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Agriculture Development Fund (ADF)
Project Final Report

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Project Title:	Process adaption and assessment of market development constrains for protein products from cold press, GM canola meal.	
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Abstract *(maximum 500 words)*

Detail an outline on overall project objectives, methods, key findings and conclusions for use in publications and in the ministry's database. The abstract should address the following (usually 1–2 sentences per topic):

- Key aspects of the literature review
- Problem under investigation or research question(s)
- Clearly stated hypothesis or hypotheses
- Methods used (including brief descriptions of the study design, sample, and sample size)
- Study results
- Conclusions

Cold pressing is a suitable canola meal feed stock generation process because of minimum alterations it causes to the protein and fiber during oil extraction. This project addressed value-chain extension potential for cold pressed canola cake (CCC) based on protein (P). Ethanol is a suitable solvent for de-oiling CCC. De-oiled meal (with ethanol, E-CCM, 40.6% P or hexane, H-CCM 35.4% P) and non-deoiled CCC (N-CCC, 31% P) were fractionated into protein-rich and fibre-rich fractions using AAFC Brassica meal fractionation. Two protein-rich fractions, namely, napin-rich protein isolate (NPI), cruciferin-rich protein concentrate (CPC), one protein and soluble fibre-rich intermediate fraction (IPF) and two fibre-rich fractions, one with liquified fibre (SSF) and the other predominantly composed of seed coat (SCF). Use of deoiled meal improved protein content of resulting fractions compared to the fractions of non-deoiled meal. NPI had the highest protein content (96.2-97.9%) followed by CPC (55.3 to 74.2%). NPI and CPC recovered 64-66% of total meal protein. Remaining fractions were low in protein levels: IPF with 25.2-29.7% and SCF 21.4 – 24.7% protein. AAFC meal fractionation process can be applied to non-deoiled CCC as well, producing same five fractions with high protein purity in napin-rich protein isolate. Residual oil of CCC was portioned into other four fractions. Protein fractions had amino acid profiles reflecting the protein type present and NPI predominantly contained napin while CPC contained other proteins including

cruciferin and comprised the largest fraction by weight. Most of the essential amino acid (EAA) of each meal exceeded the recommended value of WHO. NPI and CPC of respective meals showed the differences in protein types and purity reflect in the key functional and nutritional properties demonstrating that two protein products with differences in functional and nutritional properties can be obtained from CCC through this fractionation process and de-oiling of canola cake gives more protein purity, particularly for the large protein-containing fraction, which can be recovered more in yield-wise.

Studies of this project show the possibilities existing for cold-pressed canola cake(CCC). Ethanol (without added water) is an alternative to hexane to reduce oil content of CCC producing a suitable material for protein product preparation. Canola proteins extracted from cold-pressed meal showed better functionality than those from HE meal could mainly be due to the alterations of proteins during commercial level hexane extraction. Modification of meal proteins due to the enzymes released during fungal growth, also the changes occur in non-protein components affect recovery of proteins from depending on the conditions used.

Solid-state fermentation (SF) of CCC and desolventizer toasted hexane extracted (HE) canola meal was carried with *Aspergillus niger* NRRL 334 and *A. oryzae* NRRL 5590 fungi (both has GRAS status) for 72h with 50% starting moisture in meal. Both meals had a significant reduction (~80%) in phytic acid level, and a 65% to 81% decrease in total phenolics, and for CCC decrease in oil content from ~12% to 9% with an increase in crude protein level (~36% to ~40%) with both strains. Proteins of fermented meals was extracted with salt-extraction (SE) and alkaline extraction-isoelectric precipitation (AE-IP). SE protein products had protein content of ~95%, while AE-IP had 56.5% to 85% protein. Solubility of protein products of fermented meal decreased at pH 3 but increased at pH 7. Protein products had improved water and oil holding capacities but not the emulsifying activity index (5.6 to 21.1 m^2/g) and emulsion stability index (1.1 to 4.5 min). Values of foaming capacity were in the range of 154.4% to 480.0% and foaming stability 68.0% to 89.0%. Overall, 72-hour SF with these two organisms showed improvement in nutritional value of both CP and HE meals but resulted in beneficial and adverse impacts on functionality of protein products extracted from these substrates. The organisms used in SF was not very effective in utilizing residual oil of CCC and improving protein extraction, but partial hydrolysis of meal protein was evident. SF did not provide additional benefit to protein solubilization under the extraction conditions tested. The protein products obtained from fermentation modified meals were not significantly different in their functional properties.

Techno-economic analysis of showed the inverse relationship between capital investment and plant capacity of the protein production plant. However, the unit capital cost decreases correspondingly, clearly demonstrating the concept of economies of scale which refer to cost advantages (decrease in overall plant costs) that a processing plant can derive from expanding its operating scale. Canola meals for creating value-added protein products for suitable product markets require understanding of the acceptance level of the participant industries. The survey conducted to understand how willing the industries are to include GM canola proteins in their food products and what could be the barriers for that market. There is a greater level of acceptance by various industrial manufacturers towards plant protein in general. If canola proteins were to be extracted, produced, and exhibit exceptional functional properties compared to alternatives, and if the pricing also competitive with similar ingredients available; nearly half of the industries are willing to include canola proteins in their ingredient list. Even though there are industries that are reluctant to include canola protein considering it being GM, the survey results showed that almost 40-50% of the industries would consider canola proteins regardless of their GM status.

Extension Messages *(3 to 5 bullet point in plain language)*

Provide key outcomes and their importance for producers/processors and the relevant industry sector.

This research brought foundational level information to utilize and add value to cold-pressed GM canola cake.

Value addition to canola press cake can be through component fractionation or bio-transformation. Component fractionation can produce protein-rich and fibre-rich products, in which the nutritional and tecno-functional values are dictated by the protein types and/or fibre types present in the fraction. Solid state fermentation with Aspergillus strains with GRAS Status can be employed in bio-transforming cold-press canola cake as will as industrial hexane extracted canola meal. Compared to cold-pressed canola cake, the proteins and fibre of hexane -extracted meal is less susceptible to modifications that are needed for improving protein fraction utilization. Further research on aligning canola protein products, either fractionated or bio-transformed with plant proteins in the market, demonstrating their nutritional and functional strength in consumer products haven't happened yet. These involves working with industry who is truly willing to take canola protein or meal-based products into market. Further scientific investigations can be carried for detailed understanding of chemical- or bio-technological transformation of canola protein and fibre into new products with demonstrated functions. Developing canola proteins with exceptional functional properties superior to available alternatives and increasing consumers awareness about the benefits of GM products in general would help address decreased consumer acceptance, identified as one of the major barriers by the industries.

Introduction *(maximum 1,500 words)*

Provide a brief project background and rationale.

The seed proteins that comprise about 22% of seed weight is the most economically valuable storage component of canola after oil. Together oil and protein determine the economic value of canola industry end products. In the canola crop value-chain extension strategies, maximizing the value of protein of oil removed meal becomes a priority because of the increasing market demand for plant protein. The pressure is on to find whether a fraction of canola protein can enter the food protein market in addition to the current use in animal feed. Increasing canola production and increasing capacity of domestic seed crushing operations give locally available starting materials for generating plant proteins for the increasing global demand in the revolutionary protein transition that affect food production system as whole.

Proteins of canola seed is reported for satisfactory nutritional and functional value (Newkirk et al., 2003; Wanasundara, 2011) that can bring additional value to canola industry. Studies conducted under laboratory scale show that proteins of canola be successfully separated with 50-60% recovery yield after oil removal. Although these findings can be extended to canola meal generated from commercial oil extraction (prepressed, solvent extracted and desolventized), many studies showed it is not the case. Few medium to large scale canola protein extraction ventures had started their operations in the prairies in last 20 years and utilized distinct meal and protein processing technologies for different target markets. The most recent venture was for generating canola proteins for plant protein food market and employed unique technology to separate canola protein thus ingredient functionality is at the maximum. Global plant protein ingredient manufacturers are interested in canola/rapeseed protein showing that still the market demand for unique protein ingredients exists.

At present, canola protein production is disconnected from the existing canola meal production or the other way around. Oil being the primary product as well as the revenue stream, seed processing is optimized to obtain high quality canola oil for vegetable oil market. Canola meal production in Canada is primarily by pre-press solvent extraction generating a meal not suitable to produce high quality protein that can compete in the protein ingredient market (Mupondwa et al., 2018). Cold-pressing emerges as an option for canola for several reasons: i) to capture the market share for virgin, chemical-free edible oils; ii) generates a meal with minimum heat damage to the protein; iii) generates an energy-dense protein feed because of high (14-16%) residual oil levels. Opportunities exist to align a protein recovery process in tandem with this low temperature meal processing and consequently improve economics of cold-pressing operation. In 2007, AAFC scientists develop a meal fractionation process applicable to all brassica oilseeds, considering the differences in protein types and also to recover fibre separately from protein. This process also requires mildly processed meal so that proteins are less altered and can be efficiently separated and successfully utilized. However, technology development gaps exist for protein production using cold-press canola meal. This project proposed investigation of value chain extension of cold-pressed canola meal via utilizing protein components and, the existing gaps in exact applications or markets that can be targeted based on functional and nutritional attributes including an assessment of consumer perceptions of canola protein and GM- canola to position products in the market.

Objectives and Progress *(add additional lines as needed)*

Please list the original objectives and/or revised objectives if ministry-approved revisions have been made to original objectives. A justification is needed for any deviation from original objectives.

Objective	**Progress** *(i.e., completed/in progress)*
1. To modify AAFC Brassica protein fractionation process to use cold-press canola meal.	Completed
2. To assess solid-state fermentation (SF) as pre-treatment for cold-press and prepress-solvent (hexane) extracted canola (HE) meals to improve protein solubility. Identify fermentation conditions for enhanced protein recovery from fermentation modified CCC and HE meal.	Completed
3. Techno-economic analysis of cold-press canola operation in combination with canola protein production. Techno-economic evaluation, value-chain characterization, business cases for cold-press GM canola seed.	Completed

Methodology *(maximum of five pages)*

Specify project activities undertaken during this reporting period. Include approaches, experimental design, tests, materials, sites, etc. Please note that any significant changes from the original work plan will require written approval from the ministry.

Objective 1 (Wanasundara & Majumder) and Objective 2 (Nickerson & C. Li)

Meals, chemicals and other ingredients

Cold-press canola cake (CCC) from Pleasant Valley Oil Mills (Clive, AB) (3 batches during May and October 2019; and August 2020)and commercial hexane-extracted meal (HE) from Bunge Canada (Harobe, MB) were meal substrates used in these study.

Fungal strains *Aspergillus niger* NRRL 334 and *A. oryzae* NRRL 5590 that are Generally Regarded as Safe (GRAS) obtained from Agricultural Research Service (ARS) Culture Collection (NRRL, Peoria, Illinois, US) were used for spore suspension preparation as described by the publications by Li et al. (2023 from this project)

Plant (soy protein isolate from Bob's Red Mill) and animal protein (whey protein isolate from 100% premium whey isolate, Canadian Protein, Ontario), and Milk protein concentrate (80% from Idaho Milk Products, Jerome Idaho) were purchased from respective commercial suppliers. Canola oil obtained from a local grocery store (No-name brand of Loblaws) was used.

All media, reagents and chemicals used were analytical grade and obtained from Sigma-Aldrich Canada Ltd. Fisher, and Bio-Rad suppliers in Canada. Ethanol (99%) was obtained from Greenfield Global (Ontario, Canada).

Processes applied in meal and protein preparation

Ethanol-extracted canola cold-pressed meal (E-CCM): CCC was extracted with ethanol or aqueous ethanol according to the flowchart provided in **Figure 1.** The factors that affect oil extraction; water content in ethanol-water mixture, meal-to-solvent ratio, extraction temperature, and extraction time were studied, and 27 experimental points were obtained according to the Box-Behnken (four factors) experimental design using Design Expert®software v.11.0.3.0 64 bit (2017). Based on optimization experiments results (0% water content in ethanol, meal-to-solvent ratio of 1:4.5 w:v, an extraction time of 1.75 h, and a temperature of 25℃) several ~50 g batches of CCC were extracted with a second extraction using (**Figure 2**) to reduce the remaining oil content of E-CCM to reach less than 1 %.

Hexane-extracted canola cold pressed meal (H-CCM): H-CCM was obtained by extracting CCC with hexane (1:2.5 w:v) at room temperature to represent the effect of hexane on meal components when oil extraction is carried out at the same temperature as ethanol extraction.

Protein and co-product separation using AAFC Brassica meal fractionation process : AAFC Meal fractionation process (Wanasundara & McIntosh, 2013) is a continuous process that a meal goes through and generates at least five different fractions as illustrated in **Figure 2**. E-CCM, H-CCM, and N-CCC were used in the meal component fractionation process.

Solid-state fermentation: CCC and HE substrates (200 g of each) were fermented with *A. niger* NRRL 334 and *A. oryzae* NRRL 5590 spore suspensions after standardizing to a spore concentration of 10^7 colony forming units (CFU) to apply per gram of meal. The substrate, spore suspension and deionized water were mixed at speed 5 for approximately 3 min using a commercial stand mixer before spreading out thinly (approximately 1.5 cm) and evenly onto a stainless-steel sheet pan. Fermentation was started at 50% moisture content (w/w), at 30℃ over a 72-h period in an Isotemp incubator (Fisher Scientific, Model 650D, Waltham, MA, USA). Mill-Q water was added according to the weight loss each day to maintain the moisture content. Samples (~50 g) were collected on random spots on each batch at the time of initial inoculation (0), 24, 48 and 72 hours, dried. To stop the fermentation, the meals were frozen at –20℃ right after fermentation until further freeze-drying. Both dried powders were stored at 4℃ until further testing.

For protein product preparation, CCC and HE (each 150 g) fermented separately with *A. niger* NRRL 334 and *A. oryzae* NRRL 5590 (each at 10^7spores/g meal) for three days (72 h) were used. A sample of deoiled CCC prepared by extracting with a meal:hexane ratio of 1:3 (w/v) over a 1.5 h period of stirring at room temperature for three times, vacuum filtered with No. 1 Whatman filter paper to recover solids. This extraction was repeated twice to reach an oil content of < 2%. Un fermented, non-deoiled CCC and HE were the controls used throughout the study.

