR 6300 Double Beam UV-Vis Spectrophotometer, VWR, Palo Alto, CA, USA) using distilled water as a blank. TPC was expressed as gallic acid equivalents (mg of GAE/g dry pulse) using the calibration curve of gallic acid.

4.3.5. Assays of *in vitro* antioxidant activity

Oxygen radical absorbance capacity (ORAC) and 2,2-diphenyl-1-picryhydrazyl radical scavenging capacity (DPPH) of extracts from the pulse and their two fractions were monitored as described by Yu (Yu, 2008) using a Fluostar Optima plate reader (BMG Labtech, Chicago, IL, USA). The final ORAC and DPPH values were calculated as Trolox equivalents (TE) per mL extract (μmol TE/mL) using a standard curve prepared with 15.6-250 μmol Trolox.

4.3.6. Iron binding capacity

The ability of phenolics extracted from germinated pulses to bind iron was determined using a modified method of Chen et al (Chen et al., 2010). The iron-binding capacity was expressed as % iron bound per mL of extracts, which was calculated as (Fe concentration total - Fe concentration remained) × 100% /Fe concentration total

4.3.7. Preparation of SSO

SSO was prepared according to the method of Chen et al (Chen et al., 2014). In short, silicic acid (100 g) was washed three times with a total of 3 L of distilled water and activated at 110 °C for 20 h. A chromatographic column (3.0 cm internal diameter × 35 cm height) was packed sequentially with 22.5 g of silicic acid, followed by 5.625 g of activated charcoal and then another 22.5 g of silicic acid by the dry method. Thirty grams of soybean oil was dissolved in 30 mL of n-hexane and passed through the column. To inhibit lipid oxidation (e.g. photo-oxidation) during the 4 h stripping procedure, the collection flask oil was covered with aluminum foil. The solvent in the stripped oil solution was removed with a vacuum rotary evaporator (RE 111 Buchi, Flawil, Switzerland) at 37 °C, and the remaining trace solvent removed by flushing with nitrogen for at least one hour. Fifteen grams of colorless SSO were collected, mixed, and kept at -80 °C for subsequent studies.

4.3.8. Preparation of emulsion

SSO-in-water emulsion was prepared by homogenizing 2 wt% SSO, 0.2 wt% Tween 20 and 0.01 M phosphate buffer using a high-speed blender (M133/128-0, Biospec Products, Inc., ESGC, Switzerland) for 2 min followed by further particle size reduction with a Microfluidizer

(LM20-20 Microfluidizer Processor, Microfluidics, Newton, MA) for one pass at a pressure of 5,000 psi and two passes at a pressure of 10,000 psi. The emulsions were kept on ice over the whole procedure to minimize oxidation. After homogenization, 0.04 wt% sodium azide was added as an antibacterial agent.

Pulse extract was added into freshly prepared SSO-in-water emulsion at the concentration of 200 μg GAE/g oil to study the antioxidant activity. A 0.5 mL aliquot of mixture was removed and placed into a 20 mL vial sealed with an aluminum cap. These vials were stored in an incubator at 25 °C and covered with aluminum foil.

4.3.9. Particle size analysis

The emulsion particle size was directly measured using a laser light scattering instrument (Mastersizer 3000, Malvern Instruments Ltd., Malvern, U.K.). Measurements were reported as the surface-weighted ($d_{32} = \sum n_i d_i^3 / \sum n_i d_i^2$) mean particle diameter, where n_i was the number of droplets of diameter d_i.

4.3.10. Measurement of emulsion oxidation

Lipid hydroperoxides were determined using a method adapted from Chen and coworkers (Chen et al., 2011). The concentration of hydroperoxides was calculated from a cumene hydroperoxide standard curve. Hexanal and hexanol were determined directly from the sealed vial. Analyses were performed with Agilent Technologies 7890B GC system and 5977A mass detector equipped with a PAL RSI 120 autosampler. Subsamples (0.5 mL) in 20 mL glass vials capped with aluminum caps with PTFE/silicone septa were incubated at 55 °C for 15 min in an autosampler heating block before measurement. A 50/30 mm DVB/Carboxen/PDMS solid-phase

microextraction (SPME) fiber needle (57298U, Supelco, Bellefonte, USA) was inserted into the vial for 2 min to absorb volatiles and then transferred to the injector port (250 °C) for 3 min. The injection port was operated in split mode, and the split ratio was set at 5:1. Volatiles were separated on a ZB-Wax column (60 m × 0.25 mm i.d. × 0.25 μm film thickness). Oven temperature program set as follows: Initial temp 40 °C, hold 4 min, to 70 °C at 10 °C/min, hold 6 min, then from 70 to 250 °C at 100 °C/min, hold 3 min. The helium carrier gas was used at a flow rate of 1 mL/min. Carry over and peaks originating from the fiber was regularly assessed by running blank samples. After each analysis the fiber was immediately thermally desorbed in the GC injector for 5 min at 250 °C to prevent contaminations. The MS was operated in electron impact (EI) ionization mode at 70 eV. Ion source temperature was 230 °C. Selected ion monitoring (SIM) was used for the detection of hexanal (*m/z*: 72 and 82) and hexanol (*m/z*: 55, 56, and 69). The dwell time was set to 100 ms. Identifications of hexanal and hexanol were carried out by comparing GC retention time with those of reference standard compounds. The concentrations of hexanal and hexanol were determined in duplicate as relative area using the calibration curve.

4.3.11. Statistical analysis

Data were expressed as the mean standard deviation of duplicate or triplicate measurements. The data were statistically analyzed using statistical software, SAS version 9.4 (SAS institute Inc. Cary, NC). One-way analysis of variance (ANOVA) was conducted, and a significant difference was defined at $p < 0.05$ by Tukey's test.

4.4. Results and Discussion

4.4.1. Effect of germination time on total phenolic content in pulses

Total soluble phenolic compounds (SFPs + PSBPs) of the ungerminated chickpea, lentil, and yellow pea were 1.04, 8.43, and 1.17 mg/g dry pulse, respectively (Figures 4.1 – 4.3). These vials were close to the value summarized by Amarowicz and Pegg (Amarowicz and Pegg, 2008), with chickpea, lentil, and yellow pea being in the range of 1.41-1.67, 1.02-7.53, and 1.13-1.67 mg/g dry pulse, respectively.

After germination, the content of total phenolics has changed in both PSBPs and SFPs. For instance, germination time was directly proportional to TPC in chickpea and yellow pea, while it was inversely proportional to TPC in lentil. In chickpea (Figure 4.1) and yellow pea (Figure 4.3), the original TPC of PSBPs (0.81 mg GAE/g dry chickpea and 0.85 mg GAE/g dry yellow pea) and SFPs (0.23 mg GAE/g dry chickpea and 0.33 mg GAE/g dry yellow pea) were lower. After 6 days of germination, TPC values of both PSBPs (1.40 mg GAE/g dry chickpea and 1.95 mg GAE/g dry yellow pea) and SFPs (0.96 mg GAE/g dry chickpea and 0.75 mg GAE/g dry yellow pea) increased significantly ($p < 0.05$). This trend was the same as the germination effect on antioxidant concentration reported by Tarzi et al. (2012) and López-Amorós et al. (2006), both of whom observed the significant increase in TPC of chickpea and yellow pea after germination. In contrast, for lentil (Figure 4.2), TPC value decreased during germination, with soluble phenolic compounds of lentil decreased from 8.43 to 3.07 mg/g dry pulse, which was mainly caused by the decrease of PSBPs from 7.68 to 2.59 mg/g dry pulse. The TPC change of SFPs was relatively small, from 0.377 to 0.748 mg/g dry pulse, and the

ungerminated lentil had the highest TPC value. López-Amorós et al. (2006) also observed using DPPH assay that germination had a negative effect on antioxidant ability of lentils as ungerminated lentil (IC_{50} ~4.9-5.1) exhibit greater antioxidant activity than the germinated form (IC_{50} ~5.9-10.9).

The various pulse crop seeds had different germination attributes. It can be assumed that, in chickpea and yellow pea, the synthesis rate of PEBP was larger than the consumption rate, whereas the opposite trend was observed in lentil. This trend was also reported by some researchers (Bartolomé et al., 1997; López-Amorós et al., 2006; Tarzi et al., 2012), where chickpea and yellow pea had enhanced content of phenolic compounds, while lentil tended to have a reduced level of main phenolic compounds during germination.

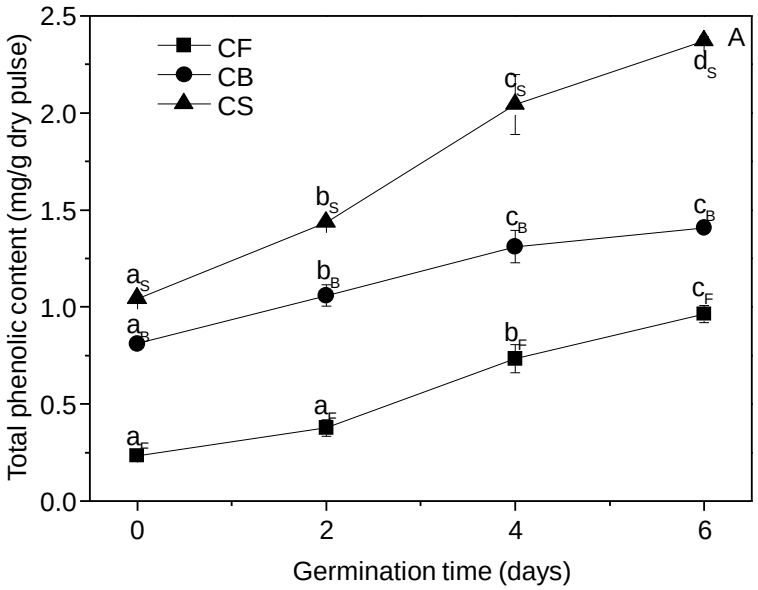

Figure 4.1. Total phenolic content of chickpea over the course of germination.
CF, CB, and CS, denotes SFPs, PSBPs, total soluble phenolics (SFPs + PSBPs) in chickpea, respectively. Data points represent means ± standard deviations. Different letters around the bars indicate statistically significant differences.

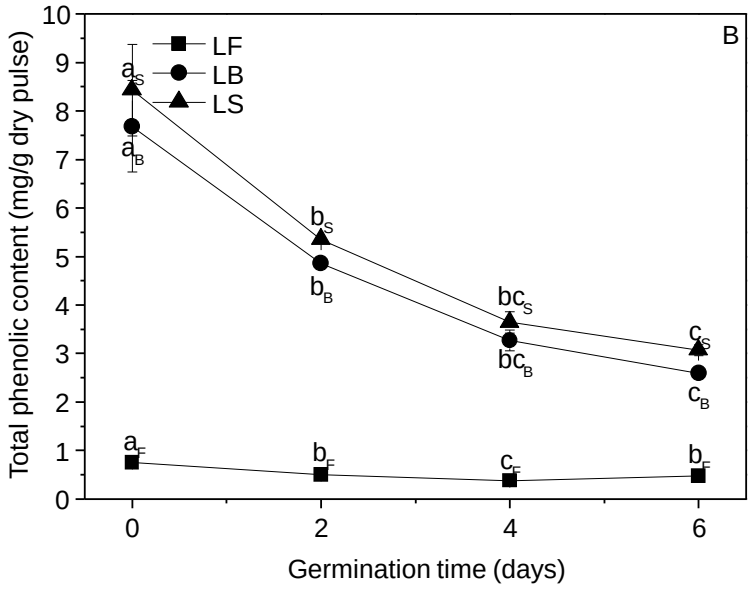

Figure 4.2. Total phenolic content of lentil over the course of germination.
LF, LB, and LS, denotes SFPs, PSBPs, total soluble phenolics (SFPs + PSBPs) in lentil, respectively. Data points represent means ± standard deviations. Different letters around the bars indicate statistically significant differences.

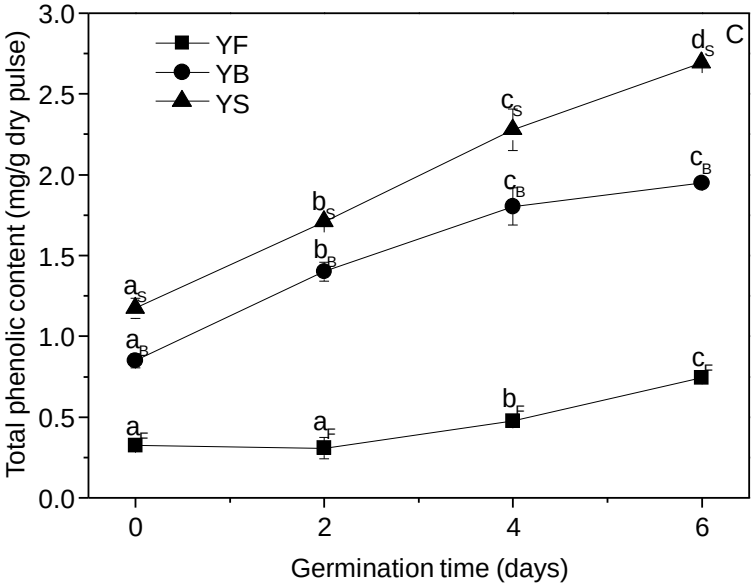

Figure 4.3. Total phenolic content of yellow pea over the course of germination.
YF, YB, and YS, denotes SFPs, PSBPs, total soluble phenolics (SFPs + PSBPs) in yellow pea, respectively. Data points represent means ± standard deviations. Different letters around the bars indicate statistically significant differences.

In addition, it was worth noting that soluble phenolic compounds in lentil were initially so high that even after 6 days of reductions, the TPC value was still greater than that in chickpea and yellow pea. This was reasonable because TPC value of ungerminated lentil was reported to be 6-8 times larger than chickpea and yellow pea (Xu and Chang, 2007; Amarowicz and Pegg, 2008; Vaz Patto et al., 2015).

4.4.2. Effect of germination time on *in vitro* antioxidant activity of pulse phenolic extracts

It was obvious that the content of phenolic compounds had changed during germination, however, the impact on antioxidant activity was not known. To sort this out, all the extracts of pulses were diluted into the same concentration of TPC, i.e., 187 μg GAE/mL extracts, and the antioxidant activity of each extract were assessed using *in vitro* assays, including free radical scavenging ability and metal chelating ability.

4.4.2.1. Free radical scavenging ability

Free radical scavenging capacity of natural antioxidants can be classified as two major mechanisms: hydrogen atom transfer (HAT) and electron transfer (ET) (Shahidi and Ambigaipalan, 2015). HAT mechanism is based on the transferring of hydrogen from antioxidants to inactivate free radicals generated in the system, thus suspending the propagation step of lipid oxidation. ET-based antioxidant reaction is a redox reaction with the transfer of electron from antioxidants to the free radicals (Galano et al., 2016).

The ORAC assay is an HAT-based radical scavenging method, which measures the inhibition ability against the peroxyl radical-induced oxidation. As the peroxyl radical is the predominant initiating radical found in the food system, ORAC values are important as an

indicator of antioxidant activity. In this experiment, the peroxyl radicals generated by AAPH can damage the fluorescent probe. Phenolic compounds extracted from pulses could react with the peroxyl radical by donating the hydrogen atom to retard the damage of fluorescent probe (Zou et al., 2011; Shahidi and Zhong, 2015).

SFPs in chickpea and yellow pea had an increasing trend of ORAC during germination, from 2.85 and 2.18 μmol TE/mL extraction to 5.82 and 4.60 μmol TE/mL extraction, respectively. With the lentil, the ORAC of SFPs remained steady within 4 days of germination ($p < 0.05$) and increased to 3.51 μmol TE/mL extraction after 6 days. Germination had little influence on PBPSs of chickpea in the first four days as observed by ORAC values compared to non-germinated chickpea. However, a 2.5-fold increase was observed after 6 days of germination. Conversely, a decline of ORAC over the course of germination was exhibited from PSBPs of both lentil and yellow pea.

DPPH radical scavenging capability is an ET based assay, although Rock and Brunswick (Rock and Brunswick, 2005) argued that HAT had also happened as a marginal pathway. DPPH is a stable radical due to the resonance stabilization and steric hindrance of three benzene rings. DPPH can be neutralized by phenolic compounds extracted from pulse based on ET, which is also called reducing power (Shahidi and Zhong, 2015). Each of pulse seeds displayed unique pattern for DPPH (Table 4.1). Much the same as ORAC value, SFPs in chickpea had an increasing trend of DPPH radical scavenging activity during germination, from 11.9 initially to 80.3 μmol TE/L extraction after 6 days germination. The DPPH radical scavenging activity of

SFPs in yellow pea was independent of germination. However, the DPPH radical scavenging activity of the SFPs in lentil decreased from 104.8 to 82.1 μmol TE/L extraction.

