VALUE ADDED FRACTIONATION OF CANARYSEED (*Phalaris canariensis* L.) STORAGE PROTEINS

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ABSTRACT

The overall goal of this research was to develop an aqueous-based protein fractionation process from canaryseed (*Phalaris canariensis* L.*),* an important specialty crop for Canada, for value addition in the food industry. Evaluation of the microstructural features of canaryseed showed that it is composed of a bran, germ (embryo) and a starch-rich (starchy) endosperm, which is a typical characteristic of a cereal grain. The germ covers ~8-12% of the total endosperm area and it is highly concentrated with oil. Except for the germ, oil is distributed in the starchy endosperm and accounts for most of the oil in the whole seed. Presence of oil distributed in the starchy endosperm would be challenging to apply aqueous-based processing techniques for protein fractionation.

The whole (full bran) canaryseed flour was high in oil, ash, fiber, phytic acid and phenolic content than that of white (low bran) flour prepared from roller-milling whereas, they contained 21.3% and 21.4% of protein concentration, respectively, which is not significantly different ($p > 0.05$). Therefore, white flour could be a purer starting material for protein fractionation than whole flour. However, whole-flour showed better oil and water holding, emulsifying capacities, and digestibility in terms of *in vitro* protein digestibility corrected amino acid score (IV-PDCAAS) compared to that of white-flour whereas, emulsion and foaming stability, and foaming capacity were similar. Both hexane and ethanol were tested as de-oiling solvents for canaryseed, and it was found that linoleic acid and phosphatidylcholine were the major fatty acid and phospholipid extracted by these two solvents. Neither hexane nor ethanol denatured the canaryseed protein (peak denaturation temperature of 107-108°C) and did not cause any negative impact on their techno-functionality and digestibility.

A laboratory-scale-enzyme-assisted-aqueous process was developed to prepare a protein concentrate (>70% protein purity) using yellow-canaryseed-white flour. The same process was successfully adapted for brown canaryseed. However, lower protein purity can be expected depending on the protein content in the white flour if the protein content in the seed is lower due to genetic and environment factors. The aqueous treatment applied for de-oiling reduced the initial oil content (6%) in yellow-seed flour to 1.2%. The enzymatic treatment applied afterward degrade the starch in the flour improving the protein content from 21% to 74.8%. It also increased the oil content from 1.2% to 12.1%, which was higher than anticipated oil content in the final product.

Same trend was observed for brown canaryseed processing. The protein concentrates from yellow and brown canaryseed prepared using this aqueous-based method showed a least gelation concentration at 16% (w/w), which is higher than commercial soy protein concentrate (CSPC, 13%), but lower than the commercial vital wheat gluten (CVWG, 19%). At the least gelation concentration, the protein gels from both yellow and brown canaryseed showed significantly lower ($p<0.05$) strength than that of CSPC even with the presence of mono- and divalent salts. On the other hand, yellow and brown canaryseed protein concentrates showed comparable bread-dough improving properties to CVWG at lower inclusion levels (1-3%). Noticeable differences of gelation properties between yellow and brown canaryseed was not observed.

During the scaling up of the lab process, some modifications to the original aqueous process was performed to address issues related to upstream decanter separation. The modified process reduced the oil content to <1% and degraded ~85% starch to prepare the final protein product. However, proteins were lost into the waste stream due to the modifications, subsequently lowering the protein recovery into the final product. Further investigations on process modifications and optimization are required to prevent protein losses and improve protein recovery.

In summary, this research was able to develop an enzyme-assisted aqueous process for canaryseed protein fractionation for value addition despite having a major fraction of oil distributed within the starchy endosperm. This process used canaryseed white flour obtained using roller milling as the starting material for protein fractionation, which is purer in chemical composition compared to whole-seed flour. The developed method can be used for both yellow and brown canaryseed to fractionate protein. Canaryseed protein has high thermal stability and the use of hexane and ethanol as de-oiling solvent does not denature the protein and affect negatively for its techno-functional and nutritional properties. The fractionated protein did not display uniquely improved techno-functional properties compared to commercial soy protein or vital wheat gluten. However, canaryseed protein showed comparable bread-dough-forming properties to that of vital wheat gluten. The enzyme-assisted aqueous process was scaled up using pilot-scale equipment and was able to successfully de-oil white canaryseed flour. However, some process modifications were required to address the issues encountered during scaling up which caused lower protein recovery and purity in the final fractionated product. Further investigations and modifications are required to improve and optimize the scaled-up process for protein recovery.

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LIST OF ABBREVIATIONS AND ACRONYMS

A	Alkali extraction
AA	Amino acids
ABTS	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid
ALC	Amylose-lipid complex
ANOVA	Analysis of variance
BCPC	Brown canaryseed protein concentrate
C	Commercial
CCD	Charge coupled device
CDC	Crop development center
CDCS	Canaryseed development commission of Saskatchewan
CFIA	Canadian food inspection agency
CS	Canaryseed
CSPC	Commercial soy protein concentrate
CVWG	Commercial vital wheat gluten
DC2	Disk stack centrifuge from GEA Westfalia Separator Group
DDT	Dough development time
DIAAS	Digestible indispensable amino acid score
DPPH	2,2-diphenyl-1-picryl-hydrazyl-hydrate
DSC	Differential scanning calorimetry
DSLR	Digital single-lens reflex
DTT	Dithiothreitol
E	Ethanol
EAA	Essential amino acids
EC	Emulsifying capacity
EC3	Decanter centrifuge from GEA Westfalia Separator Group
EDF	Ethanol-de-oiled flour
EDI	Ethanol-de-oiled isolate
ELISA	Enzyme-linked immunosorbent assay
ELSD	Evaporative light scattering detector

ES	Emulsion stability
F	Flour
FA	Fatty acids
FAME	Fatty acid methyl esters
FAOSTAT	Food and agriculture organization corporate statistical database
FC	Foaming capacity
FID	Flame ionization detector
FS	Foaming stability
G''	Loss modulus
G'	Storage modulus
GBSS	Granule-bound starch synthase
GC	Gas chromatography
GRAS	Generally recognized as safe
HDF	Hexane-de-oiled flour
HDI	Hexane-de-oiled isolate
HMW	High molecular weight
HPLC	High performance liquid chromatography
IR	Infrared
IV	*In vitro*
LC	Liquid chromatography
LGC	Least gelation concentration
LMW	Low molecular weight
LWD	Long working distance
MS	Mass spectrometry
MTI	Mixing tolerance index
MWCO	Molecular weight cut-off
MWM	Molecular weight markers
NA	Numerical aperture
NDI	Non-de-oiled isolate
OHC	Oil holding capacity
ORAC	Oxygen radical absorbance capacity

PAGE	Polyacrylamide gel electrophoresis
PD	Protein digestibility
PDA	Photo diode array
PDCAAS	Protein digestibility corrected amino acid score
PER	Protein efficiency ratio
pI	Isoelectric pH
RO	Reverse osmosis
S	Sulphur
SD1	Spray drier from Komline-Sanderson Corporation
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
TAG	Triacyl glycerol
Tc	Conclusion temperature
To	Onset temperature
Tp	Peak temperature
TPC	Total phenolic content
USDA	United States department of agriculture
UV	Ultraviolet
VS	Vibratory screen
W	Water
WGI	Wheat gluten isolate
WHC	Water holding capacity
X	Starch degrading enzyme 1 from Novozymes
Y	Starch degrading enzyme 2 from Novozymes
YCPC	Yellow canaryseed protein concentrate

1. INTRODUCTION

1.1 Overview

The glabrous canaryseed, also known as hairless canaryseed, was recently approved by Health Canada's Food Directorate and the United States Food and Drug Administration Agency for human consumption (Health Canada, 2016; GRAS notice GRN No. 529, 2015; Mason et al., 2018). There are two major types of canaryseed, namely brown canaryseed (grains with brown color seed coat) and yellow canaryseed (grains with yellow color seed coat). The brown canaryseed varieties were first to develop whereas the yellow canaryseed was developed recently with the intention of catering to the human food market. Canada is the major canaryseed producer in the world that contributes for 60% of global production with >75% market share (Achouri et al., 2020). The canaryseed production is primarily used as a constituent in the feed mixtures for caged and wild birds (Canaryseed Development Commission of Saskatchewan [CDCS], n.d. -a). Therefore, it is important to diversify the canaryseed market through value addition for human consumption to achieve a sustainable market in the long run. Adding value to canaryseed required identifying the chemical constituents in the seeds that have the potential to valorization. Canaryseed contains ~55% starch as the major chemical compound followed by protein (19-22%), fiber (6-8%) and oil (5-7%) (Abdel-Aal et al., 2011). Compared to the other cereals commonly available in the market, such as wheat, rye, barley, oats, corn, and millet, canaryseed contains extremely high protein content (Mason et al., 2018). Therefore, canaryseed has the potential to be developed as an emerging protein source and add value through protein ingredient development for different food applications.

Development of a processing method to obtain protein as an ingredient and identifying its techno-functional and nutritional properties are the initial steps towards protein ingredient development. canaryseed contains prolamin and glutelin as the major storage protein (~78% of the total storage proteins) in which the prolamins accounts for the major protein fraction (~45% of the total storage proteins; Abdel-Aal et al., 1997a). Based on the amino acid sequence, prolamin proteins are classified into sulphur-rich (S-rich), sulphur-poor (S-poor) and high molecular weight

(HMW) proteins (Shewry & Halford, 2002; Shewry et al., 1995). However, limited information is available on such classification of the canaryseed proteins and their similarities with closely related wheat, barley, rye, oat and corn prolamin or prolamins of other cereals. Knowledge on protein types (prolamins, globulins etc.) and protein structure details in comparison with other known sources will be beneficial to develop methods for protein isolation and purification, identify techno-functional and nutritional properties, and potential food applications of canaryseed protein products. In terms protein separation of method development, understanding the abundance of other chemical constituents in the seed, such as starch, oil, fiber, and their distribution is also important to identifying the techniques suitable for isolate and purify canaryseed proteins to develop protein-based ingredients.

Alkali extraction and isoelectric precipitation is a commonly used method to prepare protein isolate in the industry. This method is reported for protein isolates preparation from soy, pulses, oilseeds and some cereals, such as oat, where albumins and globulins are the major storage proteins (Nadathur et al., 2016). Previous studies carried out on canaryseed used this method to prepare protein isolates (Abdel-Aal et al., 2010; Achouri et al., 2020). In this method, ethanol de-oiling was used prior to protein to reduce the oil content in the canaryseed flour. Since canaryseed contains comparatively higher content of lipids than other cereals and solvent-de-oiling is required to prepare shelf-stable protein products. However, the impact of using ethanol on the techno-functional and nutritional properties have not been studied previously. Abdel-Aal et al. (2011a) reported that majority of the canaryseed oil is concentrated in the bran fraction and the bran contains some protein that is chemically different than the endosperm proteins. It will be beneficial to understand the differences in the proteins and oil in the endosperm and the bran and how techniques such as de-branning (removal of bran) could help to develop protein processing method without solvent-de-oiling, which could be beneficial for canaryseed protein ingredients to compete in the alternative protein market. On the other hand, if solvent-de-oiling is found to be the most feasible way to reduce the oil content for processing, it would be important to understand the effect of the solvents, such as ethanol and hexane, which are commonly used solvent in the food industry, on the protein quality. This information will be beneficial to select the suitable solvent for canaryseed protein processing without compromising the protein quality significantly.

The aim of this study was to investigate the distribution of major chemical compounds, especially oil, in the seed, effect of solvent-de-oiling on techno-functional and nutritional

properties, that could lead to development of an alternative process, preferably an aqueous-based process, to obtain canaryseed protein product that could be directed towards different food applications, while understanding the similarities and differences between yellow and brown canaryseed.

1.2 Hypotheses

1. There is no difference between yellow and brown canaryseed in terms of their microstructure and distribution of oil, protein and starch in different structural compartments of the seed.

2. Most of the oil in canaryseed will be found in the germ and bran than the endosperm, and removal of germ and bran by mechanical means could help to reduce the oil content in the resulting endosperm flour.

3. The techno-functional and nutritional properties of solvent-de-oiled flour and protein isolates are significantly different than that of non-de-oiled flour and protein isolates.

4. An aqueous-based method is capable of de-oiling canaryseed flour and prepare protein concentrate or an isolate in the laboratory scale.

5. There is no difference of gelation and bread dough forming properties of yellow and brown canaryseed protein prepared using an aqueous-based method developed in the laboratory scale, and they are not significantly different from that of commercial soy and vital wheat gluten concentrates.

6. It is possible to scale-up aqueous based process developed in the laboratory to achieve <2% oil after de-oiling canaryseed flour and produce a protein concentrate with >60% protein purity using pilot-scale equipment.

The following objectives will be used to test these hypotheses.

1.3 Objectives

1. Evaluate microstructure and chemical compositional distribution within different seed compartments of yellow and brown canaryseed.

2. Develop or adapt a mechanical (dry milling) method to effectively remove bran and the germ of the canaryseed.

3. Evaluate chemical, techno-functional and nutritional properties canaryseed full-bran flour, and low-bran (white) flour.

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4. Evaluate techno-functional and nutritional properties of solvent-de-oiled flour and protein isolates and compare them with that of non-de-oiled flour and isolates.

5. Develop a lab-scale-aqueous process to produce protein concentrate using low-bran yellow and brown canaryseed flour.

6. Evaluate gelation properties and bread dough strengthening properties of yellow and brown canaryseed protein concentrate prepared using the lab-scale-solvent-free process and compare with commercial concentrates.

7. Scaling up the lab-scale process developed using the pilot-scale equipment for canaryseed protein concentrate production.

2. LITERATURE REVIEW

2.1 Canaryseed as a crop

Canaryseed (*Phalaris canariensis* L.*)* is an annual cereal crop belonging to family *Poaceae* similar to wheat, barley, rye, oats, sorghum, millet, maize and rice. However, it is taxonomically closer to wheat, barley and rye or oat since they all belong to the sub-family *Pooidae* (Cogliatti, 2012). Canaryseed was traditionally used as a feed for caged and wild birds (Abdel-Aal et al., 2011a; Cogliatti, 2012) and predominantly grown in Canada. In addition, it is also grown in South America, Asia, Europe, North Africa, the Middle East and the United States (Abdel-Aal et al., 2011a; Abdel-Aal et al.,1997a; FAOSTAT, 2022, Irani et al., 2016a). Commercial canaryseed production in North America started in the 1950s in the United States after the World War II and advanced to Canada in 1960-1970s (Cogliatti, 2012; Patterson et al., 2018). Canaryseed typically is seeded in locations where wheat is grown and possesses some unique agronomic characteristics, such as lower input requirement, early-drought tolerance, shatter resistance and ability for late harvesting without compromising the grain quality. However, canaryseed tends to be more heat and drought sensitive than wheat (DeMilliano, 2018; Milligan, 2016).

Canada dominates the global canaryseed production and export market (Achouri et al., 2020; Patterson et al., 2018). There are two major types of canaryseed cultivated in Canada, namely "hairy" and "hairless or glabrous" canaryseed (Patterson et al., 2018). Hairy canaryseed, contains silica fiber (hair-like structure) in the hull that has a negative impact on human health (Abdel-Aal et al., 2011a; Bhatt et al., 1984). These silicious hairs cause severe skin irritations for humans and make harvesting and transportation operations quite challenging (Abdel-Aal et al., 1997a). In addition, the flour contaminated with silica fiber could potentially cause cancers in the esophagus when ingested (Abdel-Aal et al., 1997a; O'Neill et al., 1980). Therefore, consumption of canaryseed had been limited to birds for a long time and the bird feed market has been the major revenue generating opportunity for the producers even to date (CDCS, n.d. -a).

Due the potential of canaryseed being developed as food crop, the Crop Development Centre (CDC) of the University of Saskatchewan developed the "hairless" or the "glabrous" canaryseed varieties that is safe for human consumption (Mason, et al., 2018). As a result, the dehulled (hairless) seed received approval by the Health Canada's Food Directorate and the U.S. Food and Drug Administration for human consumption (*GRAS Notice (GRN) No. 529*, 2015; Heydari et al., 2018; Mason et al., 2018; Patterson et al., 2018). CDC Maria, which contains a brown seed coat was the first glabrous canaryseed variety developed by the CDC (Hucl et al., 2001a). CDC Togo, CDC Bastia and CDC Calvi are the other brown- seedcoat (glabrous) varieties commonly grown in Canada (Hursh, 2019). CDC recently developed a glabrous variety, namely CDC Cibo, which is the first yellow-seed-coat variety targeted for human food use (CDCS, 2017). According to the Western Canada Insured acers in 2018, the cultivated land percentage of Togo, Bastia and Calvi was 8%, 7% and 13%, respectively while it was only 2% for Cibo. Although the glabrous varieties are receiving more attention, the acreage for the hairy varieties such as Keet (21%) and Cantate (18%) is higher, (Hursh, 2019). Even to date, the cultivated acreage of hairless canaryseed is lower than the regular hairy varieties (Penner, 2022), Presumably, due to higher yield of the regular varieties compared to that of the glabrous varieties. Approximately 95% of the Canadian canaryseed acreage and production is from the prairie province Saskatchewan (CDCS, n.d. -a). According to Government of Saskatchewan statistics, the seeded acreage of canary seed in 2021 was 295,000 acres, which makes it the third most cultivated specialty crop in the province after lentils and peas (Specialty crop report, 2021). However, the canary seed market is stagnating (~$100 million/year) due to limited market opportunities (CDCS, n.d. -a). Therefore, adding value to this crop is important to expand the market potential and subsequently increase the canary seed acreage/production in the province. According to Specialty crop report. (2021), the total canaryseed acreage in Saskatchewan decreased from 2015 and it is currently about 10% lower than that of 2015. Moreover, there is no reported canaryseed acreage in Manitoba and Alberta (Specialty crop report, 2021), the other two prairie provinces that grows canaryseed. The statistics show the need to diversify the applications other than bird feed to promote growing this crop in the province. To reach this goal more research is required to explore the potential of this crop for value addition to direct towards human food market.

2.2 Morphology and microstructure of canaryseed

Canaryseed is a very small (~4 mm long and 1.5-2 mm wide) and elliptical grain (Hucl, et al., 1995). It is attached to a hull that accounts for 35% of the total seed (Abdel-Aal et al., 1997a), which is either hairy or hairless. The hulls are typically removed (dehulled) during post-harvest processing and the dehulled seeds could contain either brown or yellow color seed coat (Figure 2.1). Similar to other cereals, such as wheat, oats, barley and rice, canaryseed microstructure consist of a bran, starchy endosperm and germ (Patterson et al., 2018). According to Abdel-Aal et al. (2011b), the endosperm is rich in starch and contains different sizes of compound starch granules (*i.e.,* tightly packed starch granules produced at the same time in a single amyloplast) similar to starch granules in oats. They are polygonal in shape and could be 0.5-7.5 µm in size (Abdel-Aal et al.1997b; Goering & Schuh, 1967; Irani et al. 2017).

Figure 2.1 Dehulled canaryseed with brown seed coat (left) and yellow seed coat (right). Images were taken using a Cannon® EOS Rebel T7 digital single-lens reflex camera attached with Cannon® EFS 18-55 mm lens.

The bright field microscopic images of the canaryseed microstructure showed that the proteins in the endosperm mainly exist as a matrix with some distinct protein bodies distributed within the matrix (Abdel-Aal et al., 2011a). Proteins are also present in the aleurone layer. The authors suggested that the proteins in the aleurone layer are chemically different from that of the proteins in the endosperm due to the color differences resulted from protein staining observed using bright field microscopy (Abdel-Aal et al., 2011a). The bright field microscopic images of the canaryseed microstructure did not reveal the distribution of oil as it has been extracted during sample preparation process (Abdel-Aal et al., 2011a). However, chemical analysis of the de-branned seeds showed that most of the oil of canaryseed is concentrated into the bran, leaving

lower oil content in the white flour (Abdel-Aal et al., 1997a). The cell walls of the aleurone layer predominantly contains the phenolic compounds of canaryseed. Most likely ferulic and coumaric are the two major phenolic acids present in canaryseed similar to other grasses. On the other hand, phytate is located inside the aleurone cells (Abdel-Aal et al., 2011a). Further evaluation of bright field microscopy showed that the cell walls of the endosperm is composed of cellulose and/or mixed-linkage-β-glucans with 1.1 µm constant wall thickness across most of the grain, with few exceptions (Abdel-Aal et al., 2011a).

2.3 Chemical composition of canaryseed

Canaryseed contains ~20-23% protein which is comparatively higher than the other cereal and pseudocereal grains (Table 2.1). Pseudocereals refer to non-grass grains, such as quinoa, amaranth and buckwheat, which are cultivated and utilized similar to true cereal (grass of family *Poaceae*) grains and share similar chemical composition (Fletcher, 2016; Thies, 2017). Being a cereal crop, starch found to be the predominant chemical constituent in canaryseed, and it accounts for approximately 55-61% of the groat dry matter. It also contains 5-7% crude fat, 6-8% dietary fibre and 2-3% ash as the proximate composition. (Abdel-Aal et al., 2010; Abdel-Aal et al., 2011a; Abdel-Aal et al., 1997c; Abdel-Aal et al., 1997a; Mason et al., 2018). The amount of fat content in canaryseed is comparatively higher than wheat whereas, the fibre content is noticeably lower (CDCS, n.d. -b). Canaryseed contains comparatively lower content of sugars (1.7%), soluble dietary fibre (0.9%) and insoluble dietary fibre (5.1%) than that of wheat (Abdel-Aal et al., 1997c). The content of the insoluble fibre of canaryseed is significantly higher than that of soluble fiber (Patterson et al., 2018). Both hairy and hairless canaryseed reported to have higher levels of phosphorous, sulphur, magnesium, calcium, iron, manganese and zinc compared to wheat and therefore considered as richer source of minerals (Abdel-Aal et al., 2011a).

2.3.1 Proteins

Previous research has shown that canaryseed contains noticeably higher amount of protein than that of other commercially available cereals (Table 2.1). According to the Osborne's protein classification [protein solubility based on water (albumins), diluted salt (globulins), aqueous alcohol (prolamins) and weakly acid or alkaline solution (glutelins), (Osborne, 1924) canaryseed contains prolamin and glutelin as the predominant storage protein fractions (Abdel-

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Aal et al., 2010; Abdel-Aal et al., 1997a). Both prolamin and glutelin represent 78% of the total protein of canaryseed, in which the prolamin accounts for 45.5% of the total protein (Abdel-Aal et al., 1997a). The amount of prolamin in canaryseed is higher than that of wheat prolamin whereas, the glutelin content in canaryseed is lower than the wheat glutelin (Abdel-Aal et al., 1997a). The albumin and globulin content in canaryseed are significantly low (5.7% and 7.4% of the total protein, respectively). Therefore, the risk of negative health impact of proteins is presumably lower due to lower levels of albumin-type antinutritive compounds, such as enzyme inhibitors (Abdel-Aal et al., 1997a; Mason et al., 2018).

Table 2.1 Protein content of canaryseed and other cereal and pseudocereals as reported in Mason et al. (2018) and CDCS, n.d. -b.

Type	Crop name	%Protein
Cereal	Canaryseed	20-23
	Wheat	13
	Oat	10-13
	Barley	13-16
	Rye	11-16
	Millet	8.5-15
Pseudocereal	Quinoa	14.5
	Buckwheat	12.5
	Amaranth	16.5

Prolamin, rich in proline and glutamine, is the predominant storage protein in most of the cereals, except grains like rice and oats where 11S globulin-like proteins are the major storage protein (Shewry et al., 1995; Shewry & Tatham, 1990). Although prolamin (aqueous-alcohol soluble) and glutelin (weakly acid/alkaline soluble) considered as two different types of proteins according to Osborne classification, it was later found that the glutelin protein are closely related (structurally) to prolamins (Shewry & Tatham, 1990). Glutelin could not be solubilized in aqueous alcohol as it forms high molecular weight polymers stabilized by inter-chain disulphide bonds. However, the reduced subunits of the polymers are alcohol soluble and rich in proline and glutamine, which is characteristic of prolamins. Therefore, glutelins are considered as prolamin protein (Shewry et al., 1995; Shewry & Tatham, 1990). The more comprehensive classification of the prolamin proteins is based on their amino acid composition, namely sulphur-rich (S-rich), sulphur-poor (S-poor) and high molecular weight (HMW) prolamins (Shewry & Halford, 2002;

Shewry et al., 1995). Modelling of prolamin and glutelin proteins similar to that of albumins and globulins is found to be challenging due to their intrinsically disordered structure; hence, not extensively studied (Dahesh, et al., 2014). It was reported that the prolamins can be found in dimeric form (5.72 nm hydrodynamic radius), form of aggregated clusters (30 nm hydrodynamic radius) or oligomers with 68-103 nm hydrodynamic radius (Herrera et al., 2018). On the other hand, glutelin contains much larger, highly disordered and complex structure than prolamins (Shewry & Casey, 1999), making it even more difficult to study. The glutelin protein in wheat is found to form the largest protein polymer in the nature (Wrigley, 1996). Hence, it can be speculated that the glutelins in canaryseed also contains disordered structure whereas prolamins could exist either/or dimeric, aggregates or oligomeric form. Understanding the structural features and organization of prolamins and glutelins of canaryseed is important to comprehend their structure-function relationship for different food applications.

Evaluation of the polypeptide profile of canaryseed flour by Achouri et al. (2020), using sodium dodecyl sulphite polyacrylamide gel electrophoresis (SDS-PAGE), showed polypeptide bands representing albumin, globulin, prolamin and glutelin ranging from 3-100 kDa. High-intensity polypeptide bands representing prolamins were visible in 20-25 kDa range whereas polypeptide bands that represent high molecular weight glutelins and globulins were visible in 30-100 kDa range. This result confirms that prolamin is the predominant type of protein present in canaryseed. The polypeptide bands between 10-18 kDa is from low molecular weight glutelins and globulins, and <10 kDa bands represent albumins in canaryseed (Achouri et al., 2020). The authors evaluated SDS-PAGE profile of both yellow and brown canaryseed varieties and did not observe any major differences.

Previous studies have reported that the S-rich, S-poor and HMW prolamins (of wheat, barley and rye) ranges from ~36-44 kDa, ~44-78 kDa and ~67-88 kDa, respectively. The molecular weight of maize prolamins (α-Zein and γ-Zein) were reported to have a molecular weight of 19-28 kDa (Shewry & Tatham, 1990). The molecular weight of prolamins of the canaryseed reported by Achouri et al. (2020) shows close similarity to maize prolamins. A study conducted by Boye et al. (2013) to evaluate the allergenic proteins of canaryseed using LC-MS/MS showed high probability of canaryseed containing proteins similar to avenins (prolamin) and 12S globulins from oat and rice glutelins. Another study carried out by Mason et al. (2020) using LC-MS/MS analysis of canaryseed bioactive peptides showed that majority of the parent protein (14 out of 18 parent

proteins) of the peptides are homologous to barley protein while 50% of the remaining parent proteins are homologous to wheat protein. It would be interesting to understand the type of prolamins in the canaryseed proteins and their structural similarities to prolamins of closely related cereals to understand the techno-functional and nutritional properties of canaryseed proteins.

The amino acid profile of canaryseed is unique because of its higher tryptophan content (an essential amino acid), which is generally deficient in many cereals (Table 2.2; Mason et al., 2018). Other than the tryptophan, canaryseed is also rich in essential amino acids, such as leucine, isoleucine, cysteine and phenylalanine compared to other cereals (*GRAS Notice (GRN) No. 529*, 2015). However, the content of other essential amino acids, such as lysine and threonine, is lower in canaryseed, but comparable to that of wheat and other cereals (Table 2.2; Abdel-Aal et al., 1997a; GRAS Notice (GRN) No. 529, 2015; Mason et al., 2018). Since canaryseed is deficient in lysine and threonine, they have the potential to be eaten as part of a complementary diet with pulses which are rich in those amino acids (Mason et al., 2018). Overall, the essential amino acid content of the canaryseed is generally higher that wheat, but comparable to that of other cereals, such as barley, maize and oats (Table 2.1). Similar to other cereals, glutamic acid is the major non-essential amino acid present in canaryseed, and it is the most abundant one in the entire amino acid profile. The content of glutamic acid is lower than wheat however, it is higher than that of barley, maize and oats (Table 2.1).

Canaryseed protein is also reported to have many beneficial bioactive peptides (Mason et al., 2018; Mason et al., 2020). According to Mason et al. (2018), several different bioactive peptides have been identified that possess potential antidiabetic, antihypertensive and antioxidant activity. These bioactivity of canaryseed peptides could be either superior or equivalent to that of wheat and oat peptides (Mason et al., 2020). Further studies carried out by Mason et al. (2020) showed that canaryseed peptides also exhibit potential antiamnestic, antithrombotic, immunostimulating, opioid and neuro-activity. Moreover, canaryseed could be beneficial for people with coeliac disease as it is free of gluten-like protein, confirmed using enzyme-linked immunosorbent assay (ELISA), mass spectroscopy, and western blotting (Boye et al., 2013). Although canaryseed is "gluten-free", it could be sensitive to people with wheat allergies due to presence of granule-bound starch synthase (GBSS), a newly reported allergen in rice and maize, which showed cross-reactivity with sera obtained from the individuals with wheat allergies (Boye et al., 2013; Krishnan & Chen, 2013).Therefore, Canadian labelling regulations required to include

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the phrase "may not be suitable for people with wheat allergy" if canaryseed containing food is represent as gluten-free (Canadian Food Inspection Agency (CFIA), 2019).

Table 2.2 Amino acid composition of canaryseed and other cereals. Adapted from GRAS Notice (GRN) No. 529. (2015)

Amino acid (AA)	Canaryseed (g/100 g Protein)	Wheat (g/100 g Protein)	Barley (g/100 g Protein)	Maize (g/100 g Protein)	Oats (g/100 g Protein)
Essential AA					
Methionine	1.4-2.2	1.3-1.7	1.4-3.2	1.8	2.5
Cystine	2.2-3.4	1.7-2.7	1.0-1.8	1.1	1.6
Lysine	1.4-2.8	2.2-3.0	3.1-4.2	2.6	4.2
Tryptophan	2.7-3.1	1.0-2.7	1.5	0.7	1.3
Isoleucine	3.4-4.1	3.0-4.3	3.1-3.9	3.7	3.9
Histidine	1.6-1.9	2.0-2.8	1.9-3.3	2.8	2.2
Valine	4.7-4.9	4.4-4.8	3.9-5.3	5.3	5.3
Leucine	7.1-7.8	5.0-7.3	5.4-7.1	13.6	7.4
Phenylalanine	6.3-6.7	3.5-5.4	4.2-5.4	5.1	5.3
Tyrosine	3.4-3.8	1.8-3.7	1.9-2.8	4.4	3.3
Threonine	2.7-2.9	2.4-3.2	3.0-3.7	3.6	3.3
Total EAA	**36.95-43.75**	**26.3-41.6**	**30.4-42.19**	**44.7**	**40.3**
Non-essential AA					
Alanine	4.4-4.6	3.4-3.7	4.4-4.6	7.9	5.0
Arginine	6.3-6.9	4.0-5.7	4.2-6.2	3.8	6.9
Aspartic acid	4.1-4.7	4.8-5.6	6.8-7.4	6.3	8.9
Glutamic acid	25.2-26.9	29.9-34.8	21.9-26.1	18.9	23.9
Glycine	2.9-3.2	3.8-6.1	4.2-5.1	3.4	4.9
Proline	6.1-6.4	9.8-11.6	11.4-12.4	8.3	4.7
Serine	4.3-4.7	4.3-5.7	3.7-5.4	4.8	4.2

2.3.2 Starch

Canaryseed starch is reported to have a small (0.5-7.5 µm) polygonal granules with an average size of 2.6 µm (Irani et al., 2017). The granules are monomodal compared to the bimodal granules of wheat and known to contain A-type starch (Abdel-Aal et al., 1997b; Irani et al., 2016a; Irani et al., 2016b). Although the granular size ranges from 0.5-7.5 µm, majority of the granules (95-98%) lies in between 1.5-5 µm range (Abdel-Aal et al., 1997b; Irani et al., 2017). Due to the small and uniform size of starch granules, it is suggested that canaryseed starch is well suited for the cosmetic industry (Abdel-Aal et al., 2010). Goering & Schuh (1967) reported that canaryseed starch has normal amylose content based on the iodine affinity value. Later, Abdel-Aal et al. (1997b) reported that canaryseed contains relatively low amylose content (16.2-19.5%) than that of wheat. Irani et al. (2017) confirmed the lower level of amylose in canaryseed starch compared to that of wheat however, reported higher levels (22.5-23.6%). The change in the % amylose content could be due to genotype and environment conditions (Singh et al., 2006).

Canaryseed starch is subjected to two-stages of thermal transition when analyzed using differential scanning calorimetry (DSC) (Goering & Schuh, 1967). The first thermal transition occurs at 60-70 °C whereas, the second transition occurs between 85-92.5°C. Similar trend was observed by Irani et al. (2017) when they evaluated the thermal properties of canaryseed starch using DSC. They suggested that the first endothermic peak, occur ~58-80 °C, which is the principal peak (ΔH = 11.8-12.4 J/g), represent starch gelatinization whereas, the second peak, occur ~97-113 °C, which has an enthalpy (ΔH) of 2.4-2.5 J/g, is related to melting of amylose-lipid complex. Therefore, it would be better to use ≤50 °C when processing canaryseed protein and starch to avoid possible starch gelatinization and associated processing issues.

Canaryseed starch shows similar properties to wheat in dilute solutions and exhibit potential applications as thickening and stabilizing agents in food products (Heydari et al., 2018; Irani et al., 2017; Irani et al., 2016b; Mason et al., 2018). It is easily digestible. However, has a higher potential to retrograde into RS3 (a nutritionally beneficial resistant starch) and make them available for hind-gut microflora for digestion, which needs further investigation (Abdel-Aal et al., 1997b; Irani et al., 2017; Mason et al., 2018).

2.3.3 Lipids

Canaryseed contains noticeably higher amount of oil compared to other cereal grains (Mason et al., 2018). Total lipids of canaryseed, extracted with chloroform/methanol (2:1 v/v) was 11% whereas, the crude fat content, extracted with nonpolar solvent was 8.7%, showing that it contains considerable amount of polar lipids (Abdel-Aal et al., 1997a; Abdel-Aal et al., 1997c). Previous studies had used ethanol to extract canaryseed oil which proved to be a very suitable solvent (Mason et al., 2018), presumably due to the extractability and lower negative impact on the environment compared to other solvents, such as hexane. Majority of the canaryseed oil is unsaturated where linoleic acid is the major fatty acid (55%), followed by oleic (29%), palmitic (11%), linolenic (2.4%) and stearic acid (1%) (Abdel-Aal et al., 1997a). The unsaturated fatty acids in canaryseed accounts for 85% of the total fatty acid content, where ~32% is monounsaturated and ~55% polyunsaturated (*GRAS Notice (GRN) No. 529*, 2015). The unsaturated to saturate fat ratio of canaryseed is higher (~85:13) than that of other cereals, such as wheat, barley and oat (~75:25). However, the amount of polyunsaturated fatty acid is lower (~55%) compared to wheat (~66%) and barley (~60) (*GRAS Notice (GRN) No. 529*, 2015). Although canaryseed contains more unsaturated fat, oil is found to be quite stable due to presence of antioxidants such as phenolic acids and phytosterols (*GRAS Notice (GRN) No. 529*, 2015).

2.3.4 Dietary fibre

Compared to other cereals, canaryseed contains lower amount of total dietary fibre (7-8.5%; *GRAS Notice (GRN) No. 529*, 2015; Mason et al., 2018). Bran accounts for most of the fibre fraction and mainly composed of insoluble fibre (Abdel-Aal et al., 1997a; Abdel-Aal et al., 1997c). According to the GRAS notice, 2015, canaryseed contains 0.6% lignin. There is limited literature available with respect to the chemical composition of the fibre fraction of canaryseed. Understanding the types of dietary fiber is important as it could be one of the value-added streams of canaryseed processing.

2.3.5 Phytochemicals

Canaryseed reported to have number of phytochemicals, such as phenolic acid, carotenoids, tocopherols and phytosterols, phytate, trypsin inhibitors and amylase inhibitors (Abdel-Aal et al., 2011b; Chen, et al., 2016; *GRAS Notice (GRN) No. 529*, 2015; Li & Beta, 2012; Li et al., 2011). The major phenolic acid in canaryseed is ferulic acid (Abdel-Aal et al., 2011a; Li et al., 2011), followed by caffeic and coumaric acid (Li et al., 2011). However, Abdel-Aal et al. (2011a) reported higher sinapic acid content in canaryseed than that of the caffeic acids. The highest concentration of the phenolic acids was found in the bran of canaryseed (Abdel-Aal et al., 2011b; Li et al., 2011). Analysis of the phenolic content in whole flour of wheat and canaryseed showed they are comparable to each other (Abdel-Aal et al., 2011b). Neither proanthocyanidins nor condensed tannins were found in canaryseed (Abdel-Aal et al., 2011b; Li et al., 2011). Chen et al., 2016 found high correlation between the total phenolic content and the antioxidant activity of canaryseed measured using DPPH, ABTS and ORAC assays.

Canaryseed contains higher β-carotene content compared to other cereals, which could be beneficial as it has the potential to be utilized in carotenoid-enriched functional food (Li & Beta, 2012). Despite of β-carotene, canaryseed also contains lutein and zeaxanthin which adds value to total catenoid account. Evaluation of canaryseed tocopherols (mainly α-tocopherols δ-tocopherols) showed that the total tocopherol content is low than that of other cereals (*GRAS Notice (GRN) No. 529*, 2015). However, presence of the tocopherols provides oxidative stability to unsaturated oil of canaryseed.

The phytate content of canaryseed found to be significantly higher than that of wheat and most of it is concentrated in the bran (Abdel-Aal et al., 2011b). Trypsin and amylase inhibitors were also detected in canaryseed (Abdel-Aal et al., 2011a). The content of trypsin inhibitors is not significantly different than that of wheat and lies in the lower range; hence, adverse impact on the growth is not expected due to consumption. On the other hand, starchy flour of canaryseed has significantly higher content of amylase inhibitor activity compared to wheat (Abdel-Aal et al., 2011b).

2.4 Value-added processing of canaryseed

The first step of value-addition to canaryseed is to remove the hull to obtain the dehulled seed. Dehulling of canaryseed is usually carried out using an abrasive dehuller and air aspirated to separate the hulls and groats (Abdel-Aal et al., 2010; Abdel-Aal et al., 1997a). Hucl et al. (2001b) studied the effect of moisture content on dehulling efficiency. It was found that there was no significant difference between the control and the treated samples (*i.e.,* tempering to 12, 14 and 16% moisture, oven drying at 250 °C to 10% moisture and fluidized bed drying to 10% moisture) on dehulling efficiency of canaryseed. It was required to pass the hulled seeds twice through the dehuller to obtain groats with minimum hull contamination (0.29% of free hulls compared to 2.41% of free hulls from a single pass). During the dehulling process it is important optimize the equipment conditions to reduce the broken or damage seeds. Screening after air aspiration could be used to separate the damage/broken seeds from the intact dehulled seed (Hucl et al., 2001b).

Full-bran flour (whole flour) and low-bran flour (white flour) is the next potential value-added products of canaryseed that can be utilized for food applications. The dehulled seed could be milled using hammer mills and/or pin mills to prepare whole flour (CDCS, n.d. -c). It was observed that the screens of hammer mill were blinded or plugged during the milling process; presumably, due to the high oil content in canaryseed. Further investigation is required to improve the milling process and oil related issues (CDCS, n.d. -c) for whole flour preparation. Hucl et al. (2001b) used a roller mill (Barbender Quadramat Jr. mill) to fractionate bran and starchy endosperm to prepare white flour. They evaluated the effect of tempering, drying and roasting on the flour yield and color. The results suggested that tempering the groats to 14% moisture is the best obtain higher flour yields. Tempering the seeds >14%, drying or roasting the seeds prior to milling cause adverse impact on milling and flour yield. However, the color is slightly affected. Tempering the groats to 14% moisture content and roller milling could result in 25% of bran and 75% white flour fraction on the total weight basis (Abdel-Aal et al., 1997a). The bran fraction contains noticeable amount of starch (35%) and protein (21%) content which reduces the purity of the bran in terms of total dietary fibre content and cause protein and starch loss. Also, the percentage of oil in the bran (12.7%) is reported to be higher than that of the white flour (5.6%), which could be beneficial for downstream fractionation of protein and starch from the canaryseed flour.

Fractionation of canaryseed to obtain starch, protein, oil and dietary fiber is the next step of value addition towards food applications. The only method available in literature to fractionate canaryseed into these components was developed by Abdel-Aal et al. (2010). The method involves dehulling the seeds and milling the groats to prepare whole seed flour to use as the starting material for the fraction process. Then ethanol (E) was used to defat the flour to remove oil. Ethanol extraction was carried out three times at 1:1 w/v flour-to-ethanol ratio and the oil was recovered from the miscella. The ethanol-containing pallet after miscella separation was then mixed with water (W) at 1: 11.25 w/v flour-to-water ratio and homogenized. The homogenized slurry was screened three times using 210 µm (US standard mesh #70) to trap the coarse fibre. The filtrate (fine solids) was mixed with 0.05 N NaOH at 1:10 ratio and performed the alkali extraction (A) for an hour. The authors did not mention the pH that the slurry obtained after mixing with NaOH. The protein extract and the solids (starch & fine fibre) were separated, and the isoelectric precipitation (pH 4) was carried out to isolate the proteins from the extract using 4N HCl. The fine solids were screened using 52 µm (US standard mesh #270) to separate the fine fibre (retentate) and the starch (filtrate). The filtrate was centrifuged to separate the starch. The starch was washed twice with 95% ethanol and dried with acetone to obtain highly purified starch. Depending on the sequence of the solutions used, this method was named EWA method. The authors changed the sequence of the solutions and modified the method to have two more methods, namely EWWA and EAW. There were no significant differences between the purity of the starch obtained using these three methods. However, EWWA method provided the lowest starch yield. Also, the EWWA method provided the lowest protein purity and yield. The EWA method provided the highest protein purity (82.7%) followed by EAW (78.9%), albeit their yield was not significantly different. All three methods resulted same oil recovery with 100% purity. In terms of dietary fiber, the EWWA showed the highest yield with lowest purity. The dietary fiber yield was higher in EWA method than the EAW method. Opposite was observed for the dietary fiber purity. The focus of this fractionation process development was to obtain an optimized process for starch recovery and purity (Achouri et al., 2020). Therefore, Achouri et al. (2020) selected the EAW process and further optimized for protein fractionation. The authors used pH 12 and pH 5 as the optimized conditions for protein extraction and precipitation to produce a protein isolate. The resulting isolate contained 91-93% protein purity with 65-69% protein recovery, which is favorable for commercial production of canaryseed protein isolate.

2.5 Wet fractionation of proteins

Wet fractionation generally refers to aqueous-based separation and purification of proteins from other compounds, such as starch, fibre, lipids, sugars etc. in the seed. Osborne classification (Osborne, 1924) is the classical method of wet fractionation. This method isolate proteins into water-soluble albumin, salt-soluble globulin, aqueous-alcohol-soluble prolamins and weakly-acid/base-soluble glutelin. However, this method is not used in the industrial scale. Alkali extraction and isoelectric precipitation is the most widely used protein wet fractionation method that generally provides protein with >70% purity (Han & Hamaker, 2002). In this method protein from the flour/meal is solubilized at alkaline pH conditions and precipitate at their isoelectric pH to separate from the other extracted material. The precipitated protein is then dried (*e.g.* spray drying) to prepare protein isolates (Hu et al., 2010; Singhal et al., 2016). This method is commonly used to prepare protein isolates from soy, pulses, oilseeds and some cereals, such as oat, where albumins and globulins are the major storage proteins (Mäkinen et al. 2017; Singhal et al., 2016; Wanasundara, 2011). As discussed in section 2.4, alkali extraction and isoelectric precipitation was used to prepare canaryseed protein isolate from canaryseed (Abdel-Aal et al., 2010; Achouri et al., 2020). Moura et al. (2020) also used alkali extraction (pH 9) and isoelectric precipitation (pH 5) to prepare protein isolates from canaryseed. The authors were able to produce a protein isolate with 86% purity. However, the yield and the protein recovery were significantly low.

Membrane filtration (ultrafiltration/diafiltration) is another technique used in protein wet fractionation of protein in which pressure used as the driving force for protein separation (Singhal et al., 2016). The proteins extracted at alkaline pH could be purified using ultrafiltration alone or together with diafiltration using desired molecular weight cut-off (MWCO, *e.g.* 5 kDa, 10 kDa etc.) by removing compounds lower than the MWCO. Generally, the protein concentrates or isolates prepared using ultrafiltration shows good functional properties than the other methods (Fredrikson et al., 2001; Fuhrmeister & Meuser, 2003). Moreover, the extracted proteins could be isolated by changing their solubility using salts, known as salting-out method (Singhal et al., 2016). Additional washing steps or a membrane filtration step should be incorporated into this process to remove the salts and associate purity and flavor issues.