Protein extraction was conducted using fermented (72 h) and unfermented CCC (both defatted and non-defatted) and HE meals (100 g) by employing two methods.

Protein separation by alkaline extraction-isoelectric precipitation (AE-IP): Meals (~ 100 g) were extracted using alkaline solution at a 1:10 (w:v, meal: water) ratio, at pH 11 (adjusted using 2 N NaOH solution) for a 1 h period at room temperature according to Aider and Barbana (2011). Meal solubles were recovered by centrifugation (4500 × g at 4℃ for 15 min), and pH of the supernatant was then adjusted to pH 5 by adding 2 N HCl solution to precipitate the protein. Precipitated protein was collected as a pellet by centrifugation at 4500 × g at 4℃ for 15 min, followed by freeze drying.

Protein extraction by salt extraction-dialysis (SE): Protein extraction of meals was according to Klassen *et al.* (2011) and Chang *et al.* (2015) with minor modifications.

Chemical and physico-chemical property analysis

Composition: The moisture content of the meal samples was determined by (AOAC, 1990), where samples were dried at 105℃ overnight in a forced air oven (Fisher Scientific, Isotemp oven). The protein content of the meal sample was determined by combustion based N analysis (AOAC, 2006, AACCI method 46-30.01). A nitrogen to protein conversion factor of 6.25 was used. Crude lipid was determined using the Goldfisch apparatus according to AOAC method 920.39 (AOAC 2005). The residual oil content of the meal was determined by the Swedish tube method (Troëng, 1955). Ash content was determined according to AOAC method 943.05 (AOAC 2005). The amino acid composition (18 amino acids) of the samples were determined according to AOAC method 994.12 (AOAC, 2005). Polypeptide profile of samples was determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The phytic acid content of samples was analyzed by the colorimetric method using the Megazyme Phytate analysis kit (Phytate Assay Kit - Megazyme, 2019). The total phenolic content (TPC) of the samples was determined using the method described by Oomah et al. (2005) and using sinapic acid standard.

pH: Meal slurries (dried meal and Milli-Q water at 1:10 (w/v) ratio) at room temperature (21-25℃). Were used for measuring pH.

Degree of protein hydrolysis: The protein hydrolysis was measured as the released free amino groups using 2,4,6-trinitrobenze sulfonic acid (TNBS) method according to Adler-Nissen (1979) and Jung *et al.* (2005).

Protein solubility: Solubility of proteins in the prepared products was evaluated with pH changes (4.0, 7.4 & 9.5) using appropriate buffers.

Surface hydrophobicity: The surface hydrophobicity of samples was determined by generating 8-Anilino-1-napthalenesulfonic acid (ANS) binding curve of protein against soluble protein concentration. The surface hydrophobicity of each sample including BSA was calculated as the initial linear slope of fluorescence (AU/mg/mL) (Cardamone and Puritt, 1992).

Zeta potential: Samples prepared for surface hydrophobicity determination were used for the analysis of zeta potential. A Malvern Zetasizer Ultra coupled with XS Explorer software was used for obtaining data and analysis (Benitez and Lozano, 2006; Malvern Instruments, 2017).

Emulsifying properties: Emulsifying capacity (EC) was determined by the method described by (Wang and Kinsella, 1976) with some modifications. The height of the emulsified layer was taken as soon as the emulsion formed (0 min) and then placing in a water bath maintained at 80°C (30 min) to determine emulsion stability.

Foaming properties: Foam forming properties were determined as foam expansion and foam stability by employing the methods described by Patel et al., (1988), Liu *et al.* (2010) and Stone *et al.* (2015).

Oil holding capacity (OHC) and Water holding capacity (WHC): The OHC and WHC of protein samples was determined at ambient temperature according to (Pathiratne, 2014).

Thermal denaturation temperature and enthalpy of denaturation: Differential scanning calorimetry (DSC) was conducted to evaluate the thermal denaturation temperature of the protein-rich products as described by (Perera et al., 2016).

In-vitro protein digestibility: The *in-vitro* protein digestibility of NPI and CPC was determined based on the US patent # 9,738,920 (Plank, 2017) using Megazyme Protein digestibility assay procedure (Megazyme, 2019).

Statistical analysis

Data collected from all 27 experiments were analyzed using the Design Expert® software to find the optimum factor combination level of aqueous ethanol extraction conditions.

Fermentation was made in triplicate using a separate plate and spore suspension (n=3). A three-way analysis of variance (ANOVA) was used to study the statistical differences in composition and anti-nutritional factors as a function of meal type, fungal strains and fermentation time with a significance level of $p < 0.05$. A post-hoc Tukey's test (multiple comparison procedures) was used to detect statistical differences in fermentation time. All protein products were prepared in triplicate on separate fermented meals (n=3). The results were reported as mean ± one standard deviation. A one-way analysis of variance (ANOVA) was used to study the statistical differences in protein functionality using Tukey's test with a significance level of $p < 0.05$. A simple Pearson correlation was used to describe the relationship between protein functional properties. All statistics were performed using the IBM SPSS Version 28.0 software (IBM

Corp. NY, IL, USA) or Minitab software (Minitab LLC, PA, USA). The significance of the difference was determined at $p < 0.05$.

Objective 3: (Mupondwa and X. Li)

Technoeconomic evaluation, value-chain characterization, business cases for cold-press GM canola seed undertook two activities: a) technoeconomic analysis (TEA) of canola meal protein concentration and, b) evaluation of potential adoption of value-added products from cold-press GM canola seed.

Technoeconomic analysis (TEA) of canola meal production and meal dry fractionation; Comprehensive TEA and engineering process design require the generation of canola meal fractionation process mass flow balance data. This was undertaken in two phases. In the first phase, 250 kg of canola seed purchased from a local farmer and processed for de-oiling to generate meal (and harvest oil, which is the major products of canola). De-oiling process involved cold-pressing (temperature < 60°C may prevent heat-induced protein alterations) and ethanol washing (a food-grade solvent, and different than hexane used by current oil and feed grade meal-focused industry norm). The generation of mass balance for the first phase is depicted in **Figure 3** (red letters), where D-60/D-90 are diaphragm pumps with pumping capacity of 6,000 L hr^{-1} and 9,000 L hr^{-1}, respectively. Second phase included air classification of defatted canola meal. This phase involved collecting the coarse canola meal followed by feeding, milling, and air-classifying into fine and coarse fractions over repeated procedures. Air classification was performed with 3 kg batches at Saskatchewan Food Industry Development Centre.

Survey on the acceptance of Genetically Modified (GM) canola protein: The characterization of the value-chain and development of a business case for protein from cold-press GM canola seed was further supported by a quantitative industry survey of major stakeholders in the oilseed processing industry. The objective of the survey was to understand the willingness of food and beverage manufacturers in adopting GM canola proteins as functional ingredients in their products. This includes understanding factors that represent barriers to industry adoption or contribute to wider adoption. This is important because nearly 95% of Canada's canola is genetically modified.

A questionnaire, developed by AAFC researchers to achieve the research objectives and the design following principles described by Statistics Canada (2010), including the design of semi-structured questionnaires containing closed questions (binary, ordinal multiple choice, or general statements) and open-ended questions for additional information which respondents provided in free prose was used. The questionnaire had 14 questions (Appendix I) intended to elicit response from food and beverage manufacturers vis-à-vis the adoption of GM canola protein as a functional ingredient in food manufacturing. The information on potential participants for the survey was collected through web extraction methods and by contacting various food and manufacturing industry associations. Different food and beverage manufacturers within Canada were contacted to assess their willingness to participate in the survey. The survey commissioned the services of Kai Analytics and Survey Research Inc., a professional Canadian survey company, as the contractor and conducted via email between October 2022 to January 2023. The survey link was created by the contractor using the survey platform Alchemer and included a consent form and survey questions, with no linkage the responses to specific email addresses to ensure respondent confidentiality.

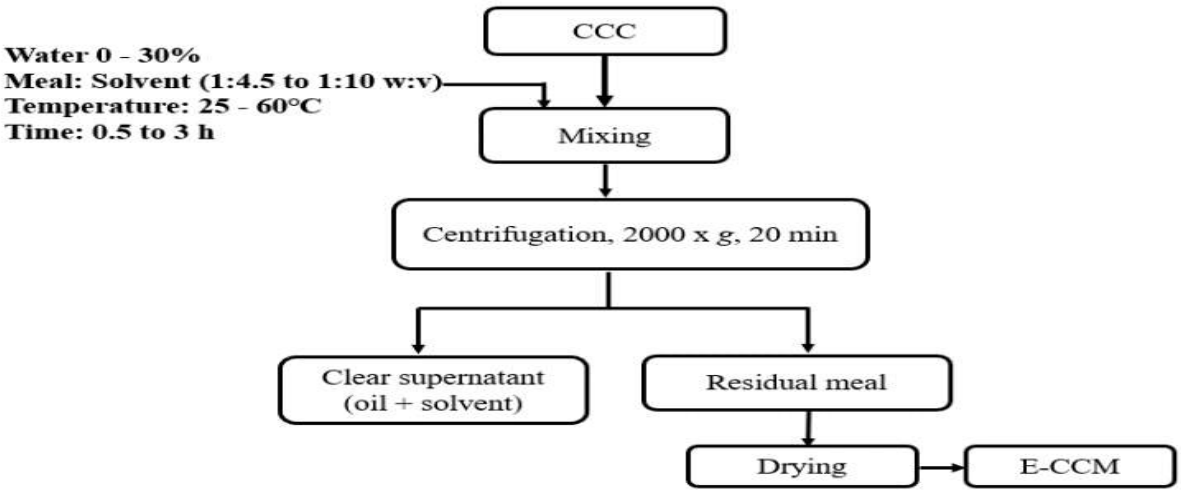

Figure 1. Process of preparing ethanol-extracted cold-pressed canola meal (E-CCM). Water content in the ethanol-water mixture, meal-solvent ratio, extraction time, and temperature of extraction were the factors considered in finding optimum conditions for removing the maximum amount of oil from N-CCC. The experimental range of these factors is in parenthesis.

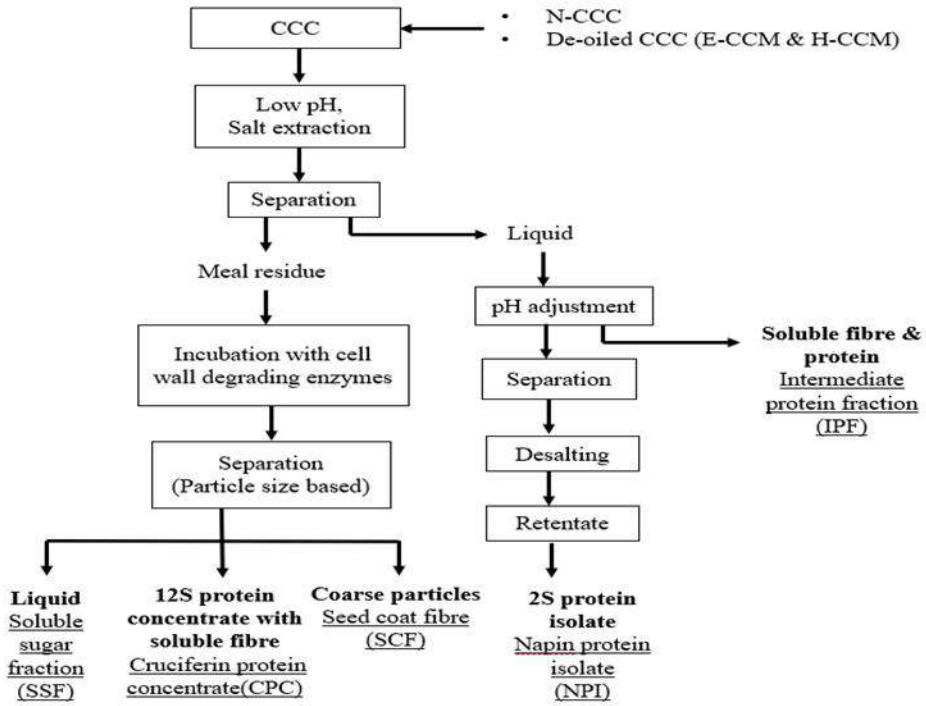

Figure 2 AAFC meal protein fractionation process. The process generates five products which are underlined. For the meals, non-deoiled CCC (N-CCC) and de-oiled CCM (E-CCM and H-CCM) were used. The products were NPI, CPC, IPF, SSF, and SCF.

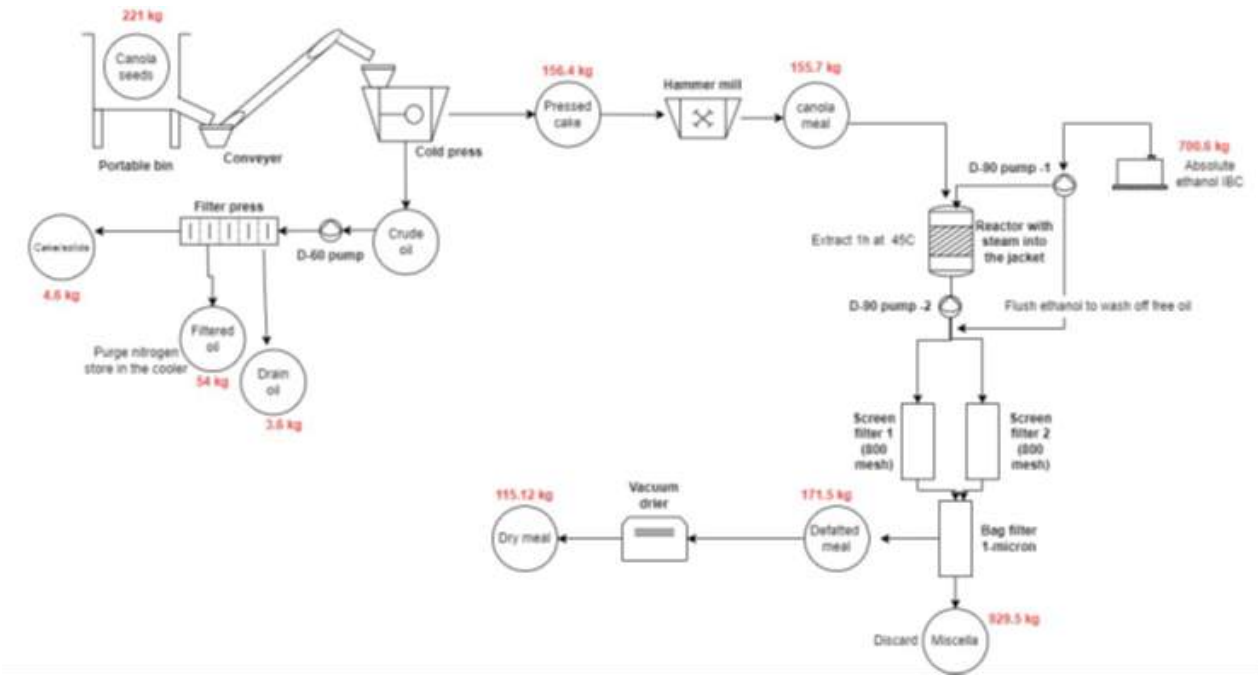

Figure 3. Process flow of canola cold-pressed oil and ethanol-de-oiled meal production including mass balance.