Table 4.1. Antioxidant activity of ungerminated and germinated pulse estimated by *in vitro* assay

	Germination (days)	DPPH (μmol TE/L extracts)	ORAC (μmol TE/mL extracts)	Iron binding capacity (μmol Fe^{2+}/mL extracts)
			------SFP------	
Chickpea	0	11.9±5.6 a	2.85±0.15 a	0.91±0.04 d
	2	62.3±1.4 b	2.96±0.48 a	0.33±0.00 b
	4	77.6±1.2 c	4.96±0.84 b	0.10±0.01 a
	6	80.3±1.6 c	5.82±0.57 b	0.49±0.02 c
Lentil	0	104.8±0.7 c	2.14±0.12 a	0.25±0.01 a
	2	101.4±1.1 c	2.10±0.09 a	0.71±0.02 d
	4	87.0±2.1 b	2.28±0.25 a	0.66±0.00 c
	6	82.1±2.3 a	3.51±0.26 b	0.53±0.01 b
Yellow pea	0	66.3±3.1 a	2.18±0.03 a	0.84±0.01 c
	2	62.0±2.4 a	1.73±0.13 a	0.63±0.01 b
	4	65.6±1.8 a	3.27±0.20 b	0.77±0.01 bc
	6	61.5±1.4 a	4.60±0.79 c	0.22±0.11 a
			------PSBP------	
Chickpea	0	45.7±3.6 a	0.61±0.04 a	Nd[d]
	2	64.9±6.4 bc	0.91±0.13 a	Nd
	4	61.1±5.1 b	0.80±0.24 a	Nd
	6	75.7±3.5 c	1.51±0.30 b	Nd
Lentil	0	122.4±0.5 a	2.49±0.40 c	Nd
	2	122.4±0.3 a	1.69±0.24 b	Nd
	4	119.3±3.7 a	0.86±0.02 a	Nd
	6	117.6±0.0 a	0.89±0.17 a	Nd
Yellow pea	0	101.4±5.6 c	0.65±0.02 d	Nd
	2	63.8±1.3 b	0.44±0.02 c	Nd
	4	39.8±1.6 a	0.14±0.05 a	Nd
	6	45.5±4.9 a	0.19±0.10 b	Nd

[*] DPPH: 2,2-diphenyl-1-picryhydrazyl radical scavenging capacity; ORAC: oxygen radical absorbance capacity; SFP: soluble free phenolic compound; PSBP: polar soluble bound phenolic compound. Nd: not detected. Data points represent mean (n=3) ± standard deviation. Different letters indicate statistically significant differences intraspecies (p<0.05).

The DPPH radical scavenging ability of PSBPs from lentil was highest among samples; however, germination had no significant influence on DPPH radical scavenging ability of lentil.

The DPPH radical scavenging activity increased in the chickpea extracts obtained from germinated samples, from 45.7 to 75.7 μmol TE/L extraction. Germination had a negative impact on DPPH of PSBPs in yellow pea since continuous loss in activity was observed, with DPPH dropping from 101.4 initially to 45.5 μmol TE/L extraction after 6 days of germination.

Conclusions drawn from radical scavenging ability assays, both ORAC and DPPH, indicated that radical scavenging ability of both extracts from chickpea and SFPs from yellow pea increased during germination. Khattak and coworkers (Khattak et al., 2007) reported that the methanol extracted phenolics increased during germination while water extracted phenolics decreased, which indicated that the composition of phenolic compounds had changed over the course of germination. In addition, the antioxidant activity of SFPs increased faster in chickpea and yellow pea than that of PSBPs, which can infer that the newly synthesized species of SFPs have higher *in vitro* antioxidant activity than that of PSBPs. The trend of *in vitro* antioxidant activity of phenolic compounds in lentil decreased during germination, which contrasted with chickpea and yellow pea. The sharp reduction in activity of (+)-catechin, *p*-coumaric acid and epicatechin was reported by Bartolomé and coworkers (Bartolomé et al., 1997) and may be the reason for the decrease in antioxidant activity of germinated lentil.

The antioxidant activity of extracts from pulses are closely related to the composition of phenolic compounds (Ayoub et al., 2016). Previous studies suggested that the major phenolic compound in chickpea (e.g. ferulic acid, caffeic acid, *p*-coumaric acid, chlorogenic acid, vanillic acid, protocatechuic acid, syringic acid, sinapic acid), lentil (e.g. catechins, *trans p*-coumaric acid, quercetin diglycoside, glycosides of flavonols, glycosides of flavones, hydroxycinnamic

acids, hydroxybenzoic acid), and yellow pea (e.g. kaempferol, ferulic acid, sinapic acid, quercetin, vanillic acid, *p*-coumaric acid, caffeic acid) were responsible for the antioxidant activity of pulses (Vaz Patto et al., 2015). Further research is needed to elucidate the relationship between phenolic composition and free radical scavenging activity of extracts from germinated pulses.

4.4.2.2. Metal chelating capacity

Metal ions are critical factors for lipid oxidation since they can actively react with hydron peroxide to produce hydroperoxides radicals (Ozen et al., 2011). In addition, metal ions can also promote the decomposition of lipid hydroperoxides to generate alkoxyl radicals and peroxide radicals (Chen et al., 2012). The antioxidative properties of a chelator can often be dependent on the synergistic effect of functional groups such as –OH, –SH, –COOH, $–PO_3H_2$, C=O, $–NR_2$, –S– and –O– (Fennema, 1996). As to the SFPs, the chelating activity of antioxidants is most probably formed by the synergism of -OH and -COOH.

After diluting all the pulse seed extracts into 187 μg GAE /mL, 10 μL of each diluted extract was added into 5 mM Fe^{2+} solution to determine their iron binding capacity. SFPs from ungerminated chickpea had the highest iron binding capacity, but the values decreased during the first 4 days of germination (Table 4.1). The iron binding capacity of SFPs extracted from lentil reached a maximum after 2 days of germination, then gradually decreased over the course of germination. Yellow pea showed similar patterns as chickpea and ungerminated one had the highest iron binding capacity. This result indicated that the structure of SFPs in chickpea had changed dramatically during germination.

It would be expected that PSBPs will carry certain iron binding capacity if no strong by virtue of the constituents that the phenolic compounds covalently bound with. For instance, some researchers have demonstrated that certain anionic polysaccharides such as xanthan gum, pectin, and sodium alginate could potentially act as antioxidants in oil-in-water emulsions due to its higher iron binding capacity (Faraji et al., 2004; Qiu et al., 2015; Salvia-Trujillo et al., 2016). In addition, some food proteins such as whey protein showed remarkable antioxidant activity in due to the strong iron binding capacity as one of the major mechanisms (Tong et al., 2000). It was unclear why all the PSBPs extracted from pulses had little to no iron binding capacity. One possible reason was that phenolic compounds were trapped by large molecule of polysaccharides or proteins resulted in the loss of iron binding capacity (Fennema, 1996; Mozuraityte et al., 2016).

4.4.3. Antioxidant activity of soluble free phenolic compounds (SFPs) from germinated pulses in oil-in-water emulsions

In vitro assays proved that the phenolic compositions of pulse extracts obtained from germinated pulses were variable, which resulted in the differences in antioxidant activity. However, *in vitro* assays mainly focused on specific or part of the antioxidant activity rather than oxidative stability of real foods. The antioxidant activity assessed by *in vitro* assay cannot be extrapolated into real food systems. Therefore, the SSO-in-water emulsion system was employed as a model food system to better understand the antioxidant activity of phenolic extracts from pulses under different germination time.

The initial average droplet size (d_{32}) of SSO emulsified by Tween 20 in phosphate buffer solution (pH 7.0) was 0.446 μm. After adding 200 μg GAE phenolic compounds/g oil, the average droplet size ranged from 0.446-0.602 μm during the storage period at 25 ^{o}C. Besides, no visual observation of creaming formation occurance in each sample emulsion during storage. Both droplet analysis and observed results indicated no major changes in the physical stability of emulsions. The oxidation kinetics of physically stable emulsions were assessed by measuring primary oxidation product, i.e., lipid hydroperoxides, integrated with secondary oxidation products hexanal and hexanol.

Similar to the *in vitro* assay, all the extracts of pulses were diluted to the same concentration of TPC, i.e., 187 μg GAE/mL extracts, and antioxidant activity of each extract were assessed in the emulsion system. SFPs extracted from ungerminated pulses exhibited no antioxidant activity retarding emulsion oxidation. However, extracts from germinated samples showed improved antioxidant activity of SFPs from chickpea and yellow pea. SFPs extracted from both 4 and 6 days germinated chickpea suppressed the formation of lipid hydroperoxides and retarded lag phase for 3 days, while SFPs of chickpea germinated for 0 and 2 days showed 2 days lag phase, which is the same as control (Figures 4.4 & 4.5). Among all the extracts of SFPs, emulsions incorporated with yellow pea extracts with 6 days of germination showed the longest lag phase of 4 days (Figures 4.8 & 4.9). Surprisingly, the lag phase of the emulsion in the presence of SFPs extracted from lentil remained the same as control regardless of germination (Figures 4.6 & 4.7).

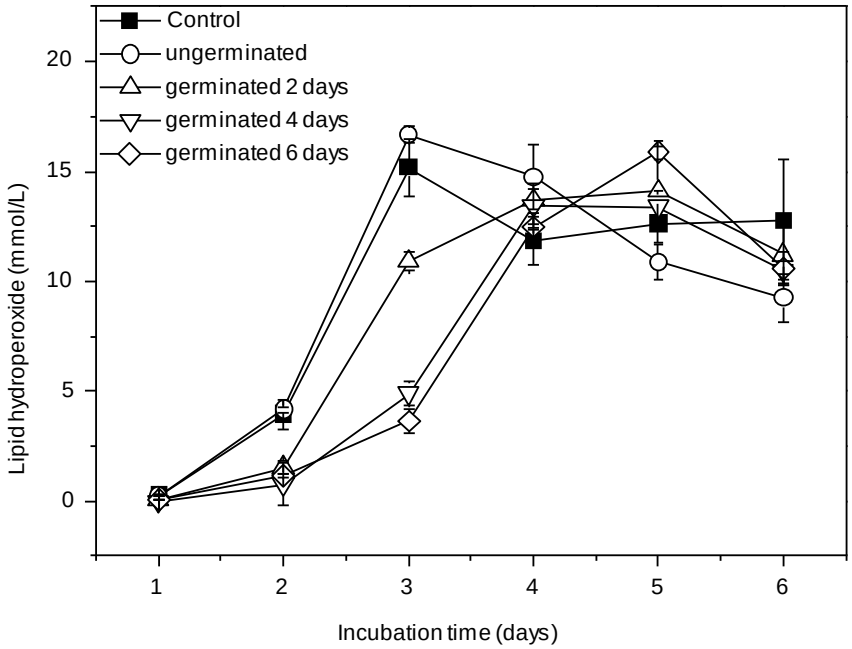

Figure 4.4. Formation of lipid hydroperoxides in SSO-in-water emulsion system with addition of SFPs extracted from chickpea. Data points represent mean (n=2) ± standard deviation.

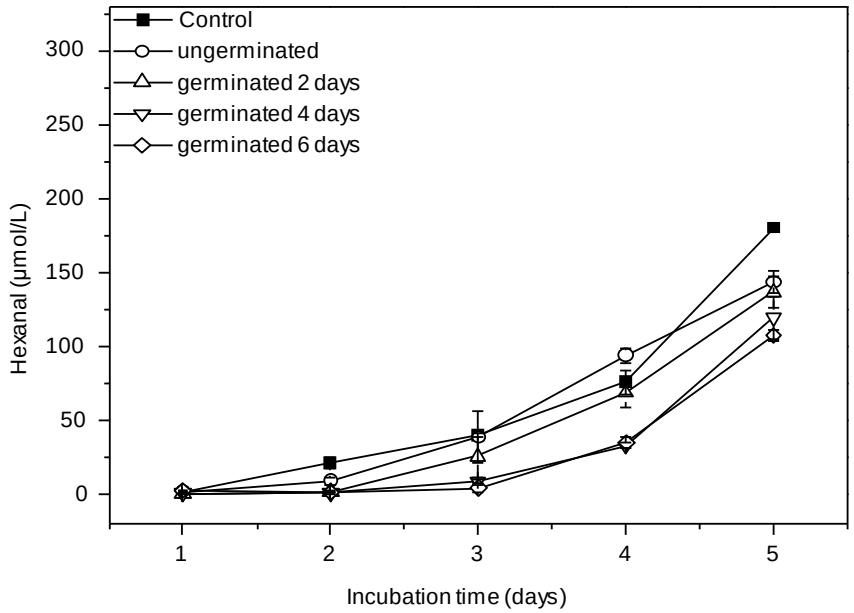

Figure 4.5. Formation of hexanal in SSO-in-water emulsion system with addition of SFPs extracted from chickpea. Data points represent mean (n=2) ± standard deviation.

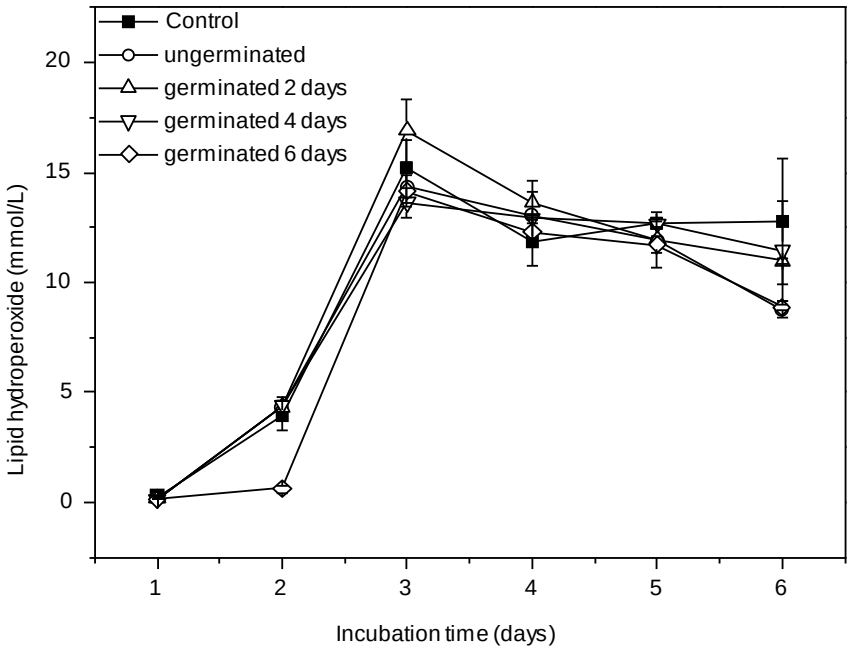

Figure 4.6. Formation of lipid hydroperoxides in SSO-in-water emulsion system with addition of SFPs extracted from lentil. Data points represent mean (n=2) ± standard deviation.

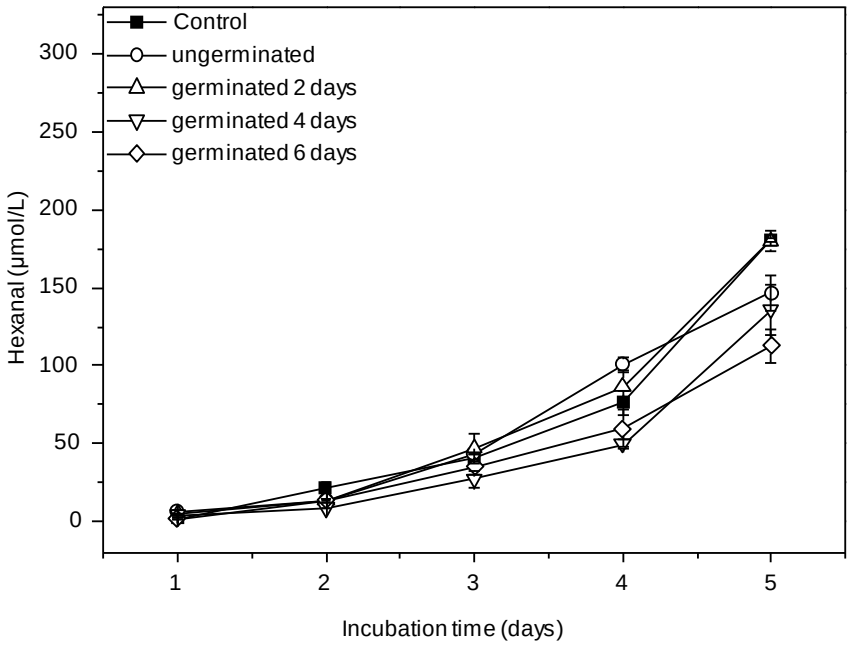

Figure 4.7. Formation of hexanal in SSO-in-water emulsion system with addition of SFPs extracted from lentil. Data points represent mean (n=2) ± standard deviation.

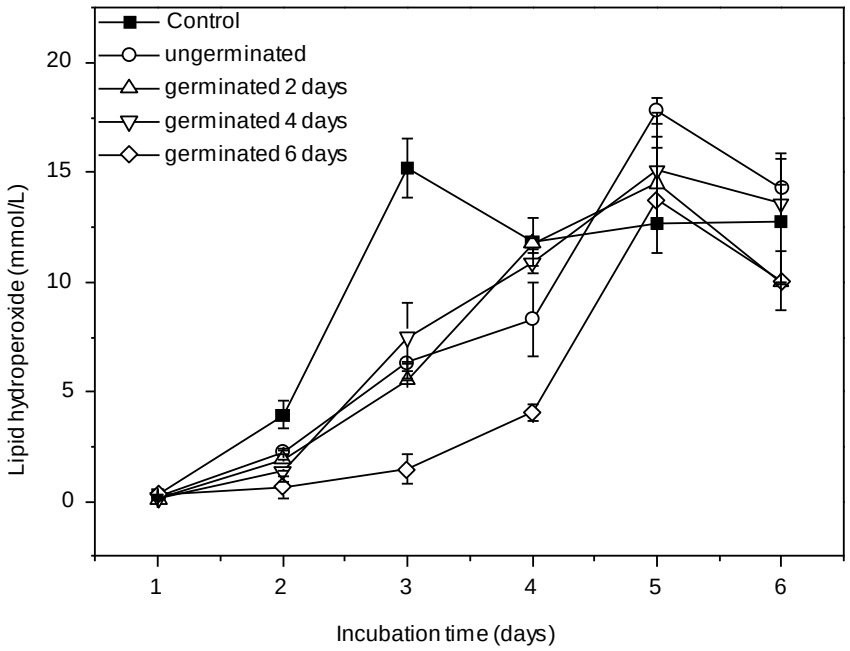

Figure 4.8. Formation of lipid hydroperoxides in SSO-in-water emulsion system with addition of SFPs extracted from yellow pea. Data points represent mean (n=2) ± standard deviation.