Ethanol leaching and acid leaching is another wet fractionation method especially used in soy industry to prepare soy protein concentrates (Ma, 2015). In the ethanol leaching process, aqueous ethanol (~60-70%) is used to wash the meal (Liu, 1997). The soluble sugars and some of

soluble proteins are washed off with ethanol while the rest of protein remain insoluble due to denaturation (Johnson, 1999). This process may improve the flavor characteristics due to removal of undesirable flavor compounds with the ethanol (Peter, 2018). However, the solubility and other functional properties may compromise due to denaturation. The acid leaching process involves washing the soluble non-protein compounds at the acidic pH where the isoelectric point of the protein lies. Since the protein shows minimum solubility at the isoelectric pH, most of the proteins remain insoluble while some of the acid-soluble proteins are lost to the waste stream. This may cause higher protein losses than that of ethanol leaching process (Peter, 2018).

Since most of the cereals contain prolamin as the major storage protein and it is different form the albumins and globulins, alkali extraction and isoelectric precipitation may not be ideal method to fractionate the proteins. However, this method has been reported in literature for some cereals where prolamin is the major storage protein, such as canaryseed (Abdel-Aal et al., 2010; Achouri et al., 2020; Bean et al., 2006; De Mesa-Stonestreet et al., 2010; Moura et al., 2020). Bean et al. (2006) used aqueous ethanol (70%) to extract the prolamin from sorghum (which was previous tested on maize) and precipitated the proteins at pH 2.5. The results showed lower yield and purity. It was necessary to add reducing agents during extraction and lower the ethanol concentration prior to precipitate proteins (~30%) to improve protein purity. Another method that has been used to wet-fractionate cereal proteins is utilizing enzymes to digest starch and cell wall material to produce soluble syrup and recover insoluble protein fraction that could result in higher protein purity (Amagliani et al., 2017).

The commonly known method used for protein fractionation in corn is wet milling. Wet milling of corn involves two-stage coarse milling of steeped corn (pre-treated using SO_2) to crack the kernel to detach the oil-containing germ from the from the endosperm and the seed coat (Jackson & Shandera, 1995; Rausch et al. 2019). The germ is then separated from the rest using liquid hydrocyclone system. The de-germed slurry is then screened to separate fibre from the starch and protein and further milled (Fine milling). After fine milling the starch and fine fibre is separated using screens and the filtrate (starch stream) is pass through a nozzle-bowl centrifuge to obtain gluten protein fraction (light phase) and starch fraction (heavy phase). The gluten protein fraction is subjected to vacuum evaporation and drying to prepare gluten meal whereas, the starch is further purified using hydrocyclones (Rausch et al., 2019). Wet fractionation of wheat is different from the corn processes. The conventional methods involve mixing wheat flour with

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water to prepare a dough or a batter as the first step (Sayaslan, 2004). Both these methods allow gluten proteins to hydrate, and then form a dough in the former case or protein aggregates in the latter case, which is then separated from starch by washing and screening. The gluten protein remains on top the screens and dried to prepare protein products. The starch is then separated from fibre by screening and purified using hydrocyclones (Sayaslan, 2004). The non-conventional methods include preparation of flour-water dispersion using shear mixing and separate gluten proteins aggregates using centrifuge or hydrocyclones. The starch fraction is further purified using screening and followed by series of hydrocyclones and drying (Sayaslan, 2004).

There are number of protein wet fractionation methods for cereals available in the literature and used in the commercial-scale processing. Each method has unique features that caters to achieve the targeted final protein product with desired purity, yield, organoleptic properties, functionality and nutrition. Therefore, learnings from these various methods/techniques are important when designing a commercial scale process for canaryseed protein ingredient development for value additional towards food applications.

2.6 Functional properties of canaryseed protein products

There is limited literature available on the functional properties of the proteins in canaryseed flour and protein isolates. Achouri et al. (2020) evaluated the emulsifying, foaming, water and oil holding capacities and solubility of the protein isolates (90.3-91.3% protein) prepared from modified and optimized EAW method in comparison to ethanol defatted soy protein isolate. The results showed that the canaryseed protein isolate has higher emulsifying and foaming capacities than that of the soy isolate. Canaryseed protein isolate showed good water and oil holding properties; however, they were lower than that of the soy isolate. Protein isolates from the yellow seeds demonstrate better emulsifying and foaming ability than that of brown seeds. The oil and water holding capacities between these two varieties were similar. Canaryseed protein isolate showed higher solubility in acidic region than that of alkaline region. The maximum solubility was <50.4% at pH 2. The minimum solubility was observed at pH 5 for both brown and yellow seeds. Overall, the solubility of the canaryseed protein isolate was lower than that of the ethanol treated soy protein isolate. Moura et al. (2020) evaluated the functional properties of canaryseed flour and protein isolates. The isolates shared similar functionality trend as observed in Achouri et al. (2020). However, the solubility of the protein isolates at neutral pH reported in Moura et al. (2020) was

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significantly higher than the solubility reported in Achouri et al. (2020). Presumably, due to the compositional differences in the protein isolates resulted from the different processing conditions used in these two studies. Most of the functional properties of canaryseed protein isolates reported in Moura et al. (2020) is superior to that of canaryseed flour, except the foaming stability at neutral and isoelectric pH. Moreover, the authors did not find significant differences of functional properties between brown and yellow canaryseed proteins, which is in agreement with Achouri et al. (2020). The use of canaryseed flour in different baked goods, pasta, breakfast cereal and snack food have been studied (Patterson et al., 2018). The maximum usage levels of canaryseed flour in these products ranged from 15-50% depending on the food type. For most of the food the acceptable inclusion levels were 15-25% (Patterson et al., 2018).

2.7 Protein quality of canaryseed flour and protein isolates

Protein quality generally refers to digestibility of a protein and availability of the essential amino acids measured using *in vitro* or *in vivo* methods (Bai, 2018). The benchtop enzymatic digestion of protein (*in vitro*) is cheap, quick, simple compared to that of using animal models (*in vivo*) and therefore acceptable for initial evaluation of protein quality. However, for better understanding of the protein quality and for regulatory purposes, *in vivo* methods, such as Protein Digestibility Corrected Amino Acid Score (PDCAAS), Digestible Indispensable Amino Acid Score (DIAAS), Protein Efficiency Ratio (PER), Net protein ratio etc. is used (Adhikari et al., 2022; Bai, 2018; Nosworthy et al., 2018). The PDCAAS and DIAAS method stands out than other methods as it considers protein utilization at amino acid level; hence, they are widely accepted than the other methods (Adhikari et al., 2022). The DIAAS method was introduced to address the shortcomings of the PDCAAS method to provide more comprehensive method of protein quality measurements. However, PDCAAS methods has been widely applied than DIAAS due to longer history of use (Adhikari et al., 2022)

Similar to the protein functionality, the information available for canaryseed protein quality is limited. An *in vitro* gastrointestinal digestibility evaluation of canaryseed flour showed that it is easily digested under sequential gastric-duodenal conditions than digestion take place in gastric or duodenal conditions separately. Also, thermal processing (roasting) found to be more effective for protein digestion than that of raw flour (Rajamohamed et al., 2013). Several *in vivo* studies carried out using rodent, swine and poultry models suggested that incorporation of

canaryseed (dehulled) into their diets at same levels of wheat has no negative impact on growth performance and was as good or as better than that of wheat. (*GRAS Notice (GRN) No. 529*, 2015; Magnuson et al., 2014; Newkirk et al., 2011; Thacker, 2003). Moura et al. (2020) evaluated the protein quality of canaryseed flour and protein isolate using *in vitro* PDCAAS method and found that the flour from both yellow and brown seed has ~29-31% digestibility whereas, it was ~55-64% for the protein isolates. Similar PDCAAS values for canaryseed flour was reported elsewhere (Mason, 2019). Lysine found to be the limiting amino acid for canaryseed protein (Moura et al., 2020; Mason et al., 2019)

3. MICROSTRUCTURE AND DISTRIBUTION OF OIL, PROTEIN, AND STARCH IN DIFFERENT COMPARTMENTS OF CANARYSEED (*Phalaris canariensis* L.)[1]

3.1 Abstract

Background and objectives: The aim of this study was to understand seed microstructure and oil, protein, and starch distribution in brown and yellow canaryseed that could provide an understanding of potential use of solvent-free technologies for protein fractionation purposes.

Findings: Varying thickness of the bran was observed from the ventral to the dorsal side of the seed. The aleurone layer is mostly a single-cell layer; however, it could occasionally be a double-cell layer. The germ, endosperm, and aleurone layer contain oil. However, more oil is distributed in the endosperm area (3.88 g of oil in roller-milled white flour) than in the germ and aleurone layer (2.83 g of oil in roller-milled bran fraction). The endosperm also contains varying sizes of compound starch granules (~4‑7 μm to 20 μm) and individual starch granules (~2‑4 μm). Protein is widely distributed in the endosperm compared to the germ, aleurone layer, and bran.

Conclusions: Microstructural differences in different regions of the canaryseed were observed. The presence of higher oil content in the endosperm suggests that the application of solvent-free technologies could be challenging for protein fractionation.

Significance and novelty: This study revealed microstructural and chemical differences in different regions of the seed. Especially, it showed the spatial distribution of oil, which is crucial in designing industrial processes, for "clean label" protein ingredients.

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[1] Perera, S. P., Hucl, P., L'Hocine, L., & Nickerson, M. T. (2021). Microstructure and distribution of oil, protein, and starch in different compartments of canaryseed (*Phalaris canariensis* L.). *Cereal Chemistry*, 98(2), 405–422. Copyright © 2020 Cereals & Grains Association.

3.2 Introduction

Cereals (Family *Poaceae*) have been a major part of the human diet, throughout the world, over many centuries (Awika, 2011). Although there are numerous types of cereals grown in the world, the most common cereal grains include wheat, corn, rice, barley, rye, oats, sorghum and millet (Awika, 2011; Papageorgiou & Skendi, 2018). In 2018/2019, the estimated total cereal production in the world was 2.653 billion tonnes (Food & Agriculture Organization of the United States [FAO], 2020), where corn, wheat and rice accounted for over two thirds of the production (United States Department of Agriculture [USDA], 2020a, 2020b). Cereals contain approximately 75% carbohydrates, predominantly in the form of starches (Laskowski et al., 2019), followed by protein, dietary fiber and lipids (Tacer-Caba et al., 2014), which are all considered important sources of macronutrients for the human diet. However, due to the relatively low protein content (6%‑15%) and extensive starch utilization, cereals are not well promoted as a plant protein source compared to pulses (~21‑>30% protein; *e.g.,* pea, lentil, faba bean etc.; Heeg, 2016, Singh, 2017) or oilseeds (~19%‑30% protein; *e.g.,* flax, canola, hemp etc.; Callaway, 2004, Martinchik et al., 2012, Wanasundara et al., 2016).

Plant proteins have gained attention from the food industry as a sustainable protein source compared to that of animal meat (Henchion et al., 2017; van der Weele et al., 2019). Moreover, the increasing consumer trend toward flexitarian, vegetarian and vegan diets (Curtain & Grafenauer, 2019; Pojić et al., 2018) and ethical issues of killing animals for meat (Henchion et al., 2017) have inspired the industry to explore alternate plant protein sources to meet growing protein demands. The food industry is currently searching for various sustainable plant protein sources and are exploring the potential of some cereals, such as oats, sorghum and millet in addition to some pseudocereals [*i.e.,* non-*Poaceae* grains that share a similar chemical composition to that of *Poaceae* cereals, such as quinoa, buckwheat and amaranth (Fletcher, 2016; Thies, 2017)]. A recent addition to the cereal group is canaryseed (*Phalaris canariensis* L.), which is a true cereal belonging to the family *Poaceae*. Canaryseed contains ~19%‑23% protein (Mason et al., 2018; Patterson et al., 2018) which is higher than that of other cereals, or pseudocereals, commonly available in the market (Bekkering & Tian, 2019; Patterson et al., 2018). Canada, especially the province of Saskatchewan, is the leading producer and exporter of canaryseed in the world (Patterson et al., 2018; Canaryseed Development Commission of Saskatchewan, n.d. -a). The

primary market for this cereal grain has been the bird seed industry (Abdel-Aal et al., 2010; Canaryseed Development Commission of Saskatchewan, n.d. -a). However, hairless (glabrous) canaryseed varieties, devoid of siliceous trichomes (hairs) that are toxic to human beings, were recently approved by the US Food and Drug Administration Agency and Health Canada for human consumption (Health Canada, 2016; Patterson et al., 2018). The higher protein content, potential to produce bioactive peptides and gluten-free nature, provides potential for the increased use of canaryseed in the food industry (Mason et al., 2018) as a plant protein source over other conventional cereal grain.

Understanding canaryseed structure and the distribution of the chemical constituents, especially protein, starch, oil and fiber within the seed is essential to designing economically viable and environmentally friendly processes to fractionate proteins. Consequently, these fractions could be introduced as "clean labeled" protein ingredients at a lower and competitive cost compared to other protein ingredients available in the market. Currently, there are limited studies available on canaryseed microstructure and chemical distribution within the seed.

Canaryseed, like other cereal grains, consists of a germ, endosperm, and bran (Abdel-Aal et al., 2011a). The bright field microscopy evaluation of canaryseed microstructure carried out by Abdel-Aal et al. (2011a) showed the presence of compound starch granules of varying size, similar to that of oats, embedded in a protein matrix of the starchy endosperm. Moreover, the presence of small protein bodies was also observed, which is unusual for other endosperm proteins found in nature. Abdel-Aal et al. (2011a) also reported that the aleurone layer consist of proteins, which are chemically different than that of the endosperm proteins. The studies carried out by Abdel-Aal et al. (2011a) and Abdel-Aal et al. (1997a), which evaluated the chemical composition of roller-milled canaryseed bran fractions, suggested that the seed-coat material, germ, and the aleurone layer contained a major proportion of the crude fat (~13%) compared to that of the endosperm (white) flour (~6%). However, detailed information on the microstructure of the germ and on the distribution of oil in the seed is lacking. Therefore, this study was carried out to further investigate the microstructure of the canaryseed and the distribution of oil, protein, and starch to address existing gaps in the current knowledge. This information is needed to identify the spatial distribution of the oil in the seed to design effective canaryseed components fractionation using solvent-free green technologies.

3.3 Materials and Methods

3.3.1 Materials

Canaryseed (dehulled) *var*. CDC Calvi (brown seed) and *var*. CDC Cibo (yellow seed) grown in Saskatchewan (SK), Canada, were utilized for this study. Seeds were kindly donated by Clancy Seeds Ltd. (Carrot River, SK, Canada) and from a local producer. All chemicals and reagents were of analytical grade and purchased from Sigma-Aldrich Canada Ltd., VWR International Inc., or otherwise mentioned in the text.

3.3.2 Scanning Electron Microscopy (SEM)

Seeds were cut into two halves along the mid-longitudinal axis using a razor blade. The seeds were then coated with gold at 10 nm thickness using a Q150T ES sample preparation system (Quorum Technologies Ltd.) and examined using a field-emission scanning electron microscope (SEM, SU8010; Hitachi High Technologies Canada Inc.). Different areas of the bran, endosperm, and the germ were observed under different magnifications ranging from $\times 300$‑4,000 as necessary for clear visualization of the structural characteristics of the respective areas. SEM image acquisition of three separate seeds of both brown and yellow seeds was carried out. The images were analyzed using ImageJ (version 1.52a,https://imagej.nih.gov/ij/).

3.3.3 Raman microscopy

3.3.3.1 Preparation of reference material

De-oiling canaryseed

Canaryseed was mixed with hexane at 1:3 w/v ratio and ground for 5 min using an explosion-proof blender (E8010—heavy duty blender, Eberbach Corporation). The resulting slurry was transferred into a beaker and the blender was washed with two parts of hexane and mixed with the slurry to recover maximum solid content. The final ratio of the canaryseed-to-hexane was 1:5 w/v. The slurry was then vacuum-filtered using a Whatman #1 filter paper and the filtrate was rotavaped (Buchi R-124, Buchi AG) at 50 °C to recover the oil. The oil recovered was centrifuged at $20,817 \times g$ for 5 min (Eppendorf Centrifuge 5417C, Brinkmann Industries (Canada) Ltd.) and the supernatant, devoid of the sediments, was recovered as purified oil fraction. The purified oil was transferred into a dark glass vial, purged with nitrogen and stored at 4 °C to prevent

lipid oxidation until analysis. The de-oiled meal was air dried under a fume hood to evaporate any residual hexane and then used for the Osborne fractionation of proteins.

Osborne fractionation

The de-oiled meal was used to fractionate the proteins into albumin, globulin, prolamin, and glutelin using the Osborne classification (Osborne, 1924) as described by Ju et al. (2001) with modifications. Briefly, the de-oiled meal was extracted with 1:4 w/v meal-to-distilled water ratio for one hour at ambient temperature. The mixture was then centrifuged at $6,000 \times g$ for 15 min (TJ-25 centrifuge; Beckman Coulter Life Sciences) and the supernatant containing albumin protein was vacuum-filtered. The pellet was re-extracted with the original volume of water and the two supernatants were combined and stored at 4 °C until further use. The post water-extracted pellet was extracted two more times with a 5% sodium chloride solution using the same conditions that were used to obtain the globulin proteins. The combined supernatant of the globulin protein was also stored at 4 °C. The remaining pellet was extracted twice at alkali pH (adjusted to pH 11 using 50% w/w sodium hydroxide) under the same conditions used to extract the glutelin proteins and stored at 4 °C. Finally, the pellet was extracted twice with a 70% ethanol solution under the same conditions that were used to extract the prolamins and stored at 4 °C until further use.

The albumins, globulins, and glutelins were precipitated at their isoelectric pH (pI) using the maximum turbidity method as described in Ju et al. (2001). Briefly, the pH of 10 mL aliquots of the combined supernatants of albumin, globulin, and glutelin fractions were adjusted to pH 2, 3, 4, 5, 6, 8, and 10 using 10% v/v HCl or 5% w/v NaOH and the turbidity (optical density) at each pH level was monitored at 320 nm using the UV-Vis spectrophotometer (Evolution™ 201; Thermo Fisher Scientific). The maximum turbidity was observed at pHs 4, 3, and 4 for albumin, globulin, and glutelin, respectively. Therefore, those pHs were selected to precipitate the corresponding protein fraction from the combined supernatant. To precipitate, the supernatants were allowed to settle for 25 min after adjusting into their pI and centrifuged for 15 min at $3,300 \times g$ (J6-MI centrifuge; Beckman Coulter Life Sciences). The supernatants were discarded, and the pellets were washed with water at 1:2 w/w wet pellet-to-water ratio for 15 min at pI and centrifuged. The clear supernatants were discarded, and the washed pellet was adjusted to pH 7 with 10 mL of distilled water. The pellets were lyophilized (Labconco FreeZoneR 6L Freeze Dryer System, Labconco Corp.) to obtain the final protein fraction for analysis. The prolamin fraction was precipitated with

four volumes of cold acetone at −20 °C, washed with water and adjusted to pH 7 and lyophilized. The lyophilized protein fractions were stored at 4 °C until further use.

Starch purification

The residual meal obtained following the Osborne fractionation was washed sequentially with water, 95% ethanol and dried with acetone to obtain starch (Abdel-Aal et al., 2010). Briefly, the meal was washed with distilled water at 1:4 w/v ratio and sieved with #60 (250 μm), #100 (150 μm), #200 (74 μm), and #325 (44 μm) US standard sieves to remove the bran particles. The filtrate containing starch was centrifuged at $3,300 \times g$ (J6-MI centrifuge; Beckman Coulter Life Sciences) and the pellet was recovered. The pellet was then washed twice with 95% ethanol at 1:2 w/v pellet-to-ethanol ratio and dried with acetone to obtain purified starch. The extractions were carried out in triplicates.

3.3.3.2 Raman spectral acquisition of reference samples

The oil, protein (albumin, globulin, prolamin, and glutelin), and starch purified from the seed were used as the reference material for the Raman spectral analysis. A minute amount of these samples was loaded on to a gold plate (~4×3 mm) to take the measurement using the InVia Reflex Raman Microscope (Renishaw Plc.) equipped with a light microscope (Leica DC2500M, Leica Camera AG). The reference samples were observed under $50 \times$ long-working-distance objective lens [$50 \times$ LWD; 0.40 numerical aperture (NA)] to collect the data. To visualize Raman scattering, a 785 nm solid-state laser was used. Prior to data collection, the internal silicon check was performed to verify the accuracy of the spectral positioning. The Raman shift was monitored between $100\text{-}3500$ cm^{-1} with 1,200 line/mm (633/780) laser grating at 1%‑100% laser power, 1 μm laser spot size and 10 s exposure time. The laser power that provided the best signal-to-noise ratio without saturating the detector (Renishaw CCD camera) due to fluorescence was selected and $4\text{-}16$ accumulative scans were performed, as necessary, with the cosmic ray removal option to reduce the noise and improve the signal of the final spectrum of the sample. After the final spectra were obtained, a baseline correction was performed using the Wire™ 3.3 software (Renishaw Plc.) using cubic spline interpolation method. Prior to spectral acquisition, the albumins, globulins, and glutelins were treated with 17% H_2O_2 to avoid fluorescence due to the color of the protein. Briefly, the proteins and H_2O_2 were mixed at a 1:2 w/w ratio in a microcentrifuge tube and vortexed for 2

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min to bleach the color of the proteins. They were then centrifuged at 20, 817 $\times$ g for 5 min and the supernatant was decanted. The sediment was mixed with reverse osmosis (RO) water at the same ratio and repeat the process to wash off the residual H_2O_2 present in the sediment. Finally, the washed sediment was used for the Raman spectral acquisition.

3.3.3.3 Raman spectral acquisition of canaryseed germ, endosperm, aleurone layer, and bran

To obtain the Raman spectra of the internal components of canaryseed, *that is,* germ, endosperm, aleurone layer, and bran, the seeds were cut into halves, using a razor blade, along the mid-longitudinal line of the seed. Then, using the $50 \times$ LWD or $20 \times$ objective lens (NA = 0.40) as necessary, the spectra were separately collected from the germ, endosperm, aleurone layer, and bran, similar to that of the above-mentioned reference samples. For each component, Raman spectra were collected from three different random spots. The fluorescence from the aleurone layer and the bran of the canaryseed was too bright and saturated for the detector, even at 1% laser power. Therefore, the spots from the aleurone layer and the bran of the yellow seed were bleached, using the laser, for 1‑5 min as necessary to reduce the fluorescence and obtain the spectra. However, the aleurone layer and the bran of the brown seed did not produce lower fluorescence even after bleaching for 5 min. Hence, the spectra for those two components of the brown seeds were not obtained since further bleaching may greatly affect the inherent bond vibrations of the molecules. After acquiring the Raman spectra, the analyses were performed as described in the reference sample section.

3.3.4 Abrasive milling and roller milling of canaryseed

To confirm the distribution of oil in canaryseed, abrasive milling and roller milling were performed to remove and/or separate the bran and germ from the seed. First, canaryseed was debranned using an abrasive mill (Satake TM05, Satake Corporation) until complete or most of the germ was removed from the seed along with the bran. Removal of the bran and the germ was evaluated and confirmed using SEM. In parallel, canaryseed was tempered to 13% moisture content, roller milled (Bradender Quadrumat Jr. mill, Brabender Instruments, Inc.) and sieved (250 µm mesh) to produce bran and endosperm (white) flour fractions. The debranned seed and the roller-milled fractions were analyzed for moisture and protein using the American Association of Cereal Chemists (AACC) approved methods of analysis 44-15.02 (AACC, 2010) and 46-30.01

(AACC, 2010), respectively. The oil content was analyzed using the Swedish tube method (AM 2-93) of the American Oil Chemists Society (AOCS, 1997). Both abrasive milling and roller milling were performed in triplicates. The general linear model procedure was used to evaluate the effect of milling on the oil-content distribution in the bran and debranned seed/flour fractions followed by the Tukey's test procedure for mean separation using R statistical software, version 3.6.3 (https://cran.r-proje ct.org/).

3.4 Results and Discussion

Canaryseed is a small (~4‑5 mm long and ~1.5‑2 mm wide) elliptical seed (Abdel-Aal et al., 1997c) either brown or yellow in color (Figure 3.1). It is composed of a bran, germ (embryo), and a starchy endosperm (Figure 2), which is a typical characteristic of seeds from plant family *Poaceae* (Abdel-Aal et al., 2011a; Holopainen-Mantila & Raulio, 2016). Bran, in general, is the outer layer of the cereal grain that covers the entire endosperm and the germ and is rich in fiber and phytochemicals (Hoseney & Delcour, 2010a; Kim & Han, 2014). The presence of numerous phytochemicals greatly influences the grain color (Lachman et al., 2017), which in fact differentiate the brown and yellow canaryseed varieties (Figure 3.1). Since both brown and yellow seeds are similar in size and shape, the obvious physical property that these two could be separated by, with the naked eye, is the color of the bran. The ImageJ analysis of the cut seeds showed that the germ covers approximately 8%‑9% of the entire endosperm area of the brown seed, whereas it covers 10%‑12% of the yellow seed (Figure 3.2). Therefore, the starchy endosperm of the brown seed occupies slightly more area (~1%‑3%) compared to that of the yellow seed. To obtain a better understanding of the structure of these seed components, the mid-longitudinal sections of the seeds were further evaluated using SEM with different magnifications.

3.4.1 Structural features of the bran, aleurone layer, germ, and the endosperm

The bran of canaryseed (brown and yellow) is composed of several cell layers (Figure 3.3a, b), which is a characteristic of a typical cereal bran. The cereal bran consists of a pericarp (*i.e.,* ovary wall firmly connected to the seed coat containing epidermis, hypodermis, intermediate cells, cross cells, and tube cells), seed coat (testa), nucellar tissue, and aleurone layer (the edge of the endosperm often separated with the bran after milling) (Evers & Millar, 2002; Hoseney &

Delcour, 2010a). Although the multilayer characteristic of the bran was evident in canaryseed (Figure 3.3 a, b), it was difficult to clearly identify the above-mentioned individual layers of the bran. It was noticed that the bran (except for the aleurone layer) on the ventral side of the seed was generally thick (~20–32 μm) and loosely packed (Figure 3a, b), whereas it was thin (~8–12 μm) and tightly packed on the dorsal side (Figure 3.3 c, d). However, the thickness of the bran layer on the ventral side gradually decreased as it moved away from the middle to the ends of the seed (Figures A1 and A2).
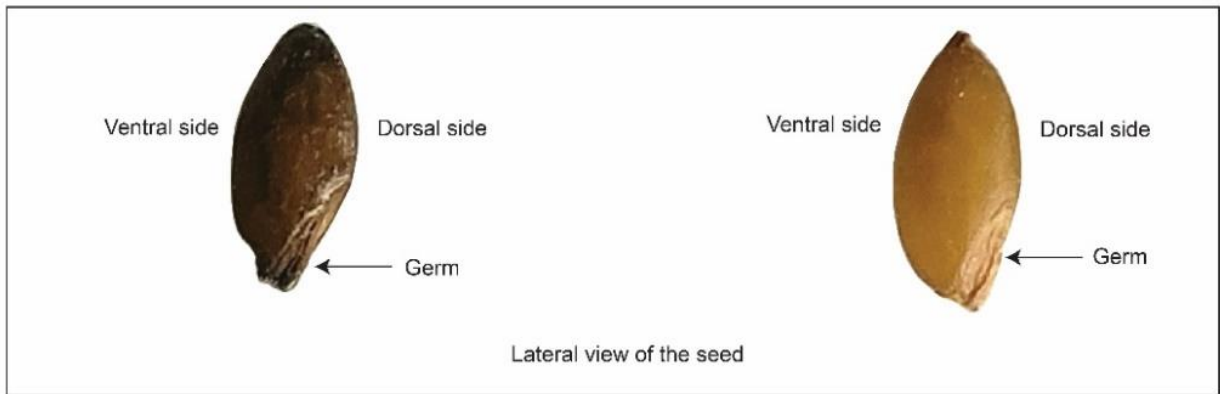
Figure 3.1 Physical appearance of brown (CDC Calvi, left) and yellow (CDC Cibo, right) canaryseed. Images were obtained using a Cannon® EOS Rebel T7 camera attached with CannonR EFS18-55 mm lens.
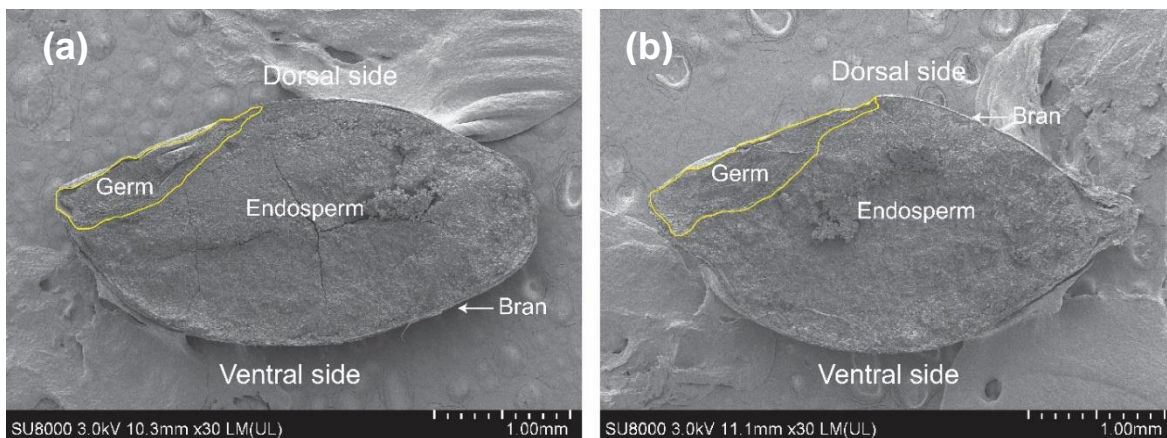
Figure 3.2 SEM images of mid longitudinal section of a canaryseed utilized for SEM and Raman microscopy. (a) Brown seed; (b) Yellow seed.
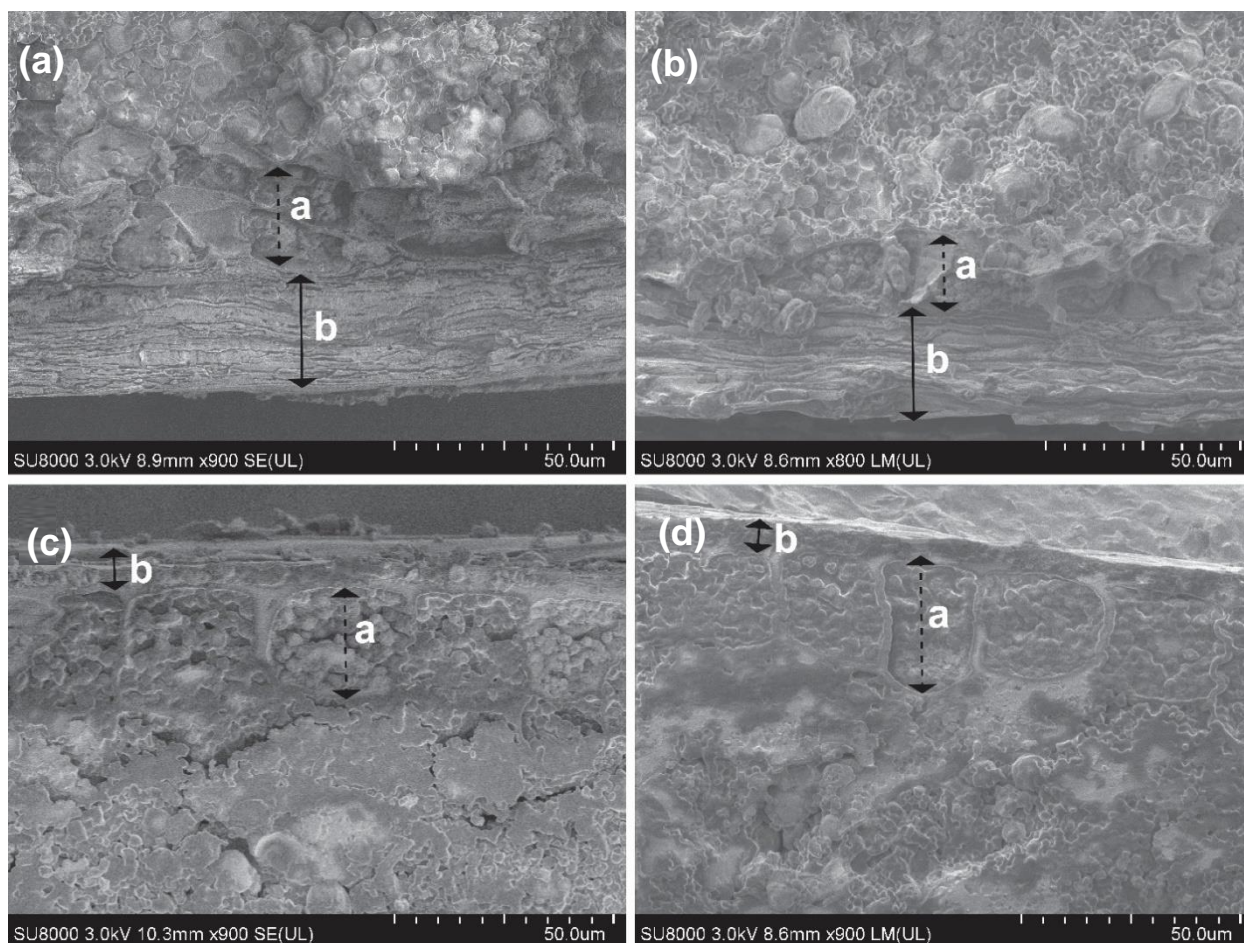
Figure 3.3 SEM images of the brown and yellow canaryseed bran. (a) Ventral side of brown seed. (b) Ventral side of yellow seed; (c) Dorsal side of brown seed; (d) Dorsal side of yellow seed. a = aleurone layer; b = bran.

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The aleurone layer of the canaryseed was mainly made of a cuboid-shaped single-cell layer (Figure 3.4), with an occasional two-cell layer in the ventral area (Figure 3.4b). The aleurone layer of a cereal grain could be a single (*e.g.,* wheat, corn) or multi-layered (*e.g.,* barley, rice) cell structure (Evers & Millar, 2002; Stone, 1996). In some rice varieties, the aleurone layer contains a mix of single to multiple cell layers depending on the location of the seed (Kasem et al., 2011). Although it is occasionally possible to observe a two-cell layer of aleurone cells in canaryseed, it was not as distinct as in the rice grain observed by Kasem et al. (2011). The aleurone cells contain aleurone grains (Figure 3.4c) that are mainly considered to be protein bodies (Garcia-Lara et al., 2019; Saulnier et al., 2007; Srivastava, 2002). These grains also could be lipid droplets (spherosomes) or phytin bodies that store the phytates in the seed (Saulnier et al., 2007; Watson, 2003). Garcia-Lara et al. (2019) reported that the aleurone protein bodies in corn are different from the proteins in the endosperm. The same was observed in canaryseed (Abdel-Aal et al., 2011a). Abdel-Aal et al. (2011a) suggested that the presence of chemically different proteins in the canaryseed aleurone layer compared to that of the endosperm is due to the observed differences in protein staining. Also, the presence of phytin bodies in the aleurone cells was revealed in this study. The Raman microscopic evaluations in the current study showed the presence of both protein and oil in the aleurone layer, which will be discussed later in this paper. Therefore, it could be suggested that canaryseed aleurone cells are composed of protein, lipid, and phytin bodies, although the SEM images are not capable of distinguishing each type of aleurone grain separately.
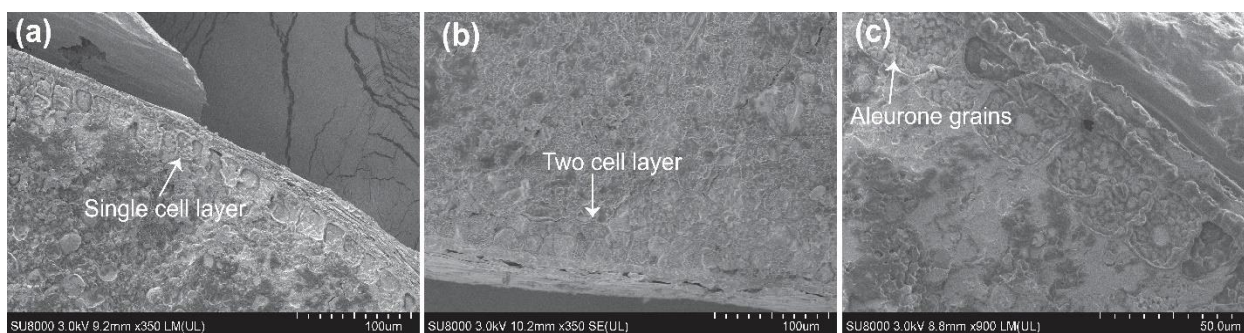
Figure 3.4 Representative SEM images of the canaryseed aleurone layer. (a) Image showing single aleurone cell layer; (b) Image showing two aleurone cell layers; (c) Image showing aleurone grains.

The endosperm of canaryseed is mainly composed of compound starch granules, which are embedded in a protein network (Figure 3.5; Abdel-Aal et al., 2011a). These granules show a round to oval shape (Figure 3.5a, b). Length of the compound starch granules vary significantly from approximately 4–7 μm to approximately 20 μm. The compound granules entangled in the protein matrix and have a crack-like appearance on the surface that demarcate the margins of the individual starch granules available inside the compound granules (Figure 3.5c, f). In addition to the compound granules, starch also presents as individual granules in canaryseed (Figure 5b, c, f; Abdel-Aal et al., 2011a). The individual starch granules are polygonal in shape, have sharp edges, and are ~2–4 μm in size. Polygonal shaped starch granules in purified canaryseed starch, with an average size of ~2.6 μm, have also been previously reported (Abdel-Aal et al., 1997a; Irani et al., 2017). The presence of protein bodies in the endosperm was first reported by Abdel-Aal et al. (2011a). The SEM images also show round-shaped structures that arise as a part of the protein network (Figure 3.5c), presumably these are the protein bodies in the protein network (Figure 3.5b, c, e). Both brown and yellow canaryseed share these characteristics of the endosperm and starch granules and no noticeable differences were observed.

The germ (embryo) of the canaryseed is bordered with the starchy endosperm from the inner edge and the aleurone layer from the outer edge (Figure 3.6). It consists of an embryonic axis and a scutellum similar to other cereals (Figure 3.6; Hoseney & Delcour, 2010a; Juliano & Tuano, 2019). The rudimentary roots and shoots of the embryonic axis are clearly visible in both brown and yellow canaryseed (Figure 3.6a, c). The scutellum is known as the food storage organ in the germ (Garcia-Lara et al., 2019; Hoseney & Delcour, 2010a). It contains granules similar to that of aleurone grains (Figure 3.6b, c). In rice, the presence of globoid-rich particles similar to that of aleurone grains was previously reported (Tanaka et al., 1977). In maize, these are mainly identified as the oil bodies (Garcia-Lara et al., 2019). The germ of the wheat kernel has a high concentration of protein and oil (Hoseney & Delcour, 2010a). Therefore, these grain-like organelles, which are also found in canaryseed, possibly contain protein and oil similar to the aleurone grains.
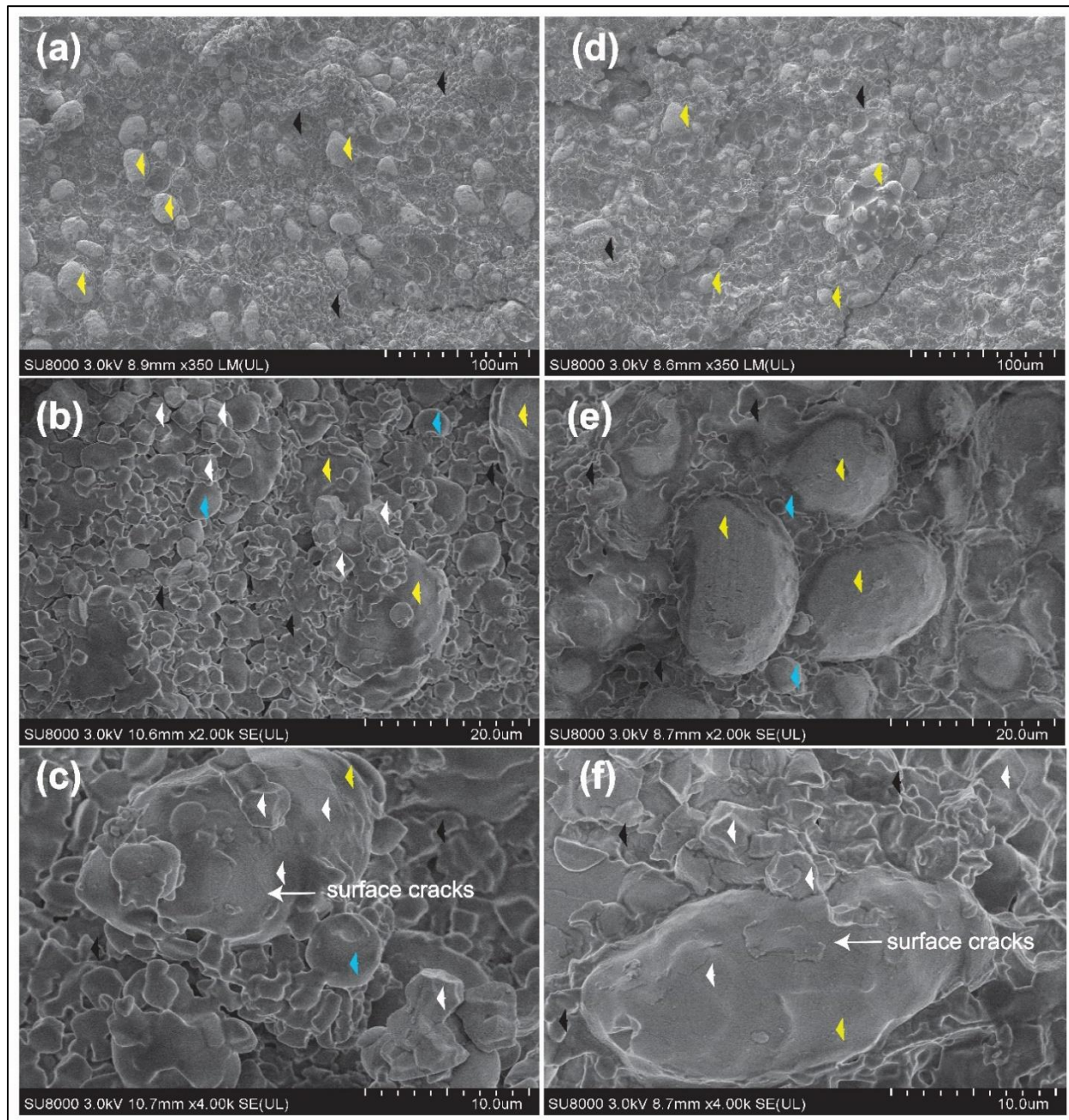
Figure 3.5 SEM images of canaryseed endosperm showing starch granules, protein network and protein bodies. (a-c) brown seed; (d-f) yellow seed. Yellow arrowheads = compound starch granules; black arrowheads = protein network; white arrowheads = individual starch granules; blue arrowheads = protein bodies.
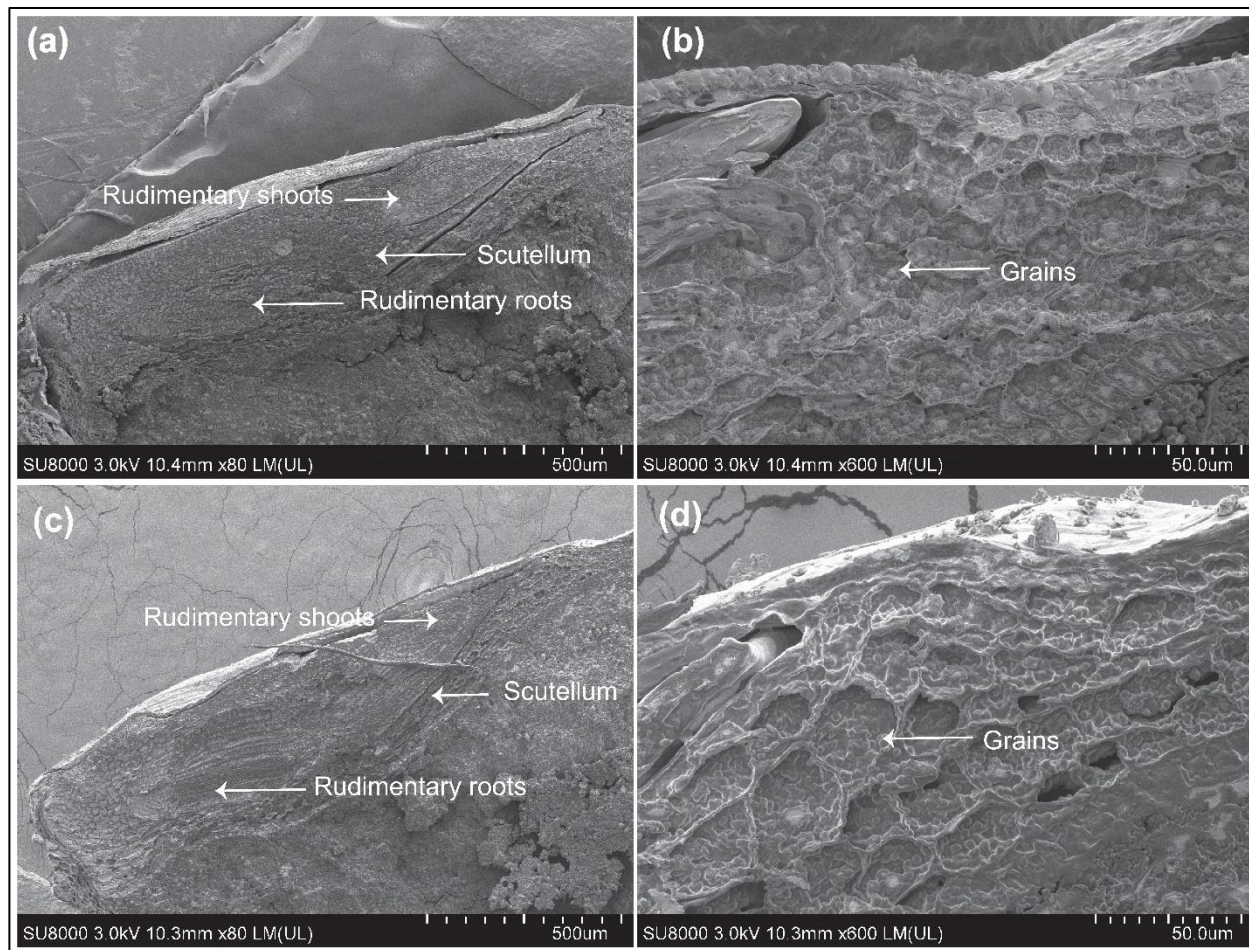
Figure 3.6 SEM images of canaryseed germ. (a) whole germ of the brown seed; (b) scutellum of the brown seed germ showing storage grains; (c) whole germ of the yellow seed; (d) scutellum of the yellow seed germ showing storage grains.

