NUTRITIONAL AND BIOACTIVE PROPERTIES OF GLABROUS CANARYSEED (*PHALARIS CANARIENSIS* L.) PROTEINS

By

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ABSTRACT

In 2015, glabrous canaryseeds were approved for human consumption by Health Canada and the FDA as a new cereal grain. Formerly, the seeds could only be used as birdseed since they were lined with inedible, hair-like silica fibers, however, the new hairless seeds are high in protein (22% w/w) and have immense potential for use by the food industry. The objective of this study was to compare the nutritional quality and bioactive properties between two yellow (C09052 & C05041) and two brown (CDC Calvi & CDC Bastia) hairless canaryseed cultivars and as compared to two common cereals (oat and wheat).

Overall, canaryseed proteins have a well-balanced amino acid profile and the seeds are high in tryptophan (2.4-2.6 g/100g protein), an essential amino acid normally deficient in other cereal grains, but low in essential amino acid lysine (2.3-2.9 g/100g protein). The protein quality of canaryseed flours were evaluated by two protein quality scoring methods; the Protein Digestible Corrected Amino Acid Score (PDCAAS) and the Digestible Indispensable Amino Acid Score (DIAAS). The PDCAAS score was calculated from the overall digestibility (*in vitro* protein digestibility) of canaryseed protein and had scores of 25.4-32.4% for infants (0-6 months). These values were comparable to those of wheat (26%), but lower than those of oat (47%). The minimal and maximal *in vitro* total ileal amino acid digestibility values and DIAAS scores were determined by measuring the bioaccessibility of each individual amino acid. The total ileal digestibility for the four canaryseed varieties ranged from minimal values of 25 to 29 % to maximal values of 65 to 71%, with the yellow varieties showing higher digestibility values. These values were comparable to those of wheat (22 to 73%) and oat (32 to 69%). The DIAAS scores showed that lysine was the limiting essential amino acid, producing *in vitro* DIAAS scores of 7.9-9.5%, 9.7-11.5%, 11.5-13.7% for infants (0-6 months), children (6 months-3 years), and older children/adults, respectively. These results indicate that even though the canaryseeds are high in tryptophan, the quantity and digestibility of the limiting amino acid lysine remains low, therefore, as for other cereals, they must be complemented by other protein sources in order to meet dietary requirements of essential amino acids.

Trypsin inhibitor activity (TIA), phytic acid, and total polyphenol content (TPC) were determined in canaryseed flours and isolates to evaluate the anti-nutritional components in the seeds and there

was no significant difference between canaryseed cultivars. TIA in canaryseed flours (0.12-0.16 mg/g) were comparable to oat (0.14 mg/g) and wheat (0.11 mg/g) flours. The phytic acid content in canaryseeds (12 mg/g flour) was significantly higher than oat (6 mg/g flour) and wheat (3 mg/g flour), but comparable to levels found in other cereals and legumes. TPC of canaryseeds (1.4 mg FAE/g flour) was higher than wheat (0.6 mg FAE/g flour) but lower than oat (2.0 mg FAE/g flour). TPC was higher in canaryseed isolates than in the flours, most likely due to higher presence of free and polyphenols-protein complexes (bounded polyphenols) in the canary protein isolates.

Finally, the health promoting effects of the four canaryseed varieties were also evaluated and compared to those of oat and wheat by measuring the potential antioxidant, (ORAC, DPPH, ABTS), chelation (Fe2+), antihypertensive (ACE), and antidiabetic (DPP-IV) activities of cereal flour hydrolysates after ultrafiltration with a 3K MWCO filter to obtain small and active peptides. Between canaryseed cultivars, there was no significant difference between brown and yellow canaryseeds except for the DPPH and Fe^{2+} assays, where the brown varieties demonstrated superior activity (IC_{50} values of 77.96-96.38 µg/mL and 0.73-0.96 mg/mL for DPPH and Fe^{2+} assays, respectively) as compared to the yellow canaryseed cultivars (IC_{50} values of 638.75-1043.55 µg/mL and 1.55-1.69 mg/mL for DPPH and Fe^{2+} assays, respectively), indicating brown cultivars may have better radical scavenging activity as compared to the yellow cultivars. As compared to oat and wheat, the antioxidant activity of canaryseed proteins was equivalent or superior for each assay tested. For the ORAC assay, canaryseeds, oat, and wheat had an activity of 1.77-1.99 µmol TE/mg protein, 1.31 µmol TE/mg protein, and 1.54 µmol TE/mg protein, respectively. For the ABTS assay, the yellow C09052 canaryseed protein had IC_{50} values 117.85 µg/mL, which was not significantly different than wheat (107.84 µg/mL), and lower than oat (176.01 µg/mL). The C09052, C05041, and Calvi canaryseed peptides had exceptional ACE inhibition activity with IC_{50} values of 333 µg/mL, 405 µg/mL, and 322 µg/mL, respectively, as compared to oat (570 µg/mL) and wheat (781 µg/mL). IC_{50} values for the DPP-IV inhibition assay were 1.0 mg/mL, 1.1 mg/mL, and 1.4 mg/mL for C09052, C05041, and Bastia peptides, respectively, and comparable to wheat (1.0 mg/mL).

To identify potential antihypertensive peptides, the C09052 3K MWCO digest was further purified by size exclusion chromatography, which showed three main peaks, and these three peaks were

collected and tested again for their ACE inhibition activity. Fraction 1 and 2 both had similar ACE inhibitory activity (32% and 29%, respectively) at a peptide concentration of 350 μg/mL, however, fraction 1 was selected for further purification because it had the highest protein content and 82% ACE inhibition at a protein concentration of 3 mg/mL. MS analysis of the C09052 fraction identified 46 peptides belonging to 18 proteins from the subfamily Pooideae. 14 of the 18 identified proteins were homologous to barley proteins and the remaining from wheat (3), and goatgrass (1). *In silico* analysis of the peptides showed all 46 peptides had potential ACE inhibitory and DPP-IV inhibitory activity, and 20 had potential antioxidant activity, which has been validated from the *in vitro* studies. However, other peptides had potential hypotensive, antiamnestic, immunostimulating, opioid, and neuro activity which have not yet been confirmed. The results indicate canaryseeds are high in digestible protein and could potentially demonstrate exceptional health promoting effects *in vivo*, particularly against cardiovascular disease, and should therefore be regarded as a functional food or ingredient.

RÉSUMÉ

En 2015, les graines de l'alpiste des Canaries glabres ont été approuvées pour l'alimentation humaine par Santé Canada ainsi que par le Secrétariat américain aux produits alimentaires et pharmaceutiques (FDA) en tant que nouveau grain céréalier. Précédemment, les graines ne pouvaient être commercialisées que pour l'alimentation des oiseaux puisqu'elles étaient pourvues de petits poils silicifiés, cependant, les nouvelles variétés glabres sont riches en protéines (22% w/w) et ont un immense potentiel d'utilisation par l'industrie alimentaire. L'objectif de cette étude était de comparer la qualité nutritionnelle et les propriétés bioactives de deux cultivars de graines d'alpiste des Canaries glabres de couleur jaune (C09052 et C05041) et de deux variétés de couleur brune (CDC Calvi et CDC Bastia), et de comparer celles-ci à deux céréales courantes (l'avoine et le blé).

Globalement, les protéines de l'alpiste des Canaries ont un profil d'acides aminés bien équilibré et les graines ont une teneur élevée en tryptophane (2.4-2.6 g/100g protéine), un acide aminé essentiel habituellement déficient dans d'autres grains céréaliers, mais ont par contre une faible teneur en lysine (2.3-2.9 g/100g protéine). La qualité protéique des farines de l'alpiste des Canaries a été évaluée par deux méthodes de mesure de la qualité des protéines; l'indice chimique corrigé de la digestibilité (PDCAAS) et l'indice de digestibilité des acides aminés indispensables (DIAAS). L'indice PDCAAS a été calculé à partir de la digestibilité globale (digestibilité protéique in vitro) des protéines de l'alpiste des Canaries et des scores de 25.4-32.4% ont été obtenus pour les nourrissons (0-6 mois). Ces valeurs étaient comparables à celles du blé (26%), mais plus faibles que celles de l'avoine (47%). Les valeurs minimales et maximales de digestibilité totale iléale *in vitro* des acides aminés et les scores DIAAS ont été déterminés en mesurant la bioaccessibilité de chaque acide aminé individuel. La digestibilité iléale totale des quatre variétés d'alpiste des Canaries était située entre des valeurs minimales de 25 à 29% jusqu'à des valeurs maximales de 65 à 71%, avec des valeurs de digestibilité plus élevées pour les variétés de couleurs jaune. Ces valeurs étaient comparables à celles du blé (22 à 73%) et de l'avoine (32 à 69%). Les valeurs de l'indice DIAAS ont montré que la lysine était l'acide aminé essentiel limitant, avec des scores *in vitro* de l'indice DIAAS de 7.9-9.5%, 9.7-11.5% et de 11.5-13.7% pour les nourrissons (0-6 mois), les enfants d'âge préscolaire (6 mois-3 ans), et les enfants plus âgés/adultes, respectivement. Ces résultats indiquent que même si les graines d'alpiste des Canaries ont une teneur élevée en

tryptophane, la quantité et la digestibilité de l'acide aminé limitant, soit la lysine, demeure faible. Ainsi, tout comme pour les autres grains céréaliers, elles devront être associées à d'autres sources de protéines afin de rencontrer l'apport nutritionnel recommandé en acides aminés.

L'activité inhibitrice de la trypsine (AIT), la teneur en acide phytique et le contenu en polyphénols totaux (TPC) ont été déterminés dans les farines et les isolats de l'alpiste des Canaries afin d'évaluer les facteurs antinutritionnels des graines, et il n'y avait pas de différence significative entre les cultivars à l'étude. L'AIT dans les farines d'alpiste des Canaries (0.12-0.16 mg/g) était comparable à l'activité dans les farines d'avoine (0.14 mg/g) et de blé (0.11 mg/g). La teneur en acide phytique dans les graines d'alpiste des Canaries (12 mg/g farine) était significativement plus élevée que celle de l'avoine (6 mg/g farine) et du blé (3 mg/g farine), mais comparable aux teneurs retrouvées dans d'autres céréales à grains et légumineuses. Le TPC était plus élevé dans les isolats d'alpiste des Canaries que dans les farines, une observation s'expliquant probablement par la présence plus importante de composés phénoliques libres et de complexes polyphénols-protéines (polyphénols liés) dans les isolats d'alpiste des Canaries.

Finalement, les effets bénéfiques pour la santé des quatre variétés d'alpiste des Canaries ont aussi été évalués et comparés à ceux de l'avoine et du blé par la mesure du potentiel antioxydant (ORAC, DPPH, ABTS), par le pouvoir chélateur du fer ($Fe^{2+)}$ ainsi que par les activités hypertensive (ACE) et antidiabétique (DPP-IV) des digestats de farine de céréale après ultrafiltration avec une membrane de poids moléculaire nominal de 3000 kilodaltons (3K MWCO) afin d'obtenir de petits peptides ayant une activité biologique. Entre les cultivars d'alpiste des Canaries, il n'y avait pas de différence significative entre les graines de couleur jaune et celle de couleur brune à l'exception des tests de DPPH et de Fe^{2+}, où les variétés brunes ont démontré une activité plus élevée (77.96-96.38 μg/mL et 0.73-0.96 mg/mL pour les tests de DPPH et de Fe^{2+}, respectivement) en comparaison aux cultivars de couleur jaune (638.75-1043.55 μg/mL et 1.55-1.69 mg/mL pour les tests de DPPH et de Fe^{2+}, respectivement), montrant ainsi que les cultivars de couleur brune pourraient avoir une meilleure capacité de neutralisation des radicaux libres par rapport aux cultivars de couleur jaune. En comparaison avec l'avoine et le blé, l'activité antioxydante des protéines de l'alpiste des Canaries était équivalente ou supérieure pour chaque méthode évaluée. Pour la méthode ORAC, les graines d'alpiste des Canaries, l'avoine et le blé avaient une activité

de 1.77-1.99 µmol TE/mg protéine, 1.31 µmol TE/mg protéine et 1.54 µmol TE/mg protéine, respectivement. Pour la méthode ABTS, les protéines des graines de l'alpiste des Canaries jaune C09052 avaient une valeur de l'IC_{50} de 117.85 µg/mL, ce qui n'est pas significativement différent du blé (107.84 µg/mL) et plus faible que l'avoine (176.01 µg/mL). Les peptides des graines d'alpiste des Canaries des cultivars C09052, C05041 et Calvi avaient une activité des inhibiteurs de l'angiotensine (ACE) exceptionnelle avec des valeurs IC_{50} de 333 µg/mL, 405 µg/mL et 322 µg/mL, respectivement, en comparaison avec l'avoine (570 µg/mL) et le blé (781 µg/mL). Les valeurs IC_{50} pour l'essai d'inhibition de la DPP-IV étaient de 1.0 mg/mL, 1.1 mg/mL et 1.4 mg/mL pour les peptides des cultivars C09052, C05041 et Bastia respectivement, des valeurs comparables à celles du blé (1.0 mg/mL).

Afin d'identifier des peptides ayant un effet antihypertenseur potentiel, le digestat du cultivar C09052 ultrafiltré sur une membrane de 3K MWCO a été purifié par chromatographie d'exclusion stérique, ce qui a montré trois pics, et ces trois pics ont été récoltés et évalués à nouveau pour l'activité d'inhibition de l'angiotensine (ACE). Les fractions 1 et 2 présentaient une activité ACE similaire (32% and 29%, respectivement) à une concentration en peptides de 350 µg/mL, cependant, la fraction 1 a été choisie afin d'être davantage purifiée puisque celle-ci avait la teneur en protéine la plus élevée et une valeur d'inhibition ACE de 82% à une concentration protéique de 3 mg/mL. L'analyse par spectrométrie de masse (MS) de la fraction du cultivar C09052 a identifié 46 peptides appartenant à 18 protéines de la sous–famille des Pooideae. Parmi les 18 protéines identifiées, 14 étaient homologues aux protéines de l'orge, tandis que les autres étaient homologues au blé (3) et à l'égilope cylindrique (1). L'analyse *in silico* des peptides a démontré que l'ensemble des 46 peptides présentaient des activités potentielles d'inhibition de l'angiotensine (ACE) et d'inhibition de l'activité de la DPP-IV, alors que 20 avaient un pouvoir antioxydant potentiel, lequel a été validé à partir des études *in vitro*. Cependant, d'autres peptides pourraient avoir un potentiel d'activité hypotensive, anti-amnésique, immunostimulant, opioïde et neurologique ce qui reste encore à confirmer. Les résultats indiquent que les graines d'alpiste des Canaries ont une teneur élevée en protéine digestible et ont potentiellement des effets exceptionnellement bénéfiques sur la santé, particulièrement contre les maladies cardiovasculaires, et devraient être considérées comme un aliment ou un ingrédient fonctionnel.

CONTRIBUTION OF AUTHORS

This thesis consists of the three following chapters:

Chapter I provides a comprehensive review of the literature on canaryseed proteins, including their composition, nutritional properties, and bioactive properties. Methodology for protein isolation and characterization were reviewed as well as food industry applications for hairless canaryseeds. Part of this chapter has been published as a review paper in the journal Nutrients (Mason, E., L'Hocine, L., Achouri, A., & Karboune, S. (2018). Hairless Canaryseed: A Novel Cereal with Health Promoting Potential. *Nutrients, 10*(9), 1327.)

Chapter II evaluates the chemical profiles of hairless canaryseed proteins as well as the nutritional quality of the seed proteins.

Chapter III assesses the potential beneficial effects of hairless canaryseed proteins on human health through the screening of bioactivities.

Connecting statements are included to provide a summary of each chapter and to introduce the subsequent chapter.

Emily Mason, the author, was responsible for the experimental work and the writing of the thesis.

Dr. Lamia L'Hocine, the MSc student's principal co-supervisor, guided all the research and critically revised the thesis prior to its submission.

Dr. Salwa Karboune, the MSc student's supervisor, critically revised the thesis prior to its submission.

Dr. Allaoua Achouri, the MSc student's collaborator, provided scientific guidance, laboratory assistance, and critically revised the thesis prior to its submission.

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LIST OF ABBREVIATIONS

ABTS:	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ACE:	Angiotensin converting enzyme
ANOVA:	Analysis of variance
DH:	Degree of hydrolysis
DIAAS:	Digestible indispensable amino acid score
DPPH :	2,2-diphenyl-1-picrylhydrazyl
DPP-IV:	Dipeptidyl peptidase-4
HMW:	High molecular weight
HPLC:	High performance liquid chromatography
HCl:	Hydrochloric acid
IVPD:	*In vitro* protein digestibility
LMW:	Low molecular weight
MS:	Mass spectroscopy
MW:	Molecular weight
MWCO:	Molecular weight cutoff
NaOH:	Sodium hydroxide
ORAC:	Oxygen radical absorption capacity
PDCAAS:	Protein digestible corrected amino acid score
SEC:	Size exclusion chromatography
SDS-PAGE:	Sodium dodecyl sulphate polyacrylamide
TID:	True ileal digestibility

INTRODUCTION

Due to the growing global demand for protein, there will be increased need for good sources of high quality plant protein for food uses. Discovering new sources of plant food proteins, besides the conventional ones (ex. wheat, soybean, pulses) provide promising opportunities in terms of environmental sustainability, economic profitability, and nutritional advantages. The consumption of different plant proteins can ensure an adequate supply of essential amino acids for meeting human physiological requirements. Opportunities are endless for using plant proteins as a functional ingredient in formulated food products to increase nutritional quality, as well as to provide desirable health promoting effects.

In 2015, Health Canada and the Food and Drug Administration (FDA) gave GRAS (Generally Regarded as Safe) status to glabrous canaryseeds (*Phalaris canariensis* L.) and approved them as a novel food product. Previously, the seeds had limited use as birdseed, because they were lined with fine, hair-like silica fibers, that were deemed hazardous to human health (Bhatt, Coombs, & O'Neill, 1984). The Crop Development Center at the University of Saskatchewan in Canada developed a new 'hairless' or 'glabrous' canaryseed from the hairy variety, which is safe for human consumption. Caged and wild birds have consumed hairy canaryseed for centuries, alone or mixed with other grains, such as millet, sunflower seeds, and flaxseeds (Cogliatti, 2012). Nonetheless, very little research regarding nutritional properties and health benefits have been conducted on the seeds, since they had no nutritional value for humans. The new glabrous canaryseed, regarded as a true cereal grain, has tremendous potential in the food industry, due to its unique properties and characteristics. Canaryseed groats contain approximately 61% (w/w) starch, 20% (w/w) protein, 8% (w/w) crude fat and 7% (w/w) total dietary fiber (Abdel-Aal, Hucl, Patterson, & Gray, 2010; Abdel-Aal, Hucl, & Sosulski, 1997b). Compared to other cereal grains in the same family such as oats (10-13% (w/w)) (Biel, Bobko, & Maciorowski, 2009), barley (13-16% (w/w) (Asare et al., 2011), wheat (13% (w/w)) (Belderok, Mesdag, & Donner, 2000), and rye (11-16% (w/w)) (Nyström et al., 2008), they are extremely high in protein. Some studies have shown the potential of hairy canaryseed proteins to produce bioactive peptides with beneficial health effects, such as antioxidant, antihypertensive, and antidiabetic activity (Estrada-Salas, Montero-Moran, Martinez-Cuevas, Gonzalez, & Barba de la Rosa, 2014; Valverde, Orona-Tamayo, Nieto-Rendón, & Paredes-López, 2017). However, no studies have evaluated the nutritional quality of canaryseeds

and none have determined the bioactive properties of proteins from the Canadian produced hairless canaryseeds.

The overall objective of the study was to investigate the nutritional quality and bioactive properties of proteins from selected hairless canaryseed varieties. This was achieved by the following specific objectives:

1. Determining the protein profiles, digestibility, amino acid profiles, and anti-nutritional components (phytic acid, trypsin inhibitor, polyphenol content) of canaryseed flour and isolates.

2. Assessing the potential human health positive effects of canaryseed proteins and their hydrolysates through the screening of bioactivities (antioxidant, anti-hypertensive, anti-diabetic, chelation activity) in canaryseed protein hydrolysates.

3. Isolating and identifying the specific canaryseed peptides responsible for the selected bioactivity.

CHAPTER I

LITERATURE REVIEW

1.1. Canaryseed Development and Production

Hairy canaryseeds, like most grass species, have seeds lined with hair-like silica fibers that were found to be causing lung damage and even esophageal cancer (Bhatt et al., 1984). Hucl et al. (2001)**,** from the University of Saskatchewan's Crop Development Center (CDC), developed a hairless canaryseed containing no fine hair to decrease skin irritations and potential cancer development by farmers involved in harvesting the crop. The new silica-free or glabrous species was not only safe for individuals manipulating the seeds but could also be safely consumed and utilized by the food industry as a new cereal grain. Using mutagenesis and breeding techniques, four hairless brown varieties have been created from the original seeds: CDC Maria, CDC Togo, CDC Bastia, and CDC Calvi (Canaryseed Development Comission of Saskatchewan, 2016). In addition, yellow colored cultivars of the glabrous seeds were developed, which are thought to be more aesthetically pleasing for food use as compared to the brown colored cultivar (Matus-Cádiz, Hucl, & Vandenberg, 2003) (**Figure 1.1**).

Figure 1.1. (**a**) Yellow (C09052) and (**b**) brown (CDC Calvi) cultivars of glabrous canaryseeds (*Phalaris canariensis* L.)

Glabrous or hairless canaryseeds are members of the family *Poaceae*, along with other prevalent cereal grains, such as wheat, oat, barley, and rye (Health Canada, 2016). The groats (hulled kernels of the grain) have an elliptical shape and measure approximately 4 mm in length and 2 mm in width, comparable to flaxseeds and sesame seeds (Abdel-Aal et al., 1997b). The seeds are

harvested from canarygrass; a grassy, herbaceous plant that grows optimally in any regions where wheat is cultivated, with growth and production cycles comparable to other winter cereals, such as spring wheat and oat. In addition, very few weeds, diseases, and insects have been reported in canarygrass, which would decrease canaryseed yields (Cogliatti, 2012). The Western provinces of Canada (Saskatchewan, Manitoba, and Alberta) cultivate the majority of canaryseeds in Canada, which produces over 80% of canaryseed exports worldwide, followed by Argentina and Hungary, mainly to countries with high proportions of caged birds (Canaryseed Development Comission of Saskatchewan, 2016). On average, about 300,000 acres of canaryseed are grown in the province of Saskatchewan every year with yields ranging between 800 to 1400 pounds per acre, representing more than 95 percent of Canadian acreage and production (Canaryseed Development Comission of Saskatchewan, 2016), and which is still comprised of only the hairy varieties. The higher yield of the older hairy varieties has limited the uptake by producers of the glabrous varieties. The brown variety CDC Calvi has the highest yield of the developed glabrous varieties (Canaryseed Development Comission of Saskatchewan, 2016). Relative to the yield of the hairless CDC Bastia cultivar, the yield of the hairless CDC Calvi was 6% higher, whereas the yield for the hairy Keet cultivar was 26% higher (Saskatchewan Seed Growers Association, 2019). The approval of glabrous canaryseed varieties for human consumption opens up new opportunities in food applications instead of the sole use as birdseed, which is expected to create more demand for the production of canaryseed.

1.2. Canaryseed Proteins

1.2.1. Protein Characteristics

Canaryseeds have been compared extensively with wheat and other cereals in the same family, and one of their distinguishing factors is their higher protein content (**Table 1.1**), which ranges between 20–23%, in comparison to 13% for wheat. Canaryseed proteins, along with other cereal proteins, can be separated into four fractions based on their solubility: prolamins, glutelins, globulins and albumins (Koehler & Wieser, 2013). The prolamin and glutelin fractions, which are principally storage proteins, are more abundant in canaryseeds than wheat, however, the globulin and albumin fractions represent the lowest amount of overall protein (Abdel-Aal et al., 2010; Abdel-Aal et al., 1997b), which is possibly indicative of a reduced amount of anti-nutritional factors, such as enzyme inhibitors (Abdel-Aal et al., 1997b). Regardless of the variations in protein

fraction proportions, wheat remains unique because of its ability to make dough, due to the exceptional viscoelastic properties of its proteins (Koehler & Wieser, 2013). Nonetheless, to date, no published data is available on the breadmaking potential of 100% canary flour, although Abdel-Aal, Hucl, Shea Miller, Patterson, and Gray (2011) reported that replacement of up to 25% of wheat flour with canaryseed flour in bread had no significant effects on bread quality and loaf volume, except for crumb color.

Table 1.1. Protein comparison between canaryseed and other cereals.

Cereal Variety	% Protein (Dry Basis)	Reference
Canaryseed	20–23%	Abdel-Aal, Hucl, Shea Miller, et al. (2011); Abdel-Aal et al. (1997b)
Wheat	13%	Belderok et al. (2000)
Oat	10–13%	Biel et al. (2009)
Barley	13–16%	Asare et al. (2011)
Rye	11–16%	Nyström et al. (2008)
Millet	8.5–15%	Abdalla, El Tinay, Mohamed, and Abdalla (1998)

A key trait of canaryseeds is their possible lack of gluten-like proteins, which elicit an allergic reaction known as coeliac disease in some sensitive individuals when they consume gluten-containing cereals, such as wheat, barley, and rye (Arendt & Zannini, 2013; Tatham & Shewry, 2008). Gluten is a complex mixture of proteins called prolamins, which play key roles in conveying dough viscosity/elasticity. Wheat prolamins are termed gliadins and glutenins, barley prolamins are hordeins, and those from rye secalin. A common characteristic of these proteins is the presence of multiple proline and glutamine residues, making them resistant to gastrointestinal digestion and more exposed to deamination by tissue transglutaminase (Comino et al., 2013). In a recent study conducted by Boye et al. (2013) to establish the safety of canaryseeds for human consumption from a food allergy perspective, glabrous canaryseeds were analyzed using three separate techniques (ELISA, mass spectroscopy, and Western blotting) which all yielded negative results for gluten, indicating the cereal is an excellent alternative for individuals with coeliac disease. Although canaryseeds do not contain gluten and may be represented as gluten-free, canaryseeds do however contain a newly reported allergen named granule-bound starch synthase (GBSS), which is present in rice and maize (Krishnan & Chen, 2013), and which cross-reacted with sera from wheat sensitive/allergic individuals (Boye et al., 2013). GBSS was simultaneously identified

through mass spectroscopy analysis in several cereals (wheat, oat, sorghum, millet, teff, quinoa, buckwheat) (Boye et al., 2013). As such, Health Canada has deemed it inappropriate for canaryseed, or food containing canaryseed, to be labelled as "wheat-free". Health Canada also requires canaryseed and foods containing canaryseed to be labelled with a statement to the effect that the product "may not be suitable for people with wheat allergy", provided the food does not also contain wheat as an ingredient (2016).

The amino acid profile of canaryseeds (**Table 1.2**) remains unique, due to its high content of tryptophan, an essential amino acid, which is usually lacking in most cereal grains. Abdel-Aal et al. (1997b) reported a higher tryptophan content in the Keet cultivar of hairy canaryseed proteins (2.8 g/100 g of protein) as compared to wheat (1.2 g/100 g) and casein (1.0 g /100 g) protein, as well as higher amounts of essential amino acids phenylalanine, leucine, and isoleucine as compared to wheat. Similarly to other cereals, canaryseeds are deficient in essential amino acids lysine, threonine, and methionine, but possess comparable levels to wheat (Abdel-Aal et al., 1997b). Glabrous canaryseeds would make an excellent addition to other cereal grain and legume products to ensure consumers meet the recommended dietary intake of essential amino acids. In addition, canaryseeds contain high amounts of glutamic acid. Glutamic acid is the most abundant amino acid in the brain, which plays significant roles in synaptic activity, memory, and learning, also, it was reported that changes in glutamic acid metabolism and regulation in the brain leads to the development of Alzheimer's disease (Esposito et al., 2013). Moreover, high content of glutamic acid in the seeds could indicate the presence of high gamma-aminobutyric acid (GABA), a functional compound produced in plants primarily by the decarboxylation of L-glutamic acid, which has several health promoting properties, including reducing blood pressure and blood cholesterol, anticancer, and anti-obesity activity (Zhang et al., 2014). GABA concentration, however, has not been directly determined in canaryseeds.

Table 1.2. Amino acid comparison between canaryseeds and other cereals

Amino Acid	Canaryseed (g/100 g protein)	Wheat (g/100 g protein)	Oat (g/16 g N or g/100 g protein)	Barley (g/100 g protein)	Millet (g/100 g protein)
Histidine	1.6	2.1	1.74	2.4	2.4
Isoleucine	3.9	2.8	2.32	3.5	4.4
leucine	7.6	5.3	5.26	7.7	11.5
lysine	2.6	1.9	2.73	3.9	2.8
Methionine	1.9	1.4	2.5	2.1	2.3
Phenylalanine	6.5	5.4	5.3	5.7	5.6
Threonine	2.7	2.8	2.46	3.9	4.2
Tryptophan	2.8	1.2	1.15	N/A	N/A
Valine	4.8	3.8	3.2	5.4	6.0
Alanine	4.5	3	3.59	4.4	8.8
Arginine	6.4	5.1	5.79	4.6	3.9
Aspartic acid	4.4	4.4	7.37	6.3	8.7
Cystine	2.5	2.3	2.74	1.4	1.2
Glutamic acid	26	33	19.12	28.1	22
Glycine	3.1	3.8	3.81	4.7	3.2
Proline	6.2	8.6	4.54	12.7	6.8
Serine	4.5	4.3	3.86	4.9	5.3
Tyrosine	3.6	3.5	1.82	2.8	2.4
Reference	Canaryseed Development Comission of Saskatchewan (2016)	Abdel-Aal et al. (1997b)	Biel et al. (2009); Pomeranz, Robbins, and Briggle (1971)	Ejeta, Hassen, and Mertz (1987)	Ejeta et al. (1987)

N/A = not available.

1.2.2. Health Promoting Properties of Canaryseed Proteins

Chronic disease is of major global concern today and includes diseases such as cardiovascular disease, cancer, and diabetes, which are leading causes of death worldwide (WHO, 2014). A balance between an active lifestyle and good eating habits are critical in the long term to prevent and combat chronic diseases. Beyond their physiological and metabolic effects, dietary proteins are intrinsically associated with health improvement and prevention of nutrition related chronic diseases (ex. cardiovascular diseases, hypertension, cancer, oxidative damage, etc.), and which need to be also considered when assessing protein quality (FAO, 2013*)*. This is particularly relevant as consumers are increasingly looking to natural food sources to help prevent specific diseases or illnesses. Some parts of world, such as Mexico, have utilized hairy canaryseeds as a traditional folk medicine for treatment of diabetes and hypertension for centuries (Estrada-Salas et al., 2014). However, because of the presence of toxic hairs, the seeds were not consumed directly but soaked in water, drained, dried and then processed to make canaryseed “milk”, which can be safely consumed.