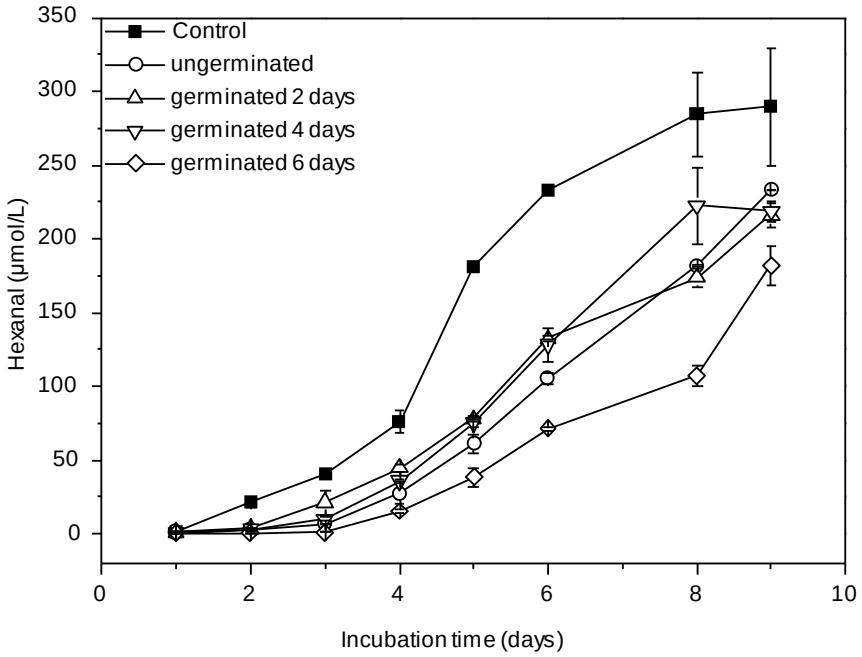

Figure 4.9. Formation of hexanal in SSO-in-water emulsion system with addition of SFPs extracted from yellow pea. Data points represent mean (n=2) ± standard deviation.

These results showed that the antioxidant activity of SFPs increased with the germination time in chickpea and yellow pea. As supported by *in vitro* assay, the antioxidant activity of SFPs of chickpea and yellow pea had increased during germination. This can account for the enhanced antioxidant activity of these extracts in emulsion system. The relationship between *in vitro* assay and emulsion system followed the same trend in germinated lentil. Both TPC and DPPH had decreased activity for germinated lentils, while the SFPs of lentil had little effect on lag phase of the emulsion system.

4.4.4. Antioxidant activity of polar soluble bound phenolic (PSBPs) extracts from germinated pulses in oil-in-water emulsions

After diluting all the extracts to 187 μg GAE/mL of TPC, antioxidant activity of PSBPs was observed in the emulsion system. The antioxidant activity of PSBPs in emulsion was much more complicated than that of SFPs (Figures 4.10-4.18). PSBPs from chickpea and yellow pea showed more sustainable antioxidant activity in emulsions than SFP forms. The PSBPs extracted from both ungerminated chickpea (Figure 4.10) and yellow pea (Figure 4.16) generated a 10-day lag phase as indicated by the formation of lipid hydroperoxides. The germination process enhanced the antioxidant activity of PSBPs in chickpea, with germination time proportional improving antioxidant activity. The longest emulsion lag phase of 14 days was observed for PSBPs extract of chickpea germinated 6 days. However, this was not the case for both lentil and yellow pea. The germination time has no impact on the antioxidant activity of PSBPs extracted from lentil when tested for lipid hydroperoxides (Figure 4.13). Conversely, germination negatively impacted the antioxidant activity of PSBPs extracted from yellow pea (Figure 4.16).

Hexanal was widely used as secondary oxidation markers for high linoleic oil. It was true in the emulsion system when SFPs were presented (Figures 4.4-4.9). Unexpectedly, hexanal did not increase significantly during storage when PSBPs were present, even though the lipid hydroperoxides had decomposed. Thus, the formation of hexanal cannot be applied to differentiate the lag phase of emulsions with different PSBPs (Figures 4.11, 4.14, and 4.17). The results of total ions scan mode via GC-MS indicated the remarkable production of hexanol during storage (data not shown). In this situation, the formation of hexanol in emulsions was also recorded along with hexanal during storage. As one can see in Figures 4.12, 4.15, and 4.18, the formation of hexanol followed the decomposition of lipid hydroperoxides and the low concentration of hexanal. We consider it was an improvement in antioxidant activity as the aroma threshold of hexanol is 4000 μg/L (Siebert et al., 2005), which is much higher than that of hexanal, 75 μg/L (Buttery et al., 1973). However, it was unclear if there were any constituents that had strong reducing power or if the β-scission resulted in greater production of hexanol instead of hexanal.

Unlike SFPs, which might also be oxidized by oxygen and transition metals rather than retarding lipid oxidation (Zhou and Elias, 2012), PSBPs extracted from chickpea and yellow pea exerted stronger antioxidant activity to prevent emulsion oxidation. One possible reason is the synergistic effect between phenolic compounds and biopolymers as they are conjugated to form PSBPs. After SFPs synthesized in the endoplasmic reticulum by shikimate pathway or flavonoid branch pathway, some of them would be transported to the cell wall and part of them would become PSBPs through the conjugation with polysaccharides or proteins (Shahidi and Yeo,

2016). Some constituents conjugated with phenolic compounds were reported to be antioxidants themselves or have synergistic effects with phenolic compounds (Xie et al., 2014). Previous iron binding study suggested that PSBPs had better antioxidant activity, but was not due to its metal chelating ability. It was more likely that hydrogen on the antioxidants was abstracted by lipid radicals may be recovered by the conjugated constituents containing lower electron reduction potentials (Gall et al., 2013). Besides, steric hindrance, caused by large molecular moieties, may happen to the phenolic compounds, which made the antioxidant activity released sustainable. Nevertheless, PSBPs from lentil had little antioxidant activity, similar to its SFP form. It may be caused by the TPC of lentil which was 2-8 times greater than that of chickpea and yellow pea, so as the dilution ratio to the final concentration (i.e., 187 μg GAE/mL), which may dilute synergistic antioxidant effect of the extract.

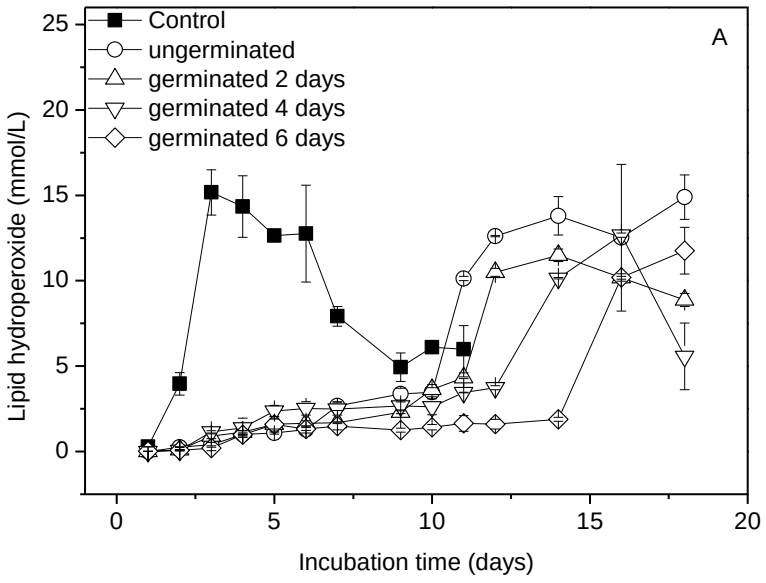

Figure 4.10. Formation of lipid hydroperoxides in SSO-in-water emulsion system with addition of PSBPs extracted from chickpea.

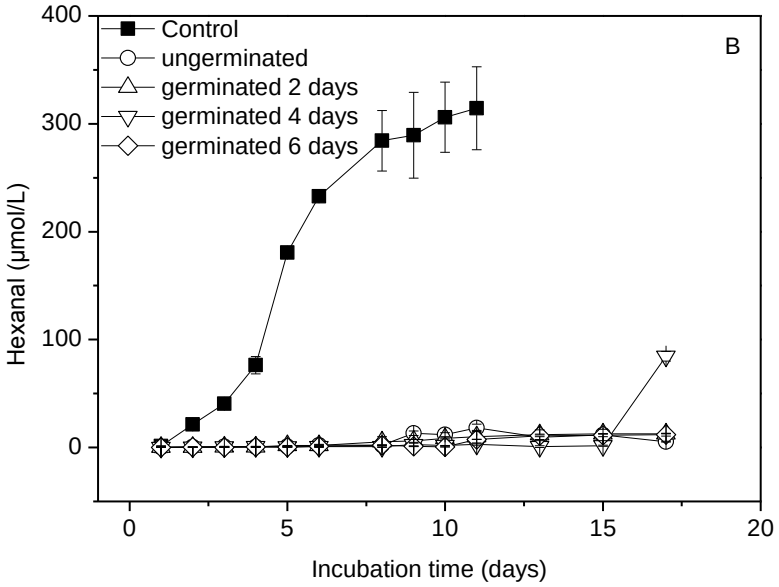

Figure 4.11. Formation of hexanal in SSO-in-water emulsion system with addition of PSBPs extracted from chickpea.

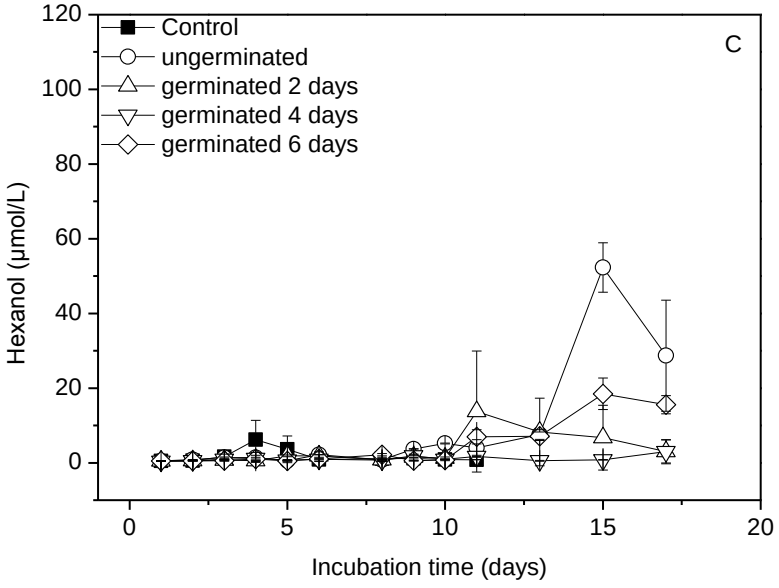

Figure 4.12. Formation of hexanol in SSO-in-water emulsion system with addition of PSBPs extracted from chickpea.

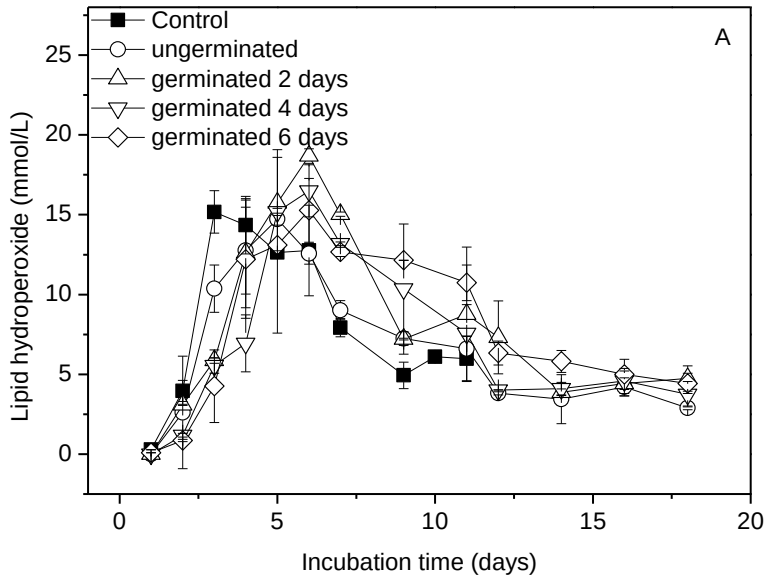

Figure 4.13. Formation of lipid hydroperoxides in SSO-in-water emulsion system with addition of PSBPs extracted from lentil.

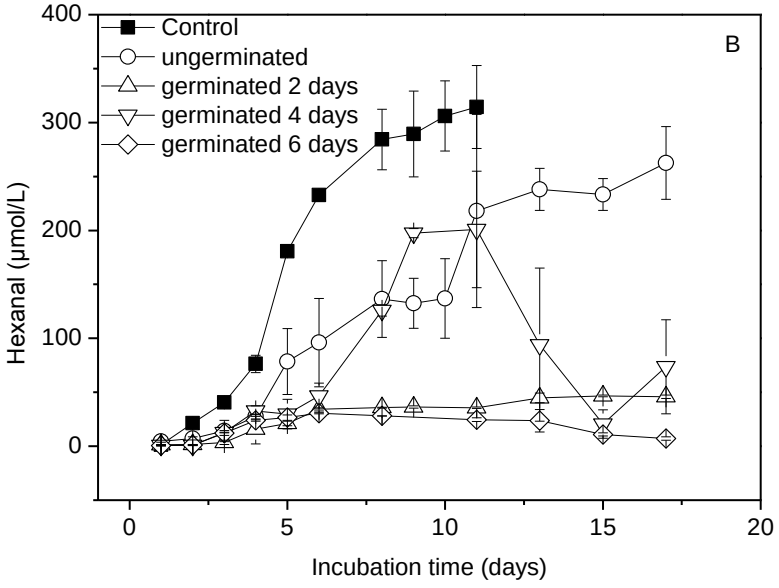

Figure 4.14. Formation of hexanal in SSO-in-water emulsion system with addition of PSBPs extracted from lentil.

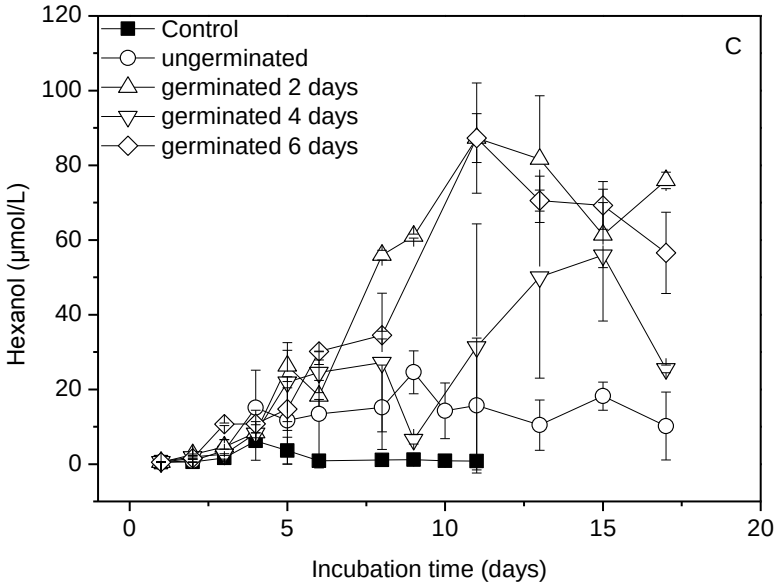

Figure 4.15. Formation of hexanol in SSO-in-water emulsion system with addition of PSBPs extracted from lentil.

