The Saponin Composition of Common Canadian Pulses

By

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Abstract

Pulses are high in nutritional value but are underutilized as foods due to their undesirable flavor attributes. These include bitterness, which may in part due to their saponin content. In this research, a simple and rapid method using high-performance liquid chromatography-mass spectrometry (HPLC-MS) and requiring the minimum sample preparation was developed for the identification and quantification of saponins. This was applied to 8 common Canadian pulses including 3 pea varieties, 4 faba bean varieties, pinto beans, black bean, kidney beans, chickpeas, and green and red lentils. The method was validated according to linearity, accuracy, detection limit, quantification limit, inter- and intra-day precision. The calibration curve for a soyasaponin Bb standard with added internal standard (ginsenoside Rb1) showed correlation coefficients of 0.994 and a linear range of 0.2-5 μg/mL. Saponin recoveries from the extraction method applied to faba bean samples ranged from 95 -105%, with n= 6 and RSD <12%.

Saponin composition and content varies depending on the pulse type and variety. Four types of group B saponins including DDMP-conjugated soyasaponin βg and αg, and non- DDMP-conjugated soyasaponin Bb and Ba were identified in several pulse samples by HPLC-MS according to their relative retention times, and their molecular and fragment ions, as compared to standards and literature. All of the pulses tested contained soyasaponin Bb and βg with different amounts and percentile distribution. Amongst all of the 8 types of pulses, the total saponin content ranged from 30 to 8566 μg/g, where the lowest saponin content was found in faba bean variety Fabelle and the highest saponin content was found in black beans. Within the 3 pea varieties, the total saponin content varied from 550 to 2144 μg/g, and for the 4 faba bean varieties tested it varied between 30 and 388 μg/g. In all pulse samples, either soyasaponin Bb or βg was the predominant

saponin type. Soyasaponin Ba and αg was only present in small amounts, except for black beans and pinto beans where 3145 and 1306 μg/g of αg was found, respectively.

The effects of pulse processing methods (sprouting, drying, baking and pressure cooking) on the saponin profile was investigated. Four cultivars of faba beans grown in Alberta, Canada were germinated and subjected to a range of sprouting times (0, 48, 54, 60, and 72 h and drying times (0, 24, 36, 48, and 60 h). The saponin profiles of raw and sprouted faba bean seeds were measured, along with those found in baked faba bean flour-based crackers. Soyasaponin Bb and soyasaponin βg were the only two types of saponin found in the faba bean varieties studied. Soyasaponin Bb reaches the highest abundance after 54 h of sprouting in most cultivars, whereas the highest for βg was observed at 60 h in most cases, except for FB9-4 (54 h). A significant reduction of soyasaponin Bb was observed after 24 h of drying the sprouted seeds at 60°C. The total saponin content after 72 h of sprouting significantly increased in Snowdrop and Fabelle, decreased in FB9-4 and no change in Snowbird compared to unsprouted seeds. Prolonged drying times of up to 60 h significantly reduced the soyasaponin Bb content of 48 hours sprouted faba beans, whereas a slight increase was observed in the soyasaponin βg content in Snowdrop and Fabelle. Regardless of the sprouting condition, both baking and cooking of faba bean flour led to significant reductions in both total saponin and individual saponin content. In addition, the combination of germination and pressure cooking is more effective in reducing saponin content in faba bean comparing to pressure cooking alone.

The present research into the saponin profile of pulses and how this is changed by common food processing methods, contributes fundamental knowledge which may be beneficial in the utilization of pulses and pulse flour in food.

Preface

This thesis is an original work by Beiyi Shen under the supervision of Dr. Jonathan Curtis. There are a total of five chapters in this thesis, where Chapter 1 is literature review provides an overall introduction on topics related to this research and the hypothesis and objective of this study; Chapter 2 developed and validated an analytical method for quantification of saponin from faba bean samples by using HPLC-MS; Chapter 3 investigated the effects of sprouting, drying and baking on the saponin composition and content of faba bean samples; Chapter 4 conducted a survey on the saponin profile of 8 common Canadian pulses and the effects of pressure-cooking on saponin content in those pulse samples; and Chapter 5 is the overall summary of the key findings and recommendations for future study.

In Chapter 2 of this thesis, the optimization of HPLC-MS was achieved with the assistance of Dr. Yuan Yuan Zhao. In Chapter 3 of this thesis, the preparation of sprouted faba bean seeds and baked cracker were prepared at Food Processing Development Centre (Leduc, AB, CA). Chapter 3 of this thesis was part of Sprouted Faba Bean Project-820039 (2017F108R), sponsored by Agriculture Funding Consortium- Alberta Pulse Growers Commission (AFC-APGC). In Chapter 4 of this thesis, the sensory test was conducted and studied at Food Processing Development Centre (Leduc, AB, CA). The performance of all the other experiments, data analysis and literature review were Beiyi Shen's original work.

Manuscripts based on Chapter 2, 3 and 4 are in the preparation for submission to Food Research International and Journal of Food Composition and Analysis for consideration of publication.

Dedication

To my parents and grandfather

For your support, love and guidance

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List of Abbreviations

Abbreviation	Definition
ACN	Acetonitrile
C	Carbon
Cys	Cystine
DDMP	2,3-Dihydro-2,5-Dihydroxy-6-Methyl-4*H*-Pyran-4-One
ELSD	Evaporative Light Scattering
ESI	Electrospray Ionization
FTIR	Fourier Transform Infrared Spectroscopy
G6PD	Glucose 6-Phosphate Dehydrogenase
GAE	Gallic Acid Equivalent
gal	Galactose
GC	Gas Chromatography
glcUA	Glucoronyl Group
glu	Glucose
Glu	Glutamic Acid
GSH	Glutathione
LC-MS	Liquid Chromatography-Mass Spectrometry
Leu	Leucine
LOD	Limit Of Detection
LOQ	Limit Of Quantification
N	Nitrogen
NMR	Nuclear Magnetic Resonance Spectroscopy
MeOH	Methanol
MRM	Multiple Reaction Monitoring
Met	Methionine
MUFA	Monounsaturated Fatty Acids
m/z	Mass-To-Charge Ratio
PUFA	Polyunsaturated Fatty Acids
QCS	Quality Control Sample
rha	Rhamnose
RP	Reverse Phase
RSD	Relative Standard Deviations
S/N	Signal-To-Noise Ratio
SFA	Saturated Fatty Acids
SIM	Selected Ion Monitoring
SPE	Solid Phase Extraction
TLC	Thin-Layer Chromatography
UPLC	Ultra Performance Liquid Chromatography
UV	Ultraviolet Spectroscopy
XIC	Extracted Ion Chromatogram

Chapter 1 Literature review

1.1 Canadian grown pulses

Pulses, also referred to as legumes belong to the family *Fabaceae* (or *Leguminosae*), which consist of more than 700 genera and 18000 species (Tiwari, Gowen, & McKenna, 2011). According to the Codex Alimentarius Commission, pulses are defined as "dry seeds of leguminous plants which are distinguished from leguminous oil seeds by their low-fat content". The Food and Agriculture Organization (FAO) has suggested that the 11 most commonly consumed pulses include dry beans, dry broad beans (faba beans), dry peas, chickpea, dry cowpea, pigeon pea, lentil, Bambara groundnut, vetch, lupins, and other minor pulses.

Pulses are well-known for their nutritional value and health-promoting properties (Derbyshire, 2011). They are a good source of carbohydrate, protein, dietary fiber, vitamins and minerals including iron, zinc, and copper. The major protein fraction in pulses are storage proteins, which are classified into albumins, globulins, prolamins and glutelins based on their solubility (Sánchez-Chino, Jiménez-Martínez, Dávila-Ortiz, Álvarez-González, & Madrigal-Bujaidar, 2015). Globulins are saline-soluble proteins that represent 70% of the total amount of proteins in pulses, followed by water-soluble albumins and dilute acid/ alkali-soluble glutelins, which represent 10-20% of the total amount of proteins, respectively. Prolamins are ethanol-soluble proteins, which are the predominant protein found in cereals whereas they are absent in pulses. Pulses contain a high amount of non-sulphur amino acid Lys, but low in Trp and sulphur amino acids. On the contrary, cereals have a low concentration of Lys and a high concentration of sulphur amino acids. Therefore, the combination of pulses and cereals in the diet provides complementary essential amino acids for adequate nutrition (Derbyshire, 2011). Pulses also contain a wide range of

bioactive compounds including phytates, lectins, phenolic compounds, flavonoids, isoflavones, saponins, and lignins (Carbonaro, 2011). According to the Canada's food guide (2020), it is yrecommended to consume ¼ plate of protein foods which include beans, peas and lentils daily. The consumption of pulses has been suggested by the Food and Agriculture Organization (FAO, 2020) to have health promotion effects such as improving immunity, cardiovascular health, managing cholesterol, digestive health and regulating energy levels. However, pulses also contain antinutritional compounds that may have adverse effects on health. Those compounds include lectins (reduce nutrient absorption), protease inhibitors (increase carcinogenesis), amylase inhibitors (reduce starch digestion), phytates and oxalates (reduce bioavailability of minerals) (Carbonaro, 2011; Derbyshire, 2011). Most of enzyme inhibitors and lectins can be inactivated by domestic processing such as soaking and cooking. The bioactive components of pulses have been used in the preparation of functional foods, particularly in ready-to-eat snacks (porridge and energy bar), extruded products (pasta and spaghetti) and bakery products (cracker, bread and cookie) (Apea-Bah, Minnaar, Bester, & Duodu, 2016; Barakat, Reim, & Rohn, 2015a; Chávez-Santoscoy, Lazo-Vélez, Serna-Sáldivar, & Gutiérrez-Uribe, 2016; Chillo, Monro, Mishra, & Henry, 2010; Huang et al., 2018; Kaczmarska, Chandra-Hioe, Frank, & Arcot, 2018).

The production of pulses play a role in promoting environmental sustainability (FAO, 2016; Watts, 2011). Pulse crops have a symbiotic relationship with microorganisms in the soil in which the bacteria simulate the growth of encapsulating nodules. Then these nodules host the bacteria colonies and collect nitrogen (N) from the air and store them for plant usage. After pulses been harvested, the nodules decompose, and the stored N is released back into the soil. In that case, the plantings require less nitrogenous fertilizer, which improve environmental sustainability.

The production and utilization of pulses have more than 20,000 years of history in Eastern cultures (Watts, 2011). Nowadays, pulses are produced all over the world, whereas India is the largest producer followed by China, Brazil, Canada, Myanmar, Australia, and Mexico. According to FAO (Table 1.1), the world production of pulses increased from 60.9 million tonnes in 2008 to 73 million tonnes in 2012. In 2012, dry beans represent 32.7% of world total pulses production, followed by chickpeas (17.0%) and dry peas (14.5%). The remaining production is made up of cowpeas, lentils, pigeon peas, broad beans, lupins, and vetches. In terms of world production of pulses by country, India remains the largest producer and chickpeas, followed by dry beans and pigeon peas are the predominant pulses. In Canada, total pulses production was 0.063 million tonnes during 1961-1963, 5.4 million tonnes during 2011-2013, and 5.9 million tonnes in 2015 (FAO, 2016; Government of Canada, 2014). Peas, lentils ,and beans are the largest pulses in terms of pulse production in Canada (Watts, 2011). The largest pulse production province in Canada is Saskatchewan, which accounts for 79.3% of the total pulse production, Ontario (38.4%), Manitoba (32.1%) and Alberta (18.8%) (Government of Canada, 2014). The majority of pulses are consumed in countries in South Asia including India, Pakistan, Bangladesh ,and Myanmar, in the Middle East such as Turkey, Iran and Egypt (Watts, 2011). India is the largest pulse consumer around the world, which account for 15 million tonnes (35% of total pulse consumption). The USA and China each consume pulses for 1.5 million tonnes annually. According to FAO, the total consumption of pulses as food is rising globally. In 1961, the global consumption of pulses was 29 million tonnes, whereas the consumption went up to 42.7 million tonnes in 2007 (48% increase). However, per capita consumption of pulses went down from 9.5 kg in 1996 to 6.5 kg in 2007.

Table 1.1.1 Major countries of global pulse production (adapted from FAO, 2016).

Country	1961-63 (mt)*	2011-13 (mt)*	Avg. ann. growth 1961-2013 (%)	Major pulse types
World	44.8	73.9	1.3	Beans, chickpeas, peas
India	12.0	17.6	1.5	Chickpeas, beans, pigeon peas
Canada	0.063	5.4	12.0	Peas, lentils
China	9.5	4.5	0.4	Beans, broad beans, peas
Nigeria	0.56	3.9	7.5	Cowpeas
Brazil	1.86	3.1	2.8	Beans
Australia	0.023	3.0	18.5	Lupines, lentils, chickpeas
USA	1.1	2.0	3.5	Beans, peas
Mexico	0.85	1.3	4.2	Beans, chickpeas, vetches
Turkey	0.60	1.3	2.0	Beans, chickpeas, lentils vetches

* = million tonnes

1.1.1 Beans

1.1.1.1 Faba bean (Vicia faba var. minor)

1.1.1.1.1 Production, consumption and significance

Faba bean (broad bean, fava bean, horse bean, field bean) is an early winter-sown legume crop that has a long history of use as feed and food. Cultivation of faba bean can be traced back about 10,000 years to the Middle East and Mediterranean regions (Ruisi et al., 2017). It is an important legume crop that mainly consumed in the Middle East, the Mediterranean region, China and Ethiopia as vegetable, green, dried or canned forms (Jensen, Peoples, & Hauggaard-Nielsen, 2010). According to FAO (2016), the total global production area of faba bean was 2.6 million ha in 2006, mainly in China (1.05 million ha), followed by the European Union (0.39 million ha), Ethiopia (0.34 million ha) and Egypt (0.18 million ha). The average world faba bean production was 5.5 million tonnes between 1961 to 63 (12.4% of total pulse production) and decreased to 4.1 million tonnes during 2011 to 13 (5.7% of total pulse production). The production of faba bean dropped by more than 50% over the past 50 years in China revealing the shift in consumption from plant-based protein towards animal-based proteins, with the growth in per capita incomes in that country

(FAO, 2016). In Italy, the production of faba bean has declined from 4% of total pulse production in 1960 to 0.5% in 2006. This decline was due to the fact that faba bean as the main animal feed in Italy was substituted to imported soybean grains and cakes (Ruisi et al., 2017). However, the average production is more stable in the UK, Australia ,and Canada from 1970 to 2005 (Jensen et al., 2010).

The productivity of most of the grains and oil crops are largely depended on fossil energy from nitrogen fertilizer (Jensen et al., 2010). The environmental concerns regarding the limitation of fossil resources, the large emission of CO_2 due to production and use of fertilizer N, and the inefficiencies use of fertilizer N due to the losses of large amounts of N from fertilized soils are arising nowadays (Jensen et al., 2010). Faba bean is beneficial to the cropping systems in terms of sustainability since they have the ability to reduce the need for fertilizer through biological nitrogen fixation thus reduced the fossil energy consumption in crop production (Giambalvo et al., 2012). Moreover, faba bean can be used as a green manure that enhances soil fertility by transferring organic N and carbon (C) into the soil (Jensen et al., 2010).

1.1.1.1.2 Plant structure and characteristics

Faba bean grows on bushy plants that have an average of 1.5 meters in height, with tapering leaves and purple, white or pink flowers. Small seeded faba bean types have approximately 60 pods, whereas large-seeded genotypes grow 15 pods, and each pod contains 3 to 6 seeds (Alberta Pulse Growers, 2019; Saskatchewan Pulse Growers, 2019). Faba bean is divided into three sub-species based on seed size: small seeds are called as tic beans (*Vicia faba L. var. minor*), medium seeds are referred as horse beans or field beans (*Vicia faba var. equina*), and large seeds are known as broad beans (*Vicia faba var.major*) (Alberta Pulse Growers, 2019). Broad beans are grown

primarily for human consumption, whereas tic beans and field beans are used both as livestock feeds and foods for human.

1.1.1.1.3 Major genotypes grown in Canada

There are two main purposes of growing different genotypes of faba beans in the present market in Canada. High or normal tannin types faba bean grown for human consumption have brown or violet flowers, and tan to brown seed coat. Low tannin types faba bean both grown for high protein livestock feed and for human consumption have white flowers and cream seed coat (Saskatchewan Pulse Growers, 2019). The high or normal tannin faba bean varieties include CDC Fatima, CDC Blitz, FB9-4, Fabelle, Florent, CDC SSNS-1, Taboar, Vertigo, 186S-11 and 247-13. Low tannin varieties include Impsosa, Snowbird, CDC Snowdrop, Tabasco, DL Tesoro and DL Rico (Saskatchewan Pulse Growers, 2019). The majority of cultivars of faba bean grown in Alberta and western Canadian is marketed as animal feed (Alberta Pulse Growers, 2019).

Snowbird: This faba bean variety originated from the cross-breeding of the varieties Alfred B and 8103 in 1989 in Lelystad, in the center of the Netherlands. The breeding selection main criteria include yield, early maturity, lodging and disease resistance were considered when the first trials for Snowbird were conducted at Westlock, Alberta during 2001 and 2002 (CFIA, 2019). Snowbird is 95 cm in height with a low tannin content that is suitable for human consumption. It has a white flower and the medium-size seeds (448g/ 1000 seeds) mature at day 104 (Government of Saskatchewan, 2019).

FB9-4: This cultivar of faba bean is also known as Malik and was bred by the Crop Diversification Center in Saskatoon, Saskatchewan (Saskatchewan Pulse Growers, 2019). The plants have

coloured flower with normal tannin content and large seed size (680g/ 1000 seeds). It has an average height of 95 cm and a mature day of 104 (Government of Saskatchewan, 2019).

Snowdrop: This variety was obtained in 2012 by Dr. Bert Vandenberg from the Crop Development Center at the University of Saskatchewan in Saskatoon. Snowdrop is a small-seeded (325 g/ 1000 seeds) with white flower low tannin faba bean variety. The plant is 97 cm tall and the maturity is 104 days (Government of Saskatchewan, 2019).

Fabelle: This variety was tested and registered in Europe in 2011 and was introduced into Canada by the DL Seeds Inc. (Morden, Manitoba). It is 104 cm in height with normal tannin and low vicine/convicine for food use. It has a maturity of 105 days when pods produce medium seeds (533g/ 1000 seeds) (Government of Saskatchewan, 2019).

1.1.1.1.4 Nutritional composition and properties

The nutritional composition of high and low tannin faba beans are presented in Table 1.1.2. The chemical composition of faba bean vary depending on genotypes, growing regions, crop year, maturity time, processing and storage conditions (Sathya Prabhu & Devi Rajeswari, 2018).

In both genotypes, starch is the dominant constituent, followed by protein. Starch is the main source of carbohydrates in the diet and provide energy for human. Other carbohydrates present in faba bean seeds are monosaccharides include ribose, glucose, galactose, and fructose, disaccharides such as sucrose and maltose. Moreover, the seeds contain water-soluble oligosaccharides such as raffinose, stachyose ,and verbascose (Sánchez-Chino et al., 2015). These oligosaccharides act as prebiotics since they can be fermented by bacteria in the colon without being digested or absorbed in the human intestinal tract due to the lack of α-galactosidase enzyme. Some beneficial effects linked with prebiotics include enhancing the immune and digestive

systems, improving glycemic regulation, reducing blood triglyceride level, anticancer and anti-inflammatory properties (Carbonaro, 2011).

The fractions of protein are composed of 79% of globulins, 7% of albumins and 7% of glutelins. Faba bean has a low concentration of methionine, cysteine ,and tryptophan but is high in arginine and glutamic acid, as compared to cereal grains (Presto, Lyberg, & Lindberg, 2011).

Faba bean is also a rich source of fiber, especially the pods part as pulse by-products can be incorporated into processed foods as functional ingredients to increase dietary fiber content. The health benefits of including fiber-rich products in daily diets have been associated with reduced breast cancer and cardiovascular disease risk, prevention of diabetes and obesity management (Carbonaro, 2011; Derbyshire, 2011).

Faba bean also contains saturated fatty acids such as palmitic acid and stearic acid; unsaturated fatty acids such as myristic, pentadecanoic, arachidic, behenic acids, oleic acid, linoleic acid and linolenic; minerals include Na, P, Ba, Co, Ca, K, Cu, Zn, Fe, Mn and Mg; vitamins include folic acid, niacin and vitamin C (Sathya Prabhu & Devi Rajeswari, 2018).

Despite the high nutritional value of faba bean, the seeds also contain many anti-nutritional constituents including vicine and convicine, which are glucosidic aminopyrimidine derivatives. Upon encountering the β-glucosidase enzyme in mammals' gut, their β-glucosidic bonds between glucose and the hydroxyl group on the pyrimidine ring at carbon (C) 5 position breaks and generates the aglycones called divicine and isouramil (Rizzello et al., 2016) (Fig. 1.1.1). When divicine and isouramil are absorbed into the blood, red blood cells will be destroyed and cause premature oxidative damage unless reduced glutathione (GSH) react and neutralize the reactive oxygen species. Individuals with low-activity variants or deficiency of erythrocytic glucose 6-phosphate dehydrogenase (G6PD) are unable to regulate the production of NADPH in the red

blood cell thus GSH is unable to be regenerated to defend against oxidative stress. Membrane proteins and hemoglobin are oxidized under oxidative stress that leads to denatured protein clumping and aggregation. This hemolytic anemia result from the ingestion of faba beans by susceptible individuals with G6PD deficiency is called favism (Getachew, Vandenberg, & Smits, 2018).

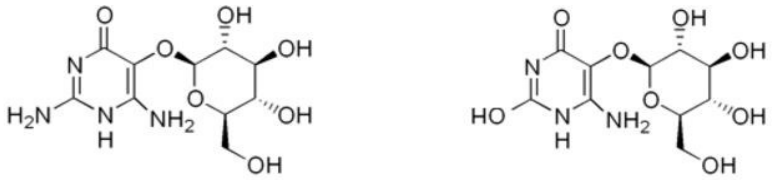
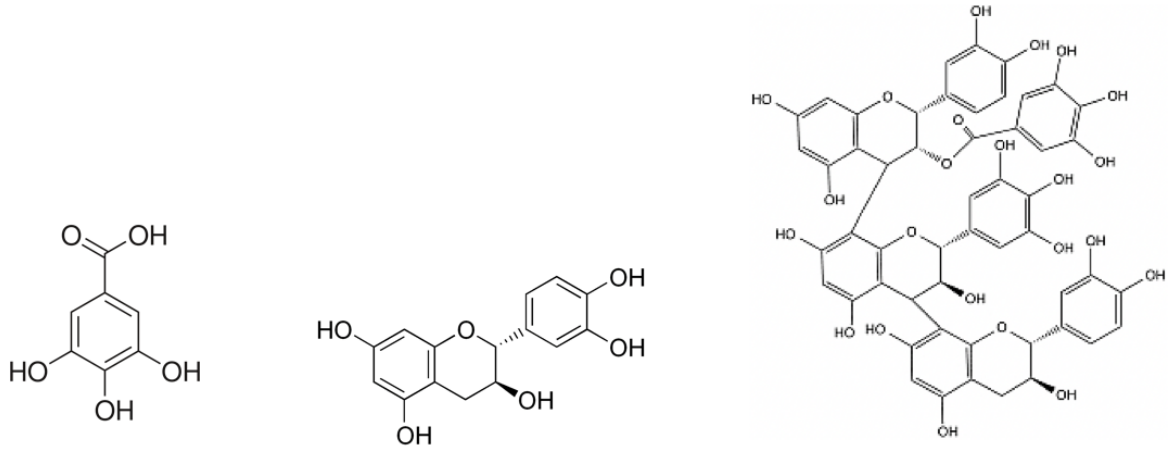


Figure 1.1.1 Molecular structures of vicine and convicine (structure information adapted from Rizzello et al., 2016).

Tannins are another major anti-nutritional factor naturally present in faba bean. They are classified into 2 groups based on chemical structure: hydrolyzable tannins (Fig. 1.1.2. (a)), which are ester form of phenolic acids, and condensed tannins, which are flavonoid polymers (catechins or proanthocyanidins) (Fig. 1.1.2. (b), (c)) (Multari, Stewart, & Russell, 2015). Tannins are able to interact with proline-rich proteins such as collagen, gelatin, casein, digestive enzymes and in particular, precipitate salivary proteins in the oral cavity to form soluble or insoluble complexes that negatively affect the palatability of foods (astringent). Tannins are considered as antinutrients because of their affinity to proteins and minerals, particularly iron, zinc ,and copper, thus reduce the bioavailability and digestibility of proteins and trace elements (Carbonaro, 2011). Moreover, condensed tannins were found out to be responsible for the astringency of pulses include faba bean (*Vicia faba var. minor*), broad bean (*Vicia faba var. major Harz*), adzuki bean, red bean, pea, green and red lentil (Ate Troszyńska, Amarowicz, Lamparski, Wołejszo, & Baryłko-Pikielna, 2006).



(a) Gallic acid (b) catechin (c) proanthocyanidins

Figure 1.1.2 Molecular structures of examples of hydrolyzable tannins (a) and condensed tannins (b) and (c) commonly found in plants. (structure information adapted from Multari, Stewart, & Russell, 2015).

Other anti-nutritional factors present in faba beans include phytic acid (reduces Ca, Cu, Mg, Zn and Fe absorption), protease inhibitors (reduce protein digestion and absorption), lipase inhibitors (reduce lipid hydrolysis and absorption), lectins (reduce nutrients absorption and interfere with digestion) and oxalates (reduce Ca absorption) (Carbonaro, 2011; Derbyshire, 2011; Ivarsson & Neil, 2018; Multari et al., 2015).

Table 1.1.2 Analyzed chemical composition of different faba bean cultivars based on dry matter (adapted from Ivarsson & Neil, 2018).

Cultivar	Starch (%)	Crude protein (%)	Crude fiber (%)	Ash (%)	Lipids (%)
High tannin[a]	43.8	34.6	9.4	3.7	0.9
Low tannin[b]	43.8	34.5	9.3	3.9	0.9

[a] 11 of high tannin cultivars, harvested at 3 different locations in southern Sweden, 54 samples
[b] 5 of low tannin cultivars, harvested at 3 different locations in southern Sweden, 30 samples

Raw faba beans contain many anti-nutrients factors that can limit the absorption of certain minerals, as well as their bitterness, astringency and undesirable beany flavor make them greatly under-ultilized. However, conventional processing such as soaking, dehulling, cooking, fermentation and sprouting have been found to reduce certain anti-nutrients such as phytate, protease inhibitors,

phenolics, condensed tannins, lectins and saponins as well as improve palatability (Patterson, Curran, & Der, 2017).

1.1.1.2 Pinto beans

Pinto bean (*Phaseolus vulgaris* L.) is a type of common beans (also called dry beans). Pinto beans originated from Andean and Mesoamerican during the New World (Balkaya & Ergün, 2008). The common varieties of pinto beans grow in Canada include CDC Pintium, Island, Mariah, CDC Marmot, Medicine Hat, Winchester and CDC WM-2 (Saskatchewan Pulse Growers, 2019). It takes 50-58 days for pinto bean seeds to grow to flower, and 100-110 days to maturity. The seed weight ranges from 293 to 367 g/ 1000 seeds depending on the varieties. Two of the most important factors need to consider in the adaption of pinto beans in Canada are temperature and moisture (Saskatchewan Pulse Growers, 2019). Pinto bean accounts for 39% of total acreage in Alberta (Alberta Pulse Growers, 2019). Pinto bean seeds contain 2.3% of moisture, 2.5% of ash, 14.4% of crude fat, 18.0% of crude protein, 3.0% of crude fiber, 59.7% of carbohydrate, and 11.5% of fatty acid (Audu & Aremu, 2011). The metabolizable energy is 1851.4 kJ/ 100 g (calculated based on protein x 17 + fat x 37 + carbohydrate x 17). Pinto bean contains large range of minerals include Mg (789.7 mg/ 100g dry matter), Ca (62.9), Na (22.0), Fe (13.3), K (8.3), Ni (4.5), P (4.2), Zn (3.8), Cr (2.8), and Cu (1.0). Total amino acids, total essential amino acids and total sulphur-containing amino acids in pinto bean seeds are 79.0, 40.1 and 2.1g /100 g crude protein, respectively. Pinto bean is high in glutamic acid (Glu) (12.0 g/ 100g crude protein dry matter) and leucine (Leu) (7.6), low in cystine (Cys) (1.0) and methionine (Met) (1.1) (Audu & Aremu, 2011).