The health benefits associated with drinking canaryseed "milk" were found to be largely related to the bioactive peptides produced during digestion. Bioactive peptides are small, specific and active protein fragments released from food proteins by proteolytic enzymes during protein digestion, which positively affect an individual's overall health (Patil, Mandal, Tomar, & Anand, 2015; Velarde-Salcedo et al., 2013). Bioactive peptides have been reported from many food sources, such as fish and crustaceans, dairy products (milk, cheese, yoghurt), eggs, meat, and vegetal sources (grains, legumes, seeds) (Sánchez & Vázquez, 2017). Depending on the amino acid composition and sequence, bioactive peptides possess different types of activity, including antioxidant, antimicrobial, antihypertensive, radical scavenging, anti-inflammatory, opioid, immunomodulatory, anticancer, chelation activity, and antidiabetic activity among others (Kitts & Weiler, 2003; Sánchez & Vázquez, 2017). In recent years, a lot of research has been focused on the ability of plant proteins from cereals, nuts, and pulses to generate bioactive peptides with measurable health benefits. Thus far, very little research has been conducted on the bioactivity of glabrous canaryseeds. Research on canaryseed proteins and peptide bioactivity has been tested exclusively *in vitro* to date, with no animal or human subjects, and predominantly using the hairy varieties. Although the nutrient profile between hairless and hairy canaryseeds are very similar, further investigation into hairless canaryseed bioactivity is required and ongoing.

1.2.2.1. Antidiabetic Activity

Dipeptidyl peptidase IV (DPP-IV) enzyme plays a major role in the development of hyperglycemia in individuals with type II diabetes, because it inactivates incretin hormones, thereby increasing blood glucose levels (Patil et al., 2015). Incretin-based therapy is a common treatment for type II diabetes, but it remains less effective, because the half-life of the hormone is very short, due to inactivation by DPP-IV enzymes (Velarde-Salcedo et al., 2013). DPP-IV inhibitors improve the efficiency of incretin-based therapy by inactivating the enzyme and increasing the activity of the incretin hormones. Estrada-Salas et al. (2014) found that peptides produced by *in vitro* gastrointestinal digestion of canaryseed milk using pepsin, trypsin, and pancreatin, displayed inhibitory activity in a dose dependent manner against DPP-IV enzyme from porcine kidney. In addition, an in vivo and *in vitro* study have demonstrated an anti-obesity effect of a lipid extract (produced by hexane extraction) of hairless canaryseed (Perez Gutierrez, Madrigales Ahuatzi, & Cruz Victoria, 2016; Perez Gutierrez et al., 2014). The anti-obesity effect of canaryseeds in

addition to the inhibitory action of DPP-IV by canaryseed peptides would make this grain an excellent nutritional approach to improve the efficiency of synthetic drugs, since food derived DPP-IV inhibitors lack the potency of synthetic drugs inhibitors (Power, Nongonierma, Jakeman, & FitzGerald, 2014). Further characterization of the DPP-IV inhibitor peptides in canaryseeds remains necessary to establish their antidiabetic effects and capacity.

1.2.2.2. Antihypertensive Activity

The angiotensin-I converting enzyme (ACE) increases blood pressure and causes hypertension in inclined individuals. ACE converts the inactive angiotensin-I into angiotensin-II (a very powerful vasoconstrictor) and inactivates bradykinin (a vasodilator), which both lead to the direct increase in blood pressure (Chen, Wang, Zhong, Wu, & Xia, 2012; Estrada-Salas et al., 2014). Synthetic ACE inhibitors are produced as a treatment for hypertension, and although effective, the synthetic inhibitors cause side effects, including coughing, food taste alterations, rashes and reduced efficiency when used in the long term (Chen et al., 2012). Food sources of ACE inhibitors are of great interest, since individuals with hypertension can consume them as part of a healthy diet to reduce their high blood pressure (Iwaniak, Minkiewicz, & Darewicz, 2014).

Recent research studies revealed that canaryseed bioactive peptides have great potential to lower blood pressure through the inhibition of the ACE enzyme. Estrada-Salas et al. (2014) showed that canaryseed flour proteins digested *in vitro* using pepsin, trypsin, and pancreatin, exhibited a maximum percent inhibition against the ACE enzyme of 73.5% and an IC_{50} value of 322 µg/mL, which was similar to the IC_{50} value of other peptides from chickpea, pea, soybean, wheat gliadin, and sardine muscle. Undigested canaryseed proteins had significantly lower inhibition activity, meaning the antihypertensive bioactive peptides are produced upon protein digestion (Estrada-Salas et al., 2014). Similarly, Valverde et al. (2017) found that canaryseed flour proteins from the prolamin fraction had the highest inhibition activity against the ACE enzyme, with an IC_{50} value of 217.4 µg/mL, after *in vitro* digestion with pepsin and pancreatin. They further identified five peptides by mass spectroscopy (LSLGT, TDQPAG, QQLQT, FEPLQLA, and KPQLYQPF) in the digested prolamin fraction that had both ACE and DPP-IV inhibition activity. Additionally, Passos et al. (2012) administered to rats an aqueous extract of canaryseeds (obtained by soaking the seeds in water), which successfully reduced systolic blood pressure in the animals while having

no renal or toxicological effects. All these studies demonstrated the potential positive effect of canaryseeds on cardiovascular disease control.

1.2.2.3. Antioxidant Activity

The antioxidant potential of plants has received a great deal of attention, because increased oxidative stress has been identified as a major causative factor in the development and progression of several life threatening diseases, including neurodegenerative and cardiovascular diseases. Free radical species that are generated in the body by various endogenous systems cause extensive damage to body tissues by destroying cell membrane structure, modifying enzyme activity, and changing DNA leading to cancer development (Chanput, Theerakulkait, & Nakai, 2009). In this regard, bioactive peptides of canaryseeds demonstrated antioxidant activity by reacting with free radical species, thereby preventing tissue damage and decay. Valverde et al. (2017) used two *in vitro* radical scavenging assays on digested canaryseed protein fractions and found that the prolamins had the overall highest antioxidant activity. Mass spectroscopy analysis of the digested prolamin fraction identified five peptides, of which only one had antioxidant activity (KPQLYQPF). Protein fractions from digested canaryseeds had higher antioxidant activity in general as compared to raw flour, because the seed proteins undergo hydrolysis, increasing their antioxidant activity (Valverde et al., 2017).

1.2.2.4. Other Bioactivities

Only very limited studies have been conducted on other bioactive properties of hairy canaryseed proteins. As an example, acetylcholinesterase inhibitors are currently employed as a form of treatment for individuals with Alzheimer's disease, because they help maintain levels of acetylcholine in the brain, which is essential for nerve impulses and transmission (Malomo & Aluko, 2016). Kchaou et al. (2015) found that a methanol extract of a hairy Tunisian canaryseed variety had a percent inhibition against acetylcholinesterase enzyme of 65% at a concentration of 1 mg/mL, which was attributed predominantly to polyphenols and flavonoids in the extract. An antibacterial activity of hairy Tunisian canaryseed extracts, especially against gram-positive bacteria, was also reported by Kchaou et al. (2015). These bioactivities could possibly be the result of canaryseed peptides, as it was previously demonstrated for hemp seed protein hydrolysates, which exhibited acetylcholinesterase inhibition (Malomo & Aluko, 2016), or for other cereal proteins, such as wheat and barley, for which antibacterial activity was reported (Cavazos &

Gonzalez de Mejia, 2013). Proteins and peptides from cereal grains and legumes (wheat, barley, amaranth, oat, rye, soybean etc.) are known to have antithrombotic, immunomodulatory, and anticancer activity (Cavazos & Gonzalez de Mejia, 2013; Dia, Bringe, & de Mejia, 2014; Jeong, Jeong, Hsieh, Hernández-Ledesma, & de Lumen, 2010; Jeong et al., 2007; Jeong et al., 2009; Maldonado-Cervantes et al., 2010; Nakurte et al., 2013; Nakurte et al., 2012; Sabbione, Nardo, Añón, & Scilingo, 2016; Tapal et al., 2016; Yu, Wang, Zhang, & Fan, 2016). Bioactivities of Canadian glabrous canaryseed peptides remain largely unknown, but because of the diverse bioactivity reported in similar cereal grains from the same family, it remains highly likely that canaryseed peptides possess additional health promoting properties, which still need to be confirmed.

1.2.3. Protein Digestibility

Protein digestibility is an important parameter to consider when assessing protein quality (Sarwar Gilani, Wu Xiao, & Cockell, 2012). The health advantages of glabrous canaryseeds depends on their digestibility and bioavailability. Several in vivo studies indicated excellent protein digestibility of canaryseed in animals. Broiler chickens fed hairless canaryseed groats and hulled seeds exhibited similar ileal protein digestibility as other feed components, including corn, wheat, sorghum, and peas (Newkirk, Ram, Hucl, Patterson, & Classen, 2011). The same study showed high apparent ileal digestibility of amino acids cysteine (86%), phenylalanine (88%), and tryptophan (93%). Furthermore, weight gain between broiler chickens fed with wheat and chickens fed with canaryseeds were similar.

Later, Classen et al. (2014) fed broiler chickens yellow glabrous canaryseeds and glabrous brown seeds and determined the seeds were equivalent in terms of feeding value. Magnuson et al. (2014) found no evidence of toxicity in rats when fed glabrous canaryseeds for a 90 day study and, furthermore, rat diets supplemented with 50% hulled and dehulled glabrous canaryseeds were comparable in terms of growth, hematology, and clinical parameters as rats with diets supplemented with 50% wheat. Thacker (2003) showed that crude protein digestibility in pigs increased linearly with increasing proportions of canaryseeds in their diets. Moreover, he found that a pig's diet containing 25% canaryseeds promoted the highest growth rates in the pigs with a crude protein digestibility of approximately 78%. All these studies indicate that hairless

canaryseeds make an excellent addition or supplement to conventional animal feed, as it promotes growth, but also enhances protein digestibility.

For human digestibility of canaryseed proteins, no in vivo study has been reported in the literature despite several *in vitro* studies that have been carried out to mimic human protein digestibility of canaryseeds under gastrointestinal conditions. Abdel-Aal et al. (1997b) used a multienzyme approach with trypsin, chymotrypsin, and peptidase and established an *in vitro* protein digestibility of 84% in hairy canaryseeds. Interestingly, Rajamohamed, Aryee, Hucl, Patterson, and Boye (2013) compared the effects of thermal treatment on canaryseed protein digestibility. The *in vitro* protein digestibility of raw, roasted, and boiled glabrous canaryseed flours was determined by gastric, duodenal, and sequential gastric-duodenal methods. The sequential gastric-duodenal method was most effective at digesting the proteins and, overall, thermal processing enhanced protein digestion. As a cereal, canaryseeds can be used in various forms, such as a whole groat, whole meal, or whole grain flour in several applications, such as a cereal, in pasta, and in baking to make products, such as bread, muffins, and cereal grain bars (Health Canada, 2016). Since thermal processing increased protein digestibility, the heating and thermal processing of canaryseeds in the development and production of baked goods will contribute to its improved nutritive value.

1.3. Other Canaryseed Components

1.3.1. Starch

Canaryseeds are comprised of 61% starch, which serves as the main energy store in the plants (Luallen, 2004). Canaryseed starch granules are small and polygonal in shape with reported sizes ranging from 0.5 to 7.5 μm (Abdel-Aal, Hucl, & Sosulski, 1997a; Goering & Schuh, 1967; Irani, Abdel-Aal, Razavi, Hucl, & Patterson, 2017). X-ray diffraction patterns of the starch exhibit the traits of an A-type starch, characteristic of most cereal grains (Abdel-Aal et al., 1997a; Irani et al., 2017). Starch is comprised of two glucose polymers; linear amylose and branched amylopectin. Abdel-Aal et al. (1997a) reported a range of amylose content in hairy canaryseeds of 16.2–19.5% of total starch and Irani et al. (2017) determined an average of 23.6% and 22.5% for a brown and yellow hairless cultivar, respectively, which is typical of most starches (Lovegrove et al., 2017). The amylose to amylopectin ratio is indicative of its digestibility because, in general, high amylose

starches are harder to digest whereas waxy starches are more readily digested (Lehmann & Robin, 2007).

Starches of the yellow and brown cultivars of glabrous canaryseeds have been extensively compared. Overall, their properties appear similar, but some researchers report differences among the two colored cultivars. Irani, Razavi, Abdel-Aal el, Hucl, and Patterson (2016) observed differences in starch granule shape between a yellow and brown hairless canaryseed variety (CO5041 and CDC Maria, respectively) in dilute solution. The yellow cultivar starch showed both spherical and ellipsoidal structure, whereas the brown cultivar and wheat starch showed only ellipsoidal structure. An investigation of the rheological properties of canaryseed starches revealed C05041 starch was less sensitive to temperature and with increasing concentration, displayed higher thixotrophy and pseudoplastic behavior as compared to CDC Maria starch (Irani, Razavi, Abdel-Aal, & Taghizadeh, 2016).

Retrogradation, the process of heating starch in the presence of water followed by cooling, results in a critical change in the ordered amylose/amylopectin structure, and hence, in changes to its physiochemical and functional properties. Although starch retrogradation is mostly considered an undesirable phenomenon, such as its involvement in the staling of bread and sensory and quality loss in high starch foods over time, it also plays a nutritionally important role (Wang, Li, Copeland, Niu, & Wang, 2015). The retrogradation process can produce resistant starch (also known as resistant starch 3 (RS3)), because the amylose and amylopectin structures become more compact and therefore resistant to enzymatic hydrolysis. Resistant starch is characterized as starch that remains mostly undigested by enzymes in the small intestine, thereby passing into the large intestine where it undergoes fermentation by the colons microflora (Masatcioglu, Sumer, & Koksel, 2017). There is no rapid release of glucose into the bloodstream and the starch acts like a prebiotic for the gut microflora. Canaryseed starch demonstrated greater rates of hydrolysis in the presence of pancreatic α-amylase as compared to wheat starch, which could be due to its small granule size and relatively low amylose composition (Abdel-Aal et al., 1997a). Nonetheless, canaryseed starch also had a higher tendency for retrogradation, potentially forming RS3, a nutritionally valuable starch. Resistant starches promote probiotic bacteria, lower the glycemic index of foods, have hypocholesterolemic effects, reduce gallstone formation, improve mineral absorption, have high satiety, and aid in weight management (Raigond, Ezekiel, & Raigond, 2015).

Overall, canaryseed starch does possess unique characteristics as compared to wheat starch. Its properties in dilute solution are similar to that of wheat and demonstrate a potential use as a thickener or stabilizer in food products (Irani et al., 2017). Canaryseed starches, although easily digestible, have a higher tendency to retrograde into RS3, which could make them more available for digestion by the colon's microflora (Abdel-Aal et al., 1997a; Irani et al., 2017). This functionality, however, would need to be further investigated.

1.3.2. Fiber

Besides starch and protein, fiber represents a minor component of the total composition of canaryseeds. Canaryseeds consist of approximately 7% dietary fiber, considerably lower compared to other cereal grains, especially wheat, which contains double the amounts on average (Abdel-Aal, Hucl, Shea Miller, et al., 2011; Abdel-Aal et al., 1997b; Robinson, 1978). The bran portion of the grain contains more dietary fiber than the whole grain and white flour portions in both canaryseeds and wheat (Abdel-Aal, Hucl, Shea Miller, et al., 2011). Several purification steps are usually required to obtain a high purity fiber, due to high contamination with starch and protein. The extraction order also plays a role on fiber extraction purity, since the removal of starch and protein prior to fiber in an ethanol, alkaline, and water wet milling extraction technique results in a higher fiber purity (Abdel-Aal et al., 2010). Overall, canaryseeds still remain a poor source of dietary fiber compared to other grains from the same cereal family.

1.3.3. Lipids

Similarly to fiber, lipids are minor components of the seeds as compared to starch and protein. To extract oil from canaryseeds, ethanol has proved a very suitable solvent. Abdel-Aal et al. (2010) reported a crude oil content of 8.3% with an extraction efficiency of 75% when the ethanol extraction step was repeated three times. Oil from canaryseed would be produced primarily as a byproduct, since its removal is necessary to obtain purified starch and protein fractions from the seeds. The crude fat content in glabrous canaryseed is high as compared to other cereal grains and the fatty acids are largely unsaturated (**Table 1.3**). Canaryseeds lipids consist of 54% linoleic, 29% oleic, 11% palmitic, 2.4% linolenic, and 1% stearic acids (Canaryseed Development Comission of Saskatchewan, 2016). In comparison, wheat grain lipids consist of 62% linoleic, 16% oleic, 17% palmitic, 4% linolenic, and 1% stearic acids (Abdel-Aal et al., 1997b). Diets high in saturated fatty

acids have been correlated with increased incidence of chronic heart disease, whereas diets higher in monounsaturated fatty acids (oleic acid) and especially polyunsaturated fatty acids (linoleic acid, linolenic acid) promote cardiovascular health, neurological function, and improved immune response (American Dietetic Association and Dietitians of Canada, 2007). Canaryseeds contain high amounts of unsaturated fatty acids, which is advantageous for a healthy diet, but could make them prone to oxidation and rancidity. However, the presence of certain antioxidants in canaryseed oil, such as caffeic acid esters, could potentially reduce these detrimental effects (Takagi & Iida, 1980). Furthermore, Ben Salah et al. (2018) reported health promoting activity in canaryseed oil, produced from a hairy Tunisian canaryseed variety, which demonstrated antioxidant, antibacterial, and antiacetylcholinesterase activity, which was largely attributed to the high total polyphenol content in the oil.

Table 1.3. Crude fat and lipid composition of canaryseed and other cereal grains.

	Canaryseed	**Wheat**	**Oat**	**Barley**	**Millet**
Crude Fat (% dry basis)	6.7	4.4	4.79	3.4	4.7
Reference	Canaryseed Development Comission of Saskatchewan (2016)	Abdel-Aal et al. (1997b)	Biel et al. (2009)	Haard (1999)	Haard (1999)
FA (% total lipids)					
Palmitic (C16)	11.38	16.6	19.2	23.0	7.42
Stearic (C18)	1.22	0.8	1.46	1.12	6.84
Oleic (C18:1)	29.1	16.2	30.8	11.4	16.11
Linoleic (C18:2)	53.39	62.1	46.4	58.8	66.68
Linolenic (C18:3)	2.42	4.0	2.13	7.78	2.48
Reference	Canaryseed Development Comission of Saskatchewan (2016)	Abdel-Aal et al. (1997b)	Welch (1975)	Welch (1975)	Zhang et al. (2015)

1.3.4. Minerals

In terms of nutrients, glabrous canaryseeds contain several essential minerals and are higher in phosphorous, magnesium, and manganese compared to wheat, oat, barley, and millet, nonetheless, although comparable to levels present in wheat, canaryseeds contain less iron and calcium as other cereal grains (**Table 1.4**). Canaryseeds contain higher amounts of vitamin B1 (thiamine) as compared to wheat and an equivalent amount of vitamin B2 (riboflavin), but are poor in niacin (Abdel-Aal, Hucl, Shea Miller, et al., 2011).

Table 1.4. Nutrient comparison between glabrous canaryseeds and other cereal grains.

Mineral	Canaryseed (mg/100 g)	Wheat grain (mg/100 g)	Oat grain (mg/100 g)	Barley (mg/100 g)	Millet (mg/100 g)
Phosphorous	640	430	340	457	288
Magnesium	200	155	140	197	149
Manganese	6.3	5.9	5.1	0.92	0.81
Iron	6.5	4.2	4.5	12.8	20
Zinc	3.9	2.5	3.5	7.4	6.6
Calcium	40	20	62	73.6	51
Potassium	385	355	420	457	280
Reference	Abdel-Aal, Hucl, Shea Miller, et al. (2011)	Abdel-Aal, Hucl, Shea Miller, et al. (2011)	Frølich and Nyman (1988)	Ragaee, Abdel-Aal, and Noaman (2006)	Ragaee et al. (2006)

1.3.5. Phytochemicals

Phytochemicals, including polyphenols, terpenoids, and alkaloids, are naturally occurring chemicals produced by plants and, when consumed, promote positive overall health. Research indicates that glabrous canaryseeds are a good source of different types of phytochemicals. Ferulic acid is the most abundant phenolic acid in canaryseeds (Abdel-Aal, Hucl, Patterson, & Gray, 2011; Chen, Yu, Wang, Gu, & Beta, 2016; Li, Qiu, Patterson, & Beta, 2011). Ferulic acid displays a broad range of health promoting effects, including anti-inflammatory, antidiabetic, antiaging, neuroprotective, radioprotective, and hepatoprotective activity, mainly due to its strong antioxidant activity (Srinivasan, Sudheer, & Menon, 2007). Li et al. (2011) compared the total phenolic and flavonoid content in nineteen different samples of brown and yellow varieties of canaryseed groats. They found the yellow and brown colored seeds had the same flavonoid profiles and that ferulic acid was the dominating phenolic acid, followed by caffeic and coumaric acid, but

unlike their flavonoid profiles, brown cultivars had higher amounts of ferulic and caffeic acid relative to the yellow cultivars (Li et al., 2011). *O*-pentosyl isovitexin, identified as the major flavonoid in canaryseeds, displays diversified activity including anti-hypotensive, anti-inflammatory, antimicrobial, antiplatelet, and antioxidant (Li et al., 2011).

Carotenoids are another class of phytochemicals that, when ingested, perform a number of beneficial biological functions, including antioxidant activity, immune response improvement, suppression of reactive oxygen species, and lowering the risk of cardiovascular disease (Mellado-Ortega & Hornero-MÈndez, 2015). Cereals in general possess only small amounts of carotenoids as compared to fruits and vegetables, nonetheless, the pigment remains present and concentrated mostly in the bran fraction. The major carotenoids present in cereals are xanthophylls like lutein, zeaxanthin, and β-cryptoxanthin with only small amounts of carotenes (Mellado-Ortega & Hornero-MÈndez, 2015). Li and Beta (2012) evaluated the total carotenoid content in brown and yellow glabrous canaryseed cultivars and determined lutein, zeaxanthin, and β-carotene were the three major carotenoids present. Surprisingly, β-carotene was present in the largest quantities in all canaryseed varieties and far outweighed the β-carotene content of other crops, including wheat, rice, barley, and corn (Li & Beta, 2012). The carotenoid content of the brown and yellow canaryseed cultivars were relatively similar, in contrast, canaryseed flour was significantly higher in total carotenoid content (11.28 mg/kg) compared to the whole meal (9.27 mg/kg), and bran (8.32 mg/kg) fractions (Li & Beta, 2012). The results indicate canaryseed flour is a good source of carotenoids. However, carotenoids are highly sensitive molecules and changes in carotenoid stability during storage and processing still need to be addressed.

1.3.6. Anti-Nutritional Components

Like all cereal grains, canaryseeds contain certain anti-nutritional factors, including enzyme inhibitors, amylase inhibitors, phytate, and heavy metals. Enzyme inhibitors play important roles in living plants by preventing proteins and carbohydrates from degradation during growth and protection against threats by animals, insects and some microorganisms (Koehler & Wieser, 2013). Trypsin inhibitor is a type of enzyme inhibitor present in raw cereals and legumes and, upon consumption, could lead to reduced protein and nutrient digestibility and even cause growth inhibition (Abdel-Aal, Hucl, Patterson, et al., 2011). Likewise, amylase inhibitors form aggregates with amylase, resulting in a reduction of starch digestion when consumed (Thompson, 1993).

Phytate can also be considered as both nutritional and anti-nutritional component in cereals. Phytate has chelating properties and could reduce the availability of some essential minerals, like calcium, iron, and zinc, thereby decreasing their absorption in the small intestine, but on the other hand, exhibits antioxidant activity showing positive effects in cancer treatment, hypercholesterolemia, hypercalcuria, and kidney stones (Abdel-Aal, Hucl, Patterson, et al., 2011). Similarly, heavy metals present in raw cereals are essential to human health and provide beneficial effects (acting as cofactors to essential enzymes and aiding in the production of amines and amino acids). Abdel-Aal, Hucl, Patterson, et al. (2011) evaluated the trypsin inhibitor, amylase inhibitor, phytate and heavy metal content in the bran, wholegrain flour, and white flour of hairy canaryseeds, hairless canaryseeds, and wheat. All hairless canaryseed fractions contained significantly more phytate than wheat (28–41%), but no significant difference in trypsin inhibitor content compared to wheat. Canaryseed amylase inhibitor content was higher in the white flour fraction, but lower in the bran fraction as compared to wheat.

With regards to heavy metals, the hairless canaryseed variety CDC Maria contained higher amounts of the essential heavy metals zinc (44.8mg/kg), nickel (2.27 mg/kg), and copper (38.0 mg/kg) as compared to the wheat control (32.24 mg/kg, 0.34 mg/kg, and 24.4 mg/kg for zinc, nickel, and copper respectively), however, the molybdenum content was higher in wheat (0.64 mg/kg) as compared to CDC Maria (0.51 mg/kg) (Abdel-Aal, Hucl, Patterson, et al., 2011). There was no significant difference in neutral metal content (antimony, cobalt, selenium, tellurium, tungsten), and toxic metal content (arsenic, cadmium, lead, mercury), between CDC Maria and the wheat control, and all toxic metals were present in acceptable levels to human health for both grains.

In summary, the anti-nutritional components of wheat and glabrous canaryseeds are very similar and the anti-nutrients are present in low enough quantities that they do not outweigh their positive health benefits. To date, no studies compare the anti-nutritional components of multiple varieties of glabrous yellow and brown seeds. Li et al. (2011) reported a difference in phenolic acid content between brown and yellow canaryseed cultivars and a similar trend could exist in terms of their anti-nutritional content.

1.4. Methods of Protein Extraction and Analysis

1.4.1. Protein Extraction and Fractionation

Extracting and isolating protein from other seed components is the first step in canaryseed protein analysis. In general, protein extraction from cereals and seeds can be accomplished without too much difficulty if the proper steps are taken. One of the major problems with seeds and cereal grains is the fact that protein is not the major component of the grain and, furthermore, the protein itself forms complexes with other components, including the cell wall and starch, which makes it more difficult to extract all the protein present (Branlard & Bancel, 2007). Before the protein and other components can be extracted, the mechanical process of dehulling the grains is carried out to remove the hull from the seeds. Afterwards, the seeds are typically soaked in water for a wet milling step, where chemicals and enzymes may also be added, assisting in the separation of seed components (Martínez-Maqueda, Hernández-Ledesma, Amigo, Miralles, & Gómez-Ruiz, 2013).

Abdel-Aal et al. (2010) evaluated three different wet-milling techniques using ethanol (E), water (W), and alkaline (A) extractions to determine which method produced the highest yields of hairless canaryseed starch, protein, fiber and oil. They concluded the EAW extraction was most efficient with high recoveries of starch (92%) and protein (75%), but by comparison, the EWA process yielded the highest amounts of protein (Abdel-Aal et al., 2010). For the EWA process, canaryseeds were first defatted with ethanol, followed by extraction with water and an alkali (Abdel-Aal et al., 2010). However, this method is unspecific as it separates protein from the grains but is unselective for the different types of proteins present. To achieve this, an Osborne fractionation is typically done to extract the storage proteins (primarily prolamin and glutelin) and the metabolically active proteins (primarily albumin and globulin).

In the late 19th century, Osborne developed a method based on solubility by sequential extraction to obtain separate protein fractions; albumin (water soluble), globulin (soluble in dilute salt solutions), gliadins (soluble in aqueous alcohol solution) and glutenins (soluble in dilute acid or alkali solution) (Arendt & Zannini, 2013). Although the method remains simplistic, it is still widely used today as an initial step in protein fractionation in order to obtain more purified protein fragments. A tiny proportion of proteins (mainly lipoproteins) are insoluble in all four Osborne fractions and remain in the insoluble residue at the end of an Osborne fractionation (Koehler & Wieser, 2013). By following the sequential extraction steps of Osborne, the four major protein

fractions of canaryseeds can be separated and several studies have utilized this technique (Abdel-Aal et al., 1997b; Estrada-Salas et al., 2014; Valverde et al., 2017).

1.4.2. Protein Purification and Quantification

1.4.2.1. Electrophoresis

Several purification and enrichment methods are used in proteomics, however, electrophoresis and liquid chromatography remain the most critical for protein and amino acid analysis. The principle behind the electrophoresis technique is relatively simple. Charged molecules, such as proteins, move towards an oppositely charged electrode within a pH gradient in the presence of an electric field. The molecules will move at different speeds due to differences in size and charge, which leads to separation of the protein fractions (Westermeier, 2016). Proteins can be analyzed using several gel electrophoresis techniques, including isoelectric focusing and sodium dodecyl sulfate (SDS) electrophoresis. The former involves the migration of proteins in a gel towards either the anode or cathode, until they reach a position where their net charge is zero and stop moving, since the electric field has no effect on uncharged molecules (Westermeier, 2016). The latter, SDS electrophoresis, is a detergent that linearizes the proteins and gives them an overall negative charge proportional to their mass, so they may be separated according to their molecular weight.

The combination of isoelectric focusing and SDS can be applied to 2D electrophoresis; the separation of protein first by their isoelectric point followed by their molecular weight. Using 2D electrophoresis, thousands of proteins can be easily separated and used for further analysis, such as by mass spectrometry (MS). Nonetheless, there remain several drawbacks to this technique. Firstly, the method lacks the ability to detect proteins present in low concentrations (Mishra, 2010). Secondly, 2D electrophoresis is less effective at separating highly hydrophobic proteins and proteins with an isoelectric points at pH extremes (James, 2001). A second type of electrophoresis, called capillary electrophoresis, can also separate proteins and peptides in a reaction executed in a slim glass tube under high voltage (Mishra, 2010). Proteins separated by capillary electrophoresis are typically injected into a mass spectrometer for further separation and identification (Mishra, 2010). To date, Valverde et al. (2017) have been the only ones to utilize 2D gel electrophoresis on canaryseed proteins. They first fractionated the protein into albumins, globulins, prolamines, and glutelins and preformed both an SDS-PAGE (polyacrylamide gel electrophoresis) and 2D-PAGE analysis. The prolamines were the most abundant protein fraction (54%) with a molecular weight

of 20-25 kda (Valverde et al., 2017). Estrada-Salas et al. (2014) reported similar results when they performed SDS-PAGE on fractionated canaryseed flour proteins, however, they reported a much lower prolamin concentration (35%). Estrada-Salas et al. (2014); (Rajamohamed et al., 2013); Valverde et al. (2017) have all utilized electrophoresis because it is an excellent tool to be able to observe the changes in canaryseed protein that occurs during digestion. At the beginning of a digestion, the electrophoresis results will show bands of larger proteins, but as the digestion continues and proteins are broken down, bands will begin to appear at lower molecular masses because of the appearance of smaller peptides.

1.4.2.2. Liquid Chromatography

Liquid chromatography techniques produce a chromatogram that shows relative intensity of the eluted sample components versus their retention time, which is different for each sample component. Using liquid chromatography techniques, the amino acids present in a sample can be determined. In previously discussed experiments, both (Abdel-Aal et al., 1997b) and Newkirk et al. (2011) used different liquid chromatography techniques to determine and quantify amino acids present in canaryseed groat proteins.

Liquid chromatography plays a significant role in protein purification. Using liquid chromatography, proteins are separated in a liquid mobile phase as they pass through a solid stationary phase. Based on differences in size, charge and affinity for the stationary phase, proteins and amino acids can be successfully separated. Several different liquid chromatography methods exists such as size exclusion (separation based on size), affinity (separation based on interaction with solid matrix material), ion-exchange (separation based on charge), and reversed phase (separation based on hydrophobicity) (Mishra, 2010). In terms of proteomics, ion-exchange and reversed phase liquid chromatography remain the most significant. Indeed, because of its excellent compatibility with MS, the majority of liquid chromatography protein analyses are done using the reversed phase technique (Shi, Xiang, Horvath, & Wilkins, 2004). Combining several chromatographic techniques can produce the same effects as 2D gel electrophoresis, where the proteins can be separated by both charge and mass. High-pressure liquid chromatography (HPLC) is commonly the final step to improve the final resolution before being injected into a mass spectrometer (Mishra, 2010). Thus far, (Abdel-Aal et al., 1997b) used reversed-phase HPLC to

determine the amino acid composition of canaryseed protein. Valverde et al. (2017) separated the prolamin fraction of canaryseed protein using HPLC to be further analyzed by LC-MS.

