A Quantitative Assessment of the Anti-Nutritional Properties of Canadian Pulses

by

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ABSTRACT

This study has assessed the effects of pulse type and processing (soaking and cooking) on antinutritional factors (α-amylase inhibitor, trypsin inhibitor, chymotrypsin inhibitor, lectins, phytic acid and oxalates) in a wide range of Canadian pulses, using soybean as a control. The contents of these antinutrients in Canadian pulses varied widely, but the levels were generally lower than those found in soybean. Analysis of variance indicated that both pulse type and processing had significant effects ($P < 0.0001$) on the investigated seeds. Soaking markedly decreased the contents of α-amylase inhibitor, trypsin inhibitor, chymotrypsin inhibitor, lectins and oxalates, but had no impact on phytic acid. Cooking of presoaked seeds appeared to be more effective; all proteinaceous antinutrients (α-amylase inhibitor, trypsin inhibitor, chymotrypsin inhibitor and lectins) were decreased by 80 – 100%, and significant reductions in phytic acid content (11 – 39%) were observed for all pulses, except common beans and soybean.

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LIST OF ABBREVIATIONS

AAFC	Agriculture and Agri-Food Canada
AIA	α-Amylase Inhibitory Activity
AIU	α-Amylase Inhibitory Unit
ANOVA	Analysis of Variance
CHD	Coronary Heart Disease
CIA	Chymotrypsin Inhibitory Activity
CIU	Chymotrypsin Inhibitory Unit
CVD	Cardiovascular Disease
DF	Degrees of Freedom
DL-BAPNA	Na-Benzoyl-D,L-arginine 4-nitroanilide hydrochloride
FAO	Food and Agriculture Organization of the United Nations
GRAS	Generally Recognized As Safe
HU	Hemagglutinating Unit
IP6	Myo-inositol 1,2,3,4,5,6, hexakis-dihydrogen phosphate
LDL	Low-density Lipoprotein
MC	Moisture Content
RDI	Recommended Daily Intake
SAS	Statistical Analysis Software
TIA	Trypsin Inhibitory Activity
TIU	Trypsin Inhibitory Unit
USA	United States of America
USDA	United States Department of Agriculture
WHO	World Health Organization

1. INTRODUCTION

As plants typically grown in tropical, subtropical and temperate areas, legumes were firstly cultivated by human beings over 3000 years ago (Tiwari, Gowen, & McKenna, 2011; Tiwari, & Singh, 2012). During this cultivation history, a wide range of non-food benefits have been discovered from legumes due to their nitrogen-fixing abilities, and their resulting use as forage, feed or cover crops (Tiwari, & Singh, 2012). More importantly, legumes have ranked second worldwide in terms of staple foods following cereal grains (Tiwari et al., 2011; Tsao, Yu, & Shahidi, 2012). Pulses, also called grain legumes, are dry edible seeds harvested from leguminous plants (Bekkering, 2014; Tiwari et al., 2011; Tiwari, & Singh, 2012). The Food and Agriculture Organization (FAO) of the United Nations (1994) classifies pulses into 11 categories: dry beans, dry broad beans, dry peas, chickpea, dry cowpea, pigeon pea, lentil, bambara groundnut, vetch, lupins and minor pulses. Although groundnut and soybean belong to legumes, they are considered as oilseeds rather than pulses (Tiwari et al., 2011; Tiwari, & Singh, 2012; Wilson, 2008).

The total world production of pulses increased by 50% for the 25 years between 1980 and 2004, achieving about 60 million tonnes (Mt) in 2004 (FAO, 2005). In 2012, approximately 75 Mt pulses were produced globally for the first time in history (FAOSTAT, 2015). Developing countries contribute approximately 74% to global pulse production whereas developed countries are responsible for the remaining 26% (Tiwari, & Singh, 2012). As the largest pulse growing country in the

world, India contributes 25% of the total world pulse production including 25% of world chickpeas production (Majumdar, 2011; Singh, & Tripathi, 1998; Tiwari, & Singh, 2012). Canada is the largest producer of lentils and dried peas, while Brazil produces the largest amount of dry beans (Tiwari, & Singh, 2012). Other major pulse-producing countries, including China, Myanmar and Australia, also make significant contributions to world production (Majumdar, 2011; Tiwari, & Singh, 2012).

Pulses play significant roles for billions of people around the world since they are rich in proteins, carbohydrates, dietary fibers, minerals, vitamins, and phytochemicals (phenolic acid and anthocyanins) (FAO, 2005; Majumdar, 2011; Oomah et al., 2011; Tiwari, & Singh, 2012). Based on their high quality and low cost, pulse proteins have served as replacements for meat proteins in many developing countries; moreover, they are also good protein sources in some regions where consumption of animal proteins is limited or restricted because of religious factors (Tiwari, & Singh, 2012; Wolf, 1976). Recently, great attention has focused on pulses in developed countries as well, where they are considered as health foods (FAO, 2005). Pulses have relatively high lysine content, whereas cereals are low in lysine, so addition of pulses to cereal diets can improve overall nutritive value and provide a balanced amino acid intake (Majumdar, 2011; Tiwari, & Singh, 2012). Additionally, reduced risk of cardiovascular disease (CVD), diabetes, obesity and cancer are all associated with frequent pulse consumption (Oomah et al., 2011; Tiwari, & Singh, 2012).

However, the food applications of pulses can be limited due to the presence of antinutritional factors, such as enzyme inhibitors (trypsin, chymotrypsin and α-amylase inhibitors), lectins, phytic acid and phenolic compounds (Khokhar, & Apenten, 2009; Tiwari, & Singh, 2012; Udahogora, 2012). These compounds may lower the nutritive value of pulses (Morrow, 1991; Mubarak, 2005). In fact, nutritive value of foods primarily depends on bioavailability and utilization of proteins, carbohydrates, vitamins and minerals (Ramakrishna, Rani, & Rao, 2006). As a result, anything that limits bioavailability and utilization such as protease inhibitors and lectins may reduce the nutritional quality of the protein (Alonso, Orúe, & Marzo, 1998). Along the same lines, starch digestion is influenced by α-amylase inhibitor (Luo, & Xie, 2013) and the ability of phytic acid to bind trace minerals affects mineral absorption (Ramakrishna et al., 2006).

Bitterness and astringency in foods and beverages can result from phenolic compounds (Bravo, 1998), which can also limit pulse consumption. To improve nutritional and sensory quality of pulses, some food processing methods and treatments, including soaking, cooking, germination, fermentation, decortication, selective extraction, irradiation and enzymatic treatment, have been applied to significantly reduce or remove antinutritive and flavour-modifying compounds from pulses (Khokhar, & Apenten, 2009; Ramakrishna et al., 2006).

The overall objective of the present research was to quantify some major classes of antinutritional factors in Canadian pulses. Specific objectives were:

1) To provide a database of antinutritional factors (trypsin inhibitor, chymotrypsin

inhibitor, α-amylase inhibitor, lectins, phytic acid and oxalates) in peas, lentils, chickpeas, fava beans and edible beans.

2) To evaluate the effect of processing (soaking and cooking) on the antinutritional properties produced from all pulses, using soybean as a control.

2. LITERATURE REVIEW

2.1 Pulses

Pulses, also known as grain legumes, are dry edible seeds harvested from leguminous plants (Bekkering, 2014; Tiwari et al., 2011; Tiwari, & Singh, 2012).

2.1.1 Pulse production

Legumes are typically grown in tropical, subtropical and temperate areas and have been cultivated by human beings for over 3000 years (Tiwari et al., 2011; Tiwari, & Singh, 2012).

2.1.1.1 World situation

In the past decade (2004 - 2013), world pulse production has followed an increasing trend from 60 to 73 million tonnes (Mt). Globally, Asia contributed to the highest pulse production (45.3%), followed by Africa (20.9%), Americas (20.4%), Europe (10%), and then Oceania (3.3%). The top 5 pulse-producing countries in 2013 are shown in Figure 2.1. Other countries that have great contributions are Turkey, Australia, Pakistan, Brazil, Russia and USA. Data on production of commonly grown and consumed pulses in the year 2013 is presented in Table 2.1.

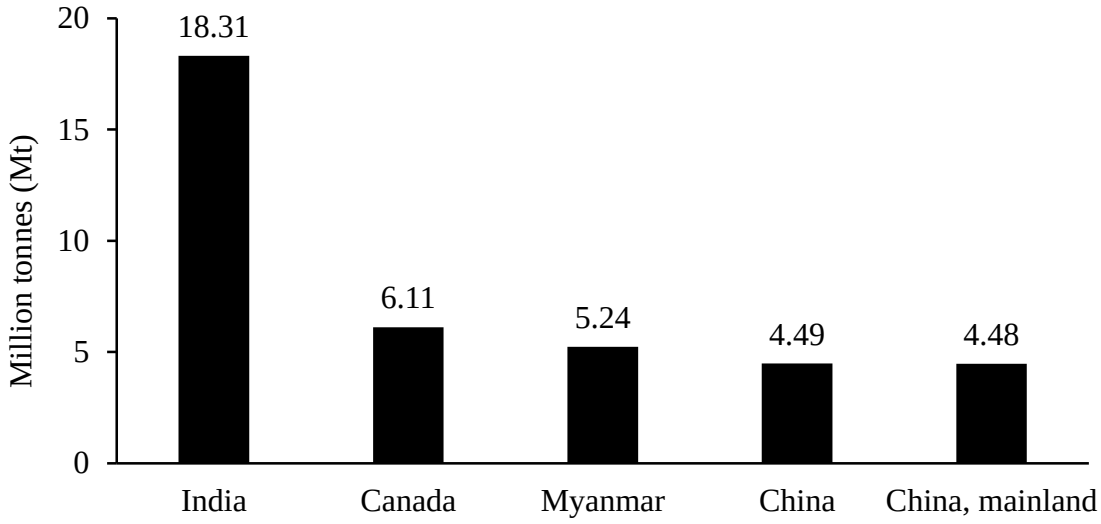

Figure 2.1. Pulse production of top 5 producers in 2013 (Adapted from FAOSTAT, 2015)

Table 2.1. Production of important pulses in major pulse-producing countries in 2013 (Adapted from FAOSTAT, 2015)

Pulses	Countries	Production (tonnes)
Lentils	Canada	1,880,500
	India	1,134,000
	Turkey	417,000
	Australia	324,100
	USA	227,658
Chickpeas	India	8,832,500
	Australia	813,300
	Pakistan	751,223
	Turkey	506,000
	Myanmar	490,000
Peas, dry	Canada	3,849,300
	China	1,566,000
	China, mainland	1,566,000
	Russian Federation	1,350,167
	USA	708,512
Broad beans, horse beans, dry	China	1,586,000
	China, mainland	1,586,000
	Australia	297,500
	France	245,546
	Egypt	158,000
Beans, dry	Myanmar	3,700,000
	India	3,630,000
	Brazil	2,892,599
	Mexico	1,294,634
	United Republic of Tanzania	1,113,541

Canada, India, Turkey, Australia and USA are the major lentil producing countries. Canada is the top producer of lentils and dry peas. China, Russia and USA are the other main producers of dry peas. India is the largest contributor to the world chickpea production, producing approximately 10 times as much as the second chickpea grower, Australia, produced. China produces the highest amount of broad beans. Dry beans are mainly grown in developing countries; Myanmar, India, Brazil, Mexico and Tanzania are primary producers.

2.1.1.2 Canadian situation

Canada is considered as a world leader in production of lentils and dry peas, one of top ten producers of chickpeas and one of top thirty producers of dry beans (Bekkering, 2014). Figure 2.2 shows the total Canadian pulse production in the past two decades (1994 - 2013). More than a three-fold increase has been observed within 20 years.

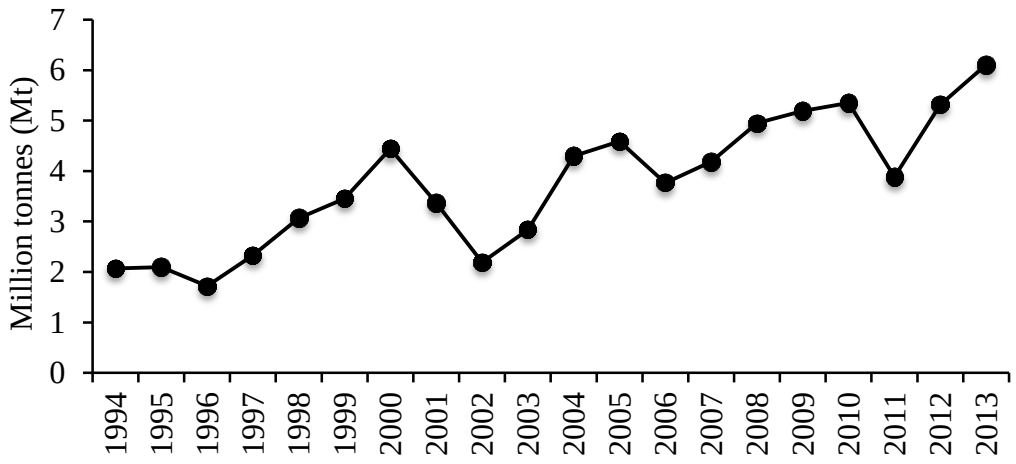

Figure 2.2. Canadian pulse production from 1994 to 2013 (Adapted from FAOSTAT, 2015)

Pulses are mainly grown in Saskatchewan, Alberta and Manitoba in Canada (Bekkering, 2014). In 2011, Saskatchewan contained the largest pulse seeded area in the country, accounting for 79.3%, with Alberta following at 16.2% and Manitoba at 2.3% (Table 2.2). Soil, climate and geographical advantages are major reasons for the Prairie Provinces to grow most of these pulse varieties (Bekkering, 2014). However, the largest seeded area of dry beans was reported in Ontario in 2011 with 38.4% of the national area, followed by Alberta and Manitoba with 32.1% and 18.8%, respectively (Bekkering, 2014).

Table 2.2. Pulse seeded area by province in Canada 2011 (Adapted from Bekkering, 2014)

Region	Area	
	Hectares	Percent
Canada total	2,157,840.6	100
Maritime provinces	327.4	0.0
Quebec	4,137.9	0.2
Ontario	39,556.8	1.8
Manitoba	49,132.9	2.3
Saskatchewan	1,711,497.9	79.3
Alberta	348,965.2	16.2
British Columbia	4,222.5	0.2

Note: No pulse area was reported in Newfoundland and Labrador.

2.1.2 Nutrient contents of pulses

Pulses consist of proteins, lipids, carbohydrates, water, mineral matters, vitamins and phytochemicals (Tiwari, & Singh, 2012). Chemical composition of pulse grains can be classified into proximate composition (protein, carbohydrate, crude fat, total ash and moisture) and ultimate composition (minerals, vitamins and other minor constitutes) (Tiwari, & Singh, 2012).

2.1.2.1 Major constitutes

Proximate compositions of some pulses vary widely; these have been summarized in Table 2.3 where they are compared to soybean. Generally, pulse grains are considered to be an important source of protein at levels of 20 – 30% protein. Carbohydrates are the most abundant constitute in pulses, ranging from 45% to 65%. Low fat content (< 2%) has been reported in most pulses, with chickpeas being an exception. Kabuli and desi chickpeas contain approximately 5 – 6% and 3 – 4.5% fat content, respectively. Additionally, 2.5 – 4% ash and 10.5 – 25% dietary fiber are present in pulse grains. As compared to pulses, soybean exhibits the highest amounts of protein (36%) and fat (19%), but the lowest amount of carbohydrate (30%).

Table 2.3. Proximate compositions of some pulse grains and soybean (g/100 g)

Seeds	Moisture	Protein	Lipid	Ash	Carbohydrate	Sugars content	Dietary fiber
Peas[a]	10.62	22.9 – 24.5	0.84 – 1.19	2.53 – 2.72	63.22	8.0	14.26 – 16.83
Kabuli chickpea[b]	7.92	20.7 – 25.5	5.00 – 5.93	2.64 – 3.96	47.57 – 56.70	10.70	15.38
Desi chickpea[c]	8.83	21.50 – 26.70	3.07 – 4.64	2.8 – 3.6	45.3 – 49.3	10.70	24.56
Faba bean[d]	10.98	26.12 – 31.24	1.27 – 1.53	4.11	56.86 – 58.29	5.70	25.0
Kidney bean[e]	11.75	23.58 – 27.06	0.83 – 1.18	4.40	60.01	2.23	19.97 – 24.90
Pinto bean[f]	11.33	21.42 – 22.40	1.01 – 1.23	3.76	62.55	2.11	15.5 – 18.91
Navy bean[g]	12.10	22.33 – 25.30	1.29 – 1.50	3.95	60.75	3.88	15.3
Mung bean[h]	9.05 – 9.75	27.5	1.85	3.76	62.3	6.60	16.3
Lentil[i]	8.26	23.10 – 30.50	0.67 – 1.23	2.46 – 3.00	63.35 – 63.36	2.03	10.70 – 14.70
Horse gram[j]	8.83	22.50	1.25	2.72	64.70	-	15.08
Soybean[k]	8.54	36.49	19.84 – 19.94	4.87	30.16	7.33	9.3 – 15.1

[a] Alonso et al. (2000b), Tiwari, & Singh (2012), Wang, Hatcher, & Gawalko (2008), Wang, & Toews (2011).

[b] Attia et al. (1994), Khan et al. (1995), Rachwa-Rosiak, Nebesny, & Budryn (2015), Rincón, Martínez, & Ibáñez (1998), Tiwari, & Singh (2012), Wang et al. (2010), Wang, & Toews (2011).

[c] Khan et al. (1995), Rincón et al. (1998), Rachwa-Rosiak et al. (2015), Tiwari, & Singh (2012), Wang et al. (2010), Wang, & Toews (2011).

[d] Alonso, Aguirre, & Marzo (2000a), Shekib, El-Iraqui, & Abo-Bakr (1988), USDA National Nutrient Database for Standard Reference (2015).

[e] Alonso et al. (2000a), Rehman, & Shah (2005), USDA National Nutrient Database for Standard Reference (2015), Wang et al. (2010).

[f] USDA National Nutrient Database for Standard Reference (2015), Wang et al. (2010).

[g] USDA National Nutrient Database for Standard Reference (2015), Wang, & Toews (2011).

[h] Mubarak (2005), USDA National Nutrient Database for Standard Reference (2015).

[i] Rehman, & Shah (2005), Shekib et al. (1988), USDA National Nutrient Database for Standard Reference (2015), Wang, & Daun (2004), Wang et al. (2009), Wang, & Toews (2011).

[j] Sreerama, Sasikala, & Pratape (2008).

[k] Canadian Nutrient File (2015), USDA National Nutrient Database for Standard Reference (2015).

2.1.2.2 Minor constitutes

In addition to the presence of macronutrients, pulse grains contain small quantities of minerals and vitamins that play significant roles in human metabolism (Tiwari, & Singh, 2012). The levels of minerals ranges from 5.1 – 13.4 mg Fe/100g, 36 – 400 mg Ca/100 g, 115 – 283 mg Mg/100 g, 304 – 451 mg P/100 g and 955 – 1724 mg K/100 g (Majumdar, 2011; Oomah et al., 2011; Tiwari, & Singh, 2012). Pulses are also good source of vitamins, especially B vitamins (Tiwari, & Singh, 2012). Folates are present in abundant amounts, varying between 23 – 622 mg/100 g while contents of thiamine, riboflavin, niacin, pantothenic acid and vitamin B_6 are 0.27 – 1.13 mg/100 g, 0.06 – 0.45 mg/ 100g, 0.48 – 3.15 mg/100 g, 0.31 – 1.91 mg/ 100g and 0.16 – 0.54 mg/100 g, respectively (Majumdar, 2011; Oomah et al., 2011; Tiwari, & Singh, 2012).

2.1.3 Pulse food use

As one of the earliest domesticated crops, lentil (*Len culinaris*) is cholesterol free, low in saturated fat and sugar, but high in dietary fiber, iron, phosphorous, thiamine, vitamin C and folic acid (Asif et al., 2013; Erskine et al., 2009). It is consumed as a major food component in the Mediterranean area and the Middle East (Raghuvanshi, & Singh, 2009). Whole and split lentils can be processed for consumption as snacks; flour is used as an ingredient in soups, stews, or combined

with cereals to produce baked goods and baby foods (AAFC, 2009; Raghuvanshi, & Singh, 2009). Due to a large vegetarian population, lentils enable to act as a meat substitute in Indian cuisine (Asif et al., 2013). More importantly, the gluten-free feature of lentil makes it become a nutritious alternative to wheat flour (Asif et al., 2013).

Dry beans (*Phaseolus vulgaris* L.) have been grown worldwide in all continents except Antarctica (Pathania, Sharma, & Sharma, 2014). Their consumption is recognized to be the highest in Latin American (Siddiq, & Uebersax, 2013). Commercially, beans can be either canned, packaged dry for home preparation or further processed into products, e.g. milled to make flour (Abu-Ghannam, & Gowen, 2011; AAFC, 2009; Siddiq, & Uebersax, 2013). In households, dry beans are usually cooked, fried, or baked and consumed as vegetables by adding into soups or combining with meat to make a main dish (Siddiq, & Uebersax, 2013).

Chickpea (*Cicer arietinum* L.), including kabuli and desi cultivar types, was originally from the Middle East (Singh et al., 2014). It has become part of traditional diets in Asia, the Mediterranean region and South America (Geil, & Anderson, 1994; Phillips, 1993). Chickpeas contain relatively high fat content as compared to other pulses and cereals (Messina, 1999; Williams, & Singh, 1987). Frying, roasting and boiling can be carried out on chickpea seeds, so they are mainly eaten in salads as well as used in snack foods and condiments; in addition, the seeds can be ground to produce flour for bread making (AAFC, 2009; Asif et al., 2013). Hummus, a typical

Middle East food dip eaten with pita and bread, is composed of chickpea (Asif et al., 2013).