3.4.2 Oil, starch, and protein distribution in the seed using Raman spectroscopy

3.4.2.1 Raman spectra of canaryseed oil

Raman spectroscopy and associated techniques, such as FT-Raman, Confocal-Raman etc., have shown potential in plant-based research and more utilization in cereal science has been found recently (Ellepola et al., 2006; Gierlinger & Schwanninger, 2007; Jaaskelainen et al., 2013; Lee et al., 2014; Ma & Phillips, 2002; Piot et al., 2002; Yang et al., 2018). It detects the vibrations (*e.g.,* stretching, wagging, bending, deformation, etc.) in molecules based on Raman scattering phenomenon that involves exiting a ground-state photon of a molecule using a laser source to a virtual state and monitoring the scattered energy when the photon is returning from the exited state (Gierlinger & Schwanninger, 2007; Ma & Phillips, 2002). Since Raman scattering relies on the changes in the polarizability of functional groups due to molecular vibrations (Gierlinger &

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Schwanninger, 2007; Ma & Phillips, 2002), it provides high intensity bands for nonpolar groups (*e.g.,* C=C, C-C) and show minimum sensitivity to the presence of water-molecules on a sample, which is one of the major advantages over the infrared (IR) spectroscopy (Gierlinger & Schwanninger, 2007; Ma & Phillips, 2002). Moreover, this technique is non-destructive, fast, requires small quantity of a sample, allows samples to be analyzed directly either in wet or dry form and could be highly beneficial to obtain structural and chemical information where IR or staining methods are problematic (De Gelder et al., 2007; Gierlinger & Schwanninger, 2007). These advantages of Raman spectroscopic analyses led us to utilize this technique to evaluate the oil, protein and starch distribution of the seed in situ.

Prior to the evaluation of the seed components (*i.e.,* germ, endosperm, aleurone layer and the bran) the oil, starch and protein isolated from the canaryseed was utilized as reference material to identify the unique Raman bands that allow us to distinguish these chemical constituents from one another inside the seed. The Raman spectra of the reference oil from brown and yellow canaryseed are almost identical and showed several high (strong signal) and low (weak signal) intensity peaks between 200 and 3,100 cm^{-1} region (Figure 3.7a). Several studies have previously been carried out to evaluate the Raman bands related to fat and oils (Baeten et al., 1998; De Gelder et al., 2007; Duraipandian et al., 2019; Sadeghi-Jorabchi et al., 1991; Weng et al., 2003) and other lipids (Anna et al., 2017; Matthews et al., 2010) that provide information to identify the bands related to the reference oils and internal seed components of canaryseed. According to this literature, the bands at ~220 cm^{-1} and ~400 cm^{-1} (Figure 3.7a) appear due to the carbon chain deformation vibrations as it is a feature of the 200-450 cm^{-1} region. The bands within the 700-1,200 cm^{-1} region are typical for C−C skeletal and C−O bond vibrations (Baeten et al., 1998). Specifically, the bands in 800-900 cm^{-1}/1,000-1,100 cm^{-1} obtained due to skeletal C−C bonds (bands at ~842 cm^{-1}, ~868 cm^{-1}, and ~1,077 cm^{-1}, Figure 3.7a) and bands near 1,150-1,160 cm^{-1}/900-970 cm^{-1} obtained due to the C−O bonds (band at ~1,153 cm^{-1} and ~970 cm^{-1}, Figure 3.7a; Baranska et al., 1987). The band at ~1,267 cm^{-1} (Figure 3.7a) is due to the =C−H deformation vibration of unconjugated *cis* double bond (Baeten et al., 1998) and could be a result of a *cis* monoene (Sadeghi-Jorabchi et al., 1991). The band at ~1,299 cm^{-1} is from a methylene twisting
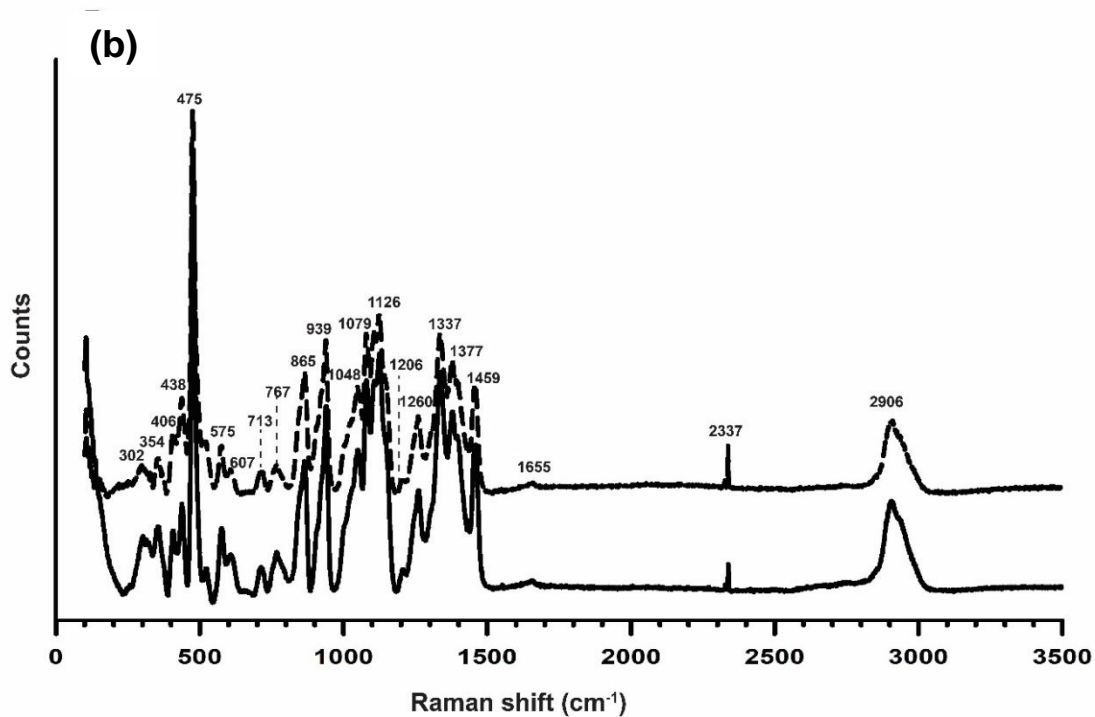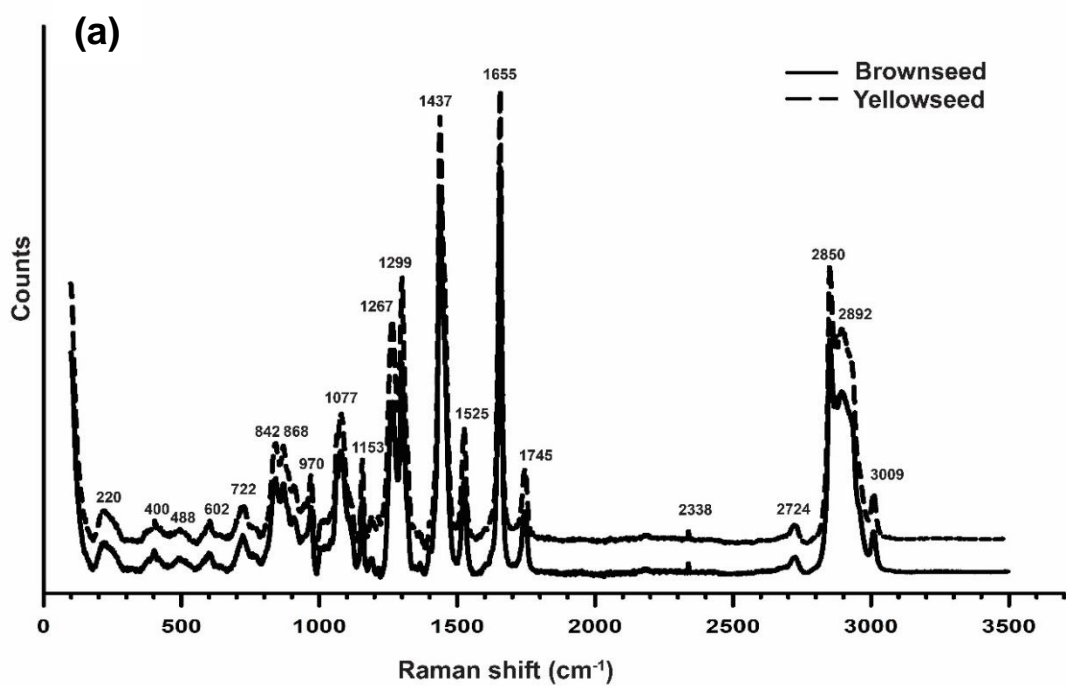
Figure 3.7 Raman spectra of the reference oil (a) and reference starch (b) from brown and yellow canaryseed. Approximate peak position of each band is marked in the figure.

deformation and is most likely due to saturated fatty acids (Baeten et al., 1998; De Gelder et al., 2007). In the $1,300‑1,500$ cm^{-1} region, there is only one major band ($\sim$1,437 cm^{-1}) with a higher intensity is visible. This band is a result of a C−H deformation vibration from methylene and provides an indication of total unsaturation (Baeten et al., 1998; Duraipandian et al., 2019; Sadeghi-Jorabchi et al., 1991). Since this band is the second most intense band (Figure 3.7a), it suggests that the canaryseed oil is highly unsaturated which is comparable with the previous literature (Abdel-Aal et al., 1997c; "GRAS Notice (GRN) No. 529," 2015). Duraipandian et al. (2019) reported a Raman band at 1527 cm^{-1} in olive oil appearing due to C=C stretching from carotenoids. Hence, the band at $\sim$1,525 cm^{-1} in Figure 7a is most likely due to the carotenoids present in canaryseed (Li & Beta, 2012) that had been co-extracted with the oil. The band at $\sim$1,655 cm^{-1} is due to the C=C stretching vibration of the unsaturated fatty acids, which is a *cis* configuration (De Gelder et al., 2007; Duraipandian et al., 2019; Weng et al., 2003). If the C=C stretching vibrations is from the *trans* configuration, the peak would have appeared near to the $\sim$1,670 cm^{-1} (Baeten et al., 1998). Since this band, at $\sim$1,655 cm^{-1}, is the most intense band in the whole spectrum, it further proves that canaryseed oil is highly unsaturated. Another important band of canaryseed oil is at $\sim$1,745 cm^{-1}, which appears due to the stretching vibrations of the C= O group of the ester bond between the fatty acids and the glycerol molecule of the triglycerides (De Gelder et al., 2007; Duraipandian et al., 2019; Weng et al., 2003). This bond is highly beneficial in distinguishing oil from protein and starch. Czamara et al. (2015) reported that the Raman bands that originate between 1,720 and 1,750 cm^{-1} are mainly from the ester bonds. The bands in the region between 2,800 and 3,100 cm^{-1} are unique and mainly occur due to the C−H stretching vibrations from CH_2 and CH_3 groups of the lipids (Baeten et al., 1998; Matthews et al., 2010). In canaryseed oil, three distinct bands, a high intensity band at 2,850 cm^{-1}, and two lower intensity bands at $\sim$2,892 cm^{-1} and $\sim$3,009 cm^{-1}, were visible and provided a unique shape (Figure 3.7a). Similar bands at the $2,800‑3,100$ cm^{-1} region were also noticed in olive oil (Baeten et al., 1998). Similarly, the overall shape of these bands is useful to distinguish oil from starch and protein.

3.4.2.2 Raman spectra of canaryseed starch

Similar to oil, the Raman spectra of the reference starch of brown and yellow canaryseed were almost identical (Figure 3.7b) and showed distinct bands with different intensities from 300 to 3,000 cm^{-1} region. The band assignment for the reference starch was performed based on previous studies (De Gelder et al., 2007; Kizil et al., 2002; Liu et al., 2004) carried out on different starches and saccharides. Bands below 800 cm^{-1} mainly appear due to the skeletal mode vibrations of the glucopyranose ring of the amylose and amylopectins of starch (Kizil et al., 2002; Liu et al., 2004). These include bands at ~302, ~354, ~406, ~438, ~475, ~575, ~607, ~713, and ~767 cm^{-1} in canaryseed starch (Figure 3.7b). However, the band at ~767 cm^{-1} could also be the result of C−C stretching vibrations (Cael et al., 1973, 1975). In this region, the most outstanding band is at ~475 cm^{-1} (Figure 3.7b), which is unique to starch and useful for identifying starch from oil and protein. The region between 800 and 1,500 cm^{-1} is quite complex (Figure 3.7b; Kizil et al., 2002) and the bands could originate from different types of vibrational modes (Sekkal et al., 1995). It has been reported that the bands between 1,300 and 1,500 cm^{-1} appear mainly due to combination of CH_2 deformations and C−O−H bending vibrations whereas the number of bands between 800 and 1,300 cm^{-1} result from the coupling modes of C−O and C−C stretching vibrations. Moreover, these studies suggested that bands in the 800‑1300 cm^{-1} could originate from C−O−C vibrations and C−O−H deformation or bending (De Gelder et al., 2007; Kizil et al., 2002; Liu et al., 2004; Sekkal et al., 1995). Similar to oil, the 2,800‑3,000 cm^{-1} band is the characteristic region of C−H stretching vibration (Kizil et al., 2002), which shows a unique shape specific to starch. As shown in Figure 3.7b, this band has a major peak around ~2,908 cm^{-1} and a small shoulder peak around ~2,937 cm^{-1}. The similar shape of Raman bands around 2,800‑3,000 cm^{-1} region was also reported by Schuster et al. (2000) and Liu et al. (2004) for an amylose/amylopectine mixture and potato starch, respectively. Therefore, the overall shape of this region of starch is useful to differentiate starch from oil and protein.

3.4.2.3 Raman spectra of canaryseed protein

Cereal grains contain albumin, globulin, prolamin, and glutelin as their main storage proteins (Hoseney & Delcour, 2010b). Prolamin and glutelin proteins are the most abundant types

in many cereals except in oats and rice, which contain globulin as their major storage protein instead (Shewry & Halford, 2002). Most of the cereals' albumins and globulins are concentrated in the bran, aleurone layer, and the germ whereas the prolamins and glutelins are found in the endosperm (Hoseney & Delcour, 2010b). Canaryseed also contains these storage proteins and prolamin was found to be the most abundant type (Abdel-Aal et al., 1997a, 2010). However, information available on the distribution of these storage proteins within the canaryseed compartments is limited. Compared to the oil and starch references, canaryseed storage protein fractions showed higher noise and the noise level varied from one protein to another. Some of the band signals were strong in one type of protein, whereas the same signal was weak in the other protein, making it difficult to identify one unique band of each protein with a great certainty.

Even though differentiation of albumin, globulin, prolamin, and glutelin using the Raman spectra was difficult, it was possible to identify a unique band, which occurs at ~1,000 cm^{-1} (Figure 3.8) and originates due to phenylalanine (David, 2012; De Gelder et al., 2007). This unique band could differentiate them from oil and starch. This band is visible in all of the above four proteins (Figure 3.8) and did not appear either in oil or starch (Figure 3.7). The spectra of proteins are quite complex as they are a mixture of 20 different amino acids. The bands originating in the Raman spectra for proteins are highly dependent on the nature of the amino acid, such as length of the R side chain, differences in the benzene ring of aromatic amino acids, reduction or oxidation state of the monomer etc., (De Gelder et al., 2007; Jenkins et al., 2005) making the assignment of a specific band, as shown in Figure 3.8, quite challenging. Other major bands that are characteristics of proteins other than phenylalanine (~1,000 cm^{-1}) are the ones that represent amide I (~1,650 cm^{-1} due to C=O stretching vibrations), II (~1,550 cm^{-1} due to N−H bending and C−N stretching vibrations), III (~1,300 cm^{-1} due to C−N stretching, N−H bending and skeleton stretches), the disulfide bridges at ~500-550 cm^{-1} due to S−S stretching vibrations and the C−S stretching vibrations either at ~640-680 cm^{-1} or 740-760 cm^{-1} (David, 2012; Rygula et al., 2013). The amide I band is prominent in all of albumin, globulin, prolamin, and glutelin tested (Figure 3.8a-d), while the amide II and III bands were only prominent in some of them (Figure 8b, d). Since canaryseed oil also contains an intense band, the same as the amide I band, it could not effectively be utilized for protein identification. The bands related to the S−S and C−S stretching vibration regions were also visible in the canaryseed reference proteins. However, they were not visible in equal

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magnitude (Figure 3.8) among the four different protein fractions. The C−H stretching vibration region of 2,800‑3,100 cm^{-1} of the Osborne protein fractions (Howell et al., 1999) showed a characteristic shape with four prominent bands (Figure 3.8). The bands at ~2,927, ~2,872, and ~3,060 cm^{-1} are clearly visible in all four proteins and albumin and only faintly visible in globulin. The overall shape of this C−H stretching vibration region is useful to distinguish protein as it is for oil and starch. However, it was assumed that the Raman spectra of albumin, globulin, prolamin, and glutelin might provide some unique vibrational bands that could differentiate these proteins from one another. To the contrary, we found it difficult to identify marked differences among these proteins and also between the yellow and brown seed varieties (Figure 3.8). Moreover, the H_2O_2 treatment and its effect on the protein molecules and the color differences of each fraction (data not shown) caused the differences in the noise level. On the other hand, it is also possible that albumin, globulin, prolamin, and glutelin do not produce Raman spectra with marked differences. Since the Osborne classification is solely based on the solubility of the proteins in different solutions and not on the structural differences, it could be expected that the bond vibrations would be similar as all these proteins contain the same functional groups in their basic chemical level.

The Raman spectral analysis of the isolated references from canaryseed show that each contain characteristic bands that are useful in identifying oil (~1,745 cm^{-1}), starch (~475 cm^{-1}), and protein (~1,000 cm^{-1}) distribution in the seed compartments. Moreover, the characteristic shape of the Raman spectra between 2,800 and 3,100 cm^{-1} further assists in identification and confirmation of these compounds. It is difficult to separately identify the seed storage protein types using the Raman spectra, and therefore, identification of the spatial distribution of albumin, globulin, prolamin, and glutelin in the seed would not be possible in this study. It was also noticed that the oil, starch, and protein from brown canaryseed do not show noticeable differences to that of yellow canaryseed.

3.4.2.4 Oil, starch, and protein distribution in the seed compartments

The Raman microscopic evaluation of the germ (Figure 3.9a) shows the unique bands for oil (~1,745 cm^{-1}) and protein (~1,000 cm^{-1}), confirming the presence of these two constituents in the germ. The shape of the C−H stretching region (2,800‑3,100 cm^{-1}) resembles the shape in the
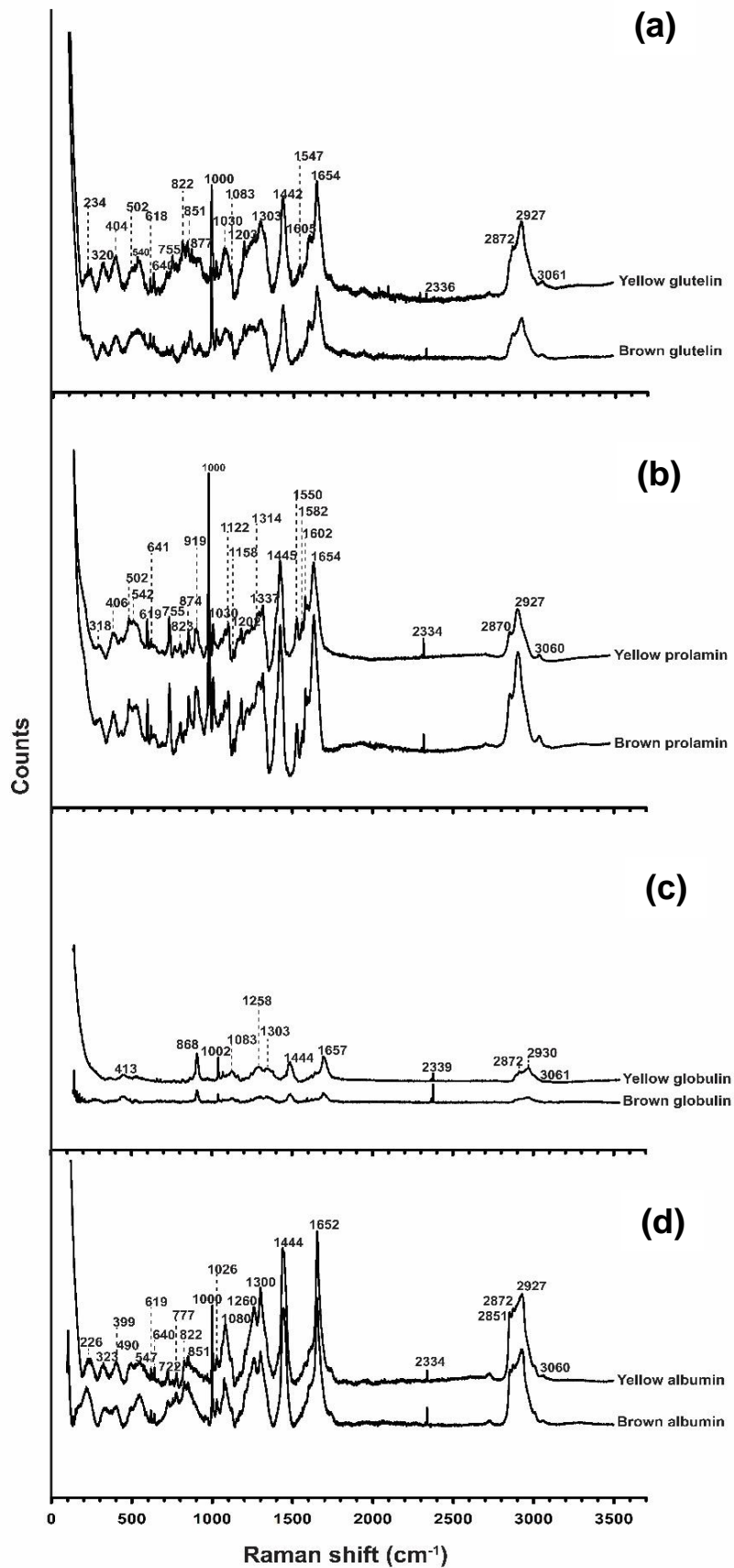
Figure 3.8 Raman spectra of brown and yellow canaryseed reference proteins. (a) glutelin; (b) prolamin; (c) globulin; (d) albumin. Approximate peak position of each band is marked in the figure.

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reference oil spectra (Figure 3.7a) with the main peaks at ~2,850 cm^{-1}. ~2,891 cm^{-1} and ~3,009 cm^{-1} suggesting that the germ is an oil dense area of the seed. The high intensity bands at ~1,438 cm^{-1} and 1,654 cm^{-1} also supports this observation. However, they could also suggest that the germ is rich in proteins, since these two bands are common for both oil and protein (Figures 3.7a and 3.8). These results suggest that the germ has a high concentration of oil and protein similar to that of other cereals such as wheat and corn (Hoseney & Delcour, 2010a; Yang et al., 2018). The grains (droplets) visible in the scutellum (Figure 3.6b, d) are a mix of oil and proteins. Abdel-Aal et al. (2011a) showed that the canaryseed endosperm is rich in starch and protein, which was further confirmed by the SEM images in the current study. As expected, the endosperm of the canaryseed showed significant bands for starch (~475 cm^{-1}) and the characteristic shape of the C−H vibrations region for a starch-rich area (Figure 3.9b). The presence of the Raman band at ~1,000 cm^{-1} also confirmed the availability of proteins in this compartment of the seed. Interestingly, the Raman band unique to triglycerides (~1,745 cm^{-1}) was also visible in the endosperm with a lower intensity compared to that of the germ (Figure 3.9b). This suggests that the endosperm also contains oil, although in a lower concentration than in the germ. However, since the endosperm occupies most of the area in the seed it may still contain a substantial amount of the total canaryseed oil content.

The aleurone layer of the yellow canaryseed also contained protein and oil similar to that of the germ (Figure 3.10a). This was confirmed by the presence of a unique protein band (~1,000 cm−1) and oil band (~1,740 cm^{-1}). The shape of the curve of the C−H vibrations region suggests a mixed characteristic of oil and proteins showing in the bands at ~2,850, ~2,873, 3,006, and ~3,058 cm^{-1} (Figure 3.10a) further suggesting that the aleurone grains (Figure 3.4c) are also a mix of proteins and oil droplets. The presence of proteins in the aleurone layer was previously reported by Abdel-Aal et al. (2011a). However, those authors were unable to visualize the oil as it was probably washed-off with ethanol during microtome preparation. The bran of the yellow canaryseed also contains proteins (Figure 3.10b; band ~1,000 cm^{-1}). The noise level of the bran spectrum was high in comparison to those of germ, endosperm, and aleurone layers. Therefore, it was difficult to identify the bands with lower intensities. The shape of the curve in the C−H vibration region resembles that of a protein more than an oil and the band at ~1,655 cm^{-1} could be either from protein or oil. This confirms the presence of proteins in the bran and made it
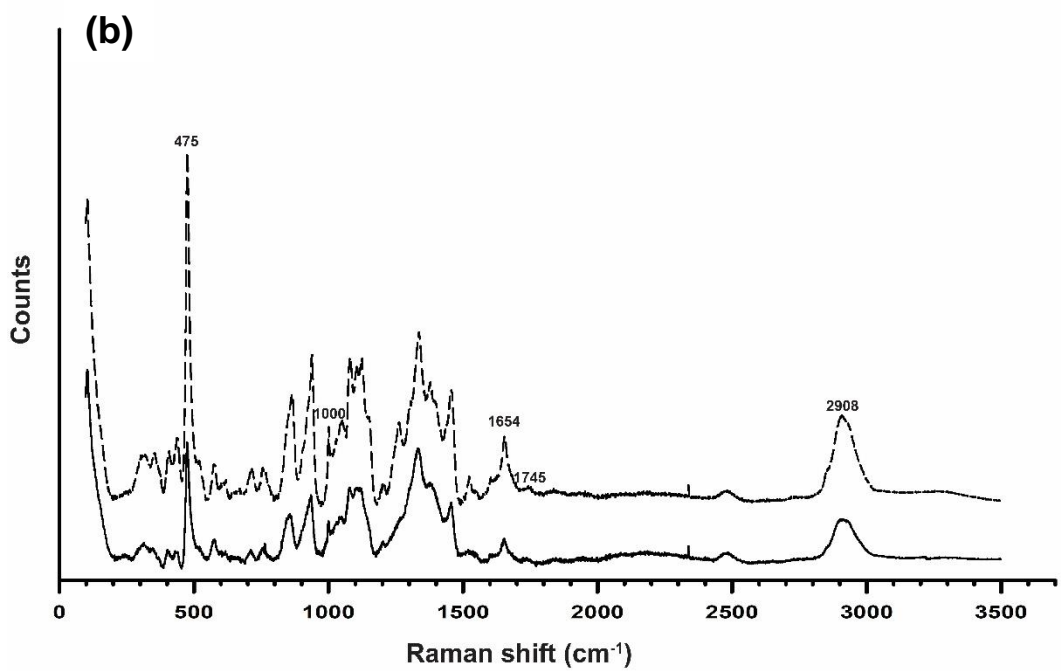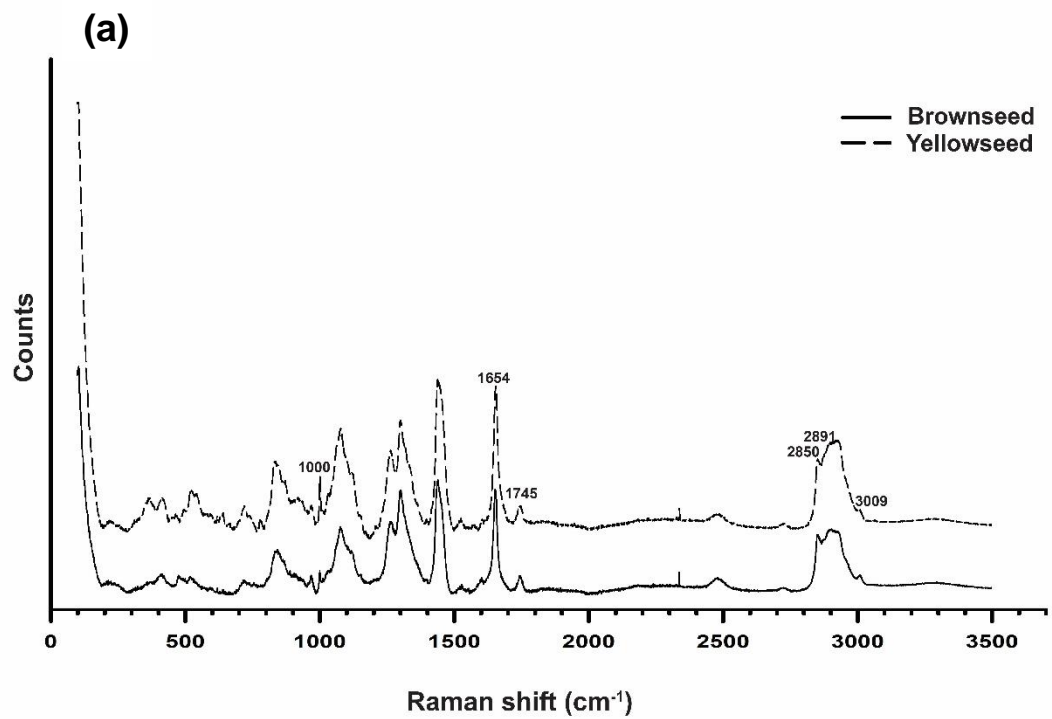
Figure 3.9 Raman spectra of the brown and yellow canaryseed germ (a) and endosperm (b). Approximate peak position of each band is marked in the figure.

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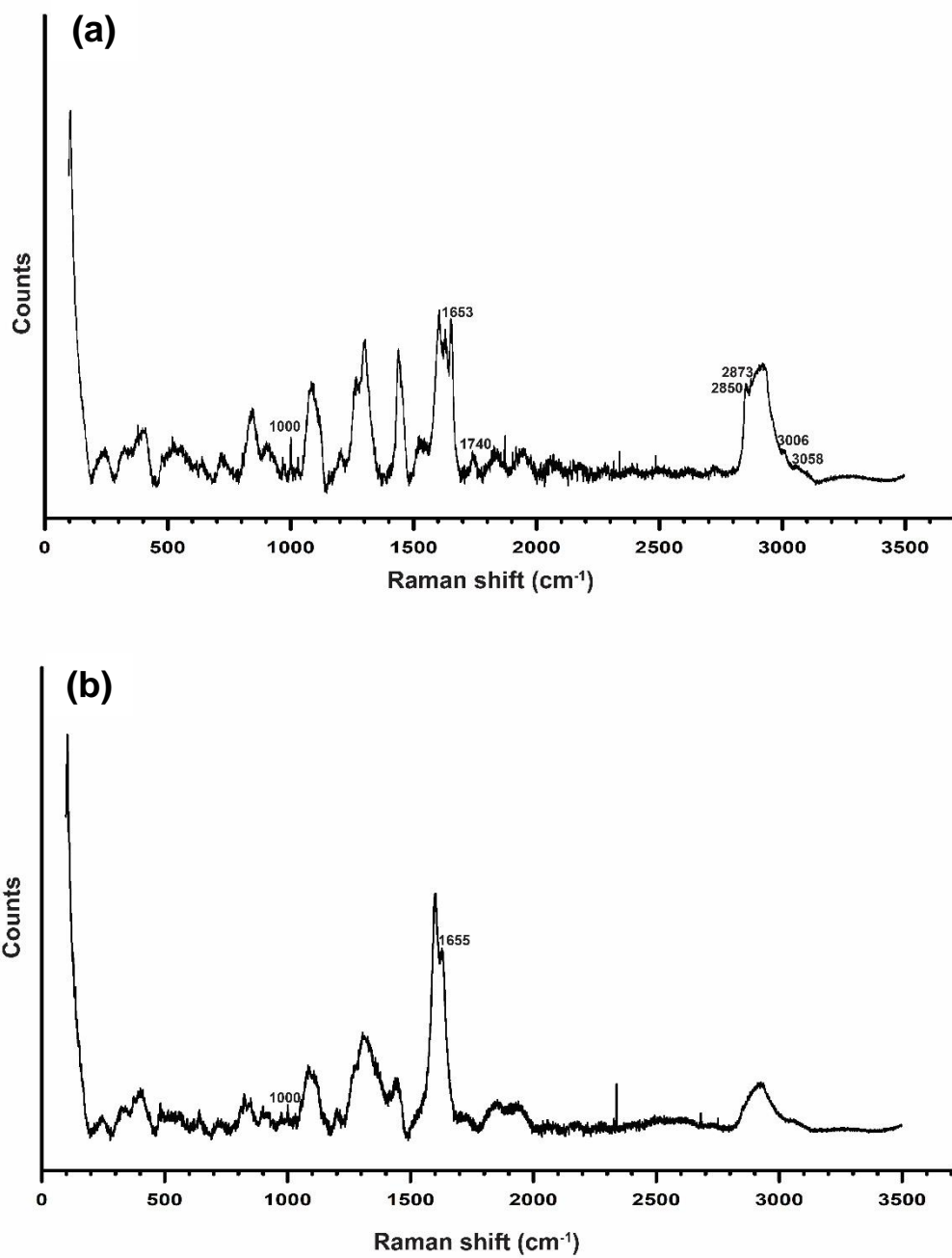
Figure 3.10 Raman spectra of the yellow canaryseed aleurone layer (a) and bran (b). Approximate peak position of each band is marked in the figure.

challenging to comment on availability of oil in this layer. The Raman spectrum of the bran (Figure 3.10b) showed an outstanding band at ~1,599 cm^{-1} that is characteristic to lignin (Yang et al., 2018). Lignin, as one of the major constituents of the bran, has been reported elsewhere ("GRAS Notice (GRN) No. 529, 2015). Evaluation of the Raman spectra of the germ and endosperm of both brown and yellow canaryseed clearly shows that they are almost identical (Figure 3.9). A similar trend was also observed for the reference oil, starch, and protein samples (Figures 3.7 and 3.8) Therefore, it is expected that the aleurone layer and bran of brown canaryseed also share similar chemical features to that of the yellow.

One of the key observations of this study was that oil in canaryseed is present in the germ, aleurone layer, and the endosperm. Even though oil appears to be more concentrated in the germ and aleurone layers, it is possible that the endosperm carries a substantial amount of oil similar to that of oats (Banas et al., 2007; Heneen et al., 2009). To evaluate this hypothesis, both abrasive and roller milling was performed for yellow canaryseed. Abrasive milling was carried out until most of the germ and the bran (including the aleurone layer) was removed. After continuous eight-minute abrasion, very little bran was left over on the seed (Figures 3.11a, c and 3.12c) and most of the germ was removed (Figures 3.11b, d and 3.12a). Milling resulted in three fractions: the bran (outer layers including aleurone layer and germ), broken seed (fractured seed after couple minutes of abrasion that passed through 1 mm mesh), and the debranned seed (mainly endosperm).

The analysis of the oil content of these fractions (Table 3.1) showed that the bran fraction has a high concentration of oil (14.53% of oil content in the bran fraction). However, the mass balances of the oil (Table 3.1) shows that the amount of oil (3.46 g) still present in the debranned seed (containing endosperm) is higher than that of the bran (1.58 g). This finding supports our hypothesis that the endosperm contains more oil than the germ due to the larger area occupied by the endosperm in the seed. The results obtained from roller milling further helped to favor this argument (Table 3.2). After roller milling, the bran fraction (outer layers including aleurone layer and germ, and some starch) contained 12.66% of oil content whereas the white flour (endosperm) contained 5.30% oil. Similar results have also been reported elsewhere (Abdel-Aal et al., 1997a, 2011a). On the other hand, the mass balances (Table 3.2) show a high oil amount (3.88 g) in white flour and a low oil content (2.83 g) in the bran fraction. This suggests that the oil is largely distributed in the endosperm and the rest is concentrated in the germ and the aleurone layers.
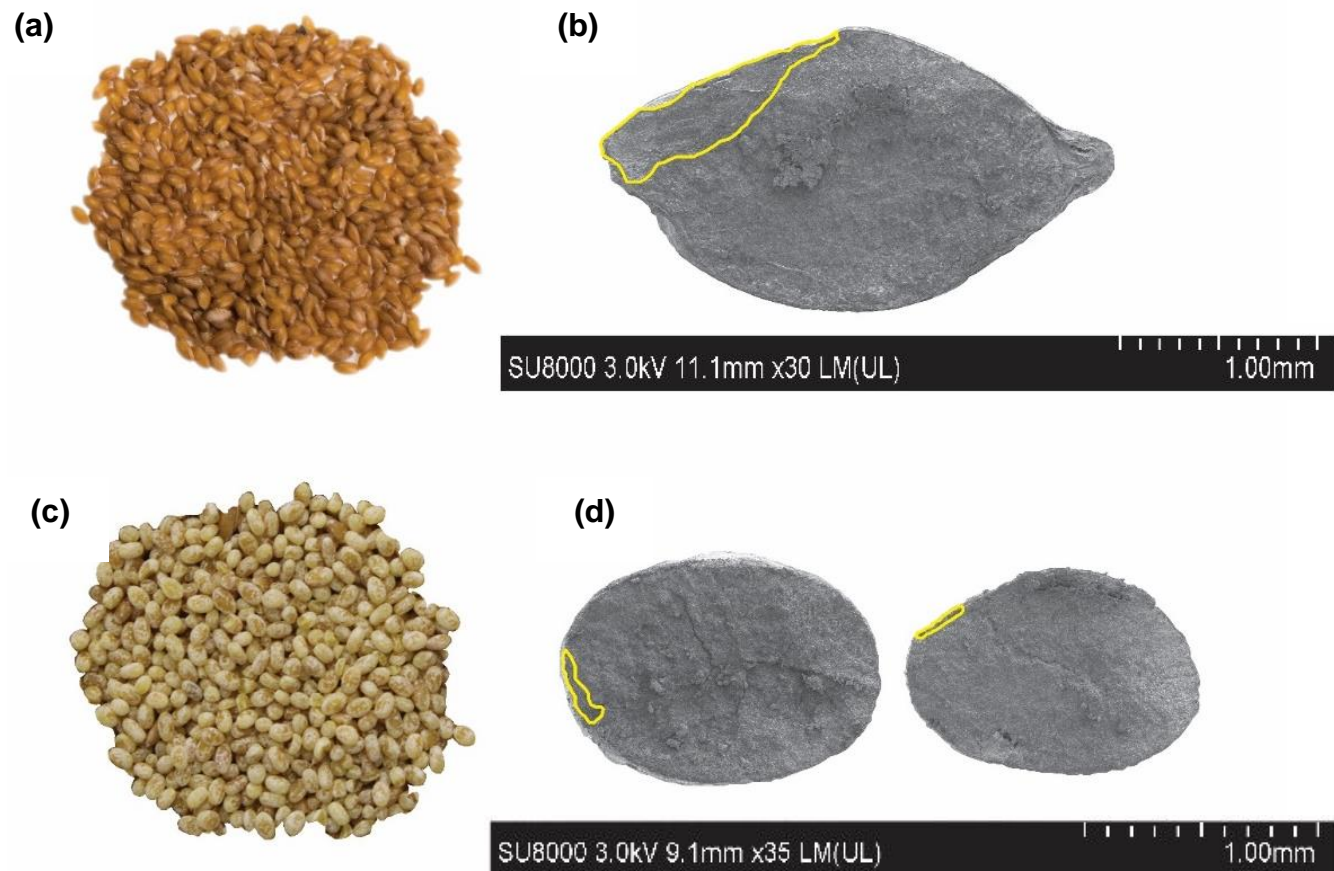
Figure 3.11 Yellow canaryseed before and after abrasive milling. (a) Seed before milling; (b) SEM image of a mid-longitudinal section showing the germ area (yellow boarder); (c) seed after milling (debranned); (d) SEM images of mid-longitudinal sections of debranned seeds showing the germ area (yellow border)
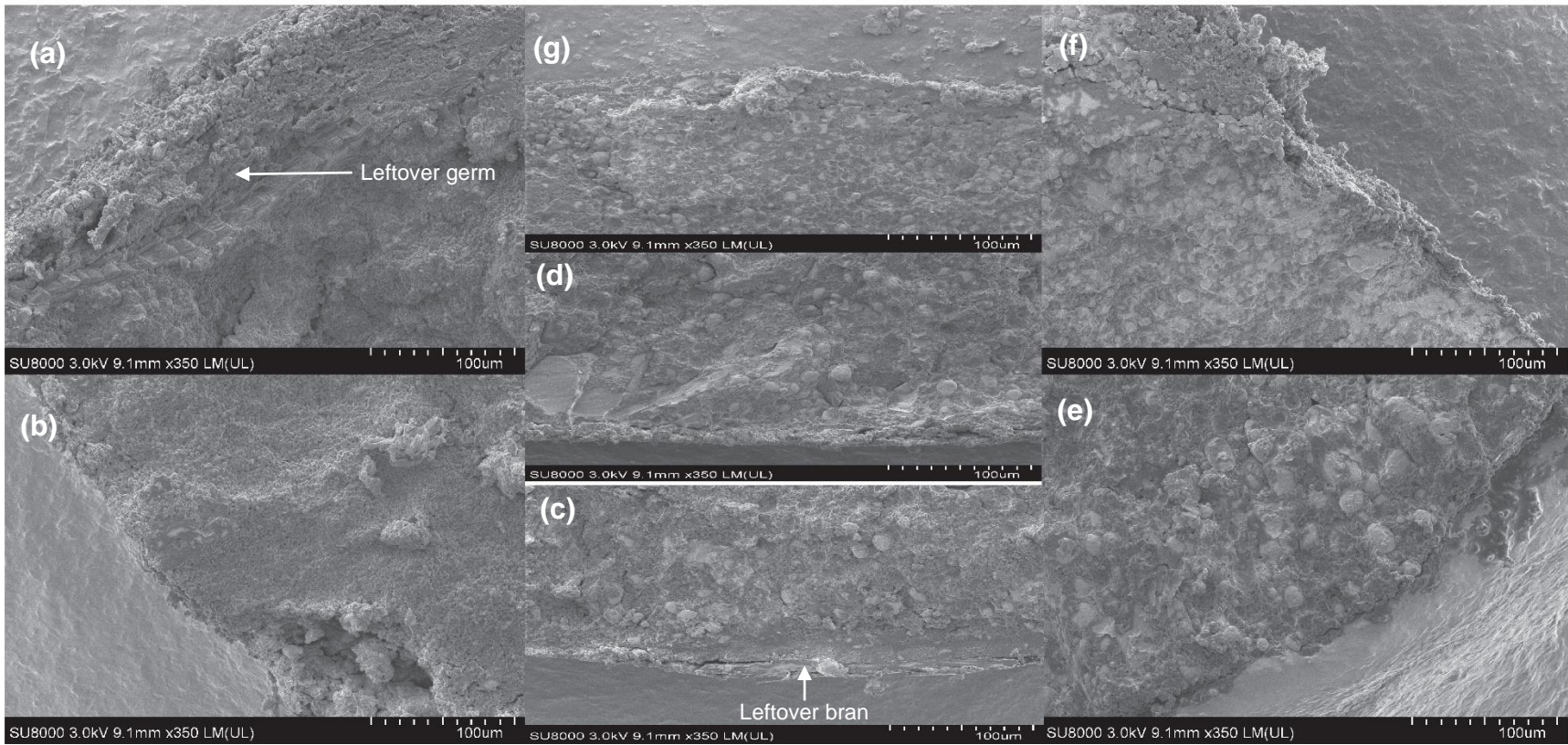
Figure 3.12 SEM images of the leftover germ, dorsal and ventral sides of the debranned yellow canaryseed. (a) leftover germ; (b–e) ventral side of the seed); (f–g) dorsal side of the seed

Moreover, the mass balance of the protein in the roller-milled fractions (Table 3.2) showed that the endosperm contains more protein than that of the germ, aleurone layer, and bran.