1.4.2.3. Mass Spectroscopy

Mass spectroscopy remains an important tool in the detection and quantification of both proteins and amino acids. Mass spectrometers first produce ions out of molecules and then separate and quantify the produced ions according to their mass to charge ratio. Because each amino acid has a separate weight, it also gives each peptide a distinct molecular weight which can be used to deduce the exact amino acid sequence in a given peptide or protein (Mishra, 2010). The MS consists of several components, including the ionizer, the mass analyzer (where ions are separated based on mass to charge ratio), a detector, and finally a powerful vacuum to permit the movement of free ions inside the system (Mishra, 2010).

In terms of mass analyzer type, two are the most significant for proteomics; the quadruple mass analyzer and the time of flight (TOF) analyzer. The former involves the use of four parallel rods at equidistance from each other that generate a controlled, oscillating electric field when specific currents and radiofrequencies are applied to the rods, causing ions with a particular mass to charge ratio to be separated as they pass between the four rods (Dass, 2006). The latter, TOF analyzer, accelerates ions with the same amount of energy, but smaller ions (smaller masses) reach the detector faster than larger ions (larger masses) resulting in separation of the ions (Mishra, 2010).

In proteomics, the most widely used mechanism of ionization is electron spray ionization (ESI) (Arnott, 2001). In ESI, the sample, containing proteins and peptides, is dissolved in a volatile liquid and sprayed while a voltage is simultaneously applied, which causes ionization of the sample components and once the solvent volatizes, only the ionized fractions remain (Mishra, 2010). Because proteins and amino acids separated by liquid chromatography and electrophoresis are in the liquid state, ESI provides excellent compatibility for MS applications (Arnott, 2001). Matrix Assisted Laser Desorption/Ionization (MALDI) is a second type of ionizer equally important in proteomics (James, 2001). For the MALDI ionization method, an analyte and the sample are adsorbed onto the surface of a matrix with the ability to absorb UV light, and when a laser beam hits the matrix surface, the analyte volatizes while the sample is ionized (Mishra, 2010). Both ESI

and MALDI are termed "soft" methods, since they do not lead to molecule fragmentation. Before the development of ESI and MALDI, MS was seldom used in protein analysis.

Although little work has been done thus far, mass spectroscopy has been successfully applied to study different aspects of canaryseed proteins. Boye et al. (2013) used tandem MS to evaluate the presence of gluten-like proteins in canaryseeds that are present in other cereals, such as wheat, rye, and barley and elicit allergic reactions in some individuals. Proteins in canaryseeds were similar to other cereal and legume proteins but celiac and gluten related proteins and peptides were absent altogether (Boye et al., 2013). Valverde et al. (2017) used MS to analyze the prolamin fraction of canaryseeds and were able to identify the sequence of five peptides with molecular weights ranging from 664 to 1019 Da. All peptides had a combination of ACE inhibition and DPP-IV inhibition activity while only one peptide (the largest) also had antioxidant activity when their sequences were compared to known peptides with known bioactivity (Valverde et al., 2017).

1.4.3. Analysis of Protein Quality

Protein quality analysis is an important parameter to consider in order to establish the ability of a food source to meet metabolic demands for amino acids and nitrogen, which depends on its amino acid composition, protein digestibility, amino acid bioavailability, and the dietary requirements of the consumer (age, health status, physiological status, and energy balance) (Boye, Wijesinha-Bettoni, & Burlingame, 2012). In 2012, the digestible indispensable amino acid score (DIAAS) replaced the previously acceptable protein digestibility-corrected amino acid score (PDCAAS) as the standard for determining protein quality, due to concerns with the PDCAAS method, including the exclusion of the bioavailability of individual indispensable amino acids, the use of true fecal protein digestibility (determined in rats instead of humans), and the truncation of the score at 1.0 (Wolfe, Rutherfurd, Kim, & Moughan, 2016). For the DIAAS method, the true ileal amino acid digestibility for each dietary indispensable amino acid is used in the calculation, whereas, for the PDCAAS method, only a single value for the fecal crude protein digestibility is considered (FAO, 2013).

1.5. Industry Applications

1.5.1. Functional Food and Food Allergen Alternative

Functional foods are a growing trend among consumers today, because consumers not only eat food to satisfy their hunger, but they eat specific foods to maintain or improve their overall health (Siró, Kápolna, Kápolna, & Lugasi, 2008). Although there is no official definition of a functional food, the general idea is their consumption provides exceptional nutritional health benefits above and beyond basic nutrition. Some food products, designated as "superfoods", offer more than one health promoting property and recent superfood trends among consumers include oats, hemp seeds, almonds, kale, acai berries, blueberries, and green tea among others (Šamec, Urlić, & Salopek-Sondi, 2018; Umme Salma, 2009; van den Driessche, Plat, & Mensink, 2018). Oats contain large proportions of beta-glucan, a type of water soluble fiber present in the grain that possess several health promoting effects, such as reducing cholesterol and lowering postprandial glucose and insulin levels in the blood, which is especially beneficial for individuals with type II diabetes (Jing & Hu, 2012). Likewise, canaryseeds demonstrate exceptional nutritional qualities, including their antioxidant, antidiabetic, antihypertensive, and even anti-obesity activity. Furthermore, their phytochemical content (phenolic acids, carotenoids, and flavonoids) and relatively low abundance of anti-nutritional factors contribute to their nutritional qualities. The grains themselves could be used as a functional ingredient in food products (such as granola bars, bread, pasta, and cereals) to improve their nutritional value. In addition, canaryseeds are gluten-free. Using canaryseed to replace wheat or gluten-containing cereals will create more options for gluten-sensitive individuals and also produces new opportunities to develop gluten-free products. Moreover, because of their size and shape, canaryseeds offer the possibility to replace sesame seeds in products, such as baked goods, snack foods, and toppings, creating new products for individuals with allergies to sesame seeds.

1.5.2. Livestock Feed

The nutritional effects of canaryseeds are also applicable to animals, hence, supplementing or substituting animal feed with the seeds will positively impact their health. Studies conducted on partial substitution of chicken feed with canaryseeds fed to broiler chickens had no negative effects on the animals and the seeds were safe for consumption by the chickens (Classen et al., 2014; Newkirk et al., 2011). Classen et al. (2014) reported the nutritive value of the canaryseeds in broiler

chickens, solely based on the retention of nutrients and the growth of the chickens, was equal to or better than that of wheat. In a separate animal study, Thacker (2003) replaced barley in the diets of pigs with either 25, 50, 75 or 100% canaryseeds and found a replacement with 25% canaryseed had the highest growth rate in the pigs. Thacker (2003) also reported the nutrients in the seeds were available in a form that was readily utilized by the animals, and although some sources describe anti-nutritional factors in the seeds, they were not abundant enough to negatively impact pig performance. Because of it functional activity, canaryseed and its bioactive peptides can aid in improving and maintaining overall animal health, which in turn, leads to higher animal yields.

1.6. Conclusion

Glabrous canaryseed, technically an ancient grain, is a new source of plant-based protein. Evaluation of its quality and confirmation of the broad spectra of its potential bioactivities and health benefits would make this cereal an excellent nutritional and therapeutic aid to help combat non-communicable diseases, including cancer, diabetes, and heart disease. Due to a lack of knowledge, and because the seed is “new”, this unique cereal is currently underutilized by consumers and the industry. However, growing trends among consumers, including the consumption of functional foods and gluten-free products, have created high demands in the food industry that can be supported with the use of glabrous canaryseeds.

CONNECTING STATEMENT I

A comprehensive literature review on the health promoting effects of canaryseed proteins and its constituents, in addition to methods of protein analysis and food industry applications of canaryseeds, were presented in Chapter I

Chapter II investigates the chemical profiles and the nutritional quality of canaryseeds proteins. The SDS-PAGE and OFFGEL electrophoretic profiles of the seeds were compared. The amino acid content, protein digestibility, and protein quality of canaryseeds and common cereals oat and wheat were evaluated. Furthermore, anti-nutritional components in cereal flours, including trypsin inhibitor activity, phytate, and total polyphenol content, were assessed.

CHAPTER II

CHARACTERIZATION OF CANARYSEED PROTEIN PROFILE AND NUTRITIONAL QUALITY

2.1. ABSTRACT

Glabrous canaryseed (*Phalaris canariensis*) protein profile analysis by one dimensional (SDS-PAGE) and bi-dimensional (2D-OFFGEL-SDS-PAGE) showed no significant varietal differences between the yellow and brown canaryseed cultivars. From the OFFGEL electrophoresis profiles, the isoelectric point of canaryseed proteins was in the neutral to basic pH range, with the majority of protein bands varying between 20 and 70kDa. Crude protein content of canaryseeds (22%) was higher than both oat (13%) and wheat (16%). Canaryseeds were higher in essential amino acid tryptophan (2.4-2.6 g/100g protein) than oat (1.5 g/100g protein) and wheat (1.1 g/100g protein). The *in vitro* true ileal digestibility for total amino acids suggested that canaryseed amino acids were digested from a minimum of 25.9-29.8% to a maximum of 64.2-70.9%. The minimum digestibility was higher than wheat (21.6%), but slightly lower than oat (32.4%), meaning that canaryseed may be more bioaccessible for absorption in the gut. For all studied cereal flours, the limiting amino acid was lysine. The calculated protein nutritional quality scores PDCAAS and DIAAS were significantly higher in the yellow C05041 cultivar than the brown Bastia. Moreover, these scores were similar to those of wheat, but lower than those of oat proteins. Anti-nutritional components in the canaryseeds were also evaluated and were found comparable in oat and wheat. Trypsin inhibitor activity was higher in Calvi flour (0.161 mg/g) than wheat (0.114 mg/g). Phytic acid content was significantly higher in canaryseeds (12 mg/g) than both oat (6 mg/g) and wheat (3 mg/g). Total polyphenol content was highest in oat (2.0 mg FAE/g), followed by canaryseeds (1.4 mg FAE/g) and then wheat (0.65 mg FAE/g).

2.2. INTRODUCTION

Non-communicable diseases, such as cardiovascular disease, diabetes, cancer, and obesity are a major concern in society today and have huge social and economic repercussions, particularly on poorer or developing countries, hence, reducing the burden of non-communicable diseases is a global priority (WHO, 2014). For this reason, there is an ever-increasing demand for functional protein ingredients, which improve the overall nutritional quality of a food product and demonstrate health promoting properties that can help reduce or control the effects of some non-communicable diseases. As the global demand for protein also increases, there is an ever-increasing interest in exploring new sources of plant-derived proteins.

Among the currently available plant-derived proteins, soy, rice and wheat proteins remain dominant (Awika, 2011). The use of pulses and ancient grains as sources of plant-based proteins has recently grown. However, animal proteins are considered "complete" as they contain the essential amino acids, whereas plant proteins are "incomplete" because they lack more than one essential amino acid (Hoffman & Falvo, 2004). To ensure all essential amino acids are acquired, individuals obtaining their protein from plant sources need to consume several types of plant foods (fruits, vegetables, cereals, legumes). Furthermore, animal protein sources (milk, meat, eggs) typically have higher ileal digestibility (>95%) as compared to plant sources such as cereals, pulses, and flours (80-90%); however, when plant proteins have been isolated from the cell wall constituents (plant protein isolates) their digestibility increases (>95%) (Tomé, 2013). The protein digestibility and amino acid bioavailability of plant proteins may be affected by the presence of many naturally occurring anti-nutritional components (phytic acid, trypsin inhibitor, and tannins). On the other side, plant protein sources have high amounts of health benefiting constituents, including fiber and phytochemicals (Sarwar Gilani et al., 2012). Individuals who obtain protein primarily from animal sources are at higher risk of developing cardiovascular disease (from a high saturated fat and cholesterol diet), as opposed to individuals who obtain their protein requirements from plant sources (Hoffman & Falvo, 2004).

In 2015, hairless canaryseeds were given GRAS status and approved for human consumption in Canada and the United States as a true cereal grain. The seeds are reported to have exceptionally high protein content and contain high amounts of essential amino acid tryptophan, which is normally deficient in cereals (Abdel-Aal et al., 1997b). Abdel-Aal, Hucl, Patterson, et al. (2011) compared the anti-nutritional components of a brown hairless canaryseed cultivar to wheat and hairy canaryseed and found canaryseeds had higher amounts of phytic acid compared to wheat, but had similar amylase inhibitor and trypsin inhibitor content. The phytochemical and heavy metal content profiles were also comparable to that of wheat (Abdel-Aal, Hucl, Patterson, et al., 2011).

The overall objective of this study was to characterize and compare the protein profile of the newly developed Canadian hairless canaryseed varieties and to evaluate their nutritional quality as compared to selected common cereal grains oat and wheat. This was achieved through the

following specific objectives: (1) Characterizing the chemical composition of canaryseed flours and isolates; (2) determining the amino acid profiles of canaryseed proteins and assessing their digestibility and nutritional quality by an *in vitro* human digestion model, and (3) finally, evaluating the possible effects of anti-nutritional components present in the seeds.

2.3. MATERIALS AND METHODS

2.3.1. Materials

Dehulled seeds from four hairless canaryseed (*Phalaris canariensis L.*) cultivars [two yellow cultivars C09052, and C05041 (now registered as cultivar CDC Cibo), and two brown CDC Calvi and CDC Bastia cultivars], one oat (*Avena sativa)* cultivar (Turcotte) and one wheat (*Triticum aestivum L.*) cultivar (Snowbird) were used in this study. The canaryseed cultivars were kindly donated by Dr. Pierre Hucl from the Crop Development Centre of the University of Saskatchewan (Saskatoon, SK). Oat and wheat seeds were purchased from Semican (Princeville, QC). All seeds were hand-cleaned to remove any broken seeds or foreign material.

Ethanol, methanol, acetone, acetonitrile, dimethyl sulfoxide (DMSO), trinitrobenzenesulfonic acid (TNBS), hydrochloric acid (HCl), norvaline, and Halt protease inhibitor, were purchased from Fisher Scientific (Fair Lawn, NJ). Sodium hydroxide (NaOH), Tris Buffered phenol pH 6.6/7.9, Tris-HCl, sodium dodecyl sulfate (SDS), sucrose, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), L-leucine, 3-[(3-as cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), sodium bicarbonate ($NaHCO_3$), monosodium phosphate (NaH_2PO_4), and disodium phosphate (Na_2HPO4) were purchased from BioShop (Burlington, ON). Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), N_α-*p*-Tosyl-L-arginine methyl ester hydrochloride (TAME), Folin-Ciocalteu's phenol reagent, *N*α-benzoyl-DL-arginine 4-nitroanilide hydrochloride (BAPNA), ferulic acid, phenol, calcium chloride ($CaCl_2$), ammonium acetate ($C_2H_7NO_2$), borax ($Na_2B_4O_7 \cdot 10H_2O$), sodium acetate ($C_2H_3NaO_2$), and sodium azide (NaN3) were purchased from Sigma (St. Louis, MO).

For the electrophoretic characterization work, the ampholytes (pH 3-10) and immobilized pH gradient (IPG) strips were purchased from GE healthcare (Chicago, IL). Pre-cast SDS-PAGE gels, 2–250 kD broad range molecular weight standard, Laemmli buffer, β-mercaptoethanol, and Coomassie blue R-250 stain were obtained from Bio-Rad (Hercules, CA).

Ethylenediaminetetraacetic acid (EDTA) was obtained from LECO (Saint Joseph, MI). For the digestion work, pancreatin from porcine mucosa (P7545), pepsin from porcine gastric mucosa (250 units/mg solid, P7000), α-amylase from porcine pancreas (10 units/mg solid, A3176), and trypsin (T0303) were purchased from Sigma (St. Louis, MO). Deionized water (Millipore) was used in all experiments.

2.3.2. Preparation of canaryseed flour and protein isolates

Canaryseed flour was prepared by grinding canaryseeds into a fine powder in liquid nitrogen. The flour was stored at room temperature in the dark until used for analysis. Canaryseed protein isolates were prepared according to the modified method of Abdel-Aal et al. (2010). Canaryseeds were defatted with 100% ethanol (1:2, w/v) by blending in a Worthington blender for 3 minutes and subsequently centrifuged at 6,000 x g, 20 °C, for 15 minutes to remove the oil phase. The defatting process was repeated four times prior to protein extraction. The recovered pellet was dissolved in 0.05 N NaOH, pH 12.0 (1:10) for alkaline solubilization of the proteins. Following 1 hour of agitation, the suspension was centrifuged at 8,000 x g, 20 °C, for 15 minutes, and the pH of the supernatant was adjusted to pH 5.0 for protein precipitation. The solution was centrifuged at 6,000 x g, 20 °C, for 15 minutes and the residue washed with water to remove impurities. Finally, the protein residue was filtered through a 300 μm sieve to remove any suspended fine fiber particles, and the filtrate pH was adjusted to pH 7.0 using 1 N NaOH, and the precipitated proteins lyophilized. The freeze-dried proteins were homogenized and stored at -20 °C.

2.3.3. Characterization of canaryseed flour proteins and protein isolates

2.3.3.1. Protein determination

Total nitrogen content was determined using a Vario MAX Cube (Elementar, Langenselbold Germany), following the Dumas combustion method using EDTA as a nitrogen standard. Crude protein content of canaryseed flour and isolates was estimated from the total nitrogen using the nitrogen conversion factor of 5.7 (Abdel-Aal et al. (1997b). For wheat and oat flours, nitrogen conversion factors of 5.7 and 5.83 were used, respectively.

2.3.3.2. Characterization of the protein profile of canaryseed flours and of their protein isolates by SDS-PAGE

SDS-PAGE was carried out according to the method of Laemmli (1970) on 10-20% precast Criterion gels. The electrophoresis was run at 120 V and the gels stained using Coomassie R-250 blue stain, following manufactures instructions. Gels were destained overnight in water and image analysis performed with a ChemiDoc imaging system (Bio-Rad, Hercules, CA). The relative proportions (%) of proteins from canaryseed flours and isolates were estimated from bands corresponding to 3-10 kDa, 10-18 kDa, 20-25 kDa, and 30-100 kDa using the ChemiDoc imaging system software.

2.3.3.3. Characterization of canaryseed flour protein by 2D off-gel protein fractionation- SDS-PAGE

2D offgel protein fractionation was carried out according to the method of Vincent (2011), with modification by Rodrigues, Torres, da Silva Batista, Huergo, and Hungria (2012). 250 mg of canaryseed flour was suspended in 0.8 mL of Tris buffered phenol, pH 6.6/7.9, and 0.8 mL of SDS buffer [0.1 M Tris-Hcl pH 8.0, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 30% sucrose (w/v), 5 mM HALT protease inhibitor] and vortexed for 10 minutes followed by centrifugation (16,000 x g, 5 minutes, 4 °C). The top phenol layer was transferred into a new tube. The pellet was re-extracted with 0.5 mL of Tris buffered phenol, pH 6.6/7.9 and the upper phenol layers combined, following centrifugation. The phenol-based extracts were precipitated with 3 volumes of cold 0.1 M ammonium acetate in absolute methanol. After 2 hours at -20 °C, the extracted proteins were pelleted by centrifugation (6,500 x g, 15 minutes, 4 °C) and washed once with cold 0.1 M ammonium acetate in methanol and once with cold 80% (v/v) acetone. After air drying at room temperature to remove residual acetone, the pellet was re-dissolved in resuspension buffer [7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 1% (w/v) DTT, 1% (v/v) β-mercaptoethanol, 10 mM TCEP, 0.5% (v/v) ampholytes (pH 3-10)]. The extracted proteins were separated by offgel isoelectric focusing using the Agilent 3100 OFFGEL fractionator (Agilent, Mississauga, ON) following the manufacturers pre-set program OG24PR00 (64kBhrs, 8000V, 50μA, 200mW). 3 mg of protein was loaded on pH 3-10 IPG strips (24 cm). After fractionation, the fractions were collected separately and combined with an equivalent volume of Laemmli buffer. Electrophoretic profile of the protein fractions and unfractionated proteins were done according to the method of Laemmli

(1970) on AnykD precast mini gels. The electrophoresis was run at 120 V and the gels stained using Coomassie R-250 blue stain, following manufactures instructions. Gels were destained overnight in water and image analysis performed with a ChemiDoc imaging system.

2.3.4. Nutritional properties and protein quality of canaryseed protein

2.3.4.1. In vitro digestion of canaryseed, oat, and wheat flours

The digestion of cereal flours was carried out according to the method of Minekus et al. (2014). In the oral phase of digestion, 1 g of flour was incubated for 2 minutes at 37 °C, pH 7.0, with simulated salivary fluid (1:1, w/v) containing α-amylase from porcine pancreas (75 U/mL of digest). Then, the mixture was diluted (1:1, v/v) with simulated gastric fluid containing pepsin from porcine gastric mucosa (2000 U/mL digest). The pH was adjusted to 3.0 and the mixture was incubated for 2 hours at 37 °C. Intestinal phase was carried out by diluting the mixture (1:1, v/v) with simulated intestinal fluid containing pancreatin from porcine mucosa (100 U trypsin activity/mL digest) and bile (10 mM). The pH was adjusted to 7.0 and the mixture was incubated for 2 hours at 37 °C. The reaction was stopped by the addition of 1 mM AEBSF (protease inhibitor). The final hydrolysates were centrifuged (15, 000 g, 30 min, 4 °C); the supernatants were collected and then frozen at -80 °C until analysis. Because proteins were the focus of the digestion, lipases were omitted. The peptidase activity of the pancreatin was tested using a fluorometric commercial enzyme kit for the determination of leucine aminopeptidase activity (BioVision, Milpitas, CA).

2.3.4.2. Degree of hydrolysis (DH)

The extent to which cereal flour proteins were hydrolyzed following the *in vitro* digestion protocol of Minekus et al. (2014) was quantified using the TNBS reagent according to the method described by Adler-Nissen (1979) and Spellman, McEvoy, O'Cuinn, and FitzGerald (2003), with modification. Briefly, 10 µL of sample and standard (both prepared in 0.1% (w/v) SDS) was added to a microplate well followed by 80 µL of 0.2125 M sodium phosphate buffer, pH 8.2, and 80 µL of 0.1% TNBS reagent, diluted with water. After mixing, the samples were incubated at 50°C for 60 minutes. 160 µL of 0.1 N HCl was added to stop the reaction and the absorbance read at 340 nm. 0-2 mM of L-Leucine, prepared and diluted in 1% SDS, was used to generate the standard curve. The DH values were calculated using the following equation:

$$DH(\%) = 100 \left(\frac{AN_2 - AN_1}{N_{pb}}\right)$$

Where AN_1 is the amino nitrogen content of the protein substrate before hydrolysis (mg/g protein), AN_2 is the amino nitrogen content of the protein substrate after hydrolysis (mg/g protein), and N_{pb} is the nitrogen content of the peptide bonds (mg/g protein) after complete hydrolysis with 6 N HCl at 110 °C for 24 hours. The values of AN_2, AN_1 and N_{pb} were determined from the standard curve of the absorbance at 340 nm versus the mg/L amino nitrogen content of L-leucine. The values obtained were then divided by the protein content in the test samples to give mg amino nitrogen per g of protein.

2.3.4.3. Amino acid content of canaryseed proteins and in vitro hydrolysates

Total amino acid analysis of the canaryseed flours and freeze-dried supernatant hydrolysates was conducted in accordance with the Agilent method (Long, 2015). Briefly, samples containing around 4 mg of protein (~ 30 mg flour) were hydrolyzed with 6 N HCl containing 0.1% (w/v) phenol and Norvaline (as internal standard) for 24 h at 110 ± 2 °C in glass tubes sealed under vacuum. The hydrolyzed samples were cooled to room temperature, and solutions evaporated with nitrogen to dryness. Once dry, the amino acids were dissolved by the addition of 10 mM borax buffer (pH 8.2, containing 0.1%w/v HCl) and then filtered with 0.22 μm PVDF filters (low protein binding) (Sigma, St. Louis, MO) prior to RP-HPLC analysis. For the *in vitro* hydrolysates, 200 μL of the supernatant was removed and analyzed for free soluble (bioaccessible) amino acids. The remaining supernatant was lyophilized and subsequently hydrolyzed following the same protocol as canaryseed flour for the determination of total amino acids in the whole *in vitro* hydrolysates, which includes free amino acids as well as polypeptides and soluble proteins.

Amino acid composition was quantified by by RP-HPLC analysis using an Agilent Poroshell HPH-C18 reversed-phase column (monitored with Agilent 1200 series HPLC system (Agilent Technologies Canada Inc., Mississauga, ON)), utilizing an automatic post-column OPA and FMOC derivatization and detection at an absorbance of 338 nm. The separation was performed at a flow rate of 1.5 mL/min employing a mobile phase of A: 10 mM Na_2HPO4, 10 mM Na_3B4O7, 5 mM NaN3, adjusted to pH 8.2 with HCl, and B: ACN: MeOH: water (45:45:10, v/v/v). The elution program was as follows: 0 min, 2 %B; 1.0 min, 2 % B; 20 min, 59 % B; 21 min, 90% B;

24 min 90% B; 29 min, 2% B; 35 min, 2% B. Five standard mixture ampoules (containing 16 amino acids) at different concentrations (10 pmoles/µL to 1 nmoles/µL) from Agilent were used for the construction of the calibration curves. The elution times of each amino acid in the analyzed samples were compared to those of the standard and the amount of each amino acid was then calculated as mg/g based on the peak area.

The content of tryptophan in the canaryseed flours was determined separately by alkali hydrolysis following the method of Yust et al. (2004), with slight modification. Samples (~ 15 mg of protein) were dissolved in 3 ml of 4 N NaOH, sealed in hydrolysis tubes, and incubated in an oven at 110 °C for 24h. Hydrolysates were cooled, neutralized to pH 7.0 using 12 N HCl, and diluted to 25 mL with 1 M borax buffer (pH 9). Aliquots of these solutions were filtered through a 0.45 µm PVDF filter, and then injected into a Nova-Pack C18 column (Waters, Mississauga, ON). An isocratic elution system consisting of 25 mM sodium acetate, 0.02% sodium azide (pH 9)/acetonitrile (91:9,v/v) delivered at 1 mL/min was used. Tryptophan standard was injected at different concentrations for calibration construction, and the amount of tryptophan in flour samples was then calculated as mg/g based on the peak area.

2.3.4.4. In vitro protein digestibility (IVPD)

The experimental work was carried out according to Tinus, Damour, van Riel, and Sopade (2012). Briefly, cereal flour equivalent to 62.5 mg of protein was rehydrated in 10 mL of water at 37 °C for 1 hour, afterwards, the pH of the solution was adjusted to 8.0 with 0.1 M NaOH and/or HCl. A 10 mL multienzyme solution was prepared fresh daily, consisting of 16 mg of trypsin (T0303 trypsin from porcine pancreas, type IX-S, 13,000-20,000 BAEE units/mg protein), 31 mg of chymotrypsin (C4129 Chymotrypsin from bovine pancreas C4129 Type II, ≥40 units/mg protein) and 13 mg protease (P5147 protease from *Streptomyces griseus*, Type XIV, ≥3.5 units/mg solids). The multienzyme solution was kept at 37 °C and its pH was adjusted to 8.0 with 0.1 M NaOH and/or HCl. After rehydration, 1 mL of the multi-enzyme solution was added to the 10 mL sample mixture, and the initial pH was immediately recorded. After 10 minutes of constant agitation at 37 °C, the final pH was recorded and the IVPD was calculated from the following equation:

$$IVPD\ (\%) = 65.66 + 18.10\Delta pH_{10min}$$

2.3.4.5 PDCAAS and DIAAS calculations for protein quality evaluation

The PDCAAS and DIAAS scores were calculated using the protein and ileal amino acid digestibility data obtained by the *in vitro* digestion models of Tinus et al. (2012) and Minekus et al. (2014), respectively (cf. sections 2.3.4.1. and 2.3.4.4.). The scores were calculated following the new FAO guidelines for the determination of dietary protein quality for infants (0-6 months), children (6 months-3 years), and older children/adolescents/adults according to the recommended reference scoring patterns (FAO, 2013), since essential amino acid requirements for maintenance and growth will not be the same for different age groups (**Table 2.1**). The amino acid content and the *in vitro* protein digestibility (IVPD) determined from the protocol by Tinus et al. (2012) were used to calculate the PDCAAS of each cereal flour from the following equation:

PDCAAS (%) = 100 x *lowest value* [(mg of indispensable amino acid in 1 g of the dietary protein)/(mg of the same dietary indispensable amino acid in 1 g of the reference protein)] x *in vitro* protein digestibility (IVPD)

Both free and total amino acid content in the *in vitro* hydrolysates were used to determine the minimum and maximum values for the DIAAS, respectively. Free amino acids estimate the minimum DIAAS value for soluble amino acids after *in vitro* digestion which are readily accessible for absorption. The maximum DIAAS value was calculated by determining the maximum digestibility of each amino acid in the digest after *in vitro* digestion by hydrolyzing all amino acids (including bioaccessible and soluble proteins/polypeptides). It is expected that the true DIAAS value lies between the minimum (free amino acids) and maximum (total amino acids) values. For the DIAAS calculation, the *in vitro* true ileal digestibility (TID) was calculated for both free and total amino acids as a percentage of intake for each amino acid as described by Havenaar et al. (2016) using the following equation:

$$\text{True ileal digestibility (TID) (\%)} = \frac{\sum AA\ content\ sample\ digestate\ (mg) - \sum AA\ content\ blank\ (mg)}{Intake_{AA\ content\ (mg)}}$$

Where ∑AA content in sample digestate is the total amino acid content (mg) in the supernatant after *in vitro* digestion; ∑AA content in sample blank is the amino acid content (mg) of the blank supernatant (containing all enzymes and solutions of the *in vitro* digestion without the addition of sample) after *in vitro* digestion; and $Intake_{AA\ content}$ is the amino acid content (mg) of the starting

material (flour). The digestible dietary indispensable amino acid content for both free and total amino acids could then be used to calculate the DIAAS for each cereal flour from the following equation:

DIAAS (%) = 100 x *lowest value* [(mg of digestible dietary indispensable amino acid in 1 g of the dietary protein)/(mg of the same dietary indispensable amino acid in 1 g of the reference protein)]

Table 2.1. Recommended amino acid reference pattern for infants, children, older children/adolescents/adults (FAO, 2013).

Essential amino acid	Age Group		
	Infant (0-6 months)	Child (6 months-3 years)	Older child/ adolescent/adult
	Reference Pattern (mg/g protein)		
His	21	20	16
Ile	55	32	30
Leu	96	66	61
Lys	69	57	48
SAA (Cyst + Met)	33	27	23
AAA (Phe + Tyr)	94	52	41
Thr	44	31	25
Trp	17	8.5	6.6
Val	55	43	40

2.3.5. Anti-nutritional components of flours and protein isolates

2.3.5.1. Trypsin inhibitor activity (TIA)

The experimental work was carried out according to Makkar, Siddhuraju, and Becker (2007), with modification. Cereal flours were defatted with ethanol. The dried, defatted flour was grinded into a fine powder and stored at -20 °C until analysis. 50 mL of 0.01 M NaOH was added to 4 g of defatted flour or protein isolates and stirred for 3 hours at room temperature. The pH of the solution was adjusted to pH 9.5 using concentrated NaOH or HCl. A 15 mg/L trypsin solution was prepared daily in 0.001 N HCl. The BAPNA substrate was prepared daily by dissolving 40 mg in 1 mL of DMSO and then diluted to 100 mL (0.921 mM) with pre-warmed (37 °C) 0.05 M Tris-HCl buffer, pH 8.2, containing 0.02 M $CaCl_2$. A reagent blank, standard enzyme solution, sample blank, and sample solution were prepared for the assay. The reagent blank (a) contained 2 mL of distilled water. The standard (b), contained 2 mL of the standard trypsin solution (15 mg/L trypsin) and 2

mL of distilled water. The sample blanks (c) contained 1 mL of diluted sample extract plus 1 mL of distilled water. The samples (d) contained 1 mL of diluted sample extract, 1 mL of distilled water and 2 mL of trypsin solution.