Results and Discussions *(maximum of 30 pages (not including figures or tables))*

Describe research accomplishments during the reporting period under relevant objectives listed under “Objectives and Progress” section. Please accompany a written description of results with tables, graphs and/or other illustrations. Provide discussion necessary to the full understanding of the results. Where applicable, results should be discussed in the context of existing knowledge and relevant literature. Detail any major concerns or project setbacks.

Objective 1 (Wanasundara & Majumder)

Obtaining low-oil meals from CCC

The CCC received in three instances had an oil content of 13.7 ± 0.7%. Oil removal efficiency was in the range of 88% to 114% with ethanol (99%) and with water to ethanol at 15% or 30% gave lower values. Over 100% efficiency may indicate not only oil but also other ethanol (99%) soluble components such as water, polyphenol, pigments and soluble sugars from CCC were removed and the calculation of oil removal efficiency based on the weight of soluble components of the extracting solvent may have also contributed.

Figures 4 (a) and (b) provide two different scenarios of the four-factor combinations obtained from data analysis (desirability 100) that can reach 100% oil removal from CCC. It was observed that the water content in ethanol for these two combinations was in the low range, 0.6% (a) and 0.07% (b). Combination (a) proposed to use a higher meal-to-solvent ratio (1:9.3, w:v), higher temperature (58℃), and shorter extraction time (0.5 h) than combination (b) in which the values were 1:7 (w:v), 32.7℃ and 2.9 h, respectively. **Figure 5** depicts the relationship between the meal-to-solvent ratio and water content in ethanol for the oil extraction efficiency while keeping extraction temperature at 32.7℃ for 2.9 h. According to the response surface and corresponding contour map, it is clear that >90% oil removal efficiency can be achieved when the water content is between 0-30% and the meal-to-solvent ratio of 4.5 to 10, w:v).

All canola protein extraction methods described in the literature including the AAFC Brassica meal fractionation process require a starting meal containing less than 1% oil. Although few experimental combinations gave residual oil content of meal <1%, combinations that use no water were selected because of practical considerations; high moisture content of recovering meal requires a higher temperature for drying and the water in ethanol makes it difficult to recover ethanol by distillation. Therefore, no added water in ethanol (0%), meal: solvent of 1:7.25 (w:v), extraction time of 1.75 h, and an extraction temperature of 25℃ were considered as extraction conditions. With large volumes (2 L) under lab conditions, sufficient oil removal didn't happen after 1st extraction and the percentage of residual oil was >1% (Table **1**). Therefore extracted meals were combined and re-extracted using the combination of no water in ethanol (0%), meal: solvent 1:4.5 (w:v), extraction time 1.75 h, and temperature 25℃ to achieve meal residual oil content <1%. The use of elevated extraction temperatures (42°C and 60℃) was avoided because of the possible effect on the protein-heat-induced aggregation that could make them less extractable. Ethanol is an effective solvent to remove the remaining oil of CCC and can be an alternative to hexane.

Composition of non-deoiled CCC (N-CCC) and de-oiled CCM (E-CCM and H-CCM)

Contents of moisture, oil, protein, total phenolic compounds, and phytates: E-CCM had a low moisture content of 1.7 % (**Table 2)** as well as the highest protein content of 40.6 % on dwb. CCC and H-CCM had a high moisture content of 7.7% and 6.8% and, a low protein content of 31.1% and 35.4% than E-CCM. Since the oil content of both E-CCM and H-CCM were in the same range (0.2-0.5%), it can be assumed that ethanol must have removed some other polar compounds that hexane as a non-polar solvent couldn't remove. Also, ethanol is a hygroscopic solvent, it can be considered that ethanol absorbs most of the moisture from the meal and takes out most of the available water in CCC.

Reported TPC of defatted canola meal is in the range of 15.9 mg/g to 18.4 mg/g (Naczk et al., 1998), however the present study reported low TPC; 1.4 mg/g for N-CCC, and 1.3 mg/g and 1.4 mg/g for E-CCM and H-CCM, respectively. The solubility (or extractability) of phenolic compounds depends on the polarity of the extracting solvent and the solubility of existing phenolic compounds in the solvents (Babbar et al., 2014). Extraction of CCC with ethanol to remove oil was able to extract some of the phenolic compounds of the meal. Ethanol has both polar and non-polar groups and therefore extracts more phenolic compounds than hexane. Canola seed contains phytates comparable to many other oilseeds. Phytate level for de-oiled canola meal has been reported as 2.0-5.0% (Uppstrom and Svensson, 1980). No significant differences were observed between non-de-oiled and de-oiled meals phytate content. Solvent treatment did not affect much on the CCC phytate removal (**Table 2**).

Amino acid profiles of CCC and low oil CCM: The values for both essential (EAA) and non-essential amino acids (NEAA) of the three canola meals (N-CCC, E-CCM, and H-CCM) were in close range. No differences were observed for the concentration of individual amino acids among the three meals indicating no effect on the defatting, in particular no loss

of protein. When these meals were compared with the reported values for canola meal and soybean meal amino acid composition, it was observed that Cys, Met, Trp, and Asp+Asn were the only AA had the lowest value in N-CCC, E-CCM and H-CCM. Other AA values of these three meals were almost similar with the canola meal and soybean meal AA. The EAA content of these samples are compared with WHO/FAO for proteins (**Figure 6**) and as a percentage of WHO adult requirements (**Figure 7)**. The values of all EAA (His, Ile, Leu, Lys, Phe+Tyr, Thr, Val) from three meals (N-CCC, E-CCM, and H-CCM) exceeded the WHO recommended requirement value except for Met and Trp. The value for Cys was similar to the WHO recommended value (WHO/FAO/UNU, 2007). When the percentage of the EAA from N-CCC, E-CCM, and H-CCM were compared (**Figure 7**), some of the EAA gave more than 100% value such as His, Ile, Leu, Lys, Phe+Tyr, Thr and Val. Cys, Met and Trp gave below 100% value.

Fractionation of N-CCC, E-CCM, and H-CCM

As expected, N-CCC, and the de-oiled CCM (E-CCM & H-CCM) gave five different products from this fractionation .The physical appearance of all these products along with the starting meal is in **Figure 8**. The two products identified as 2S napin protein isolate (NPI) and soluble fiber with protein or intermediate protein fraction (IPF) were obtained from low pH soluble liquid fraction of the meals. The permeate was the fraction that went through the 3.5 kDa membrane and belongs to the low pH soluble. The other three products, 12S protein with fiber as cruciferin protein concentrate (CPC), sugar-rich soluble derived from soluble fiber (SSF) of cell walls (liquid), and seed coat fiber (SCF) fractions were obtained by further fractionating of the insoluble remained form low-pH extraction step. The AAFC Brassica meal fractionation is different from alkali solubilization and isoelectric precipitation that is conventionally used in recovering protein from oilseed meals such as soybean and in most of the new plant protein sources. Although canola is an oil-rich seed, the proteins of canola seed differ in structure and properties from soybean. The AAFC Brassica protein fractionation process first recovers proteins with more basic characteristics (primarily napin, 2S protein) by solubilizing them at low pH (pH 3) leaving behind cruciferin (12S protein) in the meal which is not soluble under these conditions. This soluble fraction contains acid-soluble polysaccharides from cell walls, and these can be removed from the solution as a precipitate at pH 7. Some of the proteins also co-precipitate with polysaccharides therefore an intermediate fraction containing some proteins along with polysaccharides can be separated in the process. The remaining liquid is rich in protein. When salt and other small molecular weight components are removed by membrane filtration using a 3.5 kDa membrane, 2S proteins can be further concentrated with fewer non-protein contaminants. The meal residue of low-pH extraction contains primarily cruciferin, cotyledon cell walls, and seed coat particles. Enzymatic degradation of cell walls of cotyledon particles can be achieved by using multifunctional enzymes Celluclast® and Viscozyme®-L. Celluclast® breaks down the cellulosic materials into sugar units such as glucose, and cellobiose; and reduces the viscosity of soluble cellulosic substrate or increases the protein content of remaining solids. Therefore Viscozyme®-L helps to liberate bound components as well as hydrolyze non-starch polysaccharides, reducing viscosity, and increasing the yield of proteins (Cellulase-NCBC). In this step, enzymes degrade cell wall polysaccharides generating soluble sugars and at the same time, cotyledon particles get reduced in size. These enzymes are less effective in degrading the cell walls of the canola seed coat, maybe compositional differences play a role. Therefore, at the end of enzyme treatment, the liquid fraction containing sugars can be separated from the cotyledon particles along with seed coat particles. Wet separation of this solid mixture using sieves with an opening size of 250 µm allowed the separation of large seed coat particles from smaller particles. These cotyledon particles contain the remaining SSP which is primarily 12S cruciferins.

Dry matter and protein partitioning among fractions: Dry matter recovery (**Table 3)** of NPI, IPF, CPC, SSF, and SCF was based on starting meal dry weight. N-CCC contained ~13 % oil which was distributed among these fractions contributing to dry matter. Across all three meals, contribution to dry matter content was the lowest by the IPF (2.4-2.8%). The NPI fraction was between 4.2 and 4.6% and contained proteins and acid-soluble polysaccharides of the cell walls which were precipitated at pH 7.0. This neutralization step may have removed certain isoforms of napins and cruciferins that were soluble at pH 4.0 but not at neutral pH constituting proteins in the IPF. The dry matter recovery of CPC fraction (10.2% and 13.3%) included fine meal particles rich in 12S protein and cell wall fragments depleted of acid-soluble fiber. These particles are rich in insoluble fiber which could not be broken down by enzyme activities found in Viscozyme. The SCF was between 9.3-12.6% of total dry matter and derived from the insolubles at low pH and even after enzyme treatment and contained large seed coat particles. SCF particles < 250 µm may have gone to the CPC, enriching the insoluble fiber content. It is unavoidable in this process because in the CCM preparation, no seed coat removal happens, and breakage of the seed coat is random and particle size control is difficult. The soluble components due to enzyme activity make the SSF and are also derived from the cell walls (fiber fraction) of the meal. The total dry matter recovery in the SSF was 11.3-13.9%. No significant differences were found among the three meals (N-CCC, E-CCM, and H-CCM) for these dry matter recovery yields. However, H-CCM gave the lowest dry matter recovery for SCF. Since de-oiling with hexane reduced the particle size of the H-CCM, it gave the lowest dry matter recovery yield in $SCF_{H\text{-}CCM}$. The highest dry matter recovery yield was observed in the permeate from all three meals and the value was 55.6 to 59.5% (Data not shown). Permeate contained all low molecular weight components extracted from meal under low pH and also added NaCl during this process.

When protein partitioning between fractions is considered, NPI contained 17.1 - 20.1% of meal protein regardless of meal type. The CPC contained 19.8 - 30.7% of meal protein. Together these two fractions retained 36.9 - 50.8% of meal protein. The IPF had a low meal protein content which is in the range of 2.9 – 3.2% of meal protein. The SSF contained 7.1 – 9.8% of meal protein depending on the meal. SCF had 9.6-12.1% of meal protein. No significant differences were observed between the meal fractions protein recovery yield, except for $CPC_{N\text{-}CCC}$. As the N-CCC oil was not removed, most of the oil could have ended up in the CPC fraction (**Table 1.7**) and thus reduced the protein recovery yield. Some oil may have been in the permeate of membrane separation, which was discarded. It should be noted that although pH 3.0 extract permeates a considerable portion of DM. For pH 3 permeate, the total DM and protein recovery was 54.6 - 61.9% and 28.5 - 38.3%, respectively. The percentage of total dry matter recovery yield and protein recovery yield was ~100% when the permeates were considered.

Chemical composition of fractions

Protein content: The NPI recovered from low-pH and salt soluble fraction had 96.2 - 97.9% protein content regardless of the starting meal **(Table 3**). When CCC was extracted at low pH (3.0), most of the napin isoforms got into the solution. Napin is rich in basic amino acids and ionization of basic residues keeps napin soluble at acidic pH. The addition of salt increases the ionic strength of the medium and disrupts the association of napin with other molecules while increasing their solubility (Wanasundara et al., 2012). The IPF contained compounds that precipitated at pH 7.0 when low pH extract was neutralized. Some protein (25.2% N-CCC, 29.7% E-CCM, and 28.0% H-CCM) and acid soluble fiber made up most of the dry matter of this fraction. The pH 3.0 extract permeate also contain some protein 12.1-13.1%. The residual meal of low pH extraction released soluble sugars (mono-, di-, or oligo sugar units) from cell wall carbohydrates (mostly polysaccharides) because of treatment with cell wall degrading enzymes. The SSF also contained protein in the range of 15.0 to 16.3%. $SSF_{E\text{-}CCM}$ had the maximum protein content than $SSF_{N\text{-}CCC}$ and $SSF_{H\text{-}CCM}$. The remaining residue of enzymatic treatment gave two more products; the CPC and SCF which were separated based on the wet particle size. The CPC was the second protein-rich fraction obtained in this process and had 55.3%, 74.2%, and 59.2% protein for N-CCC, E-CCM, and H-CCM, respectively. When CPC is considered, $CPC_{E\text{-}CCM}$ gave the highest protein content than $CPC_{N\text{-}CCC.}$ As the oil was not treated in the N-CCC, the oil content was distributed among all the fractions. CPC was extracted from seed coat materials, most of the oil could have remained in the CPC fractions extracted from N-CCC. Therefore, it reduced the protein recovery percentage and protein content of the $CPC_{N\text{-}CCC}$. The CPC had lesser protein content than NPI because some insoluble fiber from cell walls and finer seed coat particles that escaped from the sieve were included in this fraction. The SCF had 21.4 - 24.7 % of protein content. In general, the canola seed coat contains about 18% protein (Wanasundara et al., 2012)

therefore most of the protein in this fraction may be seed coat associated. Since the starting material used in the study was from a commercial oil extraction plant, it is difficult to control seed particle size reduction going through the process and have to accept what was received from the supplier. Also, there is no understanding of canola seed coat fragmentation during the cold press oil extraction process step and how initial moisture, the temperature of the seed, etc. affect seed coat breakage. Separation of seed coat particles from cotyledon particles and the liquid was achieved according to the sieve opening size therefore larger pieces of seed coat and cotyledon fragments that did not pass through were retained in this fraction.