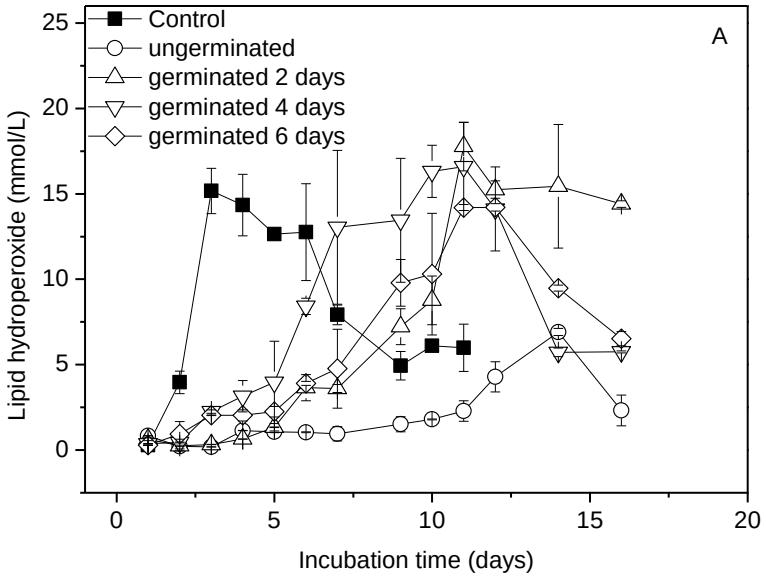

Figure 4.16. Formation of lipid hydroperoxides in SSO-in-water emulsion system with addition of PSBPs extracted from yellow pea

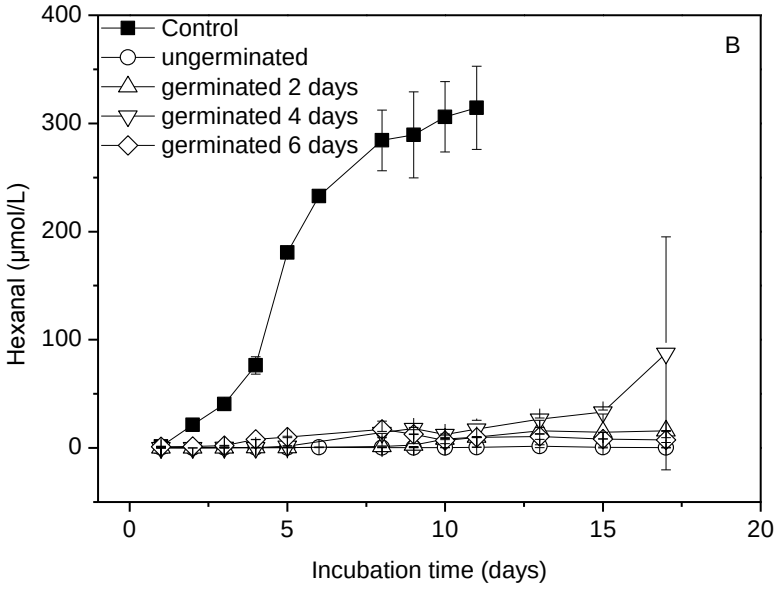

Figure 4.17. Formation of hexanal in SSO-in-water emulsion system with addition of PSBPs extracted from yellow pea

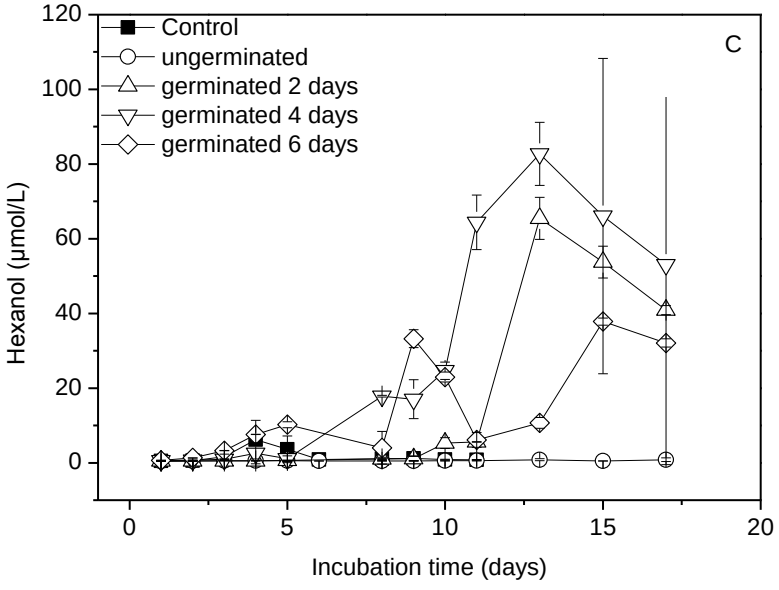

Figure 4.18. Formation of hexanol in SSO-in-water emulsion system with addition of PSBPs extracted from yellow pea

Still, antioxidant activity of PSBPs in the SSO-in-water emulsion system cannot be correlated with *in vitro* assays. The antioxidant activity of PSBPs described by ORAC and DPPH was improved by germination much less than that of SFPs in chickpea and yellow pea (Table 4.1), whereas PSBPs of chickpea (Figures 4.10-4.12) germinated 6 days and ungerminated yellow pea (Figures 4.16-4.18) had significantly better antioxidant activity, retarding emulsion oxidation for 14 and 10 days, respectively. The discrepancy of antioxidant activity between *in vitro* assay and in real food system has been reported recently. For instance, Alamed et al. reported that ascorbic acid was twice as effective at scavenging peroxyl radicals as propyl gallate in ORAC assay while propyl gallate was a much better antioxidant in oil-in-water emulsions (Alamed et al., 2009). This again suggests that *in vitro* assays have limited value in predicting the antioxidant activity in complex foods. Alternatively, the results from *in vitro* assay could be applied to unravel the mechanisms for antioxidants to inhibit lipid oxidation in food systems.

4.5. Conclusion

The composition of phenolic compounds in pulses have changed after germination. Various pulse seeds had a different trend in antioxidant activity as related to germination. In this research, extracts from chickpea and yellow pea showed ameliorable antioxidant activity while lentil had poor improvement of antioxidant activity in emulsion system. PSBPs had stronger antioxidant activity than SFPs in chickpea and yellow pea and can be exploited as novel natural antioxidants. Synergistic effects between phenolic compounds and biopolymers they conjugated with may be attributed to their stronger antioxidant activity. Compare to the *in vitro* assays, such as DPPH and ORAC which presented consistent increasing trend in antioxidant activity with the

increasing of germination time, the antioxidant activity of PSBPs in emulsion system were not correlated, while that of SFP forms had relationship between *in vitro* and emulsion system. An interesting phenomenon was observed that the oxidation of SSO-in-water emulsion system with the addition of PSBPs extracted from pulses had the ability to generate more hexanol than hexanal.

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CHAPTER 5. KEY FACTORS AFFECT THE ANTIOXIDANT ACTIVITY OF POLAR SOLVENT EXTRACTABLE PHENOLIC COMPOUNDS ELUCIDATED WITH GERMINATION OF CHICKPEA AND YELLOW PEA[1*]

5.1. Abstract

The purpose of this study was to elucidate the mechanism by which pulse germination impacts the antioxidant activity of phenolic compounds. Liquid chromatography–electrospray ionization–quadrupole time of flight–mass spectrophotometer and size exclusion chromatography–multiangle laser light scattering detector–refractive index detector were employed to evaluate the phenolic composition of polar solvent extractable phenolic compounds and molar mass of polar soluble bound phenolic compounds, respectively, over a germination of 6 days. With principal component analysis, it was revealed that four phenolic compounds were important in the antioxidant activity of chickpea extracts, while seven phenolic compounds were important phenolic compounds responsible for yellow pea extracts. Particularly, molar masses of polar soluble bound phenolic compounds had positive relationship with the antioxidant activity. Protective and co-antioxidative theories were proposed to explain how antioxidant activity of polar soluble bound phenolic compounds in oil-in-water emulsions varied with germination.

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[*] Based on two articles of Minwei Xu, Zhao Jin, Jae-Bom Ohm, Paul Schwarz, Jiajia Rao, & Bingcan Chen published in Journal of Agriculture and Food Chemistry and Food & Function on June 2018 (DOI: 10.1016/j.foodchem.2019.0100 g167) and Sep 2019 (DOI: 10.1039/C9FO00799G), respectively. Minwei Xu had primary responsibility for data collection and analysis, was the primary developer of the conclusions advanced here, and drafted and revised all versions of this chapter. Zhao Jin assisted with the experimental design. Jae-Bom Ohm, Paul Schwarz, and Jiajia Rao assisted with instruments. Bingcan Chen served as proofreader.

5.2. Introduction

Edible pulse seeds are high in antioxidant potential, dietary fiber, resistant starch, protein, vitamins, and minerals with a gluten-free status (Hall et al., 2017). Germination has been regarded as an effective process to improve nutrient digestibility and accessibility, as well as phenolic compounds of pulse seeds (Mamilla and Mishra, 2017). An overwhelming amount of publications have reported that germination can improve the quantity of total phenolic compounds, which is considered an indicator of antioxidant potential (Dueñas et al., 2016; Gan et al., 2016b; Kim et al., 2016; Guzmán-Ortiz et al., 2017). However, the effect of germination on the quality of phenolic compounds, particularly their antioxidant activity, is inconsistent.

The antioxidant activity of phenolic compounds is believed to be associated with phenolic composition. Different phenolic compounds have different antioxidative potential, e.g. chlorogenic acid > quercetin > gallic acid > *trans*-resveratrol > rutin > caffeic acid based on 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity (Helmja, 2010). During germination, a new class of phenolic compounds can be biosynthesized through the Shikimate pathway, while existing phenolic compounds can be either depleted by free radicals or as the precursors of phytochemicals (Shahidi and Yeo, 2016). Germination of pulse seeds would either cause the increase or decrease in the amount of specific phenolic compounds. Gan et al. (2017) reported that after 5 days of germination, caffeic acid, ferulic acid, and *p*-coumaric acid in black mung bean increased from 2.51 to 6.72, 2.10 to 5.57, and 1.50 to 22.0 mg/100 g dry basis (d.b.), respectively. In contrast, Kim et al. (2016) reported that after 4 days of germination, veratric

acid, protocatechuic acid, and *p*-coumaric acid in soybean decreased from 2.17 to 0.53, 85.57 to 57.36, and 2.96 to 0.39 μg/g, respectively.

In comparison to the individual phenolic compound, the moieties of phenolic compounds play a preponderant role in phenolic antioxidant activity (Shahidi and Yeo, 2016). Phenolic compounds can be classified as either extractable or non-extractable based on their solubility in different solvents, e.g. ferulic acid is extractable when bound to glucoside; however, it becomes non-extractable after covalently bound to arabinoxylans (Shahidi and Ambigaipalan, 2015). Most extractable phenolic compounds are studied with polar solvent and named as polar solvent extractable phenolic compounds, while non-polar soluble bound phenolic compounds have not been widely concerned and were regularly discarded with the defatting process (Xu et al., 2019b; Elder et al., 2019). Polar solvent extractable phenolic compounds can be further divided into soluble free (SFPs) and polar soluble bound phenolic compounds (PSBPs) depending on the absence or the presence of strong polar moieties. Polar solvent extractable phenolic compounds are great antioxidants after extraction (Naczk and Shahidi, 2004). SFPs displayed better antioxidant activity than PSBPs in the *in vitro* system (Chen et al., 2015b; Wang et al., 2016c). However, the real food system is more complicated than the *in vitro* system.

Germination increases the total phenolic content of chickpea, lentil, and yellow pea. However, the antioxidant activities of these phenolic compounds and different outcomes in *in vitro* assays. In addition, we found only the PSBPs rather than SFPs from chickpea and yellow pea can effectively prevent oil-in-water emulsion oxidation. Interestingly, germination can

improve the antioxidant activity of the PSBPs from chickpea, while decrease that from yellow pea.

Based on previous results in Chapter 4, it is hypothesized that the enhanced antioxidant activity and the different performance between SFPs and PSBPs in *in vitro* and oil-in-water emulsion may be related to the composition and structure change of phenolic compounds over the course of germination. Therefore, the objectives of this study were to (i) identify and semi-quantitative interpret the main polar solvent extractable phenolic compounds from chickpea and yellow pea with liquid chromatography–electrospray ionization–quadrupole time of flight–mass spectrophotometer (LC-ESI-QTOF-MS); (ii) investigate the molar mass of PSBPs from chickpea and yellow pea with size exclusion chromatography–multiangle laser light scattering detector–refractive index detector (SEC-MALS-RI); and (iii) propose mechanisms of how germination impact antioxidant activity of polar solvent extractable phenolic compounds in chickpea and yellow pea.

5.3. Materials and Methods

5.3.1. Chemicals

Chickpea (*Cicer aretinium* L.) and yellow pea (*Pisum sativum* L.) from the 2017 crop year was gifted from JM Grain, Viterra Inc, and AGT Food and Ingredients. The manufacturers noted that chickpea and yellow pea were the mixture of different cultivars collecting from growers. Sodium hydroxide, hydrochloric acid, acetonitrile, acetone, acetone, ethyl ether, ethyl ester, ethyl acetate, and other materials were purchased from VWR International (West Chester, Pa., U.S.A.).

5.3.2. Germination of chickpea and yellow pea

The method of pulse germination was described in section 3.3.2 without modification.

5.3.3. Extraction of phenolic compounds

Extraction of soluble free and soluble bound phenolic compounds from chickpeas and yellow peas during 0, 2, 4, and 6 days germination was followed as described in section 4.3.3 without modification.

5.3.4. Hydrolyzation of soluble bound phenolic compounds

Soluble bound phenolic compounds as described in section 2.3 were hydrolyzed for the evaluation of phenolic composition. Briefly, 30 mL soluble bound phenolic compounds were concentrated to 3 mL with freeze drying. Two hundred μL of concentrated soluble bound phenolic compounds were hydrolyzed with 2 mL 3 N sodium hydroxide purged with nitrogen and shake for 1 h with shakers. After adjusting the pH to 2-3, ethyl ether/ethyl acetate (1/1, v/v) was added to extract phenolic compounds for 3 times. Supernatants were collected and 3 mL of 6 N hydrochloric acid were added and purged with nitrogen to hydrolyze the residues at 95 °C water bath for 20 min. Three mL of ethyl ether/ethyl acetate (1/1, v/v) were added to extract phenolic compounds 3 times. The combined ethyl ether/ethyl acetate solvents were flushed with nitrogen until dry. Five hundred μL of acetonitrile were added into each sample and stored at -20 °C overnight for structure characterization.

5.3.5. LC-ESI-QTOF-MS analysis

Both SFPs and hydrolyzed PSBPs extracted from germinated yellow pea were characterized using LC-ESI-QTOF-MS according to the method described by Kadam et al.

(2017) with minor modifications. An Agilent 1290 series liquid chromatography system utilizing a Kinetex C18 (2.6 μm, 150 × 4.6 mm) column was used to separate phenolic compounds. The mobile phase comprised of water (solvent A) and acetonitrile (solvent B) with the following gradients: 0−5 min (A: 95%, B: 5%), 30−40 min (A: 0%, B: 100%), and 41−45 min (A: 95%, B: 5%). The flow rate was 0.5 mL/min, and the column temperature was 30 °C. The injection volume was 20 μL with a total run time of 45 min. A diode-array detector (DAD) with a working range from 190 to 600 nm was employed to observe the UV absorption of the separated yellow pea extracts. An Agilent G6540 UHD Accurate QTOF-MS was utilized to analyze the chromatographed yellow pea extracts. Sample ionization was achieved using an electrospray ionization (ESI) interface in negative-ion mode. The gas and vaporizer temperatures were set to 300 °C, with a drying-gas flow rate of 7 L/min. The nebulizer (N_2) was set at 50 psi, the fragmentor, skimmer, octopole RF, and capillary voltages were set at 200, 65, 750, and 4000 V, respectively. The collision energy was set as 0, 10, 25, 30, and 40 eV. The Agilent high-resolution mass spectrometer was operated for data acquisition using Data Dependent Acquisition mode (Auto MS/MS) combining a TOF scan (*m/z* 100-1000) followed by dependent TOF-MS scans (*m/z* 50-1000).

Data analysis was performed using MassHunter Qualitative Analysis software B.05.00 (Agilent Technologies). The identification of the detected compounds proceeded by the generation of candidate molecular formulas with mass-accuracy limits of 5 ppm and MS scores >80. Agilent Personal Compound Database Library (PCDL) version B.05.01 build 92 was employed to create the custom database. For the retrieval of phenolic-compound chemical

structures, the following databases were consulted: Agilent METLIN Metabolomics database, HMDB (http://www.hmdb.ca/), and Phenol-Explorer (http://www.phenol-explorer.eu).

5.3.6. SEC-MALS-RI analysis

Soluble bound phenolic compounds collected as described in Section 2.3 were concentrated 10 times, and the solutions were filtered through a 0.2 μm polytetrafluoroethylene (PTFE) disposable membrane prior to the successive SEC-MALS-RI analysis. SEC-MALS-RI analysis was carried out by a Yarra 3 μm SEC-4000, 300 × 7.8 mm SEC column connected to an Agilent G1315 C DAD detector, an Agilent 1362 A RI detector, and a DAWN® HELEOS™ II MALS detector. ASTRA® 7.1.2.5 software was used to analyze data. Deionized water was used as an eluent for the SEC-MALS-RI analysis. SEC conditions were set as follows: injection volume of 10 μL, flow rate of 0.4 mL/min, and the column temperature of 30 °C. Specific refractive index increments (dn/dc) of sample solutions with concentrations in the range of 0.8–1.5 mg/mL were determined using an Agilent 1362 A RI detector. The dn/dc values of CB0, CB2, CB4, CB6, YB0, YB2, YB4, and YB6 were 0.147, 0.145, 0.127, 0.125, 0.134, 0.109, 0.096, and 0.087 mL/g, respectively (Fredheim et al., 2002; Shibata et al., 2006).

5.3.7. Statistical analysis

The experiments were performed at least twice and data were expressed as mean± standard deviation of duplicate or triplicate measurements. Data were statistically analyzed using statistical software, SAS version 9.4 (SAS institute Inc. Cary, NC). One-way analysis of variance (ANOVA) was conducted, and significant difference was defined at $p < 0.05$ by Tukey's test. The relationships between germination time and phenolic compounds of chickpea and yellow

pea extracts were analyzed by principal-component analysis (PCA) and constellation plot-cluster analysis (CPCA) based on the counts of each phenolic compounds using JMP Pro 14.0.0 (SAS Institute Inc.).