Dry peas (*Pisum sativum* L.) share a smaller food market compared to other pulses (AAFC, 2009). They cannot only be cooked, frozen or canned and eventually consumed whole as a vegetable, but can also be processed into flour and used in soups, pastas, cereals, processed meats and bakery products due to the functional properties (emulsifying, gelling and water absorption properties) of pea proteins (AAFC, 2009; Asif et al., 2013).

2.1.4 Health benefits

Frequent pulse consumption may lower the risks of CVD, diabetes, obesity and cancer (Oomah et al., 2011; Tiwari, & Singh, 2012).

2.1.4.1 Cardiovascular health

According to the World Health Organization (WHO) (2015), coronary heart disease (CHD) is a serious form of CVDs, in which high total and low-density lipoprotein (LDL) cholesterol levels are major risk factors. Several studies have shown a relationship between pulse consumption and reduction in CHD/CVD risk. Approximately 22% lower CHD risk and 11% lower CVD risk were observed with consumption of pulses 4 or more times per week compared to those ate less than once per week (Bazzano et al., 2001). Dietary fiber in pulses may also influence

cardiovascular well-being; Bazzano et al. (2003) indicated that higher intake of dietary fiber caused lower CHD risk. Lower cholesterol levels were obtained with pinto bean consumption in an adult population (Finley, Burrell, & Reeves, 2007).

2.1.4.2 Diabetic health

In animal studies, the evidence for the anti-hyperglycemic effect of common bean is strong, particularly in diabetic animals. Khaleeva, Maloshtan and Sytnik (1987) found that diabetic rabbits experienced a remarkable reduction in glycemia in response to a common bean complex. This was in agreement with the result reported by Venkateswaran, Pari and Saravanan (2002) where oral administration of a common bean extract to diabetic rats significantly reduced blood glucose levels. However, only a few human studies have been conducted on the association between pulse intake and glycemia regulation. Campos-Vega, Loarca-Piña and Oomah (2010) indicated that mung bean slightly increased blood glycemic index in humans. More work involving human studies may be required to confirm the advantages seen in rats before recommending it for diabetic patients.

2.1.4.3 Cancer prevention

Pulses contain a wide range of components that may play significant roles in protecting against cancers (Mathers, 2002). Due to their anticarcinogenic properties, soy isoflavones have been associated with a reduction in prostate cancer risk

(Derbyshire, 2011). Isoflavones in pulses have received little attention and pulses have starch as their main component. Intake of starch in pulses might be negatively correlated with risk of colorectal cancer (Cassidy et al., 1994; Mathers, 2002). Although there is a lack of human studies, animal studies have shown that rats consuming common beans or a polysaccharide extract from common beans had lower cancer incidence (Feregrino-Perez et al., 2008; Hangen, & Bennink, 2002). This is an area where more research would be beneficial.

2.2 Antinutritional factors

Plant seeds contain a variety of constituents including basic nutrients (proteins, lipids, carbohydrates, vitamins and minerals) and some chemical compounds, which are accumulated in seeds to defend against herbivorous animals, microorganisms and viruses (Enneking, & Wink, 2000). Some of them are biologically active and known as secondary metabolites (Zenk, 1991). However, the production of these secondary metabolites affects the nutritional quality of plant materials, particularly those used in animal feeds and human foods, as is the case for grain legumes (Soetan, & Oyewole, 2009). These plant secondary metabolites, so called antinutritional factors, are toxic, unpalatable or antinutritive due to their abilities to complex nutrients, inhibit metabolism or reduce digestion (Enneking, & Wink, 2000). The amounts of some major antinutritional factors in grain legumes are summarized in Table 2.4 and Table 2.5.

Table 2.4. Amounts of enzymatic inhibitors in major pulses (dry basis)

	Phaseolus vulgaris **(Dry beans)**	***Lens culinaris*** **(Lentil)**	***Cicer arietinum*** **(Chickpea)**	***Pisum sativum*** **(Dry peas)**	***Vici faba*** **(Dry broad beans)**
Trypsin inhibitor[a]					
TIU mg^{-1}	3.10 – 31.30	3.6 – 7.6	8.10 – 20.89	0.78 – 6.32	2.31 – 7.20
mg g^{-1}	5.92 – 10.90	0	0.895 – 8.29	1.3 – 2.7	1.99 – 3.35
Chymotrypsin inhibitor[b]					
IU mg^{-1}	3.97		6.1 – 8.8	2.73 – 4.85	3.56
mg g^{-1}	0.980 – 1.700	0	0 – 0.461		
α-Amylase inhibitor[c]					
IU g^{-1}	76 – 675		3.1 – 10.5	16.8	18.9
U g^{-1}		3290	2210		4171
mg g^{-1}	1.20 – 1.43	0	0 – 0.013		0

TIU, trypsin inhibitor units; IU, International Units; U, units.

[a] Alonso et al. (1998), Alonso et al. (2000a), Attia et al. (1994), El-Adawy (2002), El-Hady, & Habiba (2003), Hernádez-Infanteet al. (1998), Khattab, & Arntfield (2009), Luo, & Xie (2012), Makkar et al. (1997), Martín-Cabrejas et al. (2009), Mondor et al. (2009), Pedrosa et al. (2012), Periago et al. (1998), Singh, & Jambunathan (1981), Sotelo, Flores, & Hernández (1987), Wang, & Daun (2004), Wang et al. (2008), Wang et al. (2010).

[b] Alonso et al. (1998), Alonso et al. (2000a), Martín-Cabrejas et al. (2009), Singh, & Jambunathan (1981).

[c] Alonso et al. (1998), Alonso et al. (2000a), El-Hady, & Habiba (2003), Deshpande et al. (1982), Martín-Cabrejas et al. (2009), Shekib et al. (1988).

Table 2.5. Amounts of non-enzymatic antinutrients in major pulses (dry basis)

	Phaseolus vulgaris **(Dry beans)**	***Lens culinaris*** **(Lentil)**	***Cicer arietinum*** **(Chickpea)**	***Pisum sativum*** **(Dry peas)**	***Vici faba*** **(Dry broad beans)**
Lectin[a]					
HU mg^{-1}	74.5		6.22	5.1 – 6.2	49.3
HU (g mg^{-1})		500	0		
mg ml^{-1}					12.5 – 37.5
g kg^{-1}	1.90 – 9.99				
Phytic acid[b]					
mg g^{-1}	9.90 – 29.30	12.5	5.8 – 12.1	3.0 – 13.3	6.40 – 21.7
Oxalate[c]					
Soluble (mg 100 g^{-1})	1.5 – 677	1.9 – 144.2	70.3	0 – 94	162
Total (mg 100 g^{-1})	13.9 – 946	13.3		0 – 103	194
Oligosaccharides[d]					
Total (g 100 g^{-1})	6.00 – 6.19	4.75	2.94	5.63 – 5.73	
Verbascose (g 100 g^{-1})	0 – 2.08	0.47 – 2.19	0.004 – 0.19	0.94 – 2.22	
Stachyose (g 100 g^{-1})	1.83 – 3.75	1.75 – 2.31	0.82 – 2.56	2.27 – 3.69	
Raffinose (g 100 g^{-1})	0.09 – 1.79	0.29 – 0.48	0.36 – 1.69	0.55 – 1.92	
Polyphenols[e]					
Total (g kg^{-1})	2.07		1.9 – 6.1	3.7 – 5.0	3.92
Tannins (g kg^{-1})	0.03 – 19.9	3.9	0.04 – 4.85		0.13 – 21.0
Condensed tannins (g kg^{-1})	0 – 3.59	5.91	1.96 – 2.19	1.35 – 2.38	0 – 35.4

Table 2.5. Amounts of non-enzymatic antinutrients in major pulses (dry basis) (Continued)

	Phaseolus vulgaris **(Dry beans)**	***Lens culinaris*** **(Lentil)**	***Cicer arietinum*** **(Chickpea)**	***Pisum sativum*** **(Dry peas)**	***Vici faba*** **(Dry broad beans)**
Saponins[f]					
g kg^{-1}	0.02 – 16		0.91 – 60	0.01 – 11	0.1 – 31.7
Vicine & convicine[g]					
mg g^{-1}	0.021 – 0.049			0 – 0.29	1.48 – 2.68

HU, hemagglutinin units.

[a] Alonso et al. (1998), Alonso et al. (2000a), Burbano et al. (1999), El-Adawy (2002), Makkar et al. (1997), Pedrosa et al. (2012), Sotelo et al. (1987).

[b] Alonso et al. (1998), Alonso et al. (2000a), Attia et al. (1994), Bishnoi, Khetarpaul, & Yadav (1994), Chitra, Singh, & Rao (1996), Deshpande et al. (1982), Duhan et al. (1989), El-Adawy (2002), El-Hady, & Habiba (2003), Khan, Zaman, & Elahi (1988), Khattab, & Arntfield (2009), Luo, & Xie (2013), Makkar et al. (1997), Nestares et al. (1999), Periago et al. (1998), Rehman, & Shah (2005), Wang, & Daun (2004), Wang et al. (2010).

[c] Hönow, & Hesse (2002), Judprasong et al. (2006), Quinteros, Farré, & Lagarda (2003), Słupski et al. (2011).

[d] Alajaji, & El-Adawy (2006), Alonso et al. (2000b), Attia et al. (1994), Burbano et al. (1999), El-Adawy (2002), Khattab, & Arntfield (2009), Pedrosa et al. (2012), Sathe, & Salunkhe (1981), Singh, Kherdekar, & Jambunathan, (1982), Wang, & Daun (2004), Wang, & Daun (2006), Wang et al. (2008), Wang et al. (2010).

[e] Alajaji, & El-Adawy (2006), Alonso et al. (1998), Alonso et al. (2000a), El-Adawy (2002), Luo, & Xie (2013), Makkar et al. (1997), Martín-Cabrejas et al. (2009), Singh, & Jambunathan (1981), Vidal-Valverde et al. (1994), Wang, & Daun (2006), Wang et al. (2010).

[f] Alajaji, & El-Adawy (2006), Burbano et al. (1999), Campos-Vega et al. (2010), El-Adawy (2002), Khokhar, & Chauhan (1986), Makkar et al. (1997), Price, Johnson, & Fenwick (1987).

[g] Champ (2002), Zdunczyk, Godycka, & Amarowicz (1997).

2.2.1 Definition and classification

Antinutritional factors may be defined as those compounds "generated in natural food stuffs by the normal metabolism of species and by different mechanisms (e.g. inactivation of some nutrients, diminution of the digestive process, or metabolic utilization of feed), which exert effects contrary to optimum nutrition" (Kumar, 1992).

According to Martín-Cabrejas et al. (2009) and Soetan, & Oyewole (2009), antinutritional factors can be classified into two groups: (1) proteins (protease inhibitors, amylase inhibitors and lectins) which are unstable at normal processing temperatures, and (2) other substances (polyphenol compounds, non-protein amino acids and galactomannan gums) which are resistant to these temperatures. Aletor (1993) listed some significant antinutritional factors found in plants used as animal feedstuffs and human foods as follows: (1) enzyme inhibitors (trypsin and chymotrypsin inhibitors, plasmin inhibitors, elastase inhibitors), (2) haemaglutinnins (concanavalin A, ricin), (3) plant enzymes (urease, lipoxygenase), (4) cyanogenic glycosides (phaseolunatin, dhurrin, linamarin, lutaustralin), (5) goitrogens (pro-goitrins and glucosinolates), (6) oestrogens (flavones and genistein), (7) saponins (soya sapogenin), (8) gossypol from *Gossypium species* e.g. cotton, (9) tannins (condensed and hydrolysable tannins), (10) amino acid analogues (BOAA, DAP, mimosine, N-methy-1-alanine), (11) alkaloids (solanine and chaconine), (12) anti-metals (phytates and oxalates), (13) anti-vitamins (anti-vitamins A, D, E, B_{12}) and (14) favism factors. Only those that have been found in pulses, including trypsin

inhibitor activity, chymotrypsin inhibitor activity, amylase inhibitor activity, hemagglutinating activity, phytates, oxalates, polyphenol content (total polyphenols, phenolic acid and tannins), saponins, oligosaccharides, vicine and convicine, have been considered in this project at the Universities of Manitoba and Saskatchewan.

Considering of the antiherbivore or antimicrobial properties of some antinutritional factors, they can be isolated and processed for use in medicine as pharmacologically-active agents or in agriculture as natural pesticides (Oakenfull, & Sidhu, 1989; Enneking, & Wink, 2000). There are two-sides to many of the antinutritional factors; whether they have beneficial or deleterious influences on human and animal health depends mainly on the amount consumed (Kersten et al., 1991; Sugano et al., 1993). Therefore, some antinutritional factors can be processed into functional foods or nutraceutical ingredients (Campos-Vega et al., 2010). For instance, Celleno et al. (2007) indicated that a bean extract named Phase 2^{TM} obtained GRAS (generally recognized as safe) and have been marketed since 2001 in terms of its impact on body composition of overweight population.

Therefore, the effects of antinutritional factors can be negative, positive or both (Champ, 2002). Table 2.6 shows the potential advantageous and harmful effects of some common antinutritional factors in legume.

Table 2.6. Potential beneficial and adverse effects of antinutritional factors in legume seeds

	Beneficial effect	**Adverse effect**
Proteins		
Protease inhibitors	Anticarcinogenic (?)	Growth inhibition (in animals); Reduction activity of (chymo)trypsin; Pancreatic hypertrophy/hyperplasia; Acinar nodules
Amylase inhibitors	Potentially therapeutic in diabetes (?)	Forming complex with amylase in salivary and pancreatic juice; Interference with starch digestion
Lectins	May help in obesity treatment (??); Reduce tumor growth (??)	Growth inhibition (in animals); Decrease nutrient absorption; Damage of gutwall; Immunological reaction; Metabolism toxicity
Miscellaneous		
Phytates	Hypocholesterolaemic effect (?); Anticarcinogenic (?)	Decrease bioavailability of minerals; Form complex with protein
Oxalates		Decrease bioavailability of minerals
Condensed tannins		Astringent taste; Reduced food intake (in animals)
Saponins	Hypocholesterolaemic effect (?); Anticarcinogenic (?)	Bitter taste; Reduced food intake (in animals)

Adapted from Campos-Vega et al. (2010), Champ (2002), Liener (1989a), Van Der Poel (1990).

2.2.2 Enzyme inhibitors

Enzyme inhibitors are present in a number of food products, including legumes, cereals (wheat, corn, barley and oat), nuts, potatoes beetroots and tomatoes (Friedman, & Brandon, 2001). They may have a significant impact on nutritional value due to interference with digestive enzymes (Molina, 2013).

2.2.2.1 Protease inhibitors

Protease inhibitors are substances that inactivate proteolytic digestive enzymes (Molina, 2013). They are widely distributed in legume seeds and can be categorized into two groups: Kunitz type and Bowman-Birk type. The Kunitz inhibitors are approximately 20 kDa in size with four cysteines and 2 disulfide bonds, but Bowman-Birk types, with molecular mass of 8 kDa, have 14 cysteines, 7 disulfide bridges and 2 reactive sites. Kunitz types show inhibition on trypsin only, whereas Bowman-Birk type inhibitors inhibit trypsin on one site and chymotrypsin on the second one. In addition, the protease inhibitors in common beans, lima beans, cowpea and lentils usually belong to the Bowman-Birk family, so this group receives the most attention (Belitz, & Weder, 1990; Bhattacharyya, Rai, & Badu, 2007; Ferrasson, Quillien, & Gueguen, 1997; Lajolo, & Genovese, 2002; Liener, 1994; Richardson, 1991). The mechanism of inhibitory action, demonstrated by Norton (1991), was the interaction between the enzyme (catalytic) site and the inhibitor reactive site, resulting in the formation of proteinase-inhibitor complex; therefore, enzyme activity declined.

2.2.2.1.1 Beneficial and antinutritional effects

A number of benefits have been identified for protease inhibitors, including protection of plants against predators (Champ, 2002). In animal studies, protease inhibitors were considered as anticarcinogenic agents due to their suppressive effects on carcinogen-induced cells (Clemente, & Domoney, 2001; Thompson, 1993). However, the presence of protease inhibitors may lower legume quality and utilizations by its negative influence on protein digestibility (Savelkoul, Van Der Poel, & Tamminga, 1992), and have adverse on human with a high intake over a long period of time; thus, they are usually considered as antinutritional factors (Champ, 2002).

2.2.2.1.2 Trypsin and chymotrypsin inhibitors

Trypsin inhibitors are low molecular weight proteins, found in a wide range of legumes (Savage, 1989; Wang, & Daun, 2004). They are capable of binding to trypsin, which is a proteolytic enzyme secreted by the pancreas (Savage, 1989; Mondor et al., 2009). Therefore, digestion of proteins is interfered with and inadequate amino acids are available for organisms (Savage, 1989). Since chymotrypsin is one of the proteolytic enzymes that is similar to trypsin, chymotrypsin inhibitor is considered to have a similar mode of action as trypsin inhibitor (Savage, 1989).

The contents of trypsin and chymotrypsin inhibitor in some common pulses are listed in Table 2.4. Different methods and units used for protease inhibitors make it difficult to compare the data among literature (Champ, 2002). It has been estimated that British diets provided 330 mg of trypsin inhibitor intake per person per day

(Doell, Ebden, & Smith, 1981). However, Billings et al. (1990) estimated the median daily intakes of trypsin inhibitor and chymotrypsin inhibitor were 4.6 mg/day (range 0 – 127.9 mg/day) and 1.6 mg/day (range 0 – 31.1 mg/day), respectively.

2.2.2.1.3 Analyses

The determination of trypsin inhibitor activity is based on indirect measurement of inhibition of trypsin activity (Makkar, Siddhuraju, & Becker, 2007): as a substrate, benzyl-DL-arginine*para*-nitroanilide (BAPNA) is hydrolyzed by trypsin to generate yellow-coloured *p*-nitroanilide, which indicates the level of residual trypsin; the yellow-coloured product is determined using a spectrophotometer; the unit of trypsin inhibitor activity can be reported in terms of trypsin units inhibited or as milligrams of pure trypsin inhibited.

Similar to the trypsin inhibitor activity measurement, the quantification chymotrypsin inhibitor activity is based on spectrophotometric determination of hydrolyzed products of casein (substrate) by chymotrypsin (Makkar et al., 2007).

2.2.2.2 Amylase inhibitors

Many plants, such as wheat, rye and legumes, naturally contain a proteinaceous inhibitor of the digestive enzyme α-amylase (α-1,4-glucan-4-glucanohydrolases, EC 3.2.1.1) (Buonocore, Petrucci, & Silano, 1977; Marshall, & Lauda, 1975; Shekib et al., 1988; Udupa, Prabhakar, & Tandon, 1989; Wang et al., 2011). The content of α-amylase inhibitors in common pulses is summarized in Table 2.4.

2.2.2.2.1 Antinutritional effects of α-amylase inhibitors

α-Amylase inhibitors in legume seeds act as antinutritional factors in both human and animal nutrition (Jaffé, Moreno, & Wallis, 1973; Svensson et al., 2004). The digestibility of starch is reduced due to inhibition of pancreatic and salivary α-amylase activity (Jaffé et al., 1973; Savelkoul et al., 1992; Wang et al., 2011). The occurrence of pancreatic hypertrophy has been observed as a result of amylase inhibitors (Champ, 2002).

2.2.2.2.2 Beneficial effects of α-amylase inhibitors

In addition to limiting amylase activity, amylase inhibitors have been shown to influence pathogens and pests (Celleno et al., 2007; Svensson et al., 2004). Extracts of amylase inhibitors can also be used as starch blockers to treat obesity and in diabetes therapy for calorie control, since they affect carbohydrate breakdown, leading to reduced glucose absorption (Obiro, Zhang, & Jiang, 2008; Svensson et al., 2004). However, there is a lack of estimated dietary exposure to amylase inhibitor.

2.2.2.2.3 Analyses

The measurement of α-amylase inhibitor activity is based on spectrophotometric determination (Makkar et al., 2007): α-amylase hydrolyzes starch molecules by breaking α-1,4 linkages to produce reducing sugars (mainly maltose); dinitrosalicylic acid is reduced by the interaction with reducing sugars to generate a brown/orange-red-coloured product, nitroaminosalicylic acid; the amount of nitroaminosalicylic acid measured by a spectrophotometer. The levels of reducing

sugar with and without α-amylase inhibitor are compared to determine α-amylase inhibitor activity.

2.2.3 Lectins

2.2.3.1 General characteristics

Lectins, also termed as hemagglutinins, are carbohydrate binding proteins or glycoproteins, distributed in most common edible plant foods, including beans, peas, soybeans, tomato and potato (Bhanu et al., 1997; Grant, 1991; Lajolo, & Genovese, 2002; Vasconcelos, & Oliveira, 2004). They show the ability to bind reversibly to specific sugars, proteins and glycoconjugates (Lajolo, & Genovese, 2002; Roberts, & Goldstein, 1984).

As one of the families of plant lectins, legume lectins can be further divided into two groups according to different composition of subunits: (a) subunits having identical molecular weights of 25,000-30,000 (one-chain lectins); (b) subunits containing a light α-chain and a heavy β-chain (two-chain lectins) (Lis, & Sharon, 1986b; Vasconcelos, & Oliveira, 2004). In many legumes, lectin dimers of 50,000 molecular weight or tetramers of 100,000 and 120,000 molecular weights are formed by combining α- and β-subunits (Savelkoul et al., 1992).

Lectins are usually found in protein bodies that are produced in the endoplasmic reticulum (Molina, 2013). Lectin content constitutes 2.4 – 5% of the total protein in kidney bean (17 – 23%), 0.8% in soybean (34%) and lima bean protein (21%), and 0.6% in garden peas (24 – 25%) (Campos-Vega et al., 2010). The amount of lectin level in major pulses is presented in Table 2.5. Great variations in lectin

content do not only reflect cultivar difference but also the different assays used for determination.