Oil content: When the oil content of the fractions from N-CCC was considered (**Table 4**), $IPF_{N\text{-}CCC}$ and $CPC_{N\text{-}CCC}$ retained the maximum amount of oil. $NPI_{N\text{-}CCC}$ and $SSF_{N\text{-}CCC}$ retained less oil. NPI was obtained by ultrafiltration (3.5 kDa MWCO), it was assumed that some amount of oil was taken by the permeate (0.61% permeate oil content, data not shown). $SSF_{N\text{-}CCC}$ contained cell wall degrading polysaccharide, thus it had less chance to retain much oil in it. $IPF_{N\text{-}CCC}$ had ~25% protein content, and protein had both polar and non-polar groups, most of the oil was retained in the IPF fractions. Residual meals may have some oil which ended up in the CPC fractions. CPC fractions were obtained from the spent meal after removing low pH-soluble proteins and fiber components.

Total phenolic content (TPC) and phytate levels: The $NPI_{N\text{-}CCC}$ had a higher TPC than the other fractions (**Table 5**). As the N-CCC was not treated with any solvent, the phenolic compounds may have remained in the meal and ended up in the NPI. Although membrane separation using 3.5 kDa was used and allowed free phenolic compounds which are much smaller than 3.5 kDa to be removed in the permeate, these phenolic compounds may have been associated with napin protein. Extraction of oil with ethanol or hexane was able to extract phenolic compounds from CCC, simultaneously. The TPC of $NPI_{E\text{-}CCM}$ and $NPI_{H\text{-}CCM}$ statistically were not different, which could be at pH 3 fewer phenolic compounds were available for interacting with protein, and less may be retained in the residue. No significant differences were observed in the TPC of $SCF_{N\text{-}CCC}$ and $SCF_{E\text{-}CCM}$ and their values were lower than the $SCF_{H\text{-}CCM}$ (0.8 mg/g). $CPC_{H\text{-}CCM}$ had a TPC of 0.5 mg/g compared to 0.4 and 0.4 mg/g, $CPC_{E\text{-}CCM}$ and $CPC_{N\text{-}CCC}$. In general, the TPC of CPC was much lower than the NPI and SCF. The CPC was recovered by the enzymatic treatment, where the enzymatic breakdown of cellular compounds may have released proteins (cruciferin) along with some fibers and a minor amount of phenolic compounds. The remaining phenolic compounds of the starting meal may have remained with the seed coat particles. $SCF_{H\text{-}CCM}$ consisted of very small size particles compared to the $SCF_{N\text{-}CCC}$ and $SCF_{E\text{-}CCM}$ because of the process (impact of steel balls in the steel tubing) used in the hexane extraction step. Thus, it may have caused more phenolic compounds to partition in the $SCF_{H\text{-}CCM}$.

No significant differences were observed in the NPIs phytate content between N-CCC, E-CCM, and H-CCM (**Table 5**). NPI had the lowest phytate content among all the fractions. Although the acidic pH and NaCl may have minimized protein-phytate interaction and allowed phytates to be released in the soluble form, the membrane separation step removed most of them in the permeate of the low-pH soluble fraction during NPI making. The napin rich Supertein™ obtained from the protein micellation process had 3.34% phytate content (GRAS, 2010) which is a much higher value than the napin-rich fraction obtained in this fractionation process. The $CPC_{E\text{-}CCM}$ contained phytates (2.8 g/100 g) more than the same fraction obtained from the other two meals; $CPC_{N\text{-}CCC}$ and $CPC_{H\text{-}CCM}$. Phytates that remained in the spent meal of low-pH extraction may have not been completely set free under the conditions of enzyme treatment, thus concentrated with CPC. The phytate content of the commercial cruciferin-rich Puratein® is 0.32% (GRAS, 2010) compared to the fractioned CPC phytate content. In this study, ethanol treatment enhances the protein content (**Table 3**) and the phytate concentration (**Table 5**) in $CPC_{E\text{-}CCM}$. After oil extraction from CCC using ethanol (99%), enhance the availability for protein and phytates. The IPFs from all meal samples had the highest phytate content among the fractions. IPF was obtained by precipitating some protein along with polysaccharides.

Amino acid composition: When the levels of EAAs of the NPIs of each meal were compared, no difference between meals could be observed. Similarly, no differences were observed for EAA levels in the CPCs and IPFs of each meal. The reported value of the AA of 2S protein isolate and 11S protein concentrate have a similar value to the NPIs and CPCs. **Figure 9 a & b** presents EAA levels of NPI and CPC in comparison with FAO/WHO recommended requirements for adults, and it was found that except for Met, NPIs exceed the requirements. CPCs also showed a similar trend except for Cys, Met, and Trp. When the percentage of all the EAA was considered (**Figure 10**), except for Leu and Met, other EAA in NPIs had >100% of the FAO/WHO requirement. In CPCs, the percentage of Cys, Met, and Trp were <100% than the other EAAs.

Polypeptide profile by SDS-PAGE electrophoresis: Polypeptide profiles of N-CCC, E-CCM, and H-CCM under non-reducing and reducing conditions, respectively (**Figure 11**). showed the protein types present in SMs, IPFs, NPIs, SSFs, CPCs, and SCFs contained respective storage proteins of canola seeds. A clear differentiation of containing proteins can be seed between NPIs (napins) and CPCs (cruciferins).

Physico-chemical and techno-functional properties of protein fractions

Protein solubility, surface hydrophobicity, and zeta potential: The solubility of canola cold-pressed NPIs at pH 4.0 was > 60%, and at pH 7.4 and 9.5 the solubility was almost 100% (**Table 6**). indicating unhindered solubility properties throughout this pH range whether the starting meal contained oil not. In comparison to other plant proteins, napin-rich protein isolates have better solubility over a wide pH range. Napin-rich protein isolates are a perfect choice for plant-based acidic beverage preparation because most proteins, including plant and animal proteins, have low solubility at pH values of 4.0 to 5.0 because of their isoelectric pH is in this pH range. When compared to NPIs, CPCs in general had low solubility values in the pH range studied and may be characteristic of this fraction. CPC fractions showed very low solubility at acidic pH compared to the other two levels (**Table 6**). Cruciferin, is the other major protein in CPC fraction which has a reported pI value of 7.25, therefore cruciferin may not have sufficient ionizable groups to solubilize around neutral pH (Gillberg and Tornell, 1976). The two protein-rich products obtained from this fractionation process have contrasting solubility properties with pH and this could be advantageous when applications are developed.

The NPIs showed the lowest surface hydrophobicity values at pH 4.0 and 7.4 compared to the values at pH 9.5 (**Table 6**). When CPCs are compared, their surface hydrophobicity values were much higher than NPI for all the pHs regardless of meal origin, indicating that more hydrophobic residues were found on the surface of protein particles. The high surface hydrophobicity values of CPC at pH 4.0, 7.4, and 9.5 may explain their low solubility values.

The zeta potential values of protein fractions were negative and in the range of -3.63 mV to -33.41 mV depending on the protein fraction and pH (**Table 6**). The $NPI_{N\text{-}CCC}$, $NPI_{E\text{-}CCM,}$ and $NPI_{H\text{-}CCM}$ had the lowest negative zeta potential values (-3.6 mV to -10.95 mV) at pH 4.0, 7.4, and 9.5. Being rich in more basic amino acids, NPI fractions may have a smaller number of charged groups. Zeta potential values of CPC gave larger negative values when pH was basic, might be some of the charged amino acid residues were exposed, such as $CPC_{N\text{-}CCC}$, $CPC_{E\text{-}CCM,}$ and $CPC_{H\text{-}CCM}$ zeta potential values were -33.4mV, -25.27mV, and -24.2mV, respectively.

Oil holding capacity (OHC) and water holding capacity (WHC): The OHC values of $NPI_{E\text{-}CCM}$ was higher than the NPI from N-CCC, H-CCM, and the commercial SPI **(Table 7**). The availability of non-polar residues on the protein surface may affect the affinity of oil for the product particles thus increasing the oil holding capacity (Kinsella, 1982). CPC had less OHC than NPI. Hexane treatment enhanced the OHC values in CPC fractions. The WHC of NPIs could not be measured because of the high solubility at a natural pH close to 7.0. The CPC of all the meals had closer WHC values of ~1 g water for 1 g material. The WHC of SPI was higher than the values of CPC. All values for the liquid holding ability (OHC and WHC) were taken at ambient temperature and at pH 7.0 and did not reflect their behavior in applications where further treatment conditions were involved such as heating.

Thermal denaturation properties: There were no significant differences in the maximum denaturation temperature, onset, and ending peak temperatures among the NPIs obtained from different meals **(Table 8)**. It showed that these protein products were quite heat stable and required temperatures close to 100ºC for complete heat denaturation. WPI had much lower values for all the parameters than NPI. CPCs denaturation temperature was not reported in this study as the CPCs could not give an endothermic peak. It could be the presence of non-protein components (seed coat materials) in

CPC that affect the thermal stability (Marcone et al., 1998).

Emulsion capacity and emulsion stability: NPIs in distilled water solutions (0.5%, /v) were able to emulsify 10 to 11 mL of canola oil regardless of the meal that it was obtained from **(Figure 12a)**. The amount of oil that NPI could emulsify was not changed with 0.5 M NaCl but reduced to 8.5 mL with 10% (w/v) sucrose in the protein solutions. Similar volumes of oil were able to emulsify with CPC of both meals and showed low (8.5-9.0 mL) oil for 10% sucrose containing samples. WPI, which was the reference protein product was able to emulsify 20 mL of oil without any additive, 18 mL with added 0.5 M NaCl, and 16 mL with 10% sucrose without emulsion inversion. Material available from H-CCM was not sufficient to do emulsion property studies and the generation of new fractions was not an option at this point of the experiments (Already delayed due to the pandemic). **Figure 12b** depicts the EC of CPC. The EC of commercial WPI was much higher than the $CPC_{N\text{-}CCC}$ and $CPC_{E\text{-}CCM}$ under any of the conditions tested. From $CPC_{N\text{-}CCC}$ and $CPC_{E\text{-}CCM}$ enough protein was not emulsified and gave a lower EC value than the WPI. Also, no significant differences were observed in the EC of $CPC_{N\text{-}CCC}$ and $CPC_{E\text{-}CCM}$. In general, NPIs had much higher EC values (~200 to 250%) than the values for CPCs (<200%).

When the stability of the formed emulsions was considered, regardless of the source of the meal or the protein fraction close to the ES values (~60%) for NPIs and CPCs were observed (**Figure 13 a & b**). Except for commercial WPI, the lowest ES values (~30%) were for 10% sucrose containing emulsions. Comparatively, napin is a smaller MW protein than cruciferin and may be able to coat more oil droplets giving larger EC values.

Foaming properties: **Figure 14a, b &c** represents the foam expansion (FE), foam stability (FS), and liquid drainage (LD), respectively of NPIs at pH 4.0 and 7.0. Comparisons were made with whey protein isolate (WPI). In general, at pH 4.0, the values of FE were smaller for all protein materials compared to the values at pH 7.0 with the lowest values observed for $NPI_{N\text{-}CCC}$. The highest FE values were for WPI and then $NPI_{E\text{-}CCM}$ at pH 4.0. At pH 7.0, there was no difference in FE values (average of 60%) of $NPI_{N\text{-}CCC}$ and $NPI_{E\text{-}CCM}$ with WPI. The foam structure created by these proteins had different stabilities depending on the pH. At pH 4.0, the FS of $NPI_{E\text{-}CCM}$ and WPI were >20% after 30 min. $NPI_{N\text{-}CCC}$ had a lower FS than the other proteins. At pH 7.0, no significant differences were observed in FS among the NPIs from N-CCC and E-CCM, and WPI. In general, about 40% stability (**Figure 14b**) was reached for the foams generated by NPIs at pH 7.0.

The NPI from N-CCC and E-CCM at pH 4.0 gave the lowest FE% and FS% compared to the pH 7.0. The lower protein solubility values of NPIs at pH 4.0 than pH 7.0 may have caused lesser availability of protein molecules to get involved in the air/ water interface stabilization to create and expand protein foam. The FE% and FS% at pH 4.0 of NPI from N-CCC was very low and at pH 7.0 the same protein has the better value. As the zeta potential of $NPI_{N\text{-}CCC}$ value was lower at pH 4.0 than the pH 7.0, it may indicate that the low zeta potential value possessing particles undergo aggregation and flocculation. High liquid drainage (**Figure 14 c**) was evident for all the proteins with higher values at pH 4.0. WPI had the lowest LD than the other protein values. The highest LD was given by $NPI_{N\text{-}CCC}$ than $NPI_{E\text{-}CCM}$. At pH 7.0, the liquid drainage % of all test samples was in similar values (~50%). The lower protein solubility values of NPI at pH 4.0 than pH 7.4, (**Table 6**) may have caused lesser availability of protein molecules to get involved in the air-water interface stabilization to create and expand protein foam. The stability of the created foam was comparable and close to the values of whey protein isolate. The foam volume generated by CPC from all three meals was quite small and the foam did not last over 5 min, therefore, did not continue for assessment. CPC had high fiber content, and low protein solubility which may be the reason a sustainable foam (at least for 5 min) could not be generated. The whey protein isolate is a known good stable foam formed across a wide range of pH.