The tubes were vortexed and preheated in a water bath at 37 °C for 10 minutes. Then, 5.0 mL of BAPNA solution (pre-incubated at 37 °C) was added to each tube. After 10 minutes of incubation at 37 °C, 1.0 mL of 30% (v/v) acetic acid was added to each tube to stop the reaction and 2.0 mL of trypsin solution was added to the reagent blank (a) and sample blank (c) tubes. All tubes were centrifuged at 3,000 x g for 10 minutes at room temperature and the absorbance of each solution was read at 410 nm. The change in absorbance (A_i) due to trypsin inhibitor/mL diluted sample extract was calculated from the following equation:

$$A_i = (A_b - A_a) - (A_d - A_c)$$

Where A_b, A_a, A_d, and A_c are the absorbance readings of the standard, reagent blank, samples, and sample blanks, respectively. The percent inhibition of each sample tube was calculated from the following equation:

$$\% \text{ Trypsin inhibition} = \frac{A_i}{A_b - A_a}$$

Because 1µg of pure trypsin gives an absorbance of 0.019, trypsin inhibitor activity (TIA) was expressed in terms of mg of pure trypsin inhibited per gram of sample (mg/g) and was calculated from the following equation:

$$\text{TIA (mg/g)} = \frac{2.362 \text{ x } A_i \text{ x DF}}{S}$$

Where DF is the dilution factor and S is the sample weight in grams.

2.3.5.2. Phytic acid (phytate) content

The determination of phytic acid content was carried out according to McKie and McCleary (2016) using a commercial assay kit (Megazyme International, Bray, Wicklow, Ireland). Briefly, 20 mL of HCl (0.66 M) was added to 1 g of cereal flours and protein isolates and stirred overnight at room temperature for acid extraction of inositol phosphates. The extracted inositol phosphates were subsequently treated with phytase and phosphatase enzymes to convert total phosphate to

inorganic phosphorous. The amount of inorganic phosphorus released was determined from its reaction with ammonium molybdate, which forms molybdate blue proportional to the amount of inorganic phosphorous present in the sample. Molybdate blue content was determined colormetrically at 655 nm from a standard curve using known concentrations of inorganic phosphorus. The assay determines the g of phosphorous in 100 g of sample material, and it is assumed the amount of phosphorous measured is exclusively released from phytic acid, which comprises of 28.2% of phytic acid. The results were then expressed as mg of phytic acid per gram of sample.

2.3.5.3. Total polyphenol content (TPC)

TPC was determined using the Folin-Ciocalteu reagent according to Singleton and Rossi (1965), with modification. Samples (flours and isolates) (5%, w/v) were extracted for 2 hours at room temperature with 70% ethanol containing 1% (v/v) concentrated HCl. The mixtures were centrifuged at 10, 000 x g for 15 minutes and the supernatants were recovered. Ferulic acid (50-500 mg/L), prepared in 70% ethanol containing 1% (v/v) concentrated HCl, was used to construct the standard curve. 1.5 mL of Folin-Ciocalteu reagent (diluted 10x with water) was added to 200 µL of blanks, standards, and samples followed by the addition of 1.5 mL sodium bicarbonate solution 7.5% (w/v) after 5 minutes (at room temperature). After an additional 90 minutes at room temperature, the sample tubes were centrifuged at 6,000 x g for 15 minutes and the absorbance of the supernatants were read at 750 nm. The TPC content in the samples was determined from the ferulic acid standard curve and results were expressed as mg ferulic acid equivalents (FAE)/g of flour or protein isolates.

2.3.6. Statistical analysis

Each experiment was run in triplicate and the data were expressed as means ± standard deviation. Statistical analyses were performed using XLSTAT software (Addinsoft, NY) in Microsoft Excel (Redmond, WA). One-way analysis of variance (ANOVA) and the Tukey's honest significant difference (HSD) test ($p < 0.05$) were performed to detect significant differences. For the true ileal digestibility and DIAAS values, statistical analysis was performed using SAS software (Cary, NC). ANOVA was determined using the MIXED procedure of the SAS system. Multiple comparisons were performed with the LSMEANS statement of the MIXED procedure using the Bonferoni option.

2.4. RESULTS AND DISCUSSION

2.4.1. Protein profiles of canaryseed flours and their protein isolates

Canaryseed flour protein content ranged from 21.9-22.5% (w/w), with no significant difference between the two yellow and brown cultivars. However, the protein content of the canaryseeds significantly exceeded ($p < 0.05$) those of oat (13%, w/w) and wheat (16%, w/w) samples (**Table 2.2**). The protein content of the prepared canaryseed isolates ranged from 96.8 to 99.9% (w/w), with the C09052 yellow cultivar having a significantly higher ($p < 0.05$) concentration as compared to the other isolates. The electrophoretic analysis under denaturing conditions showed that the protein/polypeptide profiles were similar between the studied canaryseed cultivars (**Figure 2.1**) The SDS-PAGE profile of the canaryseed flours shows bands between 3-100 kDa, corresponding to bands from albumins, prolamins, globulins, and glutelins, also known as the cereal protein Osborne fractions (Osborne, 1924). Albumins are visible at the bottom of the SDS-PAGE profile of the flours in the 3-10 kDa molecular weight range. High molecular weight (HMW) globulin and glutelin fractions are visible in the 30-100 kDa range whereas low molecular weight (LMW) globulin and glutelin fractions are visible in the 10-18 kDa range. The alcohol soluble prolamins are the predominate protein fraction in canaryseeds and visible from the prominent bands in the 20-25 kDa range. Estrada-Salas et al. (2014) and Valverde et al. (2017) both reported similar SDS-PAGE profiles for each protein fraction in hairy canaryseed flours. The prolamins (20-25 kDa) and HMW glutelins and globulins (30-100 kDa) had the highest relative proportions (%) of proteins with 36-47% and 37-52%, respectively, whereas the albumins (3-10 kDa) and LMW

glutelins and globulins (10-18 kDa) had the lowest relative proportion (%) of proteins with 6-11% and 3-9%, respectively.

In contrast, SDS-PAGE analysis of the prepared canaryseed isolates showed four clusters of protein bands ranging between 20-26 kDa, 30-35 kDa, 37-40 kDa, and 50-52 kDa, with no low molecular weight proteins visible under 20k Da. The absence of these LMW bands in the isolates could be due to the protein extraction process. Albumins are proteins of LMW and highly soluble at alkaline pH (Zayas, 1997), which makes them more difficult to extract by acid precipitation as compared to the other larger and more abundant protein fractions. Indeed, alkaline protein solubilization followed by acidic precipitation may have resulted in partial loss of soluble albumins. The electrophoretic pattern of soluble proteins at pH 5.0 showed the presence of mainly albumin bands around 5-10 kDa that did not precipitate with the other proteins (data not shown). There was small differences in the C05041 yellow isolate (lane 9) compared to the other isolates (lanes 8, 10, 11), namely, a less pronounced band at 25 kDa. Densitometric analysis of SDS-PAGE gels revealed that for all canaryseed isolates, the largest relative proportion (%) of proteins was from 20-25 kDa (prolamins) with 54-69%, followed by 30-100 kDa (HMW globulins and glutelins) with 31-44%, and then 10-18 kDa (LMW globulins and glutelins) with 0-3%.

Both two-dimensional (2D) and offgel electrophoresis are considered powerful proteomic tools that provide information regarding the isoelectric point and the molecular weight distribution of protein mixtures. Unlike the conventional 2D gel electrophoresis, where proteins are separated according to their isoelectric point within the IPG strip matrix, the isoelectric separation of proteins in an offgel fractionation occurs in liquid phase on the surface of an IPG strip, therefore, amphoteric proteins and peptides forcibly move from one compartment to another until they reach their isoelectric point and then are collected in the liquid phase (Magdeldin et al., 2015). The offgel protein fractionation of extracted canaryseed proteins revealed no considerable differences between the studied cultivars (**Figure 2.2** and **2.3**). The majority of proteins had an isoelectric point at neutral and basic pH (pH 6.9-10). No proteins were visible in the acidic region (pH 3-3.9), either because no proteins had an isoelectric point in this region or the protein load was not sufficient enough for detection. From pH 4.2-4.8 (**Figure 2.2** and **2.3**, lanes 7-9) several faint bands were visible at LMW (~15kDa) and at HMW (~37-75kDa) with a prominent band visible at 55kDa (pH 4.5, lane 8) for all canaryseed cultivars. From pH 5.4-6.9 (lanes 11-16), all visible bands were

greater than 20kDa and contain some of the higher molecular weight proteins, including proteins ranging from 100-150kDa (lanes 13-14), which were more clearly resolved in the yellow (C09052 and C05041) cultivars. From pH 5.1-10, most proteins were in the molecular weight range of 20-50kDa. Several bands are visible between pH 7.8-10 (lanes 19-26) with a molecular weight of 10-15kDa and each cultivar has two poorly resolved bands of LMW (5-10kDa) at pH 9.6-10 (lanes 25-26), the latter similar to the band pattern of the SDS-PAGE profile of the canaryseed flours (**Figure 2.1**). Prolamin proteins, corresponding to bands from 20-25 kDa, had isoelectric points mostly from pH 7.2-10, with exceptionally dark stained bands at pH 7.8 (lane 19) and pH 9.6-10 (lanes 25-26). The HMW glutelins and globulins (30-100 kDa) had isoelectric points ranging from acidic (pH 5.1) to alkaline (pH 10) pH (lanes 10-26). Few bands from the LMW globulins and glutelins (10-18 kDa) are visible and are mostly at alkaline pH. The two poorly separated bands present in lanes 25 and 26 for each canaryseed cultivar at pH 9.6 and 10 are likely albumins (3-10 kDa).

Table 2.2. Crude protein (%, DB) content and relative proportion (%) of protein fractions of cereal flours and protein isolates

Cereal Variety	Protein Content (%)	Relative Proportions (%)			
	Flours	3-10 kDa	10-18 kDa	20-25 kDa	30-100 kDa
C09052 (yellow canaryseed)	22.20 (± 0.08)^{ab}	7.0	4.1	39.3	49.6
C05041 (yellow canaryseed)	22.00 (± 0.19)^{b}	6.4	5.4	36.2	51.7
Bastia (brown canaryseed)	22.51 (± 0.11)^{a}	11.2	2.6	42.9	43.4
Calvi (brown canaryseed)	21.95 (± 0.05)^{b}	7.4	8.7	47.2	36.8
Oat	12.76 (± 0.122)^{d}	-	-	-	-
Wheat	16.05 (± 0.07)^{c}	-	-	-	-
	Isolates				
C09052 (yellow canaryseed)	99.99 (± 1.26)^{a}	0	0	68.7	31.3
C05041 (yellow canaryseed)	96.83 (± 0.55)^{b}	0	1.9	53.7	44.2
Bastia (brown canaryseed)	97.16 (± 0.38)^{b}	0	0	63.6	36.4
Calvi (brown canaryseed)	97.59 (± 0.57)^{b}	0	2.6	64.5	32.8

Means in a column with different lowercase letters are significantly different at $p < 0.05$ (n =3)
Relative proportions (%) determined from densitometric analysis of SDS-PAGE gel using the Chemidoc imaging system

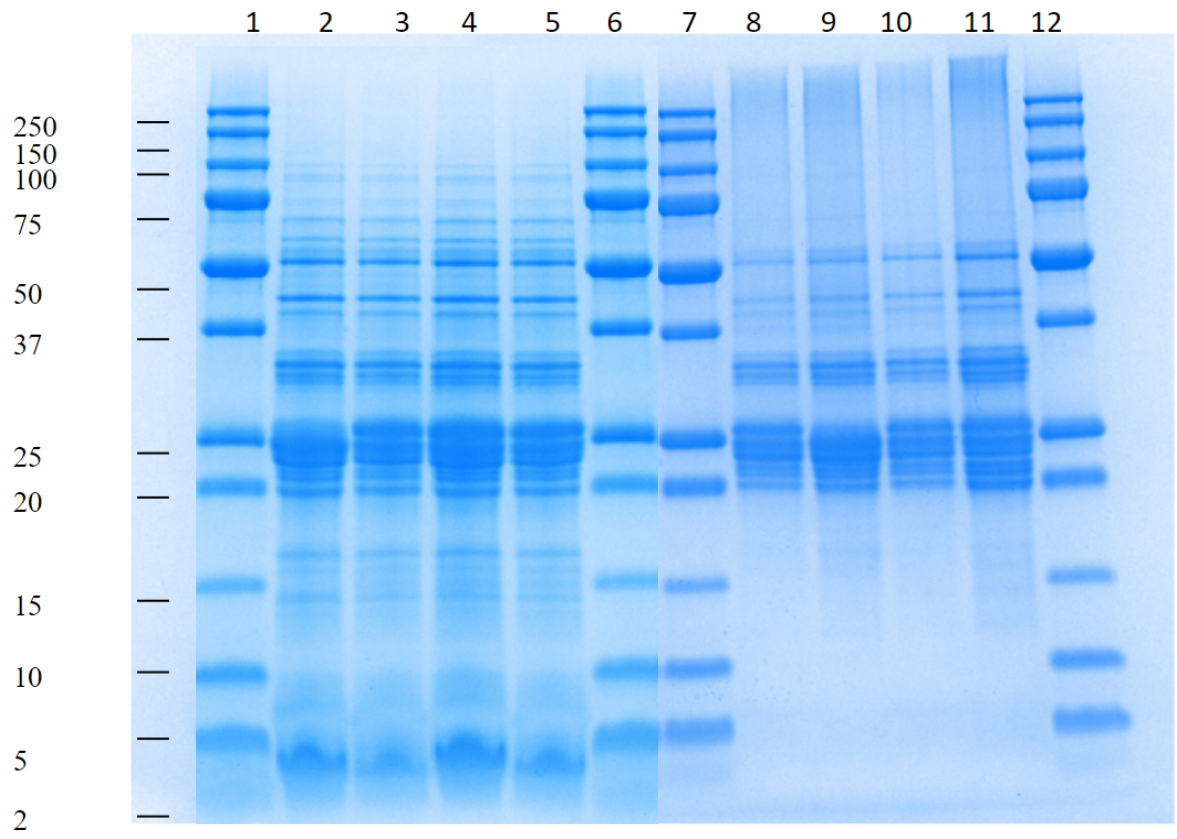


Figure 2.1. SDS-PAGE electrophoretic profile of canaryseed proteins from flours and isolates. Lanes 1, 6, 7, 12 : MWM, 2 : C05041 (yellow) flour, 3 : Bastia (brown) flour, 4 : C09052 (yellow) flour, 5 : Calvi (brown) flour, 8 : C09052 (yellow) isolate, 9 : C05041 (yellow) isolate, 10 : Bastia (brown) isolate, 11 : Calvi (brown) isolate

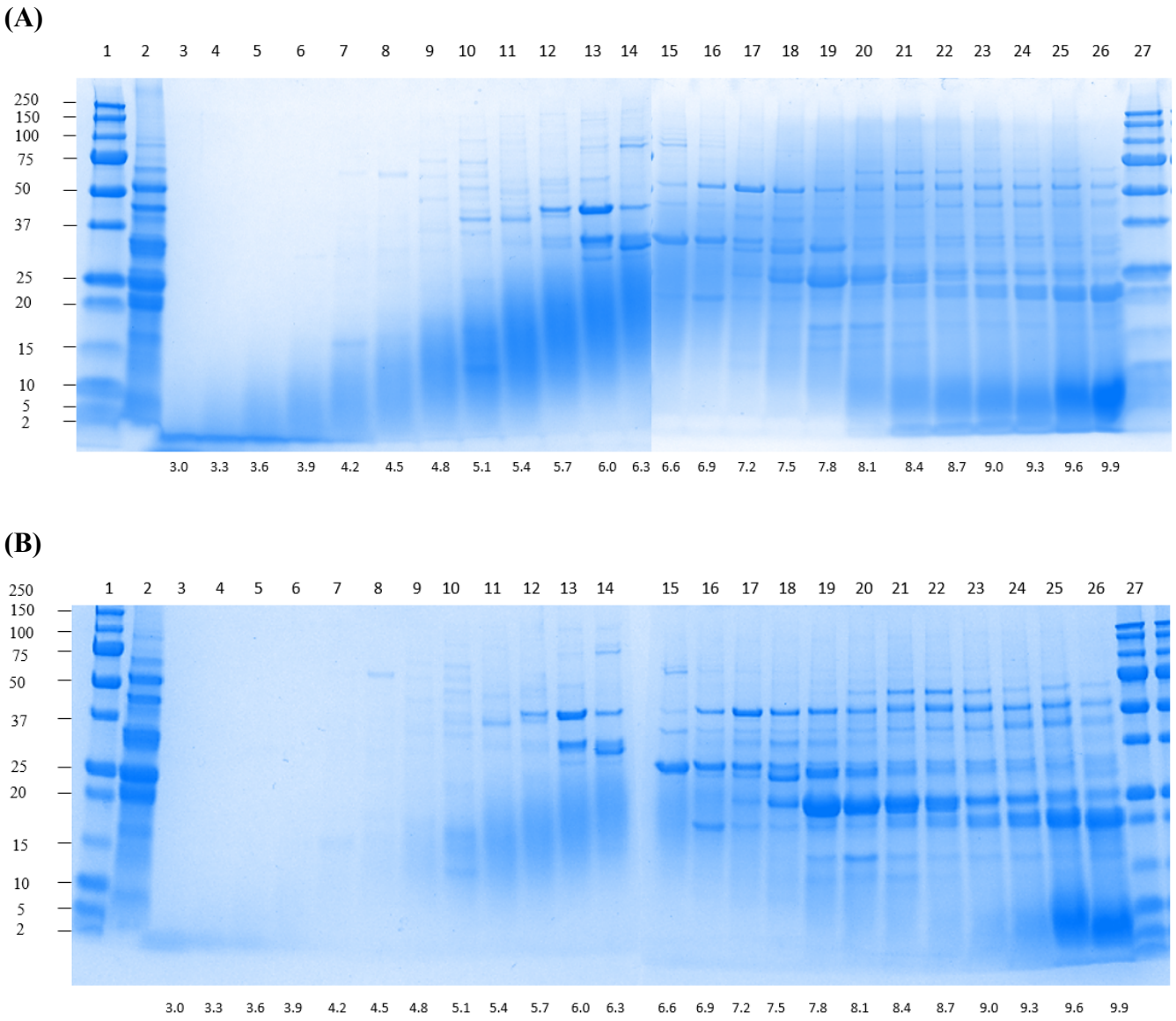
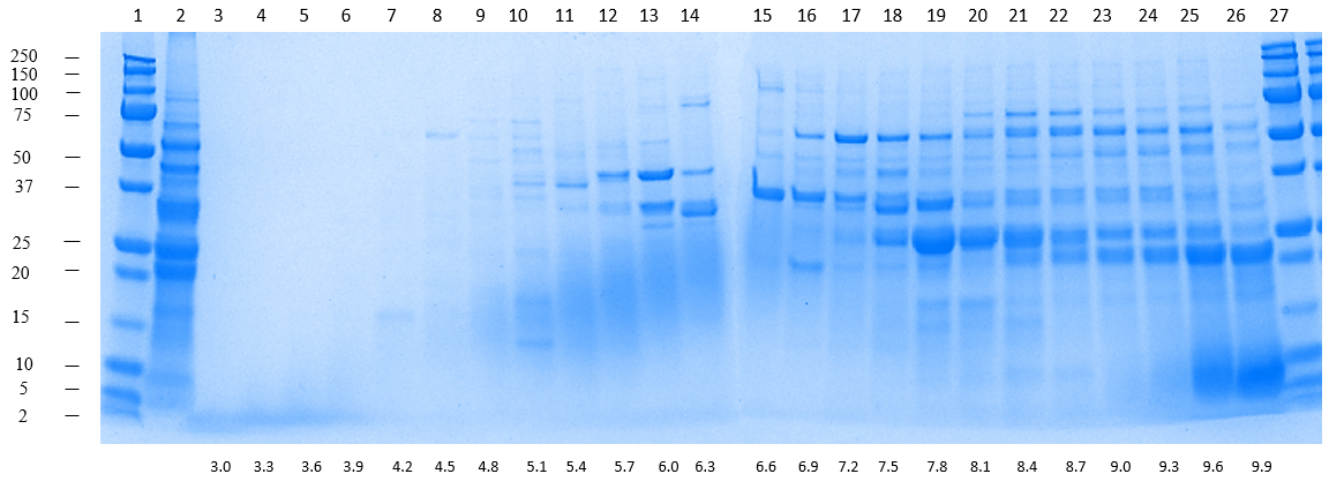


Figure 2.2. Offgel electrophoresis profile of yellow (**A**) C09052 and (**B**) C05041 glabrous canaryseed proteins. 1, 27 : MWM, 2 : unfractionated C05041 proteins, 3 : fraction 1 (pH 3.0-3.3), 4 : fraction 2 (pH 3.3-3.6), 5 : fraction 3 (pH 3.6-3.9), 6 : fraction 4 (pH 3.9-4.2), 7 : fraction 5 (pH 4.2-4.5), 8 : fraction 6 pH (4.5-4.8), 9 : fraction 7 (pH 4.8-5.1), 10 : fraction 8 (pH 5.1-5.4), 11 : fraction 9 (pH 5.4-5.7), 12 : fraction 10 (pH 5.7-6.0), 13 : fraction 11 (pH 6.0-6.3), 14 : fraction 12 (pH 6.3-6.6), 15 : fraction 13 (pH 6.6-6.9), 16 : fraction 14 (pH 6.9-7.2), 17 : fraction 15 (pH 7.2-7.5), 18 : fraction 16 (pH 7.5-7.8), 19 : fraction 17 (pH 7.8-8.1), 20 : fraction 18 (pH 8.1-8.4), 21 : fraction 19 (pH 8.4-8.7), 22 : fraction 20 (pH 8.7-9.0), 23 : fraction 21 (pH 9.0-9.3), 24 : fraction 22 (pH 9.3-9.6), 25 : fraction 23 (pH 9.6-9.9), 26 : fraction 24 (pH 9.9-10)

(A)



(B)

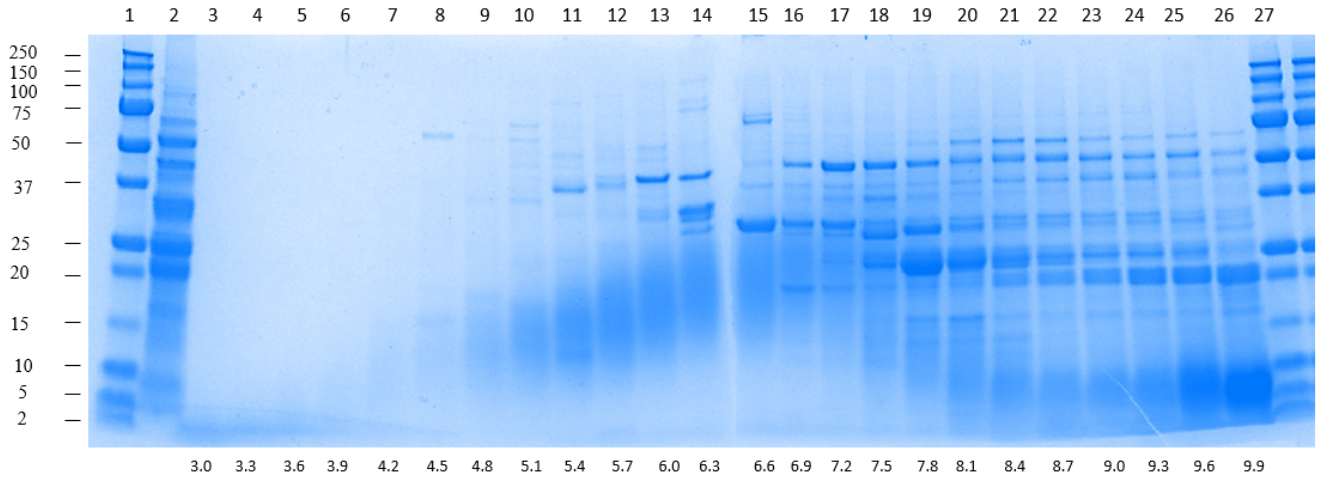


Figure 2.3. Offgel electrophoresis profile of brown (**A**) Bastia and (**B**) Calvi glabrous canaryseed proteins.1, 27 : MWM, 2 : unfractionated C05041 proteins, 3 : fraction 1 (pH 3.0-3.3), 4 : fraction 2 (pH 3.3-3.6), 5 : fraction 3 (pH 3.6-3.9), 6 : fraction 4 (pH 3.9-4.2), 7 : fraction 5 (pH 4.2-4.5), 8 : fraction 6 pH (4.5-4.8), 9 : fraction 7 (pH 4.8-5.1), 10 : fraction 8 (pH 5.1-5.4), 11 : fraction 9 (pH 5.4-5.7), 12 : fraction 10 (pH 5.7-6.0), 13 : fraction 11 (pH 6.0-6.3), 14 : fraction 12 (pH 6.3-6.6), 15 : fraction 13 (pH 6.6-6.9), 16 : fraction 14 (pH 6.9-7.2), 17 : fraction 15 (pH 7.2-7.5), 18 : fraction 16 (pH 7.5-7.8), 19 : fraction 17 (pH 7.8-8.1), 20 : fraction 18 (pH 8.1-8.4), 21 : fraction 19 (pH 8.4-8.7), 22 : fraction 20 (pH 8.7-9.0), 23 : fraction 21 (pH 9.0-9.3), 24 : fraction 22 (pH 9.3-9.6), 25 : fraction 23 (pH 9.6-9.9), 26 : fraction 24 (pH 9.9-10)

2.4.2. Nutritional properties and protein quality of canaryseed protein

2.4.2.1. Amino acid composition

The amino acid composition of the cereal flours are presented in **Table 2.3**. Between canaryseed cultivars, there were only minor differences in amino acid composition. The yellow C09052 and C05041 cultivars had a significantly higher ($p < 0.05$) valine content (5.9 g /100 g) as compared to the brown cultivars (5.2 g /100 g). The isoleucine content was significantly higher ($p < 0.05$) in the brown cultivars (4.8 g /100 g) than the yellow C09052 cultivar (4.2 g /100 g), but not significantly different ($p > 0.05$) than the yellow C05041 variety (4.3 g /100 g). In general, the overall amino acid content was comparable to what has been previously reported by Abdel-Aal et al. (1997b) in hairy brown canaryseeds and by the Canaryseed Development Comission of Saskatchewan (2016) in glabrous canaryseed groats. However, both studies reported lower amounts of certain amino acids, such as lysine, isoleucine, valine, and histidine as compared to the current study. The higher content of these specific amino acids in the currently studied hairless canary cultivars might be due to breeding induced improvement of the protein content and quality of these new experimental cultivars.

In general, the amino acid profile of canaryseeds remains comparable to that of wheat and oat except for their tryptophan and valine content. Canaryseeds contained significantly higher ($p < 0.05$) amounts of the essential amino acid tryptophan (2.4-2.6 g /100 g) than both oat (1.5 g/100 g) and wheat (1.1 g /100 g) flours. In addition, canaryseed cultivars had significantly higher ($p < 0.05$) amounts of valine (5.2-5.9 g /100 g) than both oat (3.4 g/100g) and wheat (4.1 g /100 g) flours. All cultivars were higher in arginine (5.7-6.1 g/100g) compared to wheat (3.8 g/100g). However, oat flour had significantly higher amounts of lysine (4.3 g /100g) as compared to both canaryseed (2.3-2.5 g /100g protein) and wheat (2.2 g /100g) flours. In cereal grains, the most limiting essential amino acid remains lysine. The lysine content of canaryseeds was comparable to other cereals such as millet (2.8 g /100g protein), higher than what has been reported in wheat (1.9 g /100g), but still inferior as compared to barley (3.9 g/100g protein) (Abdel-Aal et al., 1997b; Ejeta et al., 1987). Tryptophan, another essential amino acid normally lacking in cereals, makes canaryseeds a valuable source of tryptophan content (2.4-2.6 g /100g) as compared to both oat (1.5 g /100g) and wheat (1.1 g /100g). Therefore, combining canaryseeds with other cereal grains would be an excellent method to assure dietary demands for tryptophan in food formulations. In addition,

glutamic acid is considered as the most abundant amino acid in all cereal flours. Canaryseed cultivars showed significantly higher amounts than oat, barley, and millet as reported by several other studies (Abdel-Aal et al., 1997b; Biel et al., 2009; Ejeta et al., 1987). Overall, the total amino acid content of canaryseeds (86.3-89.5 g /100g) was not significantly different ($p > 0.05$) as compared to oat and wheat flours (86.2 g /100g), however, the total essential amino acids in canaryseeds ranged between 34.2-35.7 g /100g, which was significantly higher ($p < 0.05$) than the total essential amino acid content in oat (30.1 /100 g) and wheat (29.3 g /100 g) flours. The total essential amino acid content of canaryseed still remains inferior to high quality sources of protein such as casein (47.2 g /100g) (Abdel-Aal et al., 1997b).