5.4. Results and Discussion

5.4.1. Effect of germination time on major phenolic compounds of pulse seeds

5.4.1.1. Effect of germination on phenolic composition of chickpea extracts

In total, 16 phenolic compounds from germinated chickpea were identified by *m/z* value in conjunction with product ion and retention time (Table 5.1). Overall, most phenolic compounds were increased during the period of germination, with few reductions being observed by virtue of ion counts (Table 5.2). However, germination time performed a variable impact on soluble free and soluble bound phenolic compounds in terms of their content and composition.

5.4.1.1.1. Soluble free phenolic compounds (SFPs)

During germination, phenolic acids and flavonoids are synthesized for the quenching of reactive oxygen species or as the precursor to synthesize plant tissue, such as lignin (Cevallos-Casals and Cisneros-Zevallos, 2010). In consequence, individual phenolic compounds in free form is anticipated to increase with germination. The dramatic increase in the content of SFPs was observed during chickpea germination in light of the remarkable increase of both peak area and peak numbers at 260 nm (Figure 5.1A). The semi-quantification by LC-ESI-QTOF-MS also highlighted the increase in concentration of hesperetin (**C9**), 7,3',4'-trihydroxyflavone (**C7**), 8-hydroxydihydrodaidzein (**C4**), and 6-hydroxydaidzein (**C8**) during chickpea germination (Table 5.2). However, not all of SFPs increased during germination (Table 5.2). The ion counts of

Table 5.1. Profiles of phenolic compounds in germinated chickpea extracts identified by LC-ESI-QTOF-MS

Peak No.	RT (min)	Collision energy (ev)	Observed *m/z* $[M-H]^-$	Calculated *m/z* $[M-H]^-$	Molecular Formula	Diff. (ppm)	Score	Product ions	Proposed compounds
C1	9.655	10	315.0730	315.0722	$C_{13}H_{16}O_9$	-2.53	95.40	108.0209, 153.0190	Protocatechuic acid 4-O-glucoside
C2	12.047	25	153.0193	153.0193	$C_7H_6O_4$	0.01	99.80	109.0255, 135.0013	Gentisic acid
C3	12.491	25	231.0310	231.0299	$C_{12}H_8O_5$	-4.86	88.77	133.0162, 137.0101, 159.0298, 189.0008	4-Hydroxy-8-methoxy-2H-fruo[2,3-h]-1-benzopyran-2-one
C4	15.906	10	271.0300	271.0612	$C_{15}H_{12}O_5$	-1.58	81.75	109.0167, 119.0359, 150.9863	8-Hydroxydihydrodaidzein
C5	16.894	30	273.0776	273.0768	$C_{15}H_{14}O_5$	-2.86	96.28	109.0277, 125.0986, 241.1087	Afzelechin
C6	17.590	30	283.0615	283.0612	$C_{16}H_{12}O_5$	0.65	95.24	195.0228, 211.0157, 223.0137, 239.0073, 268.0064	Prunetin
C7	17.955	40	269.0528	269.0455	$C_{15}H_{10}O_5$	-1.40	97.36	195.0214, 211.0143, 223.0125, 239.0077	7,3',4'-Trihydroxyflavone
C8	19.258	25	269.0531	269.0455	$C_{15}H_{10}O_5$	-1.78	84.91	117.0350, 143.0506, 194.9254, 239.0354	6-Hydroxydaidzein
C9	19.278	10	301.0723	301.0718	$C_{16}H_{14}O_6$	-1.87	98.00	107.0013, 109.0166, 150.9857, 286.0143	Hesperetin
C10	19.442	30	285.0407	285.0405	$C_{15}H_{10}O_6$	-0.92	97.36	117.0340, 143.0514, 187.0381, 239.0375	Kaempferol
C11	19.618	10	185.0610	185.0608	$C_{12}H_{10}O_2$	0.19	98.27	141.0693	1-Naphthyl acetate
C12	19.674	10	353.1039	353.1031	$C_{20}H_{18}O_6$	-2.45	97.33	111.0082, 125.0241	Isolicoflavonol
C13	20.294	25	281.0455	281.0455	$C_{16}H_{10}O_5$	0.25	92.81	134.9930, 167.0307, 208.0270, 224.0211, 253.0217	Pseudobaptigenin
C14	20.564	25	267.0671	267.0663	$C_{16}H_{12}O_4$	-3.06	98.13	104.0216, 135.0025, 167.0418, 195.0364, 223.0302, 252.0316	Formononetin
C15	21.858	30	287.0565	287.0561	$C_{15}H_{12}O_6$	-1.52	89.09	109.0295, 125.0243	3',4',5,7-Tetrahydroxyisoflavanone
C16	22.493	30	283.0615	283.0612	$C_{16}H_{12}O_5$	-1.23	98.95	116.9955, 211.0400, 224.0409, 239.0352, 268.0375	Glycitein

*RT, retention time; Diff., difference between calculated *m/z* and observed *m/z*.

Table 5.2. Dynamic changes of proposed phenolic compounds during chickpea germination

Peak No.	Class	Proposed compounds	CF0 ($\times 10^5$ counts)	CF2 ($\times 10^5$ counts)	CF4 ($\times 10^5$ counts)	CF6 ($\times 10^5$ counts)	CB0 ($\times 10^5$ counts)	CB2 ($\times 10^5$ counts)	CB4 ($\times 10^5$ counts)	CB6 ($\times 10^5$ counts)
C1	Phenolic acid	Protocatechuic acid 4-O-glucoside	18.3 ± 0.9 b	26.9 ± 3.3 c	35.0 ± 1.7 d	42.4 ± 0.8 e	N.A.	3.1 ± 0.2 a	2.8 ± 0.3 a	2.3 ± 0.1 a
C2	Phenolic acid	Gentisic acid	N.A.	N.A.	N.A.	N.A.	31.6 ± 0.0 b	21.0 ± 0.4 a	21.4 ± 1.9 a	35.4 ± 3.8 b
C3	Coumarin	4-Hydroxy-8-methoxy-2H-fruo [2,3-h]-1-benzopyran-2-one	N.A.	N.A.	N.A.	N.A.	2.5 ± 0.3 a	3.6 ± 0.4 a	4.1 ± 0.3 ab	5.7 ± 0.6 b
C4	Isoflavone	8-Hydroxydihydrodaidzein	2.5 ± 0.1 b	3.0 ± 0.2 b	0.9 ± 0.1 a	4.6 ± 0.6 c	N.A.	0.4 ± 0.1 a	5.5 ± 0.5 c	3.1 ± 0.2 b
C5	Flavanol	Afzelechin	3.5 ± 0.2 c	2.2 ± 0.2 b	N.A.	0.8 ± 0.1 a	N.A.	2.3 ± 0.2 b	4.8 ± 0.1 d	7.2 ± 0.1 e
C6	Isoflavone	Prunetin	73.6 ± 1.3 d	22.3 ± 0.9 bc	11.2 ± 0.8 a	N.A.	N.A.	N.A.	17.8 ± 2.2 b	24.4 ± 1.1 c
C7	Flavone	7,3',4'-Trihydroxyflavone	2.8 ± 0.2 a	15.6 ± 0.2 b	13.0 ± 1.0 b	33.7 ± 2.5 c	N.A.	N.A.	2.5 ± 0.3 a	5.2 ± 0.6 a
C8	Isoflavone	6-Hydroxydaidzein	5.9 ± 0.0 b	4.8 ± 0.4 ab	5.0 ± 0.3 ab	11.3 ± 1.7 c	5.1 ± 0.2 ab	3.2 ± 0.1 ab	2.7 ± 0.2 a	N.A.
C9	Flavanone	Hesperetin	0.7 ± 0.1 a	1.8 ± 0.1 ab	2.9 ± 0.0 b	8.4 ± 1.2 c	N.A.	N.A.	0.8 ± 0.1 a	1.0 ± 0.1 ab
C10	Flavonol	Kaempferol	N.A.	N.A.	N.A.	N.A.	8.9 ± 0.7 a	8.4 ± 0.0 a	21.2 ± 1.0 b	19.2 ± 1.7 b
C11	Naphthyl ester	1-Naphthyl acetate	N.A.	N.A.	N.A.	N.A.	8.2 ± 0.0 d	5.1 ± 0.5 c	1.9 ± 0.3 b	0.4 ± 0.0 a
C12	Isoflavonol	Isolicoflavonol	N.A.	N.A.	N.A.	N.A.	99.2 ± 0.4 d	52.0 ± 3.4 c	20.7 ± 2.6 b	3.5 ± 0.1 a
C13	Isoflavone	Pseudobaptigenin	15.0 ± 1.4 d	23.4 ± 1.3 e	14.1 ± 0.5 cd	6.2 ± 0.2 a	N.A.	N.A.	7.5 ± 0.8 ab	10.9 ± 0.0 bc
C14	Isoflavone	Formononetin	84.4 ± 10.4 d	42.0 ± 1.7 c	17.6 ± 1.8 ab	11.7 ± 0.6 ab	N.A.	4.2 ± 0.6 a	26.6 ± 2.4 bc	87.4 ± 2.1 d
C15	Isoflavone	3',4',5,7-Tetrahydroxy isoflavanone	N.A.	N.A.	N.A.	1.4 ± 0.0 a	N.A.	0.5 ± 0.0 a	4.4 ± 0.5 b	7.5 ± 0.3 c
C16	Isoflavone	Glycitein	232.1 ± 29.4 c	163.9 ± 16.7 b	31.2 ± 0.2 a	N.A.	2.1 ± 0.3 a	15.7 ± 1.9 a	26.2 ± 1.4 a	43.3 ± 2.2 a

*CF and CB denoted SFPs and PSBPs, followed with different germination time: 0, 2, 4, and 6 days, respectively. Data points represent mean (n=2) ± standard deviation. Different letters indicate statistically significant differences intraspecies ($p < .05$).

afzelechin (**C5**), prunetin (**C6**), formononetin (**C14**), and glycitein (**C16**) declined during germination. Two reasons may account for such phenomenon. First, the depleted SFPs may be consumed by scavenging the radicals generated from chickpea physiological activity during germination. Second, SFPs may be transformed into PSBPs form by transferase (Hatfield et al., 2009). This can be justified by the fact that the contents of afzelechin (**C5**), prunetin (**C6**), formononetin (**C14**), and glycitein (**C16**) in soluble bound form increased over the course of germination (Table 5.2). A similar transformation between extractable and non-extractable phenolic compounds (NEPs) during lentil germination were reported by Yeo and Shahidi (Yeo and Shahidi, 2015).

Protocatechuic acids and its derivatives exist widely in cereals and legumes, such as black rice, black wheat, oats, lentils, pinto beans, and kidney bean (Shahidi and Yeo, 2016). Xu et al. (2018) reported that protocatechuic acids had strong free radicals scavenging activity in cultured neural cells. Natural source of 6-hydroxydaidzein was first identified by Hirota et al. (2004) from soybean miso and was determined to exhibit 2,2-diphenyl-1-picrylhydrazyl (DPPH)-radical scavenging activity as high as that of α-tocopherol. In addition, 6-hydroxydaidzein were more effective antioxidants than quercetin and ascorbic acid in oxygen radical absorbing capacity (ORAC) assay and the *in vitro* oxidation of low-density lipoproteins (LDL) (Rüfer and Kulling, 2006).

5.4.1.1.2. Polar soluble bound phenolic compounds (PSBPs)

Polar solvent extractable phenolic compounds of chickpea seeds are mainly present as PSBPs. PSBPs can be synthesized from SFPs through conjugation with soluble moieties.

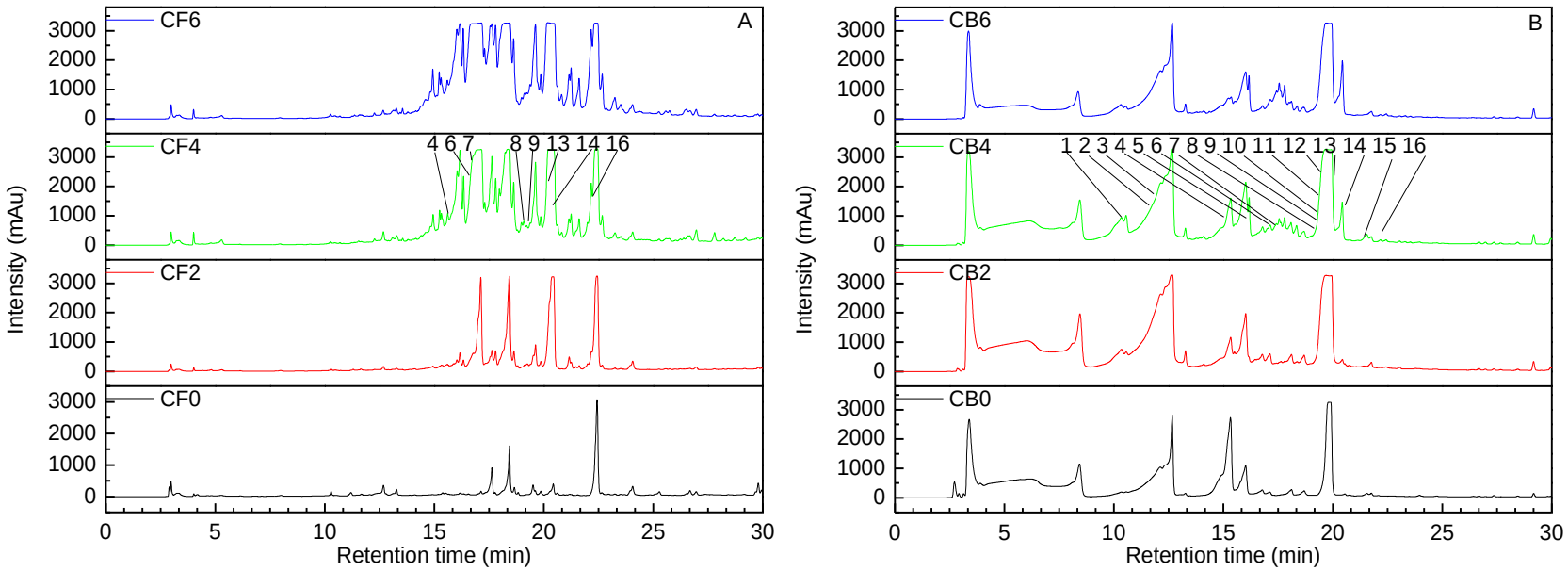

Figure 5.1. HPLC chromatograms of (A) soluble free phenolic compounds, and (B) soluble bound phenolic compounds extracted from chickpea during chickpea germination.

All these extractions were separated with C18 column and monitored with DAD at 260 nm. (Numbers on the peaks stand for the proposed phenolic compounds identified by Q-TOF-MS: **C1**, Protocatechuic acid 4-O-glucoside; **C2**, Gentisic acid; **C3**, 4-Hydroxy-8-methoxy-2H-fruo[2,3-h]-1-benzopyran-2-one; **C4**, 8-Hydroxydihydrodaidzein; **C5**, Afzelechin; **C6**, Prunetin; **C7**, 7,3',4'-Trihydroxyflavone; **C8**, 6-Hydroxydaidzein; C9, Hesperetin; **C10**, Kaempferol; **C11**, 1-Naphthyl acetate; **C12**, Isolicoflavonol; **C13**, Pseudobaptigenin; **C14**, Formononetin; **C15**, 3',4',5,7-Tetrahydroxy isoflavanone; **C16**, Glycitein)

Alternatively, PSBPs can be produced through the hydrolization of NEPs such as cell wall decomposition during germination (Shahidi and Yeo, 2016).

As can be seen, peak areas of PSBPs increased during chickpea germination based on the DAD results at 260 nm (Figure 5.1B). Peaks were further investigated with LC-ESI-QTOF-MS (Table 5.1). Most of the identified PSBPs increased, such as 4-hydroxy-8-methoxy-2H-fruo[2,3-h]-1-benzopyran-2-one (**C3**), afzelechin (**C5**), hesperetin (**C9**), 7,3',4'-trihydroxyflavone (**C7**), 8-hydroxydihydrodaidzein (**C4**), prunetin (**C6**), pseudobaptigenin (**C13**), formononetin (**C14**), 3',4',5,7-tetrahydroxy isoflavanone (**C15**), glycitein (**C16**), and gentisic acid (**C2**).

On the other hand, PSBPs can decrease due to metabolic activity, which can either be broken down into SFPs or converted into NEPs. For instance, the content of 6-hydroxydaidzein (**C8**) in soluble bound form decreased and it may decompose into the free form based on its increase in SFP form (Table 5.2). In contrast, isolicoflavonol (**C12**) and 1-naphthyl acetate (**C11**) may be transformed into their NEPs form rather than SFP form, as little of them were detected in the extracts of SFPs.