1.1.1.3 Black beans

Black beans also called black turtle beans, are a variety of the common bean (*Phaseolus vulgaris*). The variety of black beans grow in Canada includes CDC Blackstrap, CDC Jet and CDC Superjet (Saskatchewan Pulse Growers, 2019). All three varieties are indeterminate bush growth type with 3-4 branches and 10-12 nodes on the main stem. After seeding, it takes black bean seeds 53-58 days to grow flower and 110 days to mature. The seed weight ranges from 170 to 195 g/1000 seeds. The proximate chemical composition of black bean largely depending on varieties and growing conditions. On average, the black bean has 15.5% of moisture content, 22.6% of crude protein, 5.2% of crude fiber, 52.3% of carbohydrates and 3.5% of ash on dry matter basis. Black bean is high in arginine, lysine, and valine, whereas low in methionine, cystine, and tyrosine (Bressani, Elias, & Navarrete, 1961). The total starch found in cooked black bean bred in Mexico is 538 g/kg, available starch is 428 and resistant starch is 114 g/kg on dry matter basis. The total indigestible fiber, soluble indigestible fiber, and insoluble digestible fiber are 524.4, 107.8 and 416.6 g/kg, respectively. Total polyphenols and anthocyanin content found in cooked black bean are 2.54 mg/g and 48.3 mg/kg, respectively (Silva-Cristobal, Osorio-Díaz, Tovar, & Bello-Pérez, 2010).

1.1.1.4 Kidney beans

Kidney bean is another variety of common bean (*Phaseolus vulgaris*). The classification of kidney bean is based on seed colour and shape, examples include red kidney bean, white kidney bean, light speckled kidney bean, and red speckled kidney bean. The difference in the nutritional composition of kidney beans have been observed among different cultivars (Kan et al., 2017). On average, kidney bean contains 29.32-46.77% of dietary fiber, 9.16-18.09% of resistant starch, 22.06-32.63% of protein, 1.05-2.83% of lipid and 1.55-9.07% of sugars. The major soluble dietary

fiber is arabinose, galactose, mannose, and galacturonic acid. The seeds contain 20.29-43.32% of saturated fatty acids (SFA), 7.19-15.96% of monounsaturated fatty acids (MUFA), and 47.54-67.26% of polyunsaturated fatty acids (PUFA). The dominating PUFA is α-linolenic acid and linoleic acid, which are essential fatty acids have beneficial effects on human health. The major SFA are palmitic acid and stearic acid (Kan et al., 2017). Kidney bean also contains various minerals and vitamins such as Ca, Fe, Mg, P, Zn, thiamine (B1), riboflavin (B2), niacin (B3), B6, folate, vitamin C, E and K. Like other common bean varieties, kidney bean has higher amount of aspartic acid, glutamic acid, leucine, and low in cysteine, methionine and histidine content (Arija et al., 2006). The total phenolic content of kidney bean range from 0.25 to 3.79 mg gallic acid equivalent (GAE)/g on dry matter basis, and the total flavonoid content range from 0.19 to 7.05 mg rutin equivalent/g dry weight of beans (Kan et al., 2017).

1.1.2 Peas

1.1.2.1 Chickpeas

Chickpea (*Cicer arietinum* L.) is an annual plant belongs to the monogeneric tribe Cicereae of Fabaceae family. Evidence suggests that the plant is originated from the area between south-eastern Turkey and adjoining Syria in 7000 B.C. (Sobhan B. Sajja & Gaur, 2017). It was traditionally grown in semi-arid zones in India and Middle Eastern countries. Currently, chickpea is the third most important food legume grown in over 45 countries across India, North Africa, the Middle East, southern Europe, the Americas and Australis (Agriculture and Agri-Food Canada, 2009). The main annual production of chickpea is 10.1 million tonnes, behind field peas (10.4 million tonnes) and dry beans (21.5 million tonnes) from 2004 to 2013 (Muehlbauer & Sarker, 2017). The total production area of chickpea increased from 8.9 to 13.5 million hectares from 1981

to 2013. India has the largest chickpea production, which accounts for 70% of total world production, followed by Pakistan (10%), Iran (5%), Turkey (4%) and Australia (3%).

Cultivated chickpea is classified into two types, *Kabul*i and *Desi*, based on seeds colour and size. *Desi* is the more prominent type that accounts for 80% of world chickpea production and is characterized by small seed size (0.2 g/ seed) with light tan to black seed colours. It has yellow cotyledons and thick seed coats with varying colour include cream, yellow, brown, black and green based on the anthocyanin pigments content. *Kabuli* is the less prominent type that accounts for 20% of world production and is characterized by relatively large seed size (0.3-0.5 g/ seed) with cream, white or beige colour due to the lack of anthocyanin pigments (Muehlbauer & Sarker, 2017; Sobhan B. Sajja & Gaur, 2017). In Saskatchewan, the growing *Kabuli* type varieties include Amit (B-90), CDC Alma, CDC Frontier, CDC Leader, CDC Luna, CDC Orion, and CDC Palmer, whereas *Desi* type varieties include CDC Consul and CDC Cory. The plants have a height range from 40 to 47 cm and take 50-56 days to flower and 110-130 days to mature. The seed's weight ranges from 259 to 435 g/ 1000 seeds (Saskatchewan pulse growers, 2019).

In Canada, chickpeas were first grown and produced in Saskatchewan in the mid-1990s (Agriculture and Agri-Food Canada, 2009). Approximately 88% of Canadian chickpeas are produced in the brown and dark brown soil zones in Saskatchewan and the rest 12% is produced in Alberta. *Kabul*i is suitable for growing in the brown soil zone, while *Desi* is adapted to both brown and dark brown soil zones (Agriculture and Agri-Food Canada, 2009).

The chemical composition of chickpea is dependent on genotypes, growing region, harvesting conditions, crop year, processing and storage conditions (Rachwa-Rosiak, Nebesny, & Budryn, 2015; Sobhan B. Sajja & Gaur, 2017). The chemical composition of *Kabul*i and *Desi* chickpea

cultivars is listed in Table 1.1.3, and it can be seen that the main difference is protein and fiber content.

Table 1.1.3 Analyzed chemical composition of Kabuli and Desi chickpea types on dry matter (%) (Adapted from Rachwa-Rosiak, Nebesny, & Budryn, 2015)

Types	Moisture content (%)	Crude protein (%)	Crude fiber (%)	Starch (%)	Total tannin (%)	Total phenolic compounds (%)
Kabuli	7.92	24.63	6.49	39.12	0.09	0.27
Desi	8.83	22.76	9.94	38.48	0.12	0.26

Chickpea is a rich source of carbohydrates. The major monosaccharides found in chickpea are ribose, fructose, glucose, sucrose, and maltose. It contains a high content of oligosaccharides including raffinose, ciceritol, stachyose, and verbascose (Wood, Knights, Campbell, & Choct, 2014a, 2014c). The protein content of two varieties ranges from 22.76 to 24.63%, of which consist of, 53.44-60.29% of globulin, 19.38-24.40% of prolamine, 8.39-12.31% of albumin, and 3.12-6.89% of glutein. The proteins contained in chickpea are rich in lysine, leucine, aspartic acid, glutaminc acid, and arginine amino acids, as well as low in tryptophan and cystine (Rachwa-Rosiak et al., 2015). Chickpea contains a low amount of lipids (4.5-6.0 g/ 100g seed). Those lipids are mainly essential unsaturated fatty acids include linoleic acid (54.7-56.2%), oleic acid (21.6-22.2%), palmitic acid (18.9-20.4%), stearic acid (1.3-1.7%) and linolenic acid (0.5-0.9%). Chickpea is also a good source of minerals including Ca, K, Mg, P and Fe (Wood, Knights, Campbell, & Choct, 2014b). However, antinutritional compounds were also found in chickpea, which including trypsin inhibitors (11.90 mg protein/dm), trypsin inhibitor (8.29 mg/g dm), phytic acid (1.21 mg/g), polyphenol (3.39 mg/g), saponin (0.91 mg/g), and tannin (4.85 mg/g) (Rachwa-Rosiak et al., 2015; Wood et al., 2014a).

1.1.2.2 Green and yellow peas

Pea (*Pisum sativum* L.), also called garden pea, is an annual pulse crop that belongs to the Leguminosae family. The origins of peas have not been determined and confirmed, while the earliest archaeological evidence suggests the seeds were found in the Middle East back to the late Neolithic Period (Glenn,C. Sevey, 1919). Peas are mainly consumed in northern Europe, middle Europe, Syria, Turkey, Israel, Russia, Iran, Iraq, Jordan, Lebanon, and India for a long history (Agriculture and Agri-Food Canada, 2008a). Nowadays, peas are grown in all climatic zones all over the world. Canada is the largest producer of peas in the world, where the production of peas was 2.1 million tonnes (21.7% of world total pea production) in 2011 (Statistics Canada, 2011). Saskatchewan accounts for 79% of national pea production, followed by Alberta and Manitoba, which account for 18% and 2%, respectively (Agriculture and Agri-Food Canada, 2008a).

The market class of peas is classified into yellow, green, red, maple, dun and forage pea (Saskatchewan pulse growers, 2019). Each class consists of many pea varieties with its own characteristics and target markets. In Alberta and Saskatchewan, green and yellow cotyledon peas are the major production types, which are suitable for both human consumption and livestock feed (Alberta pulse growers, 2019). Yellow peas have vine length range from 75 to 90 cm and seed weight range from 210 to 300 g/ 1000 seeds, whereas green pea varieties are 70 to 85 cm in vine length and 160 to 270 g/1000 seeds in seed weight. Yellow and green pea varieties have white flowers and are semi-leafless (Saskatchewan pulse growers, 2019).

The green peas varieties grown in Canada include Blueman, AAC Comfort, Cooper, CDC Forest, CDC Greenwater, CDC Limerick, CDC Patrick, CDC Pluto, AAC Radius, CDC Raezer, AAC Royce, CDC Sage, CDC Spruce, CDC Striker, and CDC Tetris. The yellow peas varieties grown in Canada include CDC Amarillo, Abarth, Agassiz, AAC Ardill, AAC Asher, CDC Athabasca,

CDC Canary, AAC Carver, AAC Chrome, Earlystar, CDC Golden, CDC Hornet, Hyline, CDC Inca, AAC Lacombe, CDC Lewochko, CDC Meadow, AAC Profit, CDC Saffron, CDC Spectrum, Thunderbird, and CDC Treasure (Saskatchewan pulse growers, 2019).

Cooper: This pea variety originated from the cross-breeding of the varieties Baccara and Cebeco 92585 in 1997 at the Cebeco Breeding Station in Lelystad, the Netherlands. The breeding selection main criteria include yield, plant height, lodging, and powdery mildew resistance were considered as the first test for Cooper were conducted at Westlock, Alberta during 2003 and 2004 (CFIA, 2019). It has an average plant height of 80 cm with a seed weight of 270 g/ 1000 seeds. The plant has green to blue colour, white flower, and light green immature seeds (Saskatchewan pulse growers, 2019).

CDC Amarillo: This yellow pea cultivar was bred from the pedigree CDC0007, CDC 715-4, CDC 653-8 and CDC 0108 at the Crop Development Center, the University of Saskatchewan in Canada in 2010. As continuous co-op registration trials, this variety was designated to a final variety check- in 2017. Comparing to CDC Golden and Cutlass, CDC Amarillo has higher yield, lodging and powdery mildew resistance, and vine length (T. D. Warkentin et al., 2014). It is a semi-leafless, white flower, yellow-cotyledon pea variety with round and opaque seed coat. It is 86 cm in height and has a maturity of 105 days when pods produce small seeds (233g/ 1000 seeds) (T. Warkentin, 2017).

AAC Lacombe: This variety was obtained as the result of cross-breeding of CDC715S-4 and Reward in the greenhouse at the Agriculture and Agri-Food Canada Research Station in Morden, Manitoba in 2003. The breeding selection main criteria include yield, early maturity, lodging resistance, and seed quality were considered when the first trial was conducted in Lacombe, Alberta during the summers of 2012 and 2013 (CFIA, 2019). AAC Lacombe is 136 cm in height.

It has an average of 59 days to flower and 105 days to reach maturity. The immature seed of AAC Lacombe is medium green, and dry seed becomes yellow (250g/ 1000 seeds) (Saskatchewan Pulse Growers, 2019).

The chemical composition varies among pea cultivars. Analyzed chemical composition for pea (*Pisum sativum* L. cv. Maria) include carbohydrates (52.5%), crude protein (21.9%), moisture (9.88%), lipids (2.34%) and ash (3.0%). The soluble and insoluble dietary fiber content in pea is 20.3% and 1.73%, respectively (De Almeida Costa, Da Silva Queiroz-Monici, Pissini Machado Reis, & De Oliveira, 2006). The major monosaccharides and oligosaccharides in pea are sucrose (5.07%), stachyose (3.67%), verbascose (1.93%) and raffinose (1.00%) (Zia-Ul-Haq, Ahmad, Amarowicz, & Ercisli, 2013). Pea contains high amount of K (1014 mg/100g seed), P (291 mg/100g seed), Ca (111 mg/100g seed) and Na (111 mg/100g seed), as well as small amount of Cu (10.9 mg/100g seed), Mg (4.4 mg/100g seed), Zn (3.6 mg/100g seed), Mn (2.7 mg/100g seed) and Fe (2.3 mg/100g seed). Pea is a good source of vitamins including thiamine, niacin, and riboflavin. For amino acid composition, pea contain high amount of glutamic acid (20.8%), aspartic acid (11.4%), lysine (8.2%), leucine (7.4%) and arginine (7.3%), while low amount of oleic acid (28.41 mg/100g seeds) tryptophan (0.8%), methionine (1.1%) and cystine (1.5%). Linoleic acid (47.77 mg/100g seeds), oleic acid (28.41 mg/100g seeds) and palmitic acid (12.2 mg/100g seeds) are the dominating fatty acids in pea. Moreover, pea seeds are a rich source of tocopherols and sterols, which are found to have health-promoting effects such as anti-inflammatory, anti-oxidative, anticarcinogenic activities, and lowering cholesterol (Zia-Ul-Haq et al., 2013).

1.1.3 Lentils

The lentil (*Lens culinaris* L.) is a lens-shaped grain legume of the Leguminaceae family. The oldest carbonized lentil remains were dated to 11000 BC from Greece's Franchthi cave and seeds were found at Tell Mureybit in Syria traced by to 8500-7500 BC. Archaeological evidence of the presence of large storage lentils suggests that northern Israel started to grow lentils in 6800 BC (Sandhu & Singh, 2007). Lentil is originated from the near East and Central Asia and nowadays, growing all over the world, especially in India, Canada, Turkey, Australia, Nepal, United States, Bangladesh, and China. In 1970, commercial lentil production in Canada began in Saskatchewan and expanded to Manitoba and Alberta in the 1980s and early 1990s (Agriculture and Agri-Food Canada, 2008b). In 2007, Saskatchewan accounts for 100% (0.67 million tonnes) of total Canada lentil production (Agriculture and Agri-Food Canada, 2008b). Canada was the largest lentil producer in 2011, where the annual production was 1.5 million tonnes and shared 34.8% of total global production (Statistics Canada, 2011).

Lentils are classified based on seed size (small: under 40g/ 1000 seeds or large: over 50g/ 1000 seeds), cotyledon colour (yellow, red or green), and seed coat colour (green, tan, brown, grey, blotched green and black, or black) (Saskatchewan Pulse Growers, 2019). There are two lentil marketing classes, which are green and red lentils. For green market class, lentils typically have yellow cotyledons, green seed coats with different seed sizes range from small to large. Green lentils typically are consumed as whole seed, and 75% of them have large seeds and only 25% are small-seeded. As for the red market class, lentils typically have red cotyledons, grey seed coats with extra small, small and large seed sizes. To improve palatability, red lentils are typically produced as dehulled or dehulled and split (Saskatchewan Pulse Growers, 2019). Some specialty lentil market classes are grown throughout Saskatchewan in small volumes include black lentils

(Beluga), French green lentils (green marbled seed coat, yellow cotyledons with small seed size), green cotyledon lentils (green or marbled seed coat, green cotyledons with small to medium seed size), Spanish brown lentils (grey dotted seed coat, yellow cotyledons with small seed size). In Saskatchewan, there are 16 varieties of small red, 7 extra small red, 2 large red, 3 small green, 1 extra small green, 4 medium green, 5 large green and 2 French green. On average, lentil plants are short in height that ranges from 20 to 75 cm. It takes 47- 55 days to flower and 100 – 110 days to mature (Saskatchewan Pulse Growers, 2019).

The chemical composition is dependent on lentil cultivars, environmental factors such as growing region, harvest year, climate and cultivation methods (Ganesan & Xu, 2017). As can be seen in Table 1.1.4, the red and green lentil cultivars grown in Iran are a good source of carbohydrate and protein, with a higher amount present in green than red lentils.

Table 1.1.4 Analyzed chemical composition of red and green lentil varieties (Lens culinaris Medik) on dry matter (%) (Adapted from Gharibzahedi, Mousavi & Jafari, 2012)

Types	Moisture content (%)	Crude protein (%)	Crude fat (%)	Carbohydrate (%)	Crude fiber (%)	Ash (%)
Red	6.28	25.9	2.7	59.01	13.8	3.62
Green	7.78	27.3	2.5	61.5	14.4	3.41

The carbohydrate fraction in lentil seeds is made up of 35-63% of starch, mainly amylose (20-45.5%), 20% of non-starch polysaccharides, monosaccharides such as fructose, glucose and sucrose (1-2.5%), galacto-oligosaccharides such as raffinose, ciceritol, stachyose and verbascose (2-8%) (J. S. Sandhu, 2007). Lentils contain vitamins include vitamin C (3.4-4.5 mg/ 100g), niacin (2.61-3.50mg/ 100g), thiamin (0.76-0.87 mg/ 100g), pyridoxine (0.54-0.70 mg/ 100g), tocopherols (0.49-0.55 mg/ 100g), riboflavin (0.19-0.21 mg/ 100g), folate (470-555 μg/ 100g), vitamin K (4.2-5.0 μg/ 100g), and vitamin A (2.0-2.5 μg/ 100g) (Ganesan & Xu, 2017). The major fatty acids found in lentils are linoleic acid (46.81-49.11%), oleic acid (21.3-23.27%), palmitic acid (14.41-

18.10%), and linolenic acid (8.20-11.25%). The minor fatty acids present include stearic acid (1.14-1.16%), gadoleic acid (0.43-0.66%), arachidic acid (0.39-0.55%), erucic acid (0.15-0.16%), and palmitoleic acid (0.08-0.15%). Lentils contain high amount of essential minerals include P (978-1024 mg/100g), P (301-341 mg/100g), Ca (168-170 mg/100g) and Na (65-81 mg/100g), as well as small amount of Fe (6.6-7.3 mg/100g), Cu (4.62-6.12 mg/100g), Mg (4.62-5.20 mg/100g), Zn (4.2-4.3 mg/100g) and Mn (1.40-1.62 mg/100g). (Gharibzahedi, Mousavi & Jafari, 2012). Lentil also contains bioactive compounds such as phytosterols, trypsin inhibitors, protease inhibitors, lectins, defensins, polyphenols, flavonoids, phytate, triterpenoids and saponins (Ganesan & Xu, 2017).

1.2 Saponin

Saponins are a group of naturally occurring chemicals that mainly produced by plants, lower marine animals and some bacteria (Francis, Kerem, Makkar, & Becker, 2002). The name "saponin" is originated from the Latin word "sapo", meaning soap and referring to their ability to form stable, soap-like foams in aqueous solutions. They show a wide range of polarities and biological activities that have attracted much interest in research (Böttcher & Drusch, 2017).

1.2.1 Structure

Saponins are non-volatile and amphiphilic compounds consist of polar, water-soluble saccharide chains (hexose, pentose or uronic acid), and glycosidically linked to non-polar, fat-soluble aglycone (sapogenin). The saccharide chain is made up of one or more linear oligosaccharides with 2-5 sugar units in the chain length. Common sugar units include D-glucose, D-galactose, L-arabinose, D-xylose, L-rhamnose and D-glucuronic acid (Biswas & Dwivedi, 2019; Singh, Pal,

Singh, & Kaur, 2017). Most of the classification of saponins is based on their aglycone structure and the number of attached sugar chains. Two main saponin types are triterpenoidal aglycone (30 carbon atoms) and steroidal aglycone (27 carbon atoms) types (Fig.1.3). Both classes of aglycones may contain a number of functional groups such as hydroxyl group, carboxylic acid and methyl group causing large diversity. The diversity is further multiplied by the composition of sugar chains, sugar numbers, branching patterns and substitution types on the aglycones (W. A. Oleszek, 2002).

In plants, saponin biosynthesis initiated with the production of two precursors (squalene and oxidosqualene) via the mevalonic acid/ methyl erythritol pathway (MVA/MEP) within the different cellular compartments (Biswas & Dwivedi, 2019). Those two precursors are cyclized, rearranged, and degraded into different aglycone structures, and further undergo through numerous secondary modifications to introduce carbonyl or hydroxyl groups via oxidation, ether bridges or lactones, ring cleavage or homologation to form seco-skeletons and nor-bis configurations. The biological properties of saponin are depending on the number, type, and position of sugar groups. The major saponins have been identified in pulses are the triterpenoidal aglycone type (Singh et al., 2017). They can be classified into monodesmosides, where having one single sugar chain linked to the carbon C-3 aglycone position, and bidesmosides, where two sugar chains are attached to the C-3 and C-22 position on the aglycone.

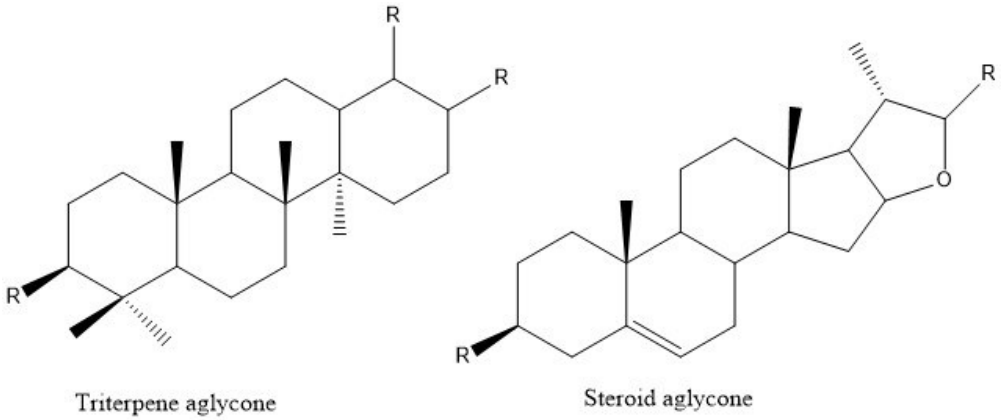


Figure 1.2.1 Molecular structures of triterpene aglycone and steroid aglycone (structure information adapted from W. A. Oleszek, 2002).

Based on the aglycone structures, triterpene saponins are categorized as group A, B and E in pulses (Fig. 1.2.1) (Singh et al., 2017). The group A saponin are bidesmosides that have two saccharide chains attached to the C-3 and C-22 position, as well as a hydroxyl group at the C-21 position on sapogenol A. The saccharide chain at the C-3 position starts with a D-glucuronic acid (glcUA) followed by an additional two or three sugar groups, whereas the saccharide chain at the C-22 position starts with L-arabinose (ara) and consists of two sugar units. Group A saponin can be classified as acetylated and non-acetylated saponins, depending on whether the terminal sugar unit of the saccharide chain at the C-22 position contains an acetyl group or not (xylosyl or glycosyl residue). Acetylated group A saponin includes Aa, Ab, Ac, Ad, Ae, Af, Ag and Ah, whereas non-acetylated group A saponin includes A_1, A_2, A_3, A_4, A_5 and A_6 (Singh et al., 2017).

The group B saponin is monodesmosides that differ from group A saponin by containing a hydrogen atom at the C-21 position on sapogenol B instead of a hydroxyl group. They have a glycosyl group attached at the C-3 position on the sapogenol B, and are named as soyasaponin Bb (I), Bc (II), Bb' (III), Bc' (IV) and Ba (V) (Singh et al., 2017). Some group B saponin contain a 2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one (DDMP) group at the C-22 position and are referred to DDMP conjugated soyasaponin, including αg, βa, βg, γa, and γg. As indicated in Fig.

1, for both non-DDMP and DDMP conjugated soyasaponin, the saccharide chain at the C-3 position of sapogenol B starts with a glucoronyl group (glcUA) and followed by two or three sugar units. DDMP saponins are unstable under high temperature, acidic and alkaline pH processing conditions (Lynn et al., 2006). As a result, DDMP moiety will be released as maltol and DDMP conjugated soyasaponin will convert to non- DDMP conjugated soyasaponin.

Group E saponins contain sapogenol E in their structure and differ from group B saponins by having a carbonyl group instead of a hydroxyl group at the C-22 position. They are monodesmosides with one single saccharide chain starts with a glcUA residue consist of three sugar units at the C-3 position of aglycone. Group E consists of two saponins, named as Bd and Be. They are known to be the photooxidation artifacts of group B saponins that arise from processing (Singh et al., 2017).

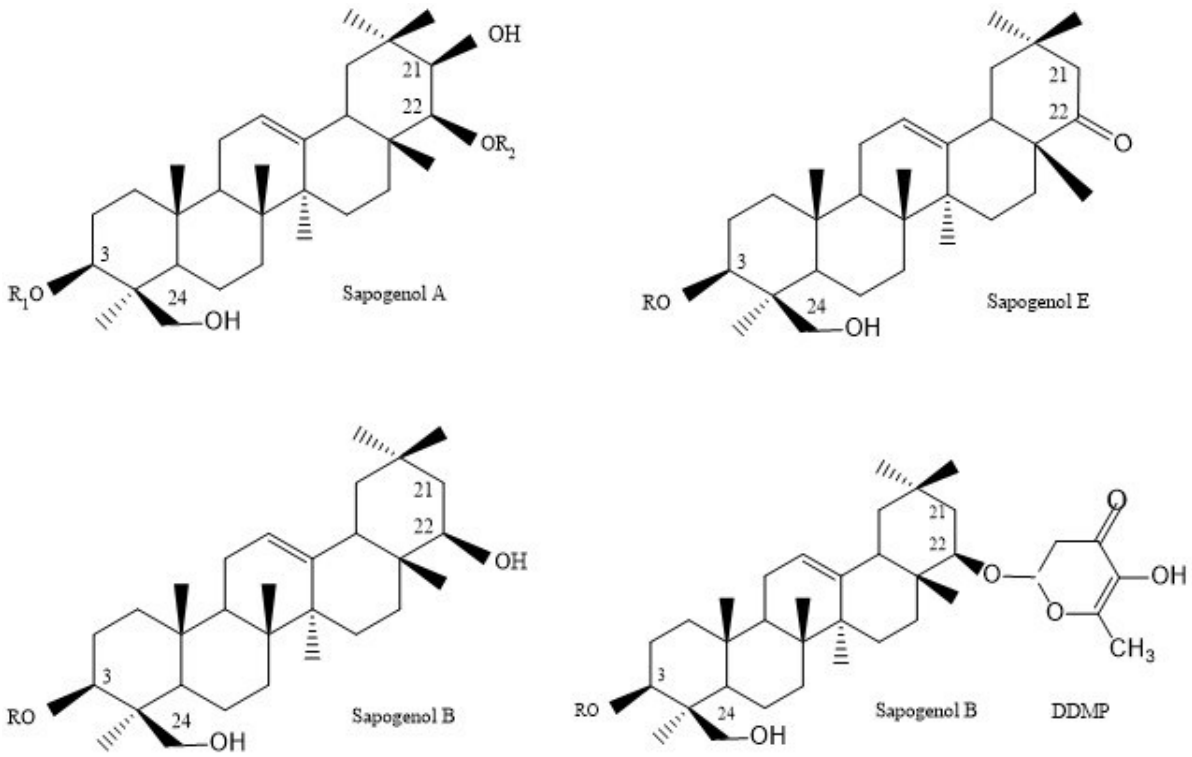


Figure 1.2.2 Chemical structures of group A, B, DDMP and E sapogenol (structure information adapted from Shiraiwa, Kudo, Shimoyamada, Harada, & Okubo (1991), Shiraiwa, Harada, & Okubo (1991) and Guglú & Mazza G. (2007).

Table 1.2.1 Chemical structures of group A, B, DDMP and E saponins.