Table 2.3. Amino acid composition comparison of glabrous canaryseed, oat, and wheat flours (g/100g protein)

Amino Acid	Cereal Variety					
	Yellow Canaryseeds		Brown Canaryseeds			
	C09052	C05041	Bastia	Calvi	Oat	Wheat
Aspartic acid	4.39 ± 0.25^{b}	4.52 ± 0.18^{b}	4.47 ± 0.07^{b}	4.47 ± 0.02^{b}	9.04 ± 0.16^{a}	4.09 ± 0.3^{b}
Glutamic acid	28.84 ± 0.79^{b}	28.60 ± 0.2^{bc}	26.56 ± 0.80^{c}	28.63 ± 0.96^{bc}	22.18 ± 0.37^{d}	35.04 ± 1.15^{a}
Serine	3.40 ± 0.12^{b}	3.25 ± 0.0^{b}	3.28 ± 0.13^{b}	4.45 ± 0.12^{b}	5.01 ± 0.06^{a}	4.64 ± 0.31^{a}
Histidine*	2.63 ± 0.23^{a}	2.65 ± 0.23^{a}	2.64 ± 0.24^{a}	2.69 ± 0.22^{a}	2.41 ± 0.03^{a}	2.56 ± 0.12^{a}
Glycine	2.52 ± 0.03^{c}	2.71 ± 0.18^{bc}	2.98 ± 0.19^{b}	2.82 ± 0.23^{bc}	4.28 ± 3.27^{a}	2.73 ± 0.17^{bc}
Threonine*	2.55 ± 0.27^{bc}	2.99 ± 0.04^{ab}	2.95 ± 0.15^{ab}	2.88 ± 0.18^{ab}	3.27 ± 0.04^{a}	2.33 ± 0.24^{c}
Arginine	5.66 ± 0.31^{a}	5.78 ± 0.09^{a}	6.02 ± 0.13^{a}	6.13 ± 0.17^{a}	6.17 ± 0.10^{a}	3.81 ± 0.41^{b}
Alanine	4.09 ± 0.10^{a}	4.07 ± 0.07^{a}	4.10 ± 0.13^{a}	4.28 ± 0.21^{a}	4.45 ± 0.04^{a}	2.88 ± 0.34^{b}
Tyrosine	2.12 ± 0.19^{b}	2.18 ± 0.17^{b}	2.37 ± 0.11^{b}	2.48 ± 0.18^{b}	3.41 ± 0.06^{a}	3.14 ± 0.17^{a}
Cystine	1.04 ± 0.11^{c}	1.15 ± 0.03^{bc}	1.36 ± 0.29^{abc}	1.59 ± 0.20^{a}	1.51 ± 0.04^{ab}	0.56 ± 0.00^{d}
Valine*	5.93 ± 0.06^{a}	5.91 ± 0.18^{a}	5.19 ± 0.20^{b}	5.15 ± 0.02^{b}	3.43 ± 0.07^{d}	4.08 ± 0.26^{c}
Methionine*	0.95 ± 0.06^{a}	1.06 ± 0.04^{a}	0.97 ± 0.12^{a}	1.04 ± 0.10^{a}	0.89 ± 0.22^{a}	0.97 ± 0.02^{a}
Tryptophan*	2.60 ± 0.02^{a}	2.44 ± 0.01^{c}	2.46 ± 0.07^{bc}	2.56 ± 0.04^{ab}	1.50 ± 0.05^{d}	1.11 ± 0.04^{e}
Phenylalanine*	5.68 ± 0.04^{ab}	6.02 ± 0.12^{a}	6.19 ± 0.17^{a}	6.23 ± 0.17^{a}	5.11 ± 0.08^{b}	5.27 ± 0.24^{b}
Isoleucine*	4.16 ± 0.28^{b}	4.31 ± 0.13^{ab}	4.83 ± 0.30^{a}	4.85 ± 0.17^{a}	2.49 ± 0.01^{c}	3.80 ± 0.20^{b}
Leucine*	7.43 ± 0.21^{ab}	7.37 ± 0.08^{ab}	7.69 ± 0.22^{ab}	7.86 ± 0.37^{a}	6.66 ± 0.09^{c}	7.02 ± 0.37^{bc}
Lysine*	2.27 ± 0.04^{b}	2.43 ± 0.19^{b}	2.48 ± 0.12^{b}	2.41 ± 0.06^{b}	4.34 ± 0.24^{a}	2.19 ± 0.11^{b}
Total AA	86.27 ± 1.91^{a}	87.44 ± 0.84^{a}	86.54 ± 0.85^{a}	89.53 ± 2.65^{a}	86.15 ± 0.60^{a}	86.24 ± 2.18^{a}
Total EAA	34.22 ± 1.35^{a}	35.18 ± 0.32^{a}	35.40 ± 0.99^{a}	35.67 ± 1.13^{a}	30.10 ± 0.15^{b}	29.34 ± 0.71^{b}

All values given are means of three determination means ± standard deviation
Means in a row with different lowercase letters are significantly different at $p < 0.05$ (n =3)
*Essential amino acid (EAA)

2.4.2.2. In vitro protein digestibility

Cereal flour proteins were digested using two *in vitro* digestion protocols. The degree of hydrolysis (DH) determined the extent to which cereal proteins were hydrolyzed or digested following the complex *in vitro* digestion protocol of Minekus et al. (2014) using enzymes amylase, pepsin, and pancreatin. The *in vitro* protein digestibility (IVPD) was also determined following the pH-drop method of Tinus et al. (2012) using enzymes trypsin, chymotrypsin, and protease. The DH and IVPD of cereal flours are presented in **Table 2.4**. Protein digestibility is an important aspect of protein quality, since it provides information regarding the capacity of a protein to provide dietary requirements of amino acids to tissues and organs in the body (Millward, Tomé, Schaafsma, & Layman, 2008). For all cereal varieties, the IVPD values were higher than the DH values. Aryee and Boye (2016) reported a similar trend in lentil flour when they compared the DH and IVPD using the same digestion protocols. The digestion protocols used to determine the DH and IVPD digestibility used different enzymes, enzyme to substrate ratios, hydrolysis times, and reaction conditions, which may attribute to the difference in protein digestibility values determined from the two methods. The degree of hydrolysis (DH) between canaryseed cultivars was not significantly different ($p > 0.05$) from each other. The DH between canaryseed (53.3-59.5%) and wheat (64.2%) flours was also not significantly different ($p > 0.05$), in addition, the DH from oat (44.3%) was not significantly different ($p > 0.05$) than the DH from the yellow C05041 (53.3%) and brown Bastia (53.3%) cultivars of canaryseeds. Similar protein digestibility values have been determined for sorghum (50.9-52.1%) (Afify, El-Beltagi, Abd El-Salam, & Omran, 2012) and buckwheat protein isolate (50.1-64.6%) (Tang, 2007). The IVPD between canaryseed flour varieties was not significantly different ($p > 0.05$) and ranged from 76.2% to 77.3%. Wheat had the highest IVPD (82.5%) whereas oat had the lowest (75.0%) but was not significantly different ($p > 0.05$) as compared to Bastia, Calvi, and C05041 varieties. Pea protein concentrate, lentil, pinto bean, and buckwheat flours led to similar IVPD values as canaryseed flours (Aryee & Boye, 2016; Çabuk et al., 2018; Nosworthy et al., 2017) using the same pH-drop method of Tinus et al. (2012).

Table 2.4. Protein digestibility of canaryseed flours as compared to oat and wheat

	DH (%)	IVPD (%)
	Digestion method	
Cereal Variety	Minekus et al. (2014)	Tinus et al. (2012)
Bastia (brown canaryseed)	53.33 (± 2.51)^{ab}	76.22 (± 0.58)^{bc}
Calvi (brown canaryseed)	58.92 (± 1.91)^{a}	76.58 (± 0.91)^{bc}
C09052 (yellow canaryseed)	59.45 (± 0.45)^{a}	77.30 (± 0.82)^{b}
C05041 (yellow canaryseed)	53.28 (± 1.55)^{ab}	76.16 (± 0.18)^{bc}
Oat	44.26 (± 2.47)^{b}	74.95 (± 0.38)^{c}
Wheat	64.22 (± 10.30)^{a}	82.49 (± 0.72)^{a}

Means in a column with different lowercase letters are significantly different at $p < 0.05$ (n =3)

DH: degree of hydrolysis, IVPD: *in vitro* protein digestibility

2.4.2.3. Protein quality (PDCAAS & DIAAS)

In this study, the PDCAAS and DIAAS values were determined from two different *in vitro* digestions protocols. The former protocol by Tinus et al. (2012) is a simple and rapid method that determines the IVPD, which is then used to calculate the digestibility of essential amino acids and their respective amino acid score. The latter protocol by Minekus et al. (2014) is the harmonized INFOGEST procedure that was created to improve experimental comparability between *in vitro* digestion results, which considers the enzymes, digestive fluids, and digestion parameters that occur during human gastrointestinal digestion. The DIAAS and the PDCAAS values are measurements of protein quality and provide information regarding the ability of a specific protein to provide sufficient amounts of essential amino acids for human requirements (Hughes, Ryan, Mukherjea, & Schasteen, 2011). In 2012, the DIAAS replaced the PDCAAS as the international standard for determining protein quality due several concerns with the PDCAAS method, particularly the lack of accountability of essential amino acid bioavailability (Marinangeli & House, 2017). Practical applications of the DIAAS and PDCAAS include evaluating and comparing the protein quality from separate dietary sources, in regulatory functions for classifying protein adequacy, such as health claims on food products purchased by consumers, and as a tool to help meet dietary demands for quality protein (because humans consume protein from several dietary sources) (FAO, 2013). High quality proteins possess DIAAS scores of at least 100%, providing all of the essential amino acid requirements if 0.66mg/kg of the protein is ingested per day (based on the average protein requirement for adult men and women) and include animal proteins such as egg, beef, casein, and whey protein isolate (Wolfe, 2015). In general, the PDCAAS and DIAAS scores from cereal sources are low. DIAAS scores of 75-99 are considered a "good source" of protein and includes chickpeas, soy protein isolate, soya flour, and herring egg protein (FAO, 2013; Havenaar et al., 2016; Marinangeli & House, 2017; Mathai, Liu, & Stein, 2017).

The FAO (2013) recommends that true ileal digestibility values be used to determine protein quality scores, which are obtained from the growing pig or laboratory rat. However, the values acquired from animal studies may not accurately reflect the true ileal digestibility of amino acids that occurs in humans. *In vivo* studies are also complex, time consuming, and extremely costly. Therefore, this study uses the complex human *in vitro* digestion protocol by Minekus et al. (2014)

to determine the *in vitro* true ileal digestibility of amino acids, and subsequently, their respective protein quality scores by the DIAAS method. To the best of our knowledge, this is the first time the INFOGEST protocol has been used to determine *in vitro* true ileal digestibility and protein quality scores (DIAAS). Furthermore, this is the first time protein quality scores have been calculated for glabrous canaryseeds.

Additionally, in order to mimic human digestion, additional enzymes from the brush border membrane, including peptidases, should be added during or after the intestinal phase of digestion. Therefore, the harmonized INFOGEST protocol still requires further standardization. Since pancreatin consists of a mixture of enzymes, including small amounts of peptidases, the leucine aminopeptidase activity in our commercial pancreatin from porcine pancreas used for *in vitro* digestion was tested and found to be 0.137 ± 0.001 mU/mg protein, where 1 unit is the amount of enzyme that generates 1 μmole of aminomethylcoumarin (AMC, substrate) per minute per mg of protein at pH 8.0 at 37°C. Mullally, O'Callaghan, FitzGerald, Donnelly, and Dalton (1994) reported slightly higher leucine aminopeptidase activity in commercial pancreatin (0.208 mU/mg protein).

The PDCAAS values determined for canaryseed, oat, and wheat flours are presented in **Table 2.5**. The results indicate that for all the cereal varieties studied, not surprisingly, lysine was the limiting essential amino acid. For each age group, the brown Bastia cultivar had lower PDCAAS values in comparison to brown Calvi, other yellow canaryseed cultivars, wheat, and oat. The latter exhibiting the highest PDCAAS values among the studied cereal grains. No significant difference ($p > 0.05$) in the PDCAAS values was observed between brown Bastia, yellow C09052 and C05041 canaryseed cultivars and wheat. Mathai et al. (2017) and Abelilla, Liu, and Stein (2018) reported PDCAAS values of 51(Lys) in wheat and 58(Lys) in oat for children (6 months-3 years), which was much higher than what was determined for wheat (32(Lys)) in this study, but similar to the PDCAAS value determined for oat (57(Lys)). Overall, the obtained PDCAAS values suggested the nutritional quality of canaryseeds was slightly better than wheat due to its higher lysine AA score (lysine content), but lower than oat. This is in good agreement with the reported higher amount of lysine in oat (**Table 2.3**) as compared to wheat and canaryseeds.

Table 2.5. Protein digestible corrected amino acid score (PDCAAS) of cereal flours

	Bastia	Calvi	C09052	C05041	Oat	Wheat
Infant (0-6 months)						
Limiting AA	Lysine	Lysine	Lysine	Lysine	Lysine	Lysine
AA score	0.33	0.42	0.41	0.40	0.63	0.32
IVPD (%)	76.22	76.58	76.16	77.30	74.95	82.49
PDCAAS (%)	25^{d}	32^{b}	32^{bcd}	30^{bc}	47^{a}	26^{cd}
Child (6 months-3 years)						
Limiting AA	Lysine	Lysine	Lysine	Lysine	Lysine	Lysine
AA score	0.40	0.51	0.50	0.48	0.76	0.38
IVPD (%)	76.22	76.58	76.16	77.30	74.95	82.49
PDCAAS (%)	31^{d}	39^{b}	38^{bc}	37^{bcd}	57^{a}	32^{cd}
Older children, adolescents, adults						
Limiting AA	Lysine	Lysine	Lysine	Lysine	Lysine	Lysine
AA score	0.48	0.61	0.59	0.58	0.90	0.46
IVPD (%)	76.22	76.58	76.16	77.30	74.95	82.49
PDCAAS (%)	36^{d}	47^{b}	46^{bc}	44^{bcd}	68^{a}	38^{cd}

Means in a row with different lowercase letters are significantly different at $p < 0.05$ (n =3)

AA score: content of the first limiting amino acid in the test protein (mg/g protein) / corresponding content of this amino acid in the FAO reference pattern (mg/g protein) (**Table 2.1**).

IVPD: *in vitro* protein digestibility

PDCAAS: product of AA score and IVPD(%)

The maximum TID values were possibly an overestimation of the true digestibility since the amino acids present in the digestate are not all in a readily bioavailable form, but could potentially be released from hydrolyzed polypeptides and other soluble proteins present in the digestate. The true digestibility could be referred as a value between the minimum and maximum TID values. For the majority of amino acids, the maximum TID values for each amino acid were greater than the calculated minimum TID values (**Table 2.6**). However, there are some discrepancies, such as the aromatic amino acids in canaryseeds and oat, where the minimum TID values range from 62-77% and the maximum TID values range from 52-67%. The observed discrepancies between minimum and maximum TID are potentially related the enzyme control which consisted of enzymes without the food matrix and was used to subtract amino acid content of the added enzymes from the total sample digest. In the absence of the substrate, an enzyme auto-digestion is likely occurring, which would increase the release of certain AA, thereby increasing their estimation in the free AA analysis in comparison to the total digestion of the whole enzyme control. There are also minimum TID values for amino acids that are not significantly different ($p > 0.05$) from their corresponding maximum TID values, such as lysine, methionine, and threonine for the canaryseed varieties and lysine for wheat, suggesting the digestibility of these particular amino acids are low for these cereal grains. Moreover, the maximum TID values reported for cysteine are far over 100% (137 – 298%) for canaryseed, oat, and wheat samples, which could be due to the AA analytical method not suitable for the determination of sulfur amino acids in protein hydrolysates.

Overall, the TID was comparable between brown and yellow canaryseed cultivars. Minimum TID values for cysteine, tyrosine, and aromatic amino acids and maximum TID values for alanine were higher for the yellow cultivars than the brown. Among the canaryseed cultivars, the yellow C05041variety had a higher ($p < 0.05$) total amino acid digestibility (30%) for the minimum TID as compared to the brown Bastia variety (26%). In terms of total amino acid digestibility, there was no significant difference ($p > 0.05$) between the investigated cereal varieties for the maximum TID values (64-72%); however, for the minimum TID, oat (32%) was significantly higher ($p < 0.05$) than Bastia, Calvi, and C09052 (26-28%) cultivars of canaryseeds; while wheat (22%) was significantly lower ($p < 0.05$) than the other cereals. For essential amino acids, lysine, histidine, and threonine, the minimum and maximum TID were significantly lower ($p < 0.05$) in canaryseeds compared to both oat and wheat.

Table 2.6. Minimum (bioaccessible) and maximum (total) *in vitro* true ileal digestibility (TID%) of glabrous canaryseed, oat, and wheat flours

Minimum TID (%)						
Amino Acid	**Cereal Variety**					
	Bastia	**Calvi**	**C09052**	**C05041**	**Oat**	**Wheat**
Alanine	27.15^{b}	28.78^{b}	32.45^{ab}	40.20^{a}	29.70^{b}	28.53^{b}
Arginine	71.59^{c}	85.22^{b}	85.00^{b}	90.79^{b}	105.20^{a}	116.00^{a}
Aspartic acid	0.90^{b}	0.83^{b}	0.40^{b}	1.19^{b}	6.29^{a}	7.19^{a}
Cystine	2.96^{c}	2.61^{c}	7.97^{c}	7.74^{c}	19.64^{b}	51.13^{a}
Glutamic acid	0.04^{b}	0.15^{b}	0.17^{b}	0.52^{b}	3.70^{b}	0.92^{ab}
Glycine	5.24^{b}	5.97^{b}	9.08^{ab}	9.39^{ab}	5.66^{b}	11.84^{a}
Histidine	5.95^{b}	5.14^{b}	5.57^{b}	5.53^{b}	27.81^{a}	19.04^{a}
Isoleucine	38.78^{c}	38.19^{c}	39.36^{bc}	44.55^{b}	61.43^{a}	38.14^{c}
Leucine	55.75^{b}	59.10^{ab}	57.26^{b}	62.40^{a}	49.43^{c}	36.83^{d}
Lysine	23.21^{b}	18.06^{bc}	17.35^{bc}	12.75^{c}	32.28^{a}	36.55^{ab}
Methionine	87.08^{bc}	105.08^{a}	90.24^{b}	73.31^{cd}	93.26^{ab}	59.58^{d}
Phenylalanine	59.23^{ab}	58.69^{b}	60.43^{ab}	66.36^{a}	57.51^{b}	35.05^{c}
Serine	9.11b	10.28b	13.38ab	16.05a	12.66ab	12.17ab
Threonine	17.99^{de}	17.65^{e}	22.75^{cd}	27.51^{bc}	45.22^{ab}	41.55^{a}
Tyrosine	75.98^{c}	70.04^{c}	106.57^{a}	104.09^{ab}	93.31^{b}	44.00^{d}
Valine	37.19^{b}	34.87^{bc}	27.22^{e}	28.93^{de}	50.91^{a}	31.36^{cd}
SAA (Cys+Met)	53.39^{a}	57.36^{a}	57.63^{a}	52.83^{a}	47.03^{a}	56.49^{a}
AAA (Phe+Tyr)	63.86^{b}	61.87^{b}	72.76^{a}	76.93^{a}	71.83^{a}	38.39^{c}
Sum of Free AA digestibility	25.93^{c}	26.45^{bc}	27.96^{bc}	29.78^{ab}	32.42^{a}	21.63^{d}
Maximum TID (%)						
Amino Acid	**Cereal Variety**					
	Bastia	**Calvi**	**C09052**	**C05041**	**Oat**	**Wheat**
Alanine	73.28^{d}	73.26^{d}	82.83^{bc}	88.93^{b}	76.35^{cd}	100.60^{a}
Arginine	90.02^{ab}	98.20^{a}	100.44^{a}	100.94^{a}	66.27^{c}	84.78^{b}
Aspartic acid	40.79^{c}	48.00^{bc}	44.50^{bc}	49.13^{bc}	58.77^{b}	88.56^{a}
Cystine	210.41^{bc}	198.19^{c}	218.71^{bc}	249.11^{b}	137.03^{d}	298.13^{a}
Glutamic acid	61.14^{b}	62.57^{b}	67.17^{ab}	65.42^{ab}	66.19^{ab}	68.80^{a}
Glycine	96.13^{bc}	108.31^{ab}	146.12^{a}	131.97^{ab}	27.12^{d}	56.41^{cd}
Histidine	22.34^{c}	19.88^{c}	25.69^{bc}	30.08^{b}	90.57^{a}	77.38^{a}
Isoleucine	47.59^{d}	47.70^{d}	52.17^{d}	57.68^{c}	114.46^{a}	72.41^{b}
Leucine	88.50^{b}	101.46^{a}	96.67^{ab}	102.65^{a}	63.22^{c}	60.14^{c}
Lysine	23.99^{b}	20.80^{b}	20.34^{b}	23.21^{b}	53.60^{a}	47.13^{a}
Methionine	87.64^{b}	81.17^{b}	84.57^{b}	90.97^{b}	149.89^{a}	135.50^{a}
Phenylalanine	65.14^{cd}	62.98^{d}	70.51^{bc}	76.59^{b}	85.30^{a}	77.29^{b}
Serine	102.24^{ab}	92.08^{b}	111.32^{a}	103.70^{a}	61.13^{c}	65.78^{c}
Threonine	16.60^{c}	17.64^{c}	20.08^{c}	21.70^{c}	77.01^{b}	98.40^{a}
Tyrosine	24.08^{d}	25.37^{cd}	35.69^{bc}	43.29^{ab}	42.35^{ab}	39.49^{a}
Valine	50.42^{c}	48.93^{c}	39.77^{d}	40.20^{d}	100.09^{a}	77.96^{b}
SAA (Cys+Met)	136.80^{b}	135.67^{b}	137.74^{b}	140.36^{b}	141.81^{b}	195.11^{a}
AAA (Phe+Tyr)	53.80^{bc}	52.45^{b}	61.21^{ab}	67.26^{a}	53.24^{c}	50.00^{c}
Sum of Total AA digestibility	64.19^{a}	65.54^{a}	69.75^{a}	70.87^{a}	67.53^{a}	71.78^{a}

Means in a row with different lowercase letters are significantly different at $p < 0.05$ (n =3)
Minimum TID was calculated as sum of free AA (mg) per g protein in supernatant digestate - (free AA (mg) per g protein of enzymes) / sum of total AA (mg) per g protein in cereal flour; while maximum TID was calculated as sum of total AA (mg) per g protein in supernatant digestate - (total AA (mg) per g protein of enzymes) / sum of total AA (mg) per g protein in cereal flour.

The minimum and maximum DIAAS values are represented in **Tables 2.7** and **2.8**, respectively. The minimum DIAAS scores in canaryseeds for infants, children, and older children/adolescents/adults ranged from 5-8%, 6-9%, and 8-11%, respectively. The maximum DIAAS scores in canaryseeds for infants, children, and older children/adolescents/adults ranged from 8-10%, 9-12%, and 11-14%, respectively. Between canaryseed cultivars there was no significant difference ($p > 0.05$) in DIAAS values except between the minimum DIAAS values for the Bastia and C05041 cultivars, where for each age group, the DIAAS score was higher in the Bastia cultivar than the C05041 variety. For minimum DIAAS scores (**Table 2.7**), C05041 is significantly lower ($p < 0.05$) than wheat, whereas, for maximum DIAAS scores (**Table 2.8**), both Bastia and C09052 are significantly lower ($p < 0.05$) than wheat. For each age group, the DIAAS scores in canaryseeds are comparable to those of wheat (12-15%, 14-18%, and 17-22% for infants, children, and adults, respectively) but significantly lower ($p < 0.05$) than DIAAS scores determined for oat (20-34%, 25-41%, and 29-49%, for infants, children, and adults, respectively).

The protein quality indicated by the DIAAS scores of canaryseed flours is comparable to that of wheat, and, in general, protein quality scores for cereal grains are low. DIAAS values have been calculated for many cereal grains, but, it remains difficult to compare these values since different methods were used to determine the TID of amino acids. Cervantes-Pahm, Liu, and Stein (2014) reported DIAAS values (determined from growing pigs) for older children, adolescents and adults for barley (51), oat (77), rye (47), sorghum (29), and wheat (43), for which lysine was the limiting amino acid in each grain. These values are significantly higher than the DIAAS values determined for oat and wheat in the present study. Han et al. (2019) reported DIAAS values (determined from growing rats) for older children, adolescents and adults for cooked brown rice (42), buckwheat (68), oats (43), millet (10), adlay (13), and whole wheat (20), for which lysine was the limiting amino acid in each grain except for buckwheat (SAA). The *in vitro* DIAAS values determined for canaryseeds in children (8-12) are similar to those reported in millet and adlay, which are all members of the grass family *Poaceae*. The *in vitro* DIAAS values determined for canaryseed flours indicate the cereal grain must be supplemented with other dietary protein sources in order to meet dietary digestible amino acid requirements.

Table 2.7. Minimum digestible indispensable amino acid score (DIAAS) of canaryseed, oat and wheat proteins determined from free (bioaccesscible) amino acids after *in vitro* digestion

Minimum DIAAS Scores									
	Indispensable amino acids (IAA)								
Cereal variety	Histidine	Threonine	Valine	Isoleucine	Leucine	Lysine	SAA	AAA	**DIAAS (%)**
Infant DIAA reference ratio (0-6 months)									
Bastia	0.08	0.11	0.34	0.33	0.49	0.08	0.29	0.66	8 (Lys)^{b}
Calvi	0.08	0.12	0.37	0.36	0.50	0.08	0.35	0.71	8 (Lys)^{bc}
C09052	0.07	0.12	0.35	0.33	0.49	0.07	0.32	0.72	7 (Lys)^{bc}
C05041	0.06	0.13	0.37	0.35	0.50	0.05	0.31	0.73	5 (Lys)^{c}
Oat	0.32	0.34	0.32	0.28	0.34	0.20	0.34	0.64	20 (Lys)^{a}
Wheat	0.23	0.22	0.23	0.26	0.27	0.12	0.26	0.34	12 (Lys)^{b}
Child DIAA reference ratio (6 months-3 years)									
Bastia	0.09	0.15	0.43	0.57	0.71	0.09	0.35	1.20	9 (Lys)^{b}
Calvi	0.09	0.17	0.48	0.62	0.73	0.09	0.42	1.29	9 (Lys)^{bc}
C09052	0.08	0.17	0.44	0.56	0.71	0.08	0.40	1.31	8 (Lys)^{bc}
C05041	0.07	0.18	0.47	0.60	0.73	0.06	0.38	1.33	6 (Lys)^{c}
Oat	0.33	0.48	0.41	0.48	0.50	0.25	0.41	1.16	25 (Lys)^{a}
Wheat	0.24	0.31	0.30	0.45	0.39	0.14	0.32	0.62	14 (Lys)^{b}
Older child, adolescent, adult DIAA reference ratio									
Bastia	0.11	0.19	0.47	0.61	0.77	0.11	0.41	1.52	11 (Lys)^{b}
Calvi	0.09	0.23	0.51	0.64	0.79	0.08	0.45	1.68	11 (Lys)^{bc}
C09052	0.10	0.21	0.48	0.60	0.77	0.10	0.46	1.66	10 (Lys)^{bc}
C05041	0.11	0.21	0.51	0.67	0.79	0.11	0.50	1.63	8 (Lys)^{c}
Oat	0.42	0.59	0.44	0.51	0.54	0.29	0.48	1.48	29 (Lys)^{a}
Wheat	0.30	0.39	0.32	0.48	0.42	0.17	0.37	0.79	17 (Lys)^{b}

Means in a row with different lowercase letters are significantly different at $p < 0.05$ (n =3)

IAA: Indispensable amino acid

DIAA reference ratio: Ratio of the digestible indispensable amino acid content (mg/g protein) in the test protein to the corresponding content of the same amino acid in the FAO reference pattern (mg/g protein) (**Table 2.1**).

Table 2.8. Maximum digestible indispensable amino acid score (DIAAS) of canaryseed, oat and wheat proteins determined from free (bioaccesscible) amino acids after *in vitro* digestion

Maximum DIAAS Scores									
	Indispensable amino acids (IAA)								
Cereal variety	Histidine	Threonine	Valine	Isoleucine	Leucine	Lysine	SAA	AAA	**DIAAS (%)**
	Infant DIAA reference ratio (0-6 months)								
Bastia	0.31	0.10	0.46	0.41	0.77	0.08	0.73	0.56	8 (Lys)^{c}
Calvi	0.32	0.12	0.52	0.45	0.86	0.09	0.82	0.60	9 (Lys)^{bc}
C09052	0.34	0.10	0.50	0.43	0.82	0.08	0.77	0.61	8 (Lys)^{c}
C05041	0.35	0.10	0.51	0.45	0.83	0.10	0.83	0.64	10 (Lys)^{bc}
Oat	1.04	0.57	0.63	0.52	0.44	0.34	1.03	0.48	34 (Lys)^{a}
Wheat	0.94	0.52	0.58	0.50	0.44	0.15	0.90	0.45	15 (Lys)^{b}
	Child DIAA reference ratio (6 months-3 years)								
Bastia	0.31	0.14	0.57	0.68	1.10	0.09	0.86	0.93	9 (Lys)^{c}
Calvi	0.33	0.17	0.67	0.78	1.25	0.11	1.00	1.09	11(Lys)^{bc}
C09052	0.36	0.15	0.65	0.74	1.20	0.10	0.94	1.10	10 (Lys)^{c}
C05041	0.37	0.14	0.66	0.77	1.20	0.12	1.02	1.16	12 (Lys)^{bc}
Oat	1.09	0.81	0.80	0.89	0.64	0.41	1.26	0.87	41 (Lys)^{a}
Wheat	0.99	0.74	0.74	0.86	0.64	0.18	1.10	0.81	18 (Lys)^{b}
	Older child, adolescent, adult DIAA reference ratio								
Bastia	0.39	0.17	0.61	0.72	1.19	0.11	1.01	1.18	11 (Lys)^{c}
Calvi	0.42	0.21	0.72	0.83	1.36	0.13	1.17	1.39	13 (Lys)^{bc}
C09052	0.45	0.18	0.69	0.79	1.30	0.12	1.11	1.39	12 (Lys)^{c}
C05041	0.46	0.18	0.70	0.83	1.30	0.14	1.20	1.47	14 (Lys)^{bc}
Oat	1.36	1.01	0.86	0.95	0.69	0.49	1.48	1.11	49 (Lys)^{a}
Wheat	1.24	0.92	0.80	0.92	0.69	0.22	1.29	1.03	22 (Lys)^{b}

Means in a row with different lowercase letters are significantly different at $p < 0.05$ (n =3)
IAA: Indispensable amino acid
DIAA reference ratio: Ratio of the digestible indispensable amino acid content (mg/g protein) in the test protein to the corresponding content of the same amino acid in the FAO reference pattern (mg/g protein) (**Table 2.1**).

2.4.3. Anti-nutritional components

Cereals possess several anti-nutritional factors that can affect its protein digestion, the most common being phytic acid, trypsin inhibitor, and tannins (Sarwar Gilani et al., 2012). In general, anti-nutritional components can reduce the digestion, bioavailability, and absorption of beneficial nutrients, including proteins, peptides, and amino acids, and hence affect the overall nutritional quality. For instance, by decreasing the enzyme activity, trypsin inhibitors can affect protein availability and nutrient absorbance, thereby inhibiting the human growth (Abdel-Aal, Hucl, Shea Miller, et al., 2011). Phytate is considered as an anti-nutritional compound because of its ability to bind proteins leading to reduced absorption during digestion. Conversely, dietary phytate has also shown to lower kidney stone formation, reduce the effects of atherosclerosis in coronary heart disease, and protect against several cancers (Greiner & Konietzny, 2005). In alkaline conditions, polyphenols are oxidized to form quinones. Quinones can further react with side chain amino groups of proteins to form irreversible complexes with sulfhydryl and amino groups of proteins, which are known to reduce the digestibility and bioavailability of protein bound lysine and cysteine, the former being an essential amino acid (Ozdal, Capanoglu, & Lokumcu Altay, 2013). In addition, flavonoids (a group of polyphenolic compounds that includes tannins), chelate metal ions such as zinc and iron, react with digestive enzymes, and can precipitate proteins, which interferes with the absorption and digestion of essential minerals and proteins, respectively (Adamczyk, Simon, Kitunen, Adamczyk, & Smolander, 2017; Karamać, 2009). Polyphenols can also bind proteins via reversible (hydrogen bonding, Van der Walls forces, hydrophobic/hydrophillic interactions) and irreversible (covalent bonds) mechanisms, which may alter the protein physio-chemical properties (solubility, thermal stability) and their nutritional properties (digestibility, amino acid modification) (Ozdal et al., 2013). Nonetheless, the high polyphenol content of some selected cereals can be considered as health beneficial property because of their strong antioxidant activity and proven effects on cancer and cardiovascular disease treatment. In these regards, the trypsin inhibitor, the phytate, and the total polyphenol content were quantified in the investigated cereals.

Trypsin inhibitor activity expressed in canaryseed flour was comparable to those present in both oat and wheat (**Table 2.9**). Wheat had a slightly lower TIA (0.114 mg/g) than the brown canaryseed cultivars (0.149 and 0.161 mg/g for Bastia and Calvi, respectively) but was not

significantly different ($p > 0.05$) as compared to the yellow cultivars (0.133 and 0.121 mg/g for C09052 and C05041, respectively). Abdel-Aal, Hucl, Patterson, et al. (2011) reported no significant difference between the trypsin inhibitor content in a hairless brown variety (CDC Maria) as compared to wheat. The TIA in all canaryseed isolates were not significantly different from one another ($p > 0.05$), but TIA in brown Calvi flour was significantly higher ($p < 0.05$) than in canaryseed protein isolates.