Table 3.1 Yield and oil content of yellow canaryseed (whole seed), debranned seed, broken seed and the bran fraction obtained from abrasive debranning.

Parameter	Whole seed	Debranned seed	Broken seed	Bran fraction
% Yield		56.9 ± 0.7	22.4 ± 0.6	10.3 ± 0.8
% Oil (dry weight basis)	7.20 ± 0.08	6.04 ± 0.08^a	5.92 ± 0.16^a	14.53 ± 0.30^b
Weight of oil (g)*	7.20 ± 0.08	3.46 ± 0.06^a	1.28 ± 0.06^b	1.58 ± 0.15^b

Mean ± standard error (SE). Means followed by the same superscript within the same row are not significantly different (p>0.05).

*Calculated based on the % value for 100g of seeds.

Table 3.2 Yield, Oil and protein content of yellow canaryseed (whole seed), white flour and the bran fraction obtained from roller milling.

Parameter	Whole seed	White flour	Bran fraction
% Yield		72.8 ± 0.4	22.3 ± 0.4
% Oil (dry weight basis)	7.20 ± 0.08	5.30 ± 0.03^a	12.66 ± 0.14^b
Weight of oil (g)*	7.20 ± 0.08	3.88 ± 0.04^a	2.83 ± 0.02^b
% Protein (dry weight basis)	22.4 ± 0.14	21.7 ± 0.03^a	22.2 ± 0.04^b
Weight of protein (g)*	22.4 ± 0.14	15.8 ± 0.02^a	5.0 ± 0.01^b

Mean ± standard error (SE). Means followed by the same superscript within the same row are not significantly different (p>0.05).

*Calculated based on the % value for 100g of seeds.

3.5 Conclusions

Canaryseed shows the common microstructural features of a cereal grain. The bran of the ventral side of the seed is broad and loosely packed whereas it is thick and tightly packed on the dorsal side. The aleurone layer is mostly a single-cell layer and occasionally appears as a two-cell layer.

The germ covers approximately ~8%‑12% area of the total endosperm area. The germ and the aleurone layer are mainly composed of oil and protein and they are stored in granular-shaped (grains) structures. The endosperm is rich in starch and the starch is organized in the form of compound granules and individual granules. SEM analysis revealed the presence of crack-like margins on the surface of the compound starch granules, which facilitates identification of the smaller compound starch granules from other entities in the endosperm. The canaryseed endosperm also contains the majority of the oil. Hence, utilizing abrasive or roller milling techniques for bran removal may not be highly effective to produce flour with the low oil content that may benefit protein fractionation and canary flour-based food application in terms of final product stability. Moreover, the presence of significant amounts of oil in the endosperm makes it difficult to effectively apply solvent-free "green" technologies for protein fractionation. Further investigation on the spatial distribution of the oil in the endosperm and in other areas of the seed is required. The proteins in canaryseed could be found in the germ, aleurone layer, bran, and endosperm while endosperm encloses the majority of them. Based on the previous literature available on cereal grains, the albumins and globulins of canaryseed should be concentrated in the germ, aleurone layer, and the bran whereas prolamin should be in the endosperm. However, it would be interesting to confirm the spatial distribution of these individual proteins to design protein fractionation processes that minimize the loss of protein yield and quality. Overall, there was no marked difference observed in the structure, composition, and composition distribution in the seed between brown and yellow canaryseed.

3.6 Connection to the next study

This study showed that the germ of the canaryseed covers a smaller area and highly concentrated with oil whereas oil is less concentrated in the endosperm that covers much larger area than that of the germ. However, due to the larger area, the total amount of oil available in the endosperm is greater than that of the germ. This makes it difficult to utilize dry milling techniques to remove germ (including aleurone layer and bran) to prepare endosperm flour (low-bran or white flour) with lower oil content to use as a starting material for protein fractionation as an alternative to the whole seed flour. Therefore, de-oiling using an organic solvent might be inevitable prior to value addition through protein fractionation, regardless of the flour-type (whole seed or white flour) used as the starting material. Organic solvents are known source for protein denaturation

that consequently alter the native techno-functional and nutritional properties. It is important to understand the native techno-functional and nutritional properties of canaryseed protein and effect of solvent de-oiling on these properties when fractionating the proteins. It would help to make a decision on the type of organic solvent required to be used based on the impact that it would pose up on different value-added protein ingredients. Previous work carried out on utilizing organic solvents for canaryseed de-oiling and their impact on the protein techno-functional and nutritional properties is very limited. Therefore, in the next study, it is expected to address this knowledge gap while evaluating techno-functional and nutritional properties of different value-added canaryseed protein ingredients. The present study also showed that there is no was no marked difference observed in the structure, composition, and composition distribution in the seed between brown and yellow canaryseed; hence, only yellow canaryseed was utilized in the next study to achieve the above-mentioned research objective.

4. TECHNO-FUNCTIONAL AND NUTRITIONAL PROPERTIES OF FULL-BRAN AND LOW-BRAN CANARYSEED FLOUR, AND THE EFFECT OF SOLVENT-DE-OILING ON THE PROTEINS OF LOW-BRAN FLOUR AND ISOLATES[2]

4.1 Abstract

Background and Objectives: The differences in functional and nutritional properties of whole and white canaryseed flour obtained by roller milling was investigated in this study. Moreover, the effect of solvent-de‑oiling on the structural, functional, and nutritional properties of the canaryseed protein present in the white flour and derived protein-isolates was also studied.

Findings: There was no significant difference between the protein content of whole (21.3%) and white (21.4%) canaryseed flours whereas, oil, ash, total starch, non-starch carbohydrates, phytic acid, and total phenolic contents showed significant differences between whole and white canaryseed flours. Water and oil holding capacity and emulsifying capacity of whole seed flour were better than that of white flour whereas, emulsion and foaming stability, and foaming capacity were similar. The IV-PDCAAS of white flour was 23%, was significantly lower than that of the whole seed flour (31.2%). Ethanol had a higher efficiency for canaryseed oil and phospholipid extraction than hexane. Linoleic acid and phosphatidylcholine were, respectively, the major fatty acid and phospholipid extracted by both ethanol and hexane. None of these solvents caused protein denaturation and showed a positive impact on certain functionalities. Canaryseed protein was found to be thermally stable, with a peak denaturation temperature of 108°C.

Conclusions: Whole canaryseed flour showed better functionality and nutrition than white flour. The white flour, however, was found to be a more favorable starting material for protein fractionation. Solvent-de-oiling did not have a negative impact on canaryseed protein, which exhibited a high thermal stability.

Significance and Novelty: This study provides information on functional and nutritional properties of different value-added protein products of canaryseed and on the effect of solvent- de-oiling on the protein quality. Such understanding is beneficial for the protein ingredient industry.

4.2 Introduction

Until 2016, canaryseed (*Phalaris canariensis* L.) was only approved as a bird feed, prevented from human consumption due to the presence of toxic siliceous trichomes (hairs) on its hull (Abdel-Aal et al., 2010, 2011a; Achouri et al., 2020; Bhatt et al., 1984; Mason et al., 2018). With the development of glabrous (hairless) seeds, which are devoid of these toxic siliceous trichomes, canaryseed received GRAS (Generally Recognized as Safe) status from the U.S. Food and Drug Administration and was recognized as a novel food product for human consumption by Health Canada (GRAS notice GRN No. 529, 2015; Health Canada, 2016; Mason et al., 2018). Currently, Canada dominates the world canaryseed market with 60% of global production and >75% market share followed by Thailand and Argentina (Achouri et al., 2020; FAOSTAT, 2019). To date, although it has been approved for human consumption, the canaryseed industry currently depends on the bird feed market (Canaryseed Development Commission of Saskatchewan (CDCS, n.d. -a). Therefore, market diversification through value addition to this crop is important for sustainable canaryseed industry in the long term. Canaryseed stands out as a true cereal crop due to its protein content (~19%‑23%; Mason et al., 2018; Patterson et al., 2018) that is significantly higher compared to other cereals (Achouri et al., 2020) and comparable to that of some pulses (Boye et al., 2010); hence, canaryseed shows the potential as an emerging protein source that needs exploration towards plant-based food applications through value addition.

There is number of emerging sources of plant-based protein, such as pulses (lentil, chickpea, faba bean, etc.), cereals (canaryseed, oat, rice, sorghum, etc.), pseudo cereals (quinoa, buckwheat, and amaranth), and oilseeds (canola, hemp, mustard, etc.) being investigated by the food industry for their protein quality, that is techno-functional and nutritional properties. The main purpose for investigating these properties is to evaluate how well these emerging sources

perform compared to the market-leading proteins, such as soybean, wheat, and pea protein (Plant Based Protein Market, 2021). To compete with these market-leading proteins and become sustainable in the plant-based protein industry, an emerging source should exhibit superior or at least similar techno-functional properties and/or possess traits that could be beneficial for marketing purposes. Among these beneficial traits are cost effectiveness, free from off‑flavors, neutral color, low allergenicity, and clean label (Akharume et al., 2021; Clayton & Specht, 2021; Ismail et al., 2020; Ohr, 2020). Although inheriting above-mentioned traits are important to emerging protein sources, it is challenging to meet some traits, such as clean label and low cost of production, especially if the protein source contains higher oil content that requires de-oiling.

If a protein source has high oil content, it is highly likely that the oil concentrates into the derived protein products (*e.g.,* protein concentrates and isolates), thereby compromising their shelf-life stability. Therefore, de-oiling is often necessary for high-oil-containing protein sources, such as canaryseed, oat, and quinoa that contains ~>5% oil in the seed (Abdel-Aal et al., 2011a; Banaś & Harasym, 2021; Banas et al., 2007; Koziol, 1993; Perera et al., 2021; Wejnerowska & Ciaciuch, 2018). Organic solvent-de-oiling is a common process in the oilseed industry and hexane, ethanol and isopropyl alcohol (IPA) are some of the major solvents used for this purpose (Russin et al., 2011). However, utilizing solvents for de-oiling affects cost of production, raises environmental concerns (Russin et al., 2011), and acts as a barrier for clean labeling. Moreover, solvents could cause structural changes in the protein by affecting the stability of hydrophobic interactions, hydrogen bonding and electrostatic interactions (Griebenow & Klibanov, 1996) and therefore, could alter its native functional properties (Bader et al., 2011; Galves et al., 2019; Teh et al., 2014). Although aqueous de-oiling is a cleaner alternative, it is not as effective as solvents in removing oil and presents processing challenges, such as the need for demulsification (Russin et al., 2011), since oil tends to emulsify with protein during processing and remain with the product. Hence, solvent de-oiling remains a practical method to remove the oil present in the protein sources to achieve high shelf-life stability for the final protein products.

Abdel-Aal et al. (2010) first developed a protein fractionation process for canaryseed and later optimized and scaled-up by Achouri et al. (2020). In this process, ethanol was used as the solvent to de-oil canaryseed flour, and the resulting protein isolate was evaluated for its techno-functional properties. Moura et al. (2020) also studied the techno-functional and nutritional properties of hexane-de-oiled canaryseed whole flour (full-bran flour), and derived protein

isolates. However, information on the effect of using different solvents on canaryseed protein quality, and oil extraction efficiency is limited. Ethanol is considered more of an environmentally friendly solvent than hexane (Abdel-Aal et al., 2010; Gandhi et al., 2003) however, hexane is still widely used for solvent de-oiling; hence, acquiring knowledge of how these solvents affect the canaryseed protein quality is important for canaryseed as an emerging protein source. Moreover, whole flour, white (low-bran) flour, protein concentrates, and isolates are the major value‑added products derived from many plant-based protein sources. Each of these products differ from one another mainly due to changes in their macromolecule composition, but also to processing induced structural changes, caused by solvents, heat, and so on. It is plausible that these compositional variations and structural changes alter the dynamics of macromolecular interactions and consequently reshape the protein quality attributes of these value-added products. Therefore, the objectives of this study were (1) to identify a difference in protein quality between whole canaryseed flour and white canaryseed flour, (2) to evaluate the efficiency of hexane and ethanol in terms of oil extraction and crude oil quality, (3) to evaluate the effect of de-oiling and solvent type used on the protein quality of flour and derived protein isolates.

4.3 Materials and Methods

4.3.1 Materials

Dehulled canaryseed (yellow) cultivar CDC Cibo grown in Saskatchewan (SK), Canada, was obtained from a local producer. Canada Western Red Spring wheat cultivar AAC Viewfield obtained from a local merchant was utilized as the control for this study. Analytical grade chemicals purchased from Sigma-Aldrich, VWR International Inc. were utilized in this study. The chemical and reagents that were not purchased from the abovementioned suppliers are included in the text.

4.3.2 Sample preparation

4.3.2.1 Whole and White flour preparation

The yellow canaryseed was tempered to 13% moisture content and roller-milled using a Bradender Quadrumat Jr. mill (Brabender Instruments, Inc.) to prepare flour. Then the flour was sieved using #18 mesh (1000 μm) to remove the contaminated hull fraction (retained on top of the mesh). The resulting flour from #18 mesh was obtained as the full-bran (whole) canaryseed flour

for this study. The whole flour was then further sieved using #60 mesh (250 μm mesh) to produce low-bran (white) flour fraction and the bran fraction (retained on top of the mesh). The same procedure was followed for wheat to prepare whole and white wheat flour; however, it was tempered to 14.5% moisture before roller milling. All the fractions obtained from the roller-milling were stored under refrigerated conditions (4 °C) until further analysis.

4.3.2.2 Solvent-de-oiling of the white flour

Iso-hexane and absolute ethanol was used as the de-oiling solvents of white canaryseed flour. The flour and solvent were mixed at 1:3 w/w flour-to-solvent ratio and stirred for 1 h. Then they were centrifuged at 3640 × g for 20min at 22°C, and the oil-containing supernatants (oil miscella) were decanted for oil recovery. The de-oiled pellet was desolventized overnight under a fume hood and vacuum dried (Vacuum oven model 8861, Napco® scientific company) at 35 °C for 1.5 h at −25 in Hg to flash-off any residual solvent trapped in the pellet. The de-oiled flour samples were stored at 4 °C until further analysis.

4.3.2.3 Preparation of protein isolates

The hexane- and ethanol-de-oiled white flours and non-de-oiled white flour were processed using proprietary process developed by Keyleaf Life-Sciences, Saskatoon, Saskatchewan, Canada to prepare protein isolates. The major steps of the process include treating with a raw starch degrading enzyme at mild temperature, centrifuging to remove degraded starch and recovering protein, screening to remove fine fibers and freeze drying to produce canaryseed protein isolates. Great care was taken to avoid using extreme/harsh processing conditions to prevent possible damage to the native protein in the flour during isolate preparation. A commercial wheat protein isolate (Vital wheat gluten) was purchased from Permolex Ltd., Alberta, Canada as the control for the isolates. The process flow diagram (Figure 4.1) briefly illustrates the experimental plan used in this study. The roller-milling and flour preparation, solvent de-oiling and protein isolate preparation were performed in triplicate.
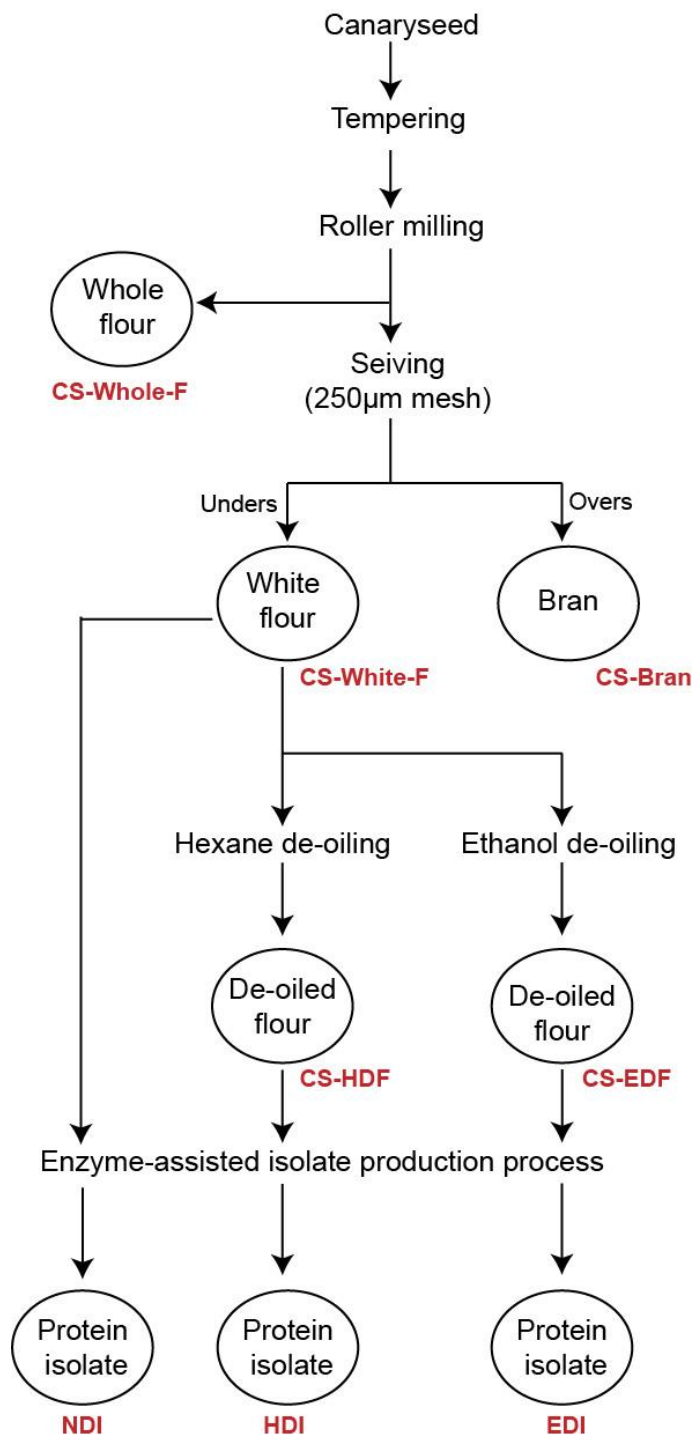
Figure 4.1 Process flow diagram of the experimental plan used in this study. Different fractions obtained for analyses are circled.

4.3.2.4 Morphology of roller milled flour

Scanning electron microscopy (SEM) was used to evaluate the morphological features of canaryseed and those of roller milled canaryseed flour as described in Perera et al. (2021).

4.3.2.5 Compositional analysis of the fractions

Proximate composition

The moisture, protein and ash content of different canaryseed fractions were analyzed according to the AOAC Official Methods: 925.10, 960.52 (%N $\times$ 5.7) and 923.03 (AOAC, 2005), respectively whereas, the Swedish tube method (AM 2‑93) of the American Oil Chemists Society (AOCS, 2017) was utilized for the oil content analysis.

Total and damage starch content

The total starch content of the canaryseed fractions was determined using the Megazyme Total Starch Assay Kit following AACC Method 76-13.01 (AACC, 2010). The percentage of the damage starch during roller milling was determined according to the Megazyme Starch Damage Assay Kit following AACC Method 76-31.01 (AACC, 2010).

Total phenolic content (TPC) and phytic acid content

Total phenolic content and phytic acid content of the whole flour, white flour, and bran fractions was evaluated using colorimetric assays according to the method described by Li et al. (2011) and analyzed using the Folin-Ciocalteau method (Li et al., 2010). The results were expressed as mg Ferulic acid equivalent per 100 g of sample. The phytic acid content was determined according to the Megazyme Phytic Acid (phytate)/Total Phosphorus Assay Kit and the results were expressed as g of phytic acid per 100 g of sample.

Fatty acid and phospholipid composition of canaryseed oil

The fatty acid composition of the hexane-extracted and ethanol-extracted canaryseed oil was determined to evaluate the oil extraction efficiency of each solvent. The oil recovered from each solvent was obtained in triplicate and a composite oil sample was obtained to analyze the fatty acid composition according to AOCS official methods Ce 1i-07 and Ce 1b-89. Briefly, the fatty acids in triacyl glycerol (TAG) were converted to fatty acid methyl esters (FAMEs) using

base hydrolysis with 0.5 N NaOH followed by an acid hydrolysis using boron trifluoride (BF3) for complete hydrolysis of the TAGs in canaryseed oil. Then, the FAMEs were analyzed using a gas chromatograph (6890 N network GC system, Agilent Technologies) equipped with flame ionization detector (FID) and an Agilent J & W DB 225 capillary column (Agilent Technologies). Helium was used as the carrier gas and the FAMEs were identified by comparing their retention time with a reference standard mixture (GLC 756 TRI, Nu-Check-prep Inc.). Methyl heneicosanoate was used as the internal standard and chromatograms were analyzed using Empower 3 chromatography software (Waters Corp.). Moreover, the phospholipid composition of each solvent-extracted oil sample was evaluated based on AOCS official method Ja 7b-91 (AOCS, 2017) to evaluate the quality of the crude oil extracted from these two different solvents. Briefly, oil samples (50‑100 mg) were weighed into a 10mL volumetric flask and volumerized with 4:1 v/v n-hexane/2-propanol mixture. A 10 μl aliquot of the prepared samples was injected into a Waters™ HPLC system (Waters Corp.) equipped with a column containing LiCrosphere® 100 DIOL HPLC sorbent (Hichrom ltd.) and evaporative light scattering detector (ELSD, Model 1200, Agilent Technologies) and a gradient elusion was performed with two mobile phases (Mobile phase A: n-hexane: 2-propanol: acetic acid: triethyamine at 1717 g: 424.2 g: 50.4 g: 1.84 g ratio; Mobile phase B: 2-propanol: water: acetic acid: triethyamine at 1976 g: 420 g: 47.4 g: 1.74 g ratio). Retention time of the peaks were compared with those of a reference standard mixture to identify the phospholipids present in canaryseed oil and the recovery of known concentration of the standards were used to quantify canaryseed phospholipid using Empower 3 chromatography software (Waters Corp.)

4.3.2.6 Differential scanning calorimetry (DSC)

Thermal properties of different fractions were determined using differential scanning calorimetry (DSC 8000, Perkin Elmer). Ten milligrams of sample, on a dry weight basis, was wetted with three volumes of distilled water in a stainless-steel DSC pan. The pans were hermetically sealed and equilibrated at room temperature for 2 h. The DSC analysis was then performed from 10 °C to 130 °C at a ramping rate of 5°C/min, with an empty DSC pan being used as the reference. The parameters of thermal transitions (T_o: onset temperature; T_p: peak temperature; T_c: conclusion temperature; and ΔH: enthalpy change) were determined using Pyris software (Perkin-Elmer).

4.3.2.7 Sodium dodecyl sulfite gel electrophoresis (SDS‑PAGE)

The polypeptide profiles of different fractions were evaluated using SDS-PAGE as described in Moura et al. (2020) with modifications. The modifications include addition of Dithiothreitol (DTT, bioWorld chemicals) as the reducing agent to provide 50 mM final concentration and utilizing FroggaBio™ BLUelf prestained protein ladder as the molecular weight markers. The protein gel images were taken using a Nikon DSLR camera and the molecular weight of the polypeptide bands were estimated using ImgeJ (version 1.52a, https://imagej.nih.gov/ij/).

4.3.2.8 Surface charge

The change in the surface charge of different fractions as a function of pH (from pH 2 to 11) was determined using a 0.05% (w/w) protein solution according to the method described by Stone et al. (2015). The measurements were obtained using a Zetasizer Nano (Malvern Instruments) and the protein solutions were stirred overnight before obtaining the measurement.

4.3.2.9 Techno-functional properties

The water holding capacity (WHC), oil holding capacity (OHC), foaming capacity (FC), foaming stability (FS), emulsifying capacity (EC), emulsion stability (ES), and solubility were determined as the techno-functional properties of different canaryseed fractions obtained. The WHC and OHC, FC and FS were determined as illustrated in Moura et al. (2020) and the foams were prepared using a polytron homogenizer (Kinematica Polytron™ PT 10-35 GT homogenizer, Kinematica Inc.) with PTA 10 TS probe (Kinematica Inc.) for 5min at 16,000 rpm. The EC was determined according to the method described by Stone et al. (2015) that involves monitoring the electrical conductivity (Model HI8733, Hanna Instruments) of series of emulsions until the inversion point, that is, the point in which the oil-in-water emulsion is converted to a water-in-oil emulsion. The ES was evaluated according to Stone and Nickerson (2012) with emulsions prepared at 1:1 w/w oil-to-protein solution ratio. Emulsions required for both EC and ES tests were prepared using a polytron homogenizer (Kinematica Polytron™ PT 10‑35 GT homogenizer, Kinematica Inc.) with PTA 10 TS probe (Kinematica Inc.) for 5min at 16,000 rpm.

The solubility of canaryseed protein in different fractions at different pHs (from pH 2 to 9) was evaluated according to the method described in Stone et al. (2015) with modifications. The modification includes dissolving 20mg of sample (protein basis) in 20 mL of distilled water and

measuring the soluble protein using the Pierce Coomassie Plus™ (Bradford) Protein Assay Kit (Thermo Fisher Scientific Inc.). The assay includes mixing 0.05 mL of sample with the Bradford reagent, incubating for 10 min and measuring the absorbance at 595 nm wavelength using a UV‑VIS Spectrophotometer (Genesys 10uv Scanning Spectrophotometer, Thermo Fisher Scientific Inc.). The protein concentration of the sample was obtained using a bovine serum albumin (Pierce Bovine Serum Albumin [BSA] Standard, Thermo Fisher Scientific Inc.) standard curve.

4.3.2.10 Color analysis

The differences in the color of canaryseed flours and isolates were measured according to the method described by Kaur and Singh (2007) using a Hunter colorimeter (Labscan II, Hunter Associates Laboratory Inc.) based on the L*, a*, b* values. L* represents brightness with a scale 0‑100 where 0 represents dark color. a* represents red-green spectrum and red is associated with highest a* value. b* represents yellow-blue spectrum and yellow is associated with higher b* value.

4.3.2.11 Nutritional properties

Amino acid profile and in vitro protein digestibility was tested to evaluate the nutritional properties of different canary seed fractions. The amino acid profile (18 primary) was determined based on to AOAC official methods 985.28 and 988.15 (AOAC, 2005). Briefly, these methods include an acid hydrolysis for 15 primary amino acids, pre-hydrolysis oxidation step with performic acid followed by an acid hydrolysis for cysteine and methionine, and base hydrolysis step for tryptophan. The hydrolysates were analyzed using a Waters™ HPLC system equipped with Pico‑Tag® C18 column (Waters Corp.) and a photodiode array (PDA) detector (Model 2998, Waters Corp.). A gradient elusion was performed with two mobile phases (Mobile phase A: 0.14 M sodium acetate containing 0.5 mL/L triethylamine titrated to pH 6.40 with glacial acetic acid; mobile phase B: 60% acetonitrile in water) and the retention times of the peaks were compared to those of reference standards (Amino acid standard H, Thermo-Fisher Scientific) to identify the amino acids present in the samples. Alpha-aminobutric acid was used as the internal standards and the peaks were quantified using Empower 3 chromatography software (Waters Corp.).

The in vitro digestibility was determined using the pH drop method and the *in vitro* protein digestibility corrected amino acid score (IV-PDCAAS) was calculated using the limiting amino acid score according to the methods illustrated in Bai et al. (2018).

4.3.2.12 Statistical analysis

The comparison between the protein quality of whole flour versus white flour, white flour versus solvent-de-oiled flours, and non-de-oiled isolate versus solvent-de-oiled isolates were performed using one-way ANOVA followed by Tukey's mean separation procedure. All the analyses were carried out in triplicate (n = 3) and the data were analyzed using R statistical software, version 4.0.3 (R Core Team, 2020). To perform Tukey's mean separation and assign the letters to denote significant (p < .05)/nonsignificant (p > .05) mean values, the R packages "emmeans" (Lenth, 2021) and "multcomp" (Hothorn et al., 2008) were utilized.

4.4 Results and Discussion
4.4.1 Whole versus white canaryseed flour

After dehulled seeds, whole-seed flour and white flour of canaryseed are the first value‐added products that could be utilized in edible product applications. In this study, the dehulled seeds were roller-milled to prepare those flours (Figure 4.1). The modern milling process in the food industry involves use of rollers for the gradual reduction of the endosperm of cereal grain to smaller particle sizes while separating the germ and the bran (Rana et al., 2014). As illustrated in Figure 4.1, the major difference of whole versus white canaryseed flours is the content of bran, that is, outer layers of the seed including the aleurone layer, and the germ particles in the flour (Perera et al., 2021). The white flour contains particles <250 micron and therefore, only contains the finer bran particles. Presence of bran particles are clearly visible in whole-flour fraction (Figure 4.2) whereas, bran particles are not prominent in white flour (Figure 2), and visually more appealing than the whole flour. The significant differences between the L*, a*, b* color values (Table 4.1) show that canaryseed white flour resulted from roller milling become more lighter and yellower in color than that of the whole flour.

Inside the canaryseed, starch granules are embedded in a protein network in the endosperm (Figure 4.3a, Abdel-Aal et al., 2011; Perera et al., 2021) and after milling, this network is broken, and the starch granules are released into the matrix (Figure 4.3b, c). During the milling

process some starch granules are damaged, that is, either granule is broken but still birefringent or it is damaged and no longer birefringent, due to the grain hardness and force applied (Hoseney & Delcour, 2010c). Some degree of damaged starch in the flour after roller milling is a desirable trait that improves water-absorption characteristics for dough formation (Hoseney & Delcour, 2010c). Previous studies have shown that some damaged starch content is desirable for best baking performance; however, excessive levels of damage starch could negatively affect baking performance (Arya et al., 2015; Dexter et al., 1994). This study showed that the level of damage starch is low ($1.4\% \pm 0.01\%$) in canaryseed white flour compared to the control-white-wheat flour sample ($7.5 \pm 0.13\%$), suggesting low level of contribution from the damaged starch to water‐absorption for dough formation and baking properties of canaryseed flour.

4.4.1.1 Fiber, mineral, and starch

The proximate analysis showed that whole flour contains a significantly higher amount of non-starch carbohydrates (mainly dietary fiber, Figure 4.2a, b) than that of white flour (Table 2). The control-wheat sample showed the same trend for whole versus white flour, as expected. Having high fiber content in the flour is not favorable as a protein-product since it reduces the protein purity and digestibility (González et al., 2020), although it provides other nutritional benefits. Moreover, high fiber content reduces the purity of the protein, which is unfavorable for protein ingredients since high purity protein isolates are high value and often preferred. The aleurone layer of the cereal grain usually has a high mineral content (Hoseney & Delcour, 2010d) and therefore, contributes to the high ash content Presence of significantly lower ash content in the white flour compared to the bran fraction in both canaryseed and wheat (Table 4.2) suggested the effectiveness of roller milling to produce white flour from both grains. Canaryseed contained significantly higher ash content in the whole flour compared to that of whole wheat flour (Table 2) confirming previous observations (Abdel-Aal et al., 2011a). As expected, the ash content of the canaryseed white flour is significantly lower than that of canaryseed whole flour but comparable to that of wheat white flour This result confirms that bran removal results in a decreased mineral content and thereby a negative impact on the nutritional value of the cereal grains. The level of starch is similar in both canaryseed whole four and control-wholewheat flour. As expected, the starch content is significantly higher in the white flours than the whole flours due to the removal of fiber, oil, ash, and some proteins into the bran fractions. The starch content in white-wheat flour
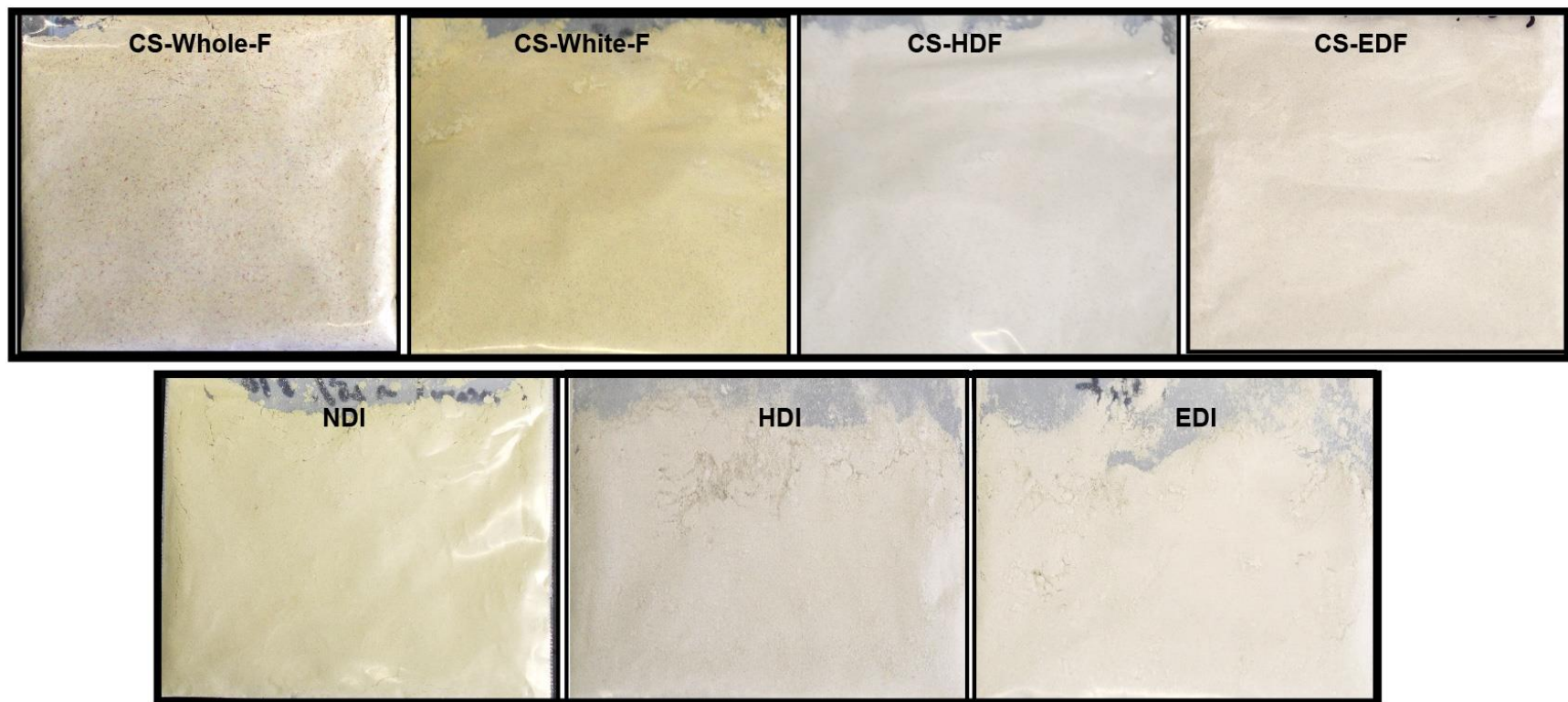
Figure 4.2 Process flow diagram of the experimental plan used in this study. Different fractions obtained for analyses are circled.

Table 4.1 Variation of the color of different canaryseed protein fractions.

Comparison	Sample	L*	a*	b*
Whole vs. roller-milled fractions	Canaryseed whole flour (CS-Whole-F)	84.43 ± 0.02^a	1.11 ± 0.14^b	15.30 ± 0.08^a
	Canaryseed white flour (CS-White-F)	88.63 ± 0.37^b	-0.11 ± 0.02^a	18.77 ± 0.29^b
Non-de-oiled vs. de-oiled flour	Canaryseed white flour (CS-White-F)	88.63 ± 0.37^p	-0.11 ± 0.02^p	18.77 ± 0.29^r
	Hexane-de-oiled white flour (CS-HDF)	90.99 ± 0.09^q	0.21 ± 0.01^q	7.69 ± 0.02^p
	Ethanol-de-oiled white flour (CS-EDF)	91.21 ± 0.06^q	0.25 ± 0.01^q	9.73 ± 0.05^q
Non-de-oiled vs. de-oiled isolates	Non-de-oiled isolate (NDI)	82.03 ± 0.31^x	0.11 ± 0.11^x	22.19 ± 0.65^y
	Hexane-de-oiled isolate (HDI)	83.81 ± 0.31^{xy}	0.25 ± 0.05^x	11.96 ± 1.47^x
	Ethanol de-oiled isolate (EDI)	84.40 ± 0.74^y	0.23 ± 0.07^x	12.94 ± 1.02^x

Note: Values are represented as means ± standard error. Means followed by the same superscript in the same column within the same comparison category are not significantly different ($p > .05$). The alphabetical order of the superscripts is arranged according to the ascending order of the mean values. L* represents brightness with a scale 0‑100 where 0 represents dark color. a* represents red ‑ green spectrum and red is associated with highest a* value. b* represents yellow ‑ blue spectrum and yellow is associated with higher b* value (Kaur & Singh, 2007).
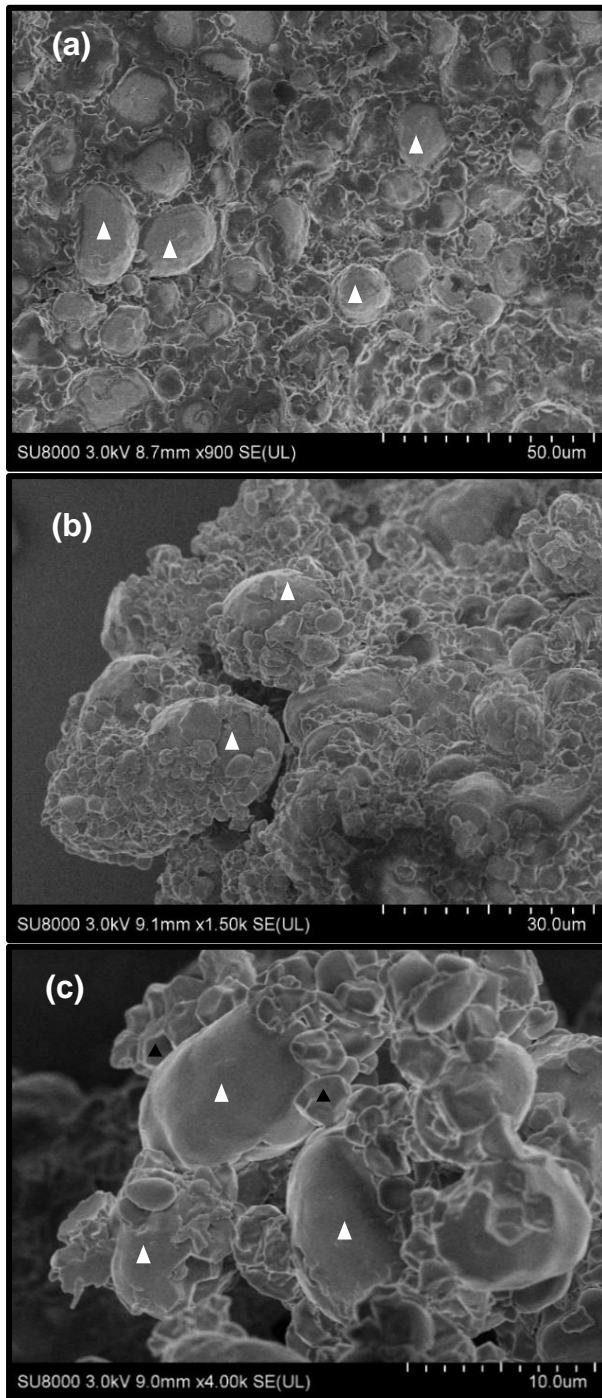
Figure 4.3 SEM images showing changes of the canaryseed endosperm subjected to roller milling. (a) Seed endosperm before roller milling; (b, c) Endosperm flour (white flour) after roller milling at different magnifications. The white triangles represent the compound starch granules while the black triangles represent individual starch granules of canaryseed.

is significantly higher than that of canaryseed-white flour (Table 4.2), mainly due to fiber removal during the roller milling process.

4.4.1.2 Other constituents

Phytic acid and phenolic compounds are largely present in the cereal bran (Dykes & Rooney, 2007; Müge et al., 2020). Phytic acid is a known antinutritive compound in cereals (Müge et al., 2020) and phenolics also could act as an antinutrient since they could block some amino acids and hinder their digestibility and utilization (Jakobek, 2015). Phenolics are often problematic as they could also be responsible for the darker color and undesirable flavor especially in plant-based protein products despite the antioxidant properties that they deliver (Sęczyk et al., 2019; Xu & Diosady, 2012). Removal of bran helps to reduce the level of these components in the resulting white flour and therefore, white flour could be a quality starting material for protein product preparation. The level of phytic acid (on dry weight basis) in canaryseed whole flour (1.28 ±0.06%) was higher than in the white flour (0.35 ±0.01%) while the bran fraction showed the highest content (3.57± 0.30%). The wheat showed similar results with 1.11±0.03%, 0.25 ±0.03%, and 2.42 ±0.01% for the whole-wheat flour, white-wheat flour, and wheat bran fraction, respectively. These results confirm those reported by Abdel-Aal et al. (2011a), where higher phytate content for canaryseed was observed in comparison to wheat (~28%‑41%). Abdel-Aal et al. (2011a) also reported higher content of phytic acid in the whole flour than that of white flour and roller milling resulted highest phytic acid content in the bran fraction. The total phenolic content (TPC) was expressed as mg of ferulic acid equivalent per 100 g of sample. Ferulic acid is the major phenolic compound present in canaryseed (Li et al., 2011). TPC of canaryseed whole flour, white flour, and bran fraction (on dry weight basis) was 137.1 ±0.3mg/100 g, 50.4 ± 1.7mg/100 g, and 215.4 ±1.2mg/100 g, respectively and it was comparable to wheat, that is, 117.9 ±1.5mg/100 g, 50.6 ± 0.6mg/100 g, and 188.7 ± 2.3mg/100 g for the whole-wheat flour, white-wheat flour, and wheat bran fraction, respectively. Li et al. (2011) reported higher TPC levels in canaryseed than the current study. Presumably, due to the changes in the cultivar and growing conditions (Frølich et al., 2013) and changes in the roller milling conditions and fractionation process. Low TPC content in the flour is important to achieve colorless/lighter colored protein product to have a competitive advantage over other proteins in the market. The results of the present study along with the literature data

Table 4.2 Proximate composition of different canaryseed protein fractions on dry weight basis.

Comparison	Sample	% Protein	% Oil	% Ash	% Total starch	% Non-starch carbohydrates*
Whole vs. roller-milled fractions	Canaryseed whole flour (CS-Whole-F)	21.3 ± 0.0^a	6.9 ± 0.1^a	2.6 ± 0.1^a	52.0 ± 1.0^a	17.2 ± 1.0^a
	Canaryseed white flour (CS-White-F)	21.4 ± 0.1^a	6.0 ± 0.0^b	1.0 ± 0.0^b	61.4 ± 1.6^b	10.3 ± 0.7^b
	Bran (CS-B)	22.1 ± 0.1^a	10.5 ± 0.1^c	7.8 ± 0.1^c	31.0 ± 1.2^c	28.7 ± 0.8^c
	Wheat whole flour (W-Whole-F)	13.9 ± 0.1^b	1.2 ± 0.1^d	1.6 ± 0.1^b	53.7 ± 1.0^a	29.5 ± 1.1^c
	Wheat white flour (W-White-F)	12.7 ± 0.0^c	0.3 ± 0.0^e	1.5 ± 0.1^b	72.3 ± 0.6^d	13.3 ± 0.5^{ab}
	Bran (W-B)	15.7 ± 0.7^d	4.2 ± 0.2^f	4.9 ± 0.2^d	34.7 ± 1.0^c	40.4 ± 0.8^d
Non-de-oiled vs. de-oiled flour	Canaryseed white flour (CS-White-F)	21.4 ± 0.1^A	6.0 ± 0.0^A	1.0 ± 0.0^A	61.4 ± 1.6^A	10.3 ± 0.7^A
	Hexane-de-oiled white flour (CS-HDF)	24.1 ± 0.1^B	1.2 ± 0.1^B	1.3 ± 0.2^A	65.5 ± 0.8^B	8.0 ± 0.8^A
	Ethanol-de-oiled white flour (CS-EDF)	23.4 ± 0.0^C	1.4 ± 0.0^B	1.8 ± 0.3^A	65.3 ± 0.8^B	8.1 ± 1.2^A
Non-de-oiled vs. de-oiled isolates	Non-de-oiled isolate (NDI)	69.4 ± 1.9^1	19.5 ± 0.2^1	0.9 ± 0.2^1	Trace[1]	10.2 ± 1.6^1
	Hexane-de-oiled isolate (HDI)	85.0 ± 0.5^2	5.8 ± 0.8^2	1.1 ± 0.1^1	Trace[1]	8.1 ± 0.4^1
	Ethanol de-oiled isolate (EDI)	85.7 ± 1.1^2	6.3 ± 0.3^3	1.4 ± 0.2^1	Trace[1]	6.6 ± 1.1^1
	Commercial wheat gluten isolate (C-WGI)	74.2 ± 0.2^1	2.0 ± 0.1^4	1.2 ± 0.0^1	6.2 ± 0.9^2	16.4 ± 0.6^2

Note: Values are represented as means ± standard error. Means followed by the same superscript in the same column within the same comparison category are not significantly different ($p > .05$). The superscripts a-f, A-C, and 1 and 2 represent comparison between whole versus roller milled fractions, non-de-oiled versus de-oiled flour and non-de-oiled versus de-oiled isolates, respectively.
*Calculated value [$100 - (\Sigma$ %protein, % oil, %ash, %total starch)].