Group A saponins	**Molecular formula**	**Molecular weight**	**Saccharide chain (R)**	
			R1	R2
Aa	$C_{64}H_{100}O_{31}$	1365	glcUA-gal-glc	ara-xyl (2,3,4-tri-*O*-Acetyl)
Ab	$C_{67}H_{104}O_{33}$	1437	glcUA-gal-glc	ara-glc (2,3,4,6-tetra-*O*-Acetyl)
Ac	$C_{67}H_{104}O_{32}$	1421	glcUA-gal-rha	ara-glc (2,3,4,6-tetra-*O*-Acetyl)
Ad	$C_{66}H_{102}O_{32}$	1407	glcUA-ara-glc	ara-glc (2,3,4,6-tetra-*O*-Acetyl)
Ae	$C_{58}H_{90}O_{26}$	1203	glcUA-gal	ara-xyl (2,3,4-tri-*O*-Acetyl)
Af	$C_{61}H_{94}O_{28}$	1275	glcUA-gal	ara-glc (2,3,4,6-tetra-*O*-Acetyl)
Ag	$C_{57}H_{88}O_{25}$	1173	glcUA-ara	ara-xyl (2,3,4-tri-*O*-Acetyl)
Ah	$C_{60}H_{92}O_{27}$	1245	glcUA-ara	ara-glc (2,3,4,6-tetra-*O*-Acetyl)
A_1	$C_{59}H_{96}O_{29}$	1269	glcUA-gal	ara-glc
A_2	$C_{53}H_{86}O_{24}$	1107	glcUA-gal	ara-glc
A_3	$C_{48}H_{78}O_{19}$	959	glcUA-ara	ara-glc
A_4	$C_{64}H_{100}O_{31}$	1365	glcUA-gal-glc	ara-xyl
A_5	$C_{58}H_{90}O_{26}$	1203	glcUA-gal	ara-xyl
A_6	$C_{57}H_{88}O_{26}$	1173	glcUA-ara	ara-xyl
Group B saponins				
Ba (V)	$C_{48}H_{78}O_{19}$	959	glcUA-gal-glc	
Bb (I)	$C_{48}H_{78}O_{18}$	943	glcUA-gal-rha	
Bb’ (III)	$C_{42}H_{68}O_{14}$	797	glcUA-gal	
Bc (II)	$C_{47}H_{76}O_{17}$	913	glcUA-ara-rha	
Bc’ (IV)	$C_{41}H_{66}O_{13}$	767	glcUA-ara	
DDMP saponins				
αg	$C_{54}H_{84}O_{22}$	1085	glcUA-gal-glc	
βa	$C_{53}H_{82}O_{20}$	1039	glcUA-ara-rha	
βg	$C_{54}H_{84}O_{21}$	1069	glcUA-gal-rha	
γa	$C_{47}H_{72}O_{16}$	893	glcUA-ara	
γg	$C_{48}H_{74}O_{17}$	923	glcUA-gal	
Group E saponins				
Bd	$C_{48}H_{76}O_{19}$	957	glcUA-gal-glc	
Be	$C_{48}H_{76}O_{18}$	941	glcUA-gal-rha	

The nomenclature of saponins used in this article is adapted from Shiraiwa, Kudo, Shimoyamada, Harada, & Okubo (1991), Shiraiwa, Harada, & Okubo (1991) and Guglú & Mazza G. (2007). Additional saponin components were reported by Panneerselvam et al. (2013) and Singh et al. (2017).

1.2.2 Properties and applications

1.2.2.1 Interfacial property

Saponins are soluble in aqueous solutions and the hydrophilic sugar chain is hydrated when dissolved. They are surface-active and adsorb at the aqueous interface. As mentioned in section 2.1, saponins are classified into triterpenoidal aglycone and steroidal aglycone types. Triterpenoid saponins have the ability to form highly viscoelastic networks at air/water- interface through the interaction of hydrogen bonds and sugar residues. Certain amounts and length of sugar chain are required to provide sufficient hydrophilicity for triterpenoid saponins to have interfacial properties (Böttcher & Drusch, 2017). Steroid saponins are unable to form those viscoelastic networks, therefore, do not have interfacial property. It is shown that the ability of saponins to form strong viscoelastic films may results in micelles with hydrophobic substances loaded inside, nano-emulsions and stable foams that can be used in dispersed systems as natural surfactants (Böttcher & Drusch, 2017).

1.2.2.2 Anticarcinogenic properties

Saponins showed anticarcinogenic properties in both *vivo* and *vitro* studies through different mechanisms (Gurfinkel & Rao, 2003; H. Y. Kim, Yu, Kim, Kim, & Sung, 2004). Recent report indicates that steroidal saponins have a wide range of antitumor activities such as blocking cell cycle, inhibiting cell proliferation, regulating cancer cell apoptosis and autophagy, suppressing tumor invasion and metastasis (Zhao et al., 2018). Apoptosis refers to programmed cell death, once apoptosis is suppressed, cell proliferation becomes uncontrolled and results in cancer development. Saponin show cytotoxic and growth inhibitory effects against tumor cells. Epidemiological studies have shown a strong relationship between colon cancer incidence and high concentration of bile

acid and cholesterol metabolites. *In vitro*, saponins form large micelles with bile acids, which reduce the free form of bile acids content in the upper gastrointestinal tract. Therefore, the presence of saponin reduces the availability of bile acids to form secondary bile acids by intestinal microflora and prevent the development of colon cancer (Rao & Sung, 1995). Moreover, saponin also interacts with free sterols and cholesterol present in the cell membrane, resulting in changes in intracellular morphology and membrane permeability (Rao & Sung, 1995). The saccharide chain attached to the C-3 position of sapogenol exhibits hepatoprotective property through suppressing the hepatitis inflammation (Singh et al., 2017). Saponin also exert hypolipidemic activity. Saponin extracted from black bean seed coat suppress lipogenesis and stimulating fatty acid oxidation and biliary cholesterol excretion through synthesis of bile acid (Chavez-Santoscoy et al., 2014). The effect of crude soyasaponins extract on phorbol 12-myristate 13-acetate (PMA)-induced inflammatory responses were examined (Kim et al., 2004). The results indicate crude saponin extract inhibits cell growth and reduces inflammatory responses by mediating the expression of inflammatory proteins through the inhibition of transcription factor nuclear factor - kappa B (NFκB). Investigation of purified saponins include soyasaponin Bb, Bb', A_1, A_2, soyasapogenol B monoglucuronide, soyasapogenol B and A were conducted on colon cells (Gurfinkel & Rao, 2003). Only soyasapogenol A and B showed inhibition effects on colon cancer cell lines, whereas soyasaponin Bb and Bb' were inactive. Soyasaponins with greater lipophilicity are more bioactive. *In vitro* study indicated that colonic microflora in digestive tract hydrolyzes inactive, acetylated or glycosylated soyasaponins into deglycosylated and bioactive form, thus, soyasaponin have the potential to generate anticancer drugs (Gurfinkel & Rao, 2003). DDMP conjugated saponins and soyasaponin Bb were found to inhibit the growth of esophageal cancer cell (Eca-9706) by activating the caspase-3 pathway and inducing apoptosis in Hep-G2 cancer

cells (W. Zhang & Popovich, 2012). Soyasaponin Bb' and Bc' contain disaccharide group was found out to be more effective than soyasaponin Bb and Bc with trisaccharide group in protecting the liver. Moreover, soyasaponins with hexosyl sugar unit are more bioactive than those with pentosyl units (W. Zhang & Popovich, 2012).

1.2.2.3 Anti-obesity properties

Saponins from plant materials have anti-obesity properties (Carbonaro, 2011; Cheok, Salman, & Sulaiman, 2014). The effect of germinated soy germ extract that is high in soyasaponin Ab (224.6 mg/g germ) on obesity was analyzed at both *in vitro* and *in vivo* levels (Kim et al., 2019). CB1 mRNA is highly expressed throughout the central nervous system and in adipose tissue to regulate food intake. CB2 expression is associated with obesity-related inflammation and brown fat storage. Both *in vitro* and *in vivo* studies showed that soyasaponin Ab treatment changed the protein expression of CB1-related factors (CB1, NAPE-PLD, FAAH) and CB2-related factors (CB2, DAGL-α, DAGL-β), thus effectively inhibiting fat accumulation, promoting beige fat conversion and activation, as well as improving obesity-related metabolic disorders in obsess mice (Kim et al., 2019). Saponins extracted from stems and leaves of *Panax ginseng* also showed strong anti-obesity properties in diet-induced obese mice (G. Chen et al., 2017). The mechanism of action includes reducing free fatty acids, total cholesterol, triglycerides, low-density lipoprotein-cholesterol, glucose, leptin, and insulin level in serum, regulating thermogenesis, lipogenesis and lipolysis. Moreover, a reduction of body, liver and epididymal adipose tissue weight was observed in obese mice treated with ginseng saponin extract.

1.2.2.4 Emulsifier, antioxidant and antimicrobial agent

Saponins can be used as a natural emulsifier, antioxidant and antimicrobial agent (Cheok et al., 2014; Choudhry et al., 2016; Y. Li, Du, & Zou, 2009). Oxidative stress induced by excessive reactive oxygen species formation can cause severe pathological conditions and aging-associated, cardiovascular, liver and lung diseases. Vitamin A and E have many potential health-promoting effects include prevent undesirable oxidation. Nano-emulsions of vitamin A and E with saponins were successfully prepared through high-pressure homogenizer. Saponin emulsions of vitamins showed good stability based on particle size, zeta potential, and diameter. Saponin encapsulated vitamins showed improved antioxidant and protective activity against oxidative stress-induced cellular damage through scavenging reactive oxygen species, reducing DNA damage and lipid peroxidation (Choudhry et al., 2016). Therefore, saponins may be used as emulsifiers to deliver bioactive compounds to commercial foods and beverages. Saponins isolated from tea (*Camellia sinensis* L.) showed high antibacterial and antioxidant activities under a wide range of pH (4.8-8.0) (Y. Li et al., 2009). Antioxidant activity was determined by reducing power, super-oxidant radical scavenging activity, and metal chelating activity, while the antibacterial property was examined using gram-negative, gram-positive bacteria and fungi. Saponin with high antibacterial property can be applied to food to achieve a longer shelf-life and higher safety with respect to pathogenic microorganisms.

1.2.2.5 Negative impact on flavor

Despite the fact that saponins are considered as bioactive and health-promoting compounds, studies have also associate undesirable bitter taste with saponin content in pulses, grains and some vegetables (Aldin, Reitmeier, & Murphy, 2006; Lynn et al., 2006; Nyembwe, Minnaar, Duodu, &

De Kock, 2015). DDMP-conjugated soyasaponin and soyasaponin Bb were determined by recognition thresholds and descriptive analysis as the main saponin types that are responsible for flavor differences among defatted soy flakes, soy protein isolate and soy germ extracts (Aldin et al., 2006). A more specific study found out that DDMP-conjugated soyasaponin is significantly more bitter than soyasaponin Bb in 16 dry pea varieties (Lynn et al., 2006). DDMP-conjugated soyasaponin is the predominating saponin in all pea varieties (in 2 varieties, it was the only saponin type present), and the bitterness perceived by trained panelists are positively correlated with their concentration. Roasting at 150°C for 20, 25, or 30 mins resulted in a change of marama beans (*Tylosema esculentum* (Burchell) A. Schreiber) (grown in Africa) sensory properties and saponin content (Nyembwe et al., 2015). Marama bean flour with 20 mins roasted time contain a higher concentration of saponins than unroasted bean flour. This may due to the increase of permeability of cell membranes by heat treatment while maximizing the extractable amount of saponins in water extracts. While when roasting time increased from 20 to 30 mins, saponin concentration was progressively decreased due to the combination of a long time and high temperature leads to hydrolysis of the glycosidic bond between the sapogenin and glycosidic residue and saponin degradation.

1.2.3 Extraction methods

There are several factors that influence the quality of saponin analysis. Sample preparation, especially the extraction method, is important in saponin profile determination, which should be carefully chosen in order to yield a representative extract from the sample (Cheok et al., 2014).

In general, the extraction methods employed in saponin extraction can be classified into conventional and green technology extraction (Cheok et al., 2014). The conventional extraction

methods include maceration, Soxhlet and reflux extraction, which require a large quantity of solvents to extract compounds from plant materials, sometimes with the aid of heating, mechanical stirring or shaking. On the other hand, green technology includes ultrasound-assisted, microwave-assisted and accelerated solvent extraction. The conventional extraction methods rely on the solubility of compounds from plant materials into solvent, which normally takes longer extraction time with low efficiency, whereas the green technology is environmentally friendly, require less amount of solvent and shorter extraction time with higher efficiency. In green technology, water is used as the extraction solvent and the properties can be altered by pressure and temperature. Each extraction method has its own advantages and disadvantages depending on the food matrix studied. Table 2.2 summarizes the advantages and drawbacks of each extraction method.

1.2.3.1 Maceration extraction

Maceration is a simple solid-liquid extraction technique where the compounds are extracted by soaking the plants/seeds directly into an extraction solvent for a certain period of time (Cheok et al., 2014). Factors influence the efficiency of maceration extraction include particle size, solid-to-solvent ratio, solvent type, extraction time and temperature (Jovanović et al., 2017). The choose of solvent type follows the general rule of "like dissolves like" which means that polar substances will dissolve into a polar solvent, whereas non-polar substances will dissolve into a non-polar solvent. Maceration extraction uses simple apparatus thus is a popular method used by many researchers. Saponins were successfully extracted by either ethanol or methanol with various concentrations (30%, 50%, 70%, 90% and 100% vol., aq. with water) from *Saponaria vaccaria L* seeds (Shrestha & Baik, 2012), alfalfa plant (*Medicago sativa* L.) (Krakowska, Rafińska, Walczak, Kowalkowski, & Buszewski, 2017), adzuki bean (Liu, Cai, & Xu, 2017), pea (*Pisum sativum* L.)

(Reim & Rohn, 2015a), faba bean (*Vicia faba*) (A. Sharma & Sehgal, 1992) and soy flour (W. Zhang, Teng, & Popovich, 2009) through maceration extraction methods. Mechanical shaker and magnetic stirring usually are applied during maceration extraction to shorten the time.

1.2.3.2 Reflux and Soxhlet extraction

Soxhlet extraction is a continuous solid-liquid extraction technique where the solid material is placed in a thimble holder inside the Soxhlet apparatus. The thimble act like a filter where the solid material will contain while the liquid solvent will pass through (Cheok et al., 2014). As the solvent placed in a distillation flask is heated at reflux, its vapor rises up and is condensed in a condenser. During operation, the thimble will be gradually filled with condensed solvent, when the solvent reaches to a certain level, it carries the extracted compounds and automatically siphons back to the distillation flask. Soxhlet extraction is a continuous process that recycles the solvent and repeats the vaporization and condensation process until full extraction is achieved. Reflux extraction has similar operating principles, except that reflux apparatus does not contain a thimble (Cheok et al., 2014). Both Soxhlet and reflux extractions are easy to perform but a large volume of solvent and long extraction time is required to achieve fully extraction. The removal of organic solvent is expensive and time-consuming, which might also cause an environmental problem (Azmin et al., 2016).Both ethanol and methanol are used as extraction solvent to reflux or Soxhlet extract saponins from soy flour (Berhow, Suk, Vermillion, & Duval, 2006; W. Zhang et al., 2009), black bean (Lee, Chen, Hwang, & Hsu, 1999), maidong seed (*Ophiopogon japonicus*) (X. E. Li, Wang, Sun, & Liao, 2016) and ginseng (*Panax notoginseng*) (Gong, Zhang, Pan, & Qu, 2014; Hu et al., 2018).

1.2.3.3 Ultrasound-assisted/ sonication extraction

Sonication extraction involves the use of ultrasound ranging from 20-2000 kHz to create longitudinal waves (Nn, 2015). When waves encounter with liquid medium, a region of altered compression and rarefaction among molecules are created. Further cavitation bubbles are formed and collapsed in this region, which increases the surface contact between solvents and plants. The permeability of cell walls is increased by ultrasound which facilitates the release of compounds from plants into extraction solvent and enhances mass transfer of the solvent into plant cells (Nn, 2015). Sonication extraction has many advantages including reduced extraction time, increased yield, reduced solvent consumption and environmentally friendly. This method has successfully extracted saponins from soy flour (W. Zhang et al., 2009), Safed musli (*Chlorophytum borivilianum*) root (Deore, Baviskar, & Rangari, 2015), arabian pea (*Bituminaria bituminosa*) (Llorent-Martinez, Spinola, Gouveia, & Castilho, 2015) and Fagioli di Sarconi bean (*Phaseolus vulgaris* L.) (Bianco, Buchicchio, & Cataldi, 2015).

1.2.3.4 Microwave-assisted extraction

Similar to sonication extraction, microwave-assisted extraction utilizes microwave energy to facilitate the diffusion of compounds from the plant matrix into the solvent (Nn, 2015). Microwave is electromagnetic radiation with a frequency range from 0.3-300 GHz, and is able to penetrate into plants and interact with dipoles of polar and polarizable molecules such as water inside the plants to generate heat (Azmin et al., 2016). Microwave induce dipole rotation of the polar molecules thus disrupt hydrogen bonds and enhance the migration of ions and promote the diffusion of bioactive compounds from the plant matrix (Cheok et al., 2014). Microwave-assisted extraction have been applied to extract saponins from ginseng (Kwon, Bélanger, & Pare, 2003;

Kwon, Lee, Bélanger, & Jocelyn Paré, 2003), chickpea (*Cicer arietinum* L.) (Cheng et al., 2017), roots of *Bupleurum falcatum* (Kwon, Choi, Chung, & Lee, 2006) and green asparagus (*Asparagus officinalis* L.) (Zhang et al., 2019). This extraction method requires less extraction time and solvent volume as compared to maceration, Soxhlet or reflux extraction and improved recovery of compounds. However, this method is not suitable for heat-liable compounds such as tannins and anthocyanins since they might be subjected to degradation under high temperatures (Nn, 2015).

1.2.3.5 Accelerated solvent extraction

Accelerated solvent extraction is a more effective solid-liquid extraction method compared to maceration, reflux or Soxhlet extraction with a reduced volume of solvent. It is operated at elevated temperature (50-200°C) and at a pressure range from 10 -15 MPa so that the solvent maintains as liquid form (Azmin et al., 2016). Plant material is packed by inert material such as sand and together with the solvent are placed in a closed container inside the pressure vessel. A thermocouple is connected to the container in order to detect the change of temperature in the container. Once the temperature changes, the heating, and cooling jacket surrounding the sample container starts to operate to maintain a constant temperature. Once the pressure increased, the pressure control valve opens and release some of the pressure, while when the pressure drops below the set value, the pressurization system operates and applies pressure to the system (Azmin et al., 2016). Accelerated solvent extraction was proven effective to extract saponin from ginseng (*Pnanx notoginseng*) (Pan, Huang, Yu, Lu, & lin, 2017), roots of *Bupleurum falcatum* (Li et al., 2010) and seeds of *Aesculus chinensis* Bunge (Chen et al., 2007).

Table 1.2.2 Summary of advantages and disadvantages of common saponin extraction methods

Type of extraction	**Advantages**	**Disadvantages**
Maceration extraction	• Simple • Inexpensive	• Long time • Large volume of solvent
Reflux and Soxhlet extraction	• Simple • Inexpensive	• Long time • Large volume of solvent • Risk of decomposition of heat-labile compounds
Ultrasound-assisted extraction	• Reduced solvent consumption • Reduced extraction time	• Not efficient for nonpolar compounds or viscous solvent • Not applicable to heat-labile compounds • Use ultrasound energy more than 20 kHz may cause formation of free radicals • Require specific equipment
Microwave-assisted extraction	• Reduced solvent consumption • Reduced extraction time • Applicable for both laboratory and industrial use • High return on capital investment	• Not efficient for nonpolar compounds or viscous solvent • Not applicable to heat-labile compounds • Require specific equipment
Accelerated solvent extraction	• Reduced solvent consumption • Reduced extraction time	• Not applicable to heat-labile compounds

1.2.4 Analytical methods

The components in the extracts from extraction are complex and contain a large variety of natural compounds. Prior to the analysis, crude saponin extract obtained from plants are usually re-concentrated by evaporating the solvent (usually either aqueous methanol or ethanol) (Zhang et al., 2009). A common preparation step of saponin extract before quantification or qualification is to partition the crude extract against ethyl acetate to remove non-polar constituents and then against *n*-butanol to remove highly water-soluble constituents including carbohydrates and salts, and to extract polar compounds including saponin (Lee et al., 1999). In some literature, petroleum ether

or chloroform is also used to remove fat from the materials (Kojima, Ohnishi, Ito, & Fujino, 1989) (Liu et al., 2017). Another alternative preparation step involves the use of solid-phase extraction (SPE) (Aldin et al., 2006).

1.2.4.1 Separation techniques

1.2.4.1.1 Thin-layer chromatography (TLC)

Thin-layer chromatography is a one-dimensional and two-dimensional mode analytical technique that has been used in the separation and determination of saponins in plant and seed materials. The most common stationary phase is silica gel and a mixture of solvent with acid as the mobile phase. Separation of saponins from other components by TLC is based on spotting samples in rows along with different concentrations of saponin standards on TLC plates. Then the plates are sprayed with a proper solvent with sulphuric acid (sometimes perchloric acid is also used), heated and dried. A densitometer is used to measure the intensity of each spot and compare them to the intensity of the saponin standard (W. Oleszek & Bialy, 2006). This detection method is more efficient when coupled with an on-line computer with a dual-wavelength flying-spot scanner and two-dimensional analytical software (W. A. Oleszek, 2002). This type of detect is also referred to TLC-densitometry, on the other hand, another type of detection is called TLC-colorimetry (W. A. Oleszek, 2002). Instead of measuring intensity, colorants include Ehrlich or vanillin reagents are applied to the plate, and measurements are made at wavelengths between 515-560 nm. The principle of TLC is based on the reaction of oxidized triterpene saponins with vanillin and sulfuric acid or perchloric acid is added as oxidant and gives rise to the distinctive colour purple to this reaction. The limitation of using TLC-colorimetry is that some components from crude extracts may contain bile acids with hydroxyl group at C-3 position and sterol, which may react with

colorants and providing false results. It might be necessary to apply the purification method such as SPE before using the colorimetric determination method (W. A. Oleszek, 2002). Some advantages of using TLC including fast analysis, ability to compare many samples simultaneously, versatile plate, support, solvent and detection reagents option.

Saponin was successfully extracted from Adzuki beans via the maceration extraction method and fractionated into individual compounds by preparative TLC on silica gel G with chloroform: methanol: water (60:35:8, v:v:v) (Kojima et al., 1989). Adzukisaponin I was the only saponin detected in Adzuki bean. In another study, saponins from many plants including saunspali (*Asparagus adscendens* Roxb.), tea (*Camellia sinensis* O. Kuntze), Indian horse chestnut (*Aesculus indica*), kanor, honeysuckle (*Lonicera japonica* Thunb), bigru root (*Silene inflata* Sm.), soapnut (*Sapindus mukorossi* Gaertn.), safed musli (*Chlorophytum borivilianum*), shatavari (*Asparagus racemosus* Willd.) and agave (*Agava americana* L.) were detected on TLC using the haemolytic property of erythrocytes (O. P. Sharma, Kumar, Singh, & Bhat, 2012). The detection was done by immersing air-dried plant samples in a suspension of sheep erythrocytes on a silica gel 60 plates in the solvent system of n-butanol: water: acetic acid (84:14:7, v:v:v). Saponins appears as white spots against pink plate background. This work is based on the reaction of saponin with blood reagent to release oxyhemoglobin which gives rise to a change of color and can be measured by spectrophotometer. Comparing to the traditional TLC method using acid spray and heating, this improved method is simpler and cheaper.

Other improved TLC methods such as high-performance TLC-densitometry have been reported and validated to quantitative determine saponins from soy (*Glycine max* L.) (Shawky & Sallam, 2017) and tea seed (*Camellia oleifera* Abel.) (Chaicharoenpong & Petsom, 2009).

1.2.4.1.2 High-performance liquid chromatography (HPLC)

HPLC is a form of column chromatography in which the mobile phase is a mixture of analyte in the solvent, and the stationary phase is packed materials in the column. HPLC is a versatile separation technique based on the partition of the analyte molecules between the stationary phase and the mobile phase. The mobile phase is pumped through the column at high pressure, and as the sample carried by gas move through the column, it interacts with both phase at a different rate due to differing in polarity (adsorption and partition), electrical charge (ion-exchange) and molecular size (size-exclusion). The compound that has a stronger interaction with the mobile phase will elute the column first, which presented by the shortest retention time. This separation method is suitable for saponins which are non-volatile and highly polar compounds. The most common types of HPLC can be divided into the reversed phase and normal phase HPLC (Stavrianidi, 2019). Normal phase HPLC consists of a polar stationary phase (eg silica) and a non-polar mobile phase (eg hexane), whereas in reversed-phase HPLC, the stationary phase is non-polar and the mobile phase is polar (eg water, methanol). For the analysis of saponins from plant material, reverse-phase HPLC is widely used. The most common columns are reversed-phase are C_8 and C_{18}, however, some other modified silica gel supports such as NH_2 and DIOL are also used (W. A. Oleszek, 2002). As shown in Fig. 1.2.3., a typical HPLC system consists of (a) a reservoir containing the mobile phase, (b) a pump to push the mobile phase and analyte through the system, (c) an injector to introduce sample, (d) a column to provide separation, (e) a detector to monitor the separated components, (f) a data collection device to interpret and store results, and (g) a waste container to collect used solvent.

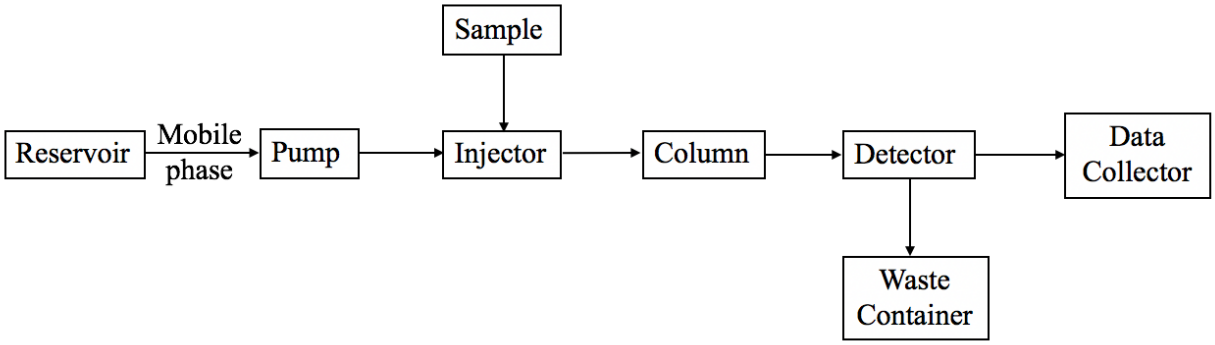


Figure 1.2.3 Schematic diagram of the basic components of an HPLC system.

Usually, HPLC is coupled with mass spectrometry to study and identify saponin from plant sources. Saponins and flavonoids in Adzuki bean (*Vigna angularis* L.) were extracted with 70% ethanol, defatted with petroleum ether then extracted with *n*-butanol via maceration at room temperature (Liu et al., 2017). HPLC with diode array detection and electrospray ionization-tandem multi-stage mass spectrometry (HPLC-DAD-ESI-MS^n) were then used to quantify and qualify saponins and flavonoids. A total of 6 saponins from adzuki bean were quantified, which are azukisaponin I, II, III, IV, V, and VI. Another study developed and validated an analytical method involving the use of an ASE 300 automated solvent extractor system for saponin extraction from soybeans and HPLC-ESI-MS for compound identification (Berhow et al., 2006). TLC was used to confirm the sapogenol aglycone structure. TLC was applied to a mixture of methanol and dichloromethane suspended with soybean powder, and the plate was developed with solvent contain methanol: dichloromethane (5:95, v:v). The developed plate was sprayed with a solution containing potassium dichromate and sulfuric acid and heated. The presence of group A and B sapogenol aglycone were confirmed by the pink/purple spots visualized by a spectrophotometer. A total of 14 soyasaponins were identified and quantified from soybeans, which include group A soyasaponin A1, A2, A4, and A7, group B soyasaponin Ba, Bb, Bb', Bc, and Bc', and DDMP conjugated soyasaponin αg, βg, βa, γg and γa (Berhow et al., 2006).