The results (**Table 2.9**) show that phytate content in canaryseed flour was the highest followed by oat flour, and then wheat. There was no significant difference ($p > 0.05$) in the phytate content between the different canaryseed flour varieties. Abdel-Aal, Hucl, Patterson, et al. (2011) also reported a higher content of phytate in a hairless brown cultivar of canaryseeds (18.8 mg/g) as compared to wheat (10.7 mg/g). However, the phytate content in canaryseed flour is comparable to that reported for other cereals such as buckwheat (9.2-16.2 mg/g), amaranth (10.6-15.1 mg/g), sorghum (5.9-11.8 mg/g), and legumes such as black beans (8.5-17.3 mg/g), kidney beans (8.3-13.4 mg/g), and soybeans (9.2-16.7 mg/g) (Greiner & Konietzny, 2005). The results (**Table 2.9**) also show that phytate in canaryseed protein isolates was significantly lower ($p < 0.05$) than canaryseed flours, but still higher than in oat and wheat, except for the C09052 yellow isolate. This last canaryseed protein isolate (C09052, 6.888 mg/g) exhibited a close phytate content to oat flour (5.938 mg/g).

TPC of canaryseed flours ranged from 1.34-1.47 mg FAE/g of flour with no significant difference in TPC between yellow and brown cultivars of canaryseed flours (**Table 2.9**), which was also reported by Li et al. (2011). Compared to wheat, canaryseed flours showed an exceptionally higher TPC ($p < 0.05$). The highest TPC was obtained in oat flour (2.04 mg FAE/g), while wheat flour exhibited the lowest TPC (0.65 mg FAE/g). Comparing the TPC of glabrous canaryseeds with previously conducted experiments is difficult because of the lack of method standardization, in particular the polyphenol extraction and their quantification. Li et al. (2011) reported an average TPC in yellow canaryseed flours of 130 mg of FAE/100 g of sample. Chen et al. (2016) reported a TPC of 55.5 mg GAE/100 g of sample in brown CDC Maria canaryseeds using gallic acid as a polyphenol standard.

The TPC in brown Bastia (2.17 mg FAE/g) and Calvi (2.02 mg FAE/g) protein isolates was significantly higher ($p < 0.05$) than the yellow C05041 protein isolate (1.77 mg FAE/g). Overall, the polyphenol content in canaryseed protein isolates (1.77-2.17 mg FAE/g) was significantly higher ($p < 0.05$) as compared to canaryseed flours. This phenomenon has been reported in a variety of other plant sources, including papaya seeds (Oseni, Gbadamosi, Olawoye, & Akanbi, 2016), amaranth (Castel, Andrich, Netto, Santiago, & Carrara, 2014), and soybean (Chamba, Hua, Dawa, Odilon, & Zhang, 2013). An organic solvent was used to extract the soluble phenolic acids, however, there are several non-extractable phenolics left behind in the residue, and in cereal grains, consist mainly of ferulic acid derivatives (Pérez-Jiménez & Torres, 2011). During the preparation of the canaryseed isolates, some of the esterified and bound phenolic acids may have been released, which increases the TPC content in the protein isolates. In addition, the Folin reagent used in the analysis reacts strongly with proteins and amino acids, particularly tyrosine and tryptophan (both present in the canaryseed protein isolates), thereby resulting in an overestimation of the TPC (Folin & Ciocalteu, 1927). The color of the canaryseed isolates produced from brown cultivars were extremely brown in color as compared to the yellow canaryseed isolates, which were light brown in comparison. This may be due to differences in color between the actual seeds or because of a higher potential of the brown cultivars to form tannins. Li et al. (2011) determined a canaryseed acetone extract was high in flavonoid glycosides, with yellow and brown cultivars of hairless canaryseeds having similar flavonoid profiles, and Abdel-Aal, Hucl, Patterson, et al. (2011) found no condensed tannins in the hairless, brown CDC Maria canaryseed.

Table 2.9. Anti-nutritional components of glabrous canaryseed, oat, and wheat flours and canaryseed protein isolates

Cereal Variety	Yellow Canaryseeds		Brown Canaryseeds		Oat	Wheat	Yellow Canaryseeds		Brown Canaryseeds	
	C09052	C05041	Bastia	Calvi			C09052	C05041	Bastia	Calvi
	Flours						Isolates			
TIA (mg/g)	0.13^{abc}	0.12^{bc}	0.15^{ab}	0.16^{a}	0.14^{abc}	0.114^{c}	0.12^{bc}	0.12^{bc}	0.12^{bc}	0.12^{bc}
Phytate (mg/g)	12.36^{a}	11.96^{a}	12.32^{a}	12.12^{a}	5.94^{e}	3.139^{f}	6.89^{de}	7.94^{cd}	8.92^{bc}	9.53^{b}
TPC (mg FAE/g)	1.47^{d}	1.34^{d}	1.43^{d}	1.42^{d}	2.04^{ab}	0.646^{e}	1.90^{bc}	1.77^{c}	2.17^{a}	2.02^{ab}

Means in a row with different lowercase letters are significantly different at $p < 0.05$ (n =3)

TIA: mg of pure trypsin inhibited per gram of sample

Phytate: mg of phytate per gram of sample

TPC: mg of ferulic acid equivalents per g of sample

2.5. CONCLUSION

The protein content of canaryseeds is high as compared to other cereals, including oat and wheat. The nutritional quality of a protein depends not on protein quantity but the ability of a protein source to provide individuals with dietary requirements of bioavailable and digestible essential amino acids. The PDCAAS and DIAAS scores of oat were highest. The scores for canaryseeds were low, but not significantly different to those of wheat. The low protein quality scores are attributed to its lower lysine content as well as lower lysine digestibility in the seeds. Cereals in general have low scoring DIAAS and PDCAAS values. This does not mean the overall protein quality of the cereal is low, however, the cereal, such as canaryseeds, must be consumed with other proteins sources in order to obtain nutritional requirements for essential amino acids. In addition to essential amino acid requirements, protein quality is also related to its health promoting effects and the presence of anti-nutritional factors. The *in vitro* digestion model used in this study is a rapid and simple approach to compare protein quality scores between different cereal varieties, however, it does have limitations and future studies should evaluate canaryseed DIAAS scores using in vivo studies. The anti-nutritional components in canaryseeds, including trypsin inhibitor activity, phytic acid and total polyphenols are comparable to oat, wheat, and other cereals and legumes. Although the protein quality scores in canaryseeds are low, their total amino acid digestibility is high and comparable to oat and wheat. In addition, they possess high amounts of tryptophan, which is generally lacking in most cereals, and could be combined with other cereals to improve their overall quality.

CONNECTING STATEMENT II

The protein quality and the anti-nutritional components of hairless canaryseed proteins were evaluated and compared between several hairless canaryseed varieties and common cereals in Chapter II.

Chapter III investigates the bioactive properties and health promoting effects of canaryseed proteins as compared to common cereal grains, including their antioxidant, chelating, antihypertensive, and antidiabetic activity *in vitro*. Canaryseed proteins exhibiting high bioactivity were further purified by size exclusion chromatography and identified by mass spectroscopy. The peptide sequences were analyzed *in silico* to determine potential peptide bioactivity.

CHAPTER III

BIOACTIVE PROPERTIES OF CANARYSEED PROTEINS AND IDENTIFICATION OF PEPTIDES RESPONSIBLE FOR HEALTH POSITIVE EFFECTS

3.1. ABSTRACT

Two yellow (C09052, C05041) and two brown (CDC Calvi, CDC Bastia) hairless canaryseed flour cultivars and two commercial cereal flours (oat and wheat) were digested via an *in vitro* digestion system using enzymes amylase, pepsin, chymotrypsin and pancreatin. The hydrolysates were filtered through a 3K MWCO membrane to isolate small peptides and screened for their antioxidant (ORAC, DPPH, ABTS), chelating (Fe^{2+}), antihypertensive (ACE inhibition), and antidiabetic (DPP-IV inhibition) activities. Between yellow and brown canaryseed cultivars, there was no significant difference in bioactivity, except for DPPH and Fe^{2+} assays, where brown cultivars had higher activity which could indicate brown cultivars of canaryseeds might be better free radical scavengers compared to the yellow cultivars. For all bioactivity assays, canaryseeds had superior or equivalent activity as compared to oat and wheat. ORAC values of Calvi hydrolysates (1.99 μmol TE/mg) was higher than oat (1.31 μmol TE/mg) and wheat (1.54 μmol TE/mg). IC_{50} values for the ABTS assay was not significantly different between the C09052 yellow cultivar (114.85 μg/mL) and wheat (107.86 μg/mL). ACE inhibition activity (expressed as IC_{50} values) in C09052, C05041, and Calvi were 333.34 μg protein/mL, 405.04 μg protein/mL, and 321.91 μg protein/mL, respectively, and significantly higher than oat (570.10 μg protein/mL) and wheat (781.19 μg protein/mL). The DPP-IV inhibitor IC_{50} values were not significantly different between C09052, C05041, Bastia, and wheat (1.00-1.35 mg protein/mL) but was significantly lower than oat (2.29 mg protein/mL). Further purification of the C09052 peptide digest by size exclusion chromatography revealed several peaks, the most abundant peaks ranging between 226-1355 Da. Each peak was collected and the ACE inhibitor activity of each peak was determined. The peptides from the peak with highest ACE inhibition activity were identified by LC-MS. Forty-six peptides were identified belonging to 18 proteins from the *Pooideae* subfamily. Fourteen of the parent proteins were from barley origins. Peptides were analyzed *in silico* to determine potential bioactivity based on their amino acid composition. All 46 peptides had potential ACE inhibitor and DPP-IV activity, and 20 had potential antioxidant activity, which has been validated *in vitro*. Canaryseed peptides also had potential hypotensive, antiamnestic, antithrombotic, immunostimulating, opioid and neuro activity. Canaryseeds demonstrate exceptional health promoting effects, particularly against cardiovascular disease.

3.2. INTRODUCTION

Plant proteins, including those from cereals (wheat, barley, oat,), pseudo-cereals (buckwheat, amaranth,), and legumes (soybean, beans, peas), are known for producing bioactive peptides with exceptional health promoting effects when digested (Malaguti et al., 2014). Bioactive peptides are small and active protein fragments produced by proteolytic enzymes during protein digestion. They have demonstrated a variety of bioactivities, including antioxidant, antihypertensive, antibacterial, antithrombic, anticancer, antidiabetic, immunomodulating, and opioid activity, which depends on the source protein, amino acid composition and sequence (Cavazos & Gonzalez de Mejia, 2013; Sánchez & Vázquez, 2017). Finding new sources of good quality plant proteins, other than the traditional sources, remains essential to meet the global growing demands for protein. The overall quality of a protein depends not only on its digestibility and amino acid bioavailability, but also the health promoting bioactive properties it demonstrates once digested (FAO, 2013; Malaguti et al., 2014). Glabrous canaryseeds are extremely high in protein (22%) as compared to other cereal grains (oat, wheat, barley) and present a new source of plant protein that could be utilized by the food industry and consumers.

Some regions of the world, including Mexico, have utilized hairy canaryseeds as a traditional folk medicine to help combat chronic societal diseases including cardiovascular disease and diabetes (Estrada-Salas et al., 2014). Until recently, no scientific evidence proved the effect of these claims but studies have since been conducted on hairy canaryseed (*Phalaris canariensis* L.) proteins demonstrating antioxidant (Valverde et al., 2017), antidiabetic (Estrada-Salas et al., 2014), and antihypertensive activity (Estrada-Salas et al., 2014; Valverde et al., 2017) due to the presence of bioactive peptides. These studies were conducted on hairy varieties of canaryseeds, and although the nutritional value is comparable to the hairless varieties, the bioactivity of the newly approved glabrous Canadian seeds remains unknown.

The overall objective of this study was to evaluate the health promoting effects associated with consuming the newly developed Canadian hairless canaryseeds. This was accomplished by the following specific objectives: (1) Determining the bioactive properties of hairless canaryseed proteins hydrolysates through the screening of bioactivity assays and, (2) Purifying and identifying the canaryseed peptide(s) responsible for demonstrating the greatest selected bioactivity.

3.3. MATERIALS AND METHODS

3.3.1. Materials

Dehulled seeds from four hairless canaryseed (*Phalaris canariensis L.*) cultivars (two yellow cultivars C09052, and C05041 (now registered as cultivar CDC Cibo), and two brown CDC Calvi and CDC Bastia cultivars), one oat (*Avena sativa)* cultivar (Turcotte) and one wheat (*Triticum aestivum L.*) cultivar (Snowbird) were used in this study. The canaryseed cultivars were kindly donated by Dr. Pierre Hucl from the Crop Development Centre of the University of Saskatchewan (Saskatoon, SK). Oat and wheat seeds were purchased from Semican (Princeville, QC). All seeds were hand-cleaned to remove any broken seeds or foreign material.

Phosphate buffered saline (PBS), sodium bicarbonate ($NaHCO_3$), monosodium phosphate (NaH_2PO_4), hydrochloric acid (HCl), sodium chloride (NaCl), and Tris-HCl were purchased from BioShop (Burlington, ON). 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH), trolox, fluorescein, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulfate ($K_2S_2O_8$), ferrous chloride ($FeCl_2$), ferrozine, borax ($Na_2B_4O_7 \cdot 10H_2O$), angiotensin converting enzyme (ACE) from rabbit lung (A6778), N-Hippuryl-His-Leu hydrate (HHL) substrate (H1635), hippuric acid, trifluoroacetic acid (TFA), gly-pro p-nitroanilide hydrochloride (G0513), and mixed standards for the size exclusion chromatography analysis (aprotinin., vitamin B_{12}, Gly-Gly-Gly, and carnosine) were all purchased form Sigma (St. Louis, MO). Dimethyl sulfoxide (DMSO), ethanol, and methanol were obtained from Fisher Scientific (Fair Lawn, NJ). The dipeptyl peptidase IV enzyme from porcine kidney (CD26, 317640) was purchased from Millipore-Sigma (Burlington, MA). Deionized water (Millipore) was used in all experiments.

3.3.2. Preparation of protein hydrolysates

The protein hydrolysates were prepared according to the standardized *in vitro* digestion method of Minekus et al. (2014), a method standardized by INFOGEST. In the oral phase of digestion, 1 g of flour (canaryseed, oat, and wheat) was incubated for 2 minutes at 37 °C, pH 7.0, with simulated salivary fluid (SSF) (1:1) containing α-amylase from porcine pancreas (75 U/mL of digest). Then, the mixture was diluted (1:1, v/v) with simulated gastric fluid (SGF) containing pepsin from porcine gastric mucosa (2000 U/mL digest). The pH of the mixture was adjusted to pH 3.0 and then incubated for 2 hours at 37 °C. Intestinal phase was carried out by diluting the mixture (1:1,

v/v) with simulated intestinal fluid (SIF) containing pancreatin from porcine mucosa (100 U trypsin activity/mL digest) and bile (10 mM). The pH of the mixture was adjusted to pH 7.0 and incubated for 2 hours at 37 °C. The reaction was stopped by placing the solutions on ice and adding 1 mM AEBSF (protease inhibitor). The final digests were centrifuged, and the supernatants filtered through an Amicon 3K molecular weight cut off (MWCO) ultrafiltration unit (Millipore, Burlington, MA). Protein content in the permeate was quantified by the Pierce BCA protein assay kit (Thermo-Scientific, Waltham-MA) using bovine serum albumin as a standard. Filtered samples were frozen at -80 °C until used for analysis.

3.3.3. Characterization of the molecular weight distribution of protein hydrolysates

SEC-HPLC was carried out according to the modified method of Achouri et al. (2010). The collected permeates of the 3K MWCO ultrafiltrations were separated using an Enrich SEC-70 column (10 × 300 mm) (Bio-Rad Laboratories, Mississauga, ON) connected to an Agilent-1200 Series HPLC system (Agilent Technologies Canada Inc., Mississauga, ON,). The protein solution (2.5 µL) was loaded on the column and eluted with 10 mM phosphate buffered saline, with 154 mM NaCl (pH 7.4) at a flow rate of 0.5 mL/min at 220 nm. Mixed standards comprising aprotinin (6,511 kDa), vitamin B_{12} (1,355 kDa), Gly-Gly-Gly (tripeptide, 189.17 Da) and carnosine (dipeptide, 226.23 Da) were mixed and used to estimate the molecular weight distribution of the canaryseed, oat, and wheat sample hydrolysates.

3.3.4 Determination of total polyphenol content (TPC) of protein hydrolysates

The total polyphenol content (TPC) of the hydrolysates was determined using the Folin-Ciocalteu reagent according to the modified method of Singleton and Rossi (1965). Ferulic acid, prepared in 70% ethanol containing 1% concentrated HCl, was used to generate the standard curve at concentrations between 0 to 500 mg/L. 1.5 mL of Folin-Ciocalteu reagent (diluted 10x with water) was added to 200 µL of blanks, standards and sample hydrolysates, followed by the addition of 7.5% [w/v] sodium bicarbonate solution after 5 minutes (at room temperature). After an additional 90 minutes incubation at room temperature, the sample tubes were centrifuged at 6,000 x g for 15 minutes and the absorbance of the supernatants were read at 750 nm. The TPC content in the samples was determined from the ferulic acid standard curve, and results were expressed as mg ferulic acid equivalents (FAE) per g of protein in the 3K MWCO permeate.

3.3.5. Determination of the antioxidant and chelation activity

3.3.5.1. Oxygen radical absorption capacity (ORAC) assay

The experimental work was carried out according to the method of Tomer et al. (2007), with modification by Garrett, Murray, Robison, and O'Neill (2010). AAPH (2,2'-azobis(2-amidino-propane) dihydrochloride; 79.65 mM) was prepared fresh in 75 mM phosphate buffer (pH 7.4). A fluorescein stock solution (1.2 µM) was prepared in advance with 10 mM PBS and stored at 4 °C, protected from light. The fluorescein working solution (0.96 nM) was prepared fresh from the fluorescein stock solution using 75 mM phosphate buffer (pH 7.4). Trolox, prepared in 75 mM phosphate buffer (pH 7.4) containing 5% DMSO, was used to generate the standard curve (6.25-100 µM). 25 µL of samples (hydrolysates), standards, and blanks were loaded onto a black, clear bottomed 96 well microplate. 150 µL of 0.96 nM fluorescein was added to each well and then incubated for 30 minutes at 37 °C. After the incubation period, 25 µL of 79.65 mM AAPH was injected into each well using an automatic injector and the fluorescence monitored using a Synergy HTX fluorescence reader (Bio-Tek, Winooski, VT) for 120 minutes at 37 °C with an excitation and emission wavelength of 485 nm and 520 nm, respectively (20 nm bandpass). The AUC (area under the curve) was calculated by the Bio-Tek software from the following equation:

$$\text{AUC} = \frac{R1}{R2} + \frac{R2}{R1} + \frac{R3}{R1} + \cdots \frac{Rn}{R1}$$

Where R1 is the initial reading and Rn is the last reading taken. The Net AUC is calculated by subtracting the AUC of the blank from the AUC of the sample or standard:

$$\text{Net AUC} = \text{AUC}_{\text{sample/standard}} - \text{AUC}_{\text{blank}}$$

The standard curve is obtained by plotting the Net AUC of the trolox dilution samples against their respective concentrations. The results are expressed as µmol of trolox equivalents (TE) per mg of protein in the 3K MWCO permeate.

3.3.5.2. DPPH assay

The DPPH assay was carried out according to Orona-Tamayo, Valverde, Nieto-Rendón, and Paredes-López (2015), with modification. Briefly, an aliquot of hydrolysate was diluted at different protein concentrations and 100 µL of diluted samples were added to a 96 well flat bottom microplate, followed by 100 µL of 0.05 mM DPPH in ethanol (stored at -20°C). The plate was

incubated for 30 minutes in the dark at room temperature, and the absorbance read at 517 nm using an Epoch microplate spectrophotometer (Bio-Tek, Winooski, VT). The data was converted into a percentage of radical scavenging activity from the following equation:

$$\text{DPPH inhibition (\%)} = 1\text{-}(\frac{Abs_{sample}}{Abs_{control}}) \text{ x } 100\%$$

Where Abs_{sample} was the absorbance of the sample and $Abs_{control}$ was the absorbance of ethanol without added inhibitor. The DPPH inhibition was plotted against its respective concentration to determine the IC_{50} value, the inhibitory concentration required to scavenge 50% of the DPPH radical, which was calculated by the Epoch software from a four-parameter logistic curve.

3.3.5.3. ABTS assay

The ABTS assay was carried out according to Re et al. (1999), with modification by Chen & Al, 2016. A 7 mM ABTS solution in water containing 2.45 mM potassium persulfate was prepared. The absorption of the $ABTS^+$ working solution was adjusted to 0.700 ± 0.02 absorbance at 734 nm using 100% ethanol at room temperature. 50 µL of appropriately diluted samples were added to a 96 well flat bottom microplate, followed by 180 µL of $ABTS^+$ working solution. After incubating the microplate for 6 minutes in the dark at room temperature, the absorbance was read at 734 nm using an Epoch microplate spectrophotometer. The data was expressed as a percentage of radical scavenging activity from the following equation:

$$\text{ABTS inhibition (\%)} = 1\text{-}(\frac{Abs_{sample}}{Abs_{control}}) \text{ x } 100\%$$

Where Abs_{sample} is the absorbance of the sample, and $Abs_{control}$ refers to the absorbance of 50% ethanol without added inhibitor. The ABTS inhibition was plotted against its respective concentration to determine the IC_{50} value; the inhibitory concentration required to scavenge 50% of the ABTS cation was calculated by the Epoch software from a four-parameter logistic curve.

3.3.5.4. Iron chelating activity (Fe^{2+}) assay

The assay was carried out according to the modified method of Orona-Tamayo et al. (2015). 50 µL of appropriately diluted sample concentrations were added to a 96 well flat bottom microplate, followed by 25 µL of 0.25 mM $FeCl_2$ and 25 µL of 0.625 mM Ferrozine (both prepared in water).

After incubating the microplate for 10 minutes in the dark at room temperature, the absorbance was read at 562 nm using an Epoch microplate spectrophotometer. The data was expressed as a percentage of chelating activity from the following equation:

$$Fe^{2+} \text{ inhibition } (\%) = 1-(\frac{Abs_{sample}}{Abs_{control}}) \text{ x } 100\%$$

Where Abs_{sample} is the absorbance of the sample, and $Abs_{control}$ refers tothe absorbance of water without added inhibitor. The Fe^{2+} inhibition was plotted against its respective concentration to determine the IC_{50} value (the inhibitory concentration required to scavenge 50% of the Ferrozine-Fe^{2+} complex), which was calculated by the Epoch software from a four-parameter logistic curve.

3.3.6. Determination of the antihypertensive activity

3.3.6.1 ACE inhibition assay

The ACE inhibition activities were determined using the modified method of Barbana and Boye (2010), with modification by Rui, Boye, Simpson, and Prasher (2012). 10 µL of ACE enzyme (8 mU) was mixed with 10 µL of appropriately diluted hydrolysate (0-8 mg/mL) prepared in 100 mM borax buffer (pH 8.3) containing 300 mM NaCl, and incubated at 37 °C for 10 minutes. Then, 50 µL of 1 mM Hippuryl-His-Leu (HHL) substrate was added, and the reaction mixture was incubated for 30 minutes at 37 °C. The concentration of ACE enzyme (8 mU) was determined by performing preliminary enzymatic assays using various concentrations of the ACE enzyme (1 to 10 mU). The reaction was stopped by adding 85 µL of 1 M HCl. 5 µL of the mixture was injected into a 4.60 x 250 mm Aqua C18 column (5 µm pore size 125Å, Phenomenex, Torrance, CA) and the elution of hippuric acid (HA) and the consumption of HHL substrate were monitored at 228 nm. Samples were eluted with 50% (v/v) methanol in water containing 0.1% TFA at a flow rate of 0.5 mL/min for 15 minutes. The ACE inhibitory activity (%) was calculated using the following equation:

$$\text{ACE inhibition } (\%) = 1-(\frac{PA_{Sample}}{PA_{control}}) \text{ x } 100\%$$

Where PA_{sample} was the peak area of the sample and $PA_{control}$ was the peak area of the enzyme (ACE) and substrate (HHL) alone without the presence of hydrolysate peptide (inhibitor). The ACE inhibition was then plotted against its respective concentration to determine the IC_{50} value, the inhibitory concentration required to scavenge 50% of the ACE enzyme. The IC_{50} value was be calculated from the equation of a four-parameter logistic curve.

3.3.7. Determination of the antidiabetic activity

3.3.7.1 DPP-IV inhibition assay

The DPP-IV inhibition assay was carried out according to Velarde-Salcedo et al. (2013), with modifications by Nongonierma and FitzGerald (2013). 25 µL of appropriately diluted hydrolysates (0-5 mg/mL), prepared in 0.1 M Tris-HCl (pH 8.0), were added to 25 µL of 1000 uM Gly-pro-p-nitroanilide in a 96 well flat bottom microplate. The mixtures were incubated for 10 minutes at 37 °C, then, 50 µL of 0.005 U/mL DPP-IV enzyme (prepared in 0.1 M Tris-HCl, pH 8.0, [2.5 mU/mL final]) was added to each well and the microplate incubated at 37 °C for 1 hour. After the incubation period, the absorbance was read at 415 nm using an Epoch microplate spectrophotometer. The percentage of DPP-IV inhibition was estimated as follows:

$$\text{DPP-IV inhibition (\%)} = 1-\left(\frac{Abs_{sample}}{Abs_{control}}\right) \times 100\%$$

Where Abs_{sample} is the absorbance of the sample, and $Abs_{control}$ is the absorbance of water, enzyme, and substrate alone without added inhibitor. The DPP-IV inhibition was plotted against its respective concentration to determine the IC_{50} value, the inhibitory concentration required to scavenge 50% of the DPP-IV enzyme. the IC_{50} value was calculated by the Epoch software from a four-parameter logistic curve.

3.3.8. Peptide identification

3.3.8.1. Mass Spectroscopy

The C09052 3K MWCO hydrolysate was fractionated by SE-HPLC and the recovered peptide fractions were lyophilized and retested for their ACE inhibition activity. The fraction exhibiting the highest ACE inhibition activity was analysed by LC-MS. The peptide sample was first solubilized in 5% acetonitrile and 0.2% formic acid and then loaded on a C18 precolumn (0.3 mm x 5 mm) followed by separation on a reversed-phase column (150 µm x 150 mm) with a gradient from 10 to 30% acetonitrile and 0.2% formic acid at 600 nL/min flow rate for 56 minutes, using an Ultimate 3000 HPLC system (Eksigent, Dublin, CA) connected to an Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific, San Jose, CA). Each full MS spectrum acquired at a resolution of 70,000 was followed by 12 tandem-MS (MS-MS) spectra on the most abundant multiply charged precursor ions. Tandem-MS experiments were performed using collision-

induced dissociation (HCD) at a collision energy of 27%. The data were processed using PEAKS 8.5 (Bioinformatics Solutions, Waterloo, ON) and a Pooideae database. Mass tolerances on precursor and fragment ions were 10 ppm and 0.01 Da, respectively. Variable selected posttranslational modifications were carbamidomethyl, oxidation, deamination, and phosphorylation.

3.3.8.2. Database Searching

All MS/MS samples were analyzed using PEAKS Studio (Bioinformatics Solutions, Waterloo, ON Canada; version 10.0). PEAKS Studio was set up to search the Pooideae 2018 database (unknown version, 506785 entries) assuming the digestion enzyme trypsin. PEAKS Studio was searched with a fragment ion mass tolerance of 0.0100 Da and a parent ion tolerance of 10.0 PPM. Carbamidomethyl of cysteine was specified in PEAKS Studio as a fixed modification. Deamination of asparagine and glutamine, oxidation of methionine, acetylation of the n-terminus, and phosphorylation of serine, threonine and tyrosine were specified in PEAKS Studio as variable modifications.

3.3.8.3. Criteria for protein identification

Scaffold (version Scaffold_4.8.9, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 97.0% probability to achieve a false discovery rate (FDR) less than 1.0% by the Peptide Prophet algorithm (Keller, Nesvizhskii, Kolker, & Aebersold, 2002) with Scaffold delta-mass correction. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii, Keller, Kolker, & Aebersold, 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

3.3.8.4. In silico analysis of potential peptide bioactivity

The peptide sequences identified by mass spectroscopy were analyzed *in silico* for their potential bioactivity using the BIOPEP database (Minkiewicz, Dziuba, Iwaniak, Dziuba, & Darewicz, 2008).

3.3.9. Statistical Analysis

Each experiment was conducted in triplicate and data expressed as means ± standard deviation. Statistical analyses were performed using XLSTAT software (Addinsoft, NY) in Microsoft Excel (Redmond, WA). One-way analysis of variance (ANOVA) and the Tukey's honest significant difference (HSD) test ($p < 0.05$) were performed to detect significant differences.

3.4. RESULTS AND DISCUSSION

3.4.1. Molecular weight distribution of in vitro hydrolysates

Bioactive peptides are typically characterized by a short chain of 2-20 amino acids (Sánchez & Vázquez, 2017); the *in vitro* hydrolysates were therefore filtered through a 3K MWCO membrane to remove HMW soluble proteins and polypeptides to recover LMW peptides with potential higher bioactivity. The soluble protein content in the collected ultrafiltration permeates was highest in canaryseeds (12.9-15.7 mg/ml) as compared to both oat (10.7 ± 1.4 mg/ml) and wheat (10.9 ± 1.0). The SE-HPLC chromatograms of the cereal flour hydrolysates are presented in **Figure 3.1**. The size exclusion profile of the four canaryseed varieties showed very similar patterns comprising of four well resolved peaks eluted at different retention times of approximately 22.7 min. (peak 1), 23.9 min. (peak 2), 26.4 min. (peak 3) and 35.8 min. (peak 4). As shown in **Figure 3.1**, peak 1 had a molecular weight greater than 1,355 kDa corresponding to Vit-B (standard reference). Peak 2 had a molecular weight closer to the dipeptide and tri-peptides corresponding to carnosine (226.23 Da) and Gly-Gly-Gly (189.17 Da), respectively. Peak 3 was of lower molecular weight than the carnosine standard (226.23 Da), since it had a higher retention time (26.4 min.). Peak 4, with the highest retention time, represents a small amount of very low molecular weight peptides that were present in the filtered hydrolysates. In addition, oat and wheat hydrolysates showed four major peaks at similar retention times as canaryseed hydrolysates, but with lower peak intensities. No larger aggregates were observed in the filtered hydrolysates, meaning the ultrafiltration step with 3K MWCO was efficient in retaining higher molecular weight polypeptides and proteins.