In-vitro protein digestibility (IVPD): Both NPI and CPC of all three meals (with heat treatment 98ºC for 15 min) gave IVPD values close to or above 100 (**Table 9**). The procedure employed for IVPD determination had enzymes for both gastric and intestinal phases. It has been noted in the method that >100% IVPD values indicate very good digestibility potential of the protein. Without heat treatment, the IVPD value of NPIs from N-CCC and H-CCM were significantly indifferent, and their digestibility value was higher than the $NPI_{E\text{-}CCM}$. The $NPI_{E\text{-}CCM}$ showed the lowest digestibility value without heat and heat treatment. It could be the structural rigidity of the protein, thus slowly releasing N in the early digestion stage (Savoie et al., 1988). In the case of CPCs, IVPD of $CPC_{E\text{-}CCM}$ was significantly higher than the CPC of N-CCC and H-CCM under heat and heat treatment.

Objective 2 (Nickerson & C. Li)

Proximate composition, pH and protein hydrolysis of fermented meals: HE meal and CCC fermented with *A. niger* NRRL 334 and *A. oryzae* NRRL 5590 over a 72-h period showed a significant hypal growth after 48 h, and showed spore formation on the surface after 72 h (**Figure 15**). Meal pH increased from ~pH 6 to pH 7-8 at 72-h fermentation (**Table 10)** may be due to the increase in organic acid release by growing organisms. Protein hydrolysis measured as the degree of hydrolysis (DH) increased from 15%-30% (24 h) to ~40% (48 h) and remained at ~45-50% after 72 h (**Table 10)**. For the first two days, the DH increased quite fast due to the large surface area that is exposed to oxygen, which helped hyphal growth. Proteins were most likely hydrolyzed partially by proteases synthesized by fungal cells, resulting in an increase in DH by releasing free amino acids.

Changes to the proximate composition was similar for both strains fermented CCC and HE meal. Crude protein level of CCC meals increased from ~34% (w/w) to ~36-38% (w/w), a ~9-11% increase after 72 h of fermentation regardless of the strains (**Table 10)**. Protein levels of HE meal remained relatively constant; ~39-41% (w/w), a ~5% increase. Compared to HE meal, fermented CCC had a lower crude protein content ($p<0.05$) due to the high oil content even after a 72-h period of SSF. The presence of high levels of residual oil in CCC can be inhibitory to fungal growth (Simon *et al.,* 2017). The increased protein levels could be the direct increase in fungi protein or the chitin from fungi cell walls as non-protein nitrogen. Conversion of meal oligosaccharides and fiber as carbon source to growing fungi cell mass during fermentation has been observed as loss of dry matter (Simon *et al.,* 2017; Rozan *et al.,* 1996), and led to an increase in nitrogen (crude protein). Fungi converted meal fibre and oligosaccharides into cell mass, which contributed to the increase in crude protein content. Crude lipid levels decreased from ~12% to ~9% (w/w) for CCC with fermentation, whereas the contents in HE meals decreased from ~3%. to ~1% (**Table 10**). A slightly increase of ash content in CCC was found from ~6% to ~7% (w/w) after 72 h fermentation, whereas HE meals ash level increased from ~8% to ~10% (w/w) (**Table 10**).

Contents of phytic acid and total phenolics upon fermentation: As for antinutrients, phytic acid (PAC, **Table 10**) of CCC meals decreased from 5.9 to 0.9-1.55 % (w/w), a~74-85% reduction with 72 h of fermentation, whereas for HE meals, a ~76% reduction was observed. The reduction of phytic acid was due to phytase synthesized by *A. niger* and *A. oryzae* during SSF. All fermented samples showed a decrease in TPC from 2.7-3.1 to ~1.0 mg GAE/ g DM (~65% reduction) with an exception for HE (*A. niger*) sample which had a greater decrease from 3.1 to 0.6 mg GAE/ g DM (~81% reduction). The degradation of antinutritional compounds in canola meals might require multiple enzymes.

Protein content of canola protein products (isolates): Protein products of AE-IP process from unfermented HE and unfermented and non-defatted CCC meals had 86.5% and 68.9% protein respectively (**Table 11**). Defatted CCC meal gave 81.6% protein content in AE-IP method. Protein isolates of CCC (CCCI) fermented with *A. niger* NRRL 334 and *A. oryzae* NRRL 5590 had protein levels of 62.7% and 57.9%, respectively (**Table 11**). The protein isolates from HE meals fermented with *A. niger* and *A. oryzae* had protein levels for 56.3% and 58.4%, respectively. Both CCC and HE meals when fermented with either of the fungal strains gave protein isolates with lower protein enrichment upon AE-IP. SE method resulted in higher protein levels for protein products of all unfermented and fermented canola than those produced by AE-IP giving protein isolates with protein levels higher than 95% with the exception of the isolates (93.3%) of CCC fermented using *A. niger* NRRL 334. SF could improve the protein extraction process when salt was used. Fermentation may lead to partial hydrolysis of proteins causing increased surface hydrophobicity (decrease in protein solubility) and stronger protein-lipid interactions. The high degree of protein hydrolysis may also lead to larger number of peptides, which could be easily lost during centrifugal separation of proteins. Also, the large number of hyphae produced by fungi may act like 'glue' to connect the solid substrates tightly, which affected the protein extraction by limiting the soluble protein dissolving into the aqueous medium. In AE-IP process, fiber that is already digested might release more insoluble or soluble carbohydrates (polysaccharides or starch) that made it difficult to separate the protein from the complex (protein-carbohydrate interaction). In addition, oil could largely affect the protein extraction process in CCC meals.

Protein solubility of protein products: AE-IP protein products of CCC control (**Table 12)** had a significantly higher ($p<0.05$) solubility (88.9%) at pH 3 compared to the defatted CCC control and HE control (76.2, 81.4% respectively). Protein solubility values of AE-IP proteins at pH 5 and 7 were low compared to at pH 3. The SE isolates of CCC (51.8%) and defatted CCC control (49.3%) had higher solubility than AE-IP CCC controls at pH 7. SE products showed higher solubility at pH 3

and 7. Canola protein products prepared from fermented meals tended to (SE) show a decreased trend in solubility at all pHs compared to the respective control products. A decrease in solubility ($p<0.05$) was found for protein products of *A. oryzae* fermented CCC and HE meals compared to their respective controls. Both *A. niger* and *A. oryzae* may be acceptable inoculums for meals that could maintain or increase the protein solubility at pH 7 for SE protein, whereas *A. niger* might be a better culture choice for AE-IP products at pH 7. Protein products extracted using SE method showed higher solubility than AE-IP products, also the protein products extracted form CCC showed higher solubility than isolates from HE meal.

Water and oil holding capacity (OHC) of protein products: AE-IP CCC control (**Table 13**) protein products showed higher WHC (2.1 g/g) value than the products of HE control (1.8 g/g). Defatted CCC control (2.7 g/g) had a higher value than non-defatted (2.1 g/g) may be due to hydrophobic nature of residual oil of the meal. SE proteins of defatted CCC control (2.1 g/g) showed higher WHC value than HE (1.3 g/g) and non-defatted CCC controls (1.1 g/g)**.** A 72-hour fermentation resulted in a significant increase ($p<0.05$) in WHC of AE-IP products without a significant difference ($p>0.05$) between strains. For the type of strains, *A. oryzae* was preferred to improve the WHC of both AE-IP and SE HE product while there was no difference between two strains. In addition, all HE products showed higher increases in WHC than CCC products. Defatted CCC controls showed higher WHC values than non-defatted CCC controls and HE controls. AE-IP protein products showed slightly higher WHC value than SE ones because of the higher amount of non-protein components (lower protein level of AE-IP products required more dry solids to correct protein content) in AE-IP products used for testing than SE products. The non-protein compounds in AE-IP products such as possible soluble fibre and polysaccharides may contribute to the ability of water absorbing and binding.

As shown in **Table 13**, AE-IP CCC control showed higher OHC value (2.9 g/g) than and HE control (2.3 g/g) and same was observed for the products obtained using SE method. CCC control had higher OHC value (3.2 g/g) than the products of HE control (2.4 g/g). The OHC value of AE-IP defatted CCC control (2.4 g/g) was lower than that of non-defatted CCC control (2.9 g/g), whereas SE defatted CCC control (3.9 g/g) had higher OHC value than CCC non-defatted control (3.2 g/g). Proteins isolated from fermented meals, the OHC values of AE-IP CCC products increased (both *A. niger* and *A. oryzae*) compared to defatted CCC control (2.4 g/g, $p<0.05$). For the type of strains, there was no significant difference ($p>0.05$) between *A. niger* and *A. oryzae* except for SE HE products where *A. niger* was preferred to increase the OHC of the products. Similar to WHC, higher increase was found in HE meal compared with CCC meal. Canola protein products obtained using SE showed better OHC compared to those prepared using AE-IP. The lower solubility and greater OHC suggest increased hydrophobic nature of the protein recovered from AE-IP process.

Emulsifying properties of protein products: AE-IP HE control had higher EAI value (13.2 m2/g) than CCC control (11.4 m^2/g) and defatted CCC control (8.3 m^2/g) at pH 3 **(Table 14)**. At pH 7, a higher EAI value was found for HE control (18.3 m^2/g) than CCC control (11.7 m^2/g) and defatted CCC control (11.6 m^2/g) at pH 3. As for pH 5, defatted CCC control (5.0 m^2/g) had higher EAI value than CCC control (4.1 m^2/g, $p>0.05$) and HE control (3.0 m^2/g, $p<0.05$). As for SE products, defatted CCC control (11.0 at pH 3, 9.4 at pH 5 and 14.5 m^2/g at pH 7) showed the highest EAI value compared to CCC (5.6 at pH 3, 5.8 at pH 5 and 12.9 m^2/g at pH 7) and HE control (5.9 at pH 3, 6.8 at pH 5 and 13.9 m^2/g at pH 7) at pH 3, 5 and 7. The difference between AE-IP and SE products may be due to the different protein fractions and nature of products extracted using AE-IP and SE. As for pH, EAI values tend to be low at pH 5 ranged from 1.3 to 5.5 m^2/g due to the low protein solubility. Higher EAI values were found at pH 7 compared to pH 3 for all the protein products obtained from unfermented control meals, which was possibly related to the higher solubility at pH 7 than pH 3. For type of meals, protein products extracted from HE meals tend to have slightly better EAI values than those from CCC meals due to the higher hydrophobicity of HE products. As for type of strains, *A. oryzae* was preferred to modify the EAI values of canola protein products.

Foaming properties of protein products: Regardless of the source of meal or fermentation treatment protein products gave a foaming capacity (FC) from 131.1 to 480.0 % and moderate foaming stability (FS) ranging from 68.0 to 89.0% at pH 3, 5 and 7. For controls, defatted AE-IP CCC control (306.7% at pH 3 and 243.8% at pH 7) had higher FC (**Table 15**) than CCC (224.4% pH 3 and 165% at pH 7) and HE control (244.4% at pH 3 and 154.4% at pH 7) possibly due to the residual oil in CCC control and low protein content (~60%) of both CCC and HE control. As for SE products, CCC control had higher foaming capacity (322.2%) than defatted CCC (246.7%) and HE (266.7%) control at pH 3. At pH 7, SE defatted CCC control showed the highest FC in all products as 480.0 %, higher than SE CCC (464.4%) and HE (145.6%) control. At pH 5, only CCC

(241.1% for AE-IP and 211.1% for SE) and defatted CCC control (131.1% for AE-IP and 222.2% for SE) showed the ability to form stable foams for both AE-IP and SE products. However, the poor solubility at pH 3 still had adverse impact on both FC and FS compared to the values at pH 3 and pH 7. SE products showed significantly higher FC values than AE-IP possibly due to the higher levels of protein in SE products (>93%) than AE-IP products (<80%) and the possible differences in protein fractions (napin and cruciferin). After SF, only AE-IP CCC protein products by *A. oryzae* showed the ability to form foams (228.9 %) with a low FS of 33.0% at pH 3. At pH 7, all AE-IP products from fermented showed foaming properties as high as 230.7% (CCC, *A. niger*), 180.0% (CCC, *A. oryzae*) 244.2% (HE, *A. niger*) and 136.7% (HE, *A. oryzae*) with comparable FC compared to controls ranging from 72.0 to 84.6%. AE-IP HE sample pre-treated using *A. niger* even showed significantly higher FC value (244.2%) compared to HE control (154.4%). As for SE products, all samples were able to generate relatively stable foams at pH 3 and 7. In detail, FC values of CCC products from fermented meals were decreased to 196.5% (*A. niger* at pH 3), 175.6% (*A. oryzae* at pH 3), 191.1% (*A. niger* at pH 7), and 153.3% (*A. oryzae* at pH 7) compared to CCC control at pH 3 and 7. For HE products, at pH 3 low FC values; 195.6% (*A. niger*) and 135.6% (*A. oryzae*), and a significant increases at pH 7 as 306.7% (*A. niger*) and 155.6% (*A. oryzae*) compared to HE control (266.7% at pH 3 and 155.6% at pH 7) was observed. The FS values decreased to ~52% for all products from fermented meals at pH 3, while remained stable as ~77.6-88.4% at pH 7. At pH 5, none of the products extracted from fermented meal showed the ability keep the foams stable. Due to the extremely low protein solubility (< 10% for protein from fermented meals) of protein from fermented meals, large foams which disappeared within 5 min (low foaming properties) was observed at pH 3 and 5. The SF could improve foaming properties of protein products extracted from HE meal at pH 7, especially when using *A. niger,* however, comparatively higher values could obtained from unfermented CCC

Objective 3 (Mupondwa & X. Li)

De-oiling and air classification: Canola seeds received had a 7.8% (w/w) moisture content which was the appropriate range for de-oiling by cold pressing. Air classification is effective for low-oil containing material. Canola seeds had 42% (w/w) oil. Partial de-oiling by pressing (limited amount of oil that can be released by pressing) followed by washing out residual oil with a solvent (hexane or ethanol) was carried out to obtain suitable meal for air classification. Cold-pressing and ethanol extraction was carried out at a local pilot plant facility resulting in a final 115.12 kg dry meal containing <2% residual oil and <1% residual ethanol level. The repeated air classification process resulted in the concentration of the protein and increased light fraction protein content from 35% to 45%. Although the separation of coarse and fine fractions was clear, separation of protein-rich particles was not. Sieving to remove large fibre-rich particles gave <60 mesh (< 250 µm) particles with enriched protein content.