Ultra-high performance liquid chromatography (UHPLC) has higher efficiency comparing to HPLC (Cheok et al., 2014). UHPLC operates at higher pressure (15,000 psi) to achieve better resolution for faster separation. The columns used for UHPLC are smaller in particle size (< 2 μm) and diameter, as well as shorter in length. In a recent study, a rapid and simple analytical approach for screening the triterpene saponins in blackberry leaves (*Rubus* cv. Loch Ness) was developed using UHPLC coupled to a quadrupole-time-of-flight mass spectrometry (UHPLC-QTOF-MS) (Gradillas, Martínez-Alcázar, Gutiérrez, Ramos-Solano, & García, 2019). Six classes of triterpene aglycones were identified based on the structure of 19α-hydroxyursane backbone with an oxygen atom attached at the C-3 position and oxidized at the C-28 position.

1.2.4.1.3 Gas chromatography (GC)

GC is a separation technique used for the quantitative or qualitative analysis of volatile compounds. In GC, the mobile phase is a carrier gas that is either an inert gas such as helium or an unreactive gas such as nitrogen. The separation of compounds in the mixture is achieved through interactions with the stationary phase coated on the column walls, which cause each compound to elute at a different time based on differences in boiling point and polarity. The identification of molecules can be done by comparing retention times of each compound from the chromatogram with the retention time of standard. For GC analysis, the target compounds should be volatile, while saponin is large and polar compound which is not easy to be volatilized upon heating (W. A. Oleszek, 2002). In that case, saponin usually is hydrolysed by acid to their triterpenoidal aglycone or steroidal aglycone type and methylated or acetylated before GC separation. However, hydrolysis of saponin may result in the formation of artifacts depending on saponin structure, hydrolysis agent, time and conditions, which may lead to misleading analysis results (W. A. Oleszek, 2002). For

instance, during acid hydrolysis, soyasaponin Bb contains soyasaponinogenol B produce by-product sapogenins such as soyasaponingenols C, D and F that originated from the rearrangement of sapogenins (W. A. Oleszek, 2002). In a recent study, five triterpene saponins with different types of aglycone and substituents were extracted and purified from *Medicago* spp. (*M. arabica* (L.) Huds., *M. arborea* L. and *M. sativa* L.) and subjected to acid hydrolysis to investigate their stability (Tava, Biazzi, Mella, Quadrelli, & Avato, 2017). Their results showed the formation of artifacts from soyasapogenols and zanhic acid under hydrolysis conditions. Use of GC analysis methods does not provide information on the sugar moieties of sugar. Nevertheless, it is a less time-consuming separation method to quantify among saponins with different aglycone comparing to HPLC (Tava et al., 2017).

Steroids and teiterpenoids from leaves and tubers of *Tamus edulis* Lowe were successfully extracted by diethyl ether via maceration extraction and dissolved into methanol via Soxhlet method and then identified by GC coupled with mass spectrometry (GC-MS) (Rogowska, Styczyński, Pączkowski, Szakiel, & Pinheiro de Carvalho, 2019). The separation was done on a HP-5MS UI column and the use of helium as a carrier gas. The results suggested the presence of steroidal aglycone saponin type such as diosgenin and yamogenin in *Tamus edulis* Lowe. In another study, the profile of triterpenoidal aglycone saponins from 28 Quinoa varieties (*Chenopodium quinoa* Willd.) was investigated (Medina-Meza, Aluwi, Saunders, & Ganjyal, 2016). Saponins were extracted with 80% MeOH (with 20% water, v:v) via Reflux method, concentrated to a certain amount under vacuum pressure, and hydrolyzed with HCl 6 N to obtain triterpenoidal aglycone. Total saponin content was measured by UV-Vis spectrometry and triterpenoidal aglycone saponins quantification was achieved by GC-MS using nitrogen as carrier gas on a Zebron ZB-5HT capillary column. The major saponin aglycone in Quinoa includes

oleanolic acid, hederagenin, serjanic acid and phytolaccagenic acid. Steroidal saponins include tigogenin, gitogenin, hecogenin and neohecogenin were extracted from *Tribulus terrestris* L. through microwave-assisted extraction method and identified by GC-MS (T. Li et al., 2009).

1.2.4.2 Detection techniques

1.2.4.2.1 Mass spectrometry (MS)

HLPC and GC are techniques used to separate individual saponin from a crude mixture, whereas MS is used to separates the ions according to their individual mass-to-charge (*m/z*) ratio, and the detector measures the number of ions for each m/z ratio. A computer then converts the signal generated in the detector into a mass spectrum (Stavrianidi, 2019). Combinations of chromatographic separation and mass spectrometry, such as in HPLC-MS, UHPLC-MS and GC-MS, are the most widely used analytical methods in saponin studies. These combinations can be further used in conjunction with different types of ionization techniques such as electrospray ionization (ESI), fast atom bombardment (FAB), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI), and scan mode includes full scan, selected ion monitoring (SIM), selected reaction monitoring (SRM), and multiple reaction monitoring (MRM) (Stavrianidi, 2019). For the detection of saponins from plant sources, full scan mode is used to scan for a desired *m/z* range to produce a spectrum consists of all the unknown sample constituents. SIM, SRM and MRM are used for targeted screening, which require knowledge of the characteristic fragments of target analyte. Those scanning modes require two or more mass analyzers, and the first one eliminates unwanted ion so that only the ion with targeted *m/z* value (called precursor ion) enter the second mass analyzer. The precursor ion dissociates into specific fragment ions by collision with an inert gas in the second mass analyzer (Stavrianidi, 2019). The

combination of chromatographic separation and mass spectrometry provides qualitative and quantitative information about the atomic and molecular composition of saponins.

1.2.4.2.2 Fourier transform infrared spectroscopy (FTIR)

FTIR is the method used to determine the structure of molecules based on the analysis of the interaction between molecules and IR light in three different ways include absorption, emission, and reflection (Türker-Kaya & Huck, 2017). The IR is divided into three regions (wavenumber cm^{-1}, wavelength nm) as near infrared (13,500-4000 cm^{-1}, 780-2500 nm), mid-infrared (4000-400 cm^{-1}, 2500-25,000 nm) and far-infrared (400-10 cm^{-1}, 25,000-1,000,000 nm) (Türker-Kaya & Huck, 2017). Comparing to traditional HPLC-MS method for saponin analysis, which is time-consuming, expensive and impractical to apply on a large number of samples, using FTIR provides some advantages include fast, easy, non-destructive, reproducible and cost-efficient analysis, which is suitable for generating a comprehensive overview of metabolites present in plant tissue. UHPLC combined with FTIR was used to determine the total steroid saponins in a total of 78 samples of 5 species of *Paris* including *P. polyphylla* var. *alba*, *P. mairei*, *P. vietnamensis*, *P. axialis*, *P. polyphylla* var. *stenophylla*, and PPY (Yang, Jin, Zhang, & Wang, 2018). In another study, the total saponin content and composition from a total 283 soybean (Glycine max Merrill) cultivars were determined by HPLC-FTIR (Ahn et al., 2016).

1.2.4.2.2 Nuclear magnetic resonance spectroscopy (NMR)

NMR is a non-destructive method that is capable of providing structural information of the compounds. This method gives information about the number of carbon and proton atoms, as well as their connectivity (Zhou, Yang, Liu, & Tu, 2007). There are mainly two types of NMR which

is ^{13}C-NMR and ^{1}H-NMR. The atomic nucleus is randomly spinning charged particles and generate a magnetic field in random directions. When a magnetic field is present externally, the nuclei align themselves either with (in a lower energy state) or against (in a higher energy state) the field of the external magnet. Both one-dimensional (1D) ^{1}H-NMR and two-dimensional (2D) ^{13}C-NMR combined with HPLC-SPE separation method have been used to identify saponin structure from the powdered roots of *Dendrobangia boliviana* (Zebiri et al., 2017). Two new oleanane saponin compounds named zebirioside M and zebirioside Q along with other 16 saponin compounds were identified by this method. In another study, both 1D and 2D NMR coupled with HPLC are applied and successfully identified two novel triterpenoid saponins named ilexhainanoside A and B from the leaves of *Ilex hainanensis* (Zhou et al., 2007).

1.3 Thesis objectives

The overall goal of this thesis is to develop and validate a rapid and sensitive analytical method, using HPLC-MS technology with simple sample preparation, to study the saponin profiles found in common Canadian pulses. The effects of different processing conditions on the saponin profile were also studied. These processing conditions include sprouting, baking, and pressure-cooking. It is hypothesized that the saponin content in different pulses will be affected by different processing conditions and their content can be quantified by HPLC-MS.

To test the hypothesis and accomplish the objective:

1. A review of the literature that is related to the different types of pulses grown in Canada; saponin structures, classifications and properties; common methods used in saponin analysis from plants (Chapter 1).

2. Develop and validate a rapid HPLC-MS analytical method with simple sample preparation (extraction, dilution and injection) for the quantitation of saponin in faba bean flour (Chapter 2).
3. Examine the effects of sprouting time, drying time and baking on the saponin content of 4 low-tannin and high-tannin faba bean varieties (Chapter 3) *.
4. Examine the effects of pressure-cooking on the saponin profile in 3 pea varieties (Amarillo, Cooper and Lacombe), black bean, chickpea, green lentil, red lentil, kidney bean, and pinto bean comparing to dry beans (Chapter 4).

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2 Chapter 2 Development of a Rapid Method for the Determination of Saponin Content in Faba Bean (*Vicia faba L. var. minor*) Flours Using High-Performance Liquid Chromatography-Mass Spectrometry

2.1 Introduction

Saponins are a diverse group of secondary metabolites containing one or more sugar moieties linked to the aglycone sapogenin. The classification of saponin is based on the structure of the sapogenin backbone, which may consist of 30-carbon triterpenoid or 27-carbon steroid (W. A. Oleszek, 2002). Saponins from legumes are generally triterpenoidal glycosides known as soyasaponins and based on the number of sugar moieties attached to the backbone, they are further categorized under group A, B, or E soyasaponin. Soyasaponin group A has two sugar moieties at C-3 and C-22 positions, as well as a hydroxyl group at the C-21 position on sapogenol A. Group B soyasaponin has one sugar moiety at C-3 and is further classified as 2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyranone (DDMP) conjugated and non-DDMP conjugated at C-22. Group B non-DDMP conjugated saponin are named as soyasaponin Bb (I), Bc (II), Bb' (III), Bc' (IV) and Ba (V), whereas DDMP conjugated saponin includes soyasaponin αg, βa, βg, γa, and γg (Singh et al., 2017). During heating, acidic and alkaline pH processing, the relatively weak covalent bond breaks and DDMP moiety will be released as maltol, so that DDMP conjugated soyasaponin will convert to non-DDMP soyasaponin (Lynn Heng et al., 2006). Group E contains a ketone group at the C-22 position and has been reported present as photooxidation artifacts of group B saponins during extraction or processing (Singh et al., 2017). The molecular formula and chemical structures of group A, B, DDMP and E saponins are listed in Table 1.2.1. in Chapter 1.

Saponins, that are believed to make an important contribution to the bitterness of the legumes, have also been detected in faba beans (Amarowicz, Yoshiki, Pegg, & Okubo, 1997). Soyasaponin

Bb (non-DDMP conjugated group B saponin) and βg (DDMP conjugated group B saponin) has been identified in faba bean (*Vicia faba* L.) obtained from Germany (Barakat, Reim, & Rohn, 2015b). The correlation between bitterness and saponin content in dry peas has been studied previously (Lynn Heng et al., 2006). In sensory testing, trained panelists tasted the bitterness in saponin extracts that contain either soyasaponin Bb alone, or a mixture of soyasaponin Bb plus soyasaponin βg at a ratio of 1:4. Statistical analysis showed that solutions containing DDMP conjugated soyasaponin βg were significantly more bitter than non-DDMP conjugated soyasaponin Bb solution. Similar results were also reported in soy bean (Aldin et al., 2006). In Barakat, Reim & Rohn's (2015) study, it was found that faba bean contains a higher amount of soyasaponin βg than soyasaponin Bb.

Various analytical methods have been reported to identify and quantify saponins in pulses (Barakat et al., 2015b; Liu et al., 2017; Sagratini et al., 2009). Common solvent extraction methods use either absolute or aqueous methanol or ethanol solution as solvents, combined with stirring, refluxing, Soxhlet, or sonication at room or elevated temperature (Amarowicz et al., 1997; Bianco et al., 2015; Liu et al., 2017; Reim & Rohn, 2015b; Sagratini et al., 2009). The application of gas chromatography (GC) is limited by the requirement of converting large, polar and non-volatile saponin compound into more volatile derivatives via acid hydrolysis. However, hydrolysis of saponin may results in formation of artifacts, which may increase the uncertainty in quantitative analysis (W. A. Oleszek, 2002). On the other hand, LC method do not need derivatization and is a more commonly used separation method for saponin extracts prior to structure identification or quantification, combined with ultraviolet spectroscopy (UV), evaporative light scattering (ELSD), electrospray ionization (ESI) mass spectrometry (MS) detectors. Nuclear magnetic resonance spectroscopy (NMR) has also been widely used to identify saponin structures (Ichikawa et al.,

2009; Liu et al., 2017; Reim & Rohn, 2015b; Sagratini et al., 2009; W. Zhang et al., 2009). The UV detection of saponin has been conducted at 206 nm for soyasaponin Bb and 292 nm for soyasaponin βg in Italian lentil seeds. Nevertheless, the weak signals obtained for both soyasaponins show the low sensitivity of UV detection due to the lack of a characteristic chromophore, which is essential for UV detection (Sagratini et al., 2009). LC/MS or LC/MS/MS is widely used in saponin analysis in pulse because high selectively and sensitivity could be achieved by coupling with MS detector.

In the present study, a simple and rapid HPLC-MS with minimum sample preparation method was developed for the identification and quantification of saponin in faba bean. Extraction methods including sonication, stirring, and dispersion were studied for higher saponin extraction yield from faba bean flour. This method was validated in terms of linearity, limits of detection (LOD), limits of quantification (LOQ), accuracy, precision and extraction recovery.

2.2 Materials and Methods

2.2.1 Plant materials and chemicals

Dehulled low-tannin faba bean flour (*Vicia faba L. var. minor*, cv. Snowbird) was obtained from the Food Processing Development Centre, Alberta Agriculture and Forestry, Alberta, Canada, and the seeds were grown in Southern Alberta from 2018 crop year. HPLC graded acetonitrile (ACN) (>99.9%), methanol (MeOH) (>99.8%), ethanol (>99.8%), and water (Optima® LC/MS) were purchased from Fisher Scientific International Inc. Soyasaponin Bb standard and ginsenoside Rb1 were purchased from AdooQ BioScience (>98%, Irvine, Canada).

2.2.2 Standard solutions preparation

Soyasaponin Bb and ginsenoside Rb1 were used as reference standard and internal standard (IS), respectively, for quantification. Soyasaponin Bb and ginsenoside Rb1 standards (5 mg each), were weighed accurately on an analytical balance (Sartorius AG, Germany), transferred into a 5mL volumetric flask, and made up with MeOH (1mg/mL stock solution). The stock solution was mixed thoroughly using a vortex-mixer (Fisher Scientific, CA), stored in a firmly closed 2 mL HPLC glass bottle and kept at 4°C in a refrigerator until analysis. The latter stock solution was used to prepare diluted standard solutions of 0.2, 0.4, 1, 2, 4, 5 μg/mL to create the calibration curve. The IS concentration was fixed at 0.5 μg/mL (spiking level) for all measurements.

2.2.3 Sample extraction methods

Three different methods were studied including sonication, stirring, and Polytron homogenization. The sonication was carried out using a sonication bath (FS60, Fisher Scientific, CA) and the extraction was performed as follows: 1g faba bean flour was accurately weighed on an analytical balance (Sartorius AG, Germany) and sonicated into 2.5 mL methanol (50/60 Hz, 335W) for 1 hour at room temperature, then centrifuged using a Centrifuge (Damon/IEC Division HN-SII, UK) for 5 min at a speed of 9000 rpm; finally the supernatant was separated and collected. The extraction and centrifugation were repeated for a second time by using another 2.5 mL methanol. The extracted fractions were then combined, and the total volume was made up 5.0 mL with methanol. A 200 μL aliquot of the methanol extract was taken for chromatographic analysis.

The stirring method was carried out using a Thermo Fisher Isotemp hot plate and magnetic stirrer (Fisher Scientific, CA) by extracting 1g faba bean flour into 5 mL methanol in a caped 25 mL of

polypropylene centrifuge tube (Falcon, Fisher Scientific, CA) at a stirring speed of 300 rpm for 24 hours at room temperature. The mixture was then centrifuged, separated, and made up to a total volume of 5 mL with methanol. Finally, a 200 µL aliquot was subjected to chromatographic analysis.

Polytron dispersion was carried out using the high-speed handheld disperser Polytron (PT 1300 D, Kinematica AG, Switzerland) at a speed of 7000 rpm. 200 mg of faba bean flour was accurately weighed onto an analytical balance, then transferred into a 7 mL centrifugation glass vial with cap containing 2 mL methanol. The mixture was extracted for 5 min, then centrifuged for another 5 min, and finally, the methanol extract was transferred into a 2 mL HPLC-MS glass vial and stored frozen (-4°C). A 100 µL aliquot of methanol extract was diluted with another 100 µL methanol and the mixture of total 200 µL was subjected to the chromatographic analysis. Different extracting solvents including pure methanol and ethanol and their aqueous mixtures of 70% each (solvent / water (HPLC-MS graded), 7:3 v/v), were used to study the extraction efficiency.

2.2.4 HPLC-MS analysis

The analysis was performed using a chromatographic system of Agilent HPLC (1200 series, Agilent Technologies, USA) attached to a linear ion trap quadrupole MS (3200 QTrap, Applied Biosystems, CA). The data was collected and processed using Analyst 1.4.2. software. An aliquot of 200 µL of methanol extract was injected directly into an Ascentis Express C18 column (15 cm x 2.1 mm, 2.7µm, Sigma-Aldrich, CA) where the injection volume was 5 µL. The flow rate was set at 0.2 mL/min. The mobile phase was modified according to the preliminary study, which consists of solvent A (water) and solvent B (acetonitrile/ methanol (3:1, v/v)). The solvent gradient

program was programmed as follow: 0-0.1 min, 85% A; 0.1-10 min, 10% A; 10-14 min, 10% A; 14-14.1 min, 10% A; 14.1-16 min, 0% A; 16-16.1 min, 85% A; 16.1-25 min, 85% A. Since only a simple extraction procedure with direct dilution was preformed, automated column valve switching was used to elute sample solvent to waste and only divert the solvent into MS during 8-14 mins of total 25 running time in order to prevent MS contamination. Electrospray ionization (turbo spray ion source) was used in the negative ion mode. Full scan mode, selected ion monitoring (SIM) and multiple reaction monitoring (MRM) scan modes were used for the quantification of saponin.

Soyasaponin Bb from the methanol extracts were identified according to their relative retention times, molecular ions and fragment ions, as compared to standards and literature.

2.2.5 Method validation

The calibration curve for soyasaponin Bb was established based on five points (0.2, 0.4, 1, 2, 4, 5 μg/mL). The linear regression equation was $y = ax + b$, where y is the ratio of analyte's peak area to the IS peak area, and x is the ratio of analyte concentration to the IS concentration. Linearity was examined based on regression equations and correlation coefficients. The limit of detection (LOD) was calculated based on the signal-to-noise ratio (S/N) of 3, and the limit of quantification (LOQ) was estimated based on a S/N of 10. Intra-day and inter-day precisions were determined by analyzing 0.2, 1, and 5 μg/mL soyasaponin Bb standard solutions three times in a single day and over three consecutive days, respectively. Accuracy was evaluated after spiking the faba bean flour samples with a known amount of soyasaponin Bb standard solution and then analyzing two times by the developed HPLC-MS method. Additionally, three standard solutions (0.2, 2, 5 μg/mL) were

prepared for quality control. Each analysis follows the sequence of blank (methanol), five calibration standards, quality control standard solutions (QCS) and faba bean samples.

2.3 Results and Discussion

2.3.1 Extraction Method development

Three different extraction methods including sonication, stirring, and Polytron dispersion the flour into the solvent (100% methanol) were chosen based on literature procedures to study the extraction method efficiency. These resulted in soyasaponin Bb recoveries, as represented by peak areas from HPLC-MS chromatograms, which were $5.29e^4$, $1.19e^4$, and $7.75e^3$ (arbitrary units, AU) for Polytron dispersion, stirring and sonication, respectively. These results show that the highest soyasaponin Bb yield was obtained when the Polytron dispersion method was used. It was found that the long extraction times (eg. 24 hours for stirring and 2 hours for sonication) resulted in a rise in temperature during extraction may be responsible for saponin degradation. Since the Polytron dispersion method is the least time-consuming extraction method, less saponin is lost by thermal degradation. In this regard, extracting saponin from faba bean using the Polytron dispersion method (200 mg flour and 2 mL methanol) was applied for further optimization steps. In order to investigate the extraction efficiency with Polytron dispersion, two different parameters including the extracting solvent (methanol, ethanol, 70% methanol, and 70% ethanol), and sample/solvent ratio (25:1, 50:1, and 100:1 (mg/mL flour:solvent)), were studied (Jovanović et al., 2017). Choosing the right extraction solvent is considered the first important step that has a great impact on saponin yields from faba bean flour (Cheok et al., 2014). As shown in Fig.2.3.1., in general, methanol was more efficient than ethanol when the pure solvent of each was used for saponin extraction from faba bean flour where the saponin content was about 12 times higher with 100%

methanol than with 100% ethanol. An increase in saponin content was observed with 100% methanol than with 70% methanol. The aqueous ethanol solvent was more efficient than the pure one. Saponin content was found out to be $1.48e^5$, $1.52e^5$, and $1.44e^5$, as it was presented in peak areas (arbitrary units, AU) when moving from 1:25, 1:50, and 1:100 mL/mg ratio and when using 100% methanol extraction solvent. This observation suggests that increasing the amount of sample in extraction solvent did not improve saponin extraction yield. This is consistent with a previous study (Xu et al., 2012), where increasing the solvent volume, thus reduce the solvent/ sample ratio had no significant effect on extraction yield of saponin from plants. It is possible that the volume of solvent tested was not sufficient enough to hydrate and penetrate all of the faba bean cell wall, so that saponin might still be kept in the matrix.

In conclusion, the extraction by Polytron dispersion at the optimum conditions of solvent/sample ratio (1:50 mg/mL, solvent:flour), 5 min extraction time, and pure methanol were chosen to be applied for sample preparation in the following studies.

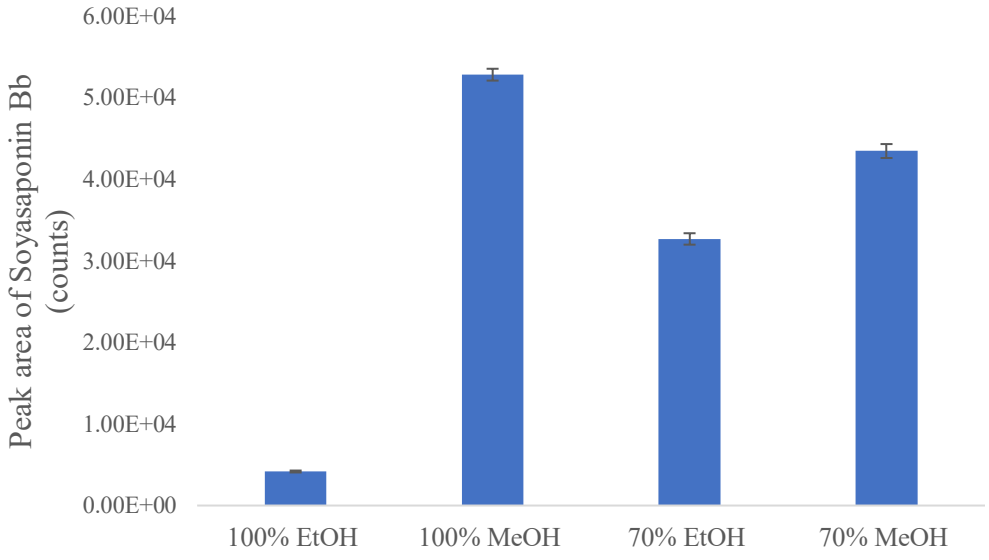

Figure 2.3.1 Soyasaponin Bb content (presented in peak areas (arbitrary units, AU)) extracted by 2 mL different solvents from 200 mg faba bean flour using Polytron.

2.3.2 Optimization of HPLC-MS parameters

Soyasaponin Bb standard solution and the IS (ginsenoside Rb1) was used to optimize the chromatographic conditions and the mass spectrometric parameters.

LC/MS was first applied full scan mode over the range of *m/z* 500-1500 in order to detect all potential saponin types present in faba bean extracts. All *m/z* value was extracted for each saponin type (group A, B, E and DDMP-conjugated, Table 1.5. Chapter 1) to exam the presence. Only soyasaponin Bb and soyasaponin βg were present in faba bean cultivars Snowbird under current study as well as in a previous study (Barakat et al., 2015b). In a preliminary study, both positive and negative electrospray ionization modes (ESI^+ and ESI^-) were used for the identification of saponins. In the ESI^+ mode, the most intense peak corresponded to the molecular ion with sodium adducts $[M+Na]^+$. In the ESI^- mode, the most intense peak corresponded to the deprotonated molecular ion $[M-H]^-$. Hence the ESI^- mode was chosen to detect saponin fragments and their relative intensities due to lack of metal adduct ions contamination. For both standards, the precursor ion was the deprotonated molecule, i.e., 941 *m/z* for soyasaponin Bb and 1107 *m/z* for ginsenoside Rb 1. Each precursor ion was then subjected to MS/MS experiments to optimize mass spectrometer conditions, as shown in Table 2.3.1 Then, MRM was used to optimize the saponin response and ultimately for quantification of saponins. To do this, precursor and product ions were determined for soyasaponin Bb and ginsenoside Rb1, for which standards are available. The peak generated by LC-MS of the extracted saponin should be separated from other signals e.g. flavonoids, in order to be identified and also quantified correctly. Due to the instability of the DDMP conjugate, an adequate standard of soyasaponin βg is not commercially available, and hence, the quantification of soyasaponin βg was based on the soyasaponin Bb response factor.

Table 2.3.1. shows MRM transitions and optimized parameters for soyasaponin Bb, soyasaponin βg and ginsenoside Rb1.

Table 2.3.1 The fragment ions for soyasaponin Bb, soyasaponin βg and ginsenoside Rb1 standards and their associated Qtrap settings.

Analyte	Parent ion (*m/z*)	Product ion (*m/z*)	Declustering potential (DP) (V)	Collision energy (CE) (V)	Entrance potential (EP) (V)	Collision entrance potential (CEP) (V)	Collison exit potential (CXP) (V)	Dwell
Soyasaponin Bb	941.5	615.2, 205.1	-120	-36	-6	-127	-6	80
Soyasaponin βg	1067.5	741.4, 583.5	-120	-60	-10	-120	-5	80
Ginsenoside Rb1	1107.2	440.4	-85	-35	-4	-52	-9	80

Soyasaponin Bb, at *m/z* 941.8 $[M-H]^-$, exhibited product ions at *m/z* 923.8, 795.8, 615.2, and 457.4, corresponding to $[M-H_2O]^-$, $[M-rha]^-$, $[795-gal-H_2O]^-$ and $[795-gal-glu]^-$, respectively, where rha= rhamnose, gal= galactose, and glu= glucose. Soyasaponin βg was observed at a deprotonated molecular of *m/z* 1067.6, and presented MS^2 fragment ions at m/z 1049.6 $[M-H_2O]^-$, 921.6 $[M-rha]^-$, 759.4 $[921-gal]^-$, 741.4 $[759-H_2O]^-$ and 583.4 $[759-glu]^-$. An example of the precursor and product ion MS/MS spectra for soyasaponin Bb and Soyasaponin βg is shown in Fig. 2.3.2. and Fig. 2.3.3.