2.2.3.2 Antinutritional effects

Lectins can have adverse biological influences on human nutrition. They are able to agglutinate red blood cells and bind to intestinal mucosa cells, impairing transportation of nutrients across the intestinal wall (Damodaran, 2008; Savelkoul, et al., 1992; Vadivel, & Pugalenthi, 2007). Absorption of amino acids, glycoprotein biosynthesis and secretion are influenced by lectins; hence, the efficiency of nutrient utilization is reduced (Damodaran, 2008; Enneking, & Wink, 2000; Pusztai, 1989). Only a few studies have been conducted investigating the toxic level of lectins for human beings. Olsnes and Pihl (1982) reported that intravenous administration of more than 5 $\mu g/m^2$ or 0.15 $\mu g/kg$ of abrin led to toxic effects.

2.2.3.3 Beneficial effects

Lectins may perform a defensive role in plants to improve resistance of plants against bacteria, fungi or insects (Pratt et al., 1993). Other beneficial effects of lectins include their role as carriers for some targeted cells (Bhanu et al., 1997) and as alternatives for skin wound treatment (Chahud et al., 2009).

2.2.3.4 Analyses

The estimation of lectins is based on agglutination of red blood cells and a serial dilution of cells was required as part of the measurement (Makkar et al., 2007).

A visual assessment has been widely used to detect agglutination (Alajaji, & El-Adawy, 2006; El-Adawy, 2002; Liener, & Hill, 1953; Morcos, Gabrial, & El-Hafez, 1976; Mubarak, 2005), but has been criticized due to a lack of precision (Makkar et al., 2007). Agglutination of red blood cells can be also determined microscopically (Makkar et al., 1997), but this too involves a visual assessment.

2.2.4 Phytic acid

2.2.4.1 General characteristics

Phytic acid, also known as myo-inositol 1,2,3,4,5,6, hexakis-dihydrogen phosphate (IP6), is commonly found in cereal grains and oilseed food products (Davies, & Reid, 1978; Singh, & Krikorian, 1982; Deshpande, & Cheryan, 1984; Knuckles, & Betschart, 1987). Phytic acid, or phytate when in salt form, accounts for 60-90% of the total phosphorus in legume seeds (Chitra et al., 1995; Chitra et al., 1996; Torre, Rodriguez, & Saura-Calixto, 1991) and therefore becomes a major reserve of phosphorus in plant seeds (Estevez et al., 1991; Latta, & Eskin, 1980; Torre et al., 1991). Fairly low amounts of other forms of phosphorus (IP3-IP5) have been detected in pulses (Tiwari, & Singh, 2012). Phytic acid is found in discrete parts of seeds, primarily in the form of globoid crystals within protein bodies, such as the aleurone layer of rice and wheat (Champ, 2002). In pulses, phytic acid is present inside protein bodies located in endosperm (Oomah et al., 2011).

Phytic acid comprises 1 – 3% of seed constituent in all nuts, cereals, legumes and oilseeds (Champ, 2002). Table 2.5 shows that phytic acid content in common pulses is lower than 3%. The phytic acid content of grains can be affected by seed

variety, various environmental or climatic conditions and application of fertilizers (Tiwari, & Singh, 2012).

2.2.4.2 Antinutritional effects

Phytic acid is regarded as an antinutritional factor due to its binding properties to mineral, protein and starch (Weaver, & Kannan, 2002). Phytic acid in foods can interfere with the bioavailability of essential minerals (e.g. calcium, zinc, iron and magnesium) by formation of insoluble compounds with them (Chitra et al., 1995; Chitra et al., 1996; Deshpande, & Cheryan, 1984; Estevez et al., 1991; Knuckles, & Betschart, 1987; O'Dell, & De Boland, 1976). The insoluble complexes are precipitated in the intestinal lumen rather than releasing minerals in the intestinal mucosa (Estevez et al., 1991; O'Dell, & De Boland, 1976); thus, these essential dietary minerals become biologically unavailable to organisms for absorption (Chitra et al., 1995; Chitra et al., 1996; Estevez et al., 1991). For instance, an iron deficiency may occur in humans (Enneking, & Wink, 2000).

Phytic acid is also able to complex with proteins, lowering solubility and bioavailability (Chitra et al., 1996; O'Dell, & De Boland, 1976). Protein digestibility is reduced due to resistance of insoluble phytate-protein complexes to proteolytic digestion (Estevez et al., 1991; Knuckles, & Betschart, 1987; O'Dell, & De Boland, 1976), resulting from inhibition of digestive enzymes, such as proteases, amylases and trypsin when associated with phytic acid (Bishnoi et al., 1994; Chitra et al., 1996; Savage, 1989).

2.2.4.3 Beneficial effects

In plants, phytic acid plays significant roles in metabolism and resistance against pathogens (Campos-Vega et al., 2010). Due to the ability of chelating metal ions, phytic acid also shows anticancer and anticarcinogenic properties as well as antioxidant activity (Campos-Vega et al., 2010; Kumar et al., 2010; Urbano et al., 2000). Consumption of approximately 25 mg of phytate or less per 100 g of phytate containing food is recommended for best health (Onomi, Okazaki, & Katayama, 2004). Recommended Daily Intake (RDI) of phytic acid varies among countries; United States and United Kingdom have reported 631 – 746 mg as daily average phytate intake, while the values 370 mg/day, 219 mg/day and as low as 180 mg/day have been reported in Finland, Italy and Sweden, respectively (Coulibaly, Kouakou, & Chen, 2011).

2.2.4.4 Analyses

The quantification of phytate can be based on precipitation of phytate (Makkar et al., 2007) in which trichloroacetic acid is used to extract phytate and precipitate it as ferric salt; the iron content of the precipitate is determined using a spectrophotometer. Phytate phosphorus content, therefore, can be calculated in terms of a constant 4 Fe/6 P molecular ratio in the precipitate.

Phytate content can be also determined by the reaction with the Wade reagent (Latta, & Eskin, 1980; Makkar et al., 2007) in which phytate is extracted using 3.5% HCl and purified by an AG1-X8 chloride anion exchange column; a reaction between ferric ion and sulfosalicylic acid in Wade reagent results in pink colour due to iron chelating with the phosphate ester in the phytate extract. As a result the iron becomes

unavailable to interact with sulfosalicylic acid, so that the pink colour intensity decreases.

2.2.5 Oxalates

2.2.5.1 General characteristics

Oxalic acid [$(COOH)_2$] and its salts, known as oxalates, are widely found in plants and plant products (Champ, 2002; Hönow, & Hesse, 2002; Judprasong et al., 2006; Noonan, & Savage, 1999; Słupski et al., 2011). Oxalates are considered as antinutritional factors due to their influence on mineral availability and formation of kidney stones (Chai, & Liebman, 2005; Judprasong et al., 2006; Massey, Roman-Smith, & Sutton, 1993; Noonan, & Savage, 1999; Quinteros et al., 2003; Ross et al., 1999; Słupski et al., 2011).

Oxalates within plants are not evenly distributed; the highest levels are in leaves, followed by seeds with the lowest levels in stems (Noonan, & Savage, 1999; Słupski et al., 2011). Oxalates in plant foods are present in two primary forms: water-soluble salts with sodium and potassium (Hönow, & Hesse, 2002; Judprasong et al., 2006; Noonan, & Savage, 1999; Słupski et al., 2011), and in the form of insoluble salts with divalent metal cations, such as magnesium and calcium (Judprasong et al., 2006; Quinteros et al., 2003; Savage, 1989; Słupski et al., 2011).

The levels of total and soluble oxalates in some selected pulses are presented in Table 2.5 and oxalate contents in these legume seeds are lower than 1%.

2.2.5.2 Antinutritional effects

Acting as a metabolic-end product in humans, oxalates display no beneficial health effects (Shekarriz, 2013). Once oxalates are produced by human metabolism or ingested from foods, they must be excreted in the urine since oxalates cannot be further metabolized by human beings (Massey et al., 1993; Ross et al., 1999). Oxalates show deleterious impacts on human nutrition and health due to their ability to chelate minerals; therefore, bioavailability of minerals decreases (Judprasong et al., 2006; Ross et al., 1999). High intake of oxalate-rich food leads to oxalosis and increased urinary oxalate excretion (Hönow, & Hesse, 2002). An elevated urinary oxalate excretion may cause calcium oxalate crystallization and formation of kidney stones (Hönow, & Hesse, 2002; Ross et al. 1999). Calcium oxalate makes up 75% of kidney stones (Massey et al., 1993; Noonan, & Savage, 1999; Williams, & Wandzilak, 1989). As an insoluble complex, calcium oxalate may pass through the digestive tract without absorption, and precipitate in the urine (Noonan, & Savage, 1999; Savage, 1989). The ingestion of approximately 5 g oxalic acid in crystal or in solution is the minimum dose capacity of causing death in human beings, and is usually associated with corrosive gastroenteritis, shock, convulsive symptoms and renal damage (Fassett, 1967).

2.2.5.3 Analyses

Oxalic acid and soluble oxalate salts are extracted by water; however, total oxalates (soluble and insoluble oxalates) can only be extracted using a strong acid (Chai, & Liebman, 2005; Ohkawa, 1985; Ross et al., 1999). The amount of insoluble

oxalates in foods can be determined by the difference between acid and water extractions (Ross et al., 1999).

The measurement of oxalate can be based on the precipitation as calcium oxalate. The precipitate interacts with dilute sulfuric acid to form a solution of oxalic acid, and is then determined by titration with a standard $KMnO_4$ solution (Makkar et al., 2007).

An enzymatic method can also be easily applied to determine oxalate content. This involves the oxidation of oxalate by oxalate oxidase, followed by determination of hydrogen peroxide produced in a peroxidase-catalyzed reaction (Chai, & Liebman, 2005; Li, & Madappally, 1989; Quinteros et al., 2003). Therefore, an oxalate kit for this purpose is available; the enzymatic reactions involved are as follows (Trinity Biotech, 2013):

$$\text{Oxalate} + O_2 \xrightarrow{\text{Oxalate Oxidase}} 2CO_2 + H_2O_2$$

$$H_2O_2 + \text{MBTH} + \text{DMAB} \xrightarrow{\text{Peroxidase}} \text{Indamine Dye} + H_2O$$

2.2.6 Other antinutritional factors

Since this research project was separated into two locations, at the University of Saskatchewan and the University of Manitoba, other antinutritional factors commonly found in pulses, including polyphenol content (total polyphenols, phenolic acid and tannins), saponins, oligassaccharides, vicine and covicine would be reviewed and addressed at University of Saskatchewan.

2.3 Processing methods

High levels of antinutritional factors have been shown to be present in raw legumes, thus processing is required before safe consumption by humans or animals (Hernádez-Infante et al., 1998; Luo, & Xie, 2013). To increase the utilization of legumes, several industrial and household processes, including dehulling, soaking, boiling, autoclaving, roasting, microwave cooking, micronization, germination and fermentation have been employed (Alonso et al., 2000b; Khattab, & Arntfield, 2009; Luo, & Xie, 2013; Mubarak, 2005).

2.3.1 Dehulling

The process of removing legume seed coats prior to consumption is termed dehulling (Deshpande et al., 1982). Dehulled cotyledons (whole or splits) with good appearance, texture and cooking qualities are obtained after the dehulling process (Tiwari, & Singh, 2012). In green and white faba bean, dehulling significantly increased the level of phytic acid (by 10.6% and 12.3%, respectively) as well as the level of trypsin inhibitor activity (by 27.8% and 18.4%, respectively) (Luo, & Xie, 2013). These results were in agreement with those reported by Wang et al. (2008) who indicated increased phytic acid content in dehulled field peas. Dehulling has also been reported to increase chymotrypsin inhibitor activity and α-amylase inhibitor activity (Alonso et al., 1998; Deshpande et al., 1982). Deshpande et al. (1982) suggested that protease inhibitors might be present in the pulse cotyledon, so that the concentration of protease inhibitors increases on a unit weight basis after seed removal. However, Mubarak (2005) stated trypsin inhibitor activity, hemagglutinin activity and phytic acid content were significantly reduced by the dehulling process,

and Wang et al. (2008) found reduced trypsin inhibitor activity in dehulled field peas. Dehulling effects might be influenced by pulse variety.

2.3.2 Soaking

As a traditional domestic treatment, soaking is often used to prepare pulse-based foods at home (Luo, & Xie, 2013). Soaking processes (36 and 48 hr) showed significant increases (23.0% and 29.4%, respectively) in trypsin inhibitor activity in green faba beans when compared to the raw seeds (Luo, & Xie, 2013); they found no significant difference with white faba beans. Trypsin inhibitor activity, hemagglutinin activity and phytic acid content in mung bean seeds were significantly decreased by 15%, 49.1% and 26.7%, respectively after soaking treatments (Mubarak, 2005). However, Alonso et al. (1998), Alonso et al. (2000) and Luo and Xie (2013) stated that soaking could not significantly affect lectin content in pea, kidney bean and faba bean seeds. According to Alonso et al. (1998) and Alonso et al. (2000), significant reductions in phytic acid, trypsin inhibitor activity and chymotrypsin inhibitor activity were achieved in all cultivars of pea seeds (Renata, Solara and Ballet), kidney and faba beans by soaking. El-Hady and Habiba (2003) also reported that soaking of peas, chickpeas, faba and kidney beans in water caused significant reductions in the contents of phytic acid, α-amylase and trypsin inhibitors. The effect of soaking varies greatly depending on soaking time, seed to water ratio and pulse crops.

2.3.3 Cooking

Heat treatments are often used for reduction or elimination of antinutritional factors in raw legume seeds, especially for destruction or inactivation of heat-labile

antinutritional substances (Mubarak, 2005; Pedrosa et al., 2012). Therefore, cooking is commonly done prior to consumption of legumes in diets (Mubarak, 2005). Trypsin inhibitor activity and phytic acid content in field peas were both decreased as a result of cooking (Wang et al., 2008). Similar observations were reported by Mubarak (2005), and Luo and Xie (2013). In mung bean, trypsin inhibitor activity was completely eliminated by cooking, but only a reduction of approximately 25% was found for phytic acid content (Mubarak, 2005). More than 40% of trypsin inhibitor activity was eliminated by Luo and Xie (2013) in green and white faba bean seeds by cooking. Alajaji and El-Adawy (2006), Mubarak (2005) and Khalil and Mansour (1995) reported that cooking of chickpea, mung bean and faba bean removed heamagglutinin completely. Akhtar et al. (2011) and Judprasong et al. (2006) found that cooking significantly decreased total oxalates in beans and soybean. Cooking process appears to be more effective than soaking. Cooking time, temperature and crop variety may influence cooking effects.

2.3.4 Other treatments

Other approaches, such as autoclaving, roasting, microwave cooking, micronization, germination and fermentation, have also been examined as ways of reducing antinutritional factors in legumes seeds, but will not be discussed further since they were not included in the current investigation.

2.3.5 Effect of processing on antinutritional factors in pulses

Generally, proteinaceous antinutritional factors, including trypsin inhibitor, chymotrypsin inhibitor, α-amylase inhibitor and lectins, are increased by dehulling.

Soaking results in slight reductions in these antinutrients in some pulses (not all), but the most effective way to remove them was through cooking where, for some crops, they were completely eliminated.

Dehulling increases phytic acid contents in pulses, but soaking and cooking decreases its level. For others, such as oxalates, cooking may reduce these levels due to leaching out of soluble oxalates. The effects of other specific processes have not been investigated on oxalates in pulses.

3. CHANGES IN LEVELS OF ENZYME INHIBITORS DURING SOAKING AND COOKING OF CANADIAN PULSES

3.1 Abstract

The effects of legume type and processing (soaking and cooking) on enzyme inhibitors (α-amylase, trypsin and chymotrypsin inhibitors) in a wide range of Canadian pulses (4 peas, 9 lentils, 3 chickpeas, 2 fava beans and 4 beans) were investigated, using soybean as a control. Analysis of variance indicated that legume type, treatment and their interaction had significant effects ($P < 0.0001$) on levels of all enzyme inhibitors. Soybean contained the highest contents of trypsin inhibitor (45.89 TIU/mg) and chymotrypsin inhibitor (30.16 CIU/mg) among all seeds. α-Amylase inhibitory activity was absent in peas, lentils, chickpeas and fava beans, but was present in beans, where values ranged from 785.58 – 1369.75 AIU/g. Trypsin inhibitor levels of raw peas, lentils, chickpeas, fava beans and beans ranged from 3.16 to 20.83 TIU/mg, with the lowest values for peas and the highest for beans. Beans contained relatively high chymotrypsin inhibitor levels followed by chickpeas, lentils, peas and fava beans with very low values (1.12 – 1.67 CIU/mg). Soaking markedly decreased the contents of enzyme inhibitors. Cooking of presoaked seeds was more effective; greater reductions (78.74 – 100%) were observed for all pulses. The content of enzyme inhibitors in Canadian pulses varied widely, but levels of protease inhibitors were generally lower that those found in soybean. Processing, in particular heat treatments, drastically reduced these levels.

3.2 Introduction

Legumes are an important food constituent for human beings and domestic animals around the world, particularly in tropical and subtropical countries. As one major category under legumes, pulses, also called grain legumes, are dry edible seeds harvested from leguminous plants (Tiwariet al., 2011). Of the total world pulse production in 2013 (73.2 million tonnes), Canada contributed approximately 6.1 million tonnnes, ranking the second highest in the world after India (18.3 million tonnes) (FAOSTAT, 2015). Canada has become a worldwide leader in the production of lentils and dry peas, one of top 10 producers of chickpeas and one of top 30 producers of dry beans (Bekkering, 2014).

Pulses are of great significance globally due to their nutritional quality. They contain adequate proportions of protein (21-25%) and starch (35-60%), along with high levels of dietary fibre (12.7-30.5 g/100 g), vitamins and minerals (Tiwari, & Singh, 2012; Wang et al., 2008). In addition to nutritional benefits, pulse consumption has also been linked to health benefits, including health-promoting and disease-preventing properties. Research has shown that increased dietary potassium may protect against risk of stroke (Larsson, Virtamo, & Wolk, 2011). Some studies have been carried out to indicate antihyperglycemic activity of common beans (Le Berre-Anton et al., 1997; Panlasigui, Panlilio, & Madrid, 1995). Adequate intake of dietary fibre has been associated with prevention of coronary heart disease (Bazzano et al., 2003), certain cancer (Park et al., 2009) and reduction in blood pressure (Streppel et al., 2005; Whelton et al., 2005).

However, underutilization of pulses is often attributed to the presence of certain heat labile and heat stable compounds, generally known as antinutritional

factors (Pusztai, Bardocz, & Martín-Cabrejas, 2004). They can be classified into two groups: (1) proteins (amylase inhibitors, protease inhibitors and lectins) which are unstable at normal processing temperatures, and (2) other substances (polyphenol compounds, non-protein amino acids and galactomannan gums) which are resistant to these temperatures (Martín-Cabrejas et al. 2009; Soetan, & Oyewole, 2009). Additionally, protease (trypsin and chymotrypsin) inhibitors can be further categorized into Kunitz type and Bowman-Birk type in terms of molecular weight (Richardson, 1991). The Kunitz inhibitors are approximately 20 kDa in size with 4 cysteines and 2 disulfide bonds, but Bowman-Birk types, with molecular mass of 8 kDa, have 14 cysteines, 7 disulfide bridges and 2 reactive sites (Bhattacharyya et al., 2007; Richardson, 1991). Kunitz types show inhibition on trypsin only, whereas Bowman-Birk type inhibitors inhibit trypsin on one reactive site and chymotrypsin on the other (Ferrasson et al., 1997). The mechanism of enzymatic inhibitory action, demonstrated by Norton (1991), was the interaction between enzyme (catalytic) site and inhibitor reactive site, resulting in the formation of enzyme-inhibitor complex; therefore, enzyme activity declined.

Proteinaceous inhibitors of α-amylase and proteases are naturally present throughout the plant kingdom (Udupa et al., 1989). Pancreatic and salivary α-amylase inhibitors have been reported to interfere with starch digestibility (Savelkoul et al., 1992; Wang et al., 2011). The occurrence of pancreatic hypertrophy was observed due to amylase inhibitors (Champ, 2002). Trypsin inhibitors are low molecular weight proteins capable of binding to trypsin, which is a proteolytic enzyme secreted by pancreas (Mondor et al., 2009; Savage, 1989). Therefore, protein degradability in the intestine tract is reduced and inadequate amino acids are available for production purpose in organisms (Makkar et al., 2007). Chymotrypsin inhibitor is considered to

have a similar mode of action as trypsin inhibitor, causing decreased digestion of protein, low availability of amino acids, and depressed growth and productive responses (Makkar et al., 2007; Savage, 1989).

To improve pulse utilization, numerous studies have been conducted on reduction or elimination of enzyme inhibitors using different food processing methods and treatments. Dehulling significantly increased α-amylase, trypsin and chymotrypsin inhibitory activities from pulses (Deshpande et al., 1982; Shekib et al., 1988). Soaking is a domestic treatment that increased enzymatic inhibitory activities (Martín-Cabrejas et al. 2009; Wang et al., 2008). This is contrary to the result from other investigators (Alonso et al., 2000a; Alonso et al., 1998; Khattab, & Arntfield, 2009; Mubarak, 2005; Shekib et al., 1988; Vadivel, & Pugalenthi, 2008) who reported a significant reduction in enzymatic inhibitory activity after soaking. Thermal processing has been proven to inactivate heat labile antinutrients (Ghorpade, Kadam, & Salunkhe, 1986). Cooking pulses in water dramatically decreased enzyme inhibitors (Alajaji, & El-Adawy, 2006; Khattab, & Arntfield, 2009; Mubarak, 2005; Shekib et al., 1988; Vadivel, & Pugalenthi, 2008; Wang et al., 2010). The levels of enzyme inhibitors in pulses are also markedly decreased by roasting, autoclaving, micronization, microwave cooking and extrusion cooking (Alajaji, & El-Adawy, 2006; Alonso et al., 1998, 2000a; Khattab, & Arntfield, 2009; Mubarak, 2005; Vadivel, & Pugalenthi, 2008).