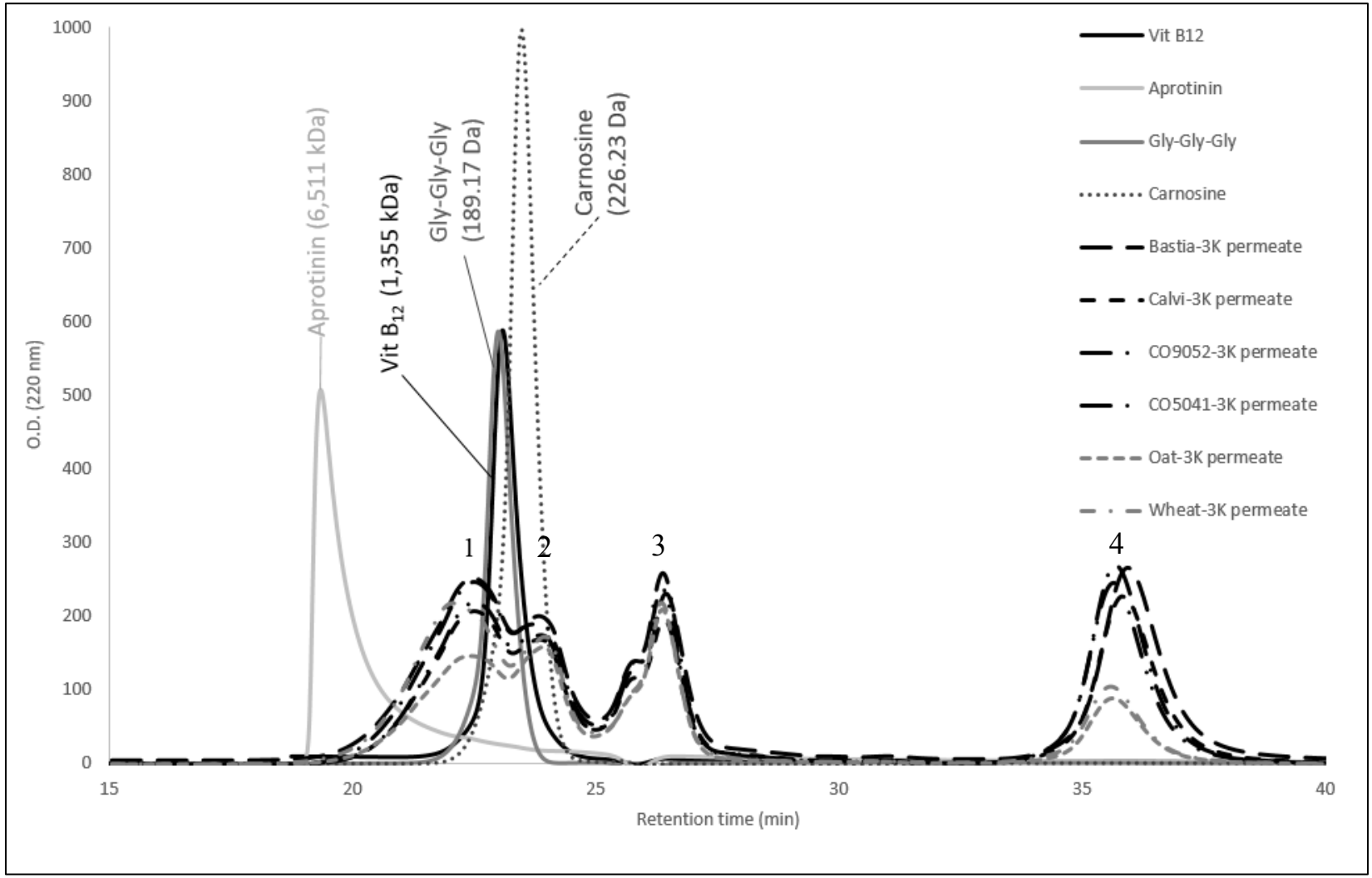


Figure 3.1. Size exclusion HPLC profile of canaryseeds, oat and wheat flours after *in vitro* digestion and 3K MWCO ultrafiltration.

3.4.2. Bioactivity Assays

3.4.2.1. Antioxidant and chelation activity

Antioxidants react with free radicals via different mechanisms that can roughly be classified into two types of reactions; hydrogen atom transfer (HAT) and single electron transfer (SET). In HAT reactions, the free radical removes a hydrogen atom from the antioxidant, whereas, in SET reactions, the antioxidant gives an electron to the free radical (Liang & Kitts, 2014). Antioxidant *in vitro* HAT tests include the ORAC and ABTS assays, while *in vitro* SET tests include DPPH and iron chelating assays (Huang, Ou, & Prior, 2005). In both types of reactions, the antioxidant becomes a free radical itself. The antioxidant assays (ORAC, DPPH, ABTS) demonstrated different inhibition profiles for all cereal varieties (**Figure 3.2**).

For the ORAC assay (**Figure 3.2 (A)**), the antioxidant activity among canaryseed cultivars was not significantly different ($p > 0.05$) and ranged from 1.77-1.99 μmol TE/mg protein. The ORAC value for C09052, C05041 and Bastia cultivars (1.77-1.96 μmol TE/mg) were not significantly different ($p > 0.05$) from wheat (1.53 μmol TE/mg); however, the Calvi cultivar showed the highest ORAC value overall (1.99 μmol TE/mg) and was significantly higher ($p < 0.05$) than wheat. Oat had the overall lowest ORAC value (1.31 μmol TE/mg) with significant difference ($p < 0.05$) to all canary cultivars. The ORAC values for canaryseeds are higher than what has been reported for the glutelin fraction from cacao seeds (0.28 μmol TE/mg) (Tovar-Pérez, Guerrero-Becerra, & Lugo-Cervantes, 2017), however, a superior ORAC value has been reported for a < 5K MWCO digest fraction from quinoa (2.72 μmol TE/mg protein) (Vilcacundo, Miralles, Carrillo, & Hernández-Ledesma, 2018).

For the ABTS assay, lower calculated IC_{50} values indicate higher antioxidant activity. As shown in **Figure 3.2 (B)**, the ABTS assay showed that the yellow C09052 canaryseed cultivar and wheat had the highest activity with IC_{50} values of 117.5 μg/ml and 107.8 μg/mL, respectively ($p > 0.05$). Calvi, Bastia, C05041, and oat IC_{50} values were not significantly different ($p > 0.05$) from one another. Recently, Valverde et al. (2017) reported the antioxidant capacity of soaked (in water for 12 hours) hairy canaryseed protein fractions (obtained by Osborne fractionation) that were digested *in vitro*. Their results indicated that digested albumin and prolamin peptides showed the best IC_{50} values, 133.2 and 181.6 μg/mL, respectively ($p < 0.05$); while globulins and glutelins showed high IC50 values. The ABTS inhibition activity of canaryseeds was also higher than the

reported IC_{50} values for chia seed prolamin (161.5 μg/mL) and glutelin (184.7 μg/mL) fractions (Orona-Tamayo et al., 2015), as well as 0.5-3K MWCO peptides from wheat (174.64 μg/mL) and mung bean (248.97 μg/mL) (Jha, Ghosal, Gupta, Ghosh, & Mandal, 2015), but significantly lower than the prolamin fraction from red beans (60 μg/mL) (Durak, Baraniak, Jakubczyk, & Swieca, 2013).

The DPPH assay results (**Figure 3.2 (C)**) showed that the brown canaryseed cultivars have the highest antioxidant activity (77.9-96.4 μg/mL), whereas the yellow C05041 (1043.5 μg/mL) and oat (742.3 μg/mL) had the lowest activity. The IC_{50} values for the DPPH inhibition activity of the brown Bastia (96.4 μg/mL) and Calvi (77.9 μg/mL) hydrolysates were comparable to results reported for chia seed albumin (124.4 μg/mL) and globulin (74.7 μg/mL) protein hydrolysates (Orona-Tamayo et al., 2015), black bean protein hydrolysate (96.2 μg/mL) purified by ultrafiltration (< 4 kDa) (Chen et al., 2018), and for the prolamin fraction of hairy canaryseeds soaked for 12 and 24 hours (114.1 and 89.7 μg/mL, respectively) (Valverde et al., 2017). Prolamins from red beans have demonstrated significantly higher activity (20 μg/mL) (Durak et al., 2013). Both the brown canaryseed cultivars also had the highest chelation activity (Fe^{2+} inhibition) with IC_{50} values of 0.73 and 0.98 mg/mL for Calvi and Bastia, respectively (**Figure 3.2 (D)**). This was also in good correlation with their high DPPH activity. Wheat (1.8 mg/mL) and the yellow C05041 (1.9 mg/mL) hydrolysates had the highest IC_{50} values and therefore the lowest chelating activity. The iron chelating activity in the brown canaryseed cultivars was higher than values reported for chia seed flour (1.6 mg/mL) (Orona-Tamayo et al., 2015) and for the prolamin fraction from red beans (2.52 mg/mL) (Durak et al., 2013).

Polyphenols are secondary metabolites produced by plants that play roles in defense mechanisms, primarily for protection against ultraviolet radiation (Pandey & Rizvi, 2009). These plant secondary metabolites are also useful as radical scavengers and possess positive biochemical effects against cardiovascular diseases, cancer growth and age-related diseases (Fekadu Gemede, 2014). Because polyphenols possess potent antioxidant activity, which has been shown in both *in vitro* and *in vivo* studies, it remains difficult to conclude if the antioxidant activity demonstrated by the cereal flour hydrolysates was due to peptides and soluble proteins or from polyphenols present in the hydrolysates. **Figure 3.3** showed that the total polyphenol content (TPC) of the canaryseed cultivars, oat and wheat flour hydrolysates (filtered through a 3K MWCO ultrafiltration

unit) ranged from 79.1-108.9 mg FAE/g protein. Among canaryseed cultivars, the yellow C09052 had significantly higher ($p < 0.05$) TPC content than the other cultivars. The TPC of the yellow canaryseed cultivars (85.0-97.1 mg FAE/g protein) was not significantly different ($p > 0.05$) compared to oat (91.5 mg FAE/g protein), while wheat showed significantly higher ($p < 0.05$) TPC content among the cereal flours studied (108.9 mg FAE/g protein). This is in good agreement with data of Zeng, Liu, Luo, Chen, and Gong (2016), which reported a significantly higher bioavailability of free and bounded phenolic acids in raw and extruded wheat compared to other cereals, namely brown rice and oat.

It is important to note, that the antioxidant potency of polyphenols depend on their bioavailability, which, in turn, depend on their release from the food matrix during digestion (Alminger et al., 2014). Thus, even though polyphenol content from a specific food source may be high, this might not necessarily be an indication of high antioxidant activity due to their poor bioavailability. Indeed, from **Figure 3.3**, the wheat hydrolysate had the highest TPC compared to the other cereal hydrolysates, however, the wheat hydrolysate did not demonstrate a higher antioxidant activity as compared to the other cereals (**Figure 3.2**). Alfieri and Redaelli (2015) recently analyzed twenty oat cultivars and reported their soluble phenol content (SPC) ranging from 0.78 to 1.09 g GAE/kg sample. On the contrary, higher values, up to 1.5 g GAE/kg d.m, were found in oat grains by Adom and Liu (2002), and a mean of 2.1 g GAE/kg d.m. was reported by Menga, Fares, Troccoli, Cattivelli, and Baiano (2010). Literature information on canaryseeds are relatively scarce, despite few studies that have investigated the phenolic profiles of hairy canaryseed (Abdel-Aal, Hucl, Patterson, et al., 2011; Li et al., 2011), as well as the phenolic profiles and antioxidant activities in germinated canaryseed (Chen et al., 2016). To the best of our knowledge, no such studies have been conducted on these four hairless canaryseeds cultivars to date.

Overall, glabrous canaryseeds have demonstrated equivalent or superior antioxidant activity as compared to common cereals wheat and oat. Brown cultivars of canaryseeds have demonstrated exceptional antioxidant activity for the DPPH and iron chelation assays. It remains difficult to conclude if the antioxidant activity in the digested flours is primarily from the peptides present or if there was a synergistic effect with the cereal polyphenols. Future *in vitro* digestion studies are necessary to investigate the bioavailability of cereal polyphenols, which is essential for their bioactivity capabilities.

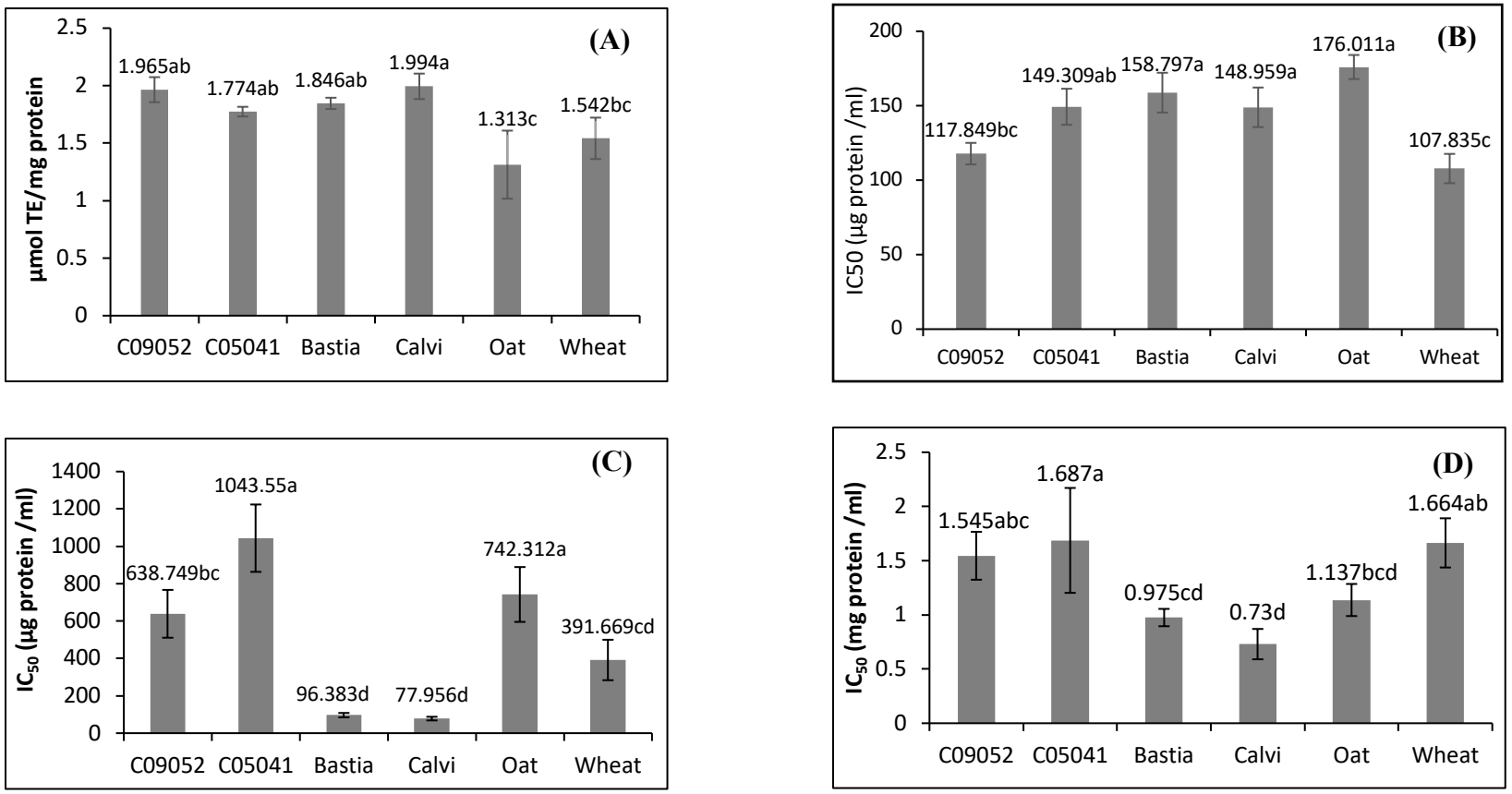


Figure 3.2. Antioxidant and chelation activity of *in vitro* canaryseed hydrolysates as compared to oat and wheat. **(A)** ORAC, **(B)** ABTS, **(C)** DPPH, **(D)** Fe^{2+} inhibition

Means with different lower-case letters are significantly different ($p < 0.05$)

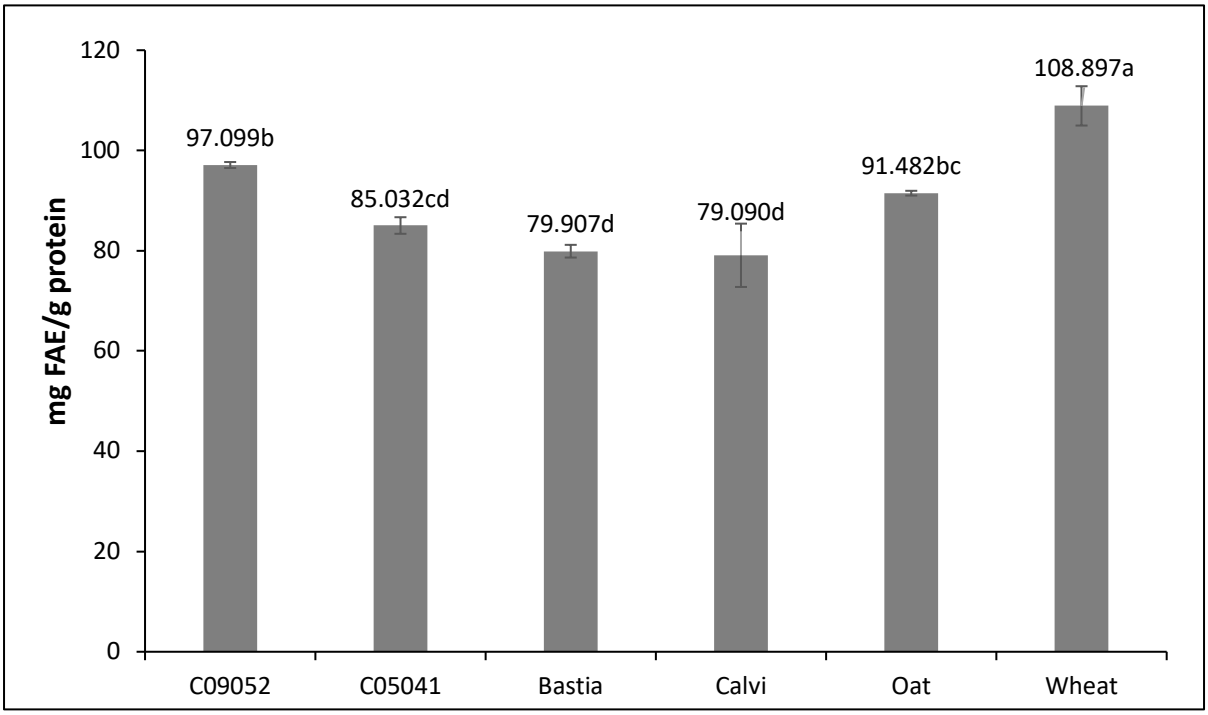


Figure 3.3. Comparison of total polyphenol content (TPC) of *in vitro* digested canaryseed cultivars, oat and wheat flours.

FAE: ferulic acid equivalents

Means with different lower-case letters are significantly different ($p < 0.05$)

3.4.2.2. Antihypertensive activity

The antihypertensive activity of flour hydrolysates was investigated using hippuryl-L-histidyl-L-leucine (HHL) as a substrate. Peptides with high antihypertensive activity can inhibit the ACE enzyme activity and limit the release of hippuric acid (HA). **Figure 3.4** shows the effects of the ACE enzyme concentration in the ACE enzyme activity assay. When no ACE enzyme is present (control), no HA is released. However, as the amount of ACE enzyme was increased, more HHL (substrate) was converted into HA, a by-product of the reaction.

The results show no significant difference ($p > 0.05$) in the IC_{50} of C09052 (334.3 µg/mL), C05041 (405.0 µg/mL), and Calvi (321.9 µg/mL) hydrolysates for their antihypertensive activity (**Figure 3.5**). However, the brown Bastia cultivar had a significantly ($p < 0.05$) higher IC_{50} value (589.9 µg/mL) as compared to the other canaryseeds cultivars, and hence lower ACE inhibition activity. Similarly, Estrada-Salas et al. (2014) reported an ACE inhibition IC_{50} of 332 µg/mL for hairy canaryseed peptides, whereas Valverde et al. (2017) reported a higher IC_{50} of 217 µg/mL for the prolamins fraction of canaryseed flour peptides. The ACE inhibitory activity determined in this study for the yellow C09052 and C05041, and brown Calvi cultivars was comparable to IC_{50} values reported for peptides from chia seed albumin (377 µg/mL) and globulin (339 µg/mL) (Orona-Tamayo et al., 2015), potato tuber (360 µg/mL) (Mäkinen et al., 2008), and flaxseed (400 µg/mL) (Udenigwe et al., 2012). The IC_{50} of Bastia (589.9 µg/mL) and oat (570.1 µg/mL) hydrolysates were not significantly different ($p > 0.05$); however, wheat hydrolysates exhibited the highest IC_{50} (781.2 µg/mL) revealing the lowest antihypertensive activity. From these results, it can be concluded that canaryseed proteins are a good source of antihypertensive peptides as compared to common cereals such as oat and wheat.

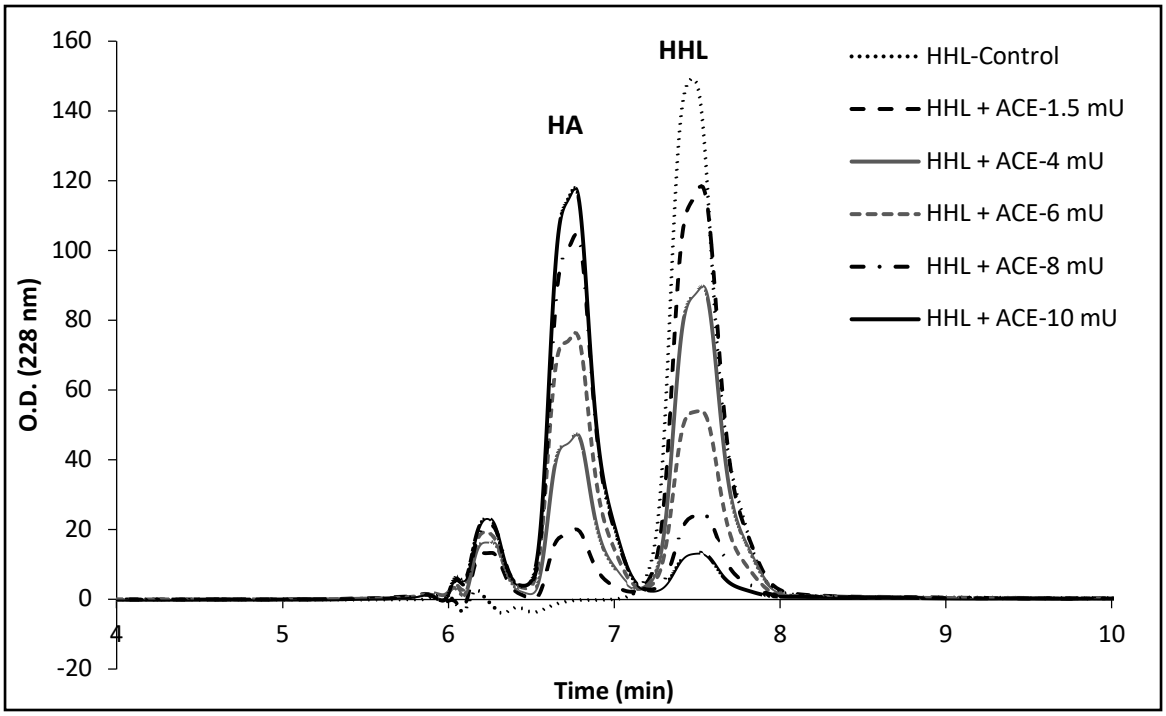


Figure 3.4. Effect of ACE enzyme concentration on hippuryl-L-histidyl-L-leucine (HHL) substrate for ACE inhibition assay

ACE: angiotensin converting enzyme, HA: hippuric acid

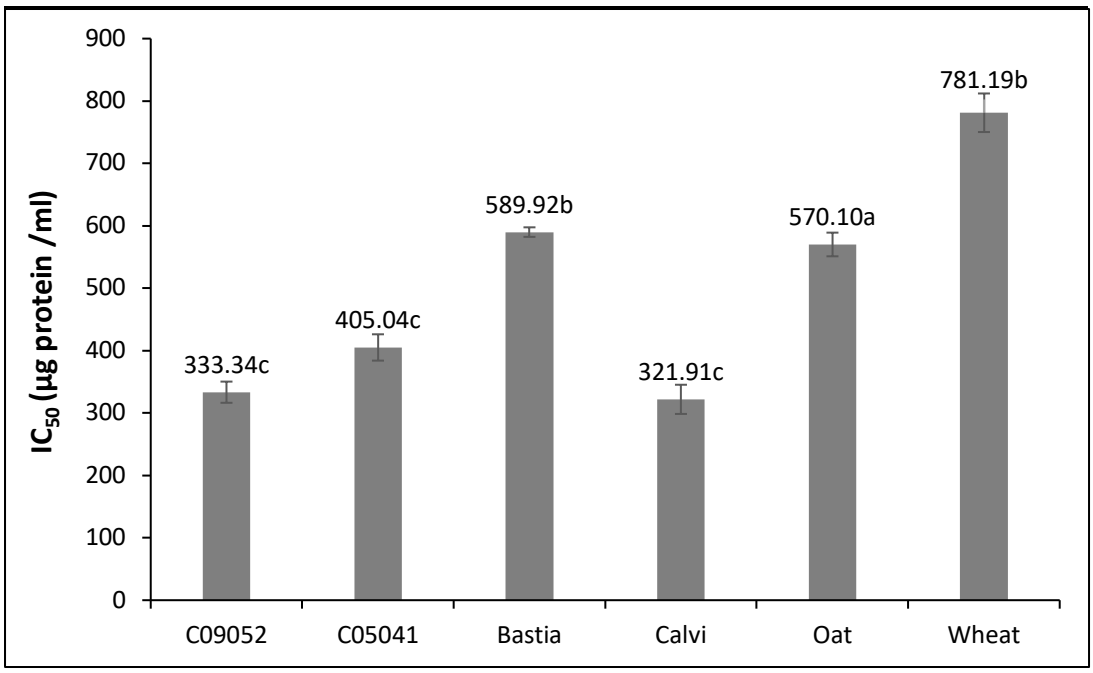


Figure 3.5. Antihypertensive activity (ACE inhibition assay) of *in vitro* canaryseed hydrolysates as compared to oat and wheat

Means with different lower-case letters are significantly different ($p < 0.05$)

3.4.2.3. Antidiabetic activity

The IC_{50} values for the inhibition of the DPP-IV enzyme, as presented in **Figure 3.6,** revealed no statistically significant differences ($p < 0.05$) among canaryseed cultivars and wheat hydrolysates, except for the brown Calvi, which exhibited the lowest inhibition capacity. Canaryseeds have demonstrated equivalent antidiabetic activity to wheat but superior activity as compared to oat. Lower IC_{50} values indicate higher antidiabetic activity, and the highest inhibition was observed for the yellow canaryseed cultivars C09052 (1.01 mg/mL), C05041 (1.14 mg/mL) and were equivalent to that of wheat (1.00 mg/mL) ($p > 0.05$), but superior ($p < 0.05$) to oat. The latter exhibited the highest IC_{50} (2.29 mg/mL) and therefore the lowest inhibition of DPP-IV enzyme activity among studied cereals. Estrada-Salas et al. (2014) reported a maximum inhibition of 43.4% at a peptide concentration of 1.4 mg/mL for hairy canaryseeds. The IC_{50} values determined in this study for yellow canaryseeds and the brown Bastia cultivar (1.01-1.35 mg/mL) are similar to those reported for amaranth peptides (1.1 mg/mL) (Velarde-Salcedo et al., 2013), germinated soybean peptides (1.49 mg/mL), and oat flour (0.99 mg/mL) (Wang, Yu, Zhang, Zhang, & Fan, 2015), and superior to those reported for barley (3.91 mg/mL) and buckwheat (1.98 mg/mL) flours (Wang, Yu, et al., 2015).

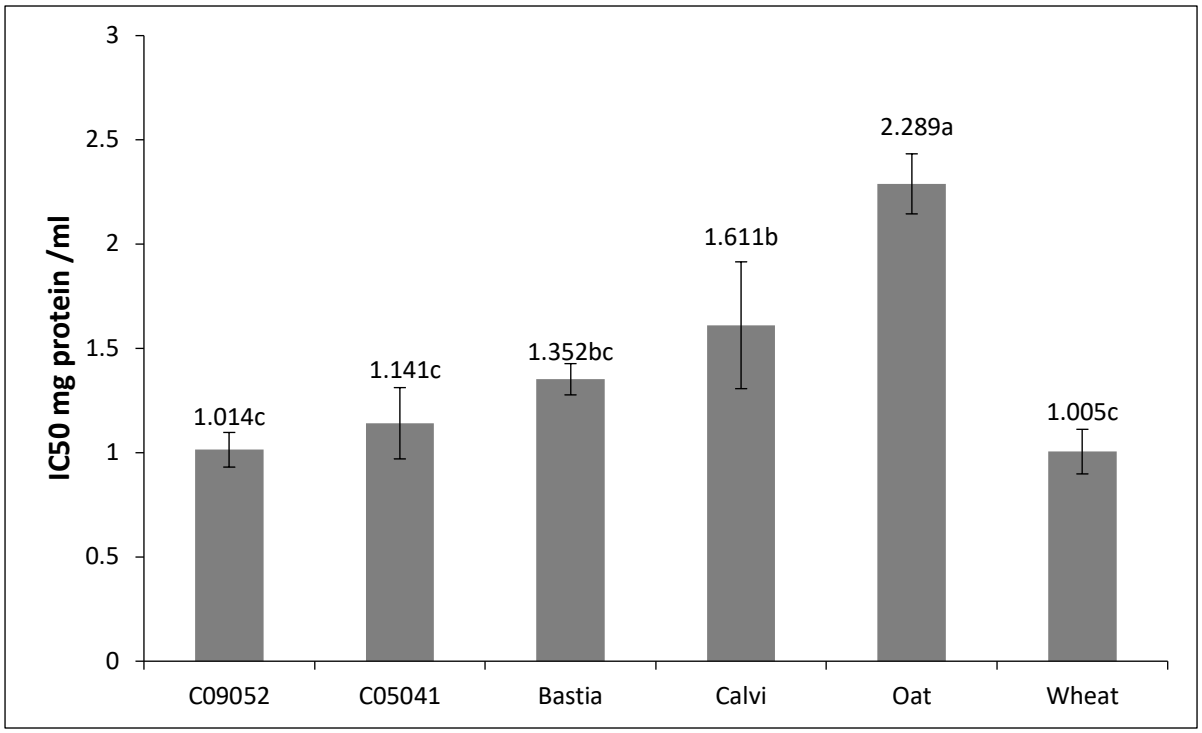


Figure 3.6. Antidiabetic activity (DPP-IV inhibition assay) of *in vitro* canaryseed hydrolysates as compared to oat and wheat

Means with different lower-case letters are significantly different ($p < 0.05$)

3.4.3. Peptide fractionation and measurement of ACE inhibitor activity

Among the four studied canaryseed cultivars, the yellow C09052 and the brown Calvi hydrolysates demonstrated excellent overall antioxidant, antihypertensive, and antidiabetic activity. However, the yellow C09052 variety was selected for further analysis since fewer analyses have been done on the newly developed variety. The peptides were fractionated by SE-HPLC, and the ACE inhibitory activity of each fraction was investigated. The size exclusion chromatogram of the C09052 hydrolysate is presented in **Figure 3.7**, showing three major peaks (3 fractions) which have been designated as F1, F2, and F3, with retention times of approximately 22.7, 26.4, and 35.8 minutes, respectively. F1 and F2 fractions had the highest inhibition activity of 32.2% and 28.8%, respectively ($p > 0.05$). F3, with the highest retention time, had very low activity (3.1%) and protein yield, corresponding to smaller molecular weight components that could possibly be assigned to single amino acids or small molecules (solvent, salts) with no ACE inhibition activity. Because the protein content of F3 was relatively small, the ACE inhibition assay was first performed at a peptide concentration of 350 μg/mL for each fraction. At lower protein concentration (350 μg/ml), only 30% of the ACE enzyme was inhibited by F1 and F2. However, when a higher concentration of peptides from F1 was used for the assay (3.0 mg/mL), the ACE inhibitory activity increased to 82.1%, as shown in **Figure 3.8**. Interestingly, before fractionation of the C09052 canaryseed hydrolysate, the whole hydrolysate showed 50% inhibition at a concentration of 333 μg/ml (**Figure 3.5**). However, after fractionation when individual fractions were tested at similar protein concentration of 350 μg/ml, lower ACE inhibitory activity was obtained for all fractions (3-32%) (**Figure 3.8**). This loss of activity after fractionation could be due to reduced synergistic interactions between peptides from different fractions, and/or the removal of other constituents in the hydrolysate which could also contribute to ACE inhibitor activity.