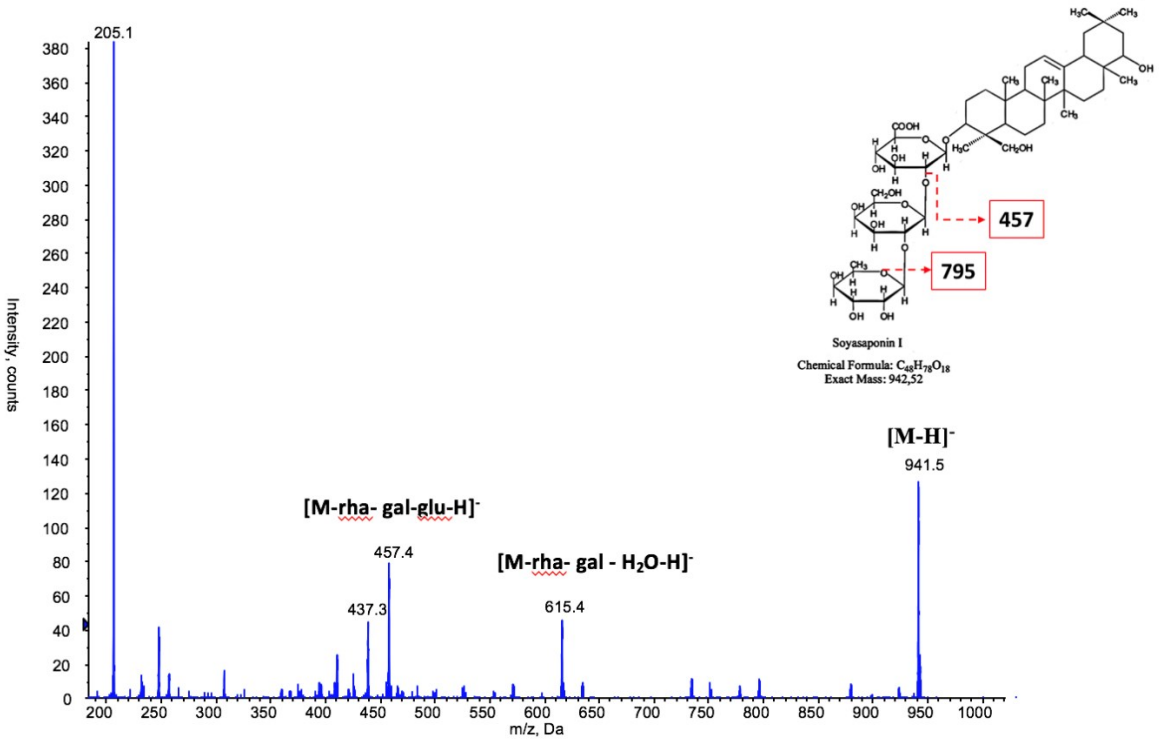

Figure 2.3.2 MS/MS product ion spectra of soyasaponin Bb standard in ESI- modes at a concentration of 0.2 mg/mL. The chemical formula, structure, fragmentation patterns, m/z value of fragment ion are shown.

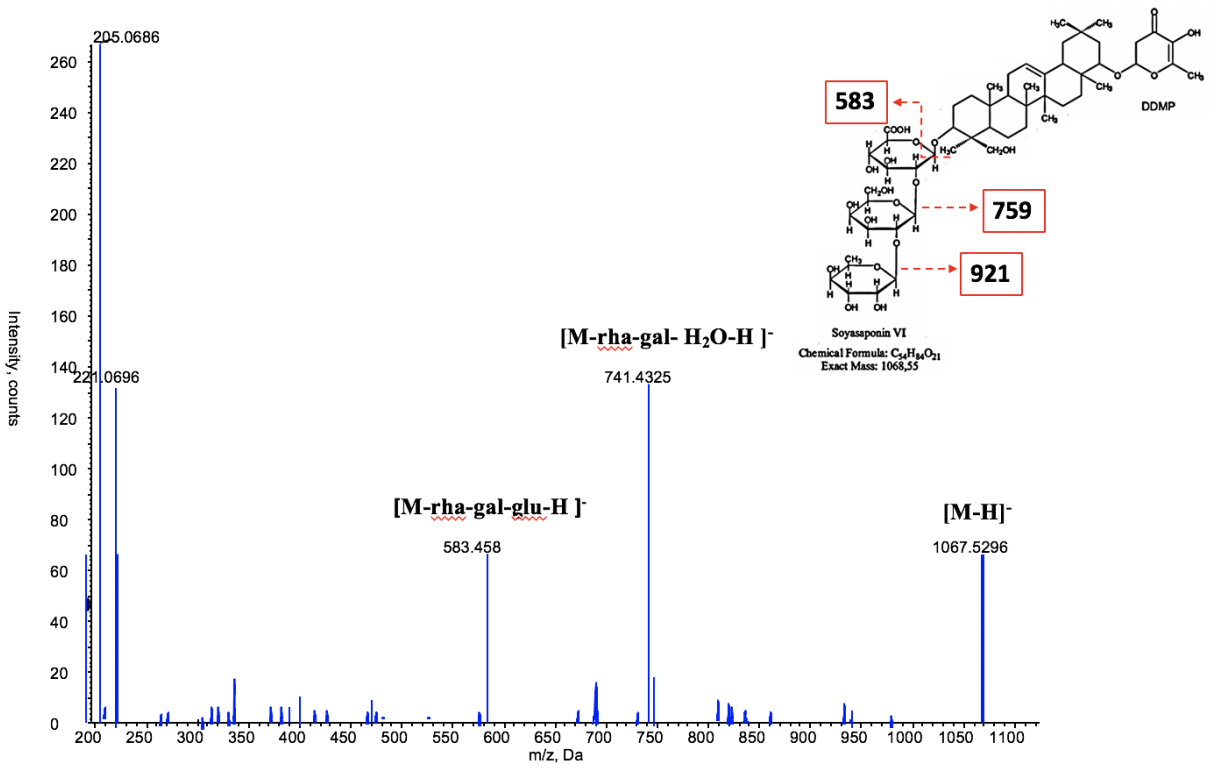

Figure 2.3.3 MS/MS product ion spectra of soyasaponin βg extracted from faba bean flour (dehulled, snowbird) in ESI- modes. The chemical formula, structure, fragmentation patterns, m/z value of fragment ion are shown.

Figure 2.3.4. shows the extracted ion chromatogram (XIC) of soyasaponin Bb standard with IS at a spiking level of 1 μg/mL. Using the developed LC-MS/MS method led to a good separation of the 3 compounds with retention times of 11.83 min (soyasaponin Bb), 13.23 (soyasaponin βg) and 11.15 min (IS). Multiple extraction and purification steps including solid phase extraction (SPE), liquid-liquid extraction, ultrasonic extraction, and Soxhlet extraction were present previously in the sample preparation, which prolongs the total analysis time (D. Guajardo-Flores, García-Patiño, Serna-Guerrero, Gutiérrez-Uribe, & Serna-Saldívar, 2012; Sagratini et al., 2009). On the other hand, in the present study, the purification, evaporation, and concentration steps were eliminated and hence an easier, faster and simpler sample preparation method was achieved.

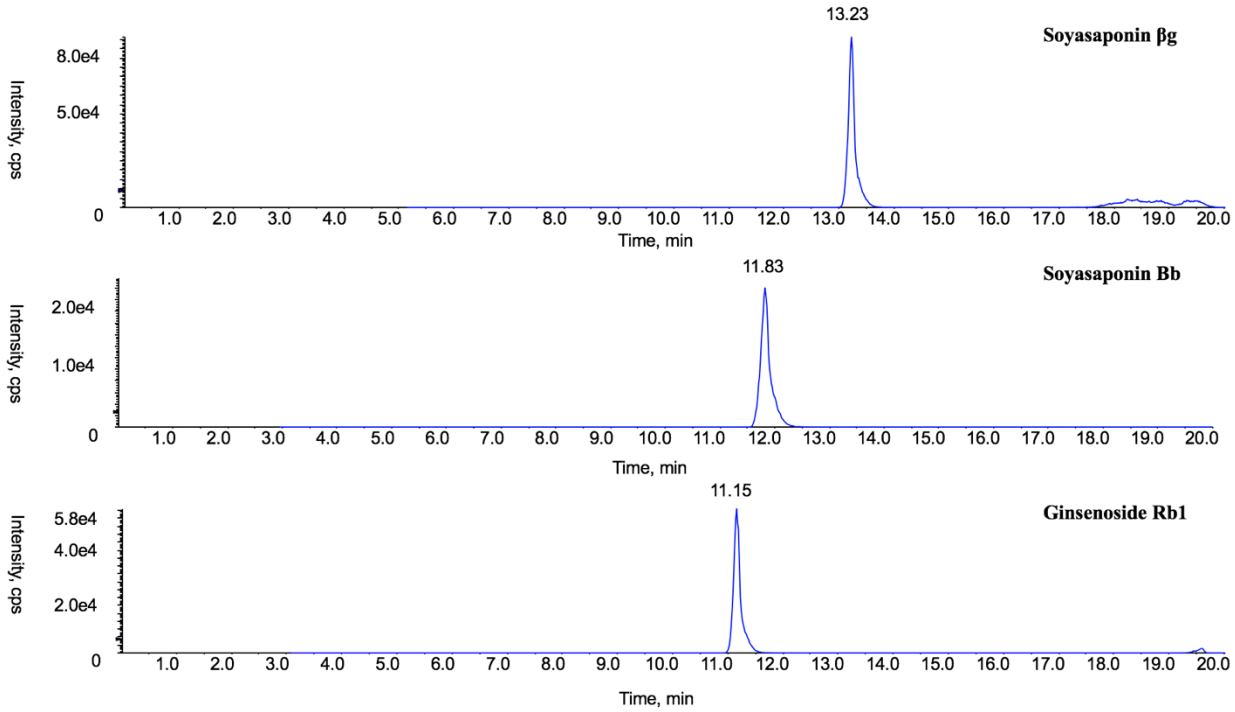

Figure 2.3.4 The extracted ion chromatogram (XIC) of saponin extract from faba bean flour (dehulled, Snowbird) at a spiking level of IS of 1 µg/mL.

2.3.3 Method validation

Table 2.3.2. and 2.3.3 summarized the results of method validation. The calibration curve of soyasaponin Bb was generated by duplicate injections of standards at 0.2, 0.4, 1, 2, 4, and 5 µg/mL using the LC/MS method described in Section 2.4. The internal standard was present at 1 µg/mL in all standard mixtures. The calibration curve shows a good linearity in the range of 0.2-5 µg/mL ($Y= 1.2319x + 0.30326$ and correlation coefficient $R^2 = 0.9936$). Accuracy was tested using quality control standards (QCS) covering the range of concentrations of 0.2, 2, and 5 µg/mL, resulting in recoveries of 98.75%, 97.53%, and 92.15%, respectively, with relative standard deviations (RSD) 1.6%, 1.9%, and 1.3%, respectively. Recovery higher than 92% and RSD value lower than 2% indicate good accuracy and precision for the developed analytical method. LOD and LOQ values

for soyasaponin Bb were 5 ng/mL and 10 ng/mL, according to the signal-to-noise ratio (S/N) of 3 and 10, respectively, as determined by serial dilution of standards to concentrations lower than 0.2 μg/mL. The LOD and LOQ results in the low μg/mL indicate that current described method is sensitive for saponin measurements. The intra-day and inter-day precisions were determined by analyzing soyasaponin Bb standard solutions three times in a single day and over three consecutive days, respectively, where the means of RSD were found to be 1.6% and 5.0%, respectively. The extraction recovery of the soyasaponin Bb was calculated by comparing the peak area of the analyte spiked at three levels of 0.5, 1, and 2 μg/mL into faba bean flour before and after the extraction with dispersion, where the mean values were found to be 95%, 103%, and 105%, respectively, with RSD < 12%.

Table 2.3.2 Calibration curve, linearity, detection limit, quantification limit and precision.

Analyte	Regression equation	Correlation coefficient (%)	Linear range (μg/mL)	Detection limit (ng/mL)	Quantification limit (ng/mL)	Intra-day precision (%)	Inter-day precision (%)
Soyasaponin Bb	y = 1.23x + 0.303	0.994	0.2-5.0	0.005	0.01	1.6	5.0

Table 2.3.3 Recoveries of soyasaponin Bb added to faba bean flour (*n=2x2).

Analyte	Original amount (mg/kg)	Spiked amount (mg/kg)	Measured amount (mg/kg)	Recovery (%)	RSD (%)
Soyasaponin Bb	0.0275	0.5	0.524	95	10
		1.0	1.028	103	5
		2.0	2.030	105	12

***** Each sample was prepared as duplicates and each replicate was analyzed twice.

2.4 Conclusion

In this study, a rapid HPLC-MS method for the identification and determination of saponin in faba bean flour samples with simple sample preparation procedure includes only Polytron dispersion and dilution has been established. Validation of the method was preformed according to linearity, accuracy, detection limit, quantification limit, inter- and intra-day precision. Calibration curve for soyasaponin Bb standard with addition of 1ppm internal standard (ginsenoside Rb1) solution showed correlation coefficients of 0.994 and a linear range of 0.2-5 μg/mL. Recoveries of extraction calculated in faba bean sample ranged from 95 -105%, with n= 6 and RSD <12%. An efficient separation of 2 saponin and IS in a single 25 min chromatographic run was achieved compared to the literature which reported a LC-MS running time of 40 mins (Berhow et al., 2006). The analytical method described in this chapter can be applied to study the processing effects on saponin content in 4 cultivars of faba bean (Chapter 3) and can be adapted to apply to study of saponin content in other type of pulses (Chapter 4).

3 Chapter 3 The Effects of Sprouting Time, Drying Time and Baking on the Saponin Content of Sprouted Faba Bean (Vicia faba var. minor) Flours

3.1 Introduction

Faba bean (*Vicia faba* L.), also called broad bean, fava bean, horse bean or field bean, are widely grown in many countries such as China, the European Union, Ethiopia and Egypt due to their high protein and starch contents along with their ability to adapt to a range of climatic zones (Crépon et al., 2010). Faba beans have been shown to contain high amount of proteins, carbohydrates, dietary fibers, vitamins, minerals, and bioactive compounds such as phenols and flavonoids (Crépon et al., 2010) (Mejri et al., 2018). At the same time, there is an increasing global interest in developing and producing plant-based healthy food products with a high protein content, and that are perceived by consumers as being healthy choices (Mejri et al., 2018). As a result of their high nutritional value, different genotypes of faba beans in the present market in Canada are valuable in both animal feed and human food. High or normal tannin types faba bean grown for human consumption have brown or violet flowers, and tan to brown seed coat. Low tannin types faba bean both grown for high protein livestock feed and for human consumption have white flowers and cream seed coat (Saskatchewan Pulse Growers, 2019). Tannins are classified into hydrolyzable tannins (ester form of phenolic acids) and condensed tannins (flavonoid polymers) (Multari et al., 2015). Condensed tannins are able to interact with proline-rich proteins, particularly to precipitate salivary proteins in the oral cavity to form soluble or insoluble complexes that negatively affect the palatability of pulses (astringency) such as faba bean, adzuki bean, red bean, pea, green and red lentil (Ate Troszyńska et al., 2006). The use of faba beans in the Western diet is limited by the undesirable bitter and astringent taste which is associated with the presence of saponins and tannins, as well as the unique beany flavor which may not be familiar to North

American consumers. DDMP-conjugated soyasaponin βg and soyasaponin Bb were determined by recognition thresholds and descriptive analysis as the main saponin types that are responsible for the bitterness attributes in soybean and pea (Aldin et al., 2006; Lynn Heng et al., 2006). In the study conducted by Heng et al., (2006) suggested that the degree of bitterness was depend on the concentration and types of saponin. They found out that both soyasaponin βg and Bb have a bitter taste at concentration as low as 2 mg/L in water, while soyasaponin βg is significantly more bitter than Bb at all concentration tested (2-12 mg/L). In addition, group A soyasaponin was found out to cause a bitter and astringent taste in soybean (Takada et al., 2013).

The sprouting process, involving both germination and subsequent drying of seeds, is known to reduce anti-nutritional factors (trypsin inhibitors, tannins, lectins, and saponins), increase micronutrient bioavailability (amino acids, peptides, vitamins, and minerals) and enzyme activity, and change the sensory properties of seeds comparing to their dry analogues (Guajardo-Flores, García-Patiño, Serna-Guerrero, Gutiérrez-Uribe, & Serna-Saldívar, 2012; Luo et al., 2013; Troszyńska et al., 2011). Luo et al. (2013) found that phytic acid and polyphenol contents in two cultivars of faba bean (Qidou 2 and Big Qinpi) were reduced by approximately 95% after 4-days germination. Such reduction in anti-nutritional compounds improve the nutritional quality of faba bean. After 7 days germination in the dark, green lentils seeds (cv. Aldona) were subjected to sensory evaluation by a trained panelists using quantitative descriptive analysis and the change in phenolic compounds were determined by HPLC-MS (Troszyńska et al., 2011). Strong correlation was found between bitterness and astringency and catechin gallate and flavonols content. The results showed a change in phenolic composition and an improvement of sensory profiles in germinated lentil seeds.

Baking is another food processing method to improve the palatability of legumes. Under high temperature, DDMP-conjugated soyasaponin βg is unstable. The relatively weak covalent bond breaks and DDMP moiety is released as maltol, so that DDMP conjugated soyasaponin is convert to non-DDMP soyasaponin. Statistical and sensory analysis showed that solutions containing DDMP conjugated soyasaponin βg were significantly more bitter than non-DDMP conjugated soyasaponin Bb solution (Lynn Heng et al., 2006). Baking at an oven temperature of 180-200°C for 5 mins significantly lowered the total saponin content in faba bean flour based vegetarian bars by 59% (Barakat et al., 2015a).

The effects of sprouting and baking on saponin composition and content in faba bean is poorly understood. Therefore, as part of a preliminary investigation into the development of a sprouted faba bean flour as a versatile food ingredient with high consumer acceptance, the effects of sprouting time and drying time on the saponin profile in faba bean was investigated. In addition, the effects of baking on the saponin composition of faba bean flour was also determined. These studies should provide a better understanding of the saponin profiles in faba bean flours, which may be used to improve their taste profiles.

3.2 Materials and Methods

3.2.1 Plant materials and chemicals

The faba bean varieties used in this study, which include Snowbird, Snowdrop, Fabelle, and FB-94, were obtained from the Food Processing Development Centre, Alberta Agriculture and Forestry, Leduc, Alberta, Canada. These seeds were grown in Southern Alberta in the 2018 crop year. HPLC graded acetonitrile (ACN) (>99.9%), methanol (MeOH) (>99.8%), and water

(Optima® LC/MS) were purchased from Fisher Scientific International Inc. Soyasaponin Bb standard and ginsenoside Rb1 were purchased from AdooQ BioScience (>98%, Irvine, Canada).

3.2.2 Standard solutions

Soyasaponin Bb and ginsenoside Rb1 were used as reference standard and internal standard (IS), respectively, for quantification. Soyasaponin Bb and ginsenoside Rb1 standards (5 mg each), were weighed accurately on an analytical balance (Sartorius AG, Germany), transferred into a 5mL volumetric flask, and made up with MeOH (1mg/mL stock solution). The stock solution was mixed thoroughly using a vortex-mixer (Fisher Scientific, CA), stored in a firmly closed 2 mL HPLC glass bottle and kept at 4°C in a refrigerator until analysis. The latter stock solution was used to prepare diluted standard solutions of 0.2, 0.4, 1, 2, 4, 5 μg/mL to create the calibration curve. The IS concentration was fixed at 0.5 μg/mL (spiking level) for all measurements.

3.2.3 Faba bean germination

Seed germination was performed at the Food Processing Development Centre (Leduc, AB, CA). The raw seeds were rinsed three times to remove dirt and then soaked in an excess of tap water for 16 h. The beans were drained and sterilized by submerging in a sanitizer (PEROX-E, 55ppm) for 10 min and then rinsed with tap water before transferring onto plastic-perforated germination trays. The trays were lined with a highly water absorbent cloth and covered with an additional cloth over the beans. The germination process was performed in an environmental chamber (model LHU-113, ESPEC) at 25°C and 80% relative humidity (RH). Throughout the sprouting process, water was constantly added to keep the beans hydrated in germination trays. Beans were removed and

either frozen flat onto a plastic tray lined and covered with aluminum foil at -18°C for at least 24 hours, or dried on metal trays lined with aluminum foil in an oven (model 1330F, SHEL-LAB) at 60°C until a moisture content of 10% or below was reached. Note that in drying time study, all of the 0 hour drying time samples were freeze-dried in order to match the water content of these seeds, and the corresponding flours, to the rest of the samples which were oven-dried to ensure a moisture content of 10% or below was reached.

3.2.4 Cracker samples preparation

The cracker preparation was performed at the Food Processing Development Centre (Leduc, AB, CA). The faba bean flour was prepared by milling using a micro-hammer cutter mill (model DFH48, GlenMills). The ingredients used for cracking-making include: 63.11% faba bean flour, 31.15% water and 5.74% vegetable shortening (All-Vegetable, Crisco). Faba bean flour and vegetable shortening were mixed with paddle on speed 2 with a stand mixer (Hobart) for 30 seconds. The speed was adjusted to 1 and water was poured in slowly. Dough samples were rolled with rolling pin, then run through pasta maker to form sheets. Crackers were cut into 4 cm squares and placed spaced apart from each other on a parchment paper. They were baked at 160°C for 13 minutes in an oven (model 1330F, SHEL-LAB).

3.2.5 Saponin extraction

The extraction procedure was adjusted and performed based on the results obtained from optimization study (Chapter 2). Finely grounded bean flour (0.2 mg) was accurately weighed on an analytical balance (Sartorius AG, Germany) and transferred into a 7 mL glass vial. 0.5 mL of internal standard (2 μg/mL), and 1.5 mL 100% methanol was added into the glass vial, and the

mixture was extracted at room temperature for 5 minutes with high-speed handheld disperser Polytron (PT 1300 D, Kinematica AG, Switzerland) at a speed of 7000 rpm. The mixture was centrifuged (Damon/IEC Division HN-SII, UK) for 5 min at a speed of 9000 rpm and the saponin extracts was transferred and stored in a 2 mL glass vial. An aliquot of 100 µL saponin extract was diluted with another 100 µL methanol and mixed thoroughly using a vortex-mixer (Fisher Scientific, CA). The mixture of total volume 200 µL was subjected to the chromatographic analysis as described below.

All faba bean seeds and cracker samples of different varieties were milled using a Micro-Mill (Science ware, Bel-Art Products, USA) at speed of 10,000 RPM for 60 s. A water-cooled system was applied to protect the mill from overheating. Liquid nitrogen was used when the frozen sprouted seeds were milled. After thawing at room temperature, the seed coats and cotyledons were separated to allow for the determination of saponin distribution within the seeds . The separated parts as well as the whole seeds were dried at 60°C for 24 hours in an oven (Jeio Tech Inc., Korea), then milled as described above, and finally, each flour was frozen until analyzed.

3.2.6 HPLC-MS

The analysis was performed using a chromatographic system consisting of Agilent HPLC (1200 series, Agilent Technologies, USA) attached to a linear hybrid ion trap quadrupole MS (3200 QTrap, Applied Biosystems, CA). The data was collected and processed using Sciex Analyst 1.4.2. software. An aliquot of 200 µL of saponin extract was injected directly into an Ascentis Express C18 column (15 cm x 2.1 mm, 2.7µm, Sigma-Aldrich, CA) where the injection volume was 5 µL. The flow rate was set at 0.2 mL/min. The mobile phase was modified according to the preliminary

study, which consists of solvent A (water) and solvent B (acetonitrile/ methanol (3:1, v/v)). The solvent gradient program was programmed as follows: 0-10 min, 85%-10% A; 10-14 min, 10% A; 14-16 min, 0% A; 16-25 min, 85% A. Since only a simple extraction procedure with direct dilution was preformed, an automated column valve switching was used to elute mobile phase to waste and only direct flow into the mass spectrometer at run times of between 8 and 14 mins (out of a total run time of 25 minutes), in order to prevent ion source contamination. Electrospray ionization (turbo spray ion source) was used in the negative ion mode. Full scan mode, selected ion monitoring (SIM) and multiple reaction monitoring (MRM) scan modes were used for the quantification of saponin. Soyasaponin Bb from the methanol extracts were identified according to their relative retention times, molecular ions and fragment ions, as compared to standards and literature.

3.2.7 Statistical analysis

Each pulse sample was extracted in duplicate, and each extract was further analyzed by LC/MS in duplicate. The resulting values (n=4) were expressed as mean ± standard deviations (mean ± SD). One-way ANOVA and Tukey's test were used to determine the significant difference of means among samples with a level of significance of $p < 0.05$. Statistical data analysis was performed by using the IBM SPSS Statistics software (ver. 26, IBM Institute Inc., Armonk, NY, USA).

3.3 Results and Discussion

3.3.1 Distribution of saponin in faba bean parts

Soyasaponins occur at different concentrations in different parts of the seeds. Dividing them into cotyledon and seed coats, the former contains usually higher amount of saponins. Guajardo-Flores

et al. (2012) found out that the concentration of soyasaponins Bb and βg in raw black bean cotyledon was 55 and 57 μg/g, respectively with no presence of soyasaponin Bb and 60 μg/g of soyasaponins βg in seed coat, The sprouted faba bean seeds (48 hours, Snowbird) were chosen to study the saponin distribution in seeds that has been split and dried as described in the Materials and Methods section. As shown in Table 3.3.1, soyasaponin Bb was predominant and roughly 10 times higher in concentration than βg either in seed coat (with hull), cotyledon, or in whole seeds.

Table 3.3.1 Concentrations of soysaponin Bb and soyasaponin βg (μg/g) in seed coat (with hull), cotyledon and whole seed of faba bean (48 hours sprouted, 24 hours dried, Snowbird) (n=4).

Tissue	Approximate weight percentage of the whole seed (%)	Analyte concentration	
		Soyasaponin Bb	Soyasaponin βg
Seed coat and hull	12.5	5.1 ± 0.2^{a}	0.6 ± 0.0^{a}
Cotyledon	87.5	49.0 ± 0.3^{b}	4.8 ± 0.1^{b}
Whole seed	100	54.5 ± 0.6^{b}	5.3 ± 0.1^{b}

The data represent the mean ± SD of at least three replicates.
Values followed by different superscripts in the column indicate significant differences with $p < 0.05$.

3.3.2 Sprouting effect on saponin content

The saponin content in seeds largely depends on the germination conditions of watering, time and light levels (Ayet et al., 1997). Time and watering have a significant influence on the saponin content based on the variance analysis data. In the current study, the germination was studied at different sprouting times of 48, 54, 60 and 72 hours followed by 72 hours drying. Figure 3.3.1 shows the changes in content of both soyasaponin Bb, βg and total saponin as a result of different sprouting times. Soyasaponin Bb was significantly increased in the sprouted flour compared to

unsprouted. In all varieties it reached its highest level at 54 hours, then decreased at different rates in each case over longer sprouting times.

The soyasaponin βg content shows similar trends to soyasaponin Bb but reached its highest level at 60 hours (54 for FB9-4); these levels decreased at longer sprouting times. A comparable increment in the saponin content of sprouts, cotyledon and seed coats was observed in an earlier study carried out on black beans. In that study, over 5 days of germination, the total amount of saponin increased 2-fold after only 24 hours sprouting then decreased at longer sprouting times (D. Guajardo-Flores et al., 2012). Ayet et al. (1997) reported an increase of soyasaponin Bb content over 6-days of germination in lentils (*Lens culinaris*), compared to unsprouted seeds. An increase in soyasaponin βg was observed after black beans (*Phaseolus vulgaris* L.) were germinated for 4 days, then decreased on day 5 (Guajardo-Flores, Serna-Saldívar, & Gutiérrez-Uribe, 2013). Soyasaponin Bb was absent in dry or soaked black beans, while after 5-days of germination, the presence of soyasaponin Bb was detected by HPLC-ELSD-UV. In contrast, the increase of saponin content observed in current study disagrees with several studies reporting a reduction in saponin content in germinated seeds (Duhan, Khetarpaul, & Bishnoi, 2001; Anita Kataria, Chauhan, & Punia, 1989; A. Sharma & Sehgal, 1992) or reported no change between unsprouted and sprouted seeds (Ruiz, Price, Fenwick, & Rhodes, 1996a). However, the results presented here are in agreement with many other authors (Ayet et al., 1997; D. Guajardo-Flores et al., 2012; D. Guajardo-Flores et al., 2013; Jyothi, Sindhu Kanya, & Appu Rao, 2007; Paucar-Menacho, Berhow, Mandarino, Chang, & Mejia, 2010).