In fact, many studies have focused on only the effect of various processing methods on selected antinutrients in a handful of pulses. Therefore, the present research was undertaken to provide a comprehensive evaluation of enzyme inhibitors in a wide range of market classes of Canadian pulses and to evaluate the effect of

limited processing (soaking and cooking) on these enzyme inhibitors using soybean for comparison.

3.3 Materials and methods

3.3.1 Materials

Seeds of 4 pea, 9 lentil, 2 fava bean, 3 chickpea, 4 bean and 1 soybean samples were received from AGT Foods and Ingredients (Regina, SK, Canada) and the Crop Development Centre at University of Saskatchewan (Saskatoon, SK, Canada). Unless otherwise noted, samples were from AGT. Samples included whole green pea, split green pea, whole yellow pea, split yellow pea, whole red lentil, split red lentil, football red lentil, split yellow lentil, split queen green lentil, French green lentil (CDC Marble), Spanish brown lentil (SB-2 3097-7 from CDC), large green lentil (CDC GreenStar), medium green lentil (CDC Imigreen), whole fava bean, split fava bean, whole chickpea B90, split chickpea B90, Desi chickpea (CDC Covy), pinto bean, dark red kidney bean, navy bean, black bean (CDC Expresso) and soybean (TH3303R2Y SB-Sorbia Preston from CDC). Flours for each seed were prepared at the University of Saskatchewan for direct measurement while seeds were subjected to different physical treatments. The raw seeds and flours were stored at -40 ^{o}C in sealed plastic bags until used.

Acetic acid, boric acid, calcium chloride dehydrate, dimethyl sulfoxide, sodium acetate anhydrous, sodium chloride, sodium hydroxide, sodium phosphate monobasic monohydrate, sodium potassium tartrate tetrahydrate, trichloroacetic acid and tris(hydroxymethyl)aminomethane were purchased from Fisher Scientific (Fair Lawn, NJ, USA). α-Amylase (from *Aspergillus oryzae*), α-amylase (from *Bacillus*

licheniformis), α-amylase (from porcine pancreas Type I-A), α-amylase (from porcine pancreas Type VI-B), casein (from bovine milk), α-chymotrypsin (from bovine pancreas Type II), 3,5-dinitrosalicylic acid, hexane, hydrochloric acid, Na-Benzoyl-D,L-arginine 4-nitroanilide hydrochloride (DL-BAPNA), maltose monohydrate, sodium phosphate dibasic dehydrate, sodium tetraborate decahydrate, starch (from potato) and trypsin (from bovine pancreas) were from Sigma-Aldrich (St. Louis, MO, USA).

3.3.2 Processing methods

3.3.2.1 Soaking

Raw seeds were soaked in distilled water at a ratio of 1:5 (seed:water, w/v) for 4 h at room temperature (~ 25 ^{o}C) (Khattab, & Arntfield, 2009). After soaking, the seeds were removed from soaking water, then rinsed with same volume of distilled water and drained.

3.3.2.2 Cooking

Presoaked seeds were cooked at 95 ^{o}C in an Isotemp Water Bath (Fisher Scientific, Hayward, CA, USA) at a seed-to-water ratio of 1:5 (w/v) for 1 h (ordinary cooking) (Vadivel, & Pugalenthi, 2008). After cooking, the water was drained.

3.3.2.3 Preparation of processed seeds and raw flours

The recoveries of Canadian pulses and soybean after soaking and cooking treatments are included in Appendix 1. The soaked and cooked seeds were dried at 55 ^{o}C in a hot air oven (Blue M Stabil-Therm Oven, Blue Island, IL, USA) overnight (Vadivel, & Pugalenthi, 2008). After drying, processed seeds were ground to pass through a 500 μm screen (USA Standard Testing Sieve) and the same step was applied to raw flours. All samples were stored at 4 ^{o}C in sealed plastic bags that were placed in a desiccator until analyzed.

3.3.2.4 Defatting of soybean flour

To obtain defatted soybean flour, the procedure reported by Chen (2015) was carried out as follows: 10 g of full fat soybean flour were suspended with 25 mL hexane in a 500 mL centrifuge bottle (Oak Ridge PPCO Centrifuge Tube, Rochester, NY, USA), and the mixture was shaken for 3 min on a Wrist Action Shaker (Burrell Scientific, PA, USA), after which the hexane layer was removed by centrifugation (Sorvall RC 6 Plus Centrifuge, Thermo Fisher Scientific Inc., NC, USA) at 600 × *g* for 1 min at 25 ^{o}C. The addition of 25 mL hexane to the centrifuge bottle and this extraction step was repeated twice. The defatted flour was dried in a fume hood at room temperature for 1 day, and then stored at 4 ^{o}C until used.

3.3.3 Analytical methods

3.3.3.1 Moisture content

Moisture in raw and processed samples was determined according to AACC International method 44-15.02 (AACC International, 1999). Aluminum dishes were first dried in a pre-heated air oven at 135 ^{o}C for 30 min, and then transferred to a desiccator. After cooling to room temperature, the weight of the dried dish was recorded accurately and approximately 2 g of sample flour was accurately weighed in the dish. After drying in a hot air oven at 100 ^{o}C for 16 h, the dishes were immediately transferred into a desiccator and weighed after 30 min. Sample moisture content can be calculated using the following formula:

$$\% \text{ Moisture} = \frac{\text{Weight of sample} + \text{Weight of dish} - \text{Weight after drying}}{\text{Weight of sample}} \times 100$$

The moisture contents of raw, soaked and cooked flours of Canadian pulses and soybean can be found in Appendix 2.

3.3.3.2 α-Amylase inhibitors

A 0.4 M sodium phosphate buffer (pH 7.0) containing 0.006 M NaCl was prepared by combining 234 mL of 0.4 M monobasic sodium phosphate and 366 mL of 0.4 M dibasic sodium phosphate with 0.2104 g of NaCl. The dinitrosalicylic acid colour reagent contained 1 g of 3,5- dinitrosalicylic acid, 30 g of sodium potassium tartrate tetrahydrate, and 20 mL of 2 N NaOH diluted up to 100 mL with distilled water. The α-amylase enzyme was diluted with sodium phosphate buffer to produce 40 unit/mL of α-amylase stock solution. One gram of soluble potato starch was

dissolved in boiling sodium phosphate buffer, cooled and then diluted to 100 mL with sodium phosphate buffer.

The method of Deshapande et al. (1982) was modified slightly to evaluate α-amylase inhibitory activity (AIA). One gram of ground sample was weighed in a 50 mL centrifuge tube (Oak Ridge PPCO Centrifuge Tube, Rochester, NY, USA) and mixed with 10 mL of distilled water. The mixture was vortexed (Vortex-Genie Mixer, Scientific Industries Inc., Bohemia, NY, and USA) for 1 min, extracted overnight on an ATR Rotamix (Appropriate Technical Resources Inc., MD, USA) at 4 ^{o}C and centrifuged at 31,920 × g for 20 min at 4 ^{o}C. α-Amylase enzyme solution, sodium phosphate buffer and a 1% starch solution were prewarmed at 37 ^{o}C in a water bath. Diluted extract (0.25 mL) containing the inhibitor was incubated with 0.25 mL of α-amylase enzyme solution in a test tube for 15 min at 37 ^{o}C. To this mixture was added 0.5 mL of starch solution and the reaction was terminated by addition of 2 mL dinitrosalicylic acid reagent after exactly 3 min. The test tube was then heated in boiling water for 10 min, cooled in running tap water and diluted with 10 mL of distilled water make a final volume of 13 mL. Whatman No. 1 paper (GE Healthcare UK Limited, Buckinghamshire, UK) was used to filter the solution before reading absorbance (Ultrospec 1100 pro, Amersham Pharmacia, and Piscataway, NJ, USA) at 540 nm. A blank was prepared in the same manner using sodium phosphate buffer rather than the α-amylase enzyme solution. A standard curve of maltose (0, 5, 10, 20, 40, 60 μmol/mL) was established to convert spectrophotometer readings into milligrams of maltose. One unit of α-amylase activity was defined as that which liberated, from soluble starch, one micromole of reducing groups (calculated as maltose) per min at 37 ^{o}C and pH 7.0 under the specified conditions. One unit of α-

amylase activity inhibited was defined as one α-amylase inhibitory unit. α-Amylase inhibitory activity was reported as AIU/g dry basis, and was calculated as following:

$$\frac{\text{AIU}}{\text{g sample}} = \frac{\text{Reduction in mg of maltose liberated}}{\text{3 min} \times \text{0.25 mL}} \times \frac{\text{10 mL of extract}}{\text{1 g of sample}} \times \text{D} \times \frac{\text{100\%}}{\text{100\%} - \text{MC}}$$

Where

D = dilution factor, and

MC= moisture content of flour.

The α-amylase inhibitor extract should be diluted to fall within 40 – 60% of α-amylase inhibition.

3.3.3.3 Trypsin inhibitors

Acetic acid solution contained 30 mL acetic acid in 70 mL distilled water. Trypsin solution was prepared by addition of 4 mg trypsin in 200 mL of 0.001 M HCl. The composition of Tris buffer (0.05 M, pH 8.2) was 6.05 g tris(hydroxymethyl)aminomethane and 2.94 g $CaCl_2$ in 1 L of distilled water. To prepare the substrate solution, 40 mg of DL-BAPNA was dissolved in 1 mL of dimethyl sulfoxide and then diluted to 100 mL with Tris buffer.

Trypsin inhibitory activity (TIA) was determined colorimetrically using an UV/visible spectrophotometer in accordance with AACC International method 22-40.01 (AACC International, 2000), with some modifications. No more than 0.5 g of finely ground flour, which passed through 140-mesh sieve with 105 μm pore size (USA Standard Testing Sieve), was placed into a 50 mL centrifuge tube. Twenty-five milliliters of 0.01 N NaOH was added to the tube, vortexed for 1 min and extracted for 3 h using an ATR Rotamix. The mixture was centrifuged at 14,190 × *g* for 10 min

at 4 ^{o}C. Aliquots of 0, 0.6, 1.0, 1.4 and 1.8 mL of diluted supernatant were pipetted into test tubes and each volume was adjusted to 2.0 mL with distilled water. Each tube was incubated with 2 mL of trypsin solution for 5 min at 37 ^{o}C. Five milliliters of prewarmed substrate solution (37 ^{o}C) was added into each test tube to initiate the reaction. The reaction was stopped by the addition of 1 mL of acetic acid solution after exactly 10 min. The mixed solution was filtered through Whatman No. 2 paper (GE Healthcare UK Limited, Buckinghamshire, UK). One trypsin unit was equivalent to an increase of 0.01 absorbance unit at 410 nm per 10 mL of reaction mixture compared to the blank sample (addition of trypsin solution after acetic acid). Trypsin inhibitor activity was defined as the amount of trypsin units inhibited per mg sample flour and expressed as TIU/mg dry basis, which can be calculated using the following formula (Mondor et al., 2009):

$$\frac{\text{TIU}}{\text{mg sample}} = \frac{\text{TIU}}{\text{mL of extract taken}} \times \frac{25 \text{ mL of extract}}{500 \text{ mg of sample}} \times \text{D} \times \frac{100\%}{100\% - \text{MC}}$$

Where

D = dilution factor, and

MC= moisture content of flour.

The trypsin inhibitor extract has to be diluted to fall within 40 – 60 % of trypsin inhibition at 1 mL.

3.3.3.4 Chymotrypsin inhibitors

Borate buffer (0.1 M) consisted of 50 mL of 0.2 M boric acid; 0.05 M borax was used to adjust pH to 7.6 and then diluted to a total of 200 mL. To prepare the substrate solution, 1 g of casein was dissolved in 100 mL of borate buffer and pH was adjusted to 7.6 with borax. Trichloroacetic acid reagent contained 18 g of

trichloroacetic acid, 18 g of anhydrous sodium acetate and 20 mL of glacial acetic acid and diluted up to 1 L with distilled water. A stock chymotrypsin solution of 4 mg of chymotrypsin was prepared in 100 mL of 0.001 M HCl containing 0.08 M $CaCl_2 \cdot 2H_2O$. .

Chymotrypsin inhibitory activity (CIA) was assayed according to the method described by Makkar et al. (2007) with the following modifications. To 1 g of flour sample in a 50 mL centrifuge tube, 10 mL of borate buffer was added, vortexed for 1 min and then extracted using an ATR Rotamix for 1 h. The slurry was centrifuged at 3000 × g for 10 min at 4 ^{o}C. In test tubes, 0, 0.25, 0.5 and 0.75 mL of diluted extract was added and diluted to 1 mL with borate buffer. These mixtures were incubated with 1 mL of stock chymotrypsin solution at 37 ^{o}C for 10 min. Subsequently 2 mL of casein solution, previously warmed to 37 ^{o}C, was added and mixed. At the end of 10 min, the reaction was stopped by the addition of 6 mL trichloroacetic acid reagent. The suspension stood at room temperature for at least 30 min and was then filtered through Whatman No. 2 paper. The absorbance of the filtrate was recorded at 275 nm against the appropriate blank. A blank contained 6 mL of trichloroacetic acid reagent before addition of 2 mL of casein solution. One chymotrypsin unit was defined as an increase of 0.01 absorbance unit at 275 nm for the reaction mixture. Chymotrypsin inhibitory activity is defined as the number of chymotrypsin units inhibited and the results were expressed as CIU per milligram of the sample:

$$\frac{\text{CIU}}{\text{mg sample}} = \frac{\text{CIU}}{\text{mL of extract taken}} \times \frac{\text{10 mL of extract}}{\text{1000 mg of sample}} \times \text{D} \times \frac{100\%}{100\% - \text{MC}}$$

Where

D = dilution factor, and

MC= moisture content of flour.

The diluted chymotrypsin inhibitor extract was optimized to obtain 40 – 60% of chymotrypsin inhibition at 0.5 mL.

3.3.4 Statistical analysis

All chemical analyses were conducted in triplicate and results are expressed as means ± standard deviation on dry matter basis. Two-way analysis of variance (ANOVA) for models with main effects and interaction was determined using the GLM procedure. Tukey's test was used to separate means and differences were considered to be significant at $P < 0.05$. The statistical analysis was performed by SAS Program version 9.3 (SAS Institute Inc., Gary, NC, USA).

3.4 Results and discussion

3.4.1 α-Amylase inhibitor activity

The α-amylase inhibitory activity in raw dark red kidney bean was tested against α-amylases of porcine pancreas Type I-A, porcine pancreas Type VI-B, *Bacillus licheniformis* and *Aspergillus oryzae* at 20 and 37 ˚C reaction temperatures (Table 3.1). Analysis of variance indicated that both α-amylase source and reaction temperature had significant ($P < 0.0001$) effects on α-amylase inhibitory activity of raw dark red kidney bean (Table 3.2). The interactive effect between α-amylase source and reaction temperature was also significant ($P < 0.0001$) (Table 3.2). At 20 ˚C, the highest inhibitory activity (380.86 AIU/g) was detected for α-amylase from porcine pancreas Type VI-B while at 37 ˚C, the inhibitor in dark red kidney bean was highest (1369.75 AIU/g) with porcine pancreas Type I-A α-amylase. There was no

inhibitory activity when using *Aspergillus oryzae* α-amylase and there was no significant different due to temperature when using *Bacillus licheniformis* α-amylase. These observations indicated that effectiveness of α-amylase inhibitors favoured pancreatic amylases rather than microbial amylases. This result was supported by Jaffé et al. (1973) who reported that higher inhibitory activity was shown by using pancreatic amylase than *Bacillus subtilis* amylase.

In addition to α-amylase source, a higher level of α-amylase inhibitory activity was found at 37 ˚C when compared with 20 ˚C for both types of porcine pancreas α-amylase. This finding revealed that the optimum temperature for porcine pancreas α-amylase was closer to 20 ˚C than 37 ˚C. A number of temperatures (20, 25, 30 and 37 ˚C) have been applied to study α-amylase inhibitory activity (Deshpande et al., 1982; Grant, Edwards, & Pusztai, 1995; Jaffé et al., 1973; Shekib et al., 1988). As a result, variations in α-amylase inhibitor activity due to temperature make it difficult to compare among investigations. In this study, porcine pancreas Type I-A α-amylase was selected for measurement at 37 ˚C, as this represented the temperature of the human body.

Table 3.1. Effect of α-amylase source and temperature on α-amylase inhibitory activity of raw dark red kidney bean

α-Amylase Source	α-Amylase inhibitory activity (AIU[1]/g dry matter)	
	20 ˚C	37 ˚C
Porcine pancreas Type I-A	337.56 ± 8.83^{bB}	1369.75 ± 82.23^{aA}
Porcine pancreas Type VI-B	380.86 ± 12.12^{bA}	1007.83 ± 5.73^{aB}
Bacillus licheniformis	6.64 ± 1.78^{aC}	7.11 ± 1.41^{aC}
Aspergillus oryzae	0.00 ± 0.00^{aC}	0.00 ± 0.00^{aC}

Row values followed by different superscript lowercase letters are significantly different ($P < 0.05$) as determined using Tukey's test.
Column values followed by different superscript uppercase letters are significantly different ($P < 0.05$) as determined using Tukey's test.
Means ± standard deviation of three determinations.
[1] AIU = α-amylase inhibitory unit.

Table 3.2. Analysis of variance of α-amylase inhibitory activity in raw dark red kidney bean using four amylase sources at two temperatures

Source	DF[1]	Mean Square	*F* Value	*P* Value
Whole model	7	831051.886	946.62	<0.0001
α-Amylase source	3	1209979.765	1378.24	<0.0001
Temperature	1	1031952.326	1175.45	<0.0001
α-Amylase source × temperature	3	385157.194	438.72	<0.0001

[1] DF = Degrees of freedom.

Data on the α-amylase inhibitory activity of Canadian pulses and soybean are presented in Table 3.3. No inhibitory activity was detected in peas, lentils, fava beans and chickpeas; only common beans and soybean contained measurable levels of α-amylase inhibitory activity. This was similar to the results of by Jaffé et al. (1973) who stated that α-amylase inhibitor in lentils and chickpeas only showed slight activity, but that in *Phaseolus vulgaris* was the most active. Grant et al. (1995) and Martín-Cabrejas et al. (2009) also found inhibitory activity in beans (pinto bean, kidney bean white bean and pink-mottled cream bean), but not in lentils or peas. Amongst common beans and soybean, the α-amylase inhibitor content was in the order: dark red kidney bean (1369.75 AIU/g) > navy bean (1079.83 AIU/g) > pinto bean (1000.91 AIU/g) > soybean (938.73 AIU/g) > black bean (785.58 AIU/g). Comparing with the results from an α-amylase inhibitor assay similar to that done in the current study, Deshpande et al. (1982) reported a range of 330 – 675 units/g for α-amylase inhibitor in 10 cultivars of *Phaseolus vulgaris* while only 248 units/g was detected in kidney bean by Alonso et al. (2000a). α-Amylase inhibitory activity of Canadian common beans was higher than these published data and may be due to the differences in climatic conditions, location, soil type and crop year.

Analysis of variance showed both legume type and treatment (soaking or cooking) had a significant effect on α-amylase inhibitory activity ($P < 0.0001$) (Table 3.4). The interactive effect of legume type × soaking or cooking on α-amylase

inhibitory activity was also significant (Table 3.4). There was about 4 – 10% reduction in α-amylase inhibitor content after soaking common beans and a 4% reduction in soybean (Table 3.3). Vadivel and Pugalenthi (2008) reported that the reduction in activity ranged between 25% and 28% in velvet bean seeds using the same soaking method. Alonso et al. (2000a) found that soaking of *Vicia faba* and *Phaseolus vulgaris* in water for 12 h caused 14.9% and 23.9% reduction in α-amylase inhibitory activity, respectively. The significant reduction in the level of α-amylase inhibitor during soaking may be due to the inhibitor leaching into steeping water (Vadivel, & Pugalenthi, 2008).

Cooking was more effective at decreasing α-amylase inhibitor content when compared with soaking. Approximately 80 – 93% reduction was caused by cooking beans; additionally, a complete inactivation was achieved in soybean (Table 3.3). These results are in accordance with those of Martín-Cabrejas et al. (2009) who stated that cooking of presoaked bean seeds caused 91 – 95% reduction in α-amylase inhibitory activity. Jaffé and Vegna (1968) reported that amylase inhibitor was completely destroyed in well-cooked *Phaseolus vulgaris*. The reduction of α-amylase inhibitory activity in the final product might be caused by a combination of leaching effect during soaking followed by inactivation of amylase inhibitor during the thermal treatment (Vadivel, & Pugalenthi, 2008).

α-Amylase inhibitory activity was only found in Canadian common beans and these values are higher than those detected in beans from USA. Soaking significantly reduced α-amylase inhibitor content; however, greater reduction has been seen after cooking. Currently, there is no estimated dietary exposure to amylase inhibitor.