Principal component analysis (PCA) were performed for the characteristic of phenolic compounds composition of chickpea (Figure 5.2). CB0 and CB2 had the opposite influence on phenolic compounds composition as compared with CB4 and CB6, based on their locations on principal component 1 (PC1) and PC2 score plot. This suggests that the composition of PSBPs changes significantly after 2 days of germination. As CB4 and CB6 were located further to the right of PC1 while to the middle of PC2, PC1 was the dominant component rather than PC2. Gentisic acid (**C2**), 7,3',4'-trihydroxyflavone (**C7**), and 1-naphthyl acetate (**C11**) were heavily

loaded on PC1, which means they should be the major phenolic components of CB4 and CB6. In regard to the previous study, the antioxidant activity of soluble bound phenolic compounds increased with germination time both in *in vitro* assay and in oil-in-water emulsions (Xu et al., 2018c). DPPH radical scavenging and ORAC value of soluble bound phenolic compounds increased from 45.7 μmol Trolox equivalence (TE)/L and 0.61 μmol TE/mL at 0 day to 75.7 μmol TE/mL and 1.51 μmol TE/mL at 6 days of germination, respectively. CB4 and CB6 could also retard lipid oxidation of stripped soybean oil-in-water emulsion to 12 and 14 days, respectively. The increased contents of gentisic acid (**C2**) and 7,3',4'-trihydroxyflavone (**C7**) during germination, potentially indicate that these compounds might be the principal phenolic compounds contributing to the improved antioxidant activity of CB4 and CB6.

Gentisic acid (**C2**) and 7,3',4'-trihydroxyflavone (**C7**) are both effective antioxidants. Villaño et al. (2007) reported that gentisic acid had a lower IC50 (the amount of antioxidant needed to decrease the DPPH radical concentration by 50%) than protocatechuic acid, siringic acid, caffeic acid, caftaric acid, and ferulic acid. Jung et al. (2003) reported that 7,3',4'-trihydroxyflavone had a 2.20 μM IC50 value in a DPPH model, lower than L-ascorbic acid (12.78 μM). In addition, 7,3',4'-trihydroxyflavone, the major active constituent extracted from *Albizzia julibrissin* bark by ethyl acetate, could quench 58.71 % of hydroxyl radicals in a model system, which was higher than the same amount of L-Ascorbic acid (Jung et al., 2003).

It should be pointed out that there might still have other unknown phenolic compounds or the interactions among the components in the extracts that may contribute to the overall antioxidant activity of the extracts.

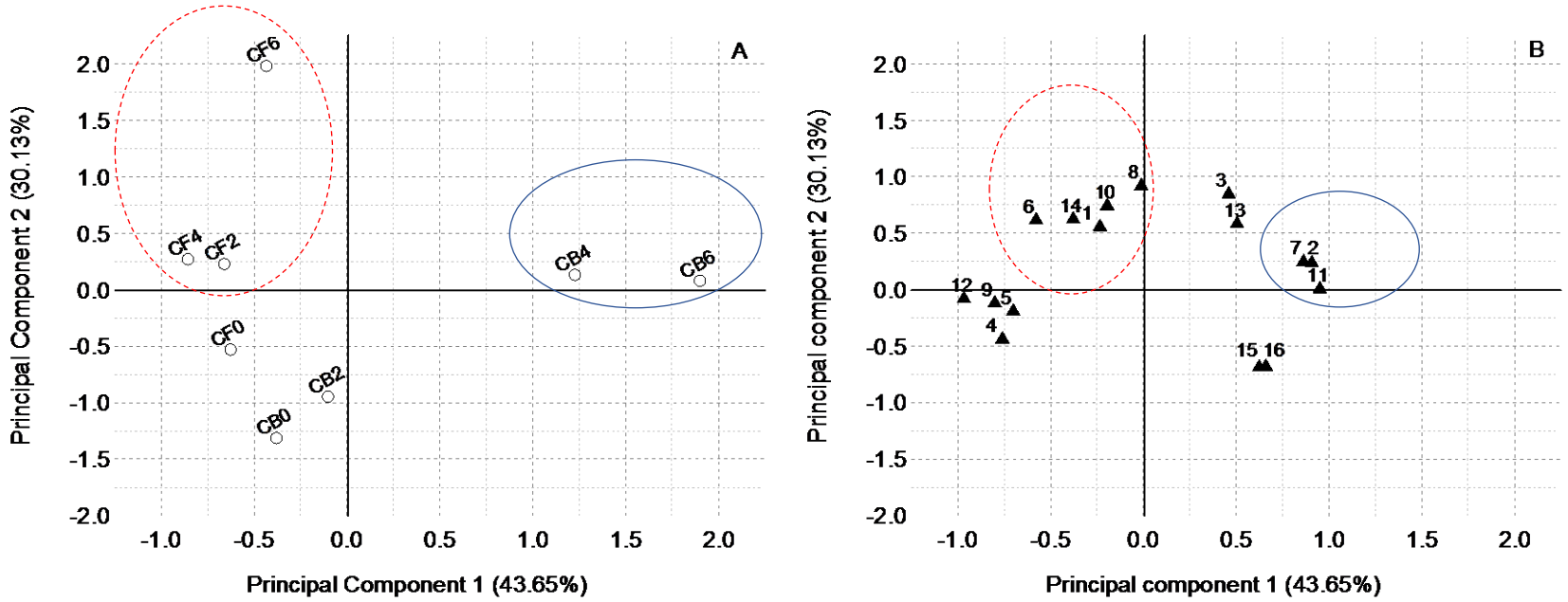

Figure 5.2. PCA (A) score plot of chickpea extracts, and (B) loading plot of phenolic compounds during chickpea germination (CF and CB denoting soluble free and soluble bound phenolic compounds at 0, 2, 4, and 6 days germination time; numbers indicating the different phenolic compounds: **C1**, Protocatechuic acid 4-O-glucoside; **C2**, Gentisic acid; **C3**, 4-Hydroxy-8-methoxy-2H-fruo[2,3-h]-1-benzopyran-2-one; **C4**, 8-Hydroxydihydrodaidzein; **C5**, Afzelechin; **C6**, Prunetin; **C7**, 7,3',4'-Trihydroxyflavone; **C8**, 6-Hydroxydaidzein; **C9**, Hesperetin; **C10**, Kaempferol; **C11**, 1-Naphthyl acetate; **C12**, Isolicoflavonol; **C13**, Pseudobaptigenin; **C14**, Formononetin; **C15**, 3',4',5,7-Tetrahydroxy isoflavanone; **C16**, Glycitein)

5.4.1.2. Effect of germination on phenolic composition of yellow pea extracts

5.4.1.2.1. Identification of phenolic compounds of yellow pea extracts

Sixteen phenolic compounds, including 1 phenolic acid, 2 chalcones, and 13 flavonoids, were identified in yellow pea extracts based on their *m/z* values in conjunction with product ions (Table 5.3). In fact, more than 16 compounds may be present in yellow pea extracts as indicated by the UV spectrum; the phenolic compounds reported here are restricted to the availability of databases and the fragments we observed. Significant variations in individual phenolic compounds were identified in both SFPs and PSBP forms during yellow pea germination.

Phenolic acids widely exist in all kinds of pulse seeds. Shikimate pathway and acetate pathway are two main metabolic pathways that are responsible for the biosynthesis of phenolic acids, which can be classified as benzoic acid and cinnamic acid (Velderrain-Rodríguez et al., 2014). *p*-Salicylic acid (**Y10**) in the SFPs decreased significantly, while it remained consistently high in PSBPs during yellow pea germination (Table 5.4). *p*-Salicylic acid (**Y10**) is a benzoic acid that has been reported in wheat, barley, sorghum, (Wang et al., 2014) and pulse seeds (Abu-Reidah et al., 2014). It is a key phytohormone for inducing the expression of the gene encoding phenylalanine ammonia-lyase (PAL) (He et al., 2010). The precursor of chalcones is coumaric acid that can be synthesized by PAL from phenylalanine (Mierziak et al., 2014). Chalconaringenin (**Y1**) and phloridzin (**Y4**), a glucoside of phloretin, are two chalcones identified in the germinated yellow pea extracts with LC-QTOF-MS. Both chalconaringenin (**Y1**) and phloridzin (**Y4**) increased in the forms of SFPs and PSBPs during yellow pea germination (Table 5.4). Chalcones such as phloretin and chalconaringenin (**Y1**) can

Table 5.3. Phenolic compounds profiles of yellow pea extracts identified by LC-ESI-QTOF-MS

Peak No.	RT (min)	Collision energy (ev)	Observed *m/z* $[M-H]^-$	Calculated *m/z* $[M-H]^-$	Molecular formula	Diff. (ppm)	Product ions	Proposed compounds	Class
Y1	15.32	10	271.0619	271.0612	$C_{15}H_{12}O_5$	-2.70	109.0166, 125.0098	chalconaringenin	chalcone
Y2	16.03	25	299.0584	299.0561	$C_{16}H_{12}O_6$	-7.62	131.0348, 147.0276, 159.0263, 201.0320, 241.0616	pratensein	isoflavone
Y3	16.89	25	273.0777	273.0768	$C_{15}H_{14}O_5$	-3.16	109.0166, 121.0151, 137.0082	(2*s*,3*s*,4*r*)-3,4,4',7-tetrahydroxyflavan	flavan
Y4	17.31	10	435.1314	435.1297	$C_{21}H_{24}O_{10}$	-3.96	273.0768, 167.0350	phloridzin	chalcone
Y5	17.44	25	305.0676	305.0667	$C_{15}H_{14}O_7$	-3.17	110.9956, 125.0094, 281.0104	epigallocatechin	flavan
Y6	17.65	25	287.0577	287.0561	$C_{15}H_{12}O_6$	-5.6	107.0016, 121.0154, 150.9858	aromadendrin	flavanonol
Y7	18.80	30	303.0869	303.0874	$C_{16}H_{16}O_6$	1.85	161.0429, 217.0263, 245.0175	arachidoside	flavan
Y8	19.42	10	285.0413	285.0405	$C_{15}H_{10}O_6$	-4.71	108.0090, 117.0205, 131.0507, 227.0081	kaempferol	flavonol
Y9	21.16	10	301.0365	301.0354	$C_{15}H_{10}O_7$	-3.75	109.0174, 134.9938, 216.9664	quercetin	flavonol
Y10	22.56	10	137.0245	137.0244	$C_7H_6O_3$	-0.68	93.0346, 65.0397	*p*-salicylic acid	phenolic acid
Y11	23.40	10	372.5577	372.5562	$C_{20}H_{20}O_7$	-4.03	255.0656, 135.0081	tangeretin	flavone
Y12	24.78	30	297.0777	297.0768	$C_{17}H_{14}O_5$	-2.99	116.9952, 130.9928, 199.0765, 225.0545, 239.0354	sayanedine	isoflavone
Y13	26.90	10	301.0734	301.0718	$C_{16}H_{14}O_6$	-5.29	286.0483, 242.0585	hesperetin	flavanone
Y14	27.06	30	297.0395	297.0405	$C_{16}H_{10}O_6$	3.10	183.0129, 255.0294	glyzaglabrin	isoflavone
Y15	31.40	10	255.0671	255.0663	$C_{15}H_{12}O_4$	-2.62	213.0557	pinocembrin	flavanone
Y16	33.47	10	283.0633	283.0612	$C_{16}H_{12}O_5$	-7.38	268.0377	genkwanin	flavone

RT, retention time; Diff., difference between calculated *m/z* and observed *m/z*.

Table 5.4. Dynamic changes of proposed phenolic compounds during yellow pea germination

Peak No.	Proposed compounds	Class	YF0	YF2	YF4	YF6	YB0	YB2	YB4	YB6
Y1	chalconaringenin	chalcone	0.13±0.01 ab	0.61±0.04 c	1.44±0.24 d	0.49±0.05 bc	0.05±0.00 a	0.25±0.02 abc	0.57±0.01 c	1.42±0.15 d
Y2	pratensein	isoflavone	0.05±0.00 a	0.16±0.02 a	0.30±0.11 a	3.00±0.11 d	1.37±0.02 c	0.65±0.13 b	0.43±0.03 a	0.17±0.02 a
Y3	(2*S*,3*S*,4*R*)-3,4,4',7-tetrahydroxyflavan	flavan	0.01±0.00 a	0.22±0.06 a	1.32±0.28 b	2.22±0.58 b	N.A.	0.36±0.02 a	1.31±0.14 b	1.83±0.05 b
Y4	phloridzin	chalcone	0.01±0.00 a	0.14±0.01 a	1.88±0.56 b	3.42±0.26 b	0.01±0.00 a	0.01±0.00 a	0.12±0.02 a	0.14±0.00 a
Y5	epigallocatechin	flavan	N.A.	0.02±0.00 a	0.27±0.04 b	0.36±0.03 bc	1.11±0.09 e	0.65±0.12 d	0.51±0.01 cd	0.42±0.02 bc
Y6	aromadendrin	flavanonol	1.35±0.2 cd	1.33±0.05 cd	1.21±0.01 bcd	0.66±0.02 a	0.91±0.03 ab	1.07±0.09 bc	1.40±0.01 d	1.43±0.00 d
Y7	arachidoside	flavan	0.02±0.01 a	0.14±0.01 ab	0.36±0.04 c	0.59±0.00 d	0.83±0.05 e	0.96±0.08 e	0.86±0.10 e	0.3±0.04 bc
Y8	kaempferol	flavonol	0.94±0.03 b	0.12±0.00 a	0.01±0.00 a	0.01±0.01 a	2.55±0.05 e	1.86±0.06 d	1.36±0.17 c	0.74±0.03 b
Y9	quercetin	flavonol	0.10±0.00 a	0.49±0.04 c	0.68±0.03 d	1.09±0.04 e	0.11±0.01 a	0.11±0.02 a	0.22±0.02 b	0.30±0.01 b
Y10	*p*-salicylic acid	phenolic acid	1.59±0.08 d	0.11±0.02 a	0.04±0.01 a	0.04±0.01 a	0.60±0.01 b	0.83±0.01 c	0.56±0.04 b	0.54±0.03 b
Y11	tangeretin	flavone	0.01±0.01 a	0.02±0.00 a	0.03±0.00 a	0.04±0.01 a	0.68±0.05 c	0.29±0.08 ab	0.51±0.15 bc	0.60±0.13 c
Y12	sayanedine	isoflavone	N.A.	0.02±0.00 a	0.14±0.03 a	0.32±0.00 b	1.46±0.18 d	1.22±0.04 d	0.78±0.04 c	0.32±0.02 b
Y13	hesperetin	flavanone	0.05±0.00 a	2.83±0.10 b	7.89±1.09 c	12.86±0.79 d	0.01±0.00 a	0.01±0.00 a	0.05±0.00 a	0.06±0.00 a
Y14	glyzaglabrin	isoflavone	1.27±0.22 b	1.65±0.23 bc	2.07±0.31 c	3.74±0.20 d	0.01±0.00 a	N.A.	0.01±0.00 a	N.A.
Y15	pinocembrin	flavanone	0.41±0.02 b	0.36±0.09 b	0.82±0.04 c	3.22±0.06 d	0.01±0.00 a	N.A.	N.A.	N.A.
Y16	genkwanin	flavone	6.29±0.99 c	2.75±0.68 b	1.42±0.24 ab	0.14±0.03 a	N.A.	N.A.	N.A.	0.01±0.00 a

YF and YB denote SFPs and PSBPs extracted from germinated yellow pea, respectively, followed by the different germination times (0, 2, 4, or 6 days). The amounts of phenolic compounds were expressed by absolute area of extracted ion chromatography (EIC) with the unit $\times 10^6$ counts; N.A., absolute area was below 10000 counts. Different letters indicate statistically significant intraspecies differences ($p<0.05$).

spontaneously cyclize to flavanones. Flavanones can be modified into isoflavanone, flavone, flavanol, and anthocyanidins by enzymes (Moss, 2018). These flavonoids are closely related to the color, fragrance, and taste of the pulse seeds (Mierziak et al., 2014). Totally, 1 flavanonol, 2 flavonols, 2 flavones, 2 flavanones, 3 isoflavans, and 3 isoflavones were identified.

5.4.1.2.2. Chemometric analysis of phenolic compounds

In the present study, constellation plot-cluster analysis (CPCA) and principal component analysis (PCA) were performed to statistically analyze the impact of germination time on the compositional changes of phenolic compounds in yellow pea. According to the results of constellation plot-cluster analysis (CPCA), phenolic compounds were separated into 6 groups at the distance 3.939 on account of the variation in the forms of SFPs and PSBPs during yellow pea germination (Figure 5.3 & Table 5.4). Cluster I, which involved pratensein (**Y2**), epigallocatechin (**Y5**), arachidoside (**Y7**), *p*-salicylic acid (**Y10**), tangeretin (**Y11**), and sayanedine (**Y12**), located at the bottom of Y-axis. The amounts of these phenolic compounds were higher in PSBPs than these in SFPs. Nevertheless, their concentration decreased over the course of germination. Cluster II was kaempferol (**Y8**) that decreased in both SFPs and PSBPs. Cluster III, involving chalconaringenin (**Y1**), (2*S*,3*S*,4*R*)-3,4,4',7-tetrahydroxyflavan (**Y3**), and aromadendrin (**Y6**), presented both in SFPs and PSBPs forms and had a significant increase in PSBPs form during yellow pea germination. Hesperetin (**Y13**), the only phenolic compound located in Cluster IV, raised sharply in SFPs, while increased subtly in PSBPs during germination. Genkwanin (**Y16**) presented in Cluster V in SFPs form and had a significant decrease during germination. Cluster VI, involving phloridzin (**Y4**), quercetin (**Y9**), glyzaglabrin

(**Y14**), and pinocembrin (**Y15**), located at the top of the Y axis, which was low in PSBP form while had an increased content of SFPs form during germination. Researchers have studied the variation of individual phenolic compounds in germinated pulse seeds. Wu et al. (2012) reported an increase of soluble free pratensein (**Y2**) in germinated chickpea. López-Amorós et al. (2006) declared that soluble quercetin (**Y9**) increased from 0 to 291−311 μg/100 g in germinated beans. Epigallocatechin (**Y5**), kaempferol (**Y8**), and pinocembrin (**Y15**) had been identified in the seed coat of pea (Amarowicz and Pegg, 2008; Stanisavljević et al., 2015). However, none of these studies separately evaluated the antioxidant activities of polar solvent extractable phenolic compounds (i.e., SFPs and PSBPs) in food systems.