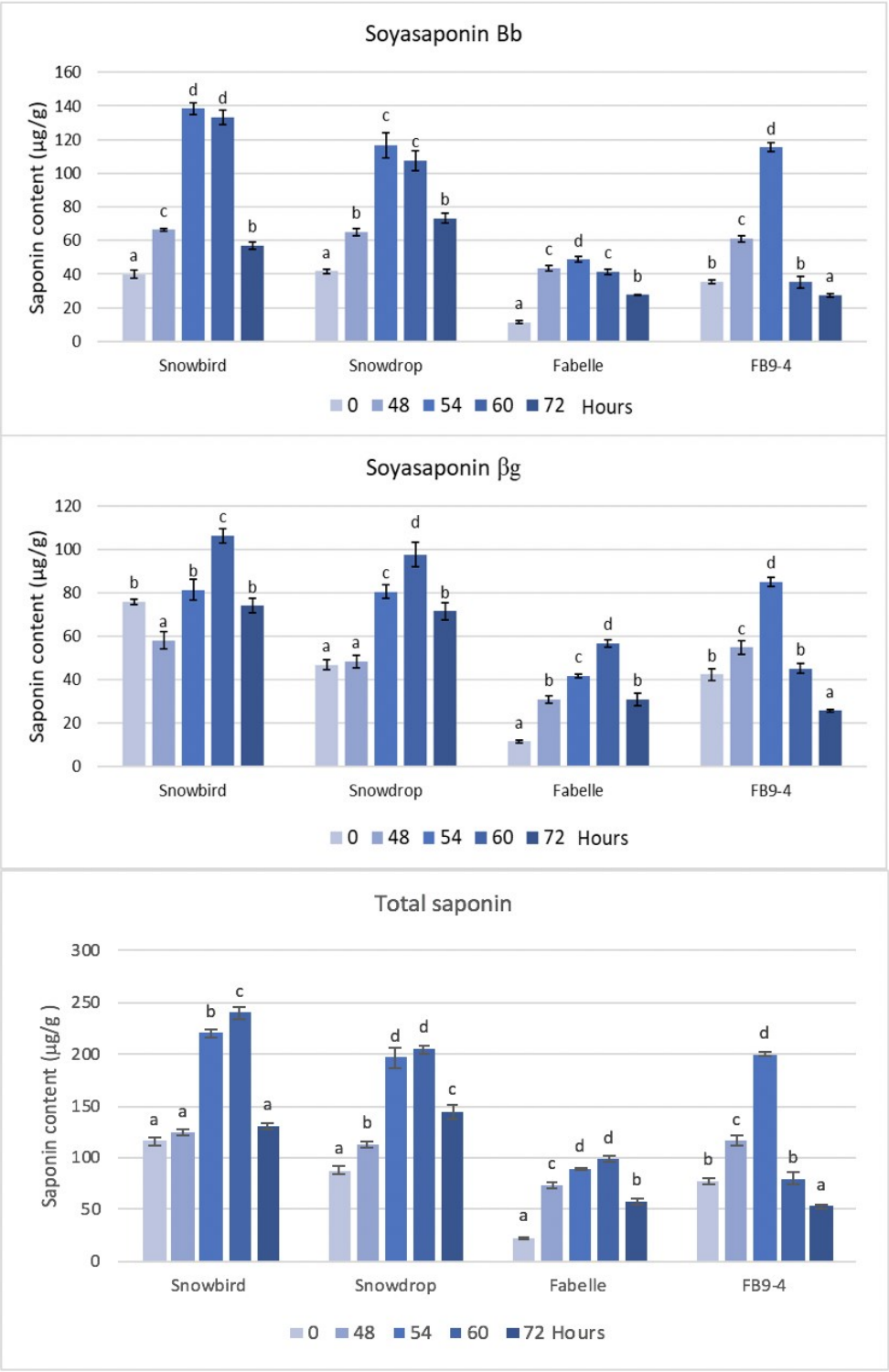

Figure 3.3.1 Effect of sprouting time on soyasaponin Bb, βg and total saponin contents (µg/g) in 4 faba bean varieties including snowbird, snowdrop, fabelle and FB9-4. The data represent the mean ± SD of two replicates and each analyzed twice by LC-MS (n=2x2=4). Values labeled with superscripts indicate the significant differences with $p < 0.05$.

The change of total saponin content varies depending on the faba bean variety. As shown in Fig. 3.1., the total saponin content significantly increased after 72 hours of sprouting in Snowdrop and Fabelle, while a significantly reduction was observed in FB9-4 and no change in Snowbird after 72 hours of sprouting. In all varieties the total saponin content reached its highest level at 60 hours of sprouting, expect for FB9-4 (54 hrs), followed by a reduction at different rates in each case over longer sprouting times.

Germination is well known to reduce some antinutritive factors such as phytic acid and tannins, thus improve the nutritive value in pulses (Luo et al., 2013). Nevertheless, it is thought to have little effect on compounds such as saponins, which participate in the plant defense systems (Ayet et al., 1997; Price, Johnson, & Fenwick, 1987). Moreover, the observed increase of saponin level is more likely due to the activation and synthesis of various enzymes that enhance the production of secondary metabolites, including the saponins. A significant increase in soyasaponin Bb and βg content was found among all 72 hrs-sprouted samples compared to unsprouted samples, except for FB9-4 (significant decrease in both soyasaponin Bb and βg content) and the soyasaponin βg content in Snowbird. On the contrary, in a study of soybeans no significant difference in soyasaponin Bb content was observed under different germination conditions of sprouting-times (0, 21, and 63 hours) and temperatures (20 or 30°C), whereas soyasaponin βg was decreased at 21 hours but increased at longer time of 63 hours (Paucar-Menacho et al., 2010). It has been suggested that germination induces DDMP saponin (soyasaponin βg) biosynthesis in wild soybeans (Krishnamurthy et al., 2014). The present study and previous results from the literature imply that the change of saponin composition and content by germination is specific to legume species, germination and analytical conditions.

3.3.3 Drying effect on saponin content

Drying the sprouted seeds is an essential step to get the final product (flour) with a better quality i.e. improving the nutritional values and removing or reducing the anti-nutrients. Seeds of each variety that had sprouted for 48 hours were dried at 60°C for times of 0, 24, 36, 48, and 60 hours. Figure 3.3.2. shows the effect of the drying times on both soyasaponins Bb, βg and total saponin content (μg/g). The former saponin was significantly decreased after only 24 hours whereas the latter one has exactly the opposite trend. Low-tannin faba beans (Snowbird and Snowdrop) have a generally higher content of both soyasaponins Bb and βg than that observed in the high-tannins seeds, either when unsprouted or sprouted for similar times (Fabelle and FB9-4). Only small or insignificant change was observed for either saponin content when longer drying times were applied (Figure 3.2). The total saponin content was significantly decreased after 24 hours of drying, further reduction in content was observed up to 36 hours of drying whereas no significant change over longer sprouting times.

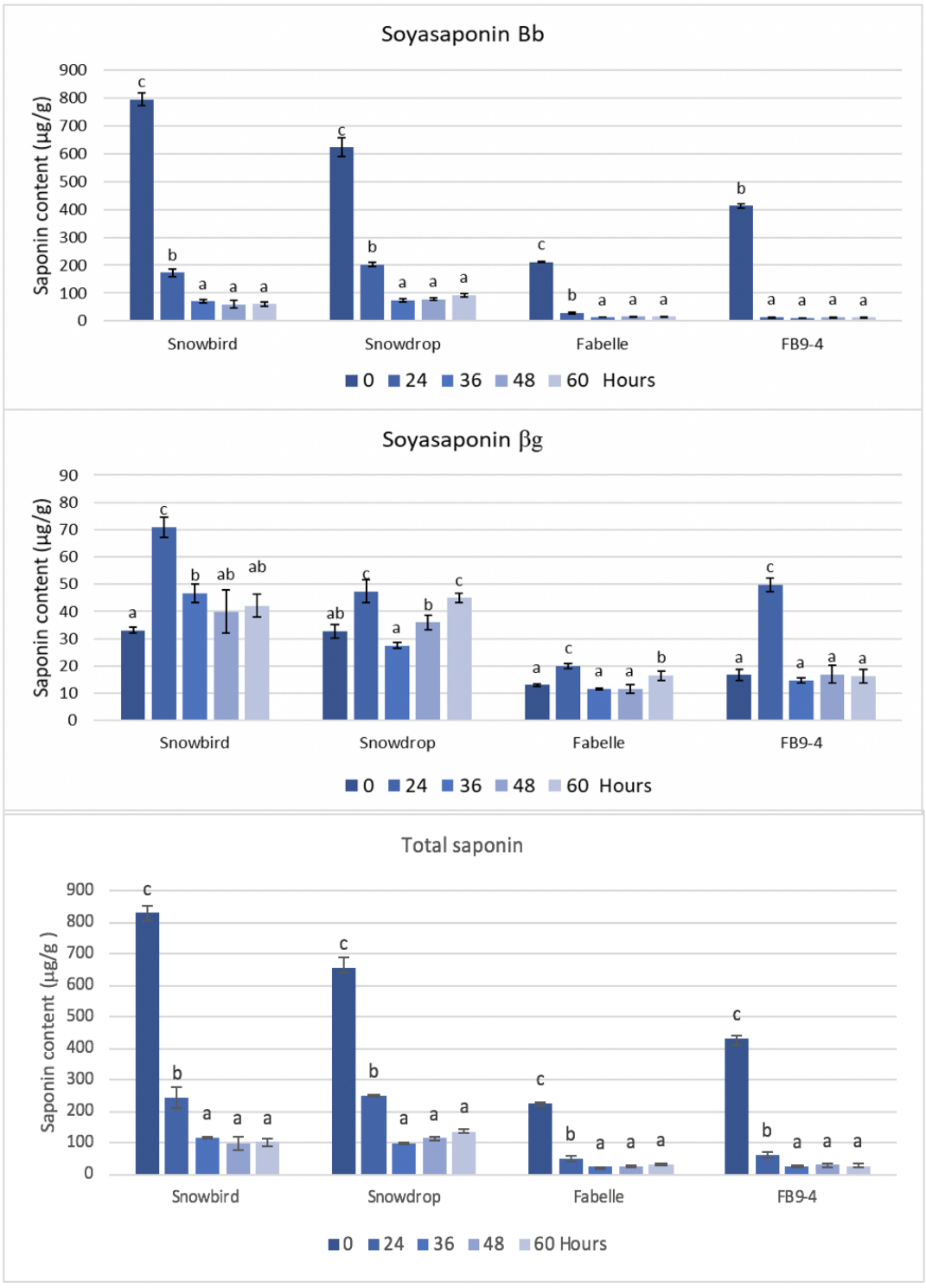

Figure 3.3.2 Effect of drying time on soyasaponin Bb, βg and total saponin contents (µg/g) in 4 faba bean varieties including snowbird, snowdrop, fabelle and FB9-4. The data represent the mean ± SD of two replicates and each analyzed twice by LC-MS (n=2x2=4). Values labeled with superscripts indicate the significant differences with $p < 0.05$.

A study was conducted on the kinetics of the extraction of saponins from *Sapindus rarak DC*, where the saponin yield increased as the extraction temperature increased, but decreased when the temperature reached 60°C (Nafiunisa, Aryanti, & Wardhani, 2019). Saponins are thought to be thermally-sensitive compounds where long exposure times to temperatures >40°C may destroy some amount of saponins, and this may explain the decrease of saponin content observed in the current study (Chindo, Adzu, & Gamaniel, 2012). To conclude, although longer drying times have a negative effect on soyasaponin Bb levels, this may seem to be beneficial in regard to bitterness, as saponins are believed to have a contribution to the bitter taste of pulses. Considering the cost-effectiveness to get the final product of sprouted faba bean flour, a 24 hour of drying time was chosen to be applied on sprouted seeds to be used in further trials.

3.3.4 Baking effect on saponin content

Figure 3.3.3 shows soyasaponin Bb, βg and the total saponin content in flour and crackers made from either sprouted or unsprouted 4 varieties of faba bean. As mentioned above, saponins are known to be thermally sensitive and hence it's expected that the total saponin content would be decreased significantly by cooking pulse flour. What was found here is that while the total saponin content was significantly decreased as a result of baking (see Table 3.3.2), this was not necessarily true for individual saponin types. Thus, soyasaponin Bb increased significantly after baking, while soyasaponin βg was quite the contrary. This large and noticeable difference in soyasaponin contents was not only observed in the crackers but also in the corresponding flours, where soyasaponin βg was dominated in the flour but not in the crackers which can be seen clearly from the ratio (Bb/βg) between both saponins (Table 3.3.2). For example, for the sprouted flours, the

ratio of Bb/βg is in the range of 0.14-0.22 whereas after baking in the crackers it changes dramatically to a range of 11-18.

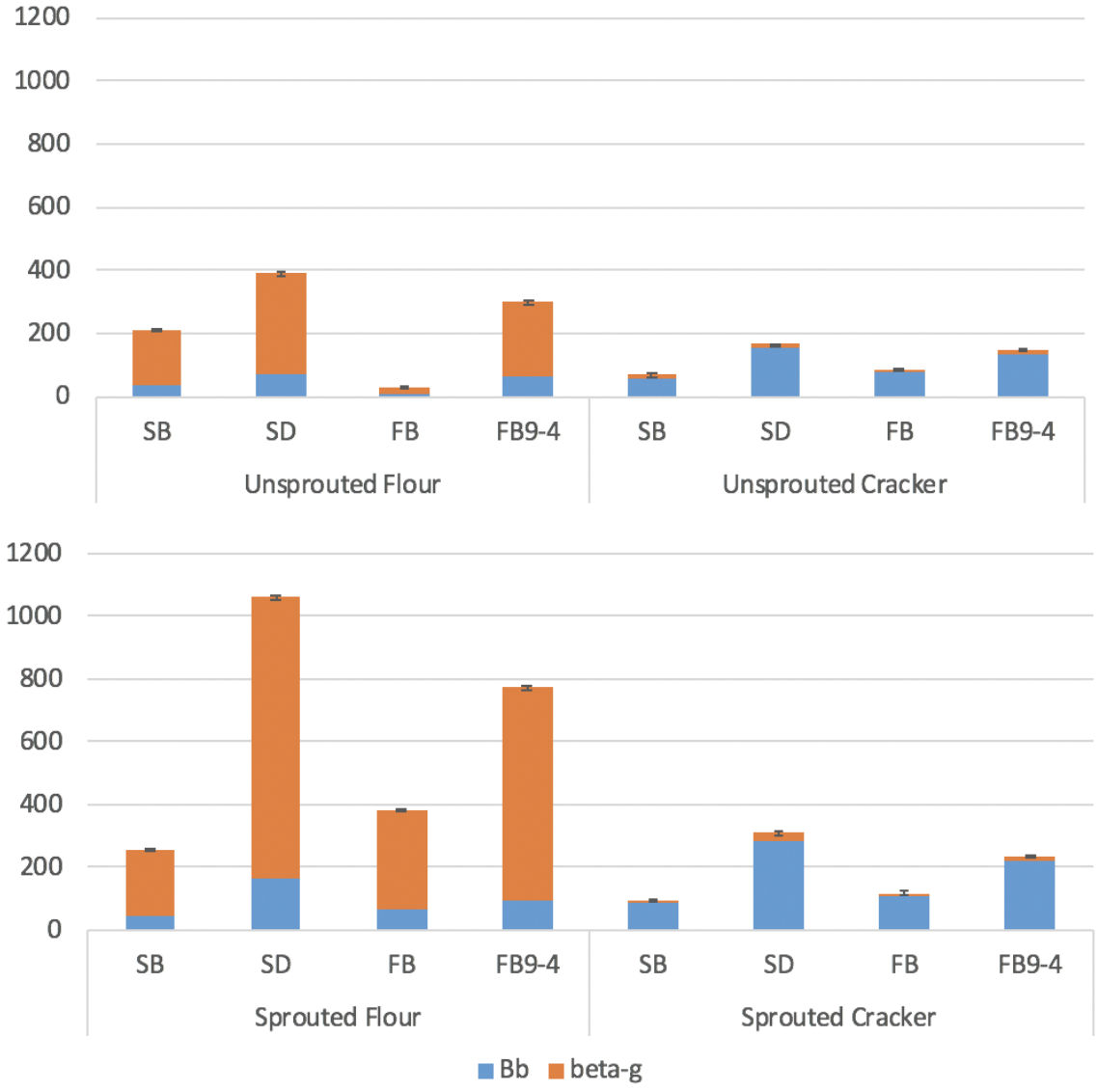

Figure 3.3.3 Soyasaponin Bb, βg and total saponin contents (μg/g) found in unsprouted flour and cracker, sprouted flour and cracker made from 4 faba bean varieties. The data represent the mean ± SD of two replicates and each analyzed twice by LC-MS (n=2x2=4).

Table 3.3.2 Baking effect on the soyasaponin Bb, βg and total saponin contents (μg/g) in cracker samples as compared to the corresponding flour, where snowbird (SB), snowdrop (SD), fabelle (FB), and FB9-4. S: sprouted, US: unsprouted. The data represent the mean ± SD of two replicates and each analyzed twice by LC-MS (n=2x2=4). Values labeled with superscripts AB indicate the significant differences within flour and cracker of the same variety (row) with $p < 0.05$. Values labeled with superscripts ab indicate the significant differences within sprouted and unsprouted of the same variety (column) with $p < 0.05$.

Saponin type		Faba bean flour				Faba bean cracker			
		SB	SD	FB	FB9-4	SB	SD	FB	FB9-4
Bb	S	47.0 ± 0.6^{bA}	165.7 ± 0.7^{bA}	63.8 ± 0.9^{bA}	94.8 ± 1.1^{bA}	87.1 ± 0.6^{bB}	282.6 ± 0.3^{bB}	110.3 ± 2.1^{bB}	219.5 ± 0.5^{bB}
	US	34.8 ± 0.5^{aA}	68.0 ± 0.7^{aA}	6.9 ± 0.5^{aA}	61.9 ± 1.2^{aA}	59.9 ± 2.1^{aB}	156.9 ± 0.5^{aB}	74.5 ± 1.1^{aB}	136.5 ± 1.7^{aB}
βg	S	207.0 ± 0.7^{bB}	892.6 ± 1.6^{bB}	315.2 ± 1.9^{bB}	677.9 ± 2.3^{bB}	5.4 ± 0.1^{bA}	25.1 ± 1.1^{bA}	6.5 ± 0.3^{bA}	12.1 ± 1.1^{aA}
	US	175.6 ± 1.1^{aB}	320.5 ± 1.4^{aB}	22.81 ± 0.7^{aB}	235.9 ± 2.0^{aB}	7.5 ± 0.3^{aA}	8.74 ± 0.1^{aA}	9.2 ± 0.5^{aA}	13.9 ± 0.5^{aA}
Total	S	254.0 ± 1.2^{bB}	1058.3 ± 1.6^{bB}	379.0 ± 2.4^{bB}	772.7 ± 3.0^{bB}	92.5 ± 0.6^{bA}	307.7 ± 0.6^{bA}	116.8 ± 2.2^{bA}	231.5 ± 1.0^{bA}
	US	210.4 ± 1.5^{aB}	388.6 ± 1.9^{aB}	29.7 ± 1.1^{aB}	297.8 ± 2.3^{aB}	67.4 ± 2.3^{aA}	165.6 ± 0.6^{aA}	83.7 ± 0.9^{aA}	150.4 ± 1.3^{aA}
Bb/ βg	S	20:80	20:80	20:80	15:85	85:15	90:10	85:15	80:20
	US	20:80	20:80	30:70	25:75	90:10	80:20	90:10	90:10

These results reflect the likely conversion of some saponin βg into saponin Bb as a result of baking. Thus, in almost all cases whilst the total saponin levels decrease on baking, the levels of saponin Bb increase. Hassan et al. (2015) reported the conversion of DDMP saponin into saponin B during domestic cooking where the loss of soyasaponin βg during baking was due to the release of DDMP as maltol moiety when temperature exceeds 30°C. The degradation of DDMP saponin may be also related to the hydrolysis happened in a solvent with high dielectric constant like water since it was used to prepare the cracker-dough in our study. The ionized intermediate could be generated and further subjected to a molecular rearrangement with subsequent conversion of soyasaponin βg into soyasaponin Bb (Barakat et al., 2015). Baking at high temperature and longer time has also been shown to lead to a transformation of polar ginsenosides into less polar ginsenosides also known as ginseng saponins (Li et al., 2018). By using HPLC-MS analysis, 7 less polar ginsenosides that were absent in raw ginseng flower were newly generated upon baking at 150°C for 4 and 6 hours, as well as at 180° for 1 hour, then decreased with longer baking time. 13 polar ginsenosides were detected in raw ginseng flower and the total content of polar ginsenosides decreased significantly when baking at 180°C. Moreover, a dramatic decrease in the total ginsenosides content was observed in flours baked at 180°C, suggesting that ginsenosides were damaged by high temperature which appears to match our results, particularly with a significantly reduction in total saponin content as shown in Table 3.3.2. Dramatic reduction of the total saponin content caused by heat treatment was also observed in marama beans roasted at 150°C for 20 mins (Nyembwe et al., 2015). One possible explanation of observed reduction in total saponin content is that severe heat treatment can cause hydrolysis of the glycosidic bond between the aglycone and glycosidic resiude (X. Li et al., 2018; Nyembwe et al., 2015).

Sensory analysis of cracker and their corresponding sprouted and unsprouted faba bean flour (paste) was carried out at the Food Processing Development Centre (FPDC) (Leduc, AB, CA). Table 3.3.3 and 3.3.4 summarizes partial sensory data based on treatment type and faba bean varieties that obtained from FPDC and total saponin content. A general conclusion from that study was that crackers made from sprouted faba bean flour were more bitter than those made from the corresponding unsprouted flour, regardless of the faba bean variety. As shown in Table 3.3.3, a higher amount of total saponin was found in crackers made from sprouted faba bean flour compared to unsprouted flour. However, for paste made from sprouted or unsprouted faba bean flour, contradictory results were obtained. In this case although a higher saponin content was present in the sprouted faba bean flour, less bitterness was actually perceived by panellists in the paste ($p=0.041$, $n=9$). Based on our results (Table 3.3.4), Snowdrop showed higher total saponin and both individual saponin (soyasaponin Bb and βg) content comparing other 3 faba bean varieties. Those findings can explain the most bitterness flavor was perceived by panellists in the paste and cracker made from Snowdrop flour. Moreover, the least bitterness flavor was perceived in Fabelle paste which corresponding to the lowest content of saponin in that faba bean varieties. Nevertheless, contradictory results were obtained in crackers made from Fabelle and Snowbird, where a significantly more bitter taste was perceived in Snowbird cracker comparing to Fabelle cracker. However, lower saponin content was determined in Snowbird cracker. This might be explained by the fact that saponin is not the only contributor to bitterness in baked foods. Faba bean contains a high amount of protein and when baking at high temperature will result in the Maillard reaction between amino acids and reducing sugars that give the brown color and flavor of faba bean cracker. It is possible that various compounds produced by Maillard reaction such as melanoidins, and furans, as well as gallic acid, protocatechuic acid, and saponin acting together

synergistically contribute to the more bitterness in faba bean cracker comparing to their paste. Overall, sensory and instrumental findings suggest that there might be a positive relationship between saponin content and bitterness.

Table 3.3.3 Sensory attributes and total saponin content related to faba bean flour and cracker based on sprouting treatment (panelists = 9). Values labeled with superscripts indicate the significant differences with $p < 0.05$. Sensory value was based on 15 cm line scale, where 0 = weakest and 15 = strongest.

	Faba bean flour (paste)		Faba bean cracker	
	Sprouted	Unsprouted	Sprouted	Unsprouted
Bitter	2.8^{b}	3.0^{a}	4.1^{a}	3.6^{b}
Astringent	2.6	2.5	2.9	2.8
Total saponin content (μg/g)	616	232	187	117

Table 3.3.4 Sensory attributes and total saponin content related to faba bean flour and cracker based on faba bean varieties (panelists = 9). Values labeled with superscripts indicate the significant differences with $p < 0.05$. Sensory value was based on 15 cm line scale, where 0 = weakest and 15 = strongest.

	Faba bean flour (paste)				Faba bean cracker			
	FB9-4	Fabelle	Snowbird	Snowdrop	FB9-4	Fabelle	Snowbird	Snowdrop
Bitter	2.7^{b}	2.2^{c}	3.0^{b}	3.7^{a}	4.0^{b}	2.4^{c}	4.3^{ab}	4.7^{a}
Astringent	2.6	2.4	2.6	2.5	3.1^{a}	2.3^{b}	2.9^{a}	3.1^{a}
Total saponin content (μg/g)	298	30	210	389	150	84	67	166

3.4 Conclusion

Saponin contents are present in different levels in 4 faba bean varieties and in different parts of seed under the present study. Saponins occur at different concentrations in cotyledon and seed coats, where higher amount of saponins has been observed in cotyledon. Germination significantly affects the total saponin content of faba bean seeds, which increased up to 60 hrs of sprouting time (54 hrs in the case of FB9-4) but decreased over longer sprouting times. However, the total saponins content showed an increase after 72 hrs of germination comparing to raw seeds, expect

for a reduction of saponin content was observed in sprouted FB9-4. Drying for 60 hours greatly reduced soyasaponin Bb content but did not greatly change the soyasaponin βg content in faba beans. Baking has a major reducing effect on the saponin content and the saponin composition as well. A conversion of DDMP-conjugated saponin (βg) to non- DDMP-conjugated saponin (Bb) was observed upon baking. The significant reduction of total saponin content induced by baking observed in the present study suggests that baked faba bean with lower saponin content can be used as an ingredient for manufacturing faba-bean based products. In addition, the sensory data obtained for both sprouted and unsprouted faba bean paste and cracker is consistent with the saponin content and the bitterness taste. The results obtained in this study provide valuable information on saponin composition and content in raw and processed faba bean flour as well as flour-based food therefore increasing faba bean utilization in foods.

3.5 Reference

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4 Chapter 4 The Effect of Pressure-cooking on Saponin Profile in Primary Pulses

4.1 Introduction

Pulses have been recommended as staple foods to provide the basic protein and carbohydrate requirements for human on a daily basis (Margier et al., 2018). Pulses contain complementary amino acid to those in cereals, which fulfill and complete the protein requirements for vegetarians. Moreover, the high nutritional value of pulses has been reported since they not only contain high amount of proteins and carbohydrates, but are also rich in dietary fibers, vitamins, minerals, and bioactive compounds. There is an increasing global interest in developing and producing plant-based healthy food products with a high protein content (Sánchez-Chino et al., 2015). However, some bioactive compounds including lectins, phytates, proteases, α-amylase, tannins, and saponins naturally exist in pulses have anti-nutritional properties (Margier et al., 2018). Saponin is known as a major anti-nutritional factor in pulses since it interferes with the absorption of dietary lipids, cholesterol, bile acids, protein and mineral such as iron and zinc by binding to the cells of small intestine (Tian et al., 2018). The biological roles of saponin in animal and human largely depends on their structure (aglycone structure, side chain composition and length, the position of attachment of sugar moieties on the aglycone). Triterpenoid saponins interfere the absorption of vitamins A and E in chicken while steroid saponins have no effect on vitamin absorption (Das et al., 2012). Dietary saponin also have negative impact on ruminant when using pulses as animal feed. Negative effects include depress animal growth, feed consumption and egg production in poultry, and those effects are caused by the astringent and bitter taste of saponin (Das et al., 2012; Gemede & Ratta, 2014). In pulses, saponins can have deleterious effects when present in a large amount. However, they have also been shown to exert beneficial effects including reducing blood

glucose levels, regulating insulin responses to foods contain high level of sugar, and exhibiting antitumor and antioxidant activities at low concentration (Gemede & Ratta, 2014).

Pulses are processed in a variety of forms for human consumption. Thermal treatments such as cooking and canning can lead to loss of nutrients and phytochemical compounds, however, it can also inactivate some heat-labile anti-nutritional factors such as phytates, tannins and saponins (Margier et al., 2018). The preparation for some common domestic pulses includes soaking the seeds in plain water or mineral salt solution overnight and cooking, pressure cooking, sprouting and cooking of the sprouted seeds. Domestic processing and cooking was shown to reduce on average around 49% of phytic acid, 30% of the saponin content, and 13% of trypsin inhibitor activity in four varieties of Moth bean (*Vigna aconitifolia* Jacq.) compared to raw seeds, which is one of the most widely grown and consumed pulse in India (Khokhar & Chauhan, 1986). Soaking for 12 hrs and pressure cooking for 15 min reduced 7.7% and 13.5% of saponin content in four varieties of rice bean (*Vigna umbellata*), respectively (Bajaj, 2014).