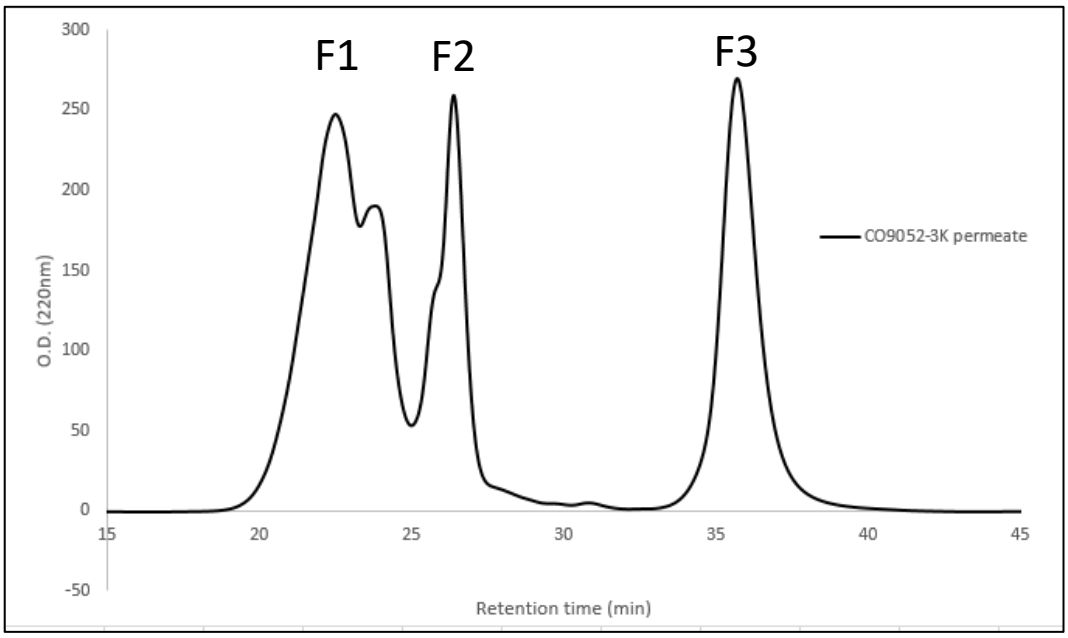


Figure 3.7. SE-HPLC peptide fractionation of C09052 hydrolysate

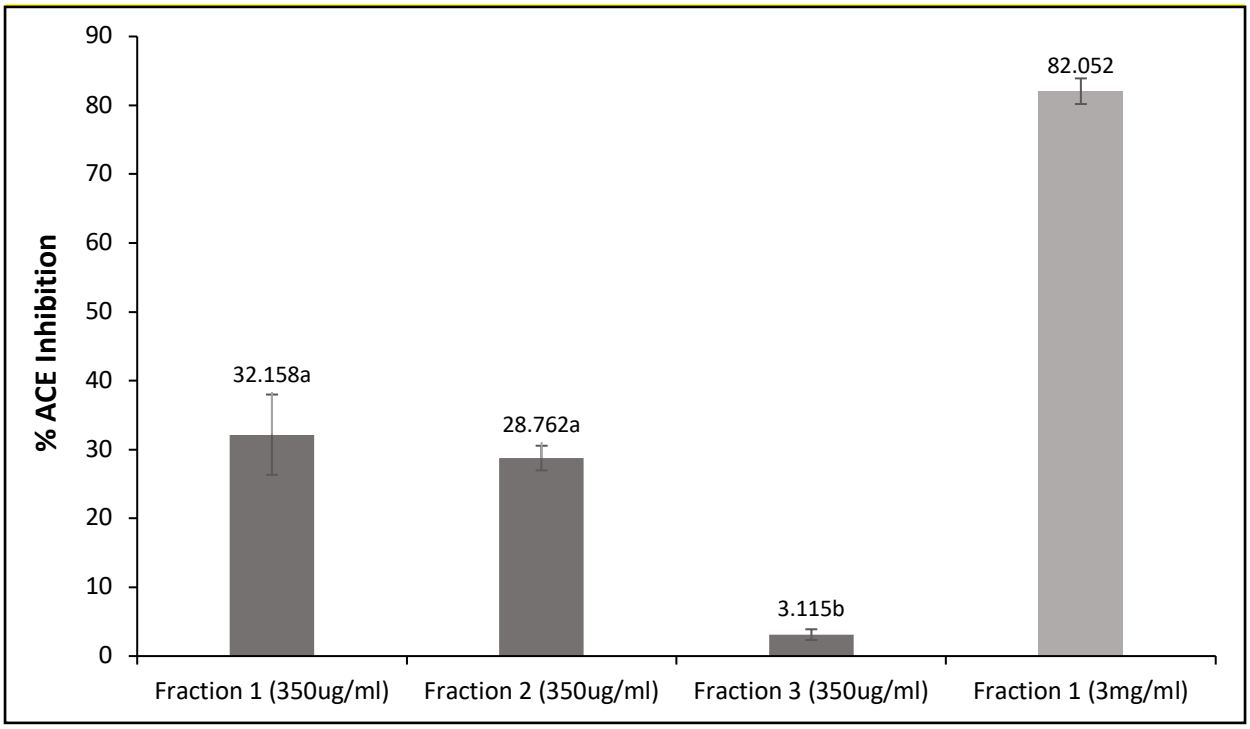


Figure 3.8. Antihypertensive activity (ACE inhibition) of collected peptide fractions from C09052 canaryseed cultivar hydrolysate following SE-HPLC separation.

Means with the same lower-case letter are not significantly different at $p < 0.05$ (n =3)

3.4.4. Peptide identification and potential bioactivity

3.4.4.1. Peptide identification

Tandem MS analysis lead to the identification of 46 peptides in the F1 size exclusion fraction. The parent protein of each identified peptide is represented in **Table 3.1**. Currently, no proteomic database exists for canaryseeds and so the identified peptides were analyzed using a *Pooideae* database, which includes proteins from cereals such as oat, wheat, barley and rye. The peptides were attributed to 18 different parent proteins in total. Canaryseeds have been extensively compared to wheat, however, 14 of the 18 proteins identified were of barley origins, indicating canaryseeds may contain several proteins similar or identical to those of barley. The shortest and longest peptides were comprised of 8 and 30 amino acids, respectively, with molecular weights ranging from 913.57 Da to 3,185.61 Da.

Some peptides (such as AVFPSIVGRPR, FPSIVGRPR, VFPSIVGRPR, and GYSFTTTAER) are redundant and present in more than one protein. The redundant peptides are found in proteins with related functions, such as both predicted proteins and uncharacterized proteins from barley. All three proteins are part of the actin cytoskeleton, filamentous proteins involved in cellular organelle and cytoplasmic transportation, which explains why they consist of several identical peptides (Breuer et al., 2017). Other proteins identified contain unique canaryseed peptides but have related functions in the plant. The ATP synthase subunit alpha protein from barley and the ATP synthase subunit beta protein from goatgrass play crucial roles in photosynthesis by producing ATP (adenosine triphosphate) and contain unique peptides GIRPAINVGLSVSR and IGLFGGAGVGK, respectively, from canaryseeds (Hahn, Vonck, Mills, Meier, & Kühlbrandt, 2018). As core components of the nucleosome, Histone H4 from einkorn and Histone H2A from wheat fold DNA to form and shape the chromatin (Dorigo, Schalch, Bystricky, & Richmond, 2003).

3.4.4.2. In silico prediction of bioactive peptide activity

Table 3.2 shows the potential bioactivity of the peptides identified by tandem MS. All 46 unique peptides that were identified had potential ACE inhibitor and DPP-IV inhibitor activity and 20 among them had potential antioxidant activity, as has been confirmed by our *in vitro* bioactivity testing. Interestingly, 22 peptides had potential hypotensive activity as renin inhibitors. Renin and ACE, two key enzymes in the renin-angiotensin aldosterone system, regulate mammalian blood

pressure; renin first converts angiotensinogen to angiotensin 1 which is in turn catalyzed by ACE to angiotensin II, a very powerful vasoconstrictor that simultaneously induces aldosterone secretion, causing increased sodium retention (Aluko, 2019). Although most studies report antihypertensive activity in terms of ACE inhibition, several studies show plant proteins have demonstrated renin inhibitor activity, including canola (Alashi et al., 2014), hempseed (Girgih, Alashi, He, Malomo, & Aluko, 2014), lima bean (Ciau-Solís, Acevedo-Fernández, & Betancur-Ancona, 2018), flaxseed (Udenigwe, Lin, Hou, & Aluko, 2009), and rapeseed (He et al., 2013).

In addition, many of the identified peptides had predicted bioactivities that have not yet been demonstrated in canaryseeds, including antiamnestic, antithrombotic, opioid, neuroprotective, and immunostimulating activities. Of particular interest is the identification of prolyl endopeptidases (PEP's) inhibition activity. PEP are a group of serine proteases that cleave internal proline residues of peptides, thereby degrading active peptides, hormones, and neuropeptides that aid in preventing neurological diseases including Alzheimer's disease, amnesia, depression, and schizophrenia (Gass & Khosla, 2007; Hsieh, Wang, Hung, Hsieh, & Hsu, 2016). PEP inhibitors are therefore of interest as potential treatments for neurodegenerative disorders. Most biologically active hormone peptides and neuroprotective peptides contain at least one internal proline residue (Polgár, 2002). Five of the identified peptides in canaryseed possessed potential antiamnestic activity as PEP inhibitors and each peptide contained at least one proline residue. Most notably is the peptide VGINYQPPTVVPGGDLAK, which contains 3 internal proline residues. The antiamnestic activity of this peptide is most likely higher than in the other 4 peptides which only contain 1 internal proline residue. All five peptides with antiamnestic activity consisted of amino acid sequences PG and/or GP, corresponding to amino acids Pro-Gly and Gly-Pro, respectively. Other identified peptides contain one or more internal proline residues but did not possess any potential antiamnestic activity because they did not have the PG and/or GP amino acid sequence, however, several of these peptides contain more than one proline residues and could still demonstrate antiamnestic activity.

In addition, 5 peptides that demonstrated potential antiamnestic activity also had antithrombotic activity. Antithrombotic peptides reduce venous platelet aggregation and coagulation, thereby helping to control and prevent cardiovascular disease incidence (Cheng, Tu, Liu, Zhao, & Du, 2019). Of these 5 peptides, FPGQLNADLR, VGINYQPPTVVPGGDLAK, and

QEYDESGPSIVHR have been isolated and identified from enzymatic blue mussel hydrolysates and have demonstrated anticoagulant activity *in silico*, furthermore, the peptide QEYDESGPSIVHR was also isolated and identified in olive oil and exhibited anticoagulant activity *in vitro* (de Roos et al., 2011; Qiao et al., 2018). Other cereals demonstrating antithrombotic activity include amaranth (Sabbione et al., 2016; Sabbione, Scilingo, & Añón, 2015), oat, barley, and buckwheat (Yu et al., 2016).

Two canaryseed peptides with similar sequences (DLYANTVLSGGTTMYPGIADR and KDLYANTVLSGGTTMYPGIADR) portrayed excellent overall activity including antiamnestic, antithrombotic, opioid, antioxidant, and hypotensive activity. Only these 2 peptides had potential opioid activity due to the peptide sequence YPG (Tyr-Pro-Gly). Wheat and soybeans have been studied extensively since they contain many peptides with opioid activity. These exogenous opioid peptides structurally resemble endogenous opioid peptides and interact with opioid like receptors, positively effecting regulatory functions in the central nervous system and gastrointestinal digestion (García, Puchalska, Esteve, & Marina, 2013; Liu & Udenigwe, 2019). Bioactive peptides with opioid activity usually contain a Tyr-Pro sequence, and many opioid peptides from wheat, called exorphins, also contain Gly residues, which coincides with the bioactive sequence Tyr-Pro-Gly that was found in 2 canaryseed peptides (Yoshikawa, 2013). Using an *in silico* approach, Garg, Apostolopoulos, Nurgali, and Mishra (2018) found the same Tyr-Pro-Gly peptide present in wheat gluten and determined it had opioid activity *in vitro*. Three peptides from canaryseeds contained the amino acid sequence GQ (Gly-Gln), giving them potential neuro activity. The endogenously produced dipeptide Gly-Gln has several biological functions in the body, including inducing lymphocytosis, enhancing the activity of natural killer cells, and helping regulate cardiovascular and hypotension functions, among others (Kecel-Gunduz, Celik, Ozel, & Akyuz, 2017). However, the neuropeptide activity of Gly-Gln has not been reported in cereals or other food sources to date.

Moreover, three of the identified canaryseed peptides had immunostimulating activity from the peptide sequences Gly-Val-Met, Gly-Phe-Leu, and Gly-Leu-Phe. Immunostimulating peptides aid the host defence system in several ways, including generating immune cells, support macrophage phagocytosis, increase antibody synthesis, and inactivate inflammatory compounds, among others (Maestri, Marmiroli, & Marmiroli, 2016). Among plant proteins, soybeans are known to possess a strong immunostimulating peptide termed ‘soymetide’; a peptide consisting of 13 amino acids

(MITLAIPVNKPGR) from its 7S globulin protein (Guijarro-Díez, García, Marina, & Crego, 2013). The soymetide peptide does contain amino acids glycine, valine, and methionine but not in the same sequence as canaryseeds. Silva-Sánchez et al. (2008) identified a biopeptide from amaranth protein with the sequence Gly-Phe-Leu that had immunomodulating activity. Although Gly-Leu-Phe and Gly-Val-Met peptides were not found in cereals, they have been identified as immunostimulating peptides from milk sources (Jaziri et al., 1992; Tsuruki & Yoshikawa, 2005; Xu, 1998).

The peptide bioactivity and potential bioactivity profiles were determined *in vitro* for canaryseeds. The biological potential of a specific peptide *in vivo* depends primarily on its ability to remain intact until its arrival at the objective organ (Segura-Campos, Chel-Guerrero, Betancur, & Hernandez-Escalante, 2011). Bioactive peptides must first be released from their parent protein during gastrointestinal digestion and remain intact or even hydrolyzed further to retain their bioactivity. Depending on their amino acid composition, some peptides are less digestible than others. Because proline is a secondary amino acid, it requires selective digestive proteases and enzymes to hydrolyze peptide bonds at proline residues, therefore, bioactive peptides containing proline residues are resistant to digestion and protect the active peptide from enzymatic degradation (Polgár, 2002). Nonetheless, some *in vitro* studies show that resistance to enzymatic hydrolysis during digestion can either increase or decrease a peptides bioactivity (Segura-Campos et al., 2011). 24 of the identified peptides in canaryseeds possess internal proline residues, potentially making them more resistant to digestion by proteolytic enzymes, which in turn, could enhance or reduce the bioactive effects of the seeds. However, the positive health effects of canaryseed proteins have not yet been determined *in vivo*.

Table 3.1. Identified peptides and parent proteins in F1 from *in vitro* digest of yellow C09052 glabrous canaryseed proteins

Protein Peptide Sequence	MW (Da)	Protein Accession Number	Organism
Predicted protein	41,787.00	F2D4P0_HORVV	*Hordeum vulgare subsp. vulgare* (barley)
AVFPSIVGRPR	1,197.71		
DLYANTVLSGGTTMYPGIADR	2,214.07		
FPSIVGRPR	1,027.60		
GYSFTTTAER	1,131.53		
HQGVMVGMGQK	1,170.57		
IWHHTFYNELR	1,514.75		
KDLYANTVLSGGTTMYPGIADR	2,342.17		
SYELPDGQVITIGNER	1,789.89		
VFPSIVGRPR	1,126.67		
WHHTFYNELR	1,401.67		
Predicted protein	42,047.70	F2DZG9_HORVV	*Hordeum vulgare subsp. vulgare* (barley)
AVFPSIVGRPR	1,197.71		
FPSIVGRPR	1,027.60		
GYSFTTTAER	1,131.53		
HQGVMVGMGQK	1,170.57		
IWHHTFYNELR	1,514.75		
QEYDESGPSIVHR	1,515.70		
VAPEEHPVLLTEAPLNPK	1,953.06		
VFPSIVGRPR	1,126.67		
WHHTFYNELR	1,401.67		
Uncharacterized protein	42,333.10	A0A287LZV9_HORVV	*Hordeum vulgare subsp. vulgare* (barley)
AVFPSIVGRPR	1,197.71		
FPSIVGRPR	1,027.60		
GYSFTTTAER	1,131.53		
IWHHTFYNELR	1,514.75		
TTGIVMDSGDGVSHTVPIYEGFTLPHAIIR	3,182.61		
VFPSIVGRPR	1,126.67		
WHHTFYNELR	1,401.67		
Tubulin alpha chain	50,071.70	F2E847_HORVV	*Hordeum vulgare subsp. vulgare* (barley)
AFVHWYVGEGMEEGEFSEAR	2,345.01		
AVFVDLEPTVIDEVR	1,700.91		
DVNAAIATIK	1,014.58		
QLFHPEQLITGK	1,409.77		
TIGGGDDSFNTFFSETGAGK	2,006.89		
VGINYQPPTVVPGGDLAK	1,823.99		
Elongation factor 1-alpha	50,936.00	A0A287P673_HORVV	*Hordeum vulgare subsp. Vulgare* (barley)
IGGIGTVPVGR	1,024.61		
LPLQDVYK	974.55		
QTVAVGVIK	913.57		
STTTGHLIYK	1,119.60		
THINIVVIGHVDSGK	1,587.88		
Tubulin beta chain	50,691.10	F2D8W7_HORVV	*Hordeum vulgare subsp. vulgare* (barley)
FPGQLNADLR	1,129.60		
IMNTFSVVPSPK	1,334.70		
ISEQFTAMFR	1,228.60		
KLAVNMVPFPR	1,270.73		
LAVNMVPFPR	1,142.63		
Uncharacterized protein	71,301.20	A0A287NGJ6_HORVV	*Hordeum vulgare subsp. vulgare* (barley)
HGSLGFLPR	982.54		
Tubulin alpha chain	40,676.70	F2DQT3_HORVV	*Hordeum vulgare subsp. vulgare* (barley)
DVNAAIATIK	1,014.58		
LISQVISSLTASLR	1,486.88		

Predicted protein (Fragment)	22,495.80	F2EJ22_HORVV	*Hordeum vulgare subsp. vulgare* (barley)
LKFPLPHR	1,006.62		
Predicted protein (Fragment)	40,285.70	F2DYG9_HORVV	*Hordeum vulgare subsp. vulgare* (barley)
FATEAAITILR	1,204.69		
Predicted protein	71,916.00	F2E5M4_HORVV	*Hordeum vulgare subsp. vulgare* (barley)
GVPQIEVTFDLDANGILNVSAVDK	2,514.29		
T-complex protein 1 subunit eta	27,260.50	A0A287TMA9_HORVV	*Hordeum vulgare subsp. vulgare* (barley)
SLHDAIMIVR	1,153.64		
Uncharacterized protein	69,103.00	A0A287QLL8_HORVV	*Hordeum vulgare subsp. vulgare* (barley)
DAGVIAGINVLR	1,196.70		
ATP synthase subunit alpha	55,307.40	A0A1C9ZNX9_HORVS	*Hordeum vulgare subsp. Spontaneum* (barley)
GIRPAINVGLSVSR	1,437.85		
Histone H4	11,367.70	M7ZMQ6_TRIUA	*Triticum urartu* (Red wild einkorn)
DNIQGITKPAIR	1,324.75		
ISGLIYEETR	1,179.62		
KTVTAMDVVYALK	1,437.80		
MDVVYALK	937.50		
TVTAMDVVYALK	1,325.70		
VFLENVIR	988.58		
Uncharacterized protein	59,868.80	A0A1D5UUD3_WHEAT	*Triticum aestivum* (Wheat)
ESTLHLVLR	1,066.62		
Histone H2A	17,470.80	A0A0C4BKM5_WHEAT	*Triticum aestivum* (Wheat)
AGLQFPVGR	943.53		
ATP synthase subunit beta, chloroplastic	53,842.70	A0A075W706_AEGBI	*Aegilops bicornis* (Spach goatgrass)
IGLFGGAGVGK	974.56		

Table 3.2. Potential peptide bioactivity of glabrous canaryseed *in vitro* hydrolysates using BIOPEP

Peptide Sequence	Potential Bioactivity								
AVFPSIVGRPR	ACE inhibitor	DPP-IV Inhibitor							
DLYANTVLSGGTTMYPGIADR	ACE inhibitor	DPP-IV Inhibitor	Antiamnestic	Antithrombotic	Opioid	Antioxidant	Hypotensive		
FPSIVGRPR	ACE inhibitor	DPP-IV Inhibitor							
GYSFTTTAER	ACE inhibitor	DPP-IV Inhibitor					Hypotensive		
HQGVMVGMGQK	ACE inhibitor	DPP-IV Inhibitor						Immunostimulating	Neuropeptide
IWHHTFYNELR	ACE inhibitor	DPP-IV Inhibitor				Antioxidant	Hypotensive		
KDLYANTVLSGGTTMYPGIADR	ACE inhibitor	DPP-IV Inhibitor	Antiamnestic	Antithrombotic	Opioid	Antioxidant	Hypotensive		
SYELPDGQVITIGNER	ACE inhibitor	DPP-IV Inhibitor				Antioxidant			Neuropeptide
VFPSIVGRPR	ACE inhibitor	DPP-IV Inhibitor							
WHHTFYNELR	ACE inhibitor	DPP-IV Inhibitor				Antioxidant	Hypotensive		
QEYDESGPSIVHR	ACE inhibitor	DPP-IV Inhibitor	Antiamnestic	Antithrombotic					
VAPEEHPVLLTEAPLNPK	ACE inhibitor	DPP-IV Inhibitor				Antioxidant			
TTGIVMDSGDGVSHTVPIYEGFTLPHAIIR	ACE inhibitor	DPP-IV Inhibitor				Antioxidant	Hypotensive		
AFVHWYVGEGMEEGEFSEAR	ACE inhibitor	DPP-IV Inhibitor				Antioxidant	Hypotensive		
AVFVDLEPTVIDEVR	ACE inhibitor	DPP-IV Inhibitor							
DVNAAIATIK	ACE inhibitor	DPP-IV Inhibitor							
QLFHPEQLITGK	ACE inhibitor	DPP-IV Inhibitor							
TIGGGDDSFNTFFSETGAGK	ACE inhibitor	DPP-IV Inhibitor					Hypotensive		
VGINYQPPTVVPGGDLAK	ACE inhibitor	DPP-IV Inhibitor	Antiamnestic	Antithrombotic					
IGGIGTVPVGR	ACE inhibitor	DPP-IV Inhibitor							
LPLQDVYK	ACE inhibitor	DPP-IV Inhibitor				Antioxidant			
QTVAVGVIK	ACE inhibitor	DPP-IV Inhibitor							
STTTGHLIYK	ACE inhibitor	DPP-IV Inhibitor				Antioxidant			
THINIVVIGHVDSGK	ACE inhibitor	DPP-IV Inhibitor							
FPGQLNADLR	ACE inhibitor	DPP-IV Inhibitor	Antiamnestic	Antithrombotic			Hypotensive		Neuropeptide
IMNTFSVVPSPK	ACE inhibitor	DPP-IV Inhibitor							
ISEQFTAMFR	ACE inhibitor	DPP-IV Inhibitor					Hypotensive		
KLAVNMVPFPR	ACE inhibitor	DPP-IV Inhibitor							
LAVNMVPFPR	ACE inhibitor	DPP-IV Inhibitor							
HGSLGFLPR	ACE inhibitor	DPP-IV Inhibitor						Immunostimulating	
LISQVISSLTASLR	ACE inhibitor	DPP-IV Inhibitor					Hypotensive		
LKFPLPHR	ACE inhibitor	DPP-IV Inhibitor				Antioxidant	Hypotensive		
FATEAAITILR	ACE inhibitor	DPP-IV Inhibitor					Hypotensive		
GVPQIEVTFDLDANGILNVSAVDK	ACE inhibitor	DPP-IV Inhibitor							
SLHDAIMIVR	ACE inhibitor	DPP-IV Inhibitor				Antioxidant			
DAGVIAGINVLR	ACE inhibitor	DPP-IV Inhibitor					Hypotensive		
GIRPAINVGLSVSR	ACE inhibitor	DPP-IV Inhibitor				Antioxidant	Hypotensive		
DNIQGITKPAIR	ACE inhibitor	DPP-IV Inhibitor				Antioxidant	Hypotensive		
ISGLIYEETR	ACE inhibitor	DPP-IV Inhibitor				Antioxidant			
KTVTAMDVVYALK	ACE inhibitor	DPP-IV Inhibitor				Antioxidant	Hypotensive		
MDVVYALK	ACE inhibitor	DPP-IV Inhibitor				Antioxidant	Hypotensive		
TVTAMDVVYALK	ACE inhibitor	DPP-IV Inhibitor				Antioxidant	Hypotensive		
VFLENVIR	ACE inhibitor	DPP-IV Inhibitor				Antioxidant	Hypotensive		
ESTLHLVLR	ACE inhibitor	DPP-IV Inhibitor				Antioxidant	Hypotensive		
AGLQFPVGR	ACE inhibitor	DPP-IV Inhibitor					Hypotensive		
IGLFGGAGVGK	ACE inhibitor	DPP-IV Inhibitor						Immunostimulating	

3.5. CONCLUSION

The bioactive properties of canaryseed protein hydrolysates were evaluated and compared to oat and wheat using *in vitro* bioactivity assays. Overall, our study indicates that glabrous canaryseed hydrolysates have antioxidant, chelating, antihypertensive, and antidiabetic activity equivalent or superior to the common cereal's oat and wheat. The antihypertensive activity of glabrous canaryseeds was especially high. 46 peptides responsible for the ACE inhibitor activity were identified from the yellow canaryseed C09052 cultivar. These peptides were identified belonging to 18 different proteins in the *Pooideae* subfamily, the majority homologous to proteins from barley origins. *In silico* analysis of the potential bioactivity shows that all 46 identified peptides had ACE inhibitor and DPP-IV inhibitor activity, and 20 had antioxidant activity, which had been validated by the *in vitro* studies. However, other peptides had antiamnestic, antithrombotic, hypotensive, and opioid/neuro activity. Chronic disease, such as heart disease, cancer, and diabetes, is a major health concern in society today. Due to the positive health promoting effects demonstrated by glabrous canaryseeds, they should be regarded as a functional food and could be used to help individuals reduce and control the effects of chronic disease, particularly cardiovascular disease.

CHAPTER IV

GENERAL SUMMARY AND CONCLUSION

The nutritional and bioactive properties of two brown (CDC Calvi, CDC Bastia) and two yellow (C09052, C05041) Canadian produced canaryseed cultivars were extensively studied and compared in this study. The crude protein content in canaryseed flours was approximately 22% and much higher than commercial wheat and oat cultivars. The SDS-PAGE profile of canaryseed flours was similar between all varieties and showed the molecular weight distribution of canaryseed prolamins, albumins, globulins, and glutelins. The OFFGEL electrophoretic profile was also similar between canaryseed varieties, with most proteins having an isoelectric point in the neutral and basic range with very few proteins having an isoelectric point in the acidic range, and the majority of proteins were in the molecular weight range of 10-50kda.

The amino acid profile of canaryseeds was determined and compared to common cereals oat and wheat. Overall, the amino acid profiles of canaryseeds are comparable to both oat and wheat and canaryseeds possess exceptionally higher amounts of tryptophan. The minimum (bioaccessible) and maximum true ileal digestibility was evaluated in this study, and the results show the minimum total amino acid digestibility is higher in canaryseeds than wheat and the maximum total amino acid digestibility is similar between all cereal varieties. Principally, the true ileal digestibility for each amino acid of canaryseeds is comparable to either oat or wheat. However, for some amino acids, such as lysine, the maximum true ileal digestibility of canaryseeds is lower than oat and wheat. The protein quality of canaryseeds was also investigated by determining the PDCAAS and DIAAS scores, and the results show that canaryseeds have similar protein quality scores as wheat, but lower scores than oat. The DIAAS and PDCAAS scores for canaryseed proteins indicate that even though the seeds are high in several essential amino acids, the quantity and digestibility of the limiting amino acid lysine remains low, therefore, canaryseeds must be consumed with other protein sources in order to meet dietary requirements of essential amino acids.

The anti-nutritional components in canaryseeds were evaluated and not found to be high enough to take away from the overall health benefits of the seeds, since they were present in quantities comparable to other cereals and legumes. Trypsin inhibitor activity was similar between all cereal varieties. The total polyphenol content was highest in oat, followed by canaryseeds, and then wheat. The total polyphenol content was higher in canaryseed isolates than the flours, since bound

polyphenols were released during isolate preparation. Phytic acid content was significantly higher in canaryseeds than both oat and wheat.

The antioxidant (ORAC, DPPH, ABTS), chelation (Fe^{2+}), antihypertensive (ACE), and antidiabetic (DPP-IV) activity of a 3K MWCO ultrafiltred *in vitro* hydrolysates of canaryseed, oat, and wheat peptides were determined to evaluate their health promoting effects. For each bioactivity assay, canaryseeds demonstrated equivalent or superior activity to both oat and wheat. The results of the DPPH and Fe^{2+} antioxidant assays suggest that brown cultivars of canaryseeds have superior free radical scavenging activity as compared to the yellow cultivars, since the IC_{50} values for both assays were significantly lower for the brown cultivars. Other than the DPPH and Fe^{2+} assays, there were no significant differences between yellow and brown varieties. Peptides from the C09052 3K MWCO permeate fraction was further purified by size exclusion chromatography and analyzed by mass spectroscopy, which identified 46 unique peptides from 18 proteins in the *Pooideae* subfamily. The results indicate that, although canaryseeds have been extensively compared to wheat, they most likely possess several proteins that are also present in barley. The 46 identified peptides were analyzed *in silico* to determine their potential bioactivity based on their amino acid sequence. The *in silico* analysis showed that all 46 peptides had potential ACE inhibitor and DPP-IV inhibitor activity, and 20 had potential antioxidant activity, which has been confirmed from the *in vitro* studies. However, 22 peptides had potential hypotensive activity, 5 had potential antiamnestic and antithrombotic activity, 2 had opioid activity, 2 had neuro activity, and 3 had immunostimulating activity, which demonstrates canaryseeds could have additional bioactivities that have not been confirmed thus far.

This study illustrates the exceptional bioactivity and health promoting effects of canaryseed proteins, particularly against cardiovascular disease. The health promoting effects of the seeds should still be confirmed further by *in vivo* studies. Canaryseeds should be regarded as a functional food since its utilization by the food industry in food products as a functional food or ingredient can help reduce the effects of non-communicable diseases, including cardiovascular disease, diabetes, and cancer as well as neurodegenerative diseases, like Alzheimer's disease.

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