Table 3.3. Effect of soaking and cooking on α-amylase inhibitory activity of Canadian pulses and soybean

Type	α-Amylase inhibitory activity (AIU[1]/g dry matter)		
	Raw	Soaked	Cooked
Pea			
Whole yellow pea	0.00 ± 0.00^{F}	nd[2]	nd
Split yellow pea	0.00 ± 0.00^{F}	nd	nd
Whole green pea	0.00 ± 0.00^{F}	nd	nd
Split green pea	0.00 ± 0.00^{F}	nd	nd
Lentil			
Whole red lentil	0.00 ± 0.00^{F}	nd	nd
Split red lentil	0.00 ± 0.00^{F}	nd	nd
Football red lentil	0.00 ± 0.00^{F}	nd	nd
Spanish brown lentil	0.00 ± 0.00^{F}	nd	nd
Split yellow lentil	0.00 ± 0.00^{F}	nd	nd
French green lentil	0.00 ± 0.00^{F}	nd	nd
Large green lentil	0.00 ± 0.00^{F}	nd	nd
Medium green lentil	0.00 ± 0.00^{F}	nd	nd
Split queen green lentil	0.00 ± 0.00^{F}	nd	nd
Fava bean			
Whole fava bean	0.00 ± 0.00^{F}	nd	nd
Split fava bean	0.00 ± 0.00^{F}	nd	nd
Chickpea			
Whole chickpea B90	0.00 ± 0.00^{F}	nd	nd
Split chickpea B90	0.00 ± 0.00^{F}	nd	nd
Desi chickpea	0.00 ± 0.00^{F}	nd	nd
Bean			
Dark red kidney bean	1369.75 ± 82.23^{aA}	1219.44 ± 30.18^{b} (10.97)	143.87 ± 3.46^{c} (89.50)
Pinto bean	1000.91 ± 35.99^{aC}	901.31 ± 19.23^{b} (9.95)	199.88 ± 1.21^{c} (80.03)
Navy bean	1079.83 ± 6.37^{aB}	1036.09 ± 5.77^{b} (4.05)	71.32 ± 0.42^{c} (93.40)
Black bean	785.58 ± 8.52^{aE}	731.70 ± 27.17^{b} (6.86)	71.24 ± 3.15^{c} (90.93)
Soybean	938.73 ± 19.17^{aD}	899.30 ± 4.55^{b} (4.20)	0.00 ± 0.00^{c} (100)

Row values followed by different superscript lowercase letters are significantly different ($P < 0.05$) as determined using Tukey's test.
Column values for raw samples followed by different superscript uppercase letters are significantly different ($P < 0.05$) as determined using Tukey's test.
Values in parentheses indicate % decrease over raw values.
Means ± standard deviation of three determinations.
[1] AIU = α-Amylase inhibitory unit.
[2] nd = not detected.

Table 3.4. Analysis of variance of effects of legume type and treatment on α-amylase inhibitory activity

Source	DF[1]	Mean Square	*F* Value	*P* Value
Whole model	14	9154663.186	922.41	<0.0001
Legume type	4	184525.302	260.29	<0.0001
Treatment	2	4063520.605	5732.05	<0.0001
Legume type × treatment	8	36190.096	51.05	<0.0001

[1] DF = Degrees of freedom.

3.4.2 Trypsin inhibitor activity

Results of trypsin inhibitory activity of raw and treated Canadian pulses and soybean are presented in Table 3.5. The trypsin inhibitor content was found to be significantly higher in raw soybean (45.89 TIU/mg) than in other pulses. This value was similar to that reported by Chen (2015) who detected 41.1 TIU/mg of trypsin inhibitory activity in defatted soybean flour. The data show that the content of trypsin inhibitor ranged from low, as in peas (3.16 – 4.92 TIU/mg), lentils (4.98 – 6.29 TIU/mg) and fava beans (5.96 – 6.10 TIU/mg), to relatively high in chickpeas (14.22 – 15.96 TIU/mg) and common beans (15.18 – 20.83 TIU/mg) (Table 3.5). These results are comparable to those stated by other researchers. Trypsin inhibitory activity of peas in this study falls within the range of the literature (2.80 – 6.32 TIU/mg) published by Alonso et al. (1998). Hernández-Infante et al. (1998) found that trypsin inhibitory activity was 3.6 TIU/mg in lentil (slightly lower than in the current work), 7.2 TIU/mg in faba bean (slightly higher than in the current work) and 17.9 TIU/mg in chickpea (slightly higher than in the current work).

When compared to whole seeds of yellow pea, green pea, red lentil, fava bean and chickpea B90, the split samples exhibited higher trypsin inhibitor content (Table 3.5). In this study, seed coat was removed as part of the splitting operation; therefore,

the split samples are actually dehulled seeds. An increase in trypsin inhibitory activity from pulses has been reported in a number of studies after dehulling process (Alonso et al., 1998; Alonso et al., 2000a; Deshpande et al., 1982; Mubarak, 2005). As Deshpande et al. (1982) suggest, this phenomenon might be due to the fact that trypsin inhibitors are present in the cotyledon fractions of pulses. After the seed coat is removed, the concentration of trypsin inhibitor increases on a unit weight basis.

Analysis of variance revealed that both legume type and treatment (soaking or cooking) had a significant effect ($P < 0.0001$) on trypsin inhibitory activity (Table 3.6). The interactive effect of legume type-by-treatment was also significant ($P < 0.0001$) (Table 3.6). Soaking seeds in water caused a significant reduction of trypsin inhibitor in peas (17.34 – 30.74%), lentils (5.57 – 19.35%), fava beans (12.73 – 22.59%), chickpeas (9.39 – 25.27%), common beans (4.88 – 9.09%) and soybean (18.58%) (Table 3.5). These results were consistent with those reported by other investigators, indicating loss of 4.47% in faba bean, 5.48% in kidney bean and 1.58 – 12.02% in peas after soaking (Alonso et al., 1998; Alonso et al., 2000a). This loss might be attributed to leaching of trypsin inhibitor into the soaking water. Cooking brought a total removal of trypsin inhibitory activity in split yellow and green peas, lentils and faba beans. A relatively high reduction was also noted in common beans (92.58 – 93.70%) and soybean (92.83%), followed by chickpeas (83.92 – 88.37%) and whole yellow and green peas (78.74 – 81.25%) (Table 3.5). These agree with the results observed by Khattab and Arntfield (2009), Martín-Cabrejas et al. (2009) and Wang et al. (2010) who reported that trypsin inhibitor content decreased by 100%, 83.3 – 100% and 79.6 – 93.5% in peas, beans and chickpeas, respectively, when heated in boiling water. Trypsin inhibitors are heat sensitive and can be inactivated by

cooking due to denaturation of these heat-liable proteins (Vidal-Valverde et al., 1994).

Canadian soybean contained the highest trypsin inhibitor level when compared with other Canadian pulses. Basically, split seeds had higher trypsin inhibitory activity then whole seeds. The levels of trypsin inhibitor in Canadian soybean and peas were similar to those found in other Canadian soybean and Spanish peas. However, trypsin inhibitor contents were lower in Canadian faba bean and chickpea, but higher in lentils when compared with same types from Mexico. Both soaking and cooking processes could significantly decrease trypsin inhibitory activity in pulses, but cooking appears to be more effective. British diets provided approximately 330 mg of trypsin inhibitor intake per person per day (Doell et al., 1981). However, it has been estimated by Billings et al. (1990) that the median daily intakes of trypsin was 4.6 mg/day (range 0 – 127.9 mg/day).

Table 3.5. Effect of soaking and cooking on trypsin inhibitory activity of Canadian pulses and soybean

Type	Trypsin inhibitory activity (TIU[1]/mg dry matter)		
	Raw	Soaked	Cooked
Pea			
Whole yellow pea	3.16 ± 0.04^{aO}	2.62 ± 0.11^{b} (17.34)	0.59 ± 0.02^{c} (81.25)
Split yellow pea	4.18 ± 0.13^{aN}	2.90 ± 0.08^{b} (30.74)	0.00 ± 0.00^{c} (100)
Whole green pea	4.65 ± 0.09^{aMN}	3.78 ± 0.07^{b} (18.56)	0.99 ± 0.00^{c} (78.74)
Split green pea	4.92 ± 0.12^{aLMN}	3.86 ± 0.09^{b} (21.61)	0.00 ± 0.00^{c} (100)
Lentil			
Whole red lentil	5.99 ± 0.19^{aHIJ}	5.42 ± 0.23^{b} (9.59)	0.00 ± 0.00^{c} (100)
Split red lentil	6.29 ± 0.35^{aH}	5.07 ± 0.36^{b} (19.35)	0.00 ± 0.00^{c} (100)
Football red lentil	5.88 ± 0.26^{aHIJK}	4.94 ± 0.58^{b} (15.89)	0.00 ± 0.00^{c} (100)
Spanish brown lentil	4.98 ± 0.08^{aLM}	4.39 ± 0.22^{b} (11.90)	0.00 ± 0.00^{c} (100)
Split yellow lentil	5.17 ± 0.21^{aLKM}	4.32 ± 0.03^{b} (16.51)	0.00 ± 0.00^{c} (100)
French green lentil	5.14 ± 0.07^{aKLM}	4.24 ± 0.14^{b} (17.64)	0.00 ± 0.00^{c} (100)
Large green lentil	6.21 ± 0.21^{aHI}	5.67 ± 0.10^{b} (8.71)	0.00 ± 0.00^{c} (100)
Medium green lentil	5.40 ± 0.09^{aJKLM}	5.10 ± 0.14^{b} (5.57)	0.00 ± 0.00^{c} (100)
Split queen green lentil	5.46 ± 0.10^{aIJKL}	4.21 ± 0.10^{b} (22.93)	0.00 ± 0.00^{c} (100)
Fava bean			
Whole fava bean	5.96 ± 0.27^{aHIJ}	5.20 ± 0.10^{b} (12.73)	0.00 ± 0.00^{c} (100)
Split fava bean	6.10 ± 0.34^{aHIJ}	4.72 ± 0.31^{b} (22.59)	0.00 ± 0.00^{c} (100)
Chickpea			
Whole chickpea B90	14.22 ± 0.13^{aG}	12.89 ± 0.26^{b} (9.39)	2.29 ± 0.07^{c} (83.92)
Split chickpea B90	16.24 ± 0.24^{aDE}	12.14 ± 0.39^{b} (25.27)	1.89 ± 0.03^{c} (88.37)
Desi chickpea	15.96 ± 0.22^{aEF}	13.85 ± 0.21^{b} (11.19)	1.88 ± 0.21^{c} (87.95)
Bean			
Dark red kidney bean	17.77 ± 0.27^{aC}	16.90 ± 0.18^{b} (4.88)	1.32 ± 0.06^{c} (92.58)
Pinto bean	15.18 ± 0.10^{aF}	13.80 ± 0.54^{b} (9.09)	0.99 ± 0.07^{c} (93.46)
Navy bean	16.44 ± 0.45^{aD}	15.02 ± 0.73^{b} (8.63)	1.36 ± 0.03^{c} (91.72)
Black bean	20.83 ± 0.33^{aB}	19.55 ± 0.26^{b} (6.18)	1.31 ± 0.08^{c} (93.70)
Soybean	45.89 ± 0.51^{aA}	37.37 ± 0.57^{b} (18.58)	3.29 ± 0.23^{c} (92.83)

Row values followed by different superscript lowercase letters are significantly different ($P < 0.05$) as determined using Tukey's test.
Column values for raw samples followed by different superscript uppercase letters are significantly different ($P < 0.05$) as determined using Tukey's test.
Values in parentheses indicate % decrease over raw values.
Means ± standard deviation of three determinations.
[1] TIU = trypsin inhibitory unit.

Table 3.6. Analysis of variance of effects of legume type and treatment on trypsin inhibitory activity

Source	DF[1]	Mean Square	*F* Value	*P* Value
Whole model	68	193.23086	30.83	<0.0001
Legume type	22	332.187333	53.01	<0.0001
Treatment	2	1733.443830	276.61	<0.0001
Legume type × treatment	44	53.742950	8.58	<0.0001

[1] DF = Degrees of freedom.

3.4.3 Chymotrypsin inhibitor activity

The results of chymotrypsin inhibitory activity in Canadian pulses and soybean are summarized in Table 3.7. Chymotrypsin inhibitor is widely distributed in legumes; soybean had the highest content (30.16 CIU/mg) among the investigated seeds. Common beans (17.77 – 24.48 CIU/mg) and chickpeas (11.78 – 13.59 CIU/mg) contained relatively high inhibitory activity, followed by lentils (3.51 – 4.89 CIU/mg) and peas (2.84 – 3.34 CIU/mg). Fava beans had the lowest chymotrypsin inhibitor content (1.12 – 1.67 CIU/mg). Limited research has been conducted on chymotrypsin inhibitory activity in legume seeds. Deshpande et al. (1982) reported a range of 217 – 345 units/mg for chymotrypsin inhibitor content in 10 cultivars of *Phaseolus vulgaris*. The range for inhibitory activity in peas was 2.73 – 4.85 units/mg (Alonso et al., 1998). Alonso et al. (2000a) also reported levels of 3.56 and 3.97 units/mg of chymotrypsin inhibitor in *Vicia faba* and *Phaseolus vulgaris*, respectively. The considerable variations noted could mainly be attributed to the different chymotrypsin inhibitor assays used. Using a similar method to the one in the current study, Singh and Jambunathan (1981) found that chymotrypsin inhibitory activity was 7.6 – 8.8 CIU/mg for desi chickpeas and 6.1 – 8.0 CIU/mg for kabuli chickpeas; these values are close to ours.

From Table 3.5 and Table 3.7, it can be seen that the activity of trypsin inhibitor is higher than that of chymotrypsin inhibitor in all seeds, except for common beans. These results are in agreement with those reported by Alonso et al. (1998), Alonso et al. (2000a), Deshpande et al. (1982), Singh and Jambunathan (1981) in peas, faba beans, common beans and chickpeas. Similar to trypsin inhibitor, chymotrypsin inhibitor content was higher in split seeds of green and yellow peas, fava bean and chickpea B90 than those whole seeds (Table 3.7). As reported by Alonso et al. (1998), Alonso et al. (2000a) and Deshpande et al. (1982), in *Phaseolus vulgaris*, *Pisum sativum* and *Vicia faba*, the dehulling process increased chymotrypsin inhibitory activity, by concentrating the inhibitors associated with the cotyledon.

The levels of chymotrypsin inhibitor in processed Canadian pulses and soybean are also shown in Table 3.7. Analysis of variance indicated that legume type, treatment (soaking or cooking) and their interaction exerted significant effects ($P < 0.0001$) on chymotrypsin inhibitory activity (Table 3.8). Soaking resulted in significant losses of chymotrypsin inhibitor content in peas (8.35 – 13.80%), lentils (5.64 – 12.68%), fava beans (11.43 – 17.51%), chickpeas (11.14 – 18.58%), beans (7.60 – 12.40%) and soybean (6.71%) (Table 3.7). This confirmed the work of Alonso et al. (1998) and Alonso et al. (2000a) who reported that 13.4 – 17.6%, 8.43% and 15.1% reductions in chymotrypsin inhibitory activity in pea, faba bean and kidney bean, respectively. The significant decreases, like those seen for α-amylase and trypsin inhibitors, might be due to leaching into the soaking water. Complete removal of chymotrypsin inhibitory activity was found for all studied seeds after cooking (Table 3.7). These results agree with those reported by Martín-Cabrejas et al. (2009) who found that cooked seeds of chickpea, white bean and pink-mottled cream bean exhibited 100% reduction in chymotrypsin inhibitory activity. Other studies have also

shown that cooking is an effective processing method to inactivate protease inhibitors in pulses (Gatta et al., 1989; Wang et al., 1997). As Wang et al. (2010) stated, pulses can be softened by sufficient cooking and the levels of protease inhibitors may also be inactivated or reduced simultaneously. Therefore, cooking enables improvement of the nutritional value of pulses. However, over-cooking may decrease nutritional quality due to loss of essential amino acids in pulses (Wang et al., 2008; Wang et al., 2010; Youssef et al., 1986).

Canadian soybean contained the highest chymotrypsin inhibitory activity among all investigated seeds. The levels of chymotrypsin inhibitor in Canadian common beans were higher than those determined in Spain but lower than those from USA. Canadian fava beans had lower chymotrypsin inhibitor content than Spanish fava beans. Canadian chickpeas contained similar chymotrypsin inhibitory activity with Indian desi and kabuli chickpeas. Soaking significantly reduced chymotrypsin inhibitor content in seeds while this enzyme inhibitor could be removed completely by cooking. Billings et al. (1990) also estimated the median daily intakes of chymotrypsin inhibitor was 1.6 mg/day (range 0 – 31.1 mg/day).

Table 7.7. Effect of soaking and cooking on chymotrypsin inhibitory activity of Canadian pulses and soybean

Type	Chymotrypsin inhibitory activity (CIU[1]/mg dry matter)		
	Raw	Soaked	Cooked
Pea			
Whole yellow pea	2.84 ± 0.09^{aL}	2.56 ± 0.21^{b} (9.83)	0.00 ± 0.00^{c} (100)
Split yellow pea	3.23 ± 0.20^{aJKL}	2.89 ± 0.09^{b} (10.28)	0.00 ± 0.00^{c} (100)
Whole green pea	3.13 ± 0.03^{aKL}	2.87 ± 0.07^{b} (8.35)	0.00 ± 0.00^{c} (100)
Split green pea	3.34 ± 0.17^{aIJKL}	2.88 ± 0.08^{b} (13.80)	0.00 ± 0.00^{c} (100)
Lentil			
Whole red lentil	4.14 ± 0.33^{aGHIJ}	3.87 ± 0.34^{b} (6.62)	0.00 ± 0.00^{c} (100)
Split red lentil	4.89 ± 0.17^{aG}	4.58 ± 0.24^{b} (6.40)	0.00 ± 0.00^{c} (100)
Football red lentil	4.66 ± 0.13^{aGH}	4.19 ± 0.45^{b} (10.21)	0.00 ± 0.00^{c} (100)
Spanish brown lentil	3.51 ± 0.10^{aIJKL}	3.15 ± 0.30^{b} (10.23)	0.00 ± 0.00^{c} (100)
Split yellow lentil	4.58 ± 0.26^{aGHI}	4.00 ± 0.24^{b} (12.68)	0.00 ± 0.00^{c} (100)
French green lentil	3.71 ± 0.12^{aHIJKL}	3.42 ± 0.18^{b} (7.82)	0.00 ± 0.00^{c} (100)
Large green lentil	4.55 ± 0.11^{aGH}	4.10 ± 0.11^{b} (9.78)	0.00 ± 0.00^{c} (100)
Medium green lentil	3.89 ± 0.09^{aHIJK}	3.42 ± 0.19^{b} (12.03)	0.00 ± 0.00^{c} (100)
Split queen green lentil	4.02 ± 0.03^{aGHIJK}	3.79 ± 0.25^{b} (5.64)	0.00 ± 0.00^{c} (100)
Fava bean			
Whole fava bean	1.12 ± 0.09^{aM}	0.99 ± 0.09^{b} (11.43)	0.00 ± 0.00^{c} (100)
Split fava bean	1.67 ± 0.11^{aM}	1.37 ± 0.10^{b} (17.51)	0.00 ± 0.00^{c} (100)
Chickpea			
Whole chickpea B90	12.29 ± 0.34^{aF}	10.83 ± 0.14^{b} (11.85)	0.00 ± 0.00^{c} (100)
Split chickpea B90	13.59 ± 0.26^{aE}	11.06 ± 0.26^{b} (18.58)	0.00 ± 0.00^{c} (100)
Desi chickpea	11.78 ± 0.22^{aF}	10.47 ± 0.32^{b} (11.14)	0.00 ± 0.00^{c} (100)
Bean			
Dark red kidney bean	21.00 ± 0.88^{aC}	18.81 ± 0.19^{b} (10.45)	0.00 ± 0.00^{c} (100)
Pinto bean	17.77 ± 0.07^{aD}	16.50 ± 0.18^{b} (9.68)	0.00 ± 0.00^{c} (100)
Navy bean	18.67 ± 0.12^{aD}	17.25 ± 0.59^{b} (7.60)	0.00 ± 0.00^{c} (100)
Black bean	24.48 ± 0.97^{aB}	21.42 ± 0.61^{b} (12.40)	0.00 ± 0.00^{c} (100)
Soybean	30.16 ± 0.17^{aA}	28.14 ± 0.45^{b} (6.71)	0.00 ± 0.00^{c} (100)

Row values followed by different superscript lowercase letters are significantly different ($P < 0.05$) as determined using Tukey's test.
Column values for raw samples followed by different superscript uppercase letters are significantly different ($P < 0.05$) as determined using Tukey's test.
Values in parentheses indicate % decrease over raw values.
Means ± standard deviation of three determinations.
[1] CIU = chymotrypsin inhibitory unit.

Table 3.8. Analysis of variance of effects of legume type and treatment on chymotrypsin inhibitory activity

Source	DF[1]	Mean Square	*F* Value	*P* Value
Whole model	68	167.80045	2729.03	<0.0001
Legume type	22	247.669715	4027.99	<0.0001
Treatment	2	1605.094526	26104.5	<0.0001
Legume type × treatment	44	62.534272	1017.03	<0.0001

[1] DF = Degrees of freedom.

3.5 Conclusion

As shown in this study, legume type, treatment (soaking or cooking) and their interaction had significant effects on the levels of all enzyme inhibitors (α-amylase, trypsin and chymotrypsin inhibitors) in Canadian pulses and soybean. Considerable differences in these enzyme inhibitors were detected among all investigated seeds. Soybean contained relatively high contents of protease inhibitors when compared with the pulses. Considering the treatments evaluated in this research, the cooking process was more effective than soaking in reducing enzyme inhibitors in seeds as a result of their heat sensitivity. Information obtained from the present study would help pulse industry and regulatory bodies in selecting the appropriate pulse for potential markets and further utilization.

4. LEVELS OF LECTINS, PHYTIC ACID AND OXALATES IN CANADIAN PULSES AS AFFECTED BY SOAKING AND COOKING METHODS

4.1 Abstract

Raw and processed (soaked or cooked) seeds of peas, lentils, chickpeas, fava beans and common beans were studied for their contents of antinutritional factors (lectins, phytic acid, total and soluble oxalates), along with soybean as a control. Analysis of variance indicated that legume type, treatment and their interactive effects were significant ($P < 0.0001$) on these antinutrients. The raw soybean seed was found to contain the highest levels of lectins (692.8 HU/mg), phytic acid (22.91 mg/g), total oxalate (370.5 mg/100 g) and water-soluble oxalate (200.7 mg/100 g) among all investigated seeds. Relatively high contents of lectins were detected in beans (87.69 – 88.59 HU/mg) and other pulses ranging from 2.73 to 11.07 HU/mg. Phytic acid in Canadian pulses varied slightly from 8.55 to 22.85 mg/g. Total oxalates were variable, ranging from 244.7 to 294.0 mg/100 g in peas, 168.6 – 289.1 mg/100 g in lentils, 241.5 – 291.4 mg/100 g in fava beans, 92.2 – 214.0 mg/100 g in chickpeas and 98.86 – 117.0 mg/100 g in common beans. Approximately 24 – 72% of total oxalate appeared to be water-soluble in all investigated pulses. Soaking the seeds in distilled water significantly decreased the contents of lectins (0.11 – 5.18%), total oxalate (17.40 – 51.89%) and soluble oxalate (26.66 – 56.29%), but had no impact on phytic acid. The cooking process was found to be more effective in reducing levels of all the investigated antinutritional factors, except phytic acid in common beans and soybean.