Most of the research conducted earlier aim to characterize the change of the overall nutrient value including proteins, fat, fibers, minerals and vitamins and antinutritional compounds such as trypsin inhibitory activity, phytic acid, polyphenols and tannins in pulses caused by domestic processing and cooking (Bajaj, 2014; Khokhar & Chauhan, 1986; Ramakrishna, Jhansi Rani, & Ramakrishna Rao, 2008). However, the complete saponin profile is only known for a limited range of pulses such as soybean (Berhow et al., 2006), black bean (Lee et al., 1999), and adzuki bean (Liu et al., 2017). The change of saponin composition and content induced by cooking is not widely known. Therefore, the present study aims to evaluate the effect of cooking on the saponin content and saponin profile on 8 selected pulses, all of which are either grown or purchased in Western Canada. This study uses a simple sample preparation and high-performance liquid chromatography coupled

with mass spectrometry (HPLC-MS) method described earlier in this thesis in Chapter 2 for saponin analysis.

4.2 Materials and methods

4.2.1 Pulses and chemicals

Seeds of yellow pea (*Pisum sativum* L., cvs. CDC Amarillo and AAC Lacombe), green pea (*Pisum sativum* L., cvs. Cooper), and faba bean (*Vicia faba L. var. minor*., cvs. Snowbird, FB9-4, CDC Snowdrop and Fabelle) were obtained from the Food Processing Development Centre, Alberta Agriculture and Forestry, Alberta, Canada, and the seeds were grown in Southern Alberta from 2018 crop year. Seeds of pinto bean (*Phaseolus vulgaris* L.), black bean (*Phaseolus vulgaris*), kidney bean (*Phaseolus vulgaris*), chickpea (*Cicer arietinum* L.), green and red lentil (*Lens culinaris* L.) were purchased from local markets (Safeway and Save-On-Foods), Edmonton, Alberta, Canada. HPLC grade acetonitrile (ACN) (>99.9%), methanol (MeOH) (>99.8%), and water (Optima® LC/MS) were purchased from Fisher Scientific International Inc. Soyasaponin Bb standard and ginsenoside Rb1 were purchased from AdooQ BioScience (>98%, Irvine, Canada).

4.2.2 Raw bean flour and cooked bean flour preparation

The seeds of each cultivar were subjected to two different preparation procedures. The raw seeds were grounded into fine flour by using Micro-Mill (Science ware, Bel-Art Products, USA) at speed of 10,000 RPM for 60 s. A water-cooled system was applied to protect the mill from overheating. The cooking procedure was modified and done according to the reference guide from Instant Pot® and Cavalcante and others (2017). Seeds were cooked without soaking in a beans to water ratio of 1:5 (*w/v*) in an electric pressure cooker (Instant Pot® Duo Mini 7-in-1 Multi-Use, 3-qt, IP-

DUOMINI, CA) under normal pressure (7.2-10.2 psi, 700W) at 115-118 °C for 13 minutes. Then the cooked beans were separated from water by using a sieve, and excess water on the surface of the beans was dried by paper towel. The cooked beans were ground into slurry by using Micro-Mill at speed of 10,000 RPM for 60 s. Then the slurry was freeze-dried under vacuum (Labconco, USA) for 24 hours. All ground flour was stored in tightly sealed polyethylene bags and stored at freezer (-4°C) until further analysis.

4.2.3 Standard solution preparation

Soyasaponin Bb and ginsenoside Rb1 were used as reference standard and internal standard (IS), respectively, for quantification. Soyasaponin Bb and ginsenoside Rb1 standards (5 mg each), were weighed accurately on an analytical balance (Sartorius AG, Germany), transferred into a 5mL volumetric flask, and made up with MeOH (1mg/mL stock solution). The stock solution was mixed thoroughly using a vortex-mixer (Fisher Scientific, CA), stored in a firmly closed 2 mL HPLC glass bottle and kept at 4°C in a refrigerator until analysis. The latter stock solution was used to prepare diluted standard solutions of 0.2, 0.4, 1, 2, 4, 5 μg/mL to create the calibration curve. The IS concentration was fixed at 0.5 μg/mL (spiking level) for all measurements.

4.2.4 Saponin extraction

The extraction procedure was performed based on the results obtained from optimization study (Chapter 2). Finely ground bean flour (0.2 mg) was accurately weighed on an analytical balance (Sartorius AG, Germany) and transferred into a 7 mL glass vial. 0.5 mL of internal standard (2 μg/mL), and 1.5 mL 100% methanol was added into the glass vial, and the mixture was extracted at room temperature for 5 minutes with high-speed handheld disperser Polytron ((PT 1300 D,

Kinematica AG, Switzerland) at a speed of 7000 rpm. The mixture was centrifuged (Damon/IEC Division HN-SII, UK) for 5 min at a speed of 9000 rpm and the saponin extracts was transferred and stored in a 2 mL glass vial. Aliquot of 100 μL saponin extract was diluted with another 100 μL methanol and was mixed thoroughly using a vortex-mixer (Fisher Scientific, CA). The mixture of total 200 μL was subjected to the chromatographic analysis.

4.2.5 Saponin analysis

The analysis was performed using a chromatographic system of Agilent HPLC (1200 series, Agilent Technologies, USA) attached to a linear ion trap quadrupole MS (3200 QTrap, Applied Biosystems, CA). The data was collected and processed using Analyst 1.4.2. software. Separation of saponin was done on an Ascentis Express C18 column (15 cm x 2.1 mm, 2.7μm). The flow rate was set at 0.2 mL/min. The mobile phase consists of MS grade water (A), and acetonitrile/ methanol (3:1, v/v) (B). The solvent gradient program was performed as follow: 0-0.1 min, 85% A; 0.1-10 min, 10% A; 10-14 min, 10% A; 14-14.1 min, 10% A; 14.1-16 min, 0% A; 16-16.1 min, 85% A; 16.1-25 min, 85% A.

Individual saponins from the methanol extracts was identified according to their relative retention time and fragment ion, as compared to standards and literature. Full scan mode, selected ion monitoring (SIM) and multiple reaction monitoring (MRM) scan modes were used for the identification and quantification of saponin. LC/MS was first applied in full scan mode over the range of *m/z* 500-1500 in order to detect all potential saponin types present in pulse extracts. All *m/z* value $[M-H]^-$ in negative electrospray mode was extracted for each saponin type (group A, B, E and DDMP-conjugated, Table 1.5. Chapter 1) to exam the presence. If the *m/z* value $[M-H]^-$ molecular ion match with saponin types reported from literature (Table 1.2.1 Chapter 1), then that

specific *m/z* value was selected and subjected to MRM to detect its product ion for identification. Quantification of each saponin was achieved by measuring the ratio of the analyte peak area and internal standard.

The calibration curve for soyasaponin Bb was established based on five points (0.2, 0.4, 1, 2, 4, 5 μg/mL). The linear regression equation was y = ax + b, where y is the ratio of analyte's peak area to the IS peak area, and x is the ratio of analyte concentration to the IS concentration. Linearity was examined based on regression equations and correlation coefficients. Additionally, three standard solutions (0.2, 2, 5 μg/mL) were prepared for quality control. Each analysis follows the sequence of blank (methanol), five calibration standards, quality control standard solutions (QCS) and pulse samples.

4.2.6 Statistical analysis

Each pulse sample was extracted in duplicate, and each extract was further analyzed by LC/MS in duplicate. The resulting values (n=4) were expressed as mean ± standard deviations (mean ± SD).

4.3 Results and discussion

4.3.1 Identification and quantification of saponins from pulse samples

For the identification of saponins in each pulse sample, the crude extract was analyzed by mass spectrometry in flow injection analysis (FIA) mode using quadrupole and ion trop analyzer (QTrap) with an ESI source in negative mode. Table 4.3.1 shows the identification of the molecular ions $[M-H]^-$ and optimum ionization conditions for each compound. Further identification of the most abundant product ion and the selection of optimum collision energies was performed in the product ion scan mode (MS/MS) for each analyte. The identification of soyasaponin Bb and βg was

mentioned in Chapter 3. Soyasaponin Ba and αg result in molecular ions as $[M-H]^-$ at *m/z* 957.5 and 1083.5, respectively. The MS/MS spectra of soyasaponin Ba shows fragment ions at *m/z* 939.5, 795.5, 633.4, 615.4 and 457.4, which correspond to $[M-H_2O-H]^-$, $[M-glc-H]^-$, $[M-glc-gal-H]^-$, $[M-glc-gal-H]^-$, $[M-glc-gal-H_2O-H]^-$, and $[SB-H]^-$, respectively, where SB= soyasapogenol B, M= monoisotopic mass, glc= glucose, gal= galactose (Fig. 4.3.1). The MS/MS spectrum of Soyasaponin αg molecular ions (*m/z* 1083.5) contains fragment ions at *m/z* 921.5, 795.4, 565.4, and 403.4. These correspond to $[M-glc]^-$, $[M-glc-gal]^-$, $[M-glc-gal-UA-H_2O-H]^-$ and $[SB-3H_2O-H]^-$, respectively, where SB= soyasapogenol B, M= monoisotopic mass, glc= glucose, gal= galactose, UA= uronic acid (Fig. 4.3.2). With respect to the HPLC separation (Fig. 4.3.3.), efficient separation of ginsenoside Rb1 (11.15 min), soyasaponin Ba (11.76 min), soyasaponin Bb (11.83 min), soyasaponin αg (12.85 min), and soyasaponin βg (13.23 min) from kidney bean were achieved within a 20 min run time and 5 min for cleaning the column. The identity for each soyasaponin was confirmed by comparing the precursor ion and MS/MS fragment ions, as well as the order of elution for each analyte with literature (Berhow et al., 2006). The elution order based on retention time of soyasaponins extracted from soybean was found out to be as follow: Ba < Bb < Bc < Be < αg < βg < βa < γg (Berhow et al., 2006; Krishnamurthy et al., 2014).

Table 4.3.1 The fragment ions for soyasaponin Bb, Ba, αg and βg, and their associated Qtrap settings

Analyte	Parent ion (Molecular	Product ion (*m/z*)	Declustering potential (DP) (V)	Collision energy (CE) (V)	Entrance potential (EP) (V)	Collision entrance potential	Collison exit potential	Dwell (ms)

	ion [M-H])$^-$ (*m/z*)					(CEP) (V)	(CXP) (V)	
Soyasaponin Bb	941.5	615.4, 457.4	-120	-36	-6	-127	-6	80
Soyasaponin Ba	957.5	939.5, 795.5, 633.4, 615.4, 457.4	-120	-40	-10	-120	-5	80
Soyasaponin βg	1067.5	741.4, 583.5	-120	-60	-10	-120	-5	80
Soyasaponin αg	1083.5	921.5, 759.4, 565.4, 403.4	-120	-60	-10	-120	-5	80
Ginsenoside Rb1	1107.2	440.4	-85	-35	-4	-52	-9	80

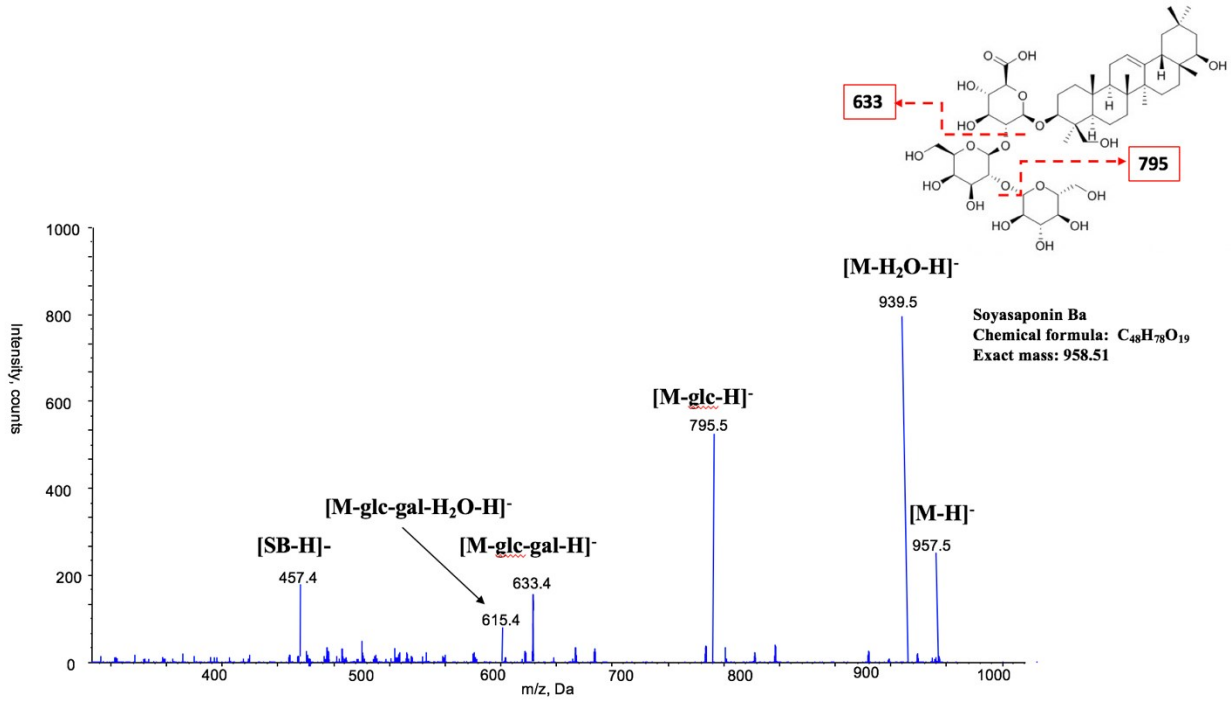

Figure 4.3.1 Full scan product ion spectra of [M-H]- for soyasaponin Ba extracted from kidney bean in ESI (-) mode by direct injection at a flow rate of 0.2 at a concentration of 0.2 mg/mL. SB= soyasapogenol B, M= monoisotopic mass, glc=glucose, gal=galactose

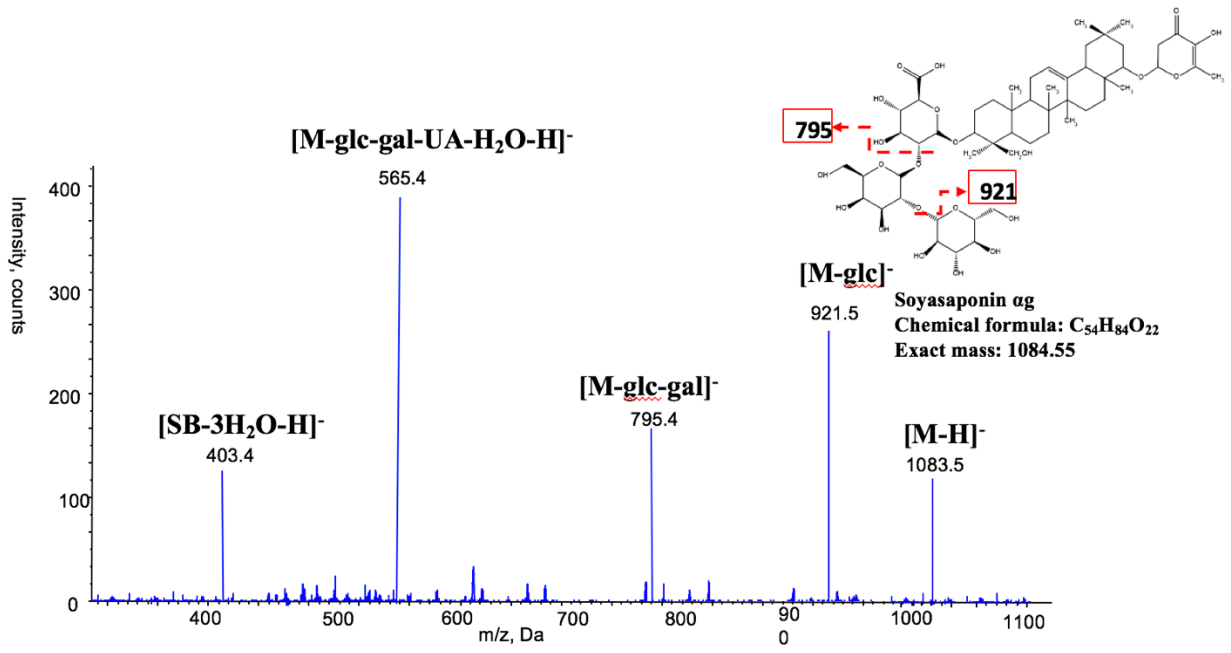

Figure 4.3.2 Full scan product ion spectra of [M-H]- for soyasaponin Ba extracted from kidney bean in ESI (-) mode by direct injection at a flow rate of 0.2 at a concentration of 0.2 mg/mL. SB= soyasapogenol B, M= monoisotopic mass, glc= glucose, gal= galactose, UA= uronic acid

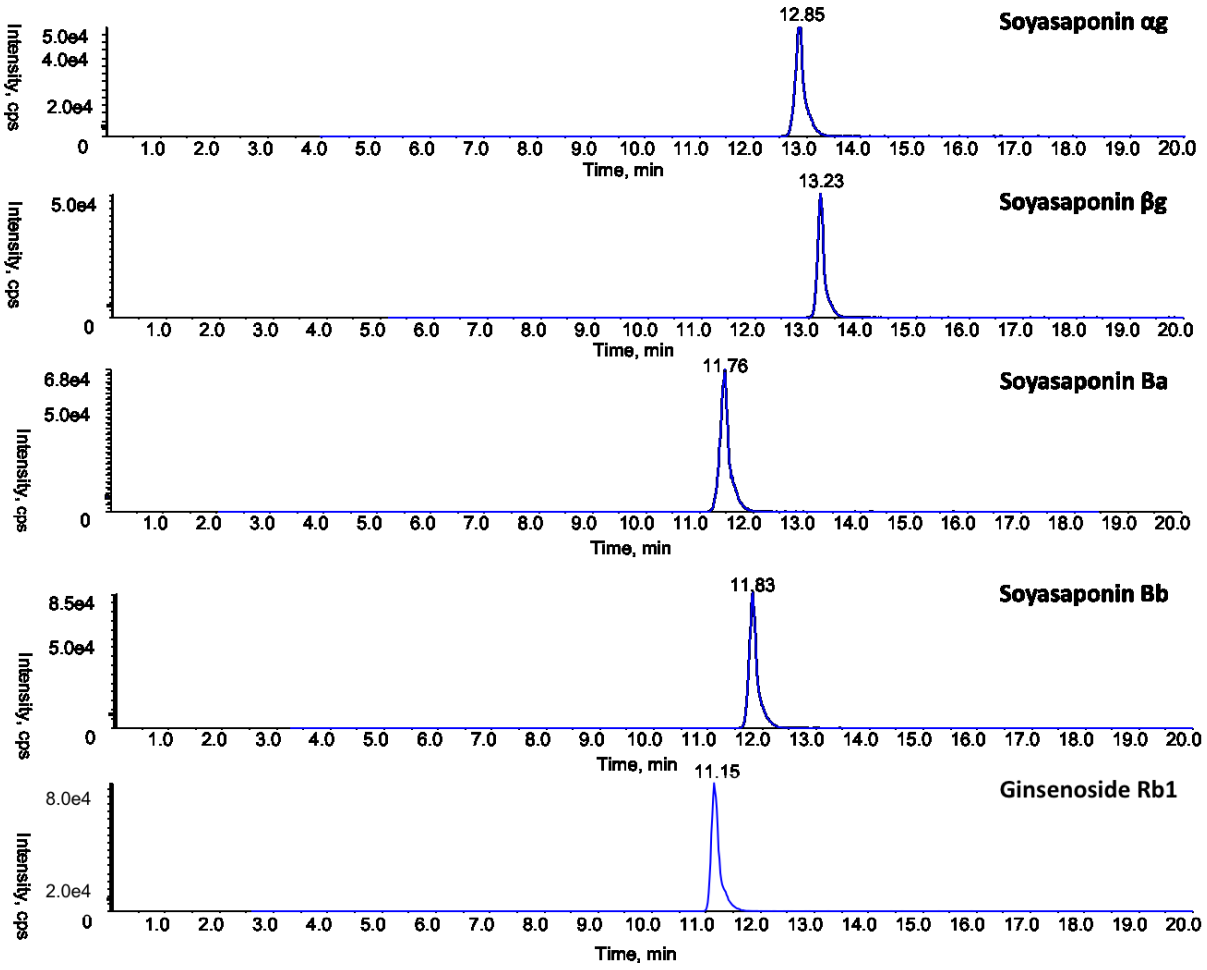

Figure 4.3.3 The extracted ion chromatogram XIC of saponin extract from kidney bean at a spiking level of IS of 1 μg/mL, where a) soyasaponin αg, b) soyasaponin βg, c) soyasaponin Ba, d) soyasaponin Bb, e) ginsenoside Rb1.

The calibration curve of soyasaponin Bb was established by duplicate injections of standards at 0.2, 0.4, 1, 2, 4, and 5 μg/mL using the same LC/MS method described in Section 2.5. The internal standard was present at 1μg/mL in all standard mixtures. The linear regression equation was Y= 1.29x + 0.0281 with correlation coefficient R^2= 0.9991. The recoveries were obtained by spiking soyasaponin Bb standard at 1ppm concentration to kidney bean sample, which were in the range of 95-99%.

4.3.2 Saponin content in different variety of pulses

The analytical data obtained from HPLC-MS for all pulses are reported in Table 4.4.1, where the content of soyasaponin Bb, βg, Ba, and αg and total saponin content expressed in micrograms per grams (μg/g) are shown. All of the pulses tested contained group B saponins (soyasaponin Bb and βg) despite the differences in the amounts and percentile distribution present.

4.3.2.1 Peas

It was reported previously that Soyasaponin Bb and βg were the only two saponins types detected in 16 pea varieties (*Pisum sativum*, *P. elatius*, and *P. arvense*) from The Netherlands (Heng et al., 2006). The average total saponin content in 16 different pea varieties is 1900 μg/g (dry matter). Soyasaponin βg was the predominant saponin in all 16 pea varieties, the Soyasaponin βg content of which varied from 700 to 1500 μg/g dry matter. In the same study , the soyasaponin Bb content varied from 0 to 400 μg/g. They also observed that yellow pea varieties had a lower saponin content compared to green and brown varieties.

In the present study, soyasaponin Bb was the predominating saponin followed by soyasaponin βg, with the content ranged from 359 to 1257 μg/g and 187 to 878 μg/g, respectively. Moreover, soyasaponin αg (3 to 11 μg/g) was found in all three pea varieties and soyasaponin Ba (1 μg/g) was present in Lacombe variety. The total saponin content of the 3 varieties tested varied considerably, from 550 to 2143 μg/g on a dry basis (Table 4.4.1).

Saponin content vary among different pea varieties and the value reported in the literature shows great difference depending on the extraction and analytical methods (Daveby, Åman, Betz, Musser, & Obermeyer, 1997; Lynn Heng et al., 2006; Reim & Rohn, 2015). In Heng et al.'s (2006) study, pea flour was defatted by hexane under refluxing for 6 hr then extracted with 70% (v/v) ethanol

for 1 hr at 25°C. The total content of saponin in 16 pea varieties determined by HPLC-ELSD ranged from 700 to 1900 μg/g. In another study, saponin was extracted from 6 pea varieties harvested in Germany in methanol for 4 hr at 50°C and characterized by high-performance thin-layer chromatography (HPTLC)-ESI-MS (Reim & Rohn, 2015). The total saponin content (Bb and βg) determined in pea ranged from 600 to 2300 μg/g (dry matter) with soyasaponin Bb being the major compound. Saponin from 40 Swedish pea varieties was extracted in 80% ethanol (ethanol/water, v/v) under refluxing for 2 hr and quantified by LC-MS (Daveby et al., 1997). The soyasaponin Bb content in the range from 820 to 2500 μg/g with an average content of 1500 μg/g have been found in 40 Swedish pea varieties. These results are in good agreement with the results of this study both in magnitude and range. Conflicting results obtained by different studies may be due the fact that the DDMP conjugated saponin (βg) is not stable under analytical and food processing conditions, and the relevant facts including temperature, extraction solvent and pH value. The degradation of soyasaponin βg results in a proportional increase in soyasaponin Bb. The mechanism of DDMP conjugated saponin decomposition was proposed, based on an acid/base-catalyzed, pseudo first order reaction with a relatively low activation energy and moderate temperature dependency (L. Heng et al., 2006). This reaction is thought to consist of a fast protonation or deprotonation reaction, followed by a rate-determining step that DDMP conjugate group is released as maltol (L. Heng et al., 2006).

4.3.2.2 Black beans

In the present study, soyasaponins Bb, βg, Ba, and αg were detected in black beans at levels of 1370, 3865, 187, and 3145 μg/g (dry matter), respectively. Thus, DDMP conjugated saponins are the major saponin type in black bean.

In other work, Soyasaponin Bb, βg, and Ba were identified in the cotyledon of black bean (*Vigna mungo* L. *Hepper*) by using liquid chromatography coupled with positive and negative ion fast atom bombardment mass spectrometry (LC-FAB-MS) (Lee et al., 1999). In addition, soyasaponin Bc, acetylsoyasaponin A_4 and two unique saponins named as saponin A (3-*O*-[α-L-rhamnopyranosyl-(1→2)-*β*-D-galactopyranosyl-(1→2)-*β*-D-glucuronopyranosyl) and saponin B (3-*O*-[α-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranosyl-(1→2)-*β*-D-glucopyranosyl) were determined by MS/MS analysis. In another study, saponins were extracted by 80% ethanol (ethanol/water, v/v) and quantified in sprouts, cotyledons and seed coats of black bean variety Sab Luis harvested in Mexico using an HPLC-TOF-MS technique (D. Guajardo-Flores et al., 2012). This identified in black beans group A saponins, including soyasaponin Af, A2, A3, A5, group B saponins including soyasaponin Ba, Bb, Bb', and DDMP conjugated saponins including soyasaponin αg, βg and γg . In that study, the total saponin content, group A and group B determined in black bean is 5260 μg/g, 2999 μg/g, and 2260 μg/g, respectively. Soyasaponin Af, αg, and Ba were the most abundant saponins in black bean. They found out that group B saponins were lower in amount than group A saponins, with DDMP conjugated saponin to be the predominate group B saponin type. Soyasaponin Ba, αg, βg, Af, and A2 were found in black bean seed coats, with soyasaponin Af, αg and βg being the major compounds (Chávez-Santoscoy et al., 2016). Saponin from black bean variety Sab Luis grown in Mexico was extracted in 80% ethanol (ethanol/water, v/v) and the presence of soyasaponin Af, Ba, Bb, αg, βg and γg was detected by HPLC-DAD-ELSD (Díaz-Sánchez, Guajardo-Flores, Serna Guerrero, Gutierrez-Uribe, & Jacobo-Velázquez, 2018).

The total saponin found out in the present study was 8566 μg/g, which is lower than the value (47640 μg/g) reported by Díaz-Sánchez et al. (2018), but higher than the content (5258 μg/g)

determined by Guajardo-Flores et al. (2012). Both studies agree with our foundings where DDMP conjugated saponin (αg and βg) are the predominate saponin type in black bean. Soyasaponin αg and βg represented 38% and 14% (Díaz-Sánchez et al., 2018), 30% and 3.5%t (D. Guajardo-Flores et al., 2012) of total saponin content in black bean, respectively. It seems like the saponin composition and content are dependent on analytical method and cultivation year since two studies used the same black bean variety Sab Luis from Mexico and same saponin extraction method. Moreover, we analyzed saponin composition in black bean as whole seeds, whereas Guajardo-Flores et al. (2012) measured the saponin profile in specific parts (sprouts, cotyledons and seed coats) of black bean. These could be some possible explanations for the different saponin composition and total content we observed in present study compared to their reports (Díaz-Sánchez et al., 2018; D. Guajardo-Flores et al., 2012).

4.3.2.3 Chickpeas

Soyasaponin Bb (718 μg/g) and βg (366 μg/g) is the main saponin found in chickpea, along with small amount of Ba (0.8 μg/g), and αg (11 μg/g). In the present study, a total saponin content of 1095 μg/g of was observed.