Oilseed proteins can be extracted using aqueous (wet) extraction or dry fractionation of oil-free meals. In general, aqueous methods involve aqueous extraction (at alkali pH or with salts) followed by aggregation (low pH or protein micelle formation) or membrane separation. In addition, chromatographic separation and meal component fractionation have been proven to work. Dry fractionation uses milling and air classification which produces protein concentrates that could be feed stock for preparing protein isolates. While both have their advantages, dry fractionation is considered to be more sustainable protein production approach because it utilizes less energy, water, and related resources while preserving the native properties of proteins. In the hybrid process of protein production, dry fractionated protein concentrate can be further processed by wet extraction for further protein enrichment making lesser use of water and other inputs as well as the cost involved. This activity conducted technoeconomic analysis to quantify the economic feasibility of dry fractionation via air classification of ethanol defatted cold-pressed canola meal.

Technoeconomic analysis: The complete engineering process design is depicted in **Figure 4 (**and process in **Figure 3)** for a plant with an annual capacity of 33,000 tonnes annum-1 (100 tonnes day-1) operating 330 days (7,920 h annum-1). The plant is hypothetically (optimally) located in Moose Jaw, SK and encompassing a collection radius of 150 km from surrounding canola producers that can supply 170,000 tonnes annum-1. The collections radius is formalized in terms of transportation distance (*L*) based on the biomass collection radius of (*r*) from nearby fields to plant of a circular area of (*A*) as specified the equation; L=23r=23√$A\pi$.

The determination of collection area is based on a similar empirical modelling of radius by Mupondwa et al. (Mupondwa,

Li, Falk, Gugel, & Tabil, 2016; Mupondwa, Li, & Tabil, 2017; Mupondwa, Li, Tabil, Falk, & Gugel, 2016). The process design is based on a small-scale processing plant which characterizes the scale of typical canola seed cold press operations in the Prairies (approximately 25,000 tonnes annum^{-1}) (Mupondwa, Li, & Wanasundara, 2018).

Capital Expenses, Operating Expenses, and Profitability: The overall technoeconomic analysis is modelled as a biorefinery circular economy concept integrating an array of unit operations spanning feedstock (canola meal) supply and logistics, process design, fractionation, and least-cost conversion into intermediate and optimized end products. This includes estimating parameters for total capital investment, operating costs, and profitability measures (Net present value; NPV, Internal rate of return; IRR) at various simulated operating plant scales.

Capital Expenses (CAPEX): **Table 16** outlines assumptions and parameters on which the estimation of CAPEX, which is the total capital investment (TCI) (Li & Mupondwa, 2021; Li, Mupondwa, & Tabil, 2018). In this case, TCI is the aggregate investment cost for the plant. It is derived by adding direct fixed capital, working capital (labor, raw materials, utilities) and start-up costs. TCI is categorized into direct fixed cost (DFC) and indirect fixed cost (IFC). DFC includes equipment, installation, piping, buildings, electrical, and instrumentation costs. Indirect fixed costs comprise engineering and construction costs, as well as contractor's fee and contingencies. These costs are derived based on equipment sizing and adjusted to current prices by reference to the chemical engineering plant cost index (CEPCI) for the year 2022. **Table 17** further illustrates the inverse relationship between capital investment and plant capacity of the protein production plant. An increase in plant capacity from 33,000 tonnes to 132,000 has a corresponding increase in total capital cost from \$15,324,000 to \$31,287,000. However, the unit capital cost decreases correspondingly from \$464 tonne-1 to \$237 tonne-1 (by 48.9%), clearly demonstrating the concept of economies of scale which refer to cost advantages (decrease in overall plant costs) that a processing plant can derive from expanding its operating scale.

Operating expenditure (OPEX): Operating expenditure (OPEX) is the cost of operating the canola protein plant. The cost include raw materials (canola seed feedstock, supplies), energy, heating, cooling, labour, royalties, insurance). maintenance fees, royalties and licensing, employee reward benefits, and insurance. OPEX is estimated as a fixed percentage of fixed capital costs. **Figure 16** summarizes OPEX for the three plant capacities. OPEX ranges from the base case of \$25 million (33,000 tonne capacity) to \$83.7 million (132,000 tonne capacity). **Figure 16** summarizes the distribution of OPEX, with raw materials accounting for nearly 72% of OPEX. Of this amount, approximately 99.75% represents the price of canola seeds, and hence major determinant of the overall economic viability of the canola protein processing plant.

Profitability: In order to determine the profitability of the plant, the net present value (*NPV*) and internal rate of return (*IRR*) was estimated, based on 20-year life of the plant, taking into account the initial capital investment, operating expenses, corresponding annual cash flow. In this case, *NPV* is derived based on the equation below as the difference between discounted annual cash flows from canola protein products and canola protein production costs (Mupondwa, Li, Tabil, et al., 2016; Mupondwa et al., 2012):

$NPV = -I0 + \Sigma CFAt(1+\delta)tNt = 1+SVN(1+\delta)N$; where, *I0* is the initial canola plant investment; *CFAt* is annual cash flow from assets given by *CFAt* = (*TRt - TCt - DEPt)*(1 - *T*) + *DEPt*; *TRt* is total revenue before tax; *TCt* is total cost before tax; *DEPt* is depreciation over the life of the canola protein plant; *T* is the corporate marginal tax rate; *SVN* is salvage value; δ is the discount rate or cost of capital; and *t = 1,2, . . .N* denotes year with *N* terminal time. Investors can use *NPV* to indicate the value of a capital investment (Mupondwa et al., 2012).

IRR is the discount rate equating discounted benefits and costs. The decision rule is to consider a capital investment if IRR exceeds an investor's minimum required rate of return or cost of capital, depending on the industry and level of acceptable risk. Investors may prefer IRR values that significantly exceed the cost of capital, as an indicator of how much value is added to the business (Mupondwa et al., 2012). **Table 18** summarizes estimated NPV for the canola protein production plant for given prices of the feedstock canola seed (\$0.50 - \$0.65 kg^{-1}), primary product canola protein fine fraction (from \$0.45 - \$0.65 kg^{-1}), and coproduct canola oil (\$1.15 - \$1.35 kg^{-1}). The NPV is evaluated at 7% cost of capital The results in **Table 18** shows that increasing plant capacity widens the range over which positive NPV is generated, even with increased feedstock cost. As table 3 shows, when the plant is operating at a 33,000 tonne annual capacity, and maximum canola seed cost of \$0.65 kg^{-1} is considered, the overall manufacturing process does not generate a positive

NPV even at the maximum selling prices of canola oil ($1.35 kg^{-1}) and maximum selling price of the protein fraction ($0.65 kg^{-1}). However, an increase in the operating capacity to 66,000 tonnes y-1 creates a wider range over which the process generates a positive NPV for given material costs and protein and coproduct selling prices.

Sensitivity Analysis: Table 18 depicts the sensitivity of canola seed cost, canola oil selling price, protein selling price, cost of capital, and plant capacity on NPV. The results show that plant capacity has the greatest impact on NPV. When plant capacity is reduced by 50% (from a baseline of 33,000 to 16,500 tonnes y^{-1}), NPV decreases by nearly eleven-fold relative to the baseline. On the other hand, increasing the capacity to 1.5 times the baseline results in an 8-fold increase in NPV, further demonstrating the significance of capacity in determining the profitability of canola protein plant. In terms of raw materials, when the cost of canola seed feedstock, a 10% increase or decrease in canola feedstock cost generates a corresponding five-fold change in NPV. Similar impacts are noted vis-à-vis the selling price of the co-product canola oil in which 10% variation its price results in a five-fold effect on NPV relative to the base value, suggesting that both parameters have similar effects the profitability of the canola protein plant, indicating that feedstock cost and coproduct price are two key variables in the profitability of the plant. Notably, the selling price of the primary product protein fraction has a modest impact on the profitability of the canola protein, as depicted in **Table 18** in which NPV only increases by two-fold from a 10% increase in canola protein selling price, while a corresponding 10% decrease in selling price results in almost 50% reduction in NPV. Finally, the cost of capital (discount rate) has the smallest impact on NPV relative to other variables, implying that variations in the cost of capital within the given range have the least impact on the profitability of the canola protein plant.

Survey on the acceptance of Genetically Modified (GM) canola protein: There were 28 responses received out of 67 survey links sent out, but only 12 respondents completed the survey, resulting in a completion rate of 18%. All the companies (100%) responding to the survey were Canadian controlled corporations operating as food and beverage manufacturers, with 50% also operating in the US, 16% in Europe, and 8% in South America, Asia, and Australia. In terms of their sizes, almost 42% of companies reported less than $1 million in annual review; 25% under $5 million; 25% between $5-$10 million; and 8% between $10-$50 million. There was no observed correlation between regional location of business, business revenue, and adoption of GM canola protein products.

Willingness to pay premium for non-GM foods: Industry participants were asked two questions:

a) Will consumers be willing to pay a premium (pay a higher price) for non-GM foods with the same nutritional properties as their GM counterparts?

b) Will industries be willing to pay a premium (pay a higher price) for non-GM foods with the same nutritional properties as their GM counterparts?

Figure 17a summarizes the results in which only 33% of the industry participants believe consumers would be willing to pay a premium for non-GM proteins, while 42% were of the view that consumers would not pay any premium for non-GM foods. About 25% were uncertain. **Figure 17b** summarizes responses for question b) and related to the industry itself would be willing to pay more for non-GM ingredients. As the results show, nearly 42% would not pay more for any non-GM products with similar functional properties as GM products while 25% would be willing to pay more for exclusive non-GM products. At least 33% were uncertain.

Major factors that would affect the choice of GM canola proteins as a functional ingredients: To understand major factors that would influence the successful incorporation of canola proteins into the manufacturing process as they enter the ingredient market, industrial participants were asked to select key factors they would consider when using GM canola proteins in their products from a list of about nine factors. The participants were given the option to choose more than one factor. **Figure 18** summarizes the responses showing that the quality of the GM canola ingredient over alternatives was considered the most important deciding factor by almost 50% of the participants. This was followed by the cost of the GM ingredient as the second most important (42%) and consumer acceptance of GM canola protein as the third most important (33%). Almost a quarter of the manufacturers stated that they would only consider canola proteins if they were non-GM, and one-sixth of participants had no interest in using either GM or non-GM canola proteins in their manufacturing process. Almost 25% consider environmental benefits or any legal regulations associated with GM canola proteins as a part of their ingredient selection process. Only one in twelve think they would consider negative media

coverage as a factor when selecting the canola ingredients. These results provide an indication that if canola protein ingredients are manufactured to have protein properties that are similar or superior to available alternatives (plant or animal), and if the price is also comparable, almost 50% of the industries would be willing to use them as a functional ingredient, even they are GM. However, approximately 25 % of the manufacturers prefer an ingredient to have non-GMO status to consider canola protein in their ingredient list. In general, the responses highlight the fact that quality and price play a major role in industry acceptance of an ingredient, rather than non-GMO status.

Barriers to the establishment of a GM canola protein market: The manufacturers were asked to identify current barriers to the development of market for GM canola protein. Participants selected several factors from a list of identified barriers. As summarized in **Figure 19**, consumer acceptance and negative media coverage of GM products are the two major obstacles, according to nearly half of the respondents. About 33% of respondents consider the absence of a well-established supply chain for GM canola protein as a barrier. Economic viability, potential cost increases associated with mandatory labeling, and a lack of proof supporting any potential advantages of canola proteins are all regarded as barriers to an established canola market by almost 25% of the participants. Only about 16% of people believe that the establishment of a market for GM canola protein is hindered by increased production and establishment costs and lack of technical expertise. None of the participants see a lack of investment or unavailability of modernized technology as a barrier. These findings demonstrate that the way in which GM products are portrayed in the media significantly influences consumer perception and acceptance of GM products. Consequently, increasing negative media coverage of GM products could potentially decrease consumer acceptance, posing a persistent barrier to the development of a market for GM canola proteins, and ultimately the overall growth of GM canola proteins. Despite the absence of current laws requiring mandatory labeling in Canada, the industry believes that any such requirements could be a barrier due to additional costs associated with further segregation requirements.

Importance of the different factors in the functional ingredient selection process: In this next section of the survey, the manufacturers were asked to rate the importance of various factors that affect the ingredient selection process on a five-point Likert scale, ranging from Extremely Important to Not Important at all. The findings, as presented in **Figure 20**, show that among the factors influencing the ingredient selection process, functional properties of an ingredient and consistency in supply and availability were rated as either extremely important, very important, or important by all participants. The cost of the ingredient, existing market and supply chain, and ease of formulation were considered as either extremely important, very important, or an important ingredient selection factor by almost 92%, and only 8% considered them less important. Almost 83% rated the ingredient being plant-based/non-animal ingredient and perceived consumer acceptance as important criteria, while only 17% considered them less or not important at all. Given the increasing demand and acceptance by consumers for products made from more natural, healthier, and less processed ingredients that fits the widely growing “clean label” trends, we wanted to understand how important it is for industries that the ingredients they include will fit within the clean label status. Surprisingly, the fit in clean label was selected as either extremely important, very important, or important by all participants. Currently, there is no well-defined description for what fits under the clean label category, but industries consider that the clean label status would improve consumer acceptance, and this might be the major reason why all the industries would like the ingredients they select to fit under the clean label category.

Another question posed in the survey was how important it is to manufacturers that the ingredient they consider to be non-GMO. According to the survey results, the ingredient's non-GMO status was either extremely important, very important, or important for nearly 58% of respondents, while it was either less important or not an important factor for the remaining 42%. Even though in one of the previous sections of the survey where only 25 % wanted specific non-GMO status for canola protein to be included as functional ingredient, here in this section almost 58% chose the non-GMO status as an important category they prefer for a functional ingredient. This helps us to have a better understanding that once canola proteins enter the market on a large scale, only about 40% would be willing to consider it without worrying about whether it comes from GM or non-GM category and for the rest of the group, they would surely require it to fall under the non-GM category to consider and include it in food development. Carefully looking into the results vis-à-vis organic status of the ingredient, only 34% consider the organic label as an important criterion; for more than 66%, the ingredient's organic status was either a less or unimportant factor. Analyzing various sections of the survey regarding how

canola protein being GM influence its inclusion in the ingredient list, more than 40% of the industries will accept it even if it is GM, as long as it meets all other functional properties and cost aspects.