Principal component analysis (PCA) was performed to further analyze the relationship between phenolic clusters and germination treatment. The principal component 1 (PC1) and principal component 2 (PC2) explained 48.2% and 30.3% of the total variance in the data set, respectively (Figure 5.4). SFPs and PSBPs can be well differentiated based on PC1 and PC2. PSBPs located in the second and third quadrant, while SFPs located in the first, third, and fourth quadrants.

SFPs varied from negative regions to positive regions of PC1 and PC2 with 6 days of germination. Phenolic compounds involving in Cluster V played important roles in the SFPs from raw yellow pea (YF0), while Cluster IV and Cluster VI involved for YF6 (Figure 5.4). In accordance to the greater antioxidant activities of YF6 than that of YF0 in SSO-in-water emulsions (Figures 4.8 and 4.9), it could be speculated that phenolic compounds in Cluster IV and Cluster VI have better antioxidant activities than those in Cluster V. Unlike SFP form,

PSBPs in raw yellow pea varied from the second quadrant to zero point after 6 days of germination (Figure 5.4A). By combining the CPCA with the loading plot (Figure 5.4B), it was

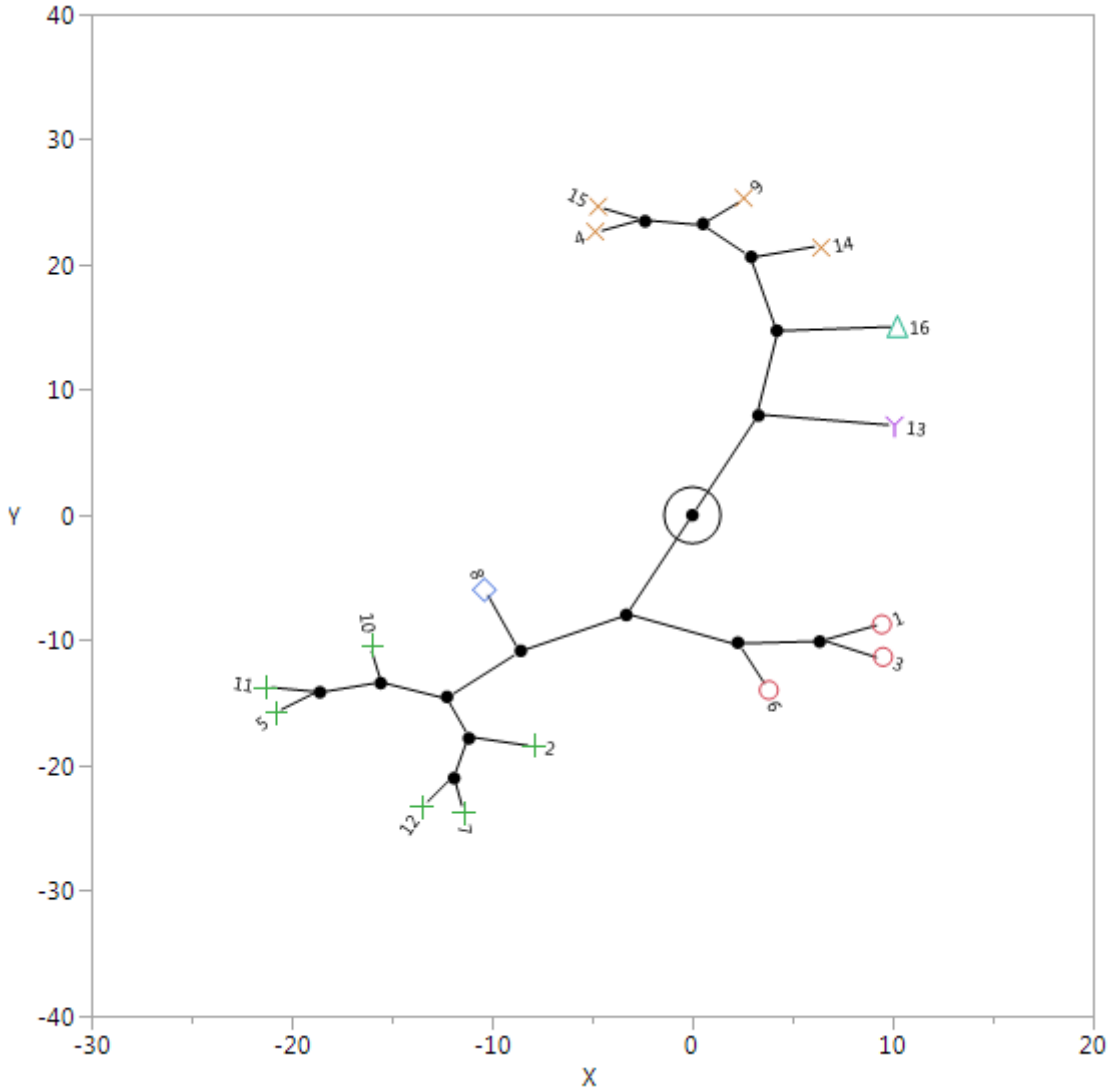

Figure 5.3. Constellation plot-cluster analysis (CPCA) representing the relation between eighteen phenolic compounds.
Six clusters were separated based on the distance 3.939 with the different symbols: cluster I +, II ◇, III ○, IV Y, V △, and VI ×. Numbers indicate the different phenolic compounds: chalconaringenin (**Y1**), pratensein (**Y2**), (2*S*,3*S*,4*R*)-3,4,4',7-tetrahydroxyflavan (**Y3**), phloridzin (**Y4**), epigallocatechin (**Y5**), aromadendrin (**Y6**), arachidoside (**Y7**), kaempferol (**Y8**), quercetin (**Y9**), *p*-salicylic acid (**Y10**), tangeretin (**Y11**), sayanedine (**Y12**), hesperetin (**Y13**), glyzaglabrin (**Y14**), pinocembrin (**Y15**), and genkwanin (**Y16**).

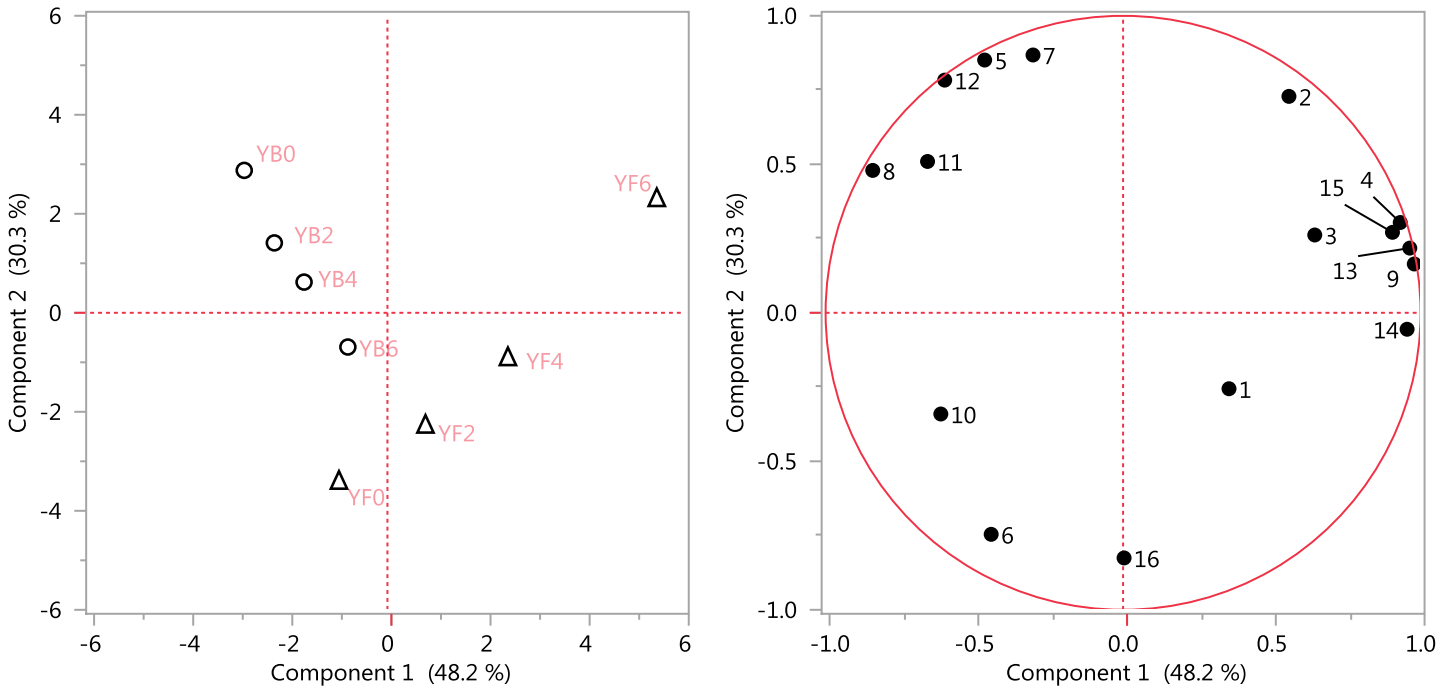

Figure 5.4. Principal component analysis (PCA) (A) score plot and (B) loading plot of phenolic compounds during chickpea germination.

YF and YB denoting SFPs and PSBPs after 0, 2, 4, and 6 days of germination; numbers indicate the different phenolic compounds: chalconaringenin (**Y1**), pratensein (**Y2**), (2*S*,3*S*,4*R*)-3,4,4',7-tetrahydroxyflavan (**Y3**), phloridzin (**Y4**), epigallocatechin (**Y5**), aromadendrin (**Y6**), arachidoside (**Y7**), kaempferol (**Y8**), quercetin (**Y9**), *p*-salicylic acid (**Y10**), tangeretin (**Y11**), sayanedine (**Y12**), hesperetin (**Y13**), glyzaglabrin (**Y14**), pinocembrin (**Y15**), and genkwanin (**Y16**).

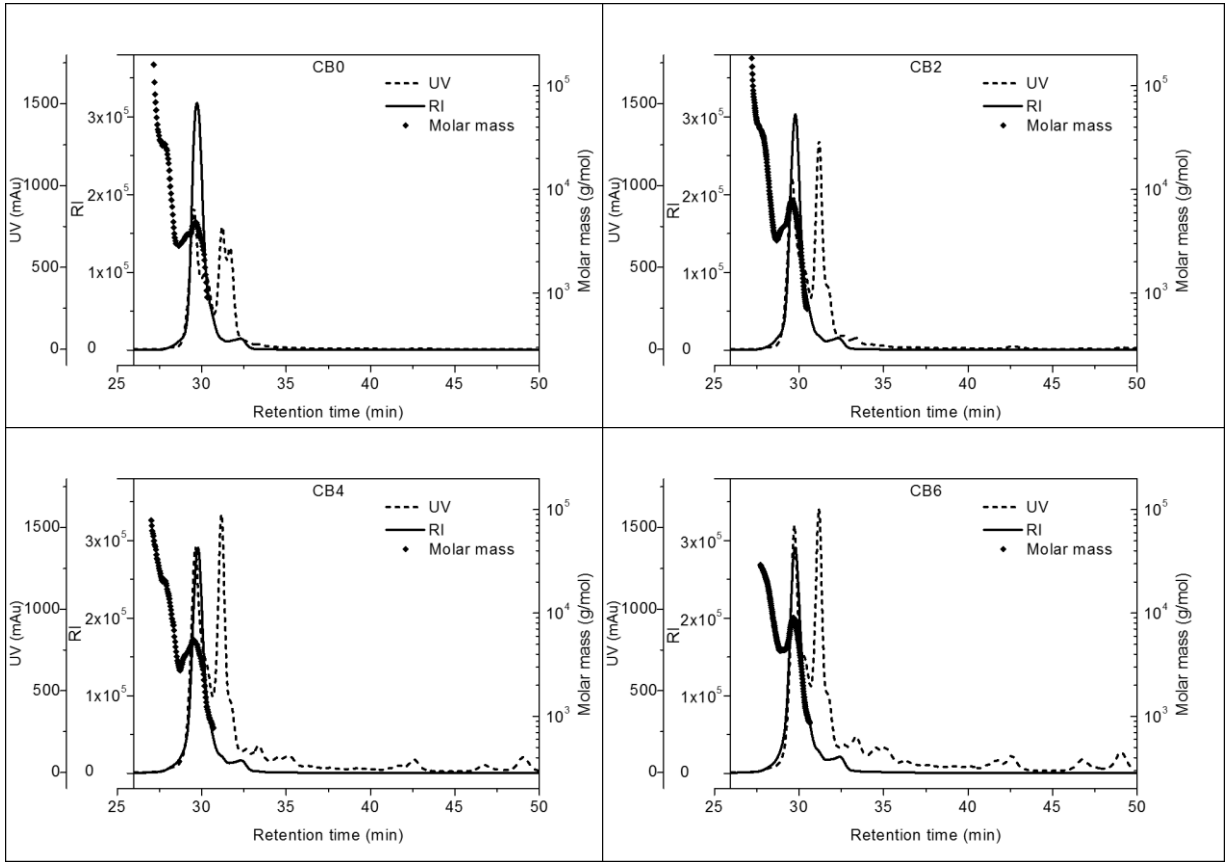

Figure 5.5. Physical characteristics of PSBPs dynamically changed during chickpea germination.
All soluble bound phenolic extractions were separated with SEC column and monitored with DAD at 260 nm, refraction index detector, and multi-angle laser light scattering detector

5.4.2. Effect of germination time on molar mass of PSBPs in pulse seeds

5.4.2.1. Effect of germination time on the molar mass of PSBPs in chickpea

SEC-MALS-RI was used to further study molecular mass change of PSPB during chickpea germination. Size exclusion chromatography separates solutes based on the particle size of compounds: large particle size elutes out faster than small particle size. As the UV results (260 nm) shown in Figure 5.5, peak area with larger particle size (RT 27-32 min) increased with germination time. In addition, smaller particle size peaks were generated during 4 to 6 days of germination. This phenomenon was attributed to the biosynthesis of PSBPs.

The detection principle of RI detector involves measuring of the change in refractive index of the column effluent passing through the detector. The main peak shown in Figure 5.5 (RT 27-31 min) elucidated that most of the solutes of soluble bound phenolic compounds are eluted out with the first peak. Similar results were reported in the fractionation of lentil extracts with SEC method (Amarowicz and Karamać, 2003). Average molar mass of the first peak in different germination time was evaluated by the combination of RI and MALS detector (Table 5.5). Results indicated the increase of both number average molecular weight (Mn) and weight average molecular weight (Mw) with germination time (Figure 5.5, Table 5.5). With 6 days germination, Mn and Mw of soluble bound phenolic compounds were statistically larger than that in any of the previous germination days.

With the previous study (Chapter 4), the antioxidant activity of PSBPs in oil-in-water emulsions increased during chickpea germination, while that of SFPs had little to no effect in the same emulsions. The molecular weight of PSBPs can be related to the antioxidant activity, as

PSBPs had an increased molar mass with germination time, while SFPs can be considered as phenolic compounds with much lower molecular weight compared to PSBPs.

Table 5.5. Molar mass of PSBPs extracted from raw and germinated chickpea

Germination time (days)	Mn (g/mol)	Mw (g/mol)	Mw/Mn
0	4003±142 [a]	4830±54 [a]	1.21
2	4548±71 [a]	5258±115 [ab]	1.16
4	4213±170 [a]	4786±642 [a]	1.14
6	5219±43 [b]	6230±60 [bc]	1.19

* Data points represent mean (n=2) ± standard deviation. Different letters indicate intraspecies statistically significant differences ($p < 0.05$).

5.4.2.2. Effect of germination time on the molar mass of PSBPs in yellow pea

Opposite to our *in vitro* assays, the antioxidant activity of PSBPs in raw yellow pea was greater than that of SFPs in SSO-in-water emulsions as reported in Chapter 4. The phenolic composition of yellow pea extracts may partly explain their antioxidant activity. According to section 5.4.2.1, molar mass difference between PSBPs and SFPs might be another factor that affects the antioxidant activity of phenolic compounds from chickpea. Therefore, molar mass of major PSBPs in germinated yellow pea was investigated by means of SEC-MALS-RI (Figure 5.6).