(Abdel-Aal et al., 2011; Li et al., 2011) shows the potential of canaryseed white flour as quality starting material to be utilized for protein ingredient development. Overall, in terms of antinutritive value, white flour is better than the whole canaryseed flour.

4.4.1.3 Protein composition

Canaryseed has higher protein content than wheat (Table 4.2) and other commonly available cereals and pseudocereals in the market (Bekkering & Tian, 2019; Patterson et al., 2018), which is the unique characteristics that makes canaryseed a potential plant-based protein source. There is no significant difference between the protein content of whole and white canaryseed flour, while the protein content in white-wheat flour was 1.2% lower than that of the whole- wheat flour. The polypeptide profiles of whole and white flours of canaryseed, and whole and white flour of wheat did not show remarkable differences (Figure 4.4a). Wheat contains albumins, globulins, prolamins (α-, β-, γ-, ω-gliadin), and high and low molecular weight glutelins as the major seed storage proteins (Siddiqi et al., 2020), and the same protein categories, are also common in canaryseed as illustrated in Figure 4.4. Whole and white canaryseed flours showed polypeptides ranging from 10 to 93 kDa and shared major polypeptide bands representative of albumins (~10 kDa) and globulins and LMW glutelins (~13, ~14, and ~16 kDa). Similar profiles were reported by Achouri et al. (2020). Highly dense protein blot at ~18‑25 kDa region is visible in both these canaryseed flours, which represents prolamins of canaryseed. This protein-dense area is visible under both reducing and nonreducing conditions, suggesting that these canaryseed prolamin-type(s) proteins are lacking disulfide bonds, similarly to the sulfur-poor-prolamin c-hordein in barley (Shewry & Tatham, 1990). The sulfur-poor (s-poor) prolamins usually exist in monomeric form (Shewry & Halford, 2002). Finnie and Svensson (2014) reported that c-hordein does not contain cysteine residues and is only available in monomeric form. Other than s-poor prolamins, the prolamins in cereal are broadly categorized into two other categories, that is, s-rich prolamins. and HMW prolamins (Shewry & Tatham, 1990). The s-rich prolamins could exist as either monomeric or polymeric form whereas, HMW prolamins are only polymers (Shewry & Halford, 2002). The HMW prolamins refer to glutelin proteins as glutelin is known as HMW polymers stabilized by interchain disulfide bonds and structurally related to prolamin in the un-polymerized
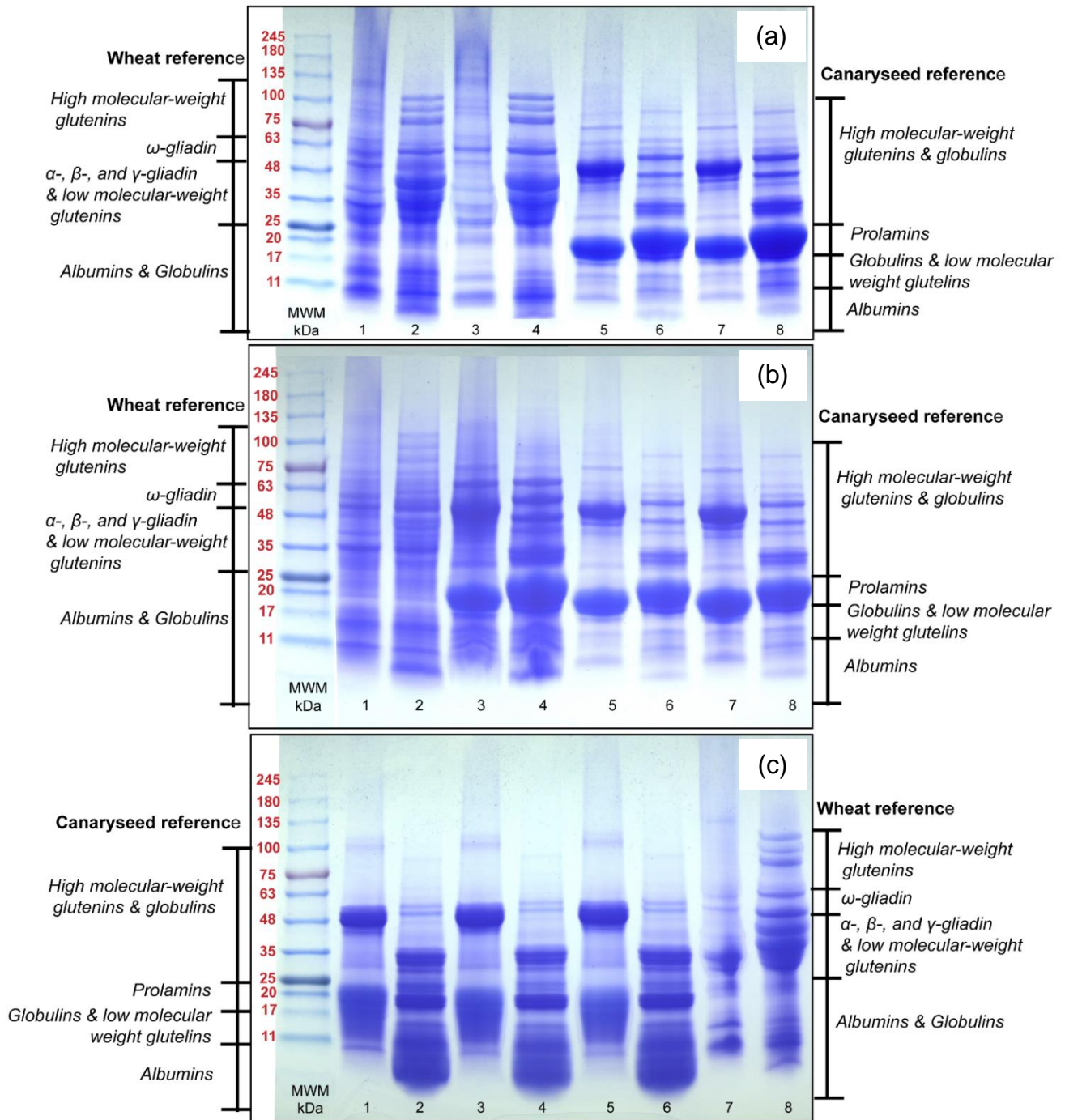
Figure 4.4 Polypeptide profiles of canaryseed and wheat protein fractions. (a) Gel A Lanes 1 and 2:W-whole-F, 3 and 4:W-White-F, 5 and 6: CS-Whole-F, 7 and 8: CS-White-F; (b) Gel B Lanes 1 and 2: W-B, 3 and 4: CS-B, 5 and 6: CS-HDF, 7 and 8: CS-EDF; (c) Gel C Lanes 1 and 2; NDI, 3 and 4: HDI, 5 and 6: EDI, 7 and 8: C-WGI. Lanes with odd number represent nonreducing conditions whereas, lanes with even numbers represent reducing conditions with DTT. MWM, molecular weight markers (FroggaBio™ BLUelf Prestained Protein ladder). The protein references for the polypeptide bands were obtained from Siddiqi et al. (2020) for wheat and Achouri et al. (2020) for canaryseed.

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form (Shewry & Tatham, 1990). The major bands representing HMW glutelins and globulins are visible at ~37, ~38, ~41, ~47, ~58, ~64, ~78, and ~92 kDa and common in both whole and white canaryseed flours (Lanes 5 and 7, Figure 4.4a). The high intensities of these bands compared to the lower molecular weight region (<18 kDa) shows that HMW glutelins are abundant in canaryseed. The glutelins are a polymeric protein type stabilized by interchain disulfide bonds (Shewry & Tatham, 1990); hence tend to disintegrate into lower molecular weight units under reducing conditions. It can be clearly seen that the intense band at ~47 kDa under nonreducing condition (Lanes 5 and 7, Figure 4.4a) is not available under reducing conditions and some high-intensity bands which were not visible under nonreducing conditions emerged at ~29 and ~31 kDa (Lanes 6 and 8, Figure 4a). This result suggests that the band ~47 kDa (under nonreducing conditions) and bands at ~29 and ~31 kDa (under reducing conditions) are representative of glutelins containing disulfide bonds. Moreover, a lower molecular weight band at ~8 kDa is also visible on the whole and white canary seed flour only under reducing conditions. Presumably, a subunit of a disulfide-bond-containing protein of canaryseed. Wheat protein profile of whole and white flours showed similar polypeptide bands between ~10 and 141 kDa as showed by Siddiqi et al. (2020). Therefore, as a value-added protein product, both whole flour and white canaryseed flour share similar potential in this context, their utilization for targeted protein-based application would therefore be differentiated based on their techno-functional and nutritional properties.

4.4.1.4 Techno-functional and nutritional properties of proteins

The oil and water holding capacity of whole canaryseed flour are significantly higher than those of the white flour and they are significantly lower than those of whole wheat and white wheat flour, respectively (Table 4.3). A previous study carried out by Moura et al. (2020) reported 0.89 g water/g flour and 1.2 g oil/g flour for WHC and OHC, respectively for whole canaryseed flour and they were comparable to the values obtained for this study. High water holding capacity is a favorable trait as it facilitates texture, nutrient, and bioactive compound retention by preventing liquid loss during processing (Boucheham et al., 2019; Lam et al., 2018). Similar to the present study, high WHC for wheat flour was reported elsewhere (Boucheham et al., 2019; Joshi et al., 2015). Moreover, the WHC of rice, millet, barley, and corn flours was also reported to be higher

than that of whole and white canaryseed flours (Boucheham et al., 2019; Joshi et al., 2015; Stone et al., 2019) showing that the WHC of canaryseed is lower compared to the other cereals that contain prolamin and glutelins as their major seed storage proteins. The emulsifying capacity of whole canaryseed flour is significantly higher than that of the white flour and was higher than that of wheat (Table 4.3). Better functionality of whole canaryseed flour could be attributed to the dietary fiber in the flour (Ashaolu & Zhao, 2020; Schneeman, 2008). Emulsion stability, foaming capacity, and stability did not show significant differences between the whole canaryseed flour and white flour. The emulsion stability of wheat was similar to canaryseed flours. Wheat had a significantly higher foaming capacity than canaryseed with similar foaming stabilities compared to its corresponding canaryseed flours. Interestingly, the data suggested that neither whole canaryseed flour nor white flour is a good former of foams. However, they showed strong stability once formed. Overall, both whole and white canaryseed flour showed low solubility (~50% or less) and shared similar solubility trend with each other (Figure 4.5a, Moura et al., 2020). The maximum solubility of 50.5% and 45.2% was obtained for whole and white four, respectively at pH 2 whereas, the minimum solubility was observed at pH 5‑7 (2%‑5.6%) for both canaryseed flours. The lower solubility of the flours is comparable with the zeta potential data observed in the pH range (pH 2‑9) tested (Figure 4.5b). Protein solubility is attributed to the net charge on the protein molecule (Achouri et al., 2020) and surface charge (zeta potential) is often used evaluate solubility characteristics of protein products. Higher solubility could be achieved with high zeta potential, usually $> +30$ or < -30 mV, due to strong electrostatic repulsion whereas, lower repulsive force is expected between +30 and −30 mV and consequently a lower solubility (Lam et al., 2017). The zeta potential of the canaryseed flours were always between +30 and −30 mV during pH 2‑9 and hence the lower solubility. The same is true for wheat flours. The white canaryseed flour showed higher solubility than the whole flour from acidic-to-neutral pH and opposite was observed in alkali pH regardless of high zeta potential of white flour during the alkali pH (Figure 4.5b). Wheat showed lower solubility than canaryseed flours at acidic pHs and higher solubility after pH 5 (Figure 4.5a). This trend did not correspond well with the zeta potential data. Neither whole flour nor white flour are merely pure proteins. They are a matrix of macromolecular compounds other than protein and matrix-effect could influence the protein solubility. Hence, it is difficult to explain the solubility trend of these flours by only using the zeta potential.

Table 4.3 Protein functionality of different canaryseed protein fractions.

Comparison	Sample	Oil holding capacity (g oil/g flour)	Water holding capacity (g water/g flour)	Emulsifying capacity (g oil/g protein)	Emulsion stability (%)	Foaming capacity (%)	Foam stability (%)
Whole vs. white flour	CS-Whole-F	1.12 ± 0.01^a	0.85 ± 0.00^a	205.21 ± 2.08^a	88.00 ± 4.16^a	37.78 ± 2.22^a	79.44 ± 2.42^a
	CS-White-F	0.96 ± 0.01^b	0.72 ± 0.00^b	186.46 ± 2.08^b	76.67 ± 1.76^a	30.00 ± 0.00^a	85.19 ± 7.41^a
	W-Whole-F	1.24 ± 0.03^c	1.10 ± 0.05^c	182.29 ± 2.08^b	89.33 ± 2.91^a	80.00 ± 0.00^b	72.22 ± 2.78^{ab}
	W-White-F	$1.17 \pm 0.00b^c$	0.96 ± 0.00^a	151.04 ± 2.08^c	81.33 ± 2.40^a	75.56 ± 4.44^b	56.11 ± 3.09^b
Non-de-oiled vs. de-oiled flour	CS-White-F	0.96 ± 0.01^A	0.72 ± 0.00^A	186.46 ± 2.08^A	76.67 ± 1.76^A	30.00 ± 0.00^A	85.19 ± 7.41^A
	CS-HDF	1.01 ± 0.02^A	0.80 ± 0.01^B	188.21 ± 4.17^{AB}	82.00 ± 5.29^{AB}	37.78 ± 2.22^B	71.67 ± 9.48^A
	CS-EDF	1.23 ± 0.01^B	0.90 ± 0.01^C	201.04 ± 2.08^B	91.33 ± 1.33^B	26.67 ± 0.00^A	79.17 ± 11.02^A
Non-de-oiled vs. de-oiled isolates	NDI	2.72 ± 0.07^1	2.07 ± 0.12^1	203.21 ± 3.61^1	92.67 ± 0.67^1	28.89 ± 1.11^1	80.56 ± 4.24^1
	HDI	3.50 ± 0.29^1	2.48 ± 0.03^2	232.29 ± 5.51^2	90.67 ± 0.67^1	38.89 ± 1.11^1	70.20 ± 3.54^1
	EDI	3.59 ± 0.03^1	2.52 ± 0.03^2	257.29 ± 2.08^3	88.67 ± 1.33^1	28.33 ± 0.96^1	74.67 ± 4.53^1
	C-WGI	2.69 ± 0.32^1	2.69 ± 0.07^2	126.04 ± 2.08^4	54.00 ± 1.15^2	97.78 ± 5.88^2	4.65 ± 2.36^2

Note: Values are represented as means $\pm$ standard error. Means followed by the same superscript in the same column are not significantly different ($p > .05$). The superscripts a-c, A-C, and 1-4 represent comparison between whole versus roller milled fractions, non-de-oiled versus de-oiled flour, and non-de-oiled versus de-oiled isolates, respectively. Abbreviations: CS-B, Bran; CS-EDF, Ethanol-de-oiled white flour; CS-HDF, Hexane-de-oiled white flour; CS-White-F, Canaryseed white flour; CS-White-F, Canaryseed white flour; CS-Whole-F; Canaryseed whole flour; C-WGI, Commercial wheat gluten isolate; EDI, Ethanol de-oiled isolate; HDI, Hexane-de-oiled isolate; NDI, Non-de-oiled isolate; W-B, Bran; W-White-F, Wheat white flour; W-Whole-F, Wheat whole flour.
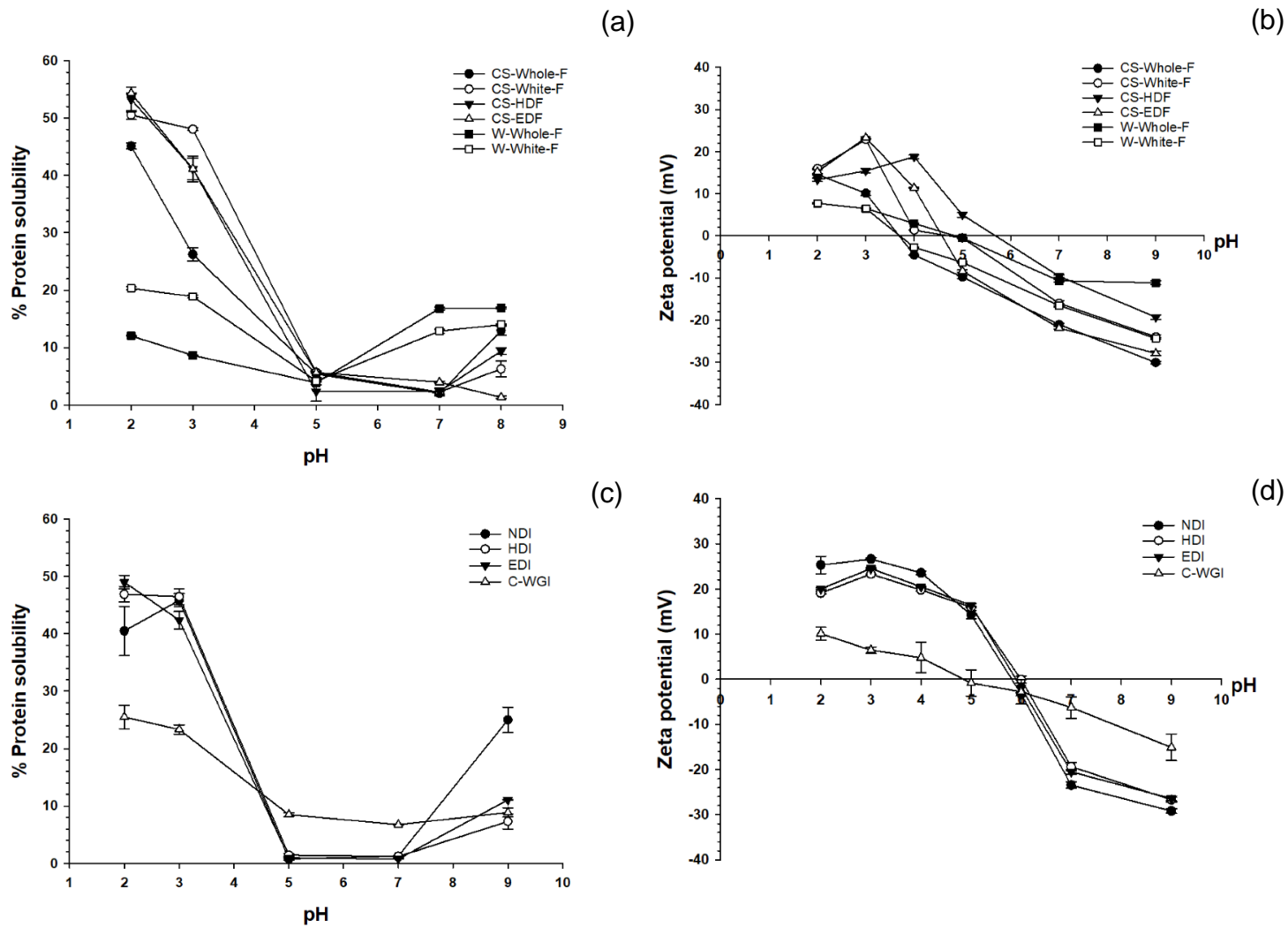
Figure 4.5 Protein solubility and zeta potential of canaryseed and wheat protein fractions. (a) solubility of flours; (b) solubility of isolates; (c) zeta potential of flours; and (d) zeta potential of isolates.

The in vitro protein digestibility of whole canaryseed flour is lower than that of white flour (Table 4.4). However, the IV-PDCAAS value is significantly higher in the whole flour than that of the white flour. Mainly due to the lower limiting amino acid score of lysine. Lysine is the limited amino acid in canaryseed and wheat (Table 4.4) similar to many cereals (Jones & Jones, 2004). During the roller milling process, some protein is lost with the bran fraction causing lower lysine content in the white flour leading to a lower IV-PDCAAS value. The same is true for wheat. This makes whole flour nutritionally superior to that of white flour. The PDCASS values reported in the literature for canaryseed is comparable to the current study (~29%‑31%, Moura et al., 2020) but lower than wheat (~42%‑45%, Joye, 2019; Stone et al., 2019), barley (~60%, Stone et al., 2019), maize (35%, Michaelsen et al., 2009), and rice (54%, Michaelsen et al., 2009). However, it is higher than sorghum (~20%, Boye et al., 2012), which is also an emerging cereal protein source.

4.4.2 Solvent de-oiling of canaryseed

Canaryseed contains significantly higher levels of oil (Table 4.2) compared to other cereals (Abdel-Aal et al., 2011a), resulting in high oil content in the derived protein ingredients, which poses a negative impact on shelf-life stability; hence, de-oiling is often necessary. Oil in wheat is mainly present in the germ (Hoseney & Delcour, 2010a) and removal of germ with the bran fraction using roller milling helps to significantly reduce the level of the residual oil in the flour (Table 4.2). However, it is difficult to achieve the same level of oil reduction in the canaryseed flour since significant level of oil is distributed throughout its endosperm (Perera et al., 2021; Table 4.2). Roller milling removes a noticeable level of oil (~10.5%‑13%, Table 4.2) into the bran fraction of canaryseed hence, the bran fraction can be utilized to produce canaryseed oil as a by-product of canaryseed value chain. The remaining oil in both whole canaryseed flour (6.9%) and white flour (6%, Table 4.2) requires de-oiling to achieve high shelf-life stability for the flours and resulting value-added protein products. De-oiling using organic solvents is one of the common/conventional methods used in vegetable oil production industry (Nde & Foncha, 2020) and selection of the right solvent is important considering the cost, efficiency of oil extraction and resulting crude oil quality, effect on protein quality, environmental impact, and marketability. Therefore, in this study, we focused on assessing two major solvents used for

Table 4.4 Limiting amino acid scores and protein quality data of different canaryseed protein fractions.

Comparison	Sample	Limiting amino acid	Limiting amino acid score[i]	IVPD[ii]	IV-PDCAAS[iii] (%)
Whole vs. white flour	CS-Whole-F	Lysine	0.43	77.9 ± 0.3^a	31.2 ± 0.1^a
	CS-White-F	Lysine	0.34	$79.3 \pm 0.5b^b$	23.0 ± 0.1^b
	W-Whole-F	Lysine	0.42	79.8 ± 0.3^b	31.9 ± 0.1^c
	W-White-F	Lysine	0.34	75.9 ± 0.2^c	23.1 ± 0.1^b
Non-de-oiled vs. de-oiled flour	CS-White-F	Lysine	0.34	79.3 ± 0.5^A	23.0 ± 0.1^A
	CS-HDF	Lysine	0.35	79.8 ± 0.3^A	25.5 ± 0.1^B
	CS-EDF	Lysine	0.36	80.0 ± 0.5^A	26.4 ± 0.1^C
Non-de-oiled vs. de-oiled isolates	NDI	Lysine	0.30	84.9 ± 0.7^1	25.5 ± 0.2^1
	HDI	Lysine	0.28	84.4 ± 0.3^1	23.6 ± 0.1^2
	EDI	Lysine	0.26	83.8 ± 0.7^1	21.8 ± 0.7^3
	C-WGI	Lysine	0.29	83.3 ± 0.2^1	24.2 ± 0.1^{12}

Note: Values are represented as means ± standard error. Means followed by the same superscript in the same column within the same comparison category are not significantly different ($p > .05$). The superscripts a-c, A-C, and 1-3 represent comparison between whole versus roller milled fractions, non-de-oiled versus de-oiled flour, and non-de-oiled versus de-oiled isolates, respectively. [i]Measurements were performed once using the corresponding amino acid composition in Appendix D-E. [ii]Measurements were performed in triplicate. Data represent the mean ± standard error. [iii]Data represent the product of the limiting amino acid score and IVPD (measured in triplicate).

Plant-seed oil extraction, that is, hexane and ethanol (Keneni et al., 2021), in terms of oil extraction efficiency, crude oil quality, and effect that they pose on the protein quality.

4.4.2.1 Fatty acid and phospholipid profiles of hexane- and ethanol-extracted oil

Hexane is a non‐polar organic solvent whereas ethanol is a more polar organic solvent with a relative polarity of 0.009 and 0.654, respectively (Reichardt & Welton, 2010), The difference in the solvent properties suggests differences in oil extraction using these solvents. The total fatty acid extracted using hexane is lower than that of ethanol and vice versa for total non-fatty acids (Table 4.5). Consequently, the level of total unsaturated, omega 3, omega 6, and omega 9 fatty

acids content in ethanol-extracted oil is higher than that of hexane-extracted oil. The level of saturated fatty acids in ethanol-extracted oil is also higher than the hexane-extracted oil. However, the increment is only 4mg/g oil and not remarkable. These results suggest better oil extraction efficiency and quality for ethanol extracted oil. Regardless of the solvent used, canaryseed oil is highly unsaturated and linoleic acid is the predominant fatty acid followed by oleic acid (Table 4.5; GRAS notice, 525, Abdel-Aal et al., 1997a), which accounts for ~80% of the total fatty acids extracted. Palmitic acid is the next most abundant fatty acid in both ethanol and hexane extracted canaryseed oil. The results obtained in the current study are comparable to the previously reported values for canaryseed oil extracted using the whole canaryseed groat (GRAS notice, 525, Abdel-Aal et al., 1997a). The hexane-extracted oil showed minute levels of caprylic and capric acids, which are medium chain fatty acids (C7-12, Schönfeld & Wojtczak, 2016), and Eicosatrienoic acid, which is a log-chain fatty acid (>C12, Schönfeld & Wojtczak, 2016) whereas they were not detected in ethanol-extracted oil (Table 4.5). Moreover, the level of other fatty acids is higher in hexane extracted oil (3.1%) than that of ethanol extracted oil (0.14%), showing more compositional diversity in hexane extracted oil.

The crude oil extracted using both these solvents showed dark color (Figure 4.6), presumably due to the carotenoid pigments (Li & Beta, 2012) present in the canaryseed. Presence of color pigments, along with some tocopherols present in canaryseed (GRAS notice, 525) could provide some antioxidative activity to the highly unsaturated canaryseed oil however, a lighter color is often associated with better quality oils (Shahidi, 2005). Moreover, total lipids extracted from whole canaryseed flour, using modified Bligh and Dyer (1959) method at chloroform-methanol-water system at 1:6:2 v/v/v ratio, showed a total phospholipid content of 5.04%, which is higher than that of soybean, rapeseed, and sunflower oil (1.0%‑3.7%) (Chew & Nyam, 2020; Nazi & Proctor, 1998; Wang et al., 1997). Due to the high polarity of ethanol compared to hexane, a higher content of phospholipids (4.38%) was extracted into the oil with ethanol whereas, only 0.82% was extracted with hexane (Table 4.6).

N-acylphosphatidylethanolamine, Phosphatidic acid, Phosphatidylethanolamine, Phosphatidylcholine, Phosphatidylinositol, and Lysophosphatidylcholine were detected as the phospholipid constituents in hexane-and ethanol-extracted canaryseed oil. Phosphatidylcholine is the major constituent of canaryseed phospholipids in both ethanol- extracted and hexane-extracted oil and accounted for 59.5% and 62.1% of the total phospholipids, respectively.

Table 4.5 Fatty acid composition of white-canaryseed-flour oil extracted with hexane and ethanol.

	Hexane	Ethanol
Fatty Acids	CS_White flour (mg FA/g oil)	CS_White flour (mg FA/g oil)
C8 Caprylic	0.3	ND
C10 Capric	0.3	ND
C14 Myristic	1.5	1.5
C16 Palmitic	94.3	98.7
C16:1n7 Palmitoleic	1	1.1
C17 Magaric	0.3	0.4
C18 stearic	11.2	11.4
C18:1n9 Oleic	212.9	238.5
C18:1n7 Vaccenic	6.1	7.1
C18:2 Linoleic	381.6	438.3
C18:3n3 alpha-Linolenic	13.7	18.3
C20 Arachidic	1.1	1
C20:1 Eicosenoic	7.7	8
C20:3n3 Eicosatrienoic	0.5	ND
C22 Behenic	0.6	0.6
C22:1n9 Erucic	0.8	0.8
C24 Lignoceric	0.4	0.4
C24:1n9 Nervonic	0.4	0.4
Others	15.2	1.2
Total Fatty Acids[a]	749.9	827.7
Total Saturates[b]	110	114
Total unsaturate[c]	624.7	712.5
Total Monounsaturates[d]	228.9	255.9
Total Polyunsaturates[e]	395.8	456.6
Total Omega 3[f]	14.2	18.3
Total Omega 6[g]	381.6	438.3
Total Omega 9[h]	221.8	247.7
Total non-fatty acids[i]	250.1	172.3

[a] Σ C8, C10, C14, C16, C16:1n7, C17, C18, C18:1n9, C18:1n7, C18:2n6, C18:3n3, C20, C20:1n9, C20:3n3, C22, C22:1n9, C24, C24:1n9, others
[b] Σ C8, C10, C14, C16, C17, C18, C20, C22, C24
[c] Σ C16:1n7, C18:1n9, C18:1n7, C20:1n9, C22:1n9, C24:1n9, C18:2n6, C18:3n3, C20:3n3
[d] Σ C16:1n7, C18:1n9, C18:1n7, C20:1, C22:1n9, C24:1n9
[e] Σ C18:2n6, C18:3n3, C20:3n3
[f] C18:3n3,
[g] C18:2n6
[h] Σ C18:1n9, C20:1n9, C22:1n9, C24:1n9
[i] 1000 – Total fatty acids[1]
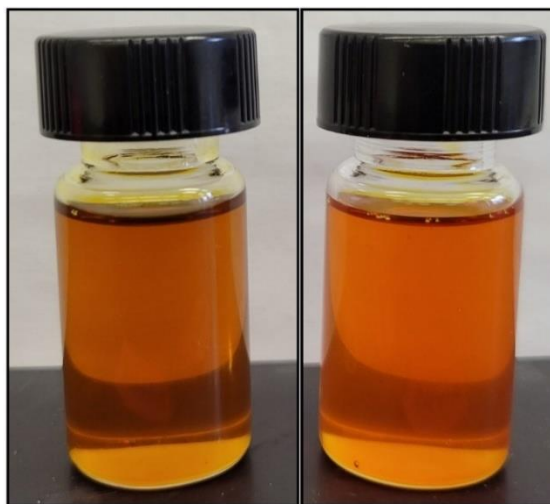
Figure 4.6. Crude canaryseed oil from white flour extracted using hexane (left) and Ethanol (right)

Lysophosphatidylcholine is the second most abundant phospholipid that represents 26.2% of the total phospholipids in ethanol-extracted oil whereas, it was phosphatidylethanolamine for hexane-extracted oil that represent 21.9% of the total phospholipids (Table 4.6). Phosphatidic acid and phosphatidylinositol were detected in lower quantities for both types of canaryseed oil. High levels of phospholipid cause undesirable effects in the refining process and storage of edible oil (Chew & Nyam, 2020). Since ethanol-extracted oil had a remarkably high level of phospholipids, lower stability of crude oil is expected when compared to hexane-extracted oil. Presence of a high level of phospholipids, dark color due to pigments and risk of oxidation of highly unsaturated fatty acids requires further refining of canaryseed crude oil to improve quality and shelf-life stability. Typically, degumming, bleaching, and deodorization are carried out as the major steps of oil refining process to remove phospholipids, pigments, volatiles, and free fatty acids from the crude oil in the industry (Chew & Nyam, 2020). The phospholipids removed from the degumming process is the source for lecithin, which is a valuable by-product in edible oil industry (Chew & Nyam, 2020). Since canaryseed possess significant level of phospholipids, lecithin could be a by-product that could add value to the emerging canaryseed industry.

4.4.2.2 Effect of de-oiling on canaryseed protein

Other than the solvent cost, requirement of explosion-proof facilities and equipment, and negative environmental impact, the major drawback of solvent de-oiling is the destabilizing effect it has on the tertiary-structural confirmation of protein (Arêas et al., 1993). Utilizing organic solvents is often associated with protein denaturation due to solubilizing non-polar side chains of

Table 4.6 Phospholipid composition of white-canaryseed-flour oil extracted from hexane and ethanol.

Phospholipid (%)	Hexane-oil	Ethanol-oil
N-acylphosphatidylethanolamine	0.13	0.22
Phosphatidic Acid	<0.01	<0.01
Phosphatidylethanolamine	0.18	0.4
Phosphatidylcholine	0.51	2.61
Phosphatidylinositol	<0.01	<0.01
Lysophosphatidylcholine	<0.01	1.15
Total Phospholipids	0.82	4.38

amino acid residues and weakening the hydrophobic interactions in protein (Damodaran, 2017). Denaturation of protein alters the native functional properties of the proteins. Nielson (1997) showed that the functionality of soy protein concentrates and isolates could be modified due to the techniques used for defatting and processing. Hence, the protein ingredient manufacturers tend to minimize or avoid the use of solvent de-oiling to retain native functionality of the protein product and increase marketability. However, the changes caused to the protein by the solvent is not always substandard. Protein denaturation could improve accessibility to digestive enzymes to improve digestibility and also potentially lower allergenicity if the allergy is caused by a conformational epitope (EFSA & European Food Safety Authority, 2014). Moreover, it could improve some functional properties, such as emulsification and foaming due to increase hydrophobicity because of structural unfolding/denaturation (Galves et al., 2019). Therefore, as an emerging protein source, it would be important to understand the effect of solvent-de-oiling on canaryseed protein and its compositional, structural, functional, and nutritional properties.

Composition of de-oiled flours and isolates

The residual oil content between hexane-de-oiled flour (CS-HDF) and ethanol-de-oiled flour (CS-EDF) was not significantly different (Table 4.2). As a result of oil removal, the protein, ash and total starch contents subsequently increased in the de-oiled flours relative to that of the

white flour (CS-White-F). However, no significant difference was observed for the non-starch carbohydrates fraction.

The isolates produced from the de-oiled flours contained significantly higher protein content and lower oil content than those produced from the white (non-deoiled) flour (Table 4.2). A de-oiling step is therefore essential to s reach the >80% of protein content of the product to allow its marketing as a high value "protein isolate." The protein content of the non-de-oiled isolate (NDI) is comparable to the commercial wheat gluten (C-WGI) tested in this study. However, the level of oil in the C-WGI is significantly lower than that of NDI, hexane-de-oiled isolate (HDI) and ethanol-de-oiled isolate (EDI), likely conferring to the C-WGI a better shelf-life stability than the canaryseed isolates. The process used in this study to develop the protein isolates from the flours involved enzyme digestion of starch and screening to remove the fine-fiber fraction. The enzyme treatment used in this process was successful and only trace level of total starch was obtained for non-de-oiled and de-oiled isolates (Table 2). On the other hand, C-WGI contained a significantly higher level of starch than that of NDI, HDI, and EDI. The non-starch carbohydrates did not show significant reduction in the NDI, HDI, and EDI compared to that of the starting CS-White-F. Since screening was used to remove fine fiber fraction, this is most likely the contribution of sugars resulting from starch digestion. The C-WGI contained a significantly higher level of non-starch carbohydrate fraction compared to any of the canaryseed isolates and it is likely the contribution of fiber as no enzymatic digestion was utilized in the commercial process to the best of authors knowledge.

Structural changes flours and isolates

The effects of utilizing hexane and ethanol on the polypeptide pattern of canaryseed flour and isolates was investigated using SDS-PAGE. The same polypeptide patterns were observed between CS-White-F (Figure 4.4a, Lanes 7 and 8), CS-HDF (Figure 4.4b, Lanes 5 and 6) and CS‑EDF (Figure 4.4b, Lanes 7 and 8), and between NDI, HDI, and EDI (Figure 4.4c), showing a lack of influence of solvents on the polypeptide profile of canaryseed flour and derived isolates. However, the number of bands between 10 and 110 kDa region of the isolates is lower than the number of bands visible 10‑93 kDa region in the white flour, especially >50 kDa that belongs to the HWM glutelin and globulin region. This shows that some of the proteins were lost into the byproduct/waste streams during the isolate production process. Since, neither weakly acid nor

bases solvents were involved in the isolate production process, these bands could presumably be the HMW globulins. On another hand, the band at ~110 kDa visible in the isolate samples was not visible in any of the canaryseed flour samples. Presumably, a protein that got concentrated during the isolation process. This high molecular weight band ~110 kDa is not visible under reducing conditions in any of the isolates. Instead, there are some new and extremely faded bands appearing in the ~50‑93 kDa region under the reducing condition. Therefore, most likely, the band ~110 kDa is a HMW glutelin containing disulfide bonds. The polypeptide bands representative of prolamins (18‑25 kDa) are distinctively visible in all three canaryseed isolates under reducing condition whereas, it was not distinctive under nonreducing conditions. The region ~12‑25 kDa is diffused, suggesting prolamins proteins are bound with non‑prolamin proteins due to high protein concentration in the isolates creating large number of overlapping protein bands in the region. Apparently, DTT facilitated breaking protein interactions in the region making prolamins more visible and making the low molecular weight region more intense with high concentration of proteins. The C-WGI showed major polypeptide bands representing, albumins, globulins, gliadins, and glutelins ranged from 8 to 157 kDa (Figure 4.4c, Lanes 7 and 8). Similar to canaryseed, lower number of polypeptide bands were visible in the gluten sample compared to the wheat flours, showing evidence of protein loss during processing. The polypeptide profiles of canaryseed isolates evaluated by Achouri et al. (2020) and Moura et al. (2020) showed clear differences in the polypeptide profiles to the current study, especially in high molecular weight glutelin and globulin region and lower molecular weight region <10 kDa, mainly representative of albumins. In these two studies, the canaryseed protein isolates were prepared using alkali extraction and isoelectric precipitation processes at different extraction pH levels whereas, a completely different process was used to prepare isolates in the present study. Therefore, differences in the type of proteins that were concentrated into the isolates in these studies could be expected. Moreover, the harsh alkaline conditions (pH 12) used to extract proteins by Achouri et al. (2020) may have caused some alkali‑induced structural damages according to the authors. This could be another factor that influences the differences in the polypeptide profiles observed in these studies.

The effect of organic solvent de-oiling on protein structure was further evaluated using DSC as it is a commonly used technique to evaluate protein denaturation/unfolding (Johnson, 2013; Mazurenko et al., 2017). The DSC thermograms of canaryseed CS-Whole-F, CS-White-F, CS-HDF, and CS-EDF showed three distinct endothermic peaks whereas, the isolates samples,

that is, NDI, HDI, and EDI, showed only one peak (Figure 4.7; Table 4.7). The peak in the protein isolates is observed at 108 °C (Tp) and is also visible in all the canaryseed flour samples; hence it is the peak related to protein denaturation. Moreover, this peak is not visible in the reheating curves (data not shown) of these samples and confirmed the identity of the protein denaturation peak and its high thermal stability. Since canaryseed flour and isolates mainly contain prolamins and glutelins (Figure 4.4), this peak is presumably attributed to these major proteins, especially the glutelins. Glutelin is reported to have high thermal stability in wheat (84 °C; Leon et al., 2003) and rice (82.2°C; Ju et al., 2001). According to Leon et al. (2003), wheat albumin and globulins denature at 55°C, and prolamins (gliadins) denature at 58 °C. Albumins and globulins of rice protein denature at 73.3 °C and 78.9 °C, respectively however, thermal denaturation peak was not observed for prolamins (Ju et al., 2001). The literature on protein denaturation of other cereals suggests that albumins, globulins, and prolamins in canaryseed have a lower denaturation temperature than that of glutelins. The DSC result of the present study showed higher thermal stability of canaryseed protein compared to other cereals and is comparable to that of oat globulins (~110 °C; Ma & Harwalkar, 1984). The protein denaturation peak of canaryseed at 108 °C is visible in all the solvent-de-oiled samples, showing that they were not capable of causing substantial changes to the canaryseed protein structure. Another study carried out related to soybean also showed that the denaturation temperatures of major storage protein, that is, glycinin (11 S) and β-conglycinin (7 S), were not affected by the solvent-de-oiling carried out in the study (L'Hocine et al., 2006). These studies suggest that upstream de-oiling of flour do not damage the structural integrity of globulin-type, prolamin, and glutelin-type proteins. However, subtle structural changes and associated functionality changes could be expected, even though these changes are not reflected in DSC thermograms. The C-WGI sample did not show any thermal transition peak, suggesting protein denaturation during processing.

In addition to the protein denaturation peak, there are two major peaks visible in the canaryseed and wheat flour samples (Figure 4.7). The first DSC peak (Tp = 70°C‑71°C) for canaryseed flour is comparable to that reported for purified canaryseed starch (Irani et al., 2017) and is therefore, attributed to starch gelatinization, which occurs at higher temperature than that of which is observed at ~60 °C (Tp) (Table 4.7) and comparable to values reported in literature (Irani et al., 2017; Kwaśniewska-Karolak et al., 2008; Leon et al., 2003). Differences of the starch gelatinization temperatures could be expected depending on the moisture content of the sample
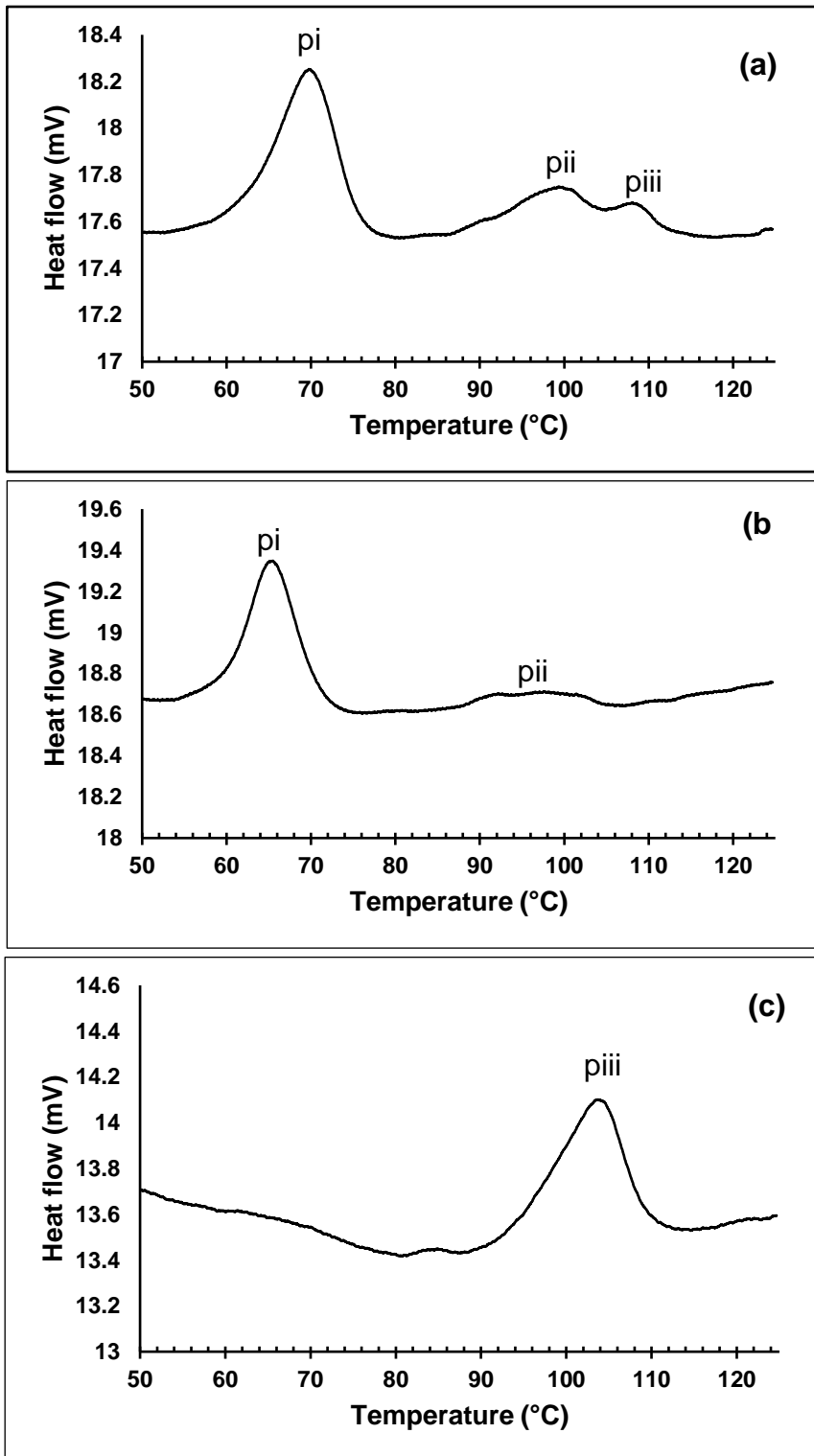
Figure 4.7 DSC thermograms of canaryseed flours and isolate fractions. a) Typical thermogram obtained for CS-Whole-F, CS-White-F, CS-HDF and CS-EDF; b) Typical thermogram obtained for W-whole-F and W-White-F; c) Typical thermogram obtained for canaryseed NDI, HDI and EDI. "p" indicates major endothermic peaks observed in each category. pi=starch gelatinization; pii=Amylose lipid complex and piii=protein denaturation.

Table 4.7 Thermal properties of different canaryseed protein fractions obtained using differential scanning calorimetry analysis.