4.2 Introduction

Pulses such as lentils, dry beans, chickpeas and dry peas are considered a significant food source for human beings. Whole and split lentils can be processed for consumption as snacks; the flour is used as an ingredient in baked goods and baby foods (AAFC, 2009). Due to a large vegetarian population, lentils act as a meat substitute in Indian cuisine (Asif et al., 2013). Commercially, beans are either canned, packaged dry for home preparation or further processed into products, e.g. milled to make flour (AAFC, 2009; Abu-Ghannam, & Gowen, 2011; Siddiq, & Uebersax, 2013). In households, dry beans are eaten as vegetables by adding to soup or combining with meat to make a main dish (Siddiq, & Uebersax, 2013). Chickpeas are mainly eaten as green vegetables in salad as well as used in snack foods and condiments; in addition, the seeds can be ground to produce flour for bread making (AAFC, 2009; Asif et al., 2013). Hummus, a typical Middle East food dip eaten with pita and bread, is composed of chickpea (Asif et al., 2013). Although dry peas share a smaller food market compared to other pulses, the functional properties (emulsifying, gelling and water absorption properties) of pea proteins enhance the utilization of pea powder in soups, bakery and puffed products (AAFC, 2009; Asif et al., 2013).

Pulse utilization, however, is often limited due to the presence of a series of compounds, known as antinutritional factors; included in this group are lectins, phytic acid and oxalates.

Lectins (or haemagglutinins/phytohaemagglutinis) are glycoporteins that are able to bind sugars or proteins (Champ, 2002; Roberts, & Goldstein, 1984). They can interact with specific sugars in cell membranes, causing agglutination of red blood cells *in vitro* (Savelkoul et al., 1992). As a result, nutrient absorption might be

disrupted when crossing the intestinal wall (Champ, 2002; Savelkoul et al., 1992). The toxicity of lectins may also lead to growth inhibition, diarrhea, nausea, bloating and vomiting (Liener, 1989b).

Phytic acid, also known as myo-inositol hexakisposphate or IP6, is the principal storage form of phosphorus in seeds and phytate is the salt form of this acid (Tiwari, & Singh, 2012). Phytic acid may complex with a number of minerals such as calcium, copper, magnesium, iron and zinc, so that it adversely affects the absorption and utilization of dietary minerals in the gastrointestinal tract (Davies, & Nightingale, 1975; Guenter, & Sell, 1974; O'Dell, & Savage, 1960; Ramakrishna et al., 2006; Tiwari, & Singh, 2012; Welch, House, & Allawy, 1974; Welch, & Van Campen, 1975). For instance, people with daily high pulse consumption may have anaemia because of iron deficiency (Tiwari, & Singh, 2012). The availability of phosphorus in phytate upon consumption is low since only small amounts of phytate can be hydrolyzed in the small intestine in monogastric species (Drewnowski, & Gomez-Carneros, 2000). Additionally, proteolytic digestion is inhibited due to binding of phytic acid and proteins, proteases or amylases (O'Dell, & De Borland, 1976; Ramakrishna et al., 2006; Tiwari, & Singh, 2012).

Oxalic acid [$(COOH)_2$] and its salts, known as oxalates, are detected in most pulses (Champ, 2002). Water-soluble (oxalic acid or potassium, sodium and ammonium oxalates) and insoluble oxalates (particularly the calcium salt) are the two major forms (Judprasong et al., 2006; Ross et al., 1999). Similar to phytates, oxalates are considered to be antinutrients since they may decrease mineral bioavailability (Hazell, & Johnson, 1987; Proulx, Weaver, & Bock, 1993). More importantly, high consumption of oxalate-rich food may cause oxalosis and increase urinary oxalate excretion (Hönow, & Hesse, 2002). An elevated urinary oxalate excretion can result

in calcium oxalate crystallization and formation of kidney stone (Hönow, & Hesse, 2002; Ross et al. 1999).

Generally, raw seeds contain higher amounts of antinutritional factors when compared with processed seeds, and therefore it is necessary to employ different processing techniques before incorporation into human food and animal diets (Aguilera et al., 2013). Previous studies (Alonso et al., 1998; Alonso et al., 2000a; Deshpande et al., 1982; Wang et al., 2008) showed that dehulling significantly increased phytic acid content, but Mubarak (2005) found a contrary result. The soaking process has been reported to reduce the levels of lectins and phytic acid (Kalpanadevi, & Mohan, 2013; Khattab, & Arntfield, 2009; Luo, & Xie, 2013; Mubarak, 2005; Vadivel, & Pugalenthi, 2008; Wang et al., 2008). Ordinary cooking caused considerable losses in lectins, phytic acid and oxalates (Akhtar et al., 2011; Alajaji, & El-Adawy, 2006; Catherwood et al., 2007; Hernández-Infante et al., 1998; Kalpanadevi, & Mohan, 2013; Khattab, & Arntfield, 2009; Luo, & Xie, 2013; Mubarak, 2005; Quinteros et al., 2003; Ramakrishna et al., 2006; Savage et al., 2000; Vadivel, & Pugalenthi, 2008; Wang et al., 2008; Wang et al., 2010). Other treatments, such as autoclaving, extrusion, fermentation, germination, micronization, microwave cooking, and roasting (Alajaji, & El-Adawy, 2006; Alonso et al., 1998; Alonso et al., 2000a; Hernández-Infante et al., 1998; Kalpanadevi, & Mohan, 2013; Khattab, & Arntfield, 2009; Luo, & Xie, 2013; Maidala, Doma, & Egbo, 2013; Mubarak, 2005; Quinteros et al., 2003; Ramakrishna et al., 2006; Vadivel, & Pugalenthi, 2008) have also affected the contents of these antinutritional factors.

Even though some reports were available on the antinutritional properties of different pulses, information on antinutritional factors of a wide range of market classes of Canadian pulses appears to be lacking. Hence, the current study was carried

out to create a database of selected antinutritional factors (lectins, phytic acid, total and soluble oxalates) in a number of Canadian pulses and also to analyze the effects of soaking and cooking treatments on the levels of these antinutrients, using soybean as a control.

4.3 Materials and methods

4.3.1 Materials

Seeds of whole green pea, split green pea, whole yellow pea, split yellow pea, whole red lentil, split red lentil, football red lentil, split yellow lentil, split queen green lentil, whole fava bean, split fava bean, whole chickpea B90, split chickpea B90, pinto bean, dark red kidney bean and navy bean were supplied from AFT Foods and Ingredients (Regina, SK, Canada). Seeds of French green lentil (Marble), Spanish brown lentil (SB-2 3097-7), large green lentil (GreenStar), medium green lentil (Imigreen), Desi chickpea (Covy), black bean (Expresso) and soybean (TH3303R2Y SB-Sorbia Preston) were provided by the Crop Development Centre at University of Saskatchewan (Saskatoon, SK, Canada). Flours, ground at the University of Saskatchewan, were used for direct determinations, but the seeds were employed for domestic processing methods. The raw seeds and flours were stored in polyethylene bags at -40 ^{o}C until use.

Dipotassium hydrogen phosphate, ferric chloride, potassium dihydrogen phosphate, sodium chloride sodium hydroxide and trichloroacetic acid were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Hexane, hydrochloric acid, phytic acid, purified red kidney bean lectin and 5-sulfosalicylic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA).

4.3.2 Processing methods

4.3.2.1 Soaking

The seeds were soaked in distilled water at room temperature (~ 25 ^{o}C) for 4 h at seed to water ratio of 1 g:5 mL (Khattab, & Arntfield, 2009). After soaking, the soaked water was drain. The seeds were rinsed with same volume of distilled water and then divided into two portions. The first portion was dried at 55 ^{o}C in a hot air oven overnight (Vadivel, & Pugalenthi, 2008). The second portion was used for the cooking treatment.

4.3.2.2 Cooking

Presoaked seeds were cooked in distilled water (seed:water, 1:5) using Isotemp Water Bath at 95 ^{o}C for 1 h (Vadivel, & Pugalenthi, 2008). Cooked seeds were then dried in a hot air oven overnight at 55 ^{o}C.

4.3.2.3 Preparation of processed seeds and raw flours

The recoveries of Canadian pulses and soybean after soaking and cooking processes can be found in Appendix 1. Soaked and cooked seeds were ground into flour using a stainless steel coffee grinder (DCG-12BCC Cuisinart Canada, Ontario, Canada). Raw and processed flours were sieved through a 500 μm screen (USA Standard Testing Sieve) and then stored in plastic bags at 4 ^{o}C prior to use.

4.3.2.4 Defatting of soybean flour

The defatting procedure of soybean flour was performed according to the method described by Chen (2015). Hexane was employed to extract fat from raw and processed soybean flours (soybean flour:hexane = 2:5, w/v). The suspension was mixed thoroughly and centrifuged (600 × *g*, 1 min, 25 ^{o}C) to remove the supernatant. Soybean flours were extracted with hexane another two times, dried 24 h in a fume hood and placed in sealed plastic bags at 4 ^{o}C until further analysis.

4.3.3 Analytical methods

4.3.3.1 Moisture content

Moisture content determination was based on AACC International (1999) method (44-15.02). Aluminum dishes were pre-dried in a hot air oven at 135 ^{o}C for 30 min, and then cooled in a desiccator prior to weighing. Approximately 2 g of sample were weighed in the dish and dried in the air oven at 100 ^{o}C for 16 h. The dried samples and dishes were transferred to a desiccator and weighed after 30 min. Calculation for sample moisture content is as follow:

$$\% \text{ Moisture} = \frac{\text{Wt of wet sample} + \text{Wt of dish} - \text{Wt of (dish} + \text{dried sample)}}{\text{Wt of wet sample}} \times 100$$

The moisture contents of raw, soaked and cooked flours of Canadian pulses and soybean are included in Appendix 2.

4.3.3.2 Hemagglutinating activity (lectins)

To extract hemagglutinin from the raw or processed sample, 10 mL of 0.9% NaCl (saline) was added into a 50 mL centrifuge tube containing 1 g of ground flour according to the procedure of Liener and Hill (1953). The mixture was vortexed for 1 min and extraction was completed after 1 h using an ATR Rotamix at speed 30. The suspension was left in the refrigerator overnight and was then centrifuged at 14,190 × *g* for 10 min at 4 ^{o}C. After filtrating through cheesecloth (Fisher Scientific, Fair Lawn, NJ, USA), the clear supernatant was serially diluted in a 96-well flat bottom microplate (Fisher Scientific, Fair Lawn, NJ, USA) based on a dilution range of none in well 1 to 1:8191 (extract:saline, v/v) in well 14. To each well was added an equal volume of 2% rabbit red blood cells (CedarLane, Burlington, ON, Canada) (final volume 0.1 mL). The mixed samples were shaken gently by a Belly Dancer (Stovall Life Science, Inc., Greensboro, NC, USA) and left at room temperature for 2 h. Hemaggutining activity was determined microscopically (Eagle CMSP240 Microscope, Burlingame, CA, USA) as described by Makkar et al. (1997). One drop of the contents from each well was transferred to a glass slide (Fisher Scientific, Fair Lawn, NJ, USA) using a Pasteur pipette and covered with a microscope cover (Fisher Scientific, Fair Lawn, NJ, USA). At least five cells aggregating together indicated a positive evidence of agglutination. Red blood cells alone in saline were prepared in same manner to serve as the blank and the purified red kidney bean lectin, diluted in phosphate buffered saline (pH 6.8) (lectin:phosphate buffered saline = 1:1, w/v) was used as a positive control for comparison (Figure 4.1).

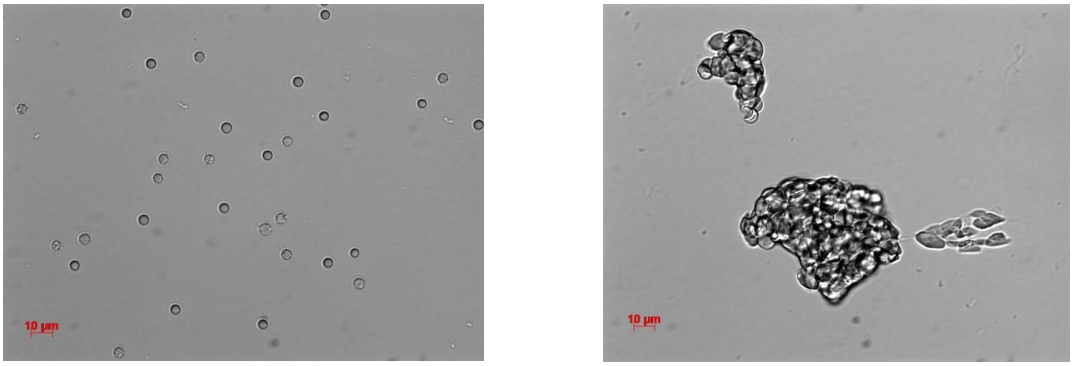

Figure 4.1. Photomicrographs of negative and positive agglutination of rabbit erthrocytes. Magnification (×400). Left: negative agglutination (blank). Right: positive agglutination (purified lectins from red kidney bean)

The last dilution of hemagglutinin producing a positive agglutination response was defined as that which contained one hemagglutinating unit (HU). The specific hemagglutinating activity (HU/mg flour on a dry basis) was calculated as follows (Liener and Hill, 1953):

$$\mathrm{HU/mg} = \frac{D_a \times D_b \times S}{V} \times \frac{100\%}{100\% - MC}$$

Where

V = volume of extract in well 1,

D_a = dilution factor of extract in well 1 (= 1 unless original extract was diluted),

D_b = dilution factor of tube containing 1 HU, and

S = mL original extract/mg flour, and

MC = moisture content of flour.

4.3.3.3 Phytic acid

Phytic acid was quantified in dried flours using the Latta and Eskin (1980) method, with minor modifications. Accurately 0.5 g of sample was mixed with 10 mL of 2.4% HCl in a 50 mL centrifuge using a Vortex-Genie Mixer for 1 min and

extracted on an ATR Rotamix at speed 30 for 1 h. The resulting mixture was centrifuged at 14,190 × *g* at 20 ^{o}C for 10 min and the supernatant was diluted with distilled water based on an appropriate dilution factor. Ion-exchange chromatography was used for phytate purification. Ten milliliters of diluted extract was passed through a 0.7×15 cm chromaflex column (Kimble Chase, Vineland, NJ, USA) filled with glass wool (Acros Organics, NJ, USA) and 0.5 g of 200-400 mesh AG 1-X8 resin in chloride form (Bio-Rad Laboratories, Inc., Herclues, CA, USA). Fifteen milliliters of 0.1 M NaCl was applied to the column to elute inorganic phosphorus; this was followed by elution of phytate with 15 mL of 0.7 M NaCl. The eluent was collected in a 25 mL volumetric flask and diluted with distilled water. One milliliter of Wade reagent (0.0328 g of $FeCl_3 \cdot 6H_2O$ and 0.3 g of sulfosalicylic acid dissolved and then diluted up to 100 mL with distilled water) was mixed with 3 mL of diluted phytate eluent and centrifuged at 20 ^{o}C for 10 min at 14,190 × *g*. The absorbance of the supernatant was determined colorimetrically using a spectrophotometer at 500 nm. A phytic acid standard curve, containing a series of phytic acid concentrations in distilled water (0 – 100 μg/mL), was created to calculate phytate concentration based on absorbance readings of assayed samples. The results were reported as mg/g of dry matter based on the following formula:

$$\text{Phytic acid concentration} = \frac{S \times 25 \times D}{0.5 \text{ g of sample}} \times \frac{100\%}{100\% - MC}$$

Where

S = sample phytic acid concentration calculated from standard curve (μg/mL),

D = dilution factor, and

MC = moisture content of flour.

4.3.3.4 Oxalates

The method proposed by Chai and Liebman (2005) was chosen for total and soluble oxalates extraction. A 1 g powdered sample was accurately weighed into a 50 mL centrifuge tube; 10 mL of 2 M H_3PO_4 was added for total oxalate extraction, or 10 mL of distilled water was used to extract soluble oxalate. The content was vortexed for 1 min to mix thoroughly. The centrifuge tube was placed in a shaking water bath (Julabo SW-22, Seelbach, Germany) at 150 rpm, 80 ^{o}C for 30 min. The suspension was cooled using running tap water, centrifuged at 14,190 × g for 10 min at 20 ^{o}C. For total oxalates 1 mL of acid-extracted supernatant was collected and mixed with 1 mL of 2 N NaOH while 1 mL of water-extracted supernatant was mixed with 1 mL of distilled water for the soluble oxalates. Total and soluble oxalate contents were determined by an oxalate kit (BioVision Inc., Milpitas, CA, USA) using a quantitative enzymatic method. The method is based on the reaction of oxalate by Oxalate Convert and Oxalate Enzyme Mix to form an intermediate, which reacts with a highly specific probe to generate colour at 450 nm. Oxalate standard were prepared into a series of 96-well flat bottom microplate to establish a standard curve (0 – 10 nmol/well). The amount of oxalate in the sample wells was determined by a multi-well spectrophotometer (Elx800 Absorbance Microplate Reader, BioTek Instruments Inc., Winooski, VT, USA) by comparing absorbance to the standard curve. Oxalate concentration (mg/100g of dry basis) in the sample was calculated using the following equation:

$$\text{Oxalate concentration} = \frac{B \times M \times 2}{1 \text{ g of sample}} \times \frac{100\%}{100\% - MC}$$

Where

B = amount of oxalate in sample well from standard curve (nmol),

M = oxalic acid molecular weight (90 g/mol), and

MC = moisture content of flour.

4.3.4 Statistical analysis

All analyses were conducted in triplicate and the results were given as means ± standard deviation. Two-way analysis of variance (ANOVA) models with legume type, treatment and their interaction were applied to the obtained analytical data using Statistical Analysis Software, Program version 9.3 (SAS Institute Inc., Gary, NC, USA). Tukey's test was used to separate means. The statistically significant differences were determined at $P < 0.05$ level.

4.4 Results and discussion

4.4.1 Hemagglutinating activity (lectins)

The results of analysis of hemagglutinating activity in raw and processed samples are given in Table 4.1. In all analyzed pulses and soybean samples, this activity was observed against rabbit erythrocyte, which was in agreement with what has been reported by Alonso et al. (1998), Alonso et al. (2000a), Grant et al. (1983), Jaffé et al. (1972), Liener and Hill (1953), Luo and Xie, (2013), Morcos et al. (1976) and Sathe and Salunkhe (1981). Raw soybean (692.8 HU/mg) showed significantly

higher activity than pulses. Comparing pulse types, common beans contained fairly high content (87.69 – 88.59 HU/mg), followed by lentils (10.91 – 11.07 HU/mg), peas (5.53 – 5.68 HU/mg), fava beans (5.52 – 5.55 HU/mg) and then chickpeas (2.73 – 2.74 HU/mg), respectively (Table 4.1). Alonso et al. (1998), Alonso et al. (2000a), El-Adawy (2002) and Pedrosa et al. (2012) reported hemagglutinating activity of Spanish pulses and soybean. The observation of Canadian peas was comparable to that found in Spanish peas (5.1 – 6.2 HU/mg). Spanish faba bean (49.3 HU/mg) and chickpeas (6.22 HU/mg) contained higher lectin content, but lentils (4.88 HU/mg), kidney bean (74.5 HU/mg) and soybean (40 HU/mg) from Spain showed lower hemagglutinating activity than Canadian legumes. The differences between Canadian and Spanish samples may result from different hemagglutination assays, type of red blood cells and definition of hemagglutinating activity unit. It can be also due to variety, climate conditions, location, irrigation conditions, soil type and crop year (Urbano et al., 2000). Determination of lectin content by erythroagglutination does not have high precision due to variable molecular properties of lectin, the properties of cell surface, the metabolic state of cells and the conditions of assay (Grant et al., 1983; Lis, & Sharon, 1986a). However, this measurement can evaluate the effectiveness of processing methods applied on toxic seeds (Grant et al., 1982).

Analysis of variance showed that legume type, treatment (soaking or cooking) and legume type × treatment had significant effects ($P < 0.0001$) on hemagglutinating activity in the samples analyzed (Table 4.2). Decreases due to soaking were significant ($P < 0.0001$); peas, lentils, fava beans, chickpeas, common beans and soybean had reductions of 3.13 – 4.09%, 1.41 – 4.33%, 0.62 – 5.18%, 0.11 – 3.51%, 0.73 – 2.44% and 0.82%, respectively (Table 4.1). These reductions were lower than that reported by Mubarak (2005), who found that hemagglutinating activity of mung

bean seeds decreased by 49.1% after soaking process. Greater decrease might be due to higher seed-to-water ratio (1:10, w/v) and longer time (12 h) used in soaking process. However, Alonso et al. (1998), Alonso et al. (2000a) and Luo and Xie (2013) stated that soaking could not significantly affect lectin content in pea, kidney bean and faba bean seeds. In the current study, decreases were small but significant because of the high precision in the measurements. Cooking was more effective at reducing hemagglutinating activity in pulses and soybean in comparison with soaking process; reductions ranged from 93.77 – 99.81% (Table 4.1). Alajaji and El-Adawy (2006), Mubarak (2005) and Khalil and Mansour (1995) reported that cooking of chickpea, mung bean and faba bean removed heamagglutinin completely. In terms of the present results, hemagglutinating activity was highly sensitive to thermal treatment. The reduction or elimination in the level of lectin content during cooking may result from breakdown of their structures into subunits or other unknown changes in conformational structure (Batra, 1987).