Opinion toward general GM questions: To gain more insight into the participants' opinions and general attitudes toward GM products, several general questions about GM products were posed of them. They were asked to choose one option from a five-point Likert scale ranging from Strongly Agree to Strongly Disagree and summarized in **Figure 21**. One key conclusion from the general opinion section is that nearly 82% of the participants agree or strongly agree that consumer acceptance is essential for the success of any GM products. Almost 75% believe that forcing GM product labels would deter consumers from purchasing GM goods. Almost 58% agreed with the statement that GM products would offer solutions to poverty and food problems, and nearly 50% agreed that they are good for farmers. However, only 33% of respondents agreed with the statement that GM products are good for society and the environment. Approximately 25% of respondents agreed and 25% disagreed with the idea that as GM products become more widely available consumers will accept them. Similarly, almost 42% agreed and 42% disagreed that consuming GM foods would lead to health issues. Likewise, almost 42% agreed with the opinion that education and awareness of consumers and industry is required for a better GM acceptance. Reviewing all these general statements collectively, it's evident that there isn't much opposition towards GM products in general. We could assume that this attitude would extend to GM canola products as well. Additionally, the industry sees many benefits or positives about GM products in general; however, the attitude of consumers towards GM foods and their rate of acceptance would eventually affect the industry's choice to include them in their products.

How is plant protein rated in comparison to animal proteins? For this question the manufacturers ranked plant-based proteins compared to animal-based proteins on a scale from excellent to poor (**Figure 22**) with only 17% ranking below average. Scores for good and excellent ranking was 58% and 25% of average ranking. This is a good indication that manufactures receive plant protein ingredients favorably.

FIGURES

Objective 1 (Wanasundara & Majumder)

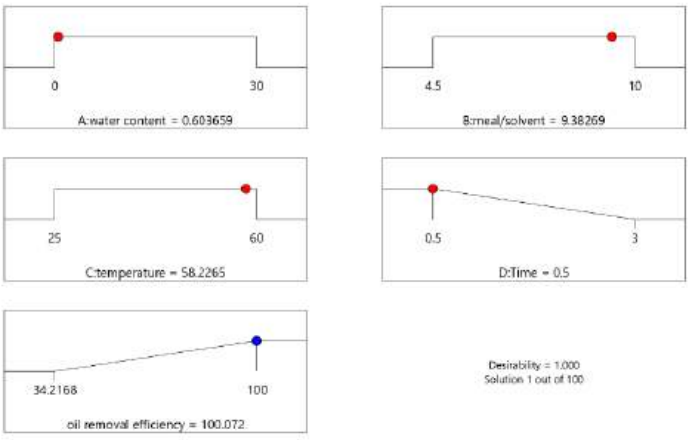

Figure 4. Two different scenarios of factor combinations **(a)** & **(b)** that can give 100% oil removal efficiency with ethanol extraction.

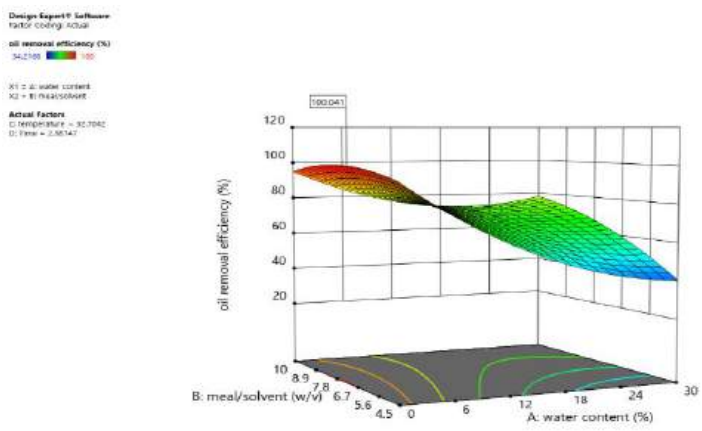

Figure 5. The 3D representation of the quadratic model obtained from data analysis showing the relationship of meal-to-solvent ratio and water content in ethanol for oil removal efficiency. The time of extraction was 2.98 h, and the temperature of extraction was 32.7°C.

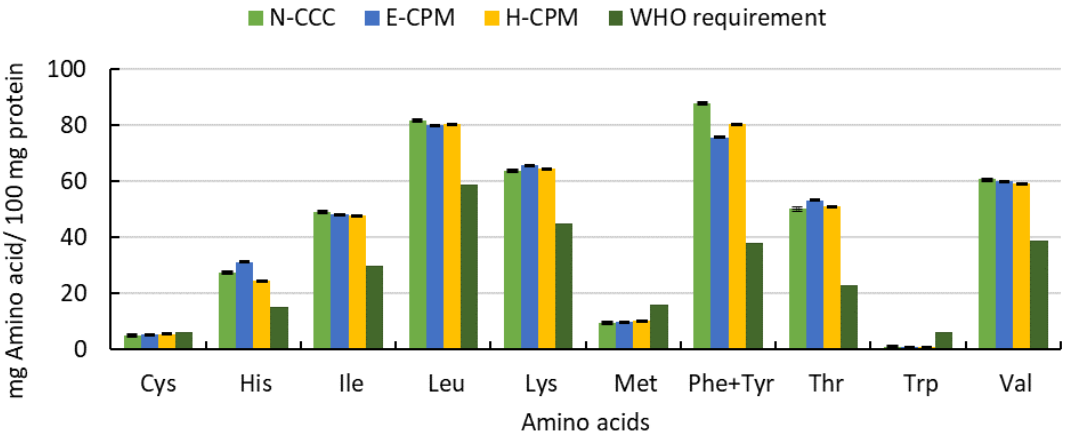

Figure 6. Levels of EAA from N-CCC, E-CCM, and H-CCM, compared with WHO recommended requirement value.

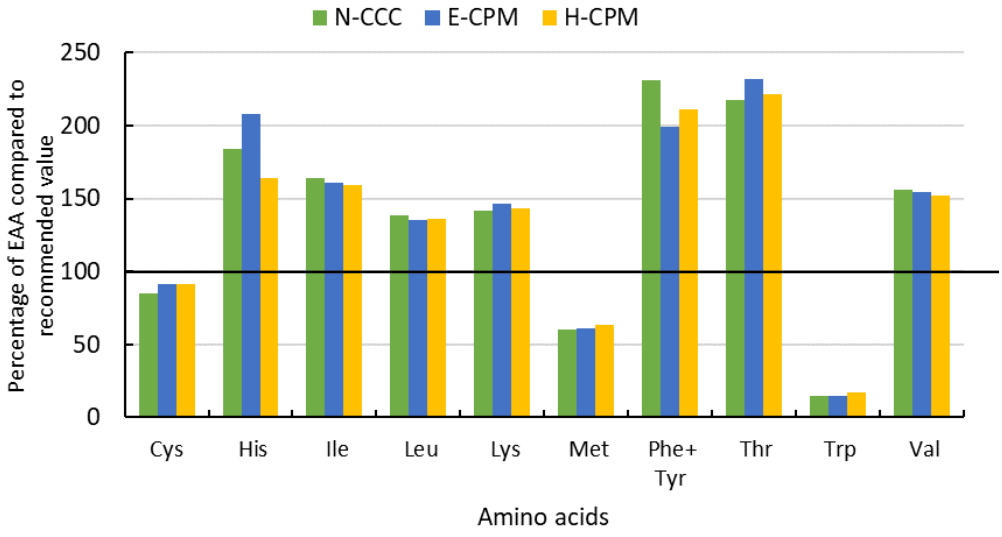

Figure 7. Comparison of EAA (mg of AA/ 100 mg of protein) of N-CCC, E-CCM, and H-CCM with WHO recommended requirement. Percentage value of 100 means (solid black line) the essential amino acid levels of the sample can provide an amount equal to the WHO recommendation.

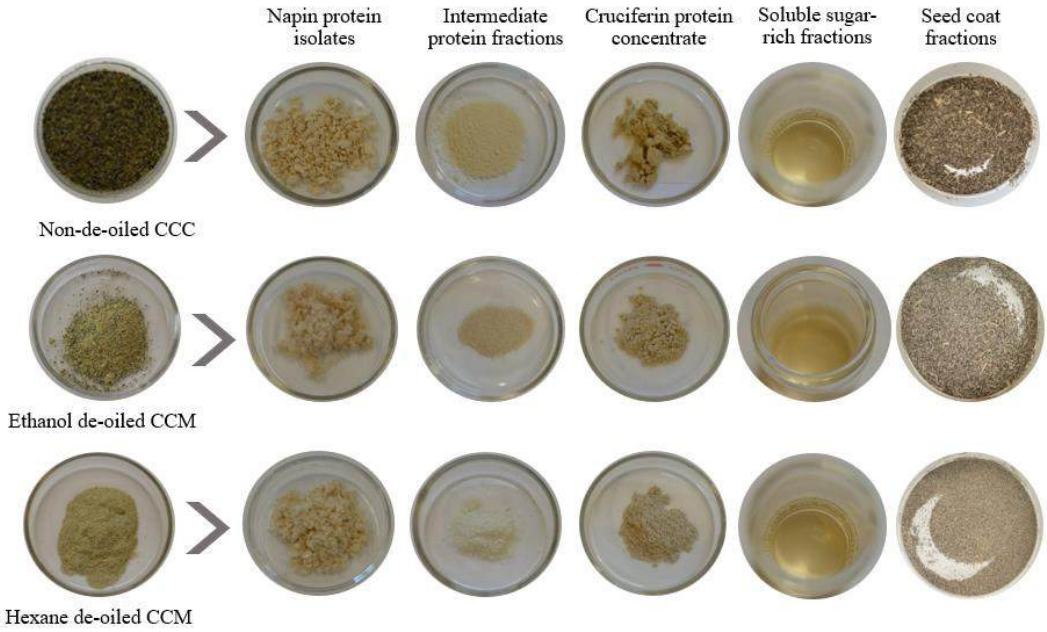

Figure 8. Starting meals (N-CCC, H-CPM and E-CPM), and their respective products obtained from the AAFC Brassica meal fractionation process.

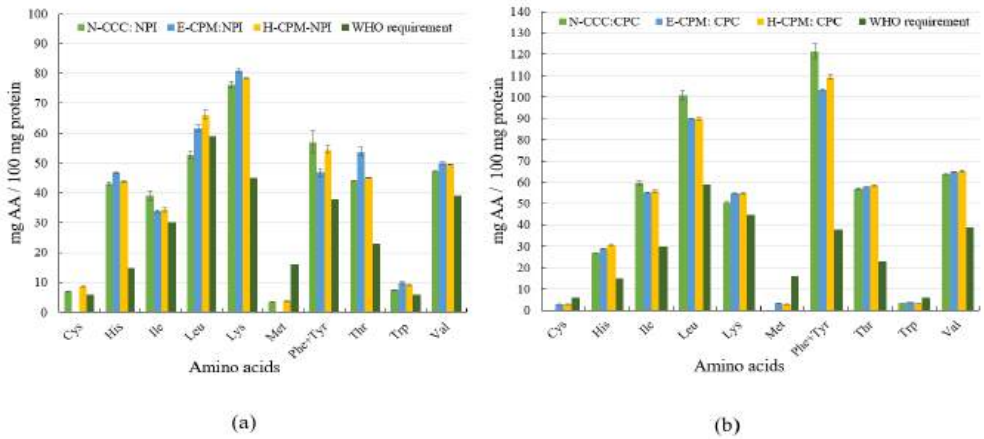

Figure 9. Levels of EAA in (a) NPI and (b) CPC obtained from three canola meals in comparison with the FAO/WHO (2007) adult requirement.

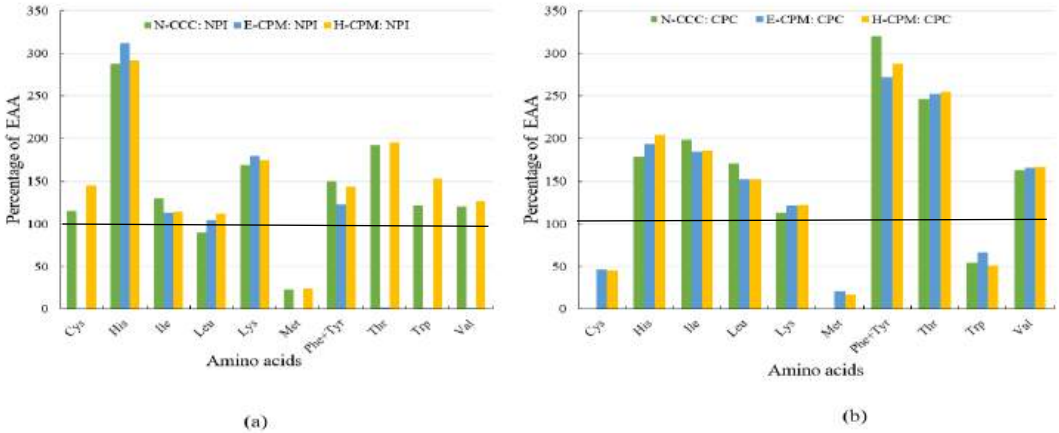

Figure 10. Comparison of EAA of NPI (a) and CPC (b) obtained from N-CCC, E-CCM, and H-CCM as a percentage value of FAO/WHO recommended requirement. Percentage value of 100 means the amino acid of the sample can provide an

amount equal to FAO/WHO recommendation for adults.

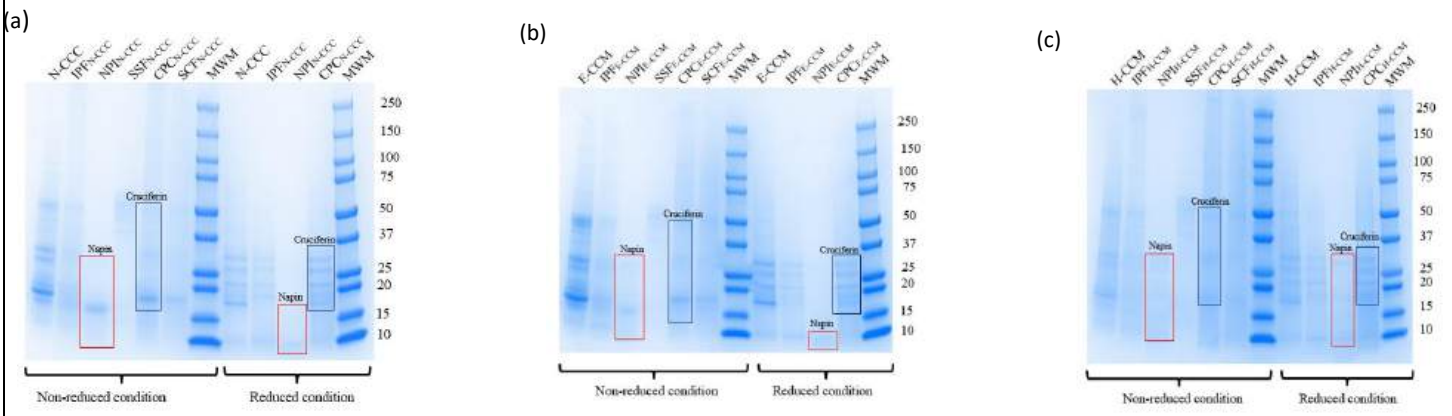

Figure 11.Polypeptide profiles of starting meals (a) N-CCC, (b) E-CCM and (c) H-CCM and their products obtained from AAFC Brassica meal fractionation process.