Chickpea varieties differ in saponin content and reported data greatly depend on genotype, seeding, harvesting and storage conditions. Moreover, saponin profile changes depend on extraction and analytical methods. Previous work reported that a 750 μg/g of soyasaponin βg content was detected in two cultivars of chickpeas (Fardon and Blanco Lechoso), and it was the only saponin detected in raw chickpea seeds grown in Spain (Ruiz, Price, Fenwick, & Rhodes, 1996b). However, they observed the presence of both soyasaponin Bb and βg in cooked chickpea seeds. The saponin content of chickpeas grown in Egypt was reported to be 910 μg/g (El-Adawy, 2002). In a more

recent study, saponins in chickpea protein isolate was extracted by 70% ethanol (ethanol/water, v/v) and analyzed by HPLC-MS (Serventi et al., 2013). The presence of soyasaponin Bb, Bb', Bc, Be, βg, βa and γa were reported, with a total saponin content of 529 μg/g. The major saponin type in chickpea protein isolate was soyasaponin βg (343 μg/g) followed by soyasaponin Bb (155 μg/g). However, saponin from chickpea obtained from Germany was extracted in methanol in water bath for 4 hr at 50°C and quantified by HPTLC-MS (Barakat et al., 2015a). Only soyasaponin Bb and βg was found out in chickpea, with the content of 171 μg/g and 126 μg/g, respectively. Saponin content determined in current study are comparable with previous studies (El-Adawy, 2002; Ruiz et al., 1996b), but higher than the results reported by the others (Barakat et al., 2015a; Serventi et al., 2013). Difference observed in saponin composition and content might be explained by the selection of various chickpea varieties and parts (whole seeds or protein isolates), as well as the different saponin extraction and analytical methods used in each study.

4.3.2.4 Kidney Beans and Pinto Beans

Four saponins include soyasaponin Bb, βg, Ba, and αg has been identified in kidney bean and pinto bean. The total content of the saponins was 4415 μg/g and 4340 μg/g for kidney bean and pinto bean, respectively. These results are consistent with earlier reports, in which a total of 4060 μg/g and 4140 μg/g (dry weight basis) saponin were found in kidney bean (Montcalm) and pinto bean (Gala) from USA (Drumm, Gray, & Hosfield, 1990). The saponin contents reported by Drumm et al. (1990) , determined using HPLC-MS techniques, appear to be higher in content in comparison with using TLC-colorimetry based on the measurement of colour reaction of saponins with vanillin and sulfuric acid (Shimelis & Rakshit, 2007). Three varieties of kidney beans (Roba, Awash and Beshbesh) grown in Africa were found to have total saponin content ranged from 940

to 1320 μg/g on a dry weight basis. In kidney bean, the major saponin is soyasaponin Bb, followed by βg and αg, whereas in pinto bean, soyasaponin βg is the predominating saponin followed by αg and Bb. Soyasaponin Bb', Bc and Bc' were found in both kidney bean and pinto bean using TLC-densitometry, while those three saponins were not found in the present study (Drumm et al., 1990). The difference in saponin composition and content observed among studies might due to the use of different bean varieties and analytical methods.

4.3.2.5 Green and Red Lentils

A total of 4187 and 1734 μg/g of saponin content were detected in green and red lentils. The predominating saponin in both green and red lentils is soyasaponin βg with a content of 3100 and 934 μg/g, respectively, followed by soyasaponin Bb (1030 and 792 μg/g). Relatively low amounts of soyasaponin αg were found in both green and red lentils (52 and 8 μg/g), and soyasaponin Ba was only found in green lentils (3.7 μg/g).

Previously, the total saponin content were found to be 1063 μg/g in peeled red lentils from Spain (Navarro del Hierro et al., 2018), 1140 μg/g in lentil (Lyda) from Spain (Ruiz et al., 1996b). A total of 32 varieties of lentils with different seed color (red, black, brown, and green) and size (giant and mignon) from Italy showed a wide distribution of total saponin content range from 180 to 1595 μg/g (Sagratini et al., 2009). Another similar study examined 30 samples of lentils from central Italy and the total amount of saponin was in the range of 541 to 1457 μg/g (Pilar et al., 2014). In both studies, only soyasaponin Bb and βg was quantified and added up to the total saponin amount, and in all cases, the content of soyasaponin βg was higher to that of soyasaponin Bb. The results obtained from those studies signified the parameters that may influence the saponin

content in lentils not only includes genotype but also related to climatic conditions and geographical tillage. For both green and red lentils selected in current study were bought in local supermarket, area of cultivation and origin were not known, only packing location (Canada) were available. In addition, red lentils were split and dehulled while green lentils were only dehulled when purchased as package from local supermarket. This might explain the higher total saponin content was observed in green lentils comparing to red lentils in the present study.

4.3.2.6 Faba Beans

Only soysaponin Bb and βg was detected in four cultivars of faba bean. The presence of both saponin in faba bean were also confirmed in the earlier studies (Amarowicz et al., 1997; Barakat et al., 2015a; Kinjo et al., 1998). The total amount of saponin present in different faba bean varieties ranged from 30 to 388 μg/g. Snowdrop displayed the highest content of total saponins (388 μg/g) and of soyasaponin Bb (68 μg/g) and βg (321 μg/g). Fabelle has the lowest content of total saponins (30 μg/g) and of soyasaponin Bb (6.9 μg/g) and βg (22.8 μg/g). In all four varieties, the content of soyasaponin βg was higher than that of soyasaponin Bb. Previous data reported 113 μg/g (dry matter) of total saponin, 39 μg/g of soyasaponin Bb, and 74 μg/g of soyasaponin βg in faba bean from Germany (Barakat et al., 2015a).

4.3.2.7 Summary of the survey of the saponin content of pulses

A summary of total saponin content found in the present study and reported from literature is shown in Fig. 4.3.4. The total saponin content in 8 different pulse samples vary from 210 to 8566 μg/g. In 3 cultivars of pea, chickpea, and kidney bean, the content of soyasaponin Bb was higher than that of soyasaponin βg, while in 4 cultivars of faba bean, black bean, green and red lentil, and

pinto bean, soyasaponin βg was the predominating saponin. Overall, saponin content and profile are largely dependent on the pulse type, variety, origin, environmental factors include growing and climatic conditions, as well as extraction and analytical methods.

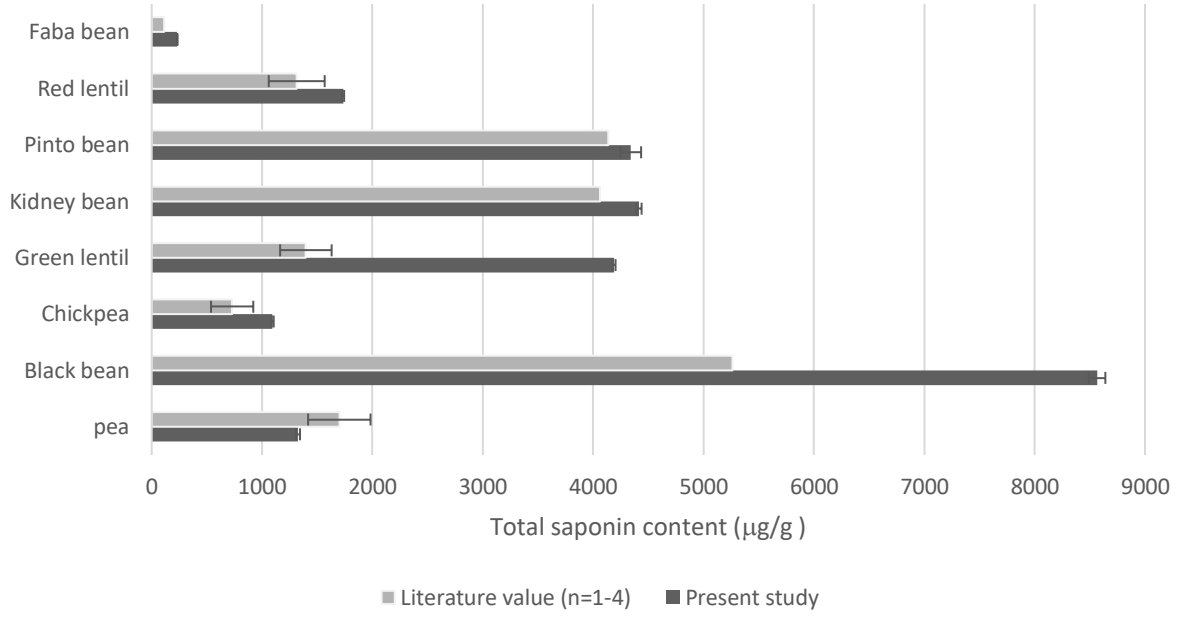

Figure 4.3.4 An average of total saponin content in different pulses determined by the present study and values reported in the literature.

4.3.3 Effect of pressure cooking on the saponin contents of pulses

Saponin contents in all pulse samples were reduced by pressure cooking (7.2-10.2 psi, 700W) at 115-118 °C for 13 minutes. The loss during cooking indicate the heat-labile nature of saponin. Table 4.4.1 shows the content of soyasaponin Bb, βg, Ba, and αg and total saponin content in pulses after pressure cooking. Figure 4.3.5 shows the average percentage reduction of total saponin content n pulses after pressure cooking. The reduction of total amount of saponin was observed to vary from 31-81% after pressure cooking. The maximum reduction effect on total saponin content was observed in green lentils (81% of loss), pea Amarillo variety (80% of loss) and kidney bean

(79% of loss). Less reduction effects were found out in the study done by Kataria, Chauhan, & Punia (1989), where 12-18% of total amount of saponin reduction was observed in four cultivars of mung bean under pressure cooking at 1.05 kg/cm^2 pressure for 15 mins. Presoaked four cultivars of moth bean seeds cooked for 20 mins at 1.05 kg/cm^2 pressure showed a 35-44% reduction of total saponin content (Khokhar & Chauhan, 1986). Sprouting at 25°C for 60hr followed by drying in hot air oven at 55°C reduced 56-66% of saponin content in moth bean and cooking of sprouted seeds reduced 61-69% of saponin content. In the present study, four cultivars of faba bean was sprouted at 25°C and 80% relative humidity for 48hr, and both soyasaponin Bb and βg content increased in the sprouted seeds. As discussed in chapter 2, we observed an increase in saponin content during the first 54 hours of germination and then a continuous reduction after 54 hours. The observed increase of saponin content may due to the fact that different enzymes are activated and synthesized during 54 hr sprouting thus enhance the production of secondary metabolites such as saponin (Ayet et al., 1997). In the present study, pressure cooking of sprouted faba bean seeds reduced total saponin level more effectively than unsprouted seeds. A complete elimination of saponin content was observed in autoclaved sprouted kidney bean (Shimelis & Rakshit, 2007). As indicated in Table 3.2, the least reduction of total saponin content by pressure-cooking was observed in red lentils (31% of reduction). As mentioned in chapter 1, literature reports indicate that red lentils contain 26% protein and 60% carbohydrate, while these values are 23% and 50% for chickpeas; 22% and 49% for kidney beans; and 22% and 52% for black beans, respectively (Bressani, Elias, & Navarrete, 1961; Kan et al., 2017; Rachwa-Rosiak, Nebesny, & Budryn, 2015; Gharibzahedi, Mousavi & Jafari, 2012). One possible explanation is that the formation of complex network among saponins, amino acids and sugars upon cooking cause saponin become poorly extractable by water (Khokhar & Chauhan, 1986). Since red lentils might contain higher amount

of free amino acids and sugars to interact with saponin, the formation of such complexes protected saponin from heat and leaching from the matrix to water during pressure cooking. This could result in a lower reduction in total saponin content in cooked red lentils compared to the other beans.

In summary, pressure cooking was effective in lowering the saponin contents to varying extents in all 8 pulse samples. Therefore, pressure cooking is an easy food processing method to improve the palatability of pulses by reducing saponin content, which is responsible for the bitterness taste in raw seeds.

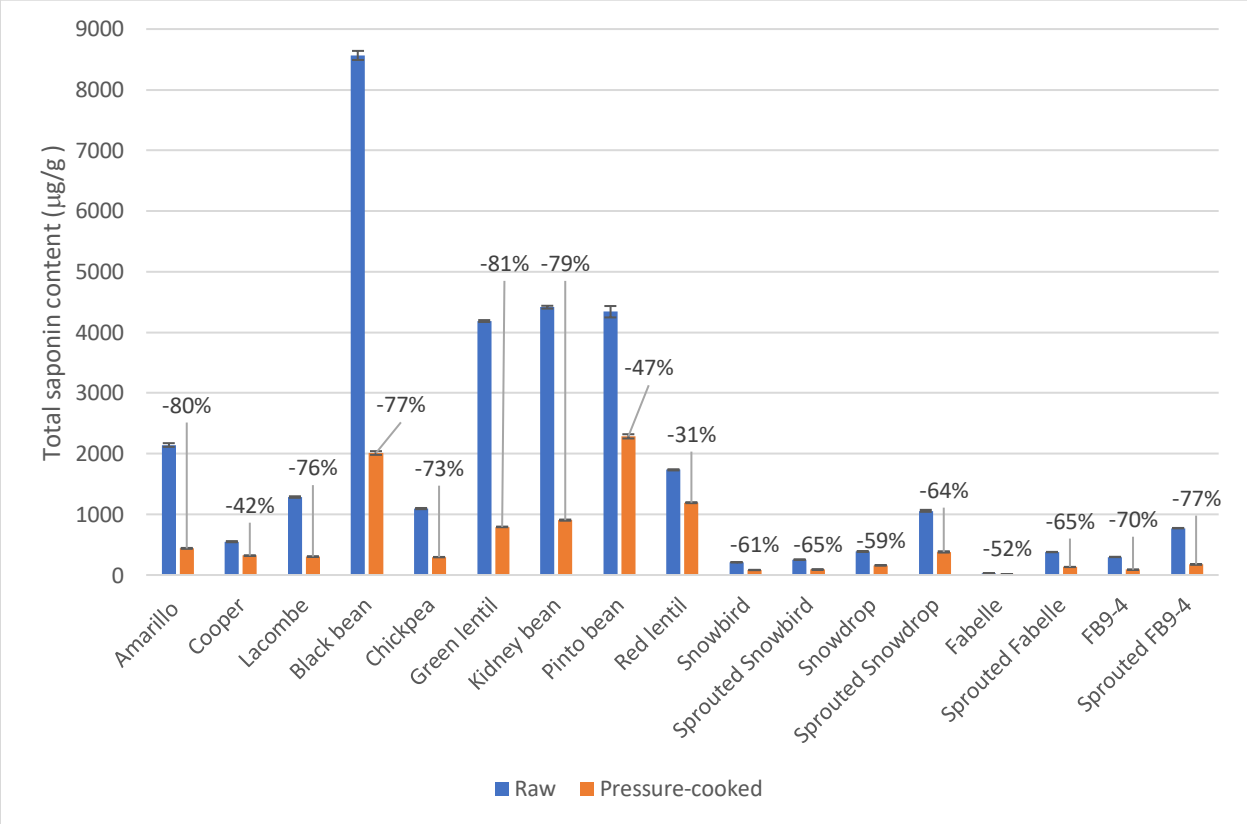

Figure 4.3.5 Loss of total saponin content in different pulses by pressure cooking.

4.4 Conclusion

The HPLC-ESI-MS/MS method was used for the simultaneous quantification of four saponins in 8 different pulses including pea, faba bean, kidney bean, pinto bean, chickpea, black bean, green and red lentils. In the present study, pressure cooking - a common processing method for pulses – was shown to be an effective way to reduce 31-81% of the total saponin content in pulses which make them more palatable for human consumption. Additionally, combination of germination and pressure cooking is more effective in reducing saponin content compare to pressure cooking alone.

Table 4.4.1 Effect of pressure cooking on the saponin content of different pulses (μg/g).

	Cultivars		Bb		βg		Ba		αg		Total	
			UC	PC	UC	PC	UC	PC	UC	PC	UC	PC
Pea	Amarillo (Y)		1257.2±25.6	85.1±1.4 (-93)	878.3±25.6	350.6±5.8 (-60)	ND	ND	8.0±0.1	3.5±0.3 (-56)	2143.6±30.3	439.2±7.5 (-80)
	Lacombe (Y)		846.8±3.4	86.2±2.2 (-90)	425.9±8.9	212.4±3.2 (-50)	1.1±0.1	ND (-100)	11.8±0.5	4.3±0.2 (-64)	1285.6±12.9	303.9±5.6 (-76)
	Cooper (G)		359.5±5.5	289.5±5.4 (-19)	187.1±3.5	27.9±0.1 (-85)	ND	ND	3.3±0.2	2.8±0.1 (-16)	550.0±9.2	320.1±5.6 (-42)
Faba bean	Snowbird (LT)	US	34.8±0.5	21.6±0.5 (-38)	175.6±1.1	61.5±2.5 (-65)	ND	ND	ND	ND	210.4±1.6	83.1±3.0 (-61)
		S	47.0±0.6	28.2±0.9 (-40)	207.0±0.7	62.1±1.3 (-70)	ND	ND	ND	ND	254.0±1.3	90.3±2.2 (-65)
	Snowdrop (LT)	US	68.0±2.3	44.2±0.7 (-35)	320.5±1.4	115.4±1.2 (-64)	ND	ND	ND	ND	388.0±3.7	159.6±1.9 (-59)
		S	165.7±0.7	104.4±0.9 (-37)	892.6±15.5	276.7±9.8 (-69)	ND	ND	ND	ND	1058.3±16.2	381.1±10.7 (-64)
	Fabelle (HT)	US	6.9±2.1	4.7±0.2 (-32)	22.8±0.8	9.6±0.5 (-58)	ND	ND	ND	ND	29.7±2.9	14.3±0.7 (-52)
		S	63.8±0.9	41.5±0.7 (-35)	315.2±1.9	91.4±1.1 (-71)	ND	ND	ND	ND	379.0±2.8	132.9±1.8 (-65)
	FB9-4 (HT)	US	61.9±1.2	36.5±0.9 (-41)	235.9±2.0	51.9±1.9 (-78)	ND	ND	ND	ND	297.8±3.2	88.4±2.8 (-70)
		S	94.8±1.1	53.1±1.1 (-44)	677.9±2.3	122.0±2.6 (-82)	ND	ND	ND	ND	772.7±3.4	175.1±3.7 (-77)
Black bean			1369.6±19.9	987.8±4.9 (-28)	3864.9±36.8	566.9±21.6 (-85)	186.6±2.3	50.4±1.6 (-73)	3144.7±15.9	406.4±2.4 (-87)	8565.9±74.9	2011.6±30.5 (-77)
Chickpea			717.5±9.0	84.9±1.5 (-88)	365.5±0.8	202.4±1.4 (-45)	0.8±0.0	0.7±0.0 (-6)	11.4±0.1	7.2±0.1 (-37)	1095.3±9.9	295.2±3.0 (-73)
Green lentil			1030.8±11.1	515.6±3.4 (-50)	3100.3±3.4	272.6±1.5 (-91)	3.7±0.1	0.1±0.0 (-96)	52.3±1.0	5.2±0.2 (-90)	4187.2±15.6	793.6±5.0 (-81)
Kidney bean			1921.7±18.9	580.3±5.7 (-70)	1487.3±18.9	185.3±1.5 (-88)	98.6±0.8	25.6±0.8 (-74)	907.8±1.8	113.9±1.5 (-87)	4415.5±23.2	905.2±9.4 (-79)
Pinto bean			1266.4±27.2	971.5±12.8 (-23)	1689.5±32.6	712.7±18.6 (-58)	78.5±1.8	64.6±0.2 (-18)	1306.1±32.0	537.6±3.2 (-59)	4340.6±93.7	2286.3±34.8 (-47)
Red lentil			791.6±5.4	669.2±5.9 (-15)	933.7±4.0	518.8±1.8 (-44)	ND	ND	8.7±0.2	4.8±0.1 (-45)	1733.9±9.6	1192.8±7.8 (-31)

US= unsprouted, S= sprouted, UC= uncooked, raw, PC= pressure-cooked, Y= yellow, G= green, LT= low-tannin, HT= high-tannin

The data represent the mean ± SD of two replicates (n=2).

Values in parentheses represent the loss of saponin after pressure cooking, as percentage of control value.

5 Chapter 5 Summary and Future Research Recommendations

5.1 Summary of key findings

Pulses have a long history of use in animal feed and human food due to their high nutritional value. However, the food use of pulses has been limited by undesirable bitter and astringent tastes and beany flavors. Saponins are a group of non-volatile secondary metabolites produced by plants and contribute to the bitter taste of pulse. In order to lower saponin levels, pulse processing methods such as dehulling, soaking, sprouting and cooking have been used but these also affect the nutritional composition of the pulses. Furthermore, only a limited number of studies have evaluated their effects on the saponin profile. In addition, the chemical composition of saponin present in pulse seeds is not well studied except for soybean.

In the present thesis research, a rapid LC/MS method for the determination of the saponin composition and content in selected pulse seeds was developed. Three different extraction methods were evaluated to compare their effect on saponin extraction efficiency. These include sonication, stirring and Polytron dispersion; in addition, four extraction solvents were tested [100% methanol, 100% ethanol, 70% methanol (methanol/water, *v/v*), and 70% ethanol (ethanol/water, *v/v*)], each at sample to solvent ratios of 25:1, 50:1 and 100:1 (w/v, mg/mL). For the saponin extraction of faba bean flour (Snowbird), Polytron dispersion for 5 min with 100% methanol in a sample to solvent ratio of 50:1 found out to be the most efficient method. Chromatographic conditions and the mass spectrometric parameters were optimized by using soyasaponin Bb and ginsenoside Rb1 standard solution. The present analytical method was validated based on linearity, accuracy, precision, detection and quantification limit. Recovery of quality control standards of higher than 92%, and RSD value lower than 2%, indicate good accuracy and precision. Low LOD and LOQ values, 5 ng/mL and 10 ng/mL respectively, demonstrate the high sensitivity of the current method for saponin measurements.

A major objective of this research was to study the saponin profile in a variety of pulses that are commonly grown in western Canada. These include 3 pea varieties, 4 faba bean varieties, pinto beans, black bean, kidney beans, chickpeas, and green and red lentils. All pulse seeds were milled into fine flour before analysis. DDMP-conjugated soyasaponin βg and αg, and non- DDMP-conjugated soyasaponin Bb and Ba were identified in several pulse samples according to their relative retention times, molecular ions and fragment ions, as compared to standards and literature. All of the pulses tested contained soyasaponin Bb and βg, despite the differences in the amounts and percentile distribution present. Saponin composition is largely dependent on pulse type and variety. The total saponin content in 8 types of pulses varied from 210 to 8566 μg/g, where the lowest saponin content was found in faba bean variety Snowbird, whereas black bean contains the highest amount of saponin. In all pulse samples, either soyasaponin Bb or βg is the predominant saponin type. Soyasaponin Ba and αg only present in small amounts with the exception of black bean (3145 μg/g of αg) and pinto bean (1306 μg/g of αg). The total saponin content in the 3 pea varieties and 4 faba bean varieties tested, varied from 550 to 2144 μg/g and 30 to 388 μg/g, respectively. Soyasaponin Ba was detected in pea variety Lacombe, while it was absent in the other two pea varieties. Our results suggest that saponin content and composition not only varies with pulse type but also differs among pulse varieties.

Another major goal of this research study was to investigate the effects of common pulse processing methods, specifically milling, sprouting, drying, baking, and cooking, on the saponin profile. The milling, sprouting, drying and banking process were done at FPDC in Leduc, Alberta. The germination of 4 varieties of faba bean was studied at sprouting times of 48, 54, 60 and 72 hours, followed by 72 hours drying. Only soyasaponin Bb and βg was detected in all faba bean

varieties. Soyasaponin Bb content was significantly increased in all varieties where reached its highest level at 54 hours then decreased at different rates for each variety over 72 hours of sprouting time. Soyasaponin βg showed a similar change patterns as Bb while reached its highest level at 60 hours, expect for variety FB9-4 (54 hours). Overall, our results showed an increase in total saponin content in the 72 hours sprouted faba bean flour compared to unsprouted. Drying of sprouted seeds is an essential step involved in the sprouting process. Seeds of each faba bean variety that had sprouted for 48 hours were dried at 60°C for times of 0, 24, 36, 48, and 60 hours. A significant decrease in soyasaponin Bb content was observed in all faba bean varieties after 24 hours of drying, whereas soyasaponin βg content significantly increased. After 24 hours of drying time, only small or insignificant change was observed in either saponin content. Overall, a significant decrease was observed in total saponin content in all faba bean varieties after 24 hours of drying.

Baking also has a significant reducing effect on total saponin content in either sprouted or unsprouted faba bean varieties in flour. However, soyasaponin Bb content increased significantly after baking, while soyasaponin βg content decreased significantly. These results suggest a possible conversion of some soyasaponin βg into Bb as a result of baking.

The effect of pressure cooking (7.2-10.2 psi, 700W, 115-118 °C, 13 minutes) on the saponin contents of pulses was also studied. A wide range of reductions (31-81%) in the total saponin content was observed in pulses after pressure cooking, as well as in individual saponin content (soyasaponin βg, αg, Bb, and Ba). The maximum reduction effect on total saponin content was observed in green lentils (81% of reduction), pea Amarillo variety (80%) and kidney bean (79%), while the minimum reduction effect was found out in red lentils (31%). In the present study,

pressure cooking of sprouted faba bean seeds reduced the total saponin level more effectively than unsprouted seeds.

Results obtained by FPDC in Leduc (Canada) from the sensory evaluation of cracker and their corresponding sprouted and unsprouted faba bean flour (paste) suggested that crackers made from sprouted faba bean flour were more bitter than those made from the corresponding unsprouted flour, regardless of the faba bean variety. This observation is in accordance with our results, where significant higher total saponin content was found in crackers made from sprouted faba bean flour. Faba bean variety Snowdrop has the highest total saponin content (388 μg/g), and the most bitterness was perceived by panellists in the paste and cracker made from Snowdrop. Thus, these results reflect the possible relationship between saponin content and bitterness in pulse.

These studies on drying and sprouting time, as well as baking effects on saponin profile in this thesis research were part of a larger project which focused on the development of sprouting processes and the characterization of aroma and flavor in Alberta grown high- and low-tannin faba bean varieties. The main objective of that bigger research project is to investigate changes of flavor aspects of both high- and low-tannin faba bean and their corresponding milled flours induced by sprouting. The anticipated outcomes of that bigger research project are developing an optimized sprouting conditions of faba beans in order to obtain maximum nutritional and functional characteristics, and to establish potential relationships among volatile and flavor impact compounds on sensory characteristics, which ultimately expand the utilization of faba bean in food use.

The results obtained from this study proved my hypothesis and met the objectives listed in Chapter 1. All of the tested processing methods changed saponin compositions in pulses. Thermal

treatments such as baking and cooking significantly reduced the saponin content in pulses, whereas sprouting caused an increase in total saponin content in the 72 hours sprouted faba bean flour compared to unsprouted.

To conclude, the present thesis work provides valuable information regarding the saponin profile in different pulse seeds, and their change induced by common pulse processing methods. The comprehensive saponin profile of different pulse samples determined by HPLC-MS can provide a basis for future study of the impact of environmental factors such as pulse varieties, growing locations and harvest year. Moreover, the results obtained from processing methods study might be valuable information for remediating flavor issues in pulse applications in foods.

5.2 Recommendations for future work

Based on the results of this thesis research, some suggestions for future studies are:

- In this study, pressure cooking time was fixed as 13 mins for each pules sample based on the average cooking time recommended for different pulses types by the pot user guide. However, in real house-cooking, each pulse type has a different recommenced cooking time which based on seed size. For example, green and red lentils usually take 2-3 mins under pressure cooking while chickpea and kidney bean require 25-35 mins cooking. Therefore, the effect of different cooking time could be studied on saponin content.
- Soaking the pulses for a certain of period time followed by boiling is another most commonly used pulses processing method besides pressure cooking. It might be useful to compare the effects of two cooking methods on saponin pulse samples.
- A potential relationship between saponin content and bitterness have been observed in different varieties of faba bean. However, the exact type of saponin might be responsible for the bitter

taste in pulse is unclear. In addition, the threshold value of saponin that can be detected by humans as bitter is unclear. The sensory properties of saponin types should be investigated in the future.

- A possible conversion of DDMP-conjugated saponin into non-DDMP-conjugated saponin induced during baking has been observed in the present study. More detailed investigations of this conversion reaction mechanism could be done in the future to provide a better understanding. For example, as a DDMP moiety is released as maltol under high temperature, the amount of maltol in faba bean flour and faba bean crackers can be determined by headspace GC-MS.

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