The highest lectin content was detected in Canadian soybean. When compared with Spanish pulses, peas contained similar lectin level; higher hemagglutinating activity was recorded in Canadian lentils and common beans while Canadian faba beans and chickpeas showed lower activity. Only a few studies investigated the toxic dose of lectin in humans. Olsnes and Pihl (1982) reported that intravenous administration of more than 5 μg/m^2 or 0.15 μg/kg of abrin led to toxic effects. Both soaking and cooking significantly decreased lectin content, but the cooking process was more effective and recommended for pulse preparation.

Table 4.1. Effect of soaking and cooking on hemagglutinating activity of Canadian pulses and soybean

Type	Hemagglutinating activity (HU[1]/mg dry matter)		
	Raw	Soaked	Cooked
Pea			
Whole yellow pea	5.64 ± 0.00^{aE}	5.46 ± 0.00^{b} (-3.13)	0.17 ± 0.00^{c} (-96.98)
Split yellow pea	5.53 ± 0.00^{aE}	5.31 ± 0.00^{b} (-4.09)	0.17 ± 0.00^{c} (-97.02)
Whole green pea	5.68 ± 0.00^{aE}	5.49 ± 0.00^{b} (-3.42)	0.17 ± 0.00^{c} (-96.99)
Split green pea	5.53 ± 0.00^{aE}	5.30 ± 0.00^{b} (-4.07)	0.17 ± 0.00^{c} (-97.02)
Lentil			
Whole red lentil	11.04 ± 0.00^{aD}	10.66 ± 0.00^{b} (-3.44)	0.33 ± 0.00^{c} (-96.99)
Split red lentil	10.91 ± 0.00^{aD}	10.65 ± 0.00^{b} (-2.39)	0.33 ± 0.00^{c} (-96.97)
Football red lentil	11.04 ± 0.00^{aD}	10.71 ± 0.00^{b} (-2.92)	0.33 ± 0.00^{c} (-97.00)
Spanish brown lentil	11.02 ± 0.01^{aD}	10.82 ± 0.01^{b} (-1.85)	0.33 ± 0.00^{c} (-96.99)
Split yellow lentil	11.07 ± 0.00^{aD}	10.59 ± 0.00^{b} (-4.33)	0.33 ± 0.00^{c} (-97.01)
French green lentil	11.01 ± 0.00^{aD}	10.79 ± 0.01^{b} (-2.01)	0.33 ± 0.00^{c} (-96.99)
Large green lentil	11.00 ± 0.01^{aD}	10.79 ± 0.01^{b} (-1.93)	0.33 ± 0.00^{c} (-96.98)
Medium green lentil	11.01 ± 0.01^{aD}	10.85 ± 0.01^{b} (-1.41)	0.34 ± 0.00^{c} (-96.96)
Split queen green lentil	11.07 ± 0.00^{aD}	10.64 ± 0.00^{b} (-3.92)	0.33 ± 0.00^{c} (-97.02)
Fava bean			
Whole fava bean	5.52 ± 0.00^{aE}	5.49 ± 0.00^{b} (-0.62)	0.09 ± 0.00^{c} (-98.46)
Split fava bean	5.55 ± 0.00^{aE}	5.26 ± 0.00^{b} (-5.18)	0.08 ± 0.00^{c} (-98.53)
Chickpea			
Whole chickpea B90	2.74 ± 0.00^{aF}	2.70 ± 0.00^{b} (-1.71)	0.17 ± 0.00^{c} (-93.77)
Split chickpea B90	2.73 ± 0.00^{aF}	2.64 ± 0.00^{b} (-3.51)	0.17 ± 0.00^{c} (-93.92)
Desi chickpea	2.73 ± 0.00^{aF}	2.72 ± 0.00^{b} (-0.11)	0.17 ± 0.00^{c} (-93.80)
Bean			
Dark red kidney bean	88.52 ± 0.00^{aB}	86.47 ± 0.00^{b} (-2.44)	0.17 ± 0.00^{c} (-99.81)
Pinto bean	88.59 ± 0.04^{aB}	87.31 ± 0.01^{b} (-1.36)	0.17 ± 0.00^{c} (-99.81)
Navy bean	88.46 ± 0.02^{aB}	86.77 ± 0.02^{b} (-1.92)	0.17 ± 0.00^{c} (-99.81)
Black bean	87.69 ± 0.01^{aC}	87.04 ± 0.05^{b} (-0.73)	0.17 ± 0.00^{c} (-99.81)
Soybean	692.82 ± 0.42^{aA}	687.50 ± 0.66^{b} (-0.82)	2.70 ± 0.23^{c} (-99.61)

Row values followed by different superscript lowercase letters are significantly different ($P < 0.05$) as determined using Tukey's test.
Column values for raw samples followed by different superscript uppercase letters are significantly different ($P < 0.05$) as determined using Tukey's test.
Values in parentheses indicate % increase or % decrease (negative sign) over raw values.
Means ± standard deviation of three determinations.
[1] HU = hemagglutinating unit.

Table 4.2. Analysis of variance of effects of legume type and treatment on hemagglutinating activity

Source	DF[1]	Mean Square	*F* Value	*P* Value
Whole model	68	41269.251	4567391	<0.0001
Legume type	22	81704.627	9042495	<0.0001
Treatment	2	59699.745	6607149	<0.0001
Legume type × treatment	44	20213.813	2237123	<0.0001

[1] DF = Degrees of freedom.

4.4.2 Phytic acid

Phytic acid contents of raw samples are summarized in Table 4.3. Comparing legume types, phytic acid contents were distributed over a wide range, 22.91 mg/g in soybean, 19.65 – 22.85 in fava beans, 15.64 – 18.82 mg/g in common beans, 8.56 – 15.56 mg/g in lentils, 11.33 – 14.00 mg/g in chickpeas and 8.55 – 12.40 mg/g in peas, respectively (Table 4.3). Approximately 18.80 – 40.67 mg/g of phytic acid were reported in different soybean cultivars (Mohamed, Mebrahtu, & Rangappa, 1991). The values obtained for Canadian pulses are also similar to those reported by Alonso et al. (1998), Alonso et al. (2000a) and El-Adawy (2002) for Spanish pea (11.9 – 13.1 mg/g), faba bean (21.7 mg/g), kidney bean (15.9 mg/g) and chickpea (12.1 mg/g) seeds. When compared with Canadian pulses in previous literature, Khattab and Arntfield (2009), Wang and Daun (2006), Wang et al. (2008) and Wang et al. (2010) reported slightly lower phytic contents in peas (6.4 – 8.3 mg/g), lentils (7.5 – 9.3 mg/g), chickpeas (9.6 – 10.6 mg/g) and beans (9.9 – 13.8 mg/g). Chinese faba beans had lower phytic acid level (8.36 – 8.57 mg/g) (Luo, & Xie, 2013) while common beans from United States contained higher values (20.4 – 29.3 mg/g) except for small white bean (11.6 mg/g) (Deshpande et al., 1982). Kumar et al. (2005) and Urbano et al. (2000) concluded that variety and/or cultivar, climatic conditions, location,

irrigation conditions, soil factors (e.g. organic phosphorus status, pH and soil temperature) and crop year were the major factors which cause differences in phytic acid contents. Compared to the content in whole seeds, split seeds of yellow pea, green pea, red lentil, fava bean and chickpea B90 contained higher phytic acid. It should be noted that as part of the splitting process, the seed coat is lost. Similar results were obtained by Alonso et al. (1998), Alonso et al. (2000a), Deshpande et al. (1982) and Wang et al. (2008) who stated that dehulling process increased phytic acid content. Phytates were mainly present in cotyledons of pulses with the seed coat making no contribution or contributing a small amount (Alonso et al., 1998; Wang et al., 2008).

Analysis of variance indicated that legume type, treatment (soaking or cooking) and their interaction had significant effects ($P < 0.0001$) on phytic acid content (Table 4.4). There was no detectable decrease in the level of phytic acid as a result of soaking any of the seeds (Table 4.3). This result confirmed the findings reported by Wang et al. (2008) for six pea varieties. However, Alonso et al. (1998), Alonso et al. (2000a), Khattab and Arntfield (2009), and Mubarak (2005) found that the soaking process could drastically decrease phytic acid contents in peas, faba bean, kidney bean and mung bean. The significant reductions in these studies might be due to the longer soaking time (12, 18, 20 or 25 h) when compared with the present study (4 h). After cooking of presoaked seeds, phytic acid content was significantly reduced by 10.64 – 26.11%, 19.12 – 80.06%, 19.09 – 38.36% and 11.14 – 29.23% in peas, lentils, fava beans and chickpeas, respectively (Table 4.3). These results are in good agreement with the observation from Alajaji and El-Adawy (2006) for chickpeas (28.93%), but higher than those found by Luo and Xie (2013) for faba beans (6.4 – 9.1%) and Wang et al. (2008) for peas (5.3 – 10.8%). These different reductions

might result from different cooking time between literature and current study. However, there was no noticeable change in the phytic acid levels in common beans and soybean (Table 4.3). These confirmed the results reported by Wang et al. (2010) that cooking did not have significant influence on phytic acid content in common beans. The reduction of phytic acid after cooking process may be partly attributed to its heat-labile nature (Udensi, Ekwu, & Isinguzo, 2007). Additionally, the formation of insoluble complexes by phytate and calcium or magnesium during heat treatment may also lead to reduction in measurable phytic acid (Crean, & Haisman, 1963; Udensi et al., 2007). In soybean and common beans, phytic acid content was not affected by the cooking process. This might be due to a different density for the cotyledon compared to other pulses. According to Tiwari and Singh (2012), pulses have slow water uptake if density of cotyledon is high. Soybean and common beans may have higher density cotyledons than other pulses, so that less boiling water penetrated into seeds. As result, there was not adequate heat to affect partially heat-sensitive portion of phytic acid or to form insoluble complexes between phytate and ions.

In comparison with soybean and pulses globally or from previous data in Canada, phytic acid contents of Canadian soybean and pulses investigated in this research varied greatly. Onomi et al. (2004) suggested that ≤ 25 mg/100 g of phytate containing food eaten is for best health. United States and United Kingdom reported 631 – 746 mg of daily average phytate intake, while the average values are 370 mg/day, 219 mg/day and a mere 180 mg/day in Finland, Italy and Sweden, respectively (Coulibaly et al., 2011). Soaking and cooking processes from the current study did not reduce phytic acid effectively; therefore, further research is required to

investigate the effect of other treatments, for example roasting, fermentation and germination, on phytic acid contents in Canadian pulses and soybean.

Table 4.3. Effect of soaking and cooking on phytic acid content of Canadian pulses and soybean

Type	Phytic acid (mg/g dry matter)		
	Raw	Soaked	Cooked
Pea			
Whole yellow pea	9.93 ± 0.21^{aJK}	9.48 ± 0.79^{a} (-4.56)	7.88 ± 0.38^{b} (-20.68)
Split yellow pea	12.40 ± 0.89^{aGHI}	12.23 ± 0.49^{a} (-1.34)	9.55 ± 0.52^{b} (-23.00)
Whole green pea	8.55 ± 0.35^{aK}	8.51 ± 0.24^{a} (-0.49)	7.64 ± 0.29^{b} (-10.64)
Split green pea	12.27 ± 0.33^{aGHI}	12.09 ± 0.27^{a} (-1.46)	9.07 ± 0.52^{b} (-26.11)
Lentil			
Whole red lentil	12.50 ± 0.19^{aGHI}	13.08 ± 0.38^{a} (4.43)	10.11 ± 0.41^{b} (-19.12)
Split red lentil	14.04 ± 1.33^{aEFG}	13.44 ± 0.35^{a} (-4.27)	8.74 ± 0.19^{b} (-37.77)
Football red lentil	17.10 ± 0.36^{aCD}	16.37 ± 0.15^{a} (-4.29)	8.87 ± 0.65^{b} (-48.14)
Spanish brown lentil	13.24 ± 0.78^{aFGH}	13.17 ± 0.95^{a} (-0.46)	4.34 ± 0.39^{b} (-67.23)
Split yellow lentil	12.17 ± 0.50^{aGHIJ}	11.87 ± 0.59^{a} (-2.39)	6.02 ± 0.26^{b} (-50.52)
French green lentil	15.56 ± 0.42^{aDE}	15.30 ± 0.83^{a} (-1.63)	6.44 ± 0.24^{b} (-58.61)
Large green lentil	14.92 ± 1.78^{aDEF}	15.34 ± 0.26^{a} (2.76)	7.42 ± 0.38^{b} (-50.27)
Medium green lentil	8.56 ± 0.25^{aK}	8.76 ± 0.74^{a} (2.25)	1.71 ± 0.18^{b} (-80.06)
Split queen green lentil	10.90 ± 0.25^{aIJ}	10.52 ± 0.24^{a} (-3.47)	6.62 ± 0.05^{b} (-39.25)
Fava bean			
Whole fava bean	19.65 ± 0.26^{aB}	19.43 ± 0.21^{a} (-1.12)	15.90 ± 0.51^{b} (-19.09)
Split fava bean	22.85 ± 0.51^{aA}	22.12 ± 0.23^{a} (-3.17)	14.08 ± 0.16^{b} (-38.36)
Chickpea			
Whole chickpea B90	11.33 ± 0.45^{aHIJ}	11.29 ± 0.43^{a} (-0.32)	10.07 ± 0.44^{b} (-11.14)
Split chickpea B90	11.53 ± 0.84^{aHIJ}	11.26 ± 0.52^{a} (-2.36)	8.16 ± 0.77^{b} (-29.23)
Desi chickpea	14.00 ± 0.66^{aEFG}	14.64 ± 0.04^{a} (4.59)	11.90 ± 0.99^{b} (-15.01)
Bean			
Dark red kidney bean	15.64 ± 0.52^{aDE}	15.55 ± 0.50^{a} (-0.54)	15.28 ± 0.79^{a} (-2.29)
Pinto bean	18.82 ± 0.79^{aBC}	19.16 ± 0.25^{a} (1.86)	18.49 ± 0.41^{a} (-1.75)
Navy bean	18.74 ± 0.33^{aBC}	18.55 ± 0.20^{a} (-1.03)	18.41 ± 1.02^{a} (-1.76)
Black bean	18.32 ± 1.18^{aBC}	18.75 ± 0.95^{a} (2.34)	18.27 ± 0.58^{a} (-0.29)
Soybean	22.91 ± 0.86^{aA}	22.27 ± 0.23^{a} (-2.82)	22.13 ± 0.45^{a} (-3.42)

Row values followed by different superscript lowercase letters are significantly different ($P < 0.05$) as determined using Tukey's test.
Column values for raw samples followed by different superscript uppercase letters are significantly different ($P < 0.05$) as determined using Tukey's test.
Values in parentheses indicate % increase or % decrease (negative sign) over raw values.
Means ± standard deviation of three determinations.

Table 4.4. Analysis of variance of effects of legume type and treatment on phytic acid content

Source	DF[1]	Mean Square	*F* Value	*P* Value
Whole model	68	67.730644	193.38	<0.0001
Legume type	22	160.232743	457.49	<0.0001
Treatment	2	330.778938	944.42	<0.0001
Legume type × treatment	44	9.522854	27.19	<0.0001

[1] DF = Degrees of freedom.

4.4.3 Oxalates

4.4.3.1 Total oxalate

The results of total oxalate content in raw and processed Canadian pulses and soybean are shown in Table 4.5. Soybean contained the highest amount of total oxalates (370.49 mg/100 g) while the lowest amount was detected in common beans, varying from 98.86 – 117.01 mg/100 g (Table 4.5). In other pulses, total oxalates were variable, ranging from 244.65 – 293.97 mg/100 g in peas, 168.62 – 289.05 mg/100 g in lentils, 241.50 – 291.42 mg/100 g in fava beans and 192.16 – 214.03 mg/100 g in chickpeas, respectively (Table 4.5). Akhtar et al. (2011) obtained higher total oxalate contents in Pakistan soybean (497 mg/100 g) while Judprasong et al. (2006) reported lower level in Thailand soybean (204 mg/100 g) as compared to the value in the present study. Ruan et al. (2013) reported that soybean and black bean from China contained 68.6 mg/100 g and 41.0 mg/100 g of total oxalates, respectively; both values are considerably lower than what was observed in this study. The level of total oxalates found in Thailand red kidney bean, however, was in accordance with the 91 mg/100 g observed by Judprasong et al. (2006). There was a lack of data on total oxalate contents was reported in raw peas, lentils, fava beans or

chickpeas. Different contents of total oxalate measured from legume seeds can be attributed to variety, growth, season, soil conditions and harvest time (Akhtar et al., 2011).

Analysis of variance indicated that legume type, treatment (soaking or cooking) and their interaction had significant effects ($P < 0.0001$) on total oxalate contents (Table 4.6). The soaking process significantly reduced their total oxalates for all samples (Table 4.5). The losses of total oxalate contents ranged from 22.51 – 31.44% in peas, 26.02 – 37.89% in lentils, 17.40 – 37.08% in fava beans, 26.86 – 29.09% in chickpeas, 21.47 – 34.30% in common beans and 51.89% in soybean, respectively (Table 4.5). Data regarding the effect of soaking on total oxalate contents in pulses and soybean were scarce. Savage and Dubois (2006) reported that soaking raw taro leaves in water for 30 min reduced approximately 10% total oxalate contents, while a 26% loss of total oxalates was detected after soaking for 18 h. The decrease in total oxalate contents might be due to the leaching of soluble oxalates during the soaking process. The reductions in total oxalates as a result of cooking presoaked seeds were, 30.83 – 41.45%, 34.45 – 54.16%, 31.85 – 45.81%, 33.48 – 39.72%, 37.81 – 44.96% and 66.15% for peas, lentils, fava beans, chickpeas, common beans and soybean, respectively (Table 4.5). However, there was no significant difference between soaking and cooking treatments on total oxalate contents (Table 4.5), as standard deviation for oxalate measurements tended to be high. Judprasong et al. (2006) reported that boiling of mung bean and red kidney bean decreased their total oxalates by 79.17% and 64.84%, respectively. Similarly a loss of total oxalate contents of 76% in white bean, 59% in soybean and 40% in red bean due to cooking was found by Akhtar et al. (2011) that cooking caused a reduction. Loss of soluble

oxalates in cooking water was considered to be the primary factor contributing to total oxalates reduction (Akhtar et al., 2011).

Table 4.5. Effect of soaking and cooking on total oxalate content of Canadian pulses and soybean

Type	Total oxalate (mg/100 g dry matter)		
	Raw	Soaked	Cooked
Pea			
Whole yellow pea	244.65 ± 30.98[a]	183.31 ± 16.86[b] (-25.07)	169.23 ± 3.96[b] (-30.83)
Split yellow pea	293.97 ± 10.87[a]	227.80 ± 26.90[b] (-22.51)	172.12 ± 34.86[b] (-41.45)
Whole green pea	280.02 ± 24.89[a]	194.99 ± 17.80[b] (-30.36)	178.13 ± 15.92[b] (-36.39)
Split green pea	287.05 ± 37.63[a]	196.81 ± 12.44[b] (-31.44)	185.02 ± 11.33[b] (-35.54)
Lentil			
Whole red lentil	234.50 ± 10.59[a]	166.90 ± 18.05[b] (-28.83)	151.77 ± 13.18[b] (-35.28)
Split red lentil	289.05 ± 33.45[a]	185.08 ± 5.80[b] (-35.97)	154.55 ± 8.44[b] (-46.53)
Football red lentil	200.10 ± 10.61[a]	145.08 ± 7.28[b] (-27.50)	131.16 ± 18.02[b] (-34.45)
Spanish brown lentil	227.88 ± 16.27[a]	141.53 ± 5.55[b] (-37.89)	107.44 ± 22.52[b] (-52.85)
Split yellow lentil	175.13 ± 16.28[a]	111.65 ± 5.43[b] (-36.25)	94.63 ± 5.70[b] (-45.96)
French green lentil	180.68 ± 40.51[a]	115.43 ± 5.11[b] (-36.12)	82.82 ± 11.68[b] (-54.16)
Large green lentil	168.62 ± 13.41[a]	124.75 ± 12.42[b] (-26.02)	97.03 ± 19.88[b] (-42.46)
Medium green lentil	193.65 ± 1.77[a]	150.35 ± 16.55[b] (-26.02)	123.25 ± 20.85[b] (-36.35)
Split queen green lentil	189.47 ± 11.13[a]	133.49 ± 4.63[b] (-29.55)	106.21 ± 15.06[b] (-43.95)
Fava bean			
Whole fava bean	241.50 ± 6.98[a]	199.49 ± 14.76[b] (-17.40)	164.58 ± 18.73[b] (-31.85)
Split fava bean	291.42 ± 21.58[a]	183.37 ± 18.38[b] (-37.08)	157.92 ± 20.71[b] (-45.81)
Chickpea			
Whole chickpea B90	192.16 ± 16.86[a]	140.55 ± 16.74[b] (-26.86)	115.83 ± 20.01[b] (-39.72)
Split chickpea B90	214.03 ± 17.17[a]	151.77 ± 5.95[b] (-29.09)	131.90 ± 23.97[b] (-38.37)
Desi chickpea	199.30 ± 31.18[a]	145.55 ± 4.98[b] (-26.97)	132.57 ± 15.19[b] (-33.48)
Bean			
Dark red kidney bean	107.79 ± 8.50[a]	72.22 ± 8.15[b] (-33.00)	59.32 ± 5.51[b] (-44.96)
Pinto bean	117.01 ± 3.16[a]	76.88 ± 6.99[b] (-34.30)	67.29 ± 5.59[b] (-42.49)
Navy bean	99.26 ± 8.70[a]	68.46 ± 11.07[b] (-31.02)	57.81 ± 4.78[b] (-41.75)
Black bean	98.86 ± 8.70[a]	77.64 ± 3.82[b] (-21.47)	61.48 ± 1.69[b] (-37.81)
Soybean	370.49 ± 41.40[a]	178.23 ± 14.77[b] (-51.89)	125.42 ± 19.01[b] (-66.15)

Row values followed by different superscript lowercase letters are significantly different ($P < 0.05$) as determined using Tukey's test.
Column values for raw samples followed by different superscript uppercase letters are significantly different ($P < 0.05$) as determined using Tukey's test.
Values in parentheses indicate % increase or % decrease (negative sign) over raw values.
Means ± standard deviation of three determinations.