Sample	Starch gelatinization (pi)				Peak 2 (pii)				Peak 3 (piii)			
	To (°C)	Tp (°C)	Tc (°C)	ΔH (J/g)	To (°C)	Tp (°C)	Tc (°C)	ΔH (J/g)	To (°C)	Tp (°C)	Tc (°C)	ΔH (J/g)
CS-Whole-F	65.0 ± 0.9[a]	71.0 ± 0.0[a]	75.8 ± 0.2[a]	7.2 ± 0.4[a]	86.4 ± 0.8[a]	95.0 ± 0.5[a]	NA	NA	NA	108.0 ± 0.0[a]	110.8 ± 0.1[a]	NA
CS-White-F	63.5 ± 0.3[a]	70.2 ± 0.1[a]	75.2 ± 0.1[a]	8.1 ± 0.5[a]	90.1 ± 1.7[a]	95.2 ± 0.4[a]	NA	NA	NA	108.5 ± 0.1[a]	111.4 ± 0.1[a]	NA
W-Whole-F	59.7 ± 0.6[b]	64.2 ± 0.3[b]	68.6 ± 0.2[b]	7.0 ± 0.6[a]	87.9 ± 1.1[a]	95.8 ± 0.6[a]	99.8 ± 2.7[a]	1.3 ± 0.0[a]	piii is not detected			
W-White-F	58.6 ± 0.2[b]	63.2 ± 0.1[c]	68.0 ± 0.0[b]	8.1 ± 0[a]	88.8 ± 0.3[a]	96.3 ± 0.1[a]	100.7 ± 0.3[a]	1.4 ± 0.0[a]	piii is not detected			
CS-White-F	63.5 ± 0.3[A]	70.2 ± 0.1[A]	75.2 ± 0.1[A]	8.1 ± 0.5[A]	90.1 ± 1.7[A]	95.2 ± 0.4[A]	NA	NA	NA	108.5 ± 0.1[A]	111.4 ± 0.1[A]	NA
CS-HDF	63.5 ± 0.3[A]	69.9 ± 0.1[AB]	74.7 ± 0.1[B]	8.5 ± 0.2[A]	85.5 ± 0.5[B]	98.9 ± 0.1[B]	NA	NA	NA	108.8 ± 0.2[A]	111.3 ± 0.2[A]	NA
CS-EDF	63.3 ± 0.3[A]	69.8 ± 0.0[B]	75.0 ± 0.0[AB]	8.8 ± 0.4[A]	94.2 ± 0.4[A]	98.9 ± 0.2[B]	NA	NA	NA	108.4 ± 0.2[A]	110.8 ± 0.1[A]	NA
NDI	pi is not detected				pii is not detected				100.7 ± 0.2[1]	108.3 ± 0.4[1]	112.8 ± 0.4[1]	5.5 ± 0.1[1]
HDI	pi is not detected				pii is not detected				100.1 ± 1.0[1]	108.9 ± 0.1[1]	114.4 ± 0.7[1]	7.3 ± 0.4[2]
EDI	pi is not detected				pii is not detected				101.2 ± 0.7[1]	108.7 ± 0.2a[1]	112.8 ± 1.2[1]	7.5 ± 0.2[2]
C-WGI	pi is not detected				pii is not detected				piii is not detected			

Note: Values are represented as means ± standard error. Means followed by the same superscript in the same column within the same comparison category are not significantly different (p > .05). The superscripts a–c, A and B, and 1 and 2 represent comparison between whole versus roller milled fractions, non-de-oiled versus de-oiled flour, and non-de-oiled versus de-oiled isolates, respectively. Abbreviations: CS-B, Bran; CS-EDF, Ethanol-de-oiled white flour; CS-HDF, Hexane-de-oiled white flour; CS-White-F, Canaryseed white flour; CS-White-F, Canaryseed white flour; CS-Whole-F; Canaryseed whole flour; C-WGI, Commercial wheat gluten isolate; EDI, Ethanol de-oiled isolate; HDI, Hexane-de-oiled isolate; NDI, Non-de-oiled isolate; W-B, Bran; W-White-F, Wheat white flour; W-Whole-F, Wheat whole flour. To, onset temperature; Tp, peak temperature; Tc, conclusion temperature; and ΔH, enthalpy change.

(Biliaderis et al., 1980; Leon et al., 2003) and differences in the other constitutes in the flour matrix that could interfere with heat distribution efficiency. Irani et al. (2017) reported that the second peak of the purified starch visible at 105 °C (Tp) for canaryseed and 103 °C (Tp) for wheat (Figure 4.7, Table 4.7) is attributed to amylose-lipid complexes (ALCs) ALCs in several wheat varieties (Kwaśniewska-Karolak et al., 2008). The ALCs dissociate during heating and reassociate during cooling and therefore, it is a reversible process (Kwaśniewska-Karolak et al., 2008). This peak related to ALCs is the only peak that was available in the reheating curve of these canaryseed and wheat flour samples (data not shown), hence, confirming its identity. The ALCs peak is partially overlapped with the protein denaturation peak of the canaryseed flour samples making it difficult to accurately calculate several thermal parameters for those peaks in the flour samples (Table 4.7).

Changes to functional and nutritional properties

One of the prominent effects of solvent-de-oiling is the improvement of the color of the de-oiled flour and subsequent protein isolates produced from those flours (Figure 4.2, Table 4.1). Color was improved in hexane and ethanol de-oiled flours and isolates (higher L* value and lower b* value) compared to that of the non-de-oiled flour and isolates (Table 4.1). Hence, the de-oiled flours and isolates appear lighter than the inherent yellowish color of the white canaryseed flour and NDI (Figure 4.2). Carotenoids (mainly β-carotene followed by lutein and zeaxanthin) are mainly responsible for the color of canaryseed flour and are lipid-soluble pigments (Li & Beta, 2012), and therefore removed during solvent de-oiling (Figure 4.2). Subtle improvement of the color with ethanol de-oiled flours and isolates was observed in comparison to hexane (Table 4.1). Improvement of protein color due to solvent de-oiling was also reported elsewhere (Galves et al., 2019; Teh et al., 2014).

De-oiling of canaryseed flour improved its water holding capacity (Table 4.3), regardless of the type of solvent used. The same trend was observed for non-de-oiled and de-oiled canaryseed isolates. Compared to hexane, ethanol-de-oiling improves the WHC of canaryseed flour however, there is no significant difference observed between hexane and ethanol at the isolate level. Achouri et al. (2020) reported 1.2‑1.4mL/g WHC for canaryseed isolates prepared using ethanol de-oiled flour. The WHC reported in the literature is lower than the EDI of this study, presumably due to changes of the process and conditions used in these two studies and differences in the analytical

method used. Improvement of WHC due to solvent-de-oiling was also reported for safflower and hemp protein (Galves et al., 2019). On the other hand, lower WHC due to de-oiling was reported for sunflower protein (Galves et al., 2019), hemp, canola, and flax flour (Teh et al., 2014) or remain unchanged as for soy protein (L'Hocine et al., 2006). The two de-oiled isolates, that is, HDI and EDI, showed similar WHC as C-WGI whereas, it is significantly lower in NDI. The oil holding capacity of canaryseed de-oiled flour increased upon solvent de-oiling according to the following order: CS-White-F> CS-HDF > CS-NDF. Similar to WHC, ethanol-de-oiling showed better OHC than that of hexane-de-oiling. The OHC of the de-oiled isolates too showed some improvement compared to that of NDI and C-WGI. However, the increment is not significant. Similar to WHC, the OHC too could be increased, decreased, or remain unchanged after solvent de-oiling (Galves et al., 2019; L'Hocine et al., 2006; Teh et al., 2014).

The EC of canaryseed flour only improved with ethanol-de-oiling in CS-EDF sample and CS-HDF did not show a significant difference compared to the CS-White-F (Table 4.3). On the other hand, both hexane and ethanol de-oiled protein samples showed improved EC at the isolate level. All the canaryseed protein isolates had a significantly higher EC than that of the C-WGI. Improved EC due to solvent de-oiling was previously reported for oilseed proteins (L'Hocine et al., 2006; Teh et al., 2014). The authors suggested that this improvement is mainly caused due to increasing hydrophobicity because of exposing the hydrophobic amino acids buried inside the core of the protein as a result of solvent-induced structural unfolding/denaturation. Although significant structural unfolding in canaryseed protein was not evident due to the solvent-de-oiling according DSC results, it's plausible that de-oiling caused a small degree of unfolding that led to increased hydrophobicity and improved functionality. Although increased hydrophobicity could favor the foaming properties similar to EC (Galves et al., 2019) there was no significant difference of FC and FS observed between non-de-oiled and de-oiled canaryseed proteins at both flour level and the isolate level. The balance between the distribution charged and hydrophobic sites on the surface of the protein determines how well the protein could align in the air-water interface to provide good foaming properties (Damodaran, 1994); perhaps, this balance is not available in canaryseed protein to provide good foaming properties despite the solvent-de-oiling. The ES of CS-White-F, CSHDF, and CS-EDF follow the same trend as with EC. However, no significant difference of EC between non-de-oiled and de-oiled canaryseed isolates was observed. Compared to C-WGI, all canaryseed isolates showed significantly higher EC, ES, and FS. However, the FC of C-WGI is

significantly higher than that of any of the canaryseed isolates. Solvent-de-oiled canaryseed flours and isolates shared a similar solubility trend as with their non-de-oiled counterparts (Figure 4.5a, b). In all cases, high solubility at acidic pH <5, minimum solubility from pH 5‑7 and increased solubility at alkaline conditions >pH 7 was observed with the inherent lower solubility nature of canaryseed (Moura et al., 2020; Achouri et al., 2020) that is correlated with the zeta potential, that is, +30 to −30 mV (Lam et al., 2017) across the pH levels tested. Similar solubility pattern for canaryseed was reported by Achouri et al. (2020) with a maximum solubility at of 50.4% at pH 2, which is comparable to the maximum solubility achieved for NDI (50.5%) and EDI (49%) at pH 2 in this study. The C-WGI showed lower solubility at <pH 5 compared to canaryseed isolates and higher solubility from pH 5‑7 (Figure 4.5b). The solubility of C-WGI was higher at alkaline pH compared to the HDI and lower than NDI and EDI after pH 7 and pH 8, respectively. The solubility of de-oiled flours and isolates of canaryseed were sometimes better at certain pH levels, for example, pH 2 (CS‑HDF = 53.2%, CS‑EDF = 54.3%, HDI= 46.9%, and EDI= 49%) than that of CS-White-F (50.5%) and NDI (40.5%), whereas it was the opposite at some other pH levels, for example, pH 9 (CS‑White-F = 6.2%, CSEDF = 1.3%, NDI = 25%, HDI= 7.2%, and EDI = 11%) compared to that of non-de-oiled flour and isolate. The same is true for hexane versus ethanol-de-oiled protein. Therefore, it is difficult conclude whether solvent-de-oiling affects the solubility positively or negatively for canaryseed protein. Depending on the pH of the medium, it could be either increased, decreased or remain similar. Overall, the functional properties of canaryseed proteins appears to be improved in most of the time due to solvent-de-oiling.

The result obtained from IVPD showed that there is no significant effect on breaking down proteins by the digestive enzymes after solvent-de-oiling, regardless of the solvent type used (Table 4.4). However, Significant improvement in IV-PDCAAS values were observed for solvent-de-oiled canaryseed flours, mainly due to improved limiting amino acid score influenced by the increased levels of lysine content. The increased levels of lysine content could be attributed to removal of oil and other solvent-soluble constituents during the de-oiling process. Compared to hexane, ethanol showed better protein quality in terms of PDCAAS values. On the other hand, the limiting amino acid score in HDI and EDI was lower than that of NDI and as a result, the IV-PDCAAS values were lower in the de-oiled isolates. It seems that lysine is lost to waste streams when the isolates were produced using solvent-de-oiled flours resulting lower amino acid score than that of NDI. It is a disadvantage of solvent-de-oiling although it was favorable at the flour

level. Despite de-oiling or not, the IVPDCASS values of the canaryseed protein isolates ranged from 21.8% to 25.5% which is in the low range and comparable with the IVPDCASS the flours (Table 4.4). On the other hand, canaryseed isolates produced from alkali extraction and isoelectric precipitation process showed improved PDCASS values ranging from 55.7% to 62.7% in comparison to that of canaryseed flour (Moura et al., 2020). Although lysine is the limiting amino acid in the present study across flour and isolates, it was histidine in the isolates produced by Moura et al. (2020). This could be mainly due to the compositional differences of the isolates in these two studies caused by the differences in the process used to prepare the isolates. The PDCAAS value of C-WGI is comparable with NDI and HDI however, significantly higher than that of EDI. Similar to canaryseed, lysine was the limiting amino acid in the C-WGI sample.

4.5 Conclusions

Overall, the whole canaryseed flour showed better functional properties than white canaryseed flour, probably due to the contribution of other macromolecules, such as fiber. The in vitro protein digestibility of white canaryseed flour is lower than that of whole flour. Hence, the whole flour was found to be better in terms of nutritional quality. The fiber, oil, phytic acid, and total phenolic content of the white canaryseed flour is lower than that of whole flour and therefore, white flour could be a better starting material for wet fractionation processes for canaryseed proteins. Even though canaryseed white flour has lower oil content than whole flour, de-oiling is still required to achieve low-oil-containing protein isolates. Canaryseed is rich in phospholipids and has a potential to become an important by-product in canaryseed value chain. The solvent de-oiling did not cause denaturation of canaryseed protein. It either improved or did not change functional and nutritional properties. This is a positive indication for using solvents for de-oiling canaryseed for protein product preparation. Based on the results obtained from this study, it is difficult to conclude which solvent is better than the other, but ethanol was found to be more efficient in canaryseed oil and phospholipid extraction than hexane. However, other factors such as cost, solvent reclaiming ability, environmental impact, effect on protein quality, and marketability should be considered when selecting a solvent for commercial operations. Last, canaryseed protein's high thermal stability (108°C), makes it feasible to use high-temperature conditions during processing and product development without protein denaturation. A limitation of the current study is the lack of particle size data and information relating to the protein profile,

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composition, and conformation, which could provide greater insight into structure-function relationships of the canaryseed protein fractions. Future studies in this area are recommended, along with further expanded functional work in the area of viscosities and gelation. Based on the findings, the isolate products displayed excellent water and oil holding properties, which may make it ideal for use as a binder in meat applications.

4.6 Connection to the next study

The present study extensively evaluated the techno-functional properties of value-added canaryseed protein fractions, such as whole flour, white flour and protein isolate. These techno-functional properties involve emulsification, foaming, solubility, water and oil holding capacities, which were also reported in some previous studies on canaryseed protein fractions. Gelation is another important techno-functional property of a protein that has not been previously studied with respect to canaryseed protein fractions. Generating information on canaryseed protein gelation is important to understand the full potential of this protein to direct towards different food applications. Therefore, the next study will be aimed at evaluating the gelation properties of canaryseed protein to fill the knowledge gap exists in this area. Moreover, the next study will aim to develop an aqueous-based de-oiling and protein fractionation process for canaryseed as an alternative process to currently using alkaline extraction and isoelectric precipitation. However, aqueous-based de-oiling is very challenging and solvent de-oiling could be the most feasible pathway of upstream de-oiling of canaryseed flour for protein fractionation. In this present study, it was shown that low-bran canaryseed flour could be a good source of starting material to fractionate protein and using hexane and ethanol for de-oiling canaryseed flour does not affect negatively on the techno-functional and nutritional properties of the flour, as well as the protein-enriched fractions derived from it. Moreover, it provides details on the oil fractions that is resulting from each solvent that could be a co-product if solvents were used for de-oiling. This information is helpful when developing a protein fractionation process for canaryseed if aqueous de-oiling found to be difficult to achieve and either hexane or ethanol should be selected as the de-oiling solvent. The protein product resulting from the developed process will be utilized to evaluate the gelation properties of canaryseed protein. Since the process resulting from the next study will be a novel process and the gelation properties have not been previously studied, both yellow and brown canaryseed will be utilized for the study.

5. GELATION AND DOUGH FORMING PROPERTIES OF CANARYSEED PROTEIN CONCENTRATES IN COMPARISON WITH COMMERCIAL SOY PROTEIN CONCENTRATE AND VITAL WHEAT GLUTEN

5.1 Abstract

Background and Objectives: A lab-scale process was developed to aqueously de-oil roller-milled canaryseed (yellow and brown-seeded) flour and produce protein concentrates. The protein concentrates prepared were evaluated for their gelation and bread-dough forming properties to identify their potential for food applications.

Findings: The aqueous process successfully reduced the oil content in both yellow-seed and brown-seed roller-milled flour. The protein concentrate prepared from yellow-seed-flour and brown-seed-flour had 74.9% and 68.2% purity (dry weight basis), respectively. Proteins in both yellow and brown canaryseed showed high resistance to thermal denaturation properties (peak denaturation at 107°C). It was found that the least gelation concentration of both yellow and brown canaryseed protein concentrates were 16% (w/w). There were no significant differences of viscoelastic properties and water holding capacity between the protein gels of these two canaryseed types and the addition of salt did not noticeably improve these properties. Compared to canaryseed, commercial soy protein showed better gelation properties. Both yellow and brown canaryseed protein showed a good potential for improving bread-dough strength when incorporated into a low-gluten-strength wheat flour and was comparable to commercial vital wheat gluten at 1-3% (w/w) inclusion levels.

Conclusions: The new canaryseed protein ingredients prepared by the aqueous-based process shows potential for application in food formulation based on their gelation and bread dough forming properties.

Significance and Novelty: In this study a novel aqueous based process was developed to fractionate canaryseed storage proteins into a protein concentrate. The resulting protein concentrate was evaluated for their gelation and bread-dough forming properties which was not

previously reported in the literature. The result generated for this study will be beneficial to direct canaryseed proteins towards different food applications.

5.2 Introduction

Glabrous canaryseed, also known as hairless Canaryseed (*Phalaris canariensis* L.), is a novel food that was recently approved for human consumption by Health Canada and received the generally recognized as safe (GRAS) status from U.S. Food and Drug Administration (Mason et al., 2018). It is a true cereal crop and contains approximately 61% starch, 20% protein and 8% crude fat and 7% dietary fiber (Mason et al., 2018) as major chemical components. The higher protein content of canaryseed as a cereal grain makes it unique among the other commonly available cereals in the market, such as wheat, rye, barley, oats, millet etc., that usually contains ~8-15% protein in their seeds (Mason et al., 2018). Similar to most of true cereals, canaryseed also contains prolamin and glutelin as the major type of storage proteins, that accounts for 78% of the total storage protein content, where prolamin found to be the most abundant protein (Abdel-Aal et al., 1997a). These proteins in canaryseed mainly exist in the endosperm as a matrix that embeds the starch granules (Abdel-Aal et al., 2011a). The level of proteins presents an opportunity to use canaryseed as potential protein source that could add value through plant-based-protein ingredient development for different food applications (Perera et al., 2022). Moreover, the gluten-free nature of glabrous canaryseed (Mason et al., 2018) provides an opportunity for canaryseed to tap into the gluten-free market, which is beneficial for canaryseed as an emerging cereal crop for human consumption.

Selection of a value-added-plant-based protein ingredient, such as protein concentrates and isolates, for a specific food application is a decision made based on different properties, such as protein purity and composition, organoleptic properties, nutritional properties and techno-functional properties. The latter playing a key role in identifying food applications, Protein solubility, emulsification properties, foaming properties, water and oil holding capacity, and gelation are the major techno-functional properties of a protein ingredient that is frequently discussed in the literature (Achouri et al., 2020; Barac et al., 2015; Nasrabadi et al., 2021). Plant-based protein ingredients are used in different food applications, such as beverage, meat analogs, bakery products, cheese, soups and desserts, where above-mentioned techno-functional properties are important in order to develop and stabilize the corresponding food system (Barac et al., 2015).

For instance, the solubility is favorable for beverage applications, whereas gelation, water and oil holding capacity is important for plant-based meat, cheese and bakery type applications (Barac et al., 2015). Therefore, when canaryseed protein ingredients are considered as a pathway for value addition, it is important to understand the techno-functional properties that they could deliver to determine the best-fit food applications to capitalize on their utilization.

Since canaryseed is an emerging protein source, the literature available on canaryseed protein concentrates or isolates and their techno-functional and nutritional properties are limited. Achouri et al. (2020) and Moura et al. (2020) studied the functional properties of protein isolates (>85% purity) that was prepared by alkali extraction, isoelectric precipitation and freeze drying and found that the isolates showed good water holding, oil holding and emulsifying properties. Similar results were obtained for canaryseed protein isolates (~84% purity) prepared by enzyme-assisted protein purification and freeze drying (Perera et al., 2022) reported with respect to water holding, oil holding and emulsifying properties. These studies showed that the solubility of the canaryseed protein isolates at neutral pH was very low (<10%) and showed ~50% maximum solubility at pH 2. On the other hand, soy protein isolate, the control sample in the study carried out by Achouri et al. (2020) showed ~60% protein solubility at pH 7 and it was ~80% at pH 2, indicating a poor applicability of canaryseed proteins in beverage-type applications where solubility plays a key role. Presumably, these differences in functional properties of canaryseed proteins is mainly attributed to the predominant prolamin proteins as opposed to globulin, the dominant component of soybean proteins (Achouri et al., 2020).

Although some information is available in the literature about emulsification, foaming, solubility, water and oil holding properties of canaryseed protein, there is no information available on their gelation properties. Gelation is an important techno-functional property that could provide an insight into the potential of using canaryseed protein in more food applications. Vital wheat gluten, which is mainly composed of prolamins and glutelins is a well-known functional ingredient to improve the dough strength for bread making where weak wheat flour is used (Schopf and Scherf, 2021). Since canaryseed also contain prolamins and glutelins, it would be interesting to explore the capability of canaryseed proteins for improving the dough strength in gluten-free bread making where food gums, such as xanthan gum and guar gum, being used to provide required dough strength (Salehi, 2019); thus, opening up new avenues for canaryseed value addition. This study was carried out to investigate the gelation properties of canaryseed protein concentrate and

evaluate its potential for improving bread-dough strength in comparison with commercial vital wheat gluten and soy protein concentrates.

5.3 Materials and Methods

5.3.1 Materials

The dehulled canaryseed, both yellow seed (var. CDC Cibo) and brown seed (var. CDC Calvi) grown in Saskatchewan, Canada, were obtained from two local producers to prepare the protein concentrates required for the study. Commercial vital wheat gluten and soy protein concentrate that were used as the controls of this study were obtained from Permolex Ltd., Alberta, Canada and Archer Daniel Midland Company, Illinois, USA, respectively. Unless specified in the text, analytical grade chemicals used in the study were purchased from Sigma Aldrich Canada Co., and VWR International Inc.

5.3.2 Methods

5.3.2.1 Preparation of canaryseed protein concentrates

The protein concentrates from yellow and brown canaryseed were produced using roller-milled-low-bran flour using a proprietary process developed by Keyleaf Life-Sciences, Saskatoon, Saskatchewan, Canada. The process includes an aqueous de-oiling step, followed by unit operations to remove fiber through screening, enzyme treatment to degrade starch to form sugar syrup, centrifuging to separate syrup and protein, and spray drying protein at neutral pH (pH 7). The same process and conditions developed using the yellow flour were used to produce brown-seed protein concentrate to evaluate the effectiveness of the same process conditions on the brown-seed flour. The spray dried protein concentrates and the control protein concentrates were kept at 4 °C until further analysis. Canaryseed grains were tempered to 13% moisture content prior to roller milling (Bradender Quadrumat Jr. mill, Brabender Instruments, Inc.) and screened using 250μm mesh to prepare low-bran flour after milling was completed.

5.3.2.2 Color analysis

The color of the yellow canaryseed protein concentrate (YCPC), brown canaryseed protein concentrate (BCPC), commercial soy protein concentrate (CSPC) and commercial vital wheat gluten (CVWG) was evaluated using a Hunter colorimeter (Labscan II, Hunter Associates

Laboratory Inc.) according to the method described by Perera et al. (2022). The color values are reported as L*, a*, b* values, and L* (0-100) represent brightness where 0 refers to dark color. a* represent red-green spectrum and red is associated with higher a*. b* represent yellow-blue spectrum and higher b* refers to yellow color (Perera et al., 2022).

5.3.2.3 Compositional analysis

The moisture, protein, oil, ash and total starch contents of the protein concentrates were measured using the AOAC International and American Oil Chemists' Society (AOCS) official methods as described in Perera et al. (2022).

5.3.2.4 Differential scanning calorimetry (DSC)

The thermal stability of the YCPC, BCPC, CSPC and CVWG was evaluated using DSC (Model 8000, PerkinElmer Inc.) as described in Perera et al. (2022).

5.3.2.5 Least gelling concentration (LGC)

The least concentration of the protein required to form a self-standing gel was evaluated according to the method described in Guldiken et al. (2021). Different protein concentrations ranging from 5%-20% (w/w) were tested to determine the least gelling concentration of YCPC, BCPC, CSPC and CVWG.

5.3.2.6 Rheological properties of the protein gels

The rheological properties, *i.e.,* storage modulus (G′), loss modulus (G″) and tan (delta) δ (the ratio of G″ vs. G′), of the YCPC, BCPC, CSPC and CVWG dispersions prepared at their corresponding LGC was tested using a rheometer (MCR 102, Modular compact rheometer, Anton Parr Canada Inc.) equipped with 40 mm parallel plate according to the method described in Guldiken et al. (2021), using a 0.1% strain. A temperature ramp of 25 °C - 95 °C was used to evaluate the rheological properties of CSPC and CVWG whereas, two temperature ramps, *i.e.,* 25°C - 95 °C and 25 °C - 112 °C was tested for YCPC and BCPC. Moreover, the effect of slat containing monovalent and divalent cations on gelation properties of these protein concentrates was also evaluated using the protein dispersions prepared at LGC in 0.5 M NaCl, 0.25 M $CaCl_2$ solutions.

Only one temperature ramp of 25°C-95 °C was used for ionic-strength experiments for all four protein concentrates.

5.3.2.7 Water holding capacity (WHC) of the protein gels

The WHC of the YCPC, BCPC, CSPC and CVWG gels prepared at their LGC was evaluated according to the method described by Nieto-Nieto et al. (2014) with modifications. Briefly, the cut sample of the gel was placed in a 16×10 mm disposable culture tube and covered with a 22-25 µm miracloth (Millipore Corp.). The covered test tube was inverted and placed in a 25 mL syringe-barrel without the plunger. Then, the syringe-barrel containing the culture tube was placed in a 50 mL centrifuge tube and processed according to the Nieto-Nieto et al. (2014) method. The effect of ionic strength on the WHC of the gels were also evaluated using the protein dispersions prepared at LGC in 0.5 M NaCl, 0.25 M $CaCl_2$ solutions.

5.3.2.8 Evaluation of bread dough forming properties

Bread-dough forming properties of the YCPC, BCPC was evaluated using Micro-DoughLAB (model 2800, Perten Instruments AB) in comparison to the CVWG. The canaryseed protein concentrates and the vital wheat gluten was mixed with a low-gluten-strength wheat flour (var. Carberry) at 0%, 1% and 3% (w/w) levels to test their effect of improving dough forming properties. The test was performed using 4 g of Carberry flour according to a standard dough-testing procedure developed at the Crop Development Centre of University of Saskatchewan, Saskatoon, Saskatchewan, Canada, at 63 rpm mixing speed, 14% moisture basis and 500 ± 20 FU peak resistance range.

5.3.2.9 Statistical analysis

Preparation of protein concentrates and all the analyses of this study were carried out in triplicates (n=3) and reported as mean ± standard error (SE). One-way analysis of variance (ANOVA) followed by Tukey's mean separation was performed using to R statistical software, version 4.0.3 (R Core Team, 2020) to evaluate the significant differences of the tested properties between YCPC, BCPC, CSPC and CVWG samples. The R packages "emmeans" and "multcomp" were used to perform Tukey's mean separation and denote the significant ($p<0.05$) and non-significant ($p>0.05$) mean values as described in Perera et al. (2022).

5.4 Results and Discussion

5.4.1 Effect of aqueous de-oiling treatment on canaryseed white flour

The roller-milled yellow and brown canaryseed flours were light yellow in color, where brown-seed flour showed slightly more intense yellow color (brown seed flour: $L^* = 88.49 \pm 0.05$, $a^* = -0.21 \pm 0.04$, $b^* = 18.66 \pm 0.04$) compared to that of the yellow-seed flour (yellow seed flour: $L^* = 88.42 \pm 0.01$, $a^* = 0.07 \pm 0.02$, $b^* = 16.98 \pm 0.02$). Aqueous de-oiling treatment resulted in removing the color of the yellow-seed flour along with the majority of the oil into oil phase (light phase/supernatant) leaving a white color pellet ($L^* = 92.24 \pm 0.01$, $a^* = -0.26 \pm 0.01$, $b^* = 6.94 \pm 0.01$) containing starch, protein and fine bran particles (Figure B1). The same was observed for brown-seed flour (data not shown). The resulting yellow-seed pellet of this treatment contained 1.2 ± 0.1 % residual oil, which is a noticeable reduction compared to that of the oil content of the roller-milled flour $6.0 \pm 0.0\%$ on dry weight basis. Similar reduction of the oil content was also observed for the brown seed, where $5.5 \pm 0.0\%$ oil content in the low-bran flour was reduced to $1.5 \pm 0.1\%$. The screening (wet sieving) of the aqueously de-oiled pellet helped to reduce the fine fiber particles to improve the protein purity of the final protein product. The white color of the de-oiled material gradually turned into yellow color due to the heat applied for enzymatic treatment to break starch, presumably due to Maillard browning reactions in the starch-sugar-protein matrix. Consequently, the final canaryseed protein concentrates after spray drying were light yellow in color (yellow-seed concentrate: $L^* = 89.55 \pm 0.02$, $a^* = -0.27 \pm 0.02$, $b^* = 15.87 \pm 0.03$; brown-seed concentrate: $L^* = 89.38 \pm 0.02$, $a^* = -0.50 \pm 0.03$, $b^* = 14.81 \pm 0.03$). However, both these concentrates looked lighter in color compared to that of the control CSPC ($L^* = 88.80 \pm 0.01$, $a^* = 1.06 \pm 0.01$, $b^* = 13.28 \pm 0.01$) and CVWG ($L^* = 84.45 \pm 0.01$, $a^* = 0.88 \pm 0.01$, $b^* = 15.90 \pm 0.02$). Color of a protein ingredient plays a key role for its acceptability (Sharan et al., 2021). Therefore, the lighter color of canary seed concentrates compared to the widely used commercial ingredients, such as CSPC and CVWG, represents a competitive advantage for canary seed in the plant-based protein market.

5.4.2 Proximate composition of the protein concentrates

The enzyme-assisted-lab-scale process developed to produce canaryseed protein concentrate resulted in almost 75% protein purity on dry weight basis for YCPC and was not significantly different to that of commercial soy (CSPC) and wheat (CVWG) protein concentrates

used in this study (Table 5.1). However, the protein purity of the BCPC was significantly lower than both yellow-seed and commercial protein concentrates (Table 5.1), presumably due to the lower protein content of the brown-seed flour (16.7%) compared to that of the yellow-seed flour (21.4%). The final YCPC contained 12.1% oil, which is lower than that of BCPC (15.7%) (Table 5.1). Despite a significant oil reduction in the white flour, the final canaryseed protein concentrates still contained >10% oil. The oil content is significantly increased in the protein concentrates compared to that of aqueous-de-oiled pellet due to the removal of the starch through enzymatic degradation. Even though the mass of the oil is similar in both aqueous-de-oiled pellet and the protein concentrates, its contribution to the proximate composition is higher in the concentrate due to lack of starch and fiber. Generally, possessing such a higher oil content adversely affect the shelf-life stability of the protein product in the commercial scale, unless the protein concentrates will be utilized for specific food applications where having a higher oil content is favorable. Therefore, depending on the end use of the protein product, the current process should be modified to obtain lower oil content in the final protein product similar to other commercial concentrates (Table 5.1). The ash content of the YCPC and the BCPC were not significantly different, while CSPC contained the highest ash content and the CVWG contained the lowest (Table 5.1). The residual starch content in the YCPC, BCPC and the CVWG were lower and not significantly different (Table 5.1), demonstrating the effectiveness in starch removal of the used processes. The non-starch carbohydrates, *i.e.,* fiber and sugars, content in both YCPC and BCPC were similar and significantly lower than that of CSPC and CVWG (Table 5.1).

5.4.3 Process-induced protein denaturation

Protein denaturation could be either advantageous for some techno-functional properties, such as gelation and emulsification, or disadvantages for some properties, such as solubility (Damodaran, 2017). Therefore, it is important to know whether proteins available in the canaryseed protein concentrates have been denatured or exist in undenatured form. The DSC analysis showed that the proteins in both yellow and brown canaryseed were not denatured whereas, both commercial proteins have denatured proteins (Table 5.2). Both canaryseed protein concentrates showed similar thermal properties (with a Tp (peak denaturation temperature) at 107 °C). A Tp of 108 °C for yellow canaryseed white flour and protein concentrates were previously reported in Perera et al. (2022). Process-induced denaturation could be either intentional if the

protein product is designed to cater gelation-based product applications to provide better performances, or unintentional and occur during processing operations, such as pasteurization. The high thermal denaturation temperature found for canary seed concentrates allows them to withstand high thermal processing conditions without denaturing the protein, which could be an advantageous trait.

5.4.4 Least gelling concentration (LGC) of the protein concentrates

The least protein concentration required to form a gel from the YCPC, BCPC, CSPC and CVWG was tested by evaluating the self-standing ability of a gel in an inverted test tube, made at different protein concentrations. The result obtained from this experiment showed that both YCPC and BCPC required 16% (w/w) minimum protein concentration to form a self-standing gel at 95 °C (Table 5.3). On the other hand, the CSPC required only 13% (w/w) protein concentration while CVWG required 19% (w/w) protein concentration at least to make a self-standing gel (Table 5.3). To the author's knowledge there is no previous studied carried out related to LGC of canaryseed protein concentrates or isolates. On the other hand, varying least gelling concentrations of soy ranging from 6% to 15%, has been previously reported (Shan et al., 2015; Wang et al., 2020). The variation in the concentration is due to the protein purity, temperature and time used for gel preparation and presence of salt. Banerjee and Bhattacharya (2012) reported that a number of factors such as, temperature, pH, ionic strength etc. could influence the gel forming ability of protein and therefore, variation in the LGC of a protein could be expected depending on the conditions used. Similarly, Wang et al. (2007) reported that commercial wheat gluten (71.5% protein) forms a gel at 22% minimum protein concentration whereas, it was reduced to 16% when the gluten was subjected to a heat treatment (100 °C for 120 seconds). It is plausible that LGC of canary seed could be lower than 16% if the gel preparation conditions are altered or subjected to a pre-treatment. Overall, the results suggest that the canaryseed protein concentrate shows better gelation capacity than wheat gluten at lower concentration whereas, it is lower compared to that of soy protein at the conditions used for gelation.

Table 5.1 Proximate composition of different canaryseed and commercial soy and wheat protein concentrates on dry weight basis.

Sample	% Protein	% Oil	% Ash	% Total starch	%Non-starch carbohydrates*
Yellow canaryseed protein concentrate (YCPC)	74.88 ± 2.10^b	12.11 ± 0.94^b	1.97 ± 0.03^b	3.52 ± 2.61^a	7.93 ± 0.93^a
Brown canaryseed protein concentrate (BCPC)	68.61 ± 2.21^a	15.76 ± 1.02^c	2.01 ± 0.06^b	6.32 ± 1.71^a	7.29 ± 0.72^a
Commercial soy protein concentrate (CSPC)	75.61 ± 0.12^b	0.50 ± 0.05^a	4.69 ± 0.08^c	NA	19.20 ± 0.20^b
Commercial vital wheat gluten (CVWG)	74.23 ± 0.25^b	1.97 ± 0.22^a	1.26 ± 0.03^a	6.16 ± 0.90^a	16.38 ± 0.64^b

Values are represented as Means ± Standard error. Means followed by the same superscript in the same column are not significantly different (p>0.05). The alphabetical order of the superscripts is arranged according to the ascending order of the mean values.

*Calculated value [100 − (Σ %protein, %, oil, %ash, %total starch)].

Table 5.2 Thermal properties of canaryseed and commercial protein concentrates.

Sample	Protein Denaturation			
	T_o (°C)	T_p (°C)	T_c (°C)	ΔH (J/g)
Yellow canaryseed protein concentrate (YCPC)	100.5 ± 0.2^a	107.4 ± 0.2^a	112.2 ± 0.1^a	6.7 ± 0.2^a
Brown canaryseed protein concentrate (BCPC)	101.3 ± 0.4^a	107.9 ± 0.2^a	112.7 ± 0.1^b	7.0 ± 0.2^a
Commercial soy protein concentrate (CSPC)	ND	ND	ND	ND
Commercial vital wheat gluten (CVWG)	ND	ND	ND	ND

Values are represented as Means ± Standard error. Means followed by the same superscript in the same column are not significantly different (p>0.05). The alphabetical order of the superscripts is arranged according to the ascending order of the mean values.

T_o = onset temperature; T_p = peak temperature; T_c = conclusion temperature; and ΔH = enthalpy change; ND=Not detected.

Table 5.3 Observations of gel formation behavior of different protein concentrations of canaryseed and commercial protein concentrates at 95 °C and neutral pH.

Sample	Concentration (w/w)	Self-supporting gel formation in an inverted test tube
Yellow canaryseed protein concentrate (YCPC)	5%	No
	10%	No
	15%	No
	16%*	Yes
	17%	Yes
	18%	Yes
Brown canaryseed protein concentrate (BCPC)	5%	No
	10%	No
	15%	No
	16%*	Yes
	17%	Yes
	18%	Yes
Commercial soy protein concentrate (CSPC)	5%	No
	10%	No
	11%	No
	12%	No
	13%*	Yes
	15%	Yes
Commercial vital wheat gluten (CVWG)	5%	No
	10%	No
	15%	No
	17%	No
	18%	No
	19%*	Yes
	20%	Yes

* Least gelation concentration of the protein sample.

5.4.5 Rheological properties of canaryseed protein concentrates

The rheological assessment showed that there is no noticeable difference between the G′ (elastic properties) and G″ (viscous properties) of the gels prepared using YCPC and BCPC at 16% (w/w) protein concentration without any salt in the medium (Table 5.4). In comparison to the gels prepared using CSPC, both G′ and G″ of YCPC were significantly lower and contained higher tan δ. Higher G′ and a lower tan δ provides a measure of strength in a gel (Guldiken et al., 2021); hence, canaryseed protein gels are significantly weaker compared to CSPC. Addition of NaCl did not improve the gel strength of YCPC whereas, $CaCl_2$ significantly improved YCPC gel strength. This could be due to the fact that divalent cations can directly bind with protein and reduce their net charge density, create salt bridges between negatively charged polypeptide chains, and the unbound cations in the medium potentially screen the electrostatic repulsions to form the gel network; hence, the effect of divalent cations on inducing gelation is three-fold (Chen et al., 2018). On the contrary, induction of gelation by the monovalent cations is mainly due to screening of electrostatic interactions between the protein aggregates to form the gel network (Chen et al., 2018). Although the salt addition improved the YCPC gel strength, it was still significantly weaker compared to CSPC gels. Neither NaCl nor $CaCl_2$ improved CSPC gel strength. The gel strength of the BCPC is not significantly different from the YCPC gels and salt type did not have a major effect on improving BCPC as it did for YCPC with $CaCl_2$. Previous studies have reported improvements of gelation properties due to addition of mono valent cations (Na+) and divalent cations (Ca2+) in soy protein and other plant protein sources (Guldiken et al., 2021; Hua et al., 2005; Wang et al., 2019). Chen et al. (2018) reported similar results in preheated aggregates of soy protein isolates. However, the data reported in this literature suggest that the addition of these salt does not always improve the gelation properties of the protein. Therefore, the difference between the salt type used in gelation could be expected depending on the protein type, conformation (native protein vs pre-heated aggregates) and concentration (Chen et al., 2018). Further investigations are required with different salt concentrations (low-to-high range) to broadly understand the effect of salt type on the canaryseed protein gels.

Table 5.4 Viscoelastic properties of canaryseed and commercial protein concentrates in different salt solutions. Values are represented as Means ± Standard error.

Sample	G′ (Pa)			G″ (Pa)			Tan δ		
	No Salt	0.5M NaCl	0.25M CaCl$_2$	No Salt	0.5M NaCl	0.25M CaCl$_2$	No Salt	0.5M NaCl	0.25M CaCl$_2$
YCPC[a]	4.4 ± 1.5[a,1]	6.6 ± 3.2[a,1]	50.3 ± 10.0[a,2]	5.7 ± 0.4[a,1]	6.2 ± 2.1[a,1]	23.5 ± 2.9[a,2]	1.53 ± 0.36[b,1]	1.23 ± 0.37[b,1]	0.49 ± 0.05[b,1]
BCPC[a]	8.6 ± 1.7[a,1]	29.5 ± 7.5[a,1]	25.0 ± 6.0[a,1]	6.6 ± 1.0[a,1]	15.2 ± 2.8[a,1]	12.0 ± 2.4[a,1]	0.80 ± 0.12[ab,1]	0.53 ± 0.04[ab,1]	0.50 ± 0.08[b,1]
YCPC[b]	344.4 ± 62.3[b]	NA	NA	153.3 ± 42.9[b]	NA	NA	0.45 ± 0.13[a]	NA	NA
BCPC[b]	417.2 ± 56.0[b]	NA	NA	194.2 ± 18.2[b]	NA	NA	0.50 ± 0.11[a]	NA	NA
CSPC[a]	1273.2 ± 98.2[c,1]	1555.7 ± 165.1[b,1]	1812.3 ± 188.2[b,1]	193.8 ± 21.1[b,1]	236.5 ± 39.9[b,1]	222.2 ± 14.1[b,1]	0.15 ± 0.01[a,1]	0.15 ± 0.01[a,1]	0.12 ± 0.01[a,1]
CVWG[a]	Test was unable to perform since gluten did not make a homogeneous solution								

[a]samples were heated from 25 °C to 95 °C and cooled to 25 °C at 2 °C/min rate.
[b]samples were heated from 25 °C to 112 °C and cooled to 25 °C at 2 °C/min rate.

In addition, protein denaturation plays an important role in gel network formation. Heating cause denaturation of protein by unfolding or dissociation of the molecules, exposing more hydrophobic and sulfhydryl groups to facilitate aggregation to form a gel (Batista et al., 2005). The DSC results (Table 5.2) indicated canaryseed protein denaturation onset at ~100°C, peaks at ~107 °C and end ~112°C. Therefore, it is plausible that at 95 °C canaryseed protein is not denatured enough to form a good gel network to provide the desired strength. Therefore, the heating and cooling cycle was changed to reach 112 °C for canaryseed to facilitate protein denaturation. As expected, the strength of YCPC and BCPC gels prepared at 112 °C significantly improved (G′ > G″ and low tan δ) compared to YCPC and BCPC gels prepared at 95 °C (Table 5.4). There were no significant differences observed between the YCPC and BCPC gels prepared at 112°C. The G′ values of YCPC and BCPC gels prepared at 112 °C were significantly lower than those of CSPC, indicating soy protein gels have more elastic nature. However, there were no significant differences observed in the tan δ values, although they were approximately three times lower. The CVWG at a 19% protein concentration did not make a homogeneous slurry when mixed and stir, even with the help of polytron blending. Therefore, it was not possible to obtain rheology data for the CVWG protein.

5.4.6 Water holding capacity (WHC) of canaryseed protein gels

The WHC of a gel-based food is an important property since lower WHC leads to loss of water over time causing quality defects, such as shrinkage and changes to mouth feel (Nieto-Nieto et al., 2014); Hence, high WHC is often desired for gel-type food to maintain its quality and consumer acceptability. The WHC of YCPC was higher than BCPC and showed moderate water holding capacity (Table 5.5). On the other hand, CSPC showed high WHC (82.3%), which is comparable to WHC of soy protein (82.2%) reported elsewhere (Wu et al., 2011). Wheat protein showed comparable WHC to canaryseed protein concentrates and was lower compared to that of CSPC. Neither NaCl nor $CaCl_2$ had a significant impact on the WHC capacity of any of the protein concentrate tested except, the WHC of the CSPC was noticeably reduced by addition of $CaCl_2$. The CVWG formed an aggregate and precipitated when $CaCl_2$ was added, and it was impossible to prepare the gels. Therefore, WHC of the gels made from CVWG was not evaluated in the present

Table 5.5 Water holding capacity of canaryseed protein concentrates in different salt solutions, in comparison to commercial soy protein concentrate and vital wheat gluten.