PSBPs were separated with size exclusion chromatography based on the size of molecules. The major peak eluted out at 27-32 min were believed to have larger molecular weights. Concentrations of the phenolic compounds, quantified by UV and RI detectors, are the fundamental for the calculation of molar mass. The resonance of chemical bonds, such as hydroxyl group and phenyl ring, is responsible for the UV absorbance. The remarkable increase of UV signal (260 nm) from 0 to 4 days of germination implied that composition of PSBPs

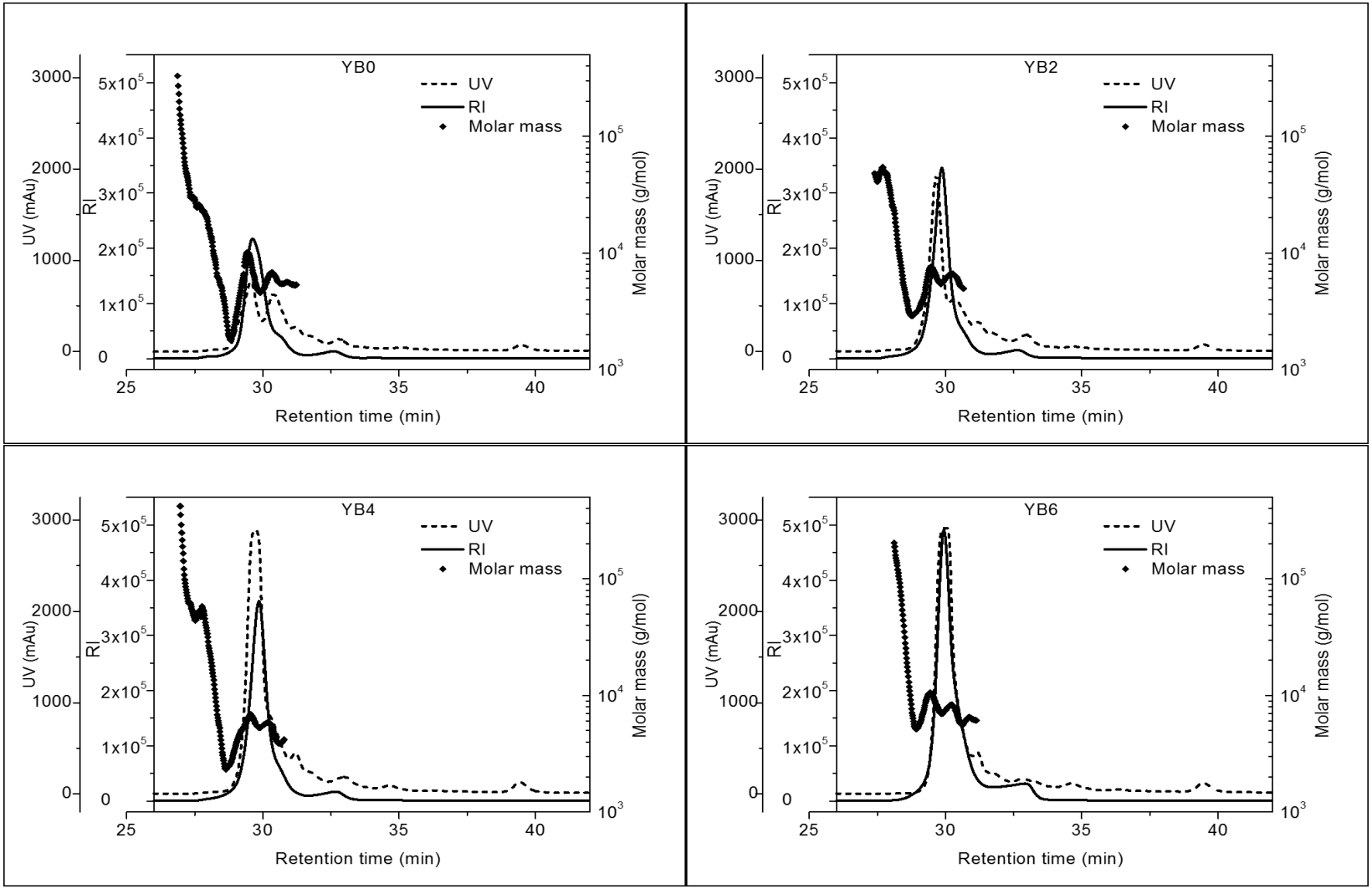

Figure 5.6. Physical characteristics of PSBPs dynamically changed during yellow pea germination.

changed substantially. The increased area of eluting peaks indicated that the type of PSBPs increased. This is most probably attributed to the biosynthesis of PSBPs originating from SFPs and/or the decomposition of NEPs over the course of yellow pea germination. RI detector is responsible for the concentration variation of solutes based on the refractive index d*n*/d*c*. As displayed in Figures 5.5 and 5.6, the major changes of RI value were on the peak eluting at 27-32 min. Area of RI peaks increased tremendously over the course of germination, which meant the concentration of the solutes had changed incremently during germination. The increase of total phenolic content (TPC) during yellow pea germination corresponded with the increase of RI peak areas.

Multiangle-laser-light-scattering (MALS) detector provides information for the molecular shape and absolute molar mass. With the detection of the molecules scattering light, coupled with the variation of molecule concentration determined by the RI detector, the instrument can calculate the absolute molar mass of molecules. Figure 5.6 and Table 5.6 provides the molar mass of major PSBPs during germination. Opposite to our previous finding of chickpea (Xu et al., 2018b), the germination decreased the molecular weight of PSBPs in yellow pea. Number average molecular weight (Mn) of major PSBPs in yellow pea dropped from 6722 ± 195 to 5271 ± 199 g/mol after 4 days of germination. Similarly, the weight average molecular weight (Mw) of major PSBPs in yellow pea declined from 7110 ± 69 to 6266 ± 14 g/mol after the same germination time. However, both Mn and Mw of PSBPs in yellow pea after 6 days of germination were statistically larger than those in the shorter germination periods. In addition, germination had no impact on the shapes of PSBPs as indicated by Mw/Mn. By correlating the

molar mass to the antioxidant activity of PSBPs in SSO-in-water emulsions, a similar variation trend was observed that both decreased from 0 to 4 days of germination and reversed after 6 days of germination.

5.4.3. Insight into the antioxidant activity of polar solvent extractable phenolic compounds in germinated pulse seeds.

Based on the results, protective and/or co-antioxidant principles (Figure 5.7) are proposed to explain the different antioxidant activities between soluble free and soluble bound phenolic compounds due to different molar mass.

It has been reported that moieties of PSBPs can protect phenolic compounds away from being oxidized (Qiu et al., 2017; Fan et al., 2018). Free radicals are generated gradually in emulsion system. Excessive phenolic compounds without protection, such as SFPs, can be rapidly oxidized and consumed by oxidants including radicals (Figure 5.7A); while PSBPs retain viability under the protection of moieties they attached against oxidants (Figure 5.7B). On the other side, free radicals were excessive at the very beginning of the *in vitro* assay (Figure 5.7C) and SFPs had higher efficacy than PSBPs (Xu et al., 2018c). This is because moieties of PSBPs may impede the proximity of active phenolic group and free radicals by steric hindrance (Figure 5.7D). Moreover, reaction time of *in vitro* assay is too short to release the whole antioxidative power of PSBPs. A well-known example for such protective mechanism is that dietary fiber can negatively affect the release and absorption of phenolic molecules (Quirós-Sauceda et al., 2014).

In addition, moieties of PSBPs can either be antioxidants or pro-antioxidants that may impose synergistic effect with phenolic compounds. Most of the moieties that phenolic

compounds conjugated were proposed as polysaccharides and alcohol soluble proteins. Many researchers reported that crude polysaccharides and protein possess potential free radical scavenging capability (Sun et al., 2005, 2010; Zhao et al., 2012; Fan et al., 2014; Wu et al., 2014). Soluble polysaccharides may donate hydrogen to the oxidized phenolic compounds with their activated reducing ends. Consequently, the durable antioxidant activity of PSBPs may be caused by the synergism of phenolic compounds and the moieties they attached.

An interesting finding on the opposite antioxidant efficacy of phenolic compounds from germinated chickpea and yellow pea against emulsion oxidation may be related to their different molecular weight changes during germination. Larger molecular weight of moieties may exert greater protection effect and synergistic effect with the phenolic compounds attached. Thus, PSBPs extracted from germinated chickpea had higher antioxidant activity than that from ungerminated chickpea, while PSBPs extracted from ungerminated yellow pea had higher antioxidant activity than that from germinated yellow pea (2 and 4 days). Nevertheless, the increased molecular weight of PSBPs after 6 days of yellow pea germination (Figure 5.6 and Table 5.6) did not follow such speculation in a review of their antioxidant activity in emulsions since it was still inferior to those extracted from the raw yellow pea. This indicates that molecular weight of PSBPs is not a sole factor to regulate the antioxidant activity; the composition of phenolic compounds also matters. Numerous researchers have found that phenolic composition and concentration are crucial to their antioxidant activity (Helmja, 2010; Shahidi and Ambigaipalan, 2015; Shahidi and Yeo, 2016). With the germination, the improved antioxidant activities of SFPs in yellow pea against SSO-in-water emulsions oxidation is

Table 5.6. Molar mass of PSBPs extracted from raw and germinated yellow pea

germination time (days)	Mn (g/mol)	Mw (g/mol)	Mw/Mn
0	6722±195 bc	7110±69 b	1.06
2	6095±117 b	6879±65 ab	1.13
4	5271±199 a	6266±14 a	1.19
6	7353±112 c	8574±546 c	1.17

*Data points represent means (n =2) ± standard deviations. Different letters indicate statistically significant intraspecies differences ($p < 0.05$). Mn, number average molecular weight; Mw, weight average molecular weight.

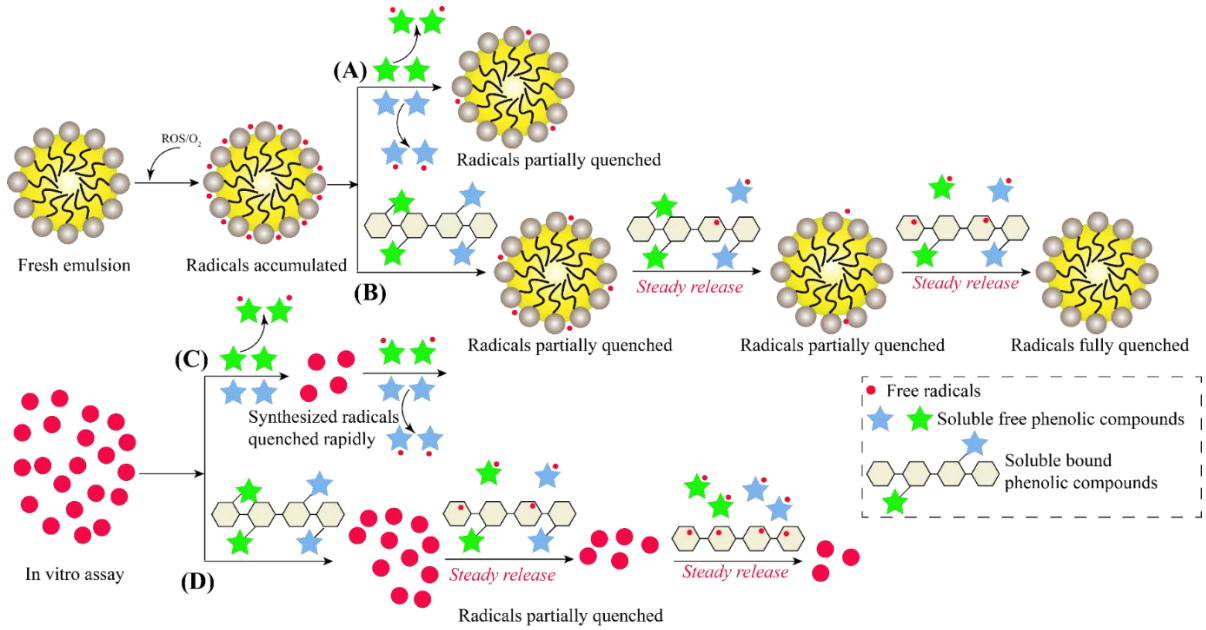

Figure 5.7. Schematic diagram for the hypothesized antioxidative mechanism of polar solvent extractable phenolic compounds extracted from germinated chickpea and yellow pea in the *in vitro* assays and oil-in-water-emulsion system

accompanied by the increased amounts of crucial phenolic compounds such as pratensein (**Y2**), phloridzin (**Y4**), quercetin (**Y9**), sayanedine (**Y12**), hesperetin (**Y13**), glyzaglabrin (**Y14**), and pinocembrin (**Y15**) (in Cluster I, IV, and VI). It is plausible that the composition of phenolic compounds is also responsible for the antioxidant activity of polar soluble bound compounds in SSO-in-water emulsions. The decrease of pivotal phenolic compounds, e.g., pratensein (**Y2**) and

sayanedine (**Y12**) in Cluster I, may be responsible for their attenuation of antioxidant activity. Such phenolic composition changes after 6 days of germination in yellow pea overruns the protective or the dual antioxidant effect stemming from the increased molecular weight of moieties, which explains its relatively poorer antioxidant activity.

5.5. Conclusion

The data indicate that both phenolic composition and the nature of the moieties phenolic compounds attached are critical to determine their antioxidant activity against lipid oxidation in emulsions. Surprisingly, molecular weight is positively related to the antioxidant activity of PSBPs. Synergistic effect between PSBPs and their moieties is proposed to explain the variation of antioxidant activity following germination. This research shed new light on the development of antioxidants with effectiveness, safety, and sustainability.

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CHAPTER 6. OVERALL SUMMARY AND CONCLUSION

6.1. Conclusions

In summary, various attributes of pulse seeds, such as proximate composition, physicochemical properties, and phenolic profiles were improved by germination. Germination is a promising process to improve the attributes and nutrients of pulse seeds to make them into food ingredients by control of germination time.

Germination improved the proximate composition of pulse seeds with an increase in crude protein, while decreases in total carbohydrate and crude lipids. The infra-structure of proximate composition contributed to the varied physicochemical properties, with changed pasting properties, thermal properties, moisture sorption isotherms, enzyme activities, and taste traits of pulse flours. Control of germination time could favor the optimal characteristics of food ingredients from raw pulse seeds.

On the other hand, the qualities of phenolic compounds affected by the germination of pulse seeds. The SFPs qualities improved by germination in chickpea, lentil, and yellow pea in *in vitro* assay. However, the antioxidant activities of SFPs is much lower than that of PSBPs in SSO-in-water emulsion system, which is the typical oil-contained food system. Interestingly, the qualities of PSBPs have different variation after germination based on the different pulse species. We observed increase, little effect, and decrease of antioxidant activities of PSBPs by germination of chickpea, lentil, and yellow pea, respectively, based on their performance in the SSO-in-water emulsion systems. The antioxidant activities of PSBPs are related to both phenolic composition and molar mass of whole molecules evidenced by LC-ESI-QTOF-MS and SEC-

MALS-RI, respectively. Several phenolic compounds played key roles in PSBPs of chickpea and yellow pea elucidated by chemometric analysis. In addition, moieties of PSBPs had both protective and co-antioxidative effect on the key phenolic compounds.

6.2. Future Research

In the future, my research plan will include, (1) developing new antioxidants by the conjugation of phenolic compounds and protein/polysaccharide; and (2) improving the pulse attributes by the combination of fermentation and germination;

6.2.1. Development of new antioxidants by the conjugation of phenolic compounds and protein/polysaccharide.

Our previous research found that soluble bound phenolic compounds can retard lipid oxidation three times longer than soluble free phenolic compounds in oil-in-water emulsion system. Both key phenolic composition and functional moieties, mainly proteins and polysaccharides, are evidenced to be responsible for the higher antioxidative activity of soluble bound phenolic compounds in the oil-in-water emulsion system. Therefore, my future research aims to develop a new type of antioxidant based on the conjugation of phenolic compounds and proteins/polysaccharides. There are three objectives: (1) to conjugate phenolic compounds with protein/polysaccharide via non-covalent bonds; (2) to conjugate phenolic compounds with protein/polysaccharides with covalent bonds; (3) to evaluate the antioxidative activity of protein-polyphenol and polysaccharide-polyphenol in the real food systems. This research could refresh our knowledge about synthetic phenolic compounds. The new antioxidants are anticipated to replace the traditional synthetic phenolic compounds originated from petroleum.

6.2.2. Improvement of grain attributes by the combination of fermentation and germination

The utilization of grains as food ingredients presents three types of challenges. First are technological challenges in baking properties, such as reduced loaf volume, increased dough stickiness, product hardness, and chewiness. Second are nutritional challenges related to the amino acid profile and antinutritional factors. Third, there are sensory challenges, such as beany or bitter flavor profiles. Fermentation is one of the oldest biotechnological processes where both beer and bread take benefit from the metabolism of yeasts and/or lactic acid bacteria. Fermentation has several advantages to improve the attributes of grains: 1, an effective process to improve loaf volume and crumb softness made by wheat or rye; 2, activates endogenous enzyme to degrade antinutritional factors; 3, an effective process to improve the aroma profiles of grains and to mask the off-odor; 4, fermented grains may influence gut health with special nutrients, such as dietary fiber complex, exopolysaccharides, and metabolites which influent gut microbiota. However, fermentation is a complicated process, which need precise control of all the factors for desired fermentation products. Most of the researchers focus on the species of microbes rather than the quality of grains in the fermentation process. Germination of grains prior to fermentation is a feasible process to improve the quality of fermentation products. First of all, germination of grains could accelerate the fermentation procedure by activation of enzymes. In addition, germination can release more nutrients for the growth of microbes. One of the classical examples that combining germination and fermentation is beer brewing. The starch of malt is converted to simple sugars (glucose, maltose, and maltotriose) by the malt amylases

during germination and converted into ethanol by yeast during fermentation. Malt serves as both a source of enzymes and a source of fermentable during beer brewing. This project has three objectives: (1) optimization of the germination conditions to obtain the desired characteristics of grains for fermentation; (2) effect of yeast (*Saccharomyces*), bacteria (*Lactobacillus plantarum*), and mold (*Aspergillus niger*) on the nutritional improvement of grains; and (3) improvement of food textures by adding the germinated and fermented grain flours. With the control of parameters during germination and fermentation, grains are expected to gain desirable textures and physicochemical properties as neo-developed food ingredients.