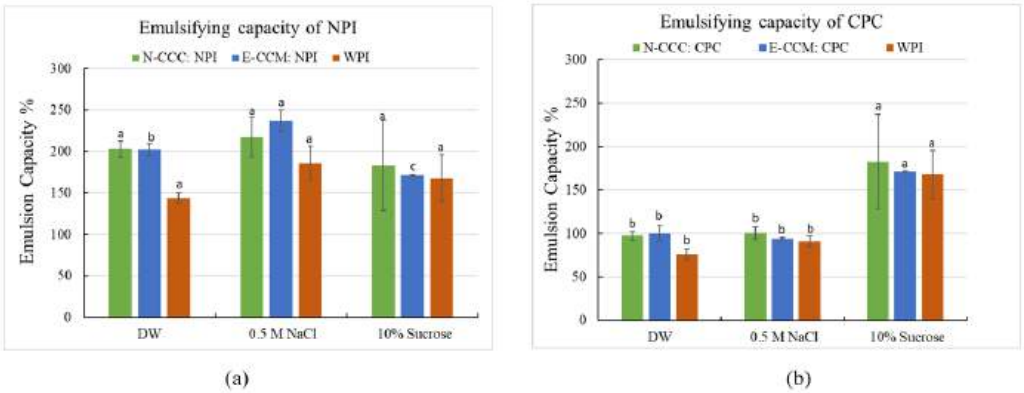

Figure 12. Emulsion capacity of **(a)** NPI and **(b)** CPC with and without additives (0.5 M NaCl or 10% sucrose) in the continuous phase.

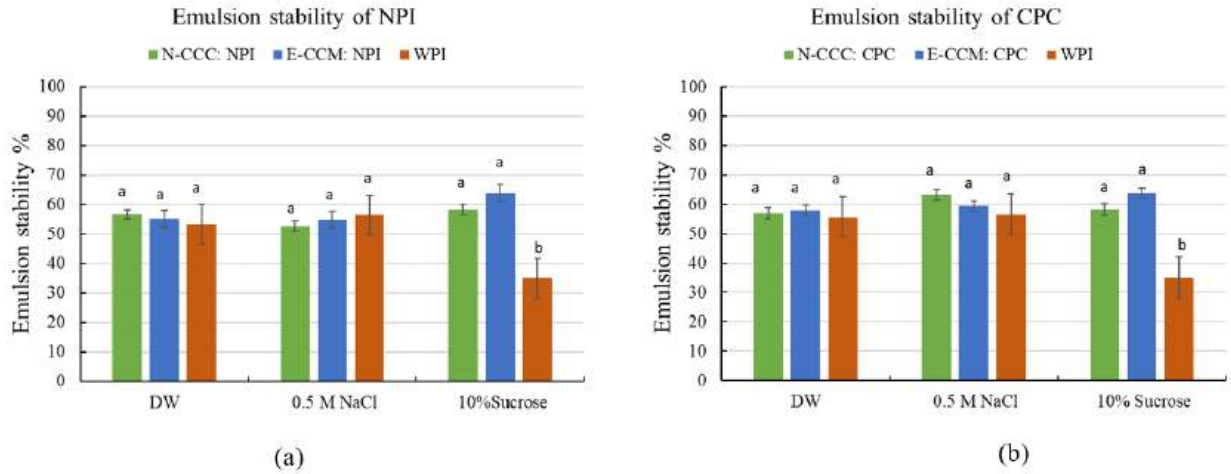

Figure 13. Emulsion stability of (a) NPI and (b) CPC obtained from different meals without any additive or under 0.5 M NaCl or 10% sucrose. *Foaming properties*

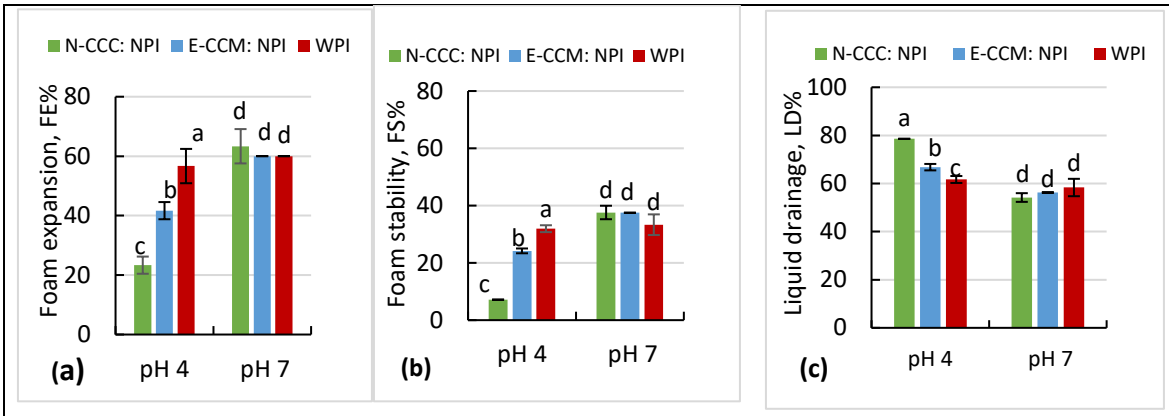

Figure 14. Foaming properties of NPI obtained from both N-CCM and E-CCM in comparison with whey protein isolate (WPI) **(a)** Foam expansion %, **(b)** Foam volume stability %, **(c)** Liquid drainage

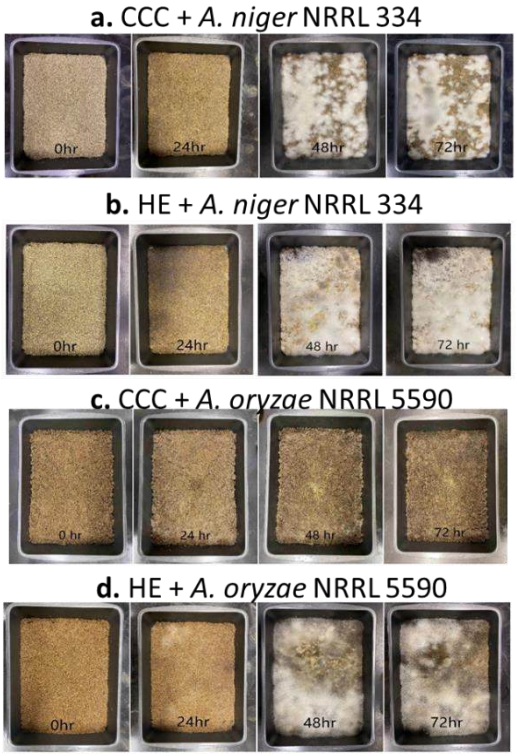

Figure 15. Cold-pressed canola cake and hexane-extracted canola meal fermented with *Aspergillus niger* NRRL 334 and *A. oryzae* NRRL 5590.

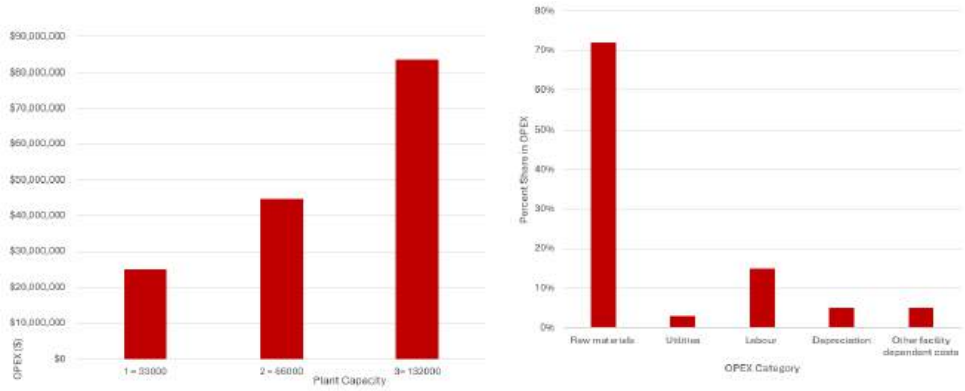

Figure 16. Total and distribution of expenses (OPEX) by category.

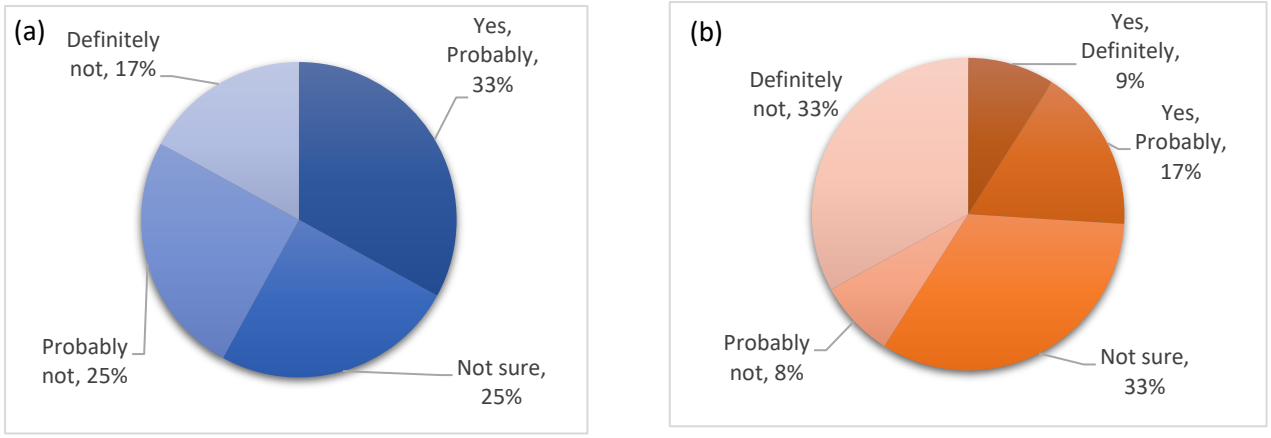

Figure 17. Distribution of responses for survey question **a)** Consumers be willing to pay a premium for non-GM foods and **b)** Will food manufacturers be willing to pay a premium for non-GM food ingredients?

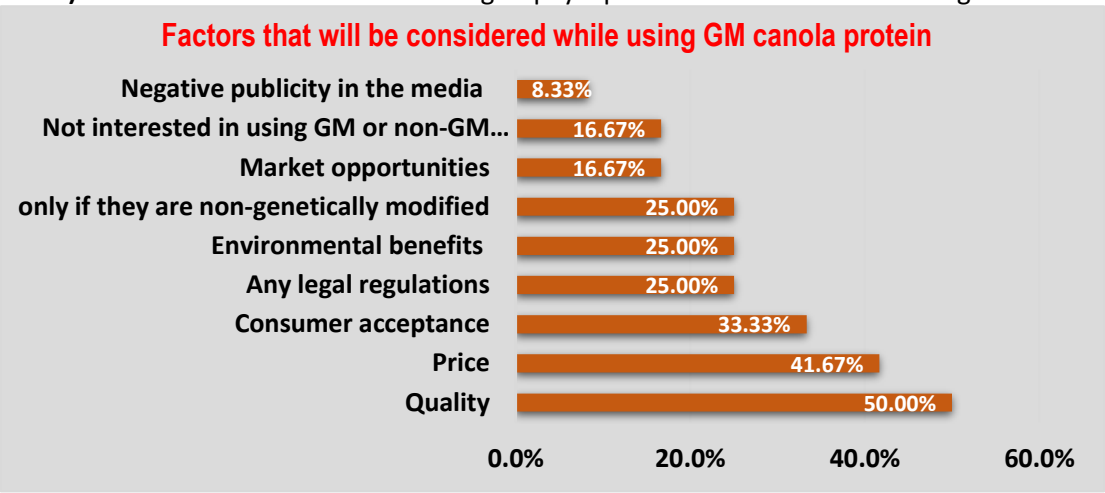

Figure 18. Major factors considered in the use of GM canola proteins.

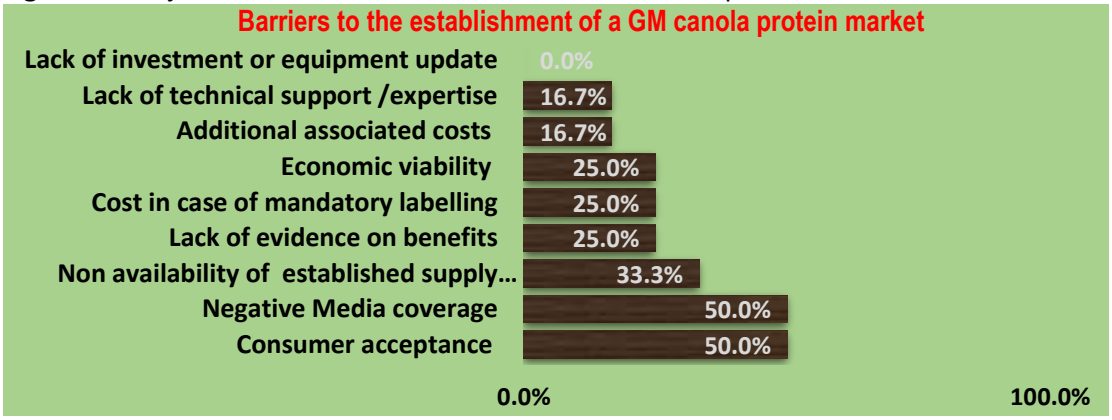