Salt type	Sample	% WHC
No salt	Yellow canaryseed protein concentrate (YCPC)	$66.6 \pm 6.2^{ab,12}$
	Brown canaryseed protein concentrate (BCPC)	$51.3 \pm 4.3^{a,1}$
	Commercial soy protein concentrate (CSPC)	$82.3 \pm 0.5^{b,2}$
	Commercial vital wheat gluten (CVWG)	$63.7 \pm 4.7^{a,12}$
0.5M NaCl	Yellow canaryseed protein concentrate (YCPC)	$70.7 \pm 1.3^{ab,12}$
	Brown canaryseed protein concentrate (BCPC)	$59.8 \pm 7.7^{a,1}$
	Commercial soy protein concentrate (CSPC)	$84.2 \pm 6.7^{b,2}$
	Commercial vital wheat gluten (CVWG)	$69.6 \pm 3.0^{ab,12}$
0.25M CaCl$_2$	Yellow canaryseed protein concentrate (YCPC)	$58.9 \pm 2.5^{a,1}$
	Brown canaryseed protein concentrate (BCPC)	$52.7 \pm 3.7^{a,1}$
	Commercial soy protein concentrate (CSPC)	$65.8 \pm 1.1^{a,12}$
	Commercial vital wheat gluten (CVWG)	NA

Values are represented as Means ± Standard error. Means followed by the same superscript letters in the same salt type are not significantly different (p>0.05). Means followed by the same superscript numbers between the salt type are not significantly different (p>0.05). The alphabetical order of the superscripts is arranged according to the ascending order of the mean values.

study. Zhang et al. (2022) reported that the WHC of the protein mixed gels prepared using wheat gluten and potato protein isolates increased with increasing NaCl and $CaCl_2$ concentration with maximum capacity attained at ~0.02M and ~0.04M, respectively. Further increment of $CaCl_2$ concentration decreases the WHC of the gels formed. Although, the concentration of NaCl used in the present study is higher than the levels reported in Zhang et al. (2022), it still showed an increasing trend. Presumably due to the difference in the protein types and composition. However, opposite trend was observed for the gels prepared using $CaCl_2$ in the present study. Perhaps, the level of calcium chloride is higher than the desired levels that caused a drop in WHC despite the protein composition. When the salt ion concentration is high, it breaks the hydration layer on the surface of proteins by salting out inducing salt-water interactions over protein-water interactions (Das Mahanta et al., 2017) resulting in reduced WHC.

5.4.7 Dough forming properties of canaryseed protein

The effect of canaryseed protein concentrates on improving bread-dough forming properties was evaluated to identify the potential of canaryseed protein as an alternative source to wheat gluten to improve low-gluten-strength flour. The water absorbance, dough development time (DDT), mixing tolerance index (MTI) and the stability of the dough formed was monitored to evaluate the performance of these protein concentrates (Table 5.6). The result showed that water absorption of both YCPC and BCPC is not significantly different from that of CVWG, and absorption gradually increases by ~2% when protein inclusion level increased from 0-3%. The amount of water affects the viscoelastic properties of the dough and sub-optimal level of water negatively affect the bread volume and uneven distribution of the protein network (Schopf and Scherf, 2021). Therefore, it is important that the alternative protein sources have a water absorption capacity comparable to that of wheat gluten. Higher DDT, higher stability and lower MTI are the preferred properties for a strong bread dough (Conforti and Jhonson, 1992; Wang et al., 2002). An increasing trend for DDT and stability with increasing protein level was observed (Table 5.6). A range of 4-12 mins in stability has been reported for doughs with a good strength where approximately 6 mins is considered as satisfactory dough stability (Koppel and Ingver, 2010). A 3% inclusion of canaryseed protein concentrate had a 7.3-7.5 min stability and was comparable to that of CVWG. This is an indication for a strong dough forming properties of canaryseed protein products used. However, at 3% inclusion the level of MTI was >50 B. U. Ideally, an MTI of ≤ 30

Table 5.6 Effect of canaryseed protein on improving dough properties compared to vital wheat gluten at different inclusion levels.

Inclusion level (w/w)	Sample	Absorbance	Dough development time (min)	Mixing Tolerance Index (B.U)	Stability (mins)
0%	Low-gluten-strength wheat flour	60.7 ± 0.1^a	4.1 ± 0.1^a	78.3 ± 1.7^b	5.3 ± 0.1^a
1%	Low-gluten-strength wheat flour + CVWG	62.6 ± 0.3^{ab}	4.8 ± 0.1^b	71.7 ± 1.7^{ab}	5.7 ± 0.3^a
	Low-gluten-strength wheat flour + YCPC	62.1 ± 0.2^{ab}	4.9 ± 0.1^b	73.3 ± 4.4^{ab}	5.9 ± 0.1^a
	Low-gluten-strength wheat flour + BCPC	62.4 ± 0.2^{bc}	4.7 ± 0.2^{ab}	73.3 ± 4.4^{ab}	5.5 ± 0.1^a
3%	Low-gluten-strength wheat flour + CVWG	64.8 ± 0.8^d	4.8 ± 0.2^b	55.0 ± 5.8^a	7.6 ± 0.5^b
	Low-gluten-strength wheat flour + YCPC	$64.3 \pm 0.^{cd}$	5.0 ± 0.1^b	58.0 ± 4.4^a	7.5 ± 0.4^b
	Low-gluten-strength wheat flour + BCPC	64.4 ± 0.4^{cd}	5.2 ± 0.2^b	58.3 ± 4.4^a	7.3 ± 0.2^b

Values are represented as Means ± Standard error. Means followed by the same superscript in the same column are not significantly different ($p > 0.05$). The alphabetical order of the superscripts is arranged according to the ascending order of the mean values.

B.U. is preferred and a value of >50 B.U. suggests a lower tolerance for mixing and difficulties with mechanical handling of the dough (https://www.ndsu.edu/faculty/ simsek/wheat/ farinograph raph.html#). Therefore, >3% inclusion level of canaryseed protein would be required to obtain a stronger dough. The same is true for the CVWG used in this study and shows that inclusion of canaryseed protein is comparable to CVWG. Since canaryseed proteins shows a potential to improve dough quality, it would act as a potential dough-strengthening agent in bread baking applications. Due to the gluten-free nature of canaryseed protein, it could be used in gluten-free bread making to improve dough quality and protein content. Developing gluten free bread and high-protein, low-carbohydrate bread are two products that falls under functional bread category with former getting more focus overs the recent years (Wójcik et al., 2022). This is a novel product application where incorporation of canaryseed protein can be explored to diversify canaryseed utilization.

5.5 Conclusions

The solvent-free lab-scale process developed in the present study is capable of producing a de-oiled roller-milled canaryseed flour from yellow canaryseed, and a protein concentrate with >70% protein purity. The method successfully reduced oil from canaryseed flour using aqueous means which is beneficial for obtaining "clean label" product claim for marketing purposes. The residual oil content in the final protein concentrate, could negatively impact product stability, but it could also be beneficial for specific product applications, such as dairy-free cheese and ice cream, egg-free mayonnaise and vegan burgers etc. Moreover, canary seed proteins exhibited high thermal stability which is advantageous during industrial processes where heat treatment is generally applied for pasteurization. This aqueous process can be applied to both yellow and brown canaryseed to produce a protein concentrate. However, the protein content in the starting flour could greatly impact in achieving >70% protein purity in the final product. Scaling up this lab-scale process using pilot-scale equipment would be necessary to assess its feasibility and reproducibility.

Yellow and brown canaryseed protein concentrates showed similar gelation properties, which were inferior to those of soy protein concentrate but comparable to those of commercial vital wheat gluten. Finally, this study demonstrates the potential of canaryseed protein concentrates to improve the properties of bread dough prepared using low-strength gluten flour at lower (1-3%)

inclusion levels, which was comparable to that of vital wheat gluten, the industry standard for improving dough strength. Further evaluation is however required to assess the effect of these protein concentrates at different inclusion levels (low-to-high range) in new product applications, such as gluten-free, high-protein, and low-carbohydrate bread formulations, to better explore the potential canaryseed proteins for different value-added applications to diversify the existing market.

5.6 Connection to the next study

In this study, a lab-scale process was developed to obtain a protein concentrate from canaryseed white flour, using aqueous de-oiling and enzymatic degradation of starch. The aqueous de-oiling significantly reduced the oil content in the white-flour and the enzymes, and the dosages used were able to degrade starch to result a protein concentrate >70% protein purity. In order to assess the viability of the lab-scale process developed in the commercial scale, it is important to carry out scaling up trials as the next step to identify potential issues, find the solutions and carry out process optimization to improve yields and reduce cost of production. The next study aims to carry out scaling up trials using pilot scale processing equipment using 100 kg canaryseed white-flour batches. It is expected to identify feasibility of scaling up the lab-scale process developed to obtain a protein concentrate from canaryseed white flour. Although this process can be applied to both yellow and brown canaryseed, only yellow canaryseed was utilized for scaling up trials due to the high cost associated with conducting scaling up trials in a contracted pilot plant facility.

6. SCALING UP ENZYME-ASSISTED-AQUEOUS PROCESS FOR CANARYSEED PROTEIN CONCENTRATE PRODUCTION

6.1 Abstract

Background and Objectives: The enzyme-assisted-aqueous process developed in the laboratory was scaled up using roller-milled-yellow canaryseed flour at KeyLeaf Life-Sciences, Saskatoon, Saskatchewan, Canada, pilot plant facility. Two scaling up trials using 100 kg canaryseed-flour batches were carried out using pilot-scale equipment including decanter and disk stack centrifuges, vibratory screens and a spray drier. The overall process was carried out with two objectives, *i.e.,* scaling up of aqueous de-oiling of canaryseed flour and enzyme-assisted starch degradation of aqueously de-oiled fraction to produce a protein concentrate with ≥60% protein purity.

Findings: The original process was modified to address the separation issues with the decanter centrifuge during the trials. With the modifications, it was possible to reduce the oil content of the roller-milled flour from 7% (dry weight basis) to 0.8% (dry weight basis) in the aqueously de-oiled fraction, achieving the first objective of the study. The enzymatic treatment applied to digest the starches in the aqueously de-oiled fraction was successful and reduced ~85% of the starch originally present in the roller-milled flour. However, the protein purity of the final protein product was 16% and the expected value of ≥60% was not achieved. Evaluation of protein contents of the in-process samples showed that majority of the protein was solubilized and lost to waste stream, which was not evident in the laboratory scale processing. The process modification performed during the scale up trials to address the centrifuging issues made the initial canaryseed-flour slurry to remain in the conditions used for de-oiling for a prolong period of time. Presumably, this caused some structural alterations in canaryseed protein causing losses into the waste stream that contains the oil.

Conclusions: It is possible to aqueously de-oil canaryseed flour and use enzyme-assisted starch digestion to concentrate protein using pilot-scale equipment. The process modifications carried out cause protein losses resulting the lower purity in the final protein product. Therefore, further investigation and process modification is required to prevent protein loss to improve protein recovery into the final product and subsequently achieve higher protein purity.

Significance and Novelty: This study showed the feasibility of aqueous de-oiling for protein fractionation at the pilot scale. The scaled-up-aqueous-based protein fractionation process lays the foundation towards "clean label" protein ingredient development that could provide canaryseed a competitive advantage in the plant-based protein market.

6.2 Introduction

Process development and scaling up are two crucial components for any new ingredient development process. The initial process development is usually carried out on a small (lab) scale, as a bench-top experience, for the proof of concept and early evaluation of the prototype properties (Penson, 2015). If the bench-top scale is successful, the next step is to carry out trials in the pilot scale where larger volumes are tested with industry-standard manufacturing equipment. This could be either mini-pilot scale or pilot scale, depending on the plant and equipment capacity. Scaling up is important since the prototype produced at the bench-top scale and its properties would not be repeatable at the pilot scale (Penson, 2015). This could be due to the differences in the lab scale vs. pilot scale, such as working mechanism of the equipment used (*e.g.,* swinging bucket type centrifuges vs. decanter/disk stack centrifuges), heat transfer characteristics (*e.g.,* hot plate heating vs. plate heat exchangers), mixing (e.g., magnetic stirrers vs. agitators), drying (*e.g.,* freeze drying vs. spray drying), material transfer (e.g., pouring vs. pumping) etc. Therefore, scaling up trials conducted in a pilot plant allows to gather accurate data, such as mass balance, energy balance, prototype characteristics and economic feasibility, and helps to identify the most desirable processing technologies (Lopez-Gomez and Barbosa-Canovas, 2005). Thus, it is prudent to carry out scaling up trials after a new process has been developed in the lab scale for any ingredient from novel sources, such as canaryseed (*Phalaris canariensis* L), a true cereal crop, that shows a potential to be developed as emerging protein source (Mason et al., 2018; Patterson et al., 2018; Perera et al., 2022).

The first lab-scale process for canaryseed protein isolation was developed by Abdel-Aal et al. (2010), which was in fact developed and optimized for starch recovery and purity (Achouri et al., 2020). In this method, the authors evaluated different solvents, *i.e.,* ethanol (E), water (W) and alkaline solution (A), in three different sequences (EWA, EWWA and EAW) to fractionate oil, protein, fiber and starch from canaryseed (Abdel-Al et al., 2010; Achouri et al., 2020). The major unit operations of these processes include de-oiling with ethanol, alkaline extraction of proteins, centrifuging to separate starch and protein, screening to remove fiber, and centrifuging to separate the purified starch, where alkaline extraction (using 0.05N NaOH) followed by isoelectric precipitation at pH 4.0 was utilized as the protein isolation method. It was found that the sequential EAW extraction is the most efficient method that delivers high purity starch (92%) and protein with a moderate purity (75%), and this method was selected by Auchori et al. (2020) for further optimization to improve protein extraction and purity. The optimized EAW method by Auchori et al. (2020) used 0.05M NaOH solution at pH 12 for protein extraction and pH 5 was used for isoelectric precipitation of the proteins. After optimization was complete, the process was scaled up using 2 kg of canaryseed and the isoelectric precipitated protein was freeze dried for further analysis. The scaled-up process resulted a protein product with >90% purity for both yellow and brown canaryseed with a 15.4%-16.3% yield.

A separate lab-scale process was developed as a part of this thesis project (Section 5.3.2.1) to prepare protein concentrate from canaryseed with ~75% purity. The objective of developing this process was to develop an alternate aqueous-based fractionation process for canaryseed, which could pave the path for canaryseed protein to obtain "clean-label" claim and reduce the cost and safety risks associated with flammable solvents and harsh pH conditions, that would benefit canaryseed as an emerging protein source to compete in the market. The major unit operations of the method developed (Section 5.3.2.1) include aqueous de-oiling, screening to remove fiber, enzymatic treatment to digest starch, centrifuging to separate protein and spray drying. This method remove starch from the matrix while concentrating the protein in contrast to extracting and isolating protein using alkaline extraction followed by isoelectric precipitation. The process resulted ~15% protein yield with ~75% purity for yellow canaryseed and ~68% purity for brown canaryseed. This process was successful in reducing the oil in canaryseed flour using aqueous means; however, it concentrates the residual oil in the final protein product due to starch degradation and result ~10-15% oil. Lower oil content is favorable in the final protein product for

shelf-life stability; however, ~10-15% oil could be beneficial if the protein is targeted for specific food applications where high oil favorable, such as dairy-free cheese and ice cream, egg-free mayonnaise, and vegan burgers etc. Although the lab scale process yielded 75% protein and 10-15% oil in the final protein product, it could entirely be different when the process is evaluated in the pilot scale. The pilot scale may result high protein purity and lower oil in the final protein product, or it could be the opposite depending on the equipment and conditions used. Therefore, this study was carried out to 1) evaluate scaling up feasibility of canary seed aqueous de-oiling, and 2) evaluate the scaling up feasibility of enzyme-assisted starch degradation to produce a protein concentrate with ≥60% (w/w) protein using roller-milled canaryseed flour. Two scaling up trials, using 100 kg of canaryseed flour, were carried out at KeyLeaf Life Science pilot plant facility in Saskatoon, Saskatchewan, Canada

6.3 Materials and methods

6.3.1 Materials

The dehulled yellow canaryseed (var. CDC Cibo) was purchased from Wiens Seed Farm, SK, Canada and utilized for the pilot scale trials. The pH adjustments during the trials were carried out using 50% sodium hydroxide (ClearTech Industries Inc.) and 85% Phosphoric acid (Univar Solutions Inc.). Two different enzymes obtained from Novozymes North America Inc., was used for starch degradation. These two enzymes are denoted as enzyme "X" and enzyme "Y" in this document since the commercial names of these enzymes are proprietary information.

6.3.2 Pilot-scale equipment

The scaling up trials were carried out using decanter centrifuge (EC3; model: CA-225-010, GEA Westfalia Separator Group), a disk stack centrifuge (DC2; model:SA14-02-076, GEA Westfalia Separator Group), two 18-inch diameter screening units (VS3 & VS4; SWECO® Vibro-Energy^{TM} Separator, SWECO Canada Ltd.) and a spray drier (SD1; model No. D-19 Spray drier, Komline-Sanderson Corp.).

6.3.3 Pilot-scale processing

6.3.3.1 Roller milling of dehulled canaryseed

Roller milling of canaryseed was carried out at Apollo Machines & Products Ltd., SK, Canada using EconoMill^TM, model 1.5. Prior to roller milling, the grains were tempered to 13% (w/w) moisture content and the tempered seeds were passed through three times to produce whole canary seed flour.

6.3.3.2 Screening whole flour to prepare low-bran (white) flour

The roller-milled whole flour (292 kg) was screened using US standard mesh size #24 (~707 µm), followed by #32 (~630 µm) to prepare white (low-bran flour) to be used as the starting material for the trials (Figure C1). The product retain on the screens (overs) is the bran fraction (Figure 6.1A-B) whereas the product passed through #32 mesh is the white flour fraction (Figure 6.1C, #32 mesh unders). The bran fraction was discarded, and the white flour fraction was taken for further processing. Overall, 70% (w/w) white-flour yield was obtained after screening. Since the yield was adequate to conduct the trials, further optimization to improve the white flour yield was not carried out.
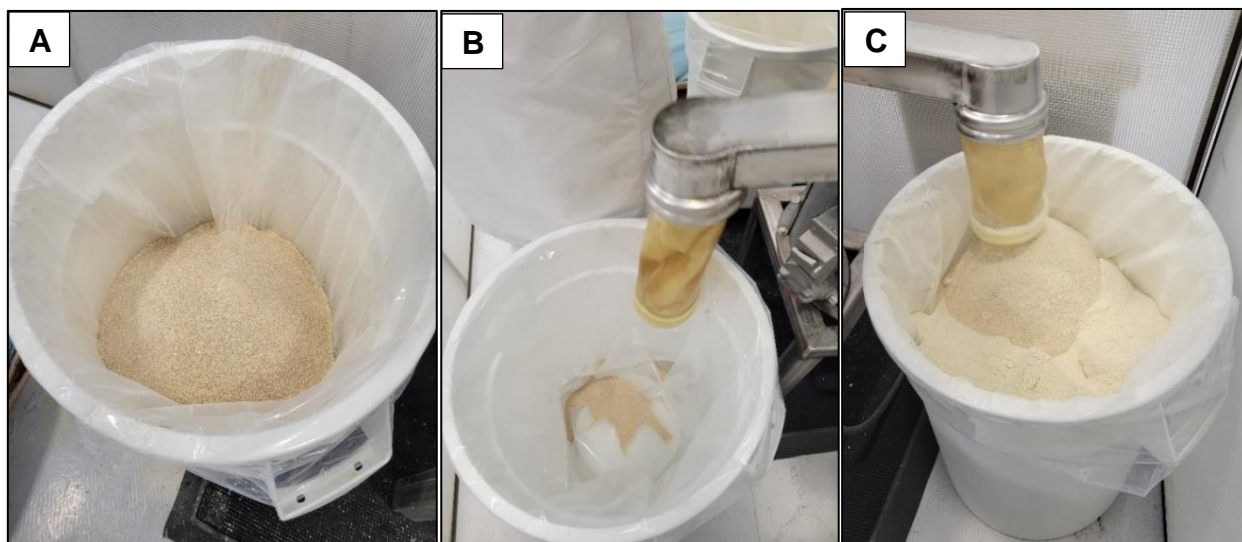
Figure 6.1 Different fractions resulting from canary seed while flour screening. A) Bran fraction 1- #24 mesh overs; B) Bran fraction 2 (#32 mesh overs); C) Canary seed white flour - #32 mesh unders.

6.3.3.3 Overall process: First scaling up trial plan for canaryseed protein production

The overall process flow originally planned for the scaling up trial, based on the lab-scale process in section 5.3.2.1, is shown in Figure 6.2. Briefly, the white-canaryseed flour was mixed with RO water at 1:10 w/w flour-to-RO water ratio for the aqueous de-oiling treatment. The slurry was held with agitation (Figure C2) for 1h for the de-oiling treatment and fed to the decanter (EC3; Figure C3-A) to separate oil-containing light phase and starch-protein-fiber-containing heavy phase. These unit operations include the aqueous de-oiling of canary seed flour. Once the aqueous de-oiling was completed, it was expected to re-slurry the heavy phase with RO water and screen (VS4; Figure C4) to remove coarse and fine fibre. The fibre-reduced filtrate that is containing starch and protein was expected to treat with the two enzymes to digest starch for converting to sugar syrup. Then, the enzyme-treated slurry is centrifuged (DC2, Figure C3-B) and the heavy-phase that contains protein and some starch is treated again with one of the two enzymes to digest residual starch to improve protein purity. After the final enzyme treatment, the slurry is centrifuged (DC2) to recover the heavy phase that contains protein. The light phase that contains sugar syrup from the two enzyme treatments were expected to be combined and sampled to evaluate the sugar profile. The protein phase was then expected to be adjusted to pH 7, pasteurized and spray dried (SD1; Figures C5-6) to produce protein concentrate. Since the amount of protein concentrate resulting from a 100 kg batch is low for other large-scale pasteurizing methods, such as shell and tube type or jet-cooking type, batch pasteurization was performed in these trials. However, there were plugging issues associated with initial decanter centrifuge separation and as a result, the original process was modified during the trials.

6.3.3.4 Modified process for canaryseed protein production trials

The decanter centrifuge was plugged soon after feeding started to the centrifuge after aqueous de-oiling treatment and caused separation issues. The plugged material that contains wet starch, protein and fiber was very thick and sticky and presumably caused separation issues. Then, a decision was made to reduce fiber in the slurry to facilitate starch and protein separation using the decanter centrifuge. Therefore, the process was modified to perform wet screening of fiber and re-fed to the decanter centrifuge. The result was the same and decanter separation was unsuccessful. Hence, it was decided to evaluate a disk stack centrifuge for starch-protein-containing heavy phase and oil-protein-containing light phase separation. Introduction of a disk

stack centrifuge for this unit operation was successful and it was utilized to continue the process. It took approximately eight hours to complete the fiber screening and disk stack separation. Until these steps are completed, the aqueous de-oiling conditions of the slurry was maintained during the entire eight-hour period. The modified process including mass balances is shown in Figure 6.3. Except these changes up to aqueous de-oiling, the remaining process was same as the original process flow (Figure 6.2) of the trial.

6.3.4 Analytical methods

6.3.4.1 Composition analysis

The protein, oil, moisture, ash and total starch content of the canaryseed flour, final product and in-process samples were analyzed as required using the AOAC International and American Oil Chemists' Society (AOCS) official methods as described in Perera et al. (2022).

6.3.4.2 Color analysis

Color of the aqueously de-oiled canaryseed fraction was evaluated using a Hunter colorimeter (Labscan II, Hunter Associates Laboratory Inc.) according to the method described in Perera et al. (2022).

6.3.4.3 Sugar profile analysis

The sugar profile of the syrup fraction was analyzed according to the method #WA64088 described in Waters XBRIDGE Amide HPLC column application notebook (https://www.waters.com/ webassets/cms/library/docs/720003438en.pdf), using Waters XBRIDGE Amide column and an evaporative light scattering detector. Ribose, xylose, glucose, fructose, lactose, sucrose and maltose purchased from Sigma -Aldrich Canada Co. were used as the sugar standards for profiling the syrup fraction.

6.4 Results and discussion

6.4.1 Aqueous de-oiling and enzyme assisted starch degradation for protein concentration

The canaryseed white flour contained 16.1% protein (Table 6.1, 18.4% dry weight basis) which is lower than the protein content of the white-flour prepared using lab-scale roller milling
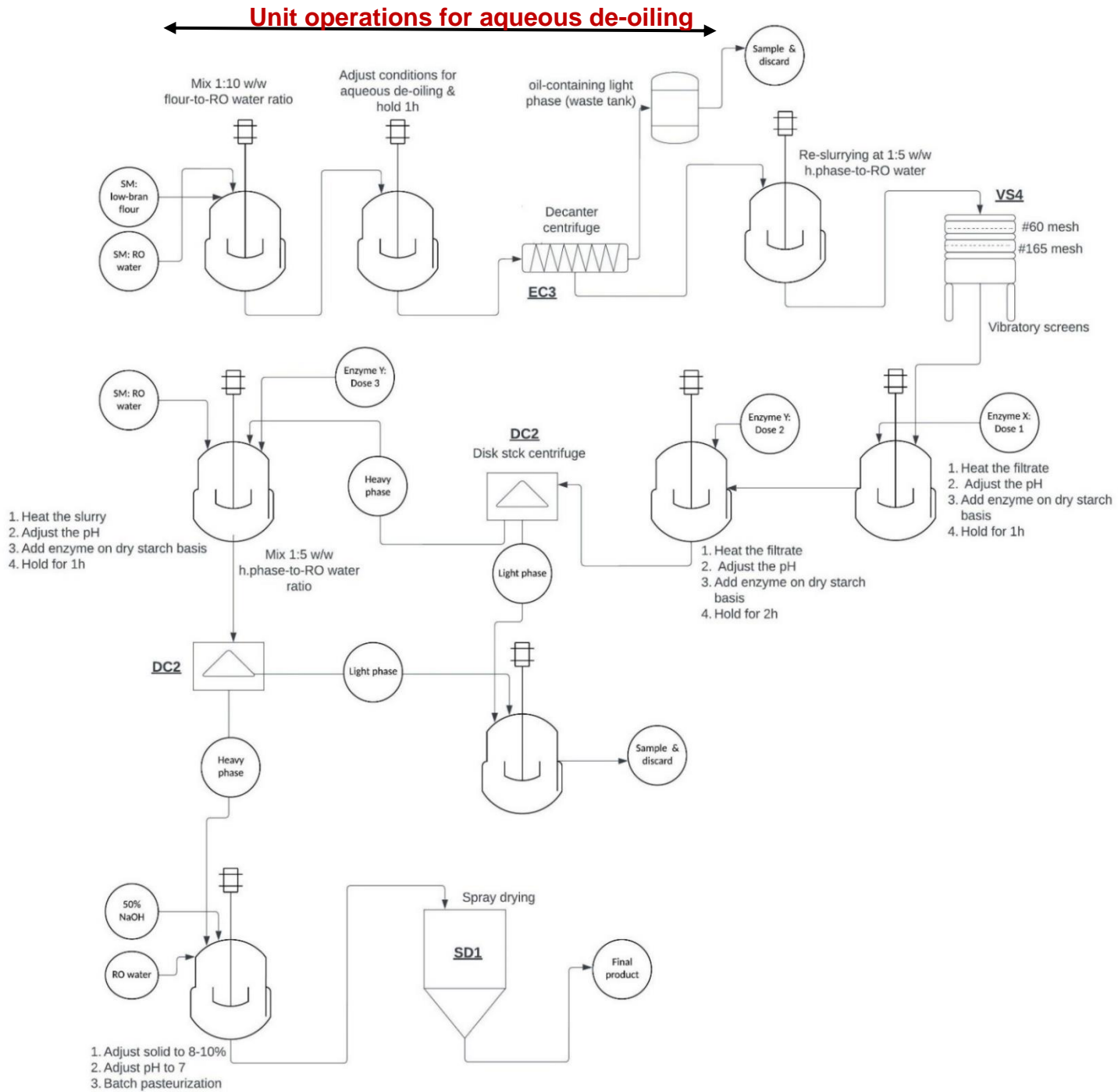
Figure 6.2 Overall process flow planned for the scaling up trials.

Unit operations for aqueous de-oiling
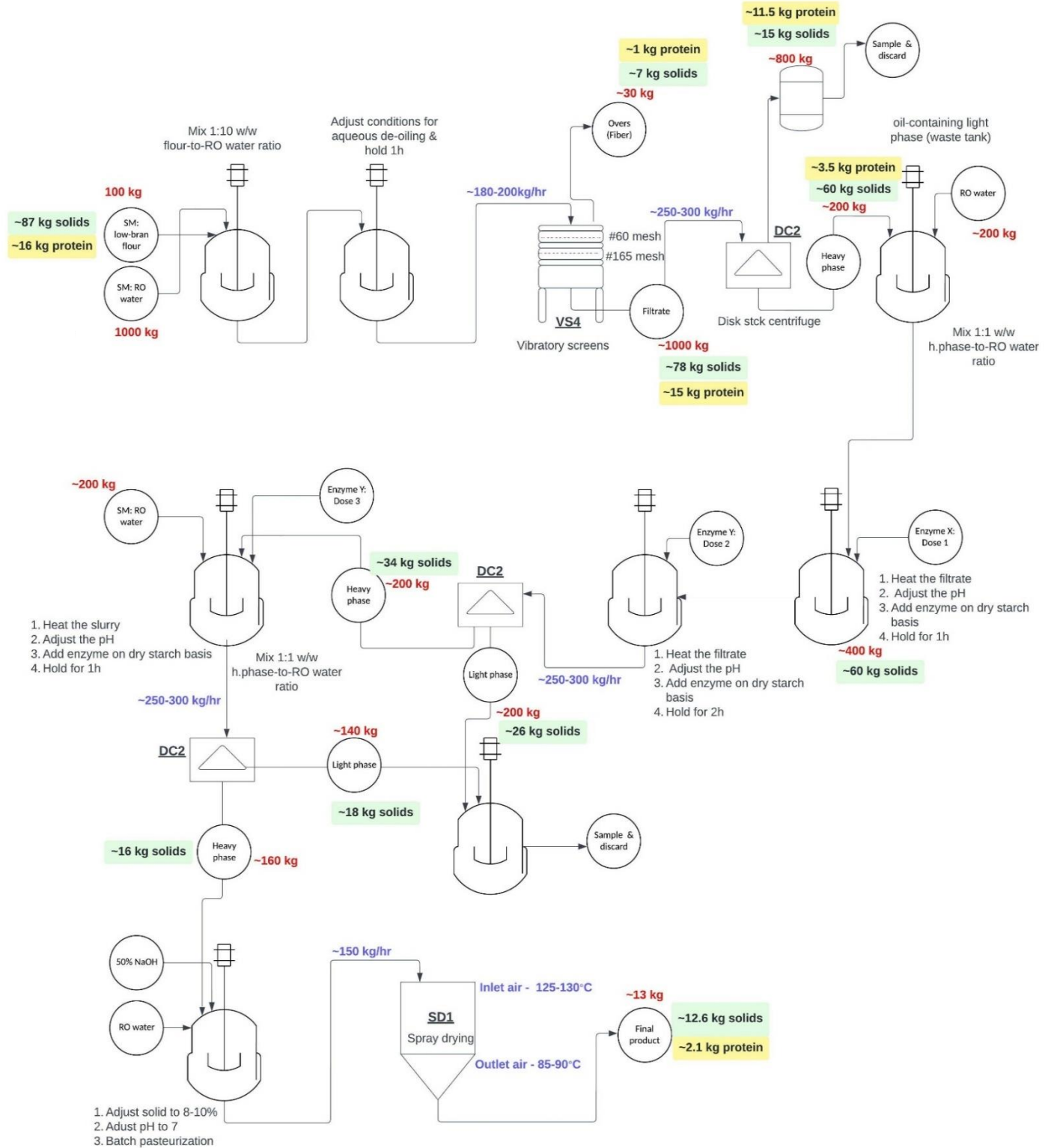
Figure 6.3 Modified process flow continued for the scaling up trials showing dry matter and protein recovery.

that was previously reported (~21%, Perera et al., 2022; Abdel-Aal et al., 2011a). The lower protein content could be attributed to the difference in the bran vs. white-flour fractions that was obtained from the lab-scale roller milling compared to the pilot-scale roller milling carried out in the present study, resulting ~3% protein loss to the bran fraction. The canaryseed flour and slurry was mixed at 1:10 w/w flour-to-water ratio using 100 kg of white flour that resulted 9.09% (w/w) solid content that required to be fed to a centrifuge after aqueous de-oiling treatment. Due to the solid content and the composition of the flour, especially fiber with larger particle size (~88-630 µm), a decanter centrifuge (EC3, ~3300 × g) was utilized to separate the oil-containing aqueous phase from the protein-starch-fiber containing insoluble phase. Decanter centrifuges are suitable for initial slurry (solid-liquid) separation, and they could handle slurries with broader range of solid levels (4%-40% w/w) with particle size ranging from 1-5000 µm whereas, the disk centrifuges are typically used as clarifiers and handle solutions containing 0.05%-10% (w/w) solid content with particles size range from 0.1-100 µm (Tarleton & Wakeman, 2007).

Table 6.1 Proximate composition of canary seed white flour (as is basis).

Composition	Spray dried protein fraction	
	Value (%)	Amount (kg) [1]
Moisture	12.60 ± 0.00	12.6
Protein (% Nitrogen × 5.7)	16.10 ± 0.00	16.1
Oil	6.10 ± 0.01	6.1
Total Starch	50.80 ± 0.00	50.8
Ash	1.60 ± 0.00	1.6
Non-starch carbohydrates	12.80 ± 0.01	12.8

Values are presented as Mean ± Standard deviation of duplicates.
[1] The amount was calculated for 100 kg of white flour that was used as the starting material for each batch.
*The non-starch carbohydrates were calculated as 100 - (% moisture + % protein + % oil + % Ash + % total starch).

With respect to the decanter centrifuges, the parameters, such as feed rate, differential speed, light phase back pressure were manipulated for better solid-liquid separation. However, the attempts carried out to set the conditions to handle the thick and sticky canaryseed material was unsuccessful. Hence, modifications to the original process were required to address this issue. It is plausible that fiber present in the canaryseed flour could contribute to this issue; therefore, wet screening of fiber was carried out to reduce the fiber (Figure C4) and re-attempt to feed the decanter centrifuge using the filtrate passed through #60 and #165 screens. The re-attempt was not unsuccessful and decanter centrifuge was plugged right after few minutes into feeding. Further

investigation is required to identify the root cause and identify the right adjustment required for the decanter or a different decanter centrifuge that possess the capacity to handle this sticky material.

The wet screening of fiber (Figure C4) resulted coarse and fine fiber fractions from #60 screen (250 µm) and #165 screen (~88 µm) overs (Figure 6.4). Approximately 6% of total fiber (#60 mesh + #165 mesh overs) fraction was yielded, with 3.3% protein content that could be dried and used as a co-product of canary seed protein processing in the future. This is approximately 47% of the non-starch carbohydrate fraction of the white flour (Table 6.1), which is mainly consist of fiber. The wet-screening was also carried out in the process developed by Abdel-Aal. (2010) and 5.6% yield was reported for fiber isolate from the EAW process. Since decanter separation was unsuccessful even with the filtrate from wet-screening (#165 screen unders), decision was made to evaluate disk stack centrifuge (DC2; ~6870 × g) for separation of aqueous oil-phase and insoluble starch-protein phase.

At this stage, the coarse fiber particles (>88 µm) have been removed by the wet screening, and the slurry is suitable to feed to the disk stack centrifuge. The feed rate, desludging interval, light phase back pressure of the centrifuge was adjusted for better separation. As expected, the disk stack separation was successful, and it was possible to obtain white color heavy phase that mainly contains the insoluble starch and protein fraction (Figure 6.5A). A sample of DC2 heavy phase was spray dried in the laboratory and the dried powder (Figure 6.5B) contained 0.8% residual oil content on dry weight basis. The starting material, *i.e.,* white flour, contained 6.1% oil (Table 6.1, 7% oil on dry weight basis). These results show that the aqueous de-oiling is successful and scalable. Therefore, these modifications performed for the original process was continued and used for the second scale up trial to evaluate repeatability.

The aqueously de-oiled fraction resulted from the disk stack heavy phase mainly consist of starch and protein, since oil and fiber has been removed during the previous unit operations. The next set of unit operations (Figure 6.3) were designed to enzymatically breakdown the starches in this de-oiled fraction and recover the remaining protein. It was expected to convert starches into sugars that can be recovered, concentrated and developed as a sugar syrup, which is the major co-product resulting from this aqueous process. Based on the laboratory testing, two enzymes were used as a split application to obtain high degree of starch digestion. Prior to the enzymatic

Figure 6.4 The fiber fraction resulting from #60 mesh overs (left) and #165 mesh overs (right)
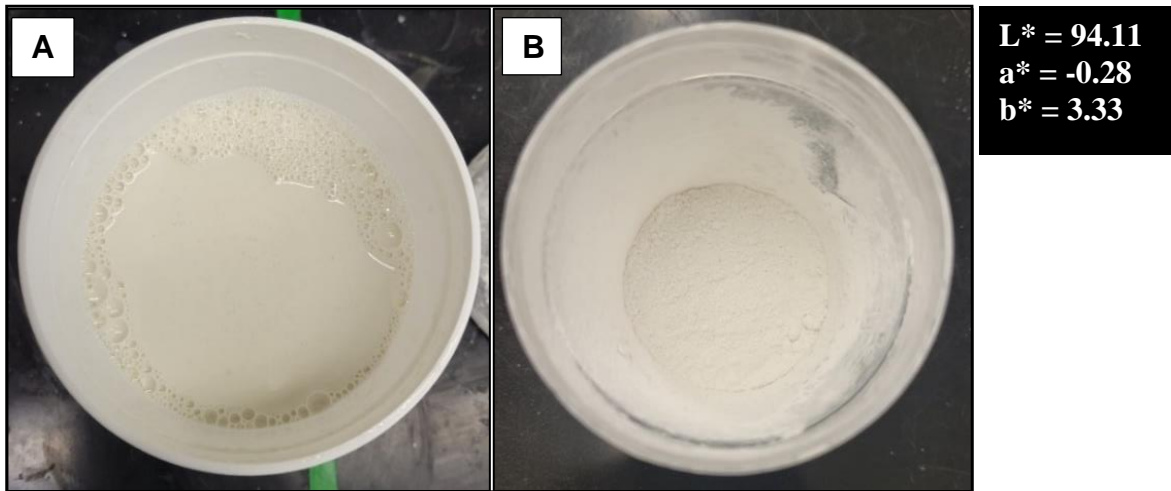
Figure 6.5 Aqueously de-oiled canary seed starch-protein fraction. A) heavy phase from DC2, the aqueously de-oiled fraction; B) Spray dried (white) powder from A showing hunter color values.

treatment, native starches were clearly visible in de-oiled fraction and accounted for approximately 23% of the total slurry volume (Figure 6.6A). The first enzyme dosage using enzyme "X" was able to slightly reduce the native starches to approximately 20% of the total volume (Figure 6.6B) and it was significantly reduced after applying the second enzyme dosage using enzyme "Y". The native starches were no longer visible at this stage (Figure 6.6C). Although starch was not visible as in native form similar to what was observed in Figure 6.6A-B, it was noticed that some undigested starch is mixed with protein and exist as a hazy white cloud, which represent gelatinize
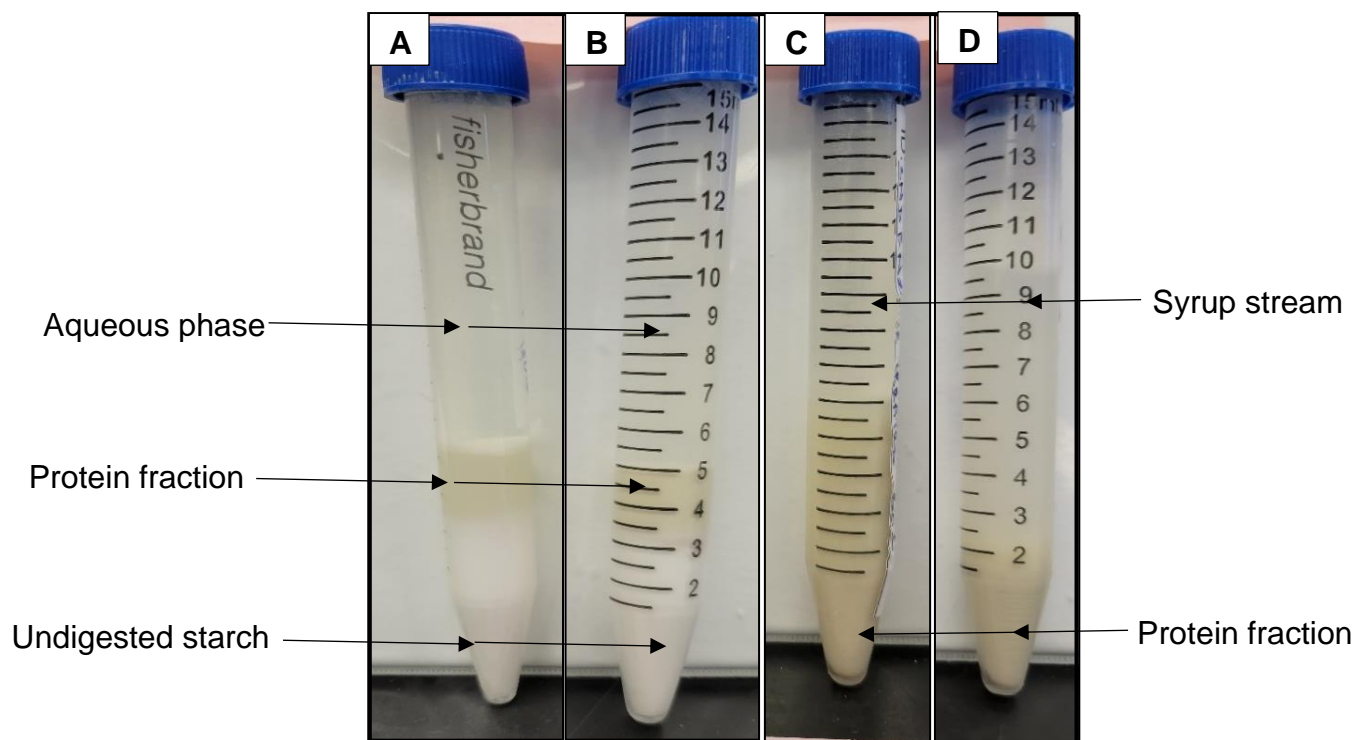
Figure 6.6 De-oiled canary seed starch-protein fraction after split enzyme treatment. A) Before enzyme treatments starts; B) After enzyme "X" treatment; C) After enzyme "Y" treatment; D) After enzyme "Y" split (3ʳᵈ dose) treatment

starch. The onset of canaryseed gelatinization occur ~60°C, peaks ~ 70 °C and ends ~75-80 °C depending on the purity of starch (Irani et al., 2017; Perera et al. 2022). The enzyme "Y" was used at ~70 °C to act on gelatinize starch whereas, it was ~50 °C for enzyme "X" where canaryseed starch remain ungelatinized. After this treatment was complete, there were some undigested starches that required further digestion to achieve higher protein purity. Hence the split application of enzyme (the third dose) was applied using enzyme "Y" to further degrade starch and improve the protein purity. After the final enzymatic treatment, majority of the remaining starch was degraded, and only protein was visible in the mixture (Figure 6.6D).

Up on completion of the enzymatic treatment, the protein fraction was recovered by centrifuging and the pH was adjusted to pH 7. The sugar syrup stream resulted from enzymatic treatment was combined and sampled to evaluate the sugar profile. The remaining sugar syrup stream was discarded and did not further process to obtain the concentrated syrup. The protein curd obtained after centrifuging (Figure 6.7A) was at 8-9% solid content and did not require further adjustment for spray drying. Prior to spray drying, batch pasteurization was carried out by heating

the protein-curd-containing tank using low-pressure steam up to 72 °C for and held for 5 mins. After the hold was complete, the protein curd was cooled down to ambient temperature and spray dried (Figures C5-6) to obtain protein concentrate (Figure 6.7B). The spray drying was carried out at ~150 kg/hr feed rate using 125-130 °C inlet and 85-90 °C outlet temperatures to target <5% moisture content in the final product. Overall, the yield of the protein concentrate was 13% of the starting white flour weight.
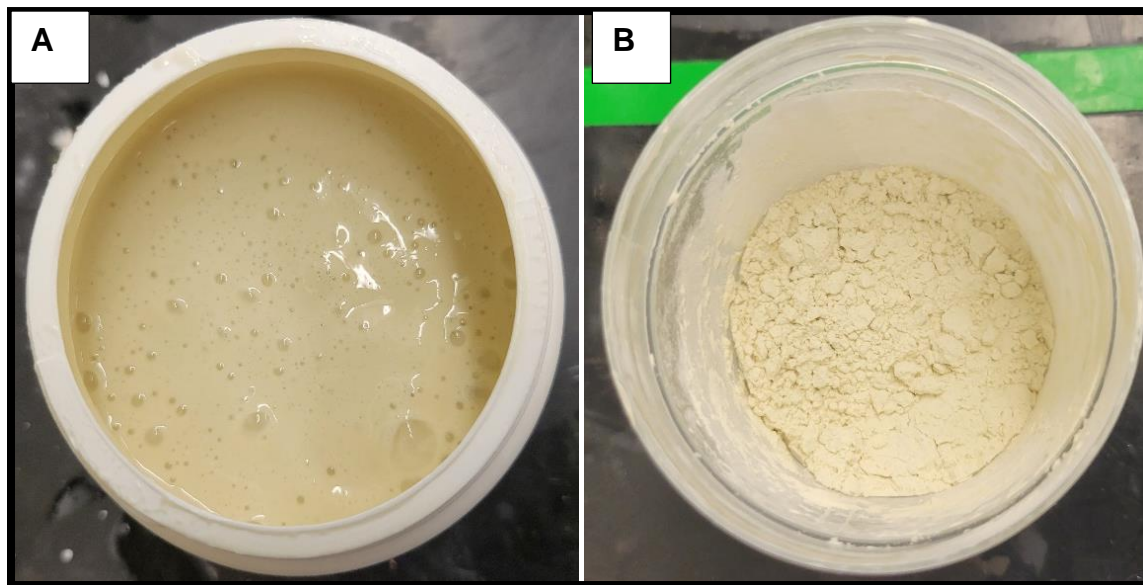
Figure 6.7 Canary seed protein fraction before spray drying (A) and after spray drying (B).