Table 4.6. Analysis of variance of effects of legume type and treatment on total oxalate content

Source	DF[1]	Mean Square	*F* Value	*P* Value
Whole model	68	12683.4853	41.36	<0.0001
Legume type	22	22263.6153	72.60	<0.0001
Treatment	2	150165.4177	489.66	<0.0001
Legume type × treatment	44	1644.2415	5.36	<0.0001

[1] DF = Degrees of freedom.

4.4.3.1 Soluble oxalate

Table 4.7 shows the results of soluble oxalate contents in raw, soaked and cooked Canadian pulses and soybean as well as the proportion of soluble oxalates in each seed. The highest soluble oxalate value was observed in soybean (200.66 mg/100 g) (Table 4.7). The soluble oxalate contents were found to be relatively high in lentils (77.98 – 129.39 mg/100 g), moderate in peas (69.38 – 87.50 mg/100 g), fava beans (67.04 – 79.78 mg/100 g) and chickpeas (46.52 – 86.51 mg/100 g), while low in common beans (36.51 – 43.73 mg/100 g) (Table 4.7). The observation of common was close to that reported by Akhtar et al. (2011) for Pakistan red bean (37 mg/100 g), but soybean (155 mg/100 g) was lower than in the current study. Quinteros et al. (2003) reported that Spanish chickpeas and lentils contained 56.5 mg/100 g and 164.4 mg/100 g of soluble oxalates, respectively. Variable percentages of the total oxalates were detected to be soluble in the different seeds; values ranged from 24.78 – 31.57% in peas, 43.67 – 71.60% in lentils, 27.38 – 34.89% in fava beans, 24.21 – 40.42% in chickpeas, 31.20 – 44.23% in common beans and 54.16% in soybean (Table 4.7). Akhtar et al. (2011) and Judprasong et al. (2006) indicated that approximately 29 – 33% of the total oxalates in beans was found to be soluble in beans, which is similar to the values presently reported. The percentage of soluble oxalates in the study for

soybean, however, is higher than the results (28.43 – 31.19%) found by Akhtar et al. (2011) and Judprasong et al. (2006).

Analysis of variance revealed that legume type, treatment (soaking or cooking) and their interaction exerted significant effects ($P < 0.0001$) on soluble oxalate contents in Canadian pulses and soybean (Table 4.8). The soaking process caused significant reduction in soluble oxalates in peas (36.51 – 47.62%), lentils (26.66 – 48.79%), fava beans (45.34 – 45.82%), chickpeas (29.92 – 35.53%), beans (36.56 – 39.65%) and soybean (56.29%) (Table 4.7). Savage and Dubois (2006) reported that soaking raw taro leaves for 30 min marginally decreased soluble oxalate content due to leaching-out effect while greater loss was detected after soaking for 18 h. Clearly the conditions during soaking, including the time of soaking, influence the ability to remove this soluble material from the seeds. Significant reductions of soluble oxalates were also noted in cooked in peas (53.75 – 63.63%), lentils (45.74 – 66.27%), fava beans (56.73 – 58.36%), chickpeas (31.43 – 44.25%), beans (52.58 – 57.38%) and soybean (73.95%) (Table 4.7). These agreed with the results of Akhtar et al. (2011), Judprasong et al. (2006) and Quinteros et al. (2003) who found that 42.58%, 19.63%, 59.65 – 61.54% and 58.71% reductions in soluble oxalate contents in lentils, chickpeas, beans and soybean, respectively. The differences in soluble oxalate reduction might be due to different pre-soaking processes and cooking times applied in literature. The cooking process, however, did not show any significant differences when compared with soaking (Table 4.7). The loss of soluble oxalates in water can be used to explain decreased oxalate contents after soaking/cooking treatment. In addition, the decreased percentage soluble oxalate has been seen in all samples (Table 4.7), which indicates that the reductions in total oxalates after soaking/cooking were in fact due to the removal of soluble oxalates. Soluble oxalates

were more easily affected by the cooking process than the insoluble fraction; oxalates content can be decreased by cooking if discarding cooking water (Chai, & Liebman, 2005; Judprasong et al., 2006; Noonan, & Savage, 1999; Słupski et al., 2011).

To summarize, total oxalate content is high in Pakistan soybean, moderate in Canadian soybean and low in Thailand and Chinese samples. Total oxalate level of Canadian common beans investigated was close to and/or higher than the reported values for Chinese and Thailand beans. Canadian soybean contains higher soluble oxalates than Pakistan soybean, but the value of Pakistan red bean was in the range of Canadian common beans. Soluble oxalate content of Spanish chickpeas and lentils were within and/or higher than the range of Canadian samples. The ingestion of approximately 5 g oxalic acid in crystal or in solution is the minimum dose capacity of causing death in human beings, and is usually associated with corrosive gastroenteritis, shock, convulsive symptoms and renal damage (Fassett, 1967). Both soaking and cooking treatments are capable of markedly reducing total oxalate content, but cooking can mostly reduce soluble oxalates in seeds. Therefore, the cooking process is recommended to treat seeds prior to their consumption.

Table 4.7. Effect of soaking and cooking on soluble oxalate content (mg/100 g dry matter) of Canadian pulses and soybean

Type	Raw	% Soluble oxalate	Soaked	% Soluble oxalate	Cooked	% Soluble oxalate
Pea						
Whole yellow pea	77.23 ± 7.72^{aDEFGH}	31.57	49.03 ± 10.91^{b} (-36.51)	26.75	28.09 ± 6.60^{b} (-63.63)	16.60
Split yellow pea	80.82 ± 6.50^{aDEFG}	27.50	45.13 ± 5.51^{b} (-44.15)	19.81	33.43 ± 3.02^{b} (-58.63)	19.42
Whole green pea	69.38 ± 3.79^{aEFGHI}	24.78	36.34 ± 7.93^{b} (-47.62)	18.64	32.09 ± 3.93^{b} (-53.75)	18.01
Split green pea	87.50 ± 12.80^{aDEFG}	30.48	47.16 ± 8.84^{b} (-46.10)	23.96	32.37 ± 7.88^{b} (-63.01)	17.50
Lentil						
Whole red lentil	109.33 ± 15.61^{aBCD}	46.62	66.29 ± 15.19^{b} (-39.36)	39.72	36.87 ± 7.90^{b} (-66.27)	24.29
Split red lentil	126.22 ± 16.03^{aBC}	43.67	79.50 ± 6.28^{b} (-37.01)	42.95	56.02 ± 13.00^{b} (56.61)	36.25
Football red lentil	107.04 ± 7.58^{aBCD}	53.49	61.63 ± 12.04^{b} (-42.43)	42.48	47.56 ± 1.96^{b} (-55.57)	36.26
Spanish brown lentil	125.05 ± 9.32^{aIJKL}	54.88	64.28 ± 11.52^{b} (-48.60)	45.42	47.14 ± 9.58^{b} (-62.30)	43.88
Split yellow lentil	94.14 ± 7.36^{aCDEF}	53.75	51.18 ± 7.74^{b} (-45.63)	45.84	40.65 ± 1.56^{b} (-56.82)	42.96
French green lentil	129.37 ± 11.86^{aB}	71.60	66.25 ± 13.47^{b} (-48.79)	57.39	46.46 ± 6.49^{b} (-64.09)	56.10
Large green lentil	77.98 ± 12.49^{aDEFGH}	46.25	57.19 ± 2.56^{b} (-26.66)	45.84	39.58 ± 2.40^{b} (-49.25)	40.79
Medium green lentil	102.07 ± 6.83^{aBCDE}	52.71	70.54 ± 10.30^{b} (-30.89)	46.92	55.38 ± 5.20^{b} (-45.74)	44.93
Split queen green lentil	110.07 ± 14.73^{aBCD}	58.09	65.14 ± 12.87^{b} (-40.82)	48.80	41.24 ± 5.81^{b} (-62.53)	38.83
Fava bean						
Whole fava bean	67.04 ± 3.16^{aFGHI}	34.89	36.64 ± 8.41^{b} (-45.34)	18.37	29.01 ± 0.91^{b} (-56.73)	17.63
Split fava bean	79.78 ± 17.55^{aDEFGH}	27.38	43.23 ± 6.79^{b} (-45.82)	23.58	33.22 ± 0.95^{b} (-58.36)	21.04
Chickpea						
Whole chickpea B90	46.52 ± 3.26^{aHI}	24.21	30.00 ± 0.75^{b} (-35.53)	21.34	31.90 ± 0.80^{b} (-31.43)	19.38
Split chickpea B90	86.51 ± 12.28^{aDEFG}	40.42	57.34 ± 1.21^{b} (-33.72)	37.78	48.22 ± 6.63^{b} (-44.25)	36.56
Desi chickpea	66.98 ± 4.53^{aGHI}	33.61	46.94 ± 4.86^{b} (-29.92)	32.25	38.93 ± 1.94^{b} (-41.88)	29.37
Bean						
Dark red kidney bean	40.99 ± 4.91^{aI}	38.03	24.74 ± 5.74^{b} (-39.65)	34.26	17.83 ± 4.04^{b} (-56.51)	30.06
Pinto bean	36.51 ± 4.04^{aI}	31.20	23.16 ± 3.37^{b} (-36.56)	30.12	17.31 ± 1.85^{b} (-52.58)	25.72
Navy bean	43.47 ± 3.80^{aI}	43.79	27.28 ± 6.16^{b} (-37.25)	39.85	18.98 ± 4.40^{b} (-56.35)	32.83
Black bean	43.73 ± 1.80^{aI}	44.23	26.42 ± 6.41^{b} (-39.58)	34.03	18.64 ± 3.87^{b} (-57.38)	30.32
Soybean	200.66 ± 27.14^{aA}	54.16	87.70 ± 18.53^{b} (-56.29)	49.21	52.31 ± 11.76^{b} (-73.93)	41.71

Row values followed by different superscript lowercase letters are significantly different ($P < 0.05$) as determined using Tukey's test.
Column values for raw samples followed by different superscript uppercase letters are significantly different ($P < 0.05$) as determined using Tukey's test.
Values in parentheses indicate % decrease over raw values.
Means ± standard deviation of three determinations.

Table 4.8. Analysis of variance of effects of legume type and treatment on soluble oxalate content

Source	DF[1]	Mean Square	*F* Value	*P* Value
Whole model	68	12683.4853	41.36	<0.0001
Legume type	22	22263.6153	72.60	<0.0001
Treatment	2	150165.4177	489.66	<0.0001
Legume type × treatment	44	1644.2415	5.36	<0.0001

[1] DF = Degrees of freedom.

4.5 Conclusion

The levels of lectin, phytic acid and oxalate in Canadian pulses varied widely, but were generally lower than those found in soybean. Common beans were high in hemagglutinating activity, but low in oxalates. The least lectin content was recorded in chickpeas while peas showed the lowest phytic acid. Fava beans contained moderate levels of lectins and oxalates, but relatively high phytic acid. In general, the contents of lectin, phytic acid and oxalate in Canadian soybean and pulses were comparable to those samples reported in previous literature. Analysis of Variance indicated that legume type, treatment (soaking or cooking) and legume type-by-treatment interaction had significant effects on the contents of these antinutritional factors. Soaking markedly decreased the levels of lectins, total and soluble oxalates, but had no impact on phytic acid. Cooking of presoaked seeds was more effective; hemagglutinating activity was reduced by more than 93%, and significant reductions in phytic acid content (11 – 67%) were observed in all samples, except for common beans and soybean. Cooked samples also had dramatically decreased oxalate contents, but had no detectable changes when compared with the soaking treatment. Results from this research will help pulse industry deal with their pulse products or fractions for potential market diversification.

5. GENERAL CONCLUSIONS

In the present research, the effects of pulse type and processing (soaking and cooking) on antinutritional factors (trypsin inhibitor, chymotrypsin inhibitor, α-amylase inhibitor, lectins, phytic acid and oxalates) in a wide market class of Canadian pulses (4 peas, 9 lentils, 3 chickpeas, 2 fava beans and 4 common beans) were investigated. Results were compared to those of soybean, which was used as a control.

Soybean contained the highest contents of trypsin inhibitor (45.89 TIU/mg), chymotrypsin inhibitor (30.16 CIU/mg), lectins (692.8 HU/mg), phytic acid (22.91 mg/g), total oxalate (370.5 mg/100 g) and soluble oxalate (200.7 mg/100 g) among all seeds investigated. α-Amylase inhibitory activity was absent in peas, lentils, chickpeas and fava beans, but was present in common beans, ranging from 785.6 – 1370 AIU/g. Using raw dark red kidney bean as an example, α-amylase source, reaction temperature and their interaction showed significant ($P < 0.0001$) effects on α-amylase inhibitory activity. Trypsin inhibitor levels of raw peas, lentils, chickpeas, fava beans and common beans ranged from 3.16 – 20.83 TIU/mg, with the lowest values in peas and the highest in common beans. Relatively high content of chymotrypsin inhibitor was detected in common beans (17.77 – 24.48 CIU/mg) while the levels in other pulses varied from 1.12 – 13.59 CIU/mg. Beans also contained high levels of lectins (87.69 – 88.59 HU/mg) followed by lentils, peas, fava beans and chickpeas with very low values (2.73 – 2.74 HU/mg). Phytic acid was found in all pulse samples with the lowest contents in peas (8.55 – 12.40 mg/g) and the highest in fava beans (19.65 – 22.84 mg/g). Total oxalates were also variable, ranging from 244.7 – 294.0 mg/100 g in peas, 168.6 – 289.1 mg/100 g in lentils, 241.5 – 291.4

mg/100 g in fava beans, 92.2 – 214.0 mg/100 g in chickpeas and 98.9 – 117.0 mg/100 g in common beans, respectively. Approximately 24 – 72% of total oxalates appeared to be soluble in all investigated pulses. The levels of these antinutritional factors in Canadian pulses and soybean were comparable to those found in literature from other parts of the world. Any variations might be due to variety and/or cultivar, climatic conditions, location, irrigation conditions, soil factors (e.g. organic phosphorus status, pH and soil temperature) and crop year.

Analysis of variance indicated that legume type, treatment (soaking or cooking) and their interaction had significant effects ($P < 0.0001$) on levels of α-amylase inhibitor, trypsin inhibitor, chymotrypsin inhibitor, lectins, phytic acid and oxalates. Overall, soaking markedly decreased contents of α-amylase inhibitor (4.05 – 10.97%), trypsin inhibitor (4.88 – 25.27%), chymotrypsin inhibitor (5.64 – 17.51%), lectins (0.11 – 5.18%), total oxalates (17.40 – 51.89%) and soluble oxalates (26.66 – 56.29%), but had no significant impact on phytic acid level. Cooking of presoaked seeds was more effective in reducing antinutritional factors; all proteinaceous antinutrients (α-amylase inhibitor, trypsin inhibitor, chymotrypsin inhibitor and lectins) were decreased drastically by 78.74 – 100% due to denaturation and significant reductions in total (30.83 – 66.15%) and soluble oxalates (31.43 – 73.93%) contents were observed for all pulses and soybean. However, significantly reduced phytic acid contents after cooking were found only in peas (10.64 – 26.11%), lentils (19.12 – 80.06%), chickpeas (11.14 – 29.23%) and fava beans (19.09 – 38.36%). The effects of soaking and cooking treatments on these antinutritional factors in Canadian pulses and soybean appear to follow the same trend as has been reported in the literature. The slight differences may result from processing conditions and crop types.

The levels of antinutritional factors in Canadian pulses varied widely, but the contents were generally lower than those found in soybean. Processing, specifically heat treatment, drastically reduced these levels (not all) although the effect of heat did vary with the crop and the antinutritional factor. Further research is required to better investigate the effects of more different processing methods on these antinutritional factors. It is also possible that different cultivars will respond differently; samples used in this study were either commercial blends or one specific cultivar.

6. APPLICATIONS AND RECOMMENDATIONS

This project has created a database that provides quantifiable contents of antinutritional factors (trypsin inhibitor, chymotrypsin inhibitor, α-amylase inhibitor, lectins, phytic acid and oxalates) relative to soybean for current market class varieties of Canadian pulses (peas, lentils, chickpeas, fava beans and common beans). It has also provided methodologies that can be applied to further work on these compounds. The effects of the processing methods examined (soaking and cooking) on changes to antinutritional factors will assist Canadian pulse companies in understanding the importance of pre-treatment with moisture and heat on antinutritional factors for both whole and split seed products. Information obtained from the present study will help the Canadian pulse industry and regulatory bodies in selecting the appropriate pulse for potential market diversification and further utilization. However, future research is still required to expand this database by including more pulses, which are not currently grown in Canada at a significant level. These include cowpea, pigeon pea, bambara groundnut, vetch, lupins and minor pulses (Jack bean, winged bean, velvet bean, yam bean). In addition to soaking and cooking, more processing methods, for example roasting, autoclaving, microwave cooking, micronization, fermentation and germination, can be applied to evaluate the fate of antinutritional properties in pulses. Therefore, sufficient technical data relating to the stability of antinutritional factors within pulses could be provided to Canadian pulse processors to aid in preparation of food and feed products.

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8. APPENDICES

Appendix 1. Recovery of Canadian pulses and soybean after soaking and cooking

Seeds	Recovery	
	Soaking	Cooking
Pea		
Whole Green Pea	90.03%	85.44%
Split Green Pea	87.41%	79.02%
Whole Yellow Pea	91.16%	88.48%
Split Yellow Pea	88.83%	80.05%
Chickpea		
Whole Chickpea B90	92.54%	89.10%
Split Chickpea B90	86.53%	78.93%
Desi Chickpea	95.36%	90.33%
Fava bean		
Whole Fava Bean	92.31%	89.54%
Split Fava Bean	86.39%	75.29%
Lentil		
Whole Red Lentil	91.68%	83.57%
Football Red Lentil	82.97%	74.65%
Split Red Lentil	83.49%	76.00%
Split Yellow Lentil	84.77%	73.18%
Spanish Brown Lentil	93.89%	85.58%
Split Queen Green Lentil	83.43%	71.60%
French Green Lentil	92.12%	83.58%
Large Green Lentil	93.98%	86.68%
Medium Green Lentil	94.01%	86.48%
Bean		
Pinto Bean	94.44%	88.24%
Navy Bean	92.83%	85.11%
Dark Red Kidney Bean	93.91%	85.66%
Black Bean	94.78%	87.93%
Soybean	93.18%	85.17%

$$\% \text{ Recovery} = \frac{\text{Weight of soaked/cooked seed}}{\text{Weight of raw seed}} \times 100$$

Appendix 2. Moisture content of raw, soaked and cooked flours of Canadian pulses and soybean

Type	Moisture content (%)		
	Raw	Soaked	Cooked
Pea			
Whole yellow pea	9.19 ± 0.13	6.21 ± 0.07	5.96 ± 0.05
Split yellow pea	7.34 ± 0.06	3.46 ± 0.03	2.71 ± 0.03
Whole green pea	9.88 ± 0.12	6.65 ± 0.05	6.27 ± 0.03
Split green pea	7.34 ± 0.07	3.41 ± 0.08	2.68 ± 0.07
Lentil			
Whole red lentil	7.17 ± 0.05	3.88 ± 0.04	3.48 ± 0.04
Split red lentil	6.07 ± 0.04	3.79 ± 0.05	2.94 ± 0.03
Football red lentil	7.13 ± 0.04	4.38 ± 0.01	3.01 ± 0.02
Spanish brown lentil	7.10 ± 0.03	5.38 ± 0.07	3.54 ± 0.02
Split yellow lentil	7.39 ± 0.02	3.27 ± 0.04	3.04 ± 0.00
French green lentil	7.00 ± 0.03	5.03 ± 0.02	3.43 ± 0.02
Large green lentil	6.95 ± 0.02	5.01 ± 0.03	3.80 ± 0.02
Medium green lentil	7.02 ± 0.02	5.63 ± 0.04	4.38 ± 0.02
Split queen green lentil	7.47 ± 0.03	3.67 ± 0.03	2.95 ± 0.03
Fava bean			
Whole fava bean	7.30 ± 0.07	6.71 ± 0.02	5.65 ± 0.06
Split fava bean	7.77 ± 0.03	2.72 ± 0.02	2.10 ± 0.05
Chickpea			
Whole chickpea B90	6.69 ± 0.09	5.06 ± 0.03	6.20 ± 0.07
Split chickpea B90	6.21 ± 0.03	2.84 ± 0.06	3.53 ± 0.02
Desi chickpea	6.18 ± 0.06	5.98 ± 0.03	5.40 ± 0.00
Bean			
Dark red kidney bean	7.48 ± 0.07	5.21 ± 0.05	4.25 ± 0.06
Pinto bean	7.44 ± 0.06	6.15 ± 0.02	5.65 ± 0.04
Navy bean	7.35 ± 0.13	5.55 ± 0.04	3.60 ± 0.04
Black bean	6.62 ± 0.03	5.83 ± 0.02	5.12 ± 0.02
Soybean, full fat	4.93 ± 0.02	3.85 ± 0.04	4.89 ± 0.09
Soybean, defatted	5.40 ± 0.03	4.66 ± 0.02	5.17 ± 0.03

Means ± standard deviation of three determinations.