6.4.2 Final product composition and process evaluation

The proximate composition analysis of the spray-dried protein fraction (final product; Table 6.2) showed that it contained only ~16% protein and the total starch content was ~59%. The starting white flour contained 16.1% protein and 50.8% total starch that accounted for 16.1 kg of initial protein and 50.8 kg of starch, respectively (Table 6.1). The proximate composition of the final spray dried product showed that it only contained ~2 kg of protein (at 13% yield) whereas it contained ~7.6 kg of undigested starch (Table 6.2). The result suggested a significant protein loss into a co-product (syrup) or waste stream (oil-containing aqueous phase). Further investigations showed that the aqueously de-oiled-starch-protein fraction (Figure 6.6A) only contained ~3.3 kg of protein (~0.84% protein of ~400 kg of product stream) and ~2 kg of that protein was recovered from rest of the process into the final product. This result shows that approximately 13 kg of

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protein, *i.e.,* ~80% of protein in the white flour was solubilized into the oil-containing light phase during aqueous de-oiling process at mild alkaline pH. The prolamins and glutelin proteins in cereals are not known as soluble proteins in water and dilute salt solutions, and typically soluble in aqueous alcohol solutions in their native form (Shewry & Tatham, 1990). Therefore, high solubility of canaryseed proteins with the aqueous conditions used for de-oiling was not expected.

The lab-scale process had been repeatedly resulted >70% protein in the final spray dried product and such protein loss into the oil-containing light phase was not previously observed. Hence, it appears that the process modification performed during scaling up have caused this change. Due to the upstream process modification of fiber screening and disk stack centrifuging for aqueous de-oiling caused the white-flour slurry to remain at the pH used for aqueous de-oiling for ~8h, until those unit operations were completed. Presumably, prolong hold of canary seed proteins at this aqueous de-oiling conditions have caused some degree of structural changes that significantly improved its solubility, resulting protein loss into the light phase. Otherwise, it is also plausible that the proteins in canaryseed is fragile in nature or became fragile due to process modifications associated structural changes causing physical damage during high-speed agitation, pumping and centrifugation through disk centrifuge due to the hydrodynamic shear. Negative impact on protein recovery due to the structural alterations by hydrodynamic shear has been previously reported (Bekard et al., 2011; Elias & Joshi, 1998). Further investigations are required to understand the structural changes of canary seed major seed storage proteins and their solubility changes due to extended holding times with the pH of the medium and hydrodynamic shear. This information will be beneficial for designing processes and defining the process parameters for canaryseed protein ingredient development.

Table 6.2 Proximate composition of spray dried protein fraction (as is basis)

Composition	Spray dried protein fraction	
	Value (%)	Amount (kg) [1]
Moisture	2.58 ± 0.43	0.33
Protein (%Nitrogen × 5.7)	16.38 ± 0.15	2.13
Oil	4.60 ± 0.07	0.60
Total Starch	58.95 ± 0.35	7.70
Ash	2.73 ± 0.14	0.35
Non-starch carbohydrates	14.76 ± 0.37	1.90

Values are presented as Mean ± Standard deviation of duplicate of each batch.
[1] The amount was calculated using 13% spray dried product yield. Hence, 13 kg product out of 100 kg white flour.
*The non-starch carbohydrates were calculated as 100 - (%moisture + %protein + % oil + % Ash + %total starch).

Although the process modification caused loss of protein into the waste stream, this phenomenon resulted an aqueous-de-oiled-starch-protein fraction that contains ~83% total starch and ~5.3% protein (on dry weight basis) up on drying (Figure 6.5B). It is possible that this starch-rich fraction could be further purified by aqueous washing to produce high-purity, clean-labelled starch product that could be directed towards some other applications, such as in cosmetic products. Moreover, this starch fraction was white in color (L*= 94.11, a*= -0.28, b*=3.33; Hunter color system) which could be an added advantage for the starch for cosmetic applications. Neither solvent washing nor bleaching is required to improve the color of starch fraction resulting from this process. Hence, the current process could be modified to prepare aqueous starch and protein for clean label purposes.

The enzymatic treatment of the current process was able to degrade ~85% starch that was originally available in the white flour, resulting ~7.7 kg starch (out of ~50.8 kg starch in white flour) in the spray dried protein product. If the protein loss (~13 kg) did not take place, and recovered into the spray dried powder, it seems feasible to achieve at least >60% protein purity, considering potential losses. This will also improve the final yield ~20% or more and potentially reduce the %oil content in the final product. The %oil in the current spray dried product was 4.6% (Table 6.2), which accounts for ~0.6 kg of residual oil out of ~6 kg of initial oil available in the white flour (Table 6.1). Therefore, it is important to minimize the amount of time that the white flour slurry is holding at aqueous de-oiling conditions. Further lab-scale investigations are required to identify the changes required to optimize the scaled-up process. The ash content in the white flour (1.6 kg, Table 6.1) was reduced during processing and the final product only contained 0.35 kg (Table 6.2), presumably due to the removal of coarse and fiber fraction during when screening. The non-starch carbohydrate fraction of the final product accounted for 14.76%, which is ~2 kg of the total weight (Table 6.2). This fraction is mainly composed of fine fiber that was <88 µm and some sugars that retained from starch digestion. Further analysis to quantify amount of dietary fiber and simple sugar was not carried out since the final product resulted was not the expected protein concentrate and further process modification and optimization is required to produce a protein concentrate with >60% protein purity.

6.4.3 Sugar syrup (co-product) stream

In this present study, the canaryseed syrup stream was not further processed in the pilot scale. Instead, a sample was obtained and concentrated in the lab for profiling the sugars. The sample obtained from the syrup stream in the pilot plant (~16 kg at 14% solids) was concentrated in the lab using a rotavapor at 50 °C and 80-100 mbar vacuum level to obtain concentrated sugar syrup. The concentrated syrup contained 65% solids content and it was viscous. The viscosity of the syrup at this solid content was 4,100 cP and yellow in color (Figure 6.8; L*=64.72, a*=2.27, b*=33.35, Hunter color system). The major type of sugar present in the concentrated canary seed syrup was maltose and it accounts for ~17% of the dry solid basis (Table 6.3). It also contained glucose ~2% and rest of the sugars accounted for <0.01%. The sugar profile should be further investigated to evaluate the oligosaccharide composition to find out rest of the solid composition. Further investigation of this syrup stream will be useful to develop different food application and explore potential market for canary seed value addition.
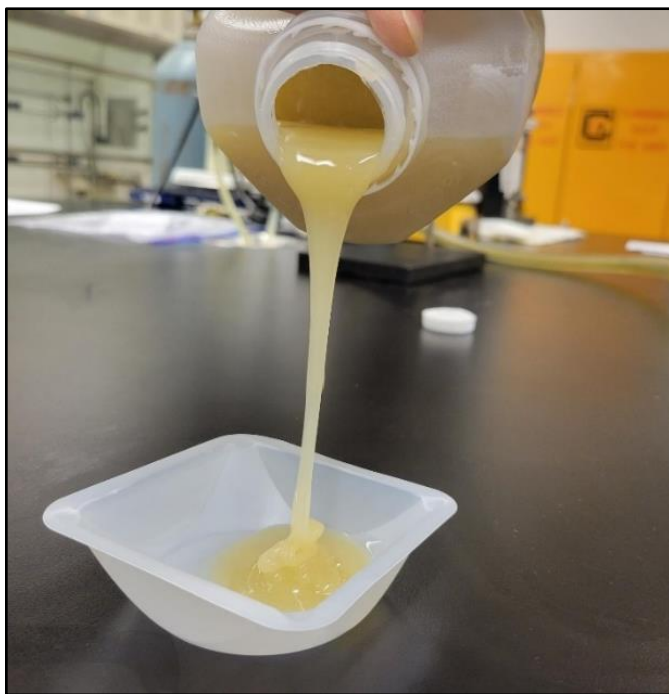
Figure 6.8 Concentrated canary seed sugar syrup.

Table 6.3 Sugar profile of concentrated canary seed syrup at 65% solid content

Composition	Value (%)
Ribose	<0.01
Xylose	<0.01
Fructose	<0.01
Glucose	1.30
Sucrose	<0.01
Maltose	11.15
Lactose	<0.01

6.5 Conclusions

Scaling up aqueous-de-oiling of canary seed flour is successful. The aqueous de-oiling treatment significantly reduces (~90%) oil present in the canary seed flour. The enzymatic treatment applied was effective and was able to degrade ~85% canary seed starch to produce sugar syrup stream. However, there is still room for improvement since there is ~15% undigested starch available in the spray dried product. Further investigation with other different enzymes and dosages should be carried out to produce canary seed sugar syrup if it is targeted as a major co-product.

It is important to investigate oligosaccharide composition of the resulting syrup to understand modifications required to obtain desired sugar composition in the syrup. Moreover, methods of concentration and purification of syrup stream to make it marketable ingredient should be investigated. Although, the enzyme-assisted starch degradation is successful it was not possible to achieve the second objective of this scaling up, which is obtaining a protein concentrate with at least >60% protein purity. The major reason for not achieving minimum protein purity expected was the unexpected loss of protein into the oil-containing light phase. It is important to understand the reason behind this phenomenon. Therefore, further investigation is required to evaluate solubility of protein at different pHs at different time (1-10 h range) and molecular weight profiling of the proteins solubilized at each pH-time combination.

If the expectation is to produce a clean labelled protein ingredient from canary seed, the current process could be adapted with modifications to prevent protein losses to increase the purity of the protein concentrate.

7. GENERAL DISCUSSION

The overall goal of this research was to develop a scaled-up aqueous process for canaryseed protein fractionation. It was expected that this process developed will result a protein product, *i.e.,* either a protein concentrate or an isolate, that could be directed towards different food applications based on their key techno-functional properties. Moreover, a protein ingredient that is derived from an aqueous-based process could be marketed as a "clean label" product that could provide canaryseed a competitive advantage to compete with other numerous plant-based protein ingredients available in the market. To develop an aqueous-based process it is important to understand the oil distribution in the seed. Typically, cereal grains have oil concentrated in the germ area than the endosperm, such as wheat and corn (Hoseney & Delcour, 2010a; Yang et al., 2018) where dry milling techniques could be applied (*e.g.,* corn dry milling; Barrera-Arellano et al., 2019) to separate the germ and bran to result low-oil-containing endosperm flour that can be utilized as the starting material for protein fractionation. On the contrary, some cereals, such as oat, has oil distributed throughout the endosperm (Heneen et al., 2009) that would make it difficult to utilize similar dry-milling techniques to generate low-oil flour suitable for protein fractionation. In such situations, solvent-de-oiling would be the path forward for de-oiling. Previous studies carried out by Abdel-Aal et al. (1997a; 2011a) showed that as in many cereals, crude fat content in the canaryseed flour fraction obtained from roller milling contain lower oil concentration than that of the bran fraction. However, there are limited information available on the oil distribution in the different seed compartments (*i.e.,* germ, aleurone layer, bran and endosperm) that could provide further insight on deciding a pathway towards developing a process to obtain a low-oil-containing canaryseed flour for protein fractionation.

The first study (Chapter 3) was carried out to explore more details on the canaryseed microstructure and investigate the distribution of oil, protein and starch in the seed compartments that will be complementary to the microstructural details previously reported by Abdel-Aal. (2011a). In this study both brown and yellow canaryseed was used and no marked differences

observed in the structure, composition, and composition distribution in the seed between brown and yellow canaryseed. The germ of canaryseed covers ~8-12% of the total endosperm area (Figure 3.2) and it contains granule-like structures in the scutellum (Figure 3.6). It was interesting to observe site-specific microstructural variations within a single seed compartment. The bran layer is appears to be broad and loosely packed in the ventral side of the seed whereas it is tick and tightly packed in the dorsal side of the seed (Figure 3.3). The aleurone layer is mostly a single-cell layer throughout the seed except, a two-cell layer was occasionally observed in the ventral side of the seed (Figure 3.4). These changes may result in uneven removal of bran fraction from the seed when dry milling techniques are applied to separate these from the endosperm fraction. The endosperm contains compound starch granules embedded in a protein matrix (Figure 3.6) as shown in Abdel-Aal et al. (2011a) and occupies majority (~88-92%) of the total endosperm area. The Raman spectral analysis showed that the germ contains oil and protein as the major chemical constituents (Figure 3.9a) similar to other cereals, such as wheat and corn (Hoseney & Delcour, 2010a; Yang et al., 2018). Similar trend was observed for the aleurone layer (Figure 3.10a). Presence of oil is also evident in the endosperm, along with proteins and starch (Figure 3.9b), where starch is the predominant chemical constituent. Abdel-Aal. (2011a) showed that the protein present in the endosperm and the aleurone layer is chemically different. Most likely, the endosperm contains the prolamin and glutelins, which are the predominant storage proteins in canaryseed (Abdel-Aal et al., 2010; Abdel-Aal et al., 1997a) whereas the aleurone layer and germ would mostly contains albumin and globulin type proteins as in other cereal seeds (Hoseney & Delcour, 2010b). The key finding of this study is that the oil is highly concentrated in the germ area than that of the endosperm area (Figure 3.9); however, the endosperm would contain more oil content than the germ due to the larger area that the endosperm occupies as suggested by the subsequent abrasive and roller milling study (Tables 3.1-3.2). These findings suggest that the oil distribution in canaryseed is more similar to oat than that of wheat or corn like typical cereal seeds. Therefore, application of dry milling techniques, such as abrasive milling and roller milling, would not merely help to prepare low-oil containing flour for protein fractionation and solvent-de-oiling may be the feasible path to achieving this goal.

Organic solvents are capable of denaturing the proteins (Damodaran, 2017) when used for de-oiling and therefore altering its native techno-functional and nutritional properties. The denaturation and associated changes in functional and nutritional properties could be either

favorable or unfavorable depending on the product application. Therefore, it is important to understand the effects of organic solvents on the protein structure and consequent property changes when selecting a suitable solvent for de-oiling. Achouri et al. (2020) used absolute ethanol for de-oiling canaryseed (whole/full bran) flour and evaluated the techno-functional properties of the protein isolate derived from the ethanol-de-oiled flour. In another study, Moura et al. (2020) used hexane for de-oiling canaryseed (whole/full bran) flour and evaluated the techno-functional properties of the hexane-de-oiled flour and isolates derived from the de-oiled flour. However, there is limited information available on the impact that these solvents pose on canaryseed proteins, and its properties compared to that of un-de-oiled flour and protein products derived from it. Ethanol and hexane are two widely utilized solvents for de-oiling and generating information on their usage and effect on the proteins and their properties is important for the industry when developing processes for canaryseed protein fractionation, especially if aqueous-based de-oiling is difficult to achieve due to the nature of oil distribution within the seed.

The second study (Chapter 4) was carried out to evaluate the effect of utilizing hexane and ethanol on denaturing the proteins and associated changes in their properties. Only yellow canaryseed was used in this study. The polypeptide profiles of non-de-oiled and de-oiled flours and protein isolates did not show any significant differences (Figure 4.4), showing lack of influence of solvents on the polypeptide profile of canaryseed flour and derived isolates. The DSC analysis showed that both hexane and ethanol do not denature the protein. The denaturation peak observed (108 °C peak denaturation temperature) in the non-de-oiled flour was observed in both de-oiled flour (hexane and ethanol) and the protein isolate derived from those flours (Figure 4.7a, C). However, there were some differences observed in techno-functional properties between the non-de-oiled and de-oiled flours and protein isolates (Table 4.3), suggesting some degree of structural modifications, which cannot be captured by DSC analysis. However, it did not cause any negative change on the functional properties. The functional properties were either improved or remained unchanged after solvent de-oiling. The IV-PDCAAS values showed that solvent de-oiling improved the digestibility in the flour level and the opposite was observed for the protein isolate, compared to that of their non-de-oiled counterparts (Table 4.4). The change is mainly influenced by the limiting amino acid score. De-oiling reduced the oil content and consequently increased the limiting amino acid content (lysine) and contributed to higher limiting amino acid score. On the other hand, the limiting amino acid score was decreased in the isolates prepared from

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de-oiled flour compared to the isolate prepared using non-de-oiled flour, plausibly due to loss of some lysine into the waste stream during processing. The changes to the IV-PDCAAS values, whether it is increased or decreased, the change is ~3% difference, which is not remarkable (Table 4.4). These results show that solvent de-oiling did not cause significant changes to canaryseed protein structure and the techno-functional and nutritional properties. Moreover, it was observed that ethanol is efficient in extracting oil than hexane and result higher phospholipid content, which could be a value-added co-product of canaryseed oil processing. It was difficult to conclude whether hexane is better than ethanol or *vice versa*. Therefore, other factors, such as solvent cost, solvent reclaiming ability, environmental impact, marketability and regulations on residual solvent levels in the product should be consider when selecting a solvent if solvent de-oiling is required.

The third study (Chapter 5) was focused on developing an aqueous-based de-oiling and protein fractionation process for canaryseed as an alternative process to currently using alkaline extraction and isoelectric precipitation. If aqueous de-oiling found to be difficult, the plan was to use either hexane or ethanol for de-oiling and use solvent-de-oiled flour for protein fractionation. Since the techno-functional properties, such as emulsification, foaming, solubility, water and oil holding capacities of canaryseed proteins were studied in Chapter 4, and also reported elsewhere (Achouri et al., 2020; Moura et al., 2020), the gelation properties of canaryseed protein resulting from developed process was evaluated. After a number of preliminary trials, it was possible to develop a lab scale process to aqueously de-oil canaryseed flour. The aqueously de-oiled fraction from this process (yellow seed) had 1.2% residual oil content (Section 5.4.1) and comparable to that of hexane de-oiled flour (1.2 % residual oil) and ethanol-de-oiled flour (1.4% residual oil) (Table 4.2), showing the effectiveness of the aqueous treatment. When the same method was applied to brown canaryseed flour it resulted 1.5% residual oil content in the aqueously de-oiled fraction (Section 5.4.1). Hence, the method could be used for the brown canaryseed similar to the yellow seed. The aqueously de-oiled wet fraction was then treated with two different enzymes to digest the starch and produce a protein product with 75% protein purity on dry weight basis; hence, called a protein concentrate. However, the protein purity was 69% for the brown canaryseed protein concentrate. The detail of the process is discussed in Chapter 6; however, due to the proprietary nature of this work, the aqueous de-oiling treatment, enzymes used, and their dosages are not revealed. Although the process developed was successful in terms of removing oil aqueously and produce a protein concentrate, it also resulted higher oil content (12-15%, Table

5.1) for both yellow and brown canaryseed, which was unexpected and unfavorable for the shelf-life stability. Further process modification is required to reduce the final oil content in the protein concentrate resulting from this process. Perhaps, a lower oil content could be resulted when this process is scaled up using pilot-scale equipment due to the differences in their operating mechanism compared to the laboratory equipment. However, having high oil content could favor some product applications, such as plant-based cheese, ice cream, mayonnaise etc. Evaluating this canaryseed protein ingredient in some of these product formulations will be beneficial to understand potential utilization of this "clean labelled" canaryseed protein concentrate. Both yellow and brown canaryseed protein concentrate showed a 16% (w/w) least gelation concentration at 95 °C and neutral pH (Table 5.3). At this concentration the canaryseed did not make strong gels and was significantly weaker compared to the gels prepared using commercial soy protein concentrate, even with addition of mono or divalent salts (Table 5.4). The result was the same even if the gels were prepared using 112°C, which is above the peak denaturation temperature of the protein concentrates (Table 5.2). Canaryseed protein did not show promising gelation properties and further investigation is required in this area to understand the canaryseed gelation properties and gelation-based applications. On the other hand, promising results were obtained in terms of using canaryseed protein concentrate on strengthening the bread-dough properties of low-gluten wheat flour even at 1-3% w/w inclusion levels and it is comparable to vital wheat gluten at same inclusion levels (Table 5.6). This is an indication of the potential of canaryseed protein to be incorporated in gluten-free bread baking and high-protein, low-carbohydrate bread applications as an alternative to food gums gum and wheat gluten used in these product categories, respectively (Hoehnel et al., 2019; Wójcik et al., 2022). Gluten-free bread and high-protein, low-carbohydrate bread are two product categories that has recently gained attention in the food industry (Hoehnel et al., 2019; Taghdir et al., 2017; Wójcik et al., 2022). Further investigations with higher inclusion levels of protein in MicroDough LAB testing similar to the present study, incorporation of protein in bread formulations and baking is necessary to understand the full potential of canaryseed protein fractions in this product categories. Since a novel aqueous-based process was developed and gelation properties were evaluated for the first time, both yellow and brown canaryseed was utilized in this study to generate information on both the canaryseed types. Significant differences of gelation or dough forming properties between yellow and brown canaryseed was not observed.

The final study of this research (Chapter 6) was to scale-up the laboratory-scale process developed in Chapter 4 to evaluate the scaling up feasibility and understand the processing issues for commercial scale operation. Only yellow canaryseed four (low bran/white) was used in this study. The scaling up was carried out at Key Leaf Life Science pilot plant facility in Saskatoon, Saskatchewan, Canada, using pilot-scale equipment, such as decanter centrifuge, vibratory screens, disk stack centrifuge and spray drier. Decanter separation of aqueously de-oiled canaryseed flour slurry caused issues and was unsuccessful. Therefore, modification to the original (Figure 6.2) process planned was applied and decanter centrifugation was omitted from the process and fiber removal using vibratory screens was moved ahead of the process (Figure 6.3). After that the process was executed without any issue and enzyme treatment successfully removed ~85% of the starch originally present in the white flour (Section 6.4.2; Figure 6.6). The process (with modifications) was able to reduce the oil content of the flour from 7% to 0.8% on dry weight basis (Section 6.4.1), which shows that aqueous de-oiling can be scaled up for canaryseed protein processing. However, the process modifications caused the aqueous flour slurry to remain in the aqueous de-oiling treatment conditions ~8 h and made protein soluble and lost into the waste stream, which was unexpected. This phenomenon caused lower protein concentration (16%, Table 6.2) in the final product. Further investigation is required to understand the potential structural modifications to the canaryseed proteins at prolong holding times in the aqueous de-oiling conditions and apply required modifications to obtain an aqueously de-oiled protein concentrate at least with >60% protein purity. The major co-product of this scaled-up process is a sugar syrup (Figure 6.8). Further processing and process optimization is required to obtain a purified syrup stream with more analytical information on its complete sugar profile to understand its potential for food application to add value to make this process cost effective and commercially viable. This modified process resulted a white color starch fraction with >80% starch purity (Figure 6.5). With further washing it is plausible to further improve its purity. Hence, it could be a high-purity canaryseed starch, a co-product, derived aqueously from an oil-containing source. Due to the smaller granular size, this could be a co-product that could be directed towards cosmetic applications (Abdel-Aal et al., 2010). Therefore, it is important to define the co-product stream of the protein fractionation process, *i.e.,* either sugar syrup or starch, and apply modification to the process accordingly to produce a "clean label" protein concentrate in the future.

8. GENERAL CONCLUSIONS

The microstructural features of yellow and brown canaryseed do not have any remarkable differences. The bran and aleurone layers of canaryseed has structural difference between the ventral and dorsal side of the seed, which could be occasionally seen in some other cereals. The germ occupies 8-12% total endosperm area and highly concentrated with oil and protein. Aleurone layer also contained oil and protein while endosperm contains protein, oil and majority of starch where compound starch granules are embedded in a protein matrix. The concentration of oil is lower in the endosperm compared to that of germ; however, higher oil content is available in the endosperm than the germ due to its larger area occupied in the seed. This phenomenon invalidates the original hypothesis of this research that the germ is containing more oil than the endosperm similar to other cereals, such as wheat and corn. Hence, it is difficult to prepare low-oil-containing flour using dry milling techniques, such as abrasive milling or roller milling. However, these milling techniques helps to reduce the original oil load to a certain extent, and help reducing bran fraction and associated fiber, phenolics, phytates and ash, which is favorable for resulting low-bran (white) flour as a starting material for protein fractionation.

Since oil is distributed in the endosperm, solvent-de-oiling may be necessary to prepare de-oiled flour and would be easier pathway than carrying out aqueous de-oiling. Both hexane and ethanol can be used as a de-oiling solvent. Ethanol provides higher oil extraction efficiency and phospholipid recovery. It is difficult to conclude which solvent is best in terms of preserving native protein structure and its techno-functional ad nutritional properties. Both act similar manner and overall, they either improved these properties or remained unchanged. Neither hexane nor ethanol denature the canaryseed protein and it contained very high denaturation temperature (~108°C). These results show that canaryseed proteins has high tolerance for harsh processing conditions which is beneficial in terms of selecting processing conditions for protein fractionation. Due to the higher oil extraction efficiency, no adverse effect on protein structure and functional properties and known lower impact on the environment, ethanol could be the solvent ideal for de-oiling

canaryseed. However, other factors, such as solvent cost, solvent reclaim ability and regulatory implications should be considered when selecting a suitable solvent for de-oiling.

Despite containing high oil in the low-bran flour, it was possible to reduce the oil content using the laboratory process developed for aqueous de-oiling, and the method can be utilized for both yellow and brown canaryseed. The enzymatic digestion of the starch in the aqueously de-oiled fraction resulted in a protein concentrate with ~70% or more protein purity; hence, the laboratory-scale enzyme-assisted aqueous process is capable of producing a "clean label" canaryseed protein ingredient, which was a major milestone of this research. The canaryseed protein resulted from this process did not show strong gelation properties compared to that of commercial soy protein concentrate. However, it was comparable to wheat gluten in terms of improving bread dough quality prepared from low-gluten-strength wheat flour. Therefore, canaryseed protein concentrate shows a potential to be utilized in bread baking applications, such as gluten-free bread and high-protein, low-carbohydrate bread, which are some of the novel food categories.

The aqueous de-oiling is possible to carry out in the pilot scale with some modifications to the originally developed process in the laboratory. However, the process modifications change the canaryseed protein properties to improve its solubility and negatively impact on the protein recovery subsequently lowering the protein purity of the final protein product. Further investigations are required to understand the plausible structural changes to apply process modifications to improve protein recovery and purity of the "clean label" protein product resulting from this aqueous process.

9. FUTURE STUDIES

In this research it was evident that the oil in canaryseed is distributed in the germ, aleurone layer and the endosperm. However, the intensity of oil distribution in different areas within each compartment is not known. It would be helpful if a chemical map for oil content distribution within the seed could be generated. A similar chemical map will be helpful for proteins distribution as well. It was expected to generate such a chemical map using Raman microscopy in this study. However, it was difficult to obtain suitable microtomes due to the starchy endosperm even with multiple attempts with different techniques, including cryomicromes. Traditional resin embedding techniques tend to interfere with the signal getting from oil and using alcohols cause washing the oils in the seed sections making it a difficult task to map oil within the seed. Therefore, obtaining suitable microtomes and evaluating different techniques, such as synchrotron light beams, to map oil and protein could be a future area of canaryseed research.

There is limited information available on the structural features of prolamin and glutelin proteins of canaryseed. According to the literature, prolamins can be categorized as S-rich, S-poor and HWM proteins and it could exist either/or dimeric, aggregates or oligomeric form. There are different prolamins identified in different cereals, such as α- and γ-Zein in maize, α-, β-, and ω-gliadins in wheat etc. On the other hand, glutelin is a polymerized protein and has a highly disordered structure. Generating such information on canaryseed prolamins and glutelins is important to understand different protein types, structural properties and their structure and function relationship. This is another important area of future research to improve the scientific understanding of the canaryseed proteins.

Improving techno-functional and nutritional properties of canaryseed proteins to diversify their utilization should be further investigated. Technologies, such as protein modification and fermentation can be tested on improving these properties. Moreover, the fractionated protein products, as it is or improved, should be included in product formulation, such as gluten-free bread

or high-protein, low-carbohydrates bread or other products and evaluated to understand their potential for real-world food applications.

Another key area of research for canaryseed proteins is characterizing the flavor profiles in flour and fractionated proteins, using techniques such as GC-MS analysis or electronic nose testing. Moreover, sensory evaluations could be carried out to understand how consumer perceive canaryseed protein flavor as a food ingredient. Since canaryseed has oil distributed throughout the seed, it is highly likely that oil is subjected to oxidation and affect negatively on the flavor. Therefore, lipid oxidation and associated flavor changes in canaryseed flour, factors affecting lipid oxidation, such as lipoxygenase activity, and ways to mitigate lipid oxidation should be investigated.

Finding applications for co-products resulting from protein fractionation process is important for a commercially viable process. Hence, characterizing the co-products and exploring potential applications are important. Starch, dietary fiber, sugar syrup and oil are the co-products that results from existing canaryseed protein fractionation processes. Therefore, further investigations on these co-products, such as their chemical composition, physical and chemical properties, techno-functionality and nutrition is essential for the future of the canaryseed industry.

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11. APPENDIX A: SUPPLEMENTARY MATERIAL FOR CHAPTER 3

Figure A1 Differences of the bran and aleurone layer microstructure in various regions of a brown grain. A) Whole seed; B) Ventral side near the germ end; C-D) Middle of the ventral side at different magnification; E) Ventral side opposite to the germ end; F) Dorsal side opposite to the germ end; G-H) Middle of the dorsal side at different magnification; I) Germ area. a=Aleurone layer; b=Bran layer; e=Endosperm; g=Germ.
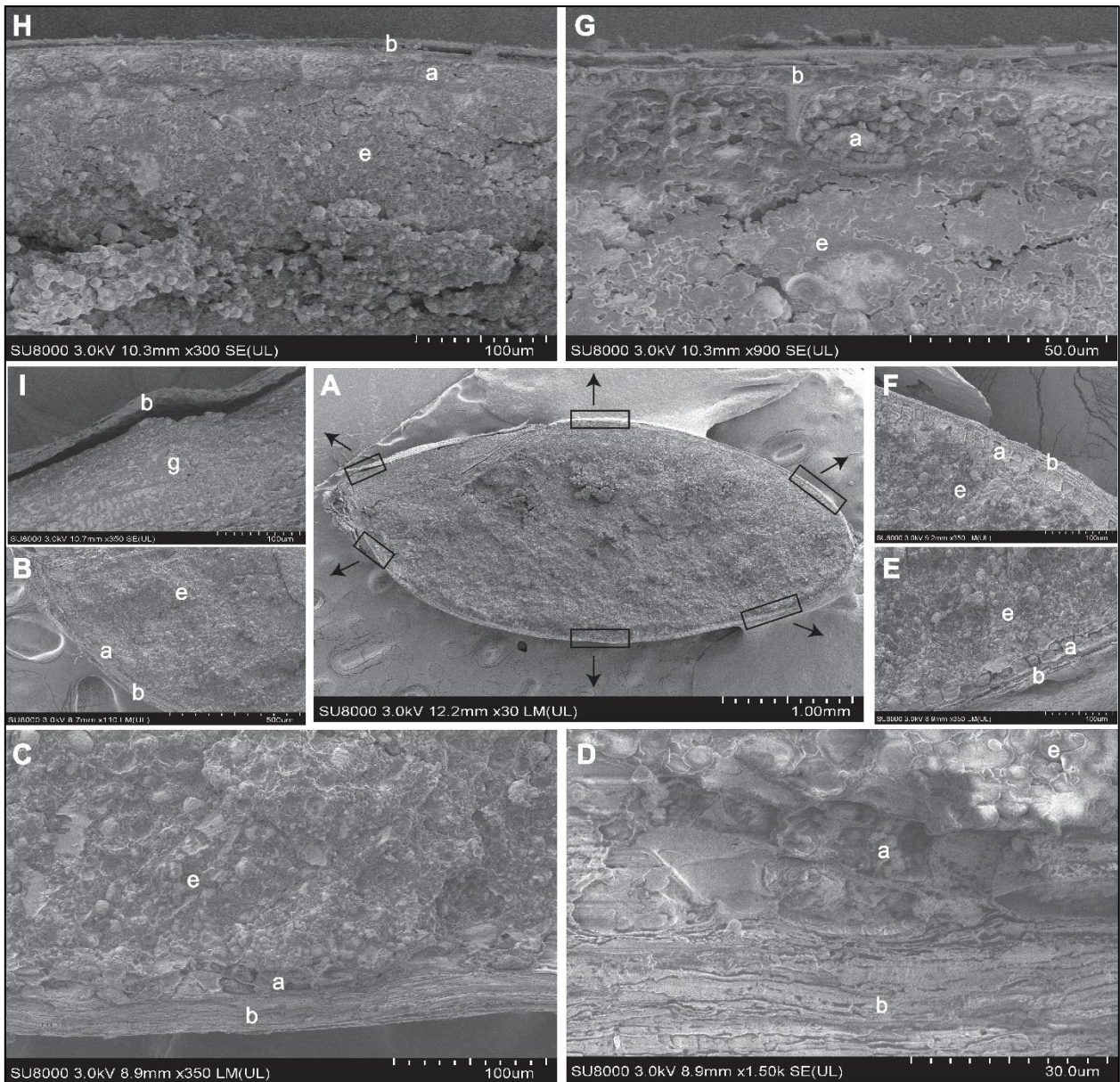
Figure A2 Differences of the bran and aleurone layer microstructure in various regions of a yellow grain. A) Whole seed; B) Ventral side near the germ end; C-D) Middle of the ventral side at different magnification; E) Ventral side opposite to the germ end; F) Dorsal side opposite to the germ end; G-H) Middle of the dorsal side at different magnification; I) Germ area. a=Aleurone layer; b=Bran layer; e=Endosperm; g=Germ.
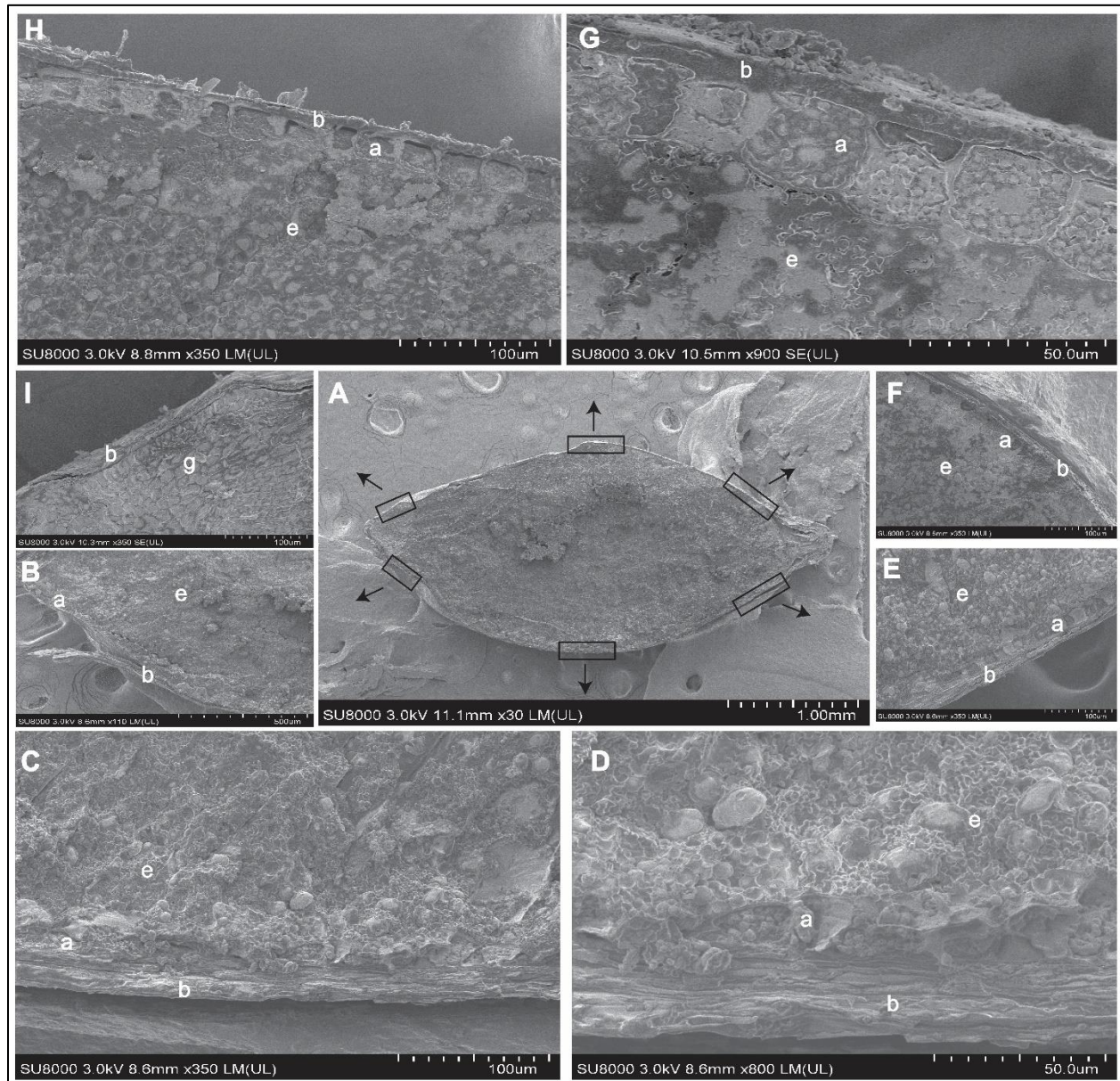
12. APPENDIX B: SUPPLEMENTARY MATERIAL FOR CHAPTER 5
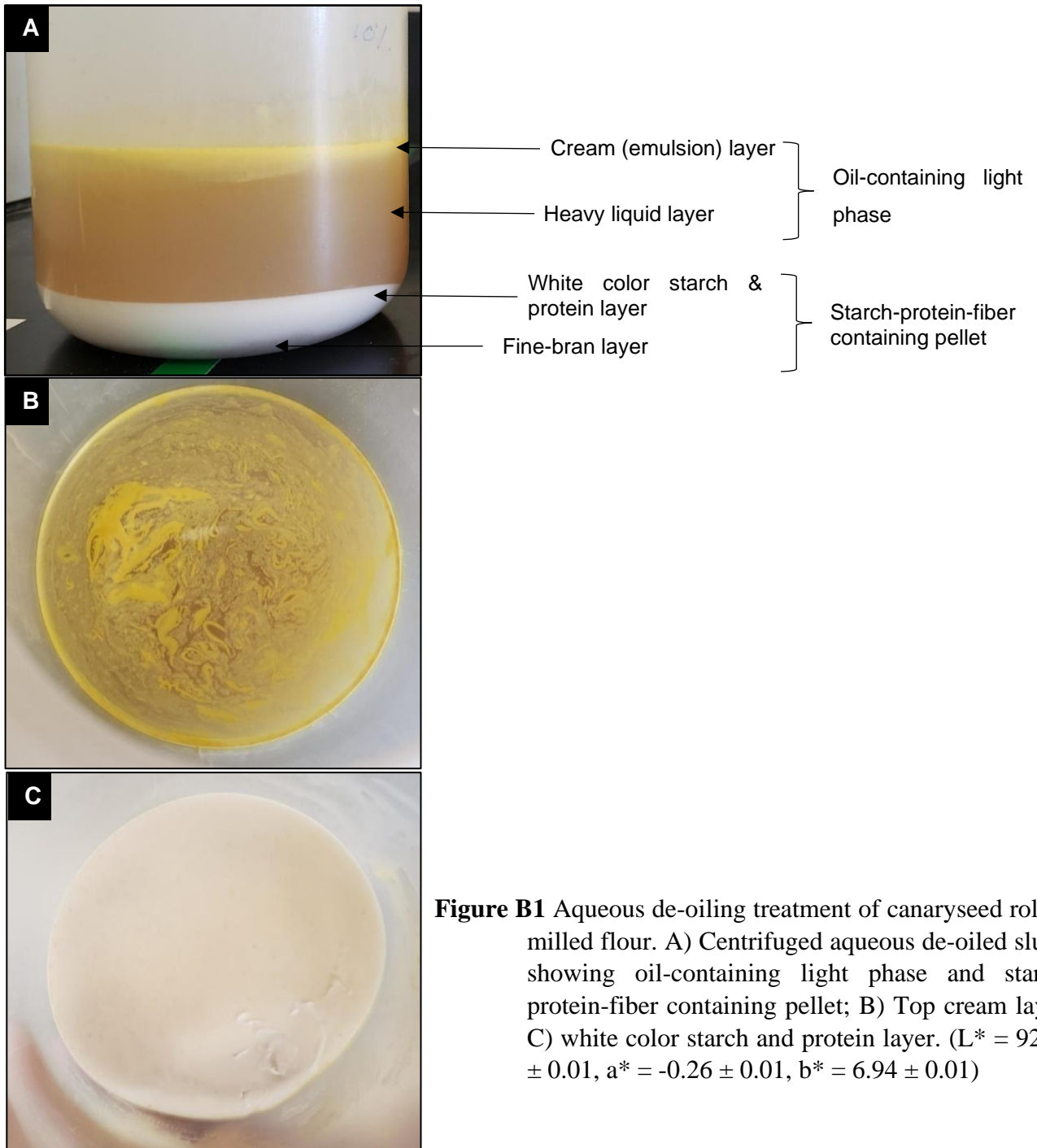
Figure B1 Aqueous de-oiling treatment of canaryseed roller-milled flour. A) Centrifuged aqueous de-oiled slurry showing oil-containing light phase and starch-protein-fiber containing pellet; B) Top cream layer; C) white color starch and protein layer. (L* = 92.24 ± 0.01, a* = -0.26 ± 0.01, b* = 6.94 ± 0.01)

13. APPENDIX C: SUPPLEMENTARY MATERIAL FOR CHAPTER 6

Figure C1 SWECO® Vibro-Energy™ Separator (VS3) set up for canary seed white flour preparation with #24 mesh on the top and #32 mesh on the bottom

Figure C2 Canaryseed white-flour slurry holding with agitation at aaqueous de-oiling conditions

Figure C3 Centrifuges used in the scaling up trials. A) Decanter centrifuge (EC3) and B) disk stack centrifuge (DC2)

Figure C4 SWECO® Vibro-Energy™ Separator (VS4) set up for screening fiber in the white-flour slurry.

Figure C5 Komline-Sanderson No. D-19 Spray drier (SD1) with the tent covering spray dried powder collection area

Figure C6 Spray drier outlet showing dried product collection set up (inside the tent shown in Figure 5). A SWECO® Vibro-Energy™ Separator was used to screen clumped particles and any larger foreign material

14. APPENDIX D

Table D1 Amino acid composition of different canaryseed protein flours and control wheat flours

Category	Amino acid (AA) (g/100 g Protein)	Canaryseed				Wheat	
		Whole flour	White flour	Hexane de-oiled flour	Ethanol de-oiled flour	Whole flour	White flour
Essential AA	Methionine	1.19	1.55	1.35	1.36	1.7	1.5
	Cystine	3.78	4.56	4.19	3.78	3.9	3.2
	Lysine	2.34	1.68	1.88	1.91	2.4	1.7
	Tryptophan	2.49	2.70	2.92	2.84	1.5	0.8
	Isoleucine	3.54	3.45	3.53	3.48	3.2	2.9
	Histidine	2.25	2.52	2.18	2.25	2.9	2.0
	Valine	3.97	3.72	3.88	3.82	3.8	3.2
	Leucine	7.22	6.90	7.15	7.26	6.1	6.1
	Phenylalanine	5.93	5.71	6.02	6.07	4.5	4.4
	Tyrosine	2.49	2.52	2.49	2.38	2.4	2.5
	Threonine	2.44	2.17	2.14	2.29	2.1	2.5
	Total EAA	**37.61**	**37.46**	**37.73**	**37.43**	**34.53**	**30.69**
Non-essential AA	Alanine	4.06	3.76	3.84	3.82	3.0	2.6
	Arginine	6.21	6.06	5.93	5.86	4.1	3.6
	Aspartic acid	5.07	4.69	4.45	4.71	3.5	3.6
	Glutamic acid	29.20	30.70	30.36	30.68	30.5	35.8
	Glycine	3.01	2.65	2.66	2.63	4.0	3.2
	Proline	6.69	6.50	7.02	6.79	10.3	12.1
	Serine	5.54	5.62	5.45	5.43	5.8	6.0

15.   APPENDIX E

Table E1 Amino acid composition of different canaryseed protein isolates and commercial wheat gluten isolate

| Category | Amino acid (AA) (g/100 g Protein) | Canaryseed | | | Wheat |
		Non-de-oiled isolate	Hexane de-oiled isolate	Ethanol de-oiled isolate	Commercial gluten isolate
Essential AA	Methionine	1.21	1.40	1.21	1.70
	Cystine	2.52	2.99	2.40	3.13
	Lysine	1.72	1.61	1.52	1.66
	Tryptophan	2.53	2.71	2.49	1.05
	Isoleucine	4.28	4.23	4.22	3.25
	Histidine	2.08	1.77	1.98	2.17
	Valine	4.63	4.63	4.62	3.44
	Leucine	8.20	8.28	8.21	6.35
	Phenylalanine	6.24	5.86	5.81	4.85
	Tyrosine	2.84	2.82	2.79	2.86
	Threonine	2.08	2.31	2.45	1.73
	Total EAA	**38.33**	**38.61**	**37.69**	**32.17**
Non-essential AA	Alanine	3.96	4.02	4.1	2.28
	Arginine	6.68	6.80	6.9	3.21
	Aspartic acid	5.47	5.32	5.5	2.92
	Glutamic acid	28.76	29.59	29.4	37.60
	Glycine	2.56	2.65	2.7	3.40
	Proline	6.75	6.78	7.2	12.04
	Serine	5.50	5.92	6.0	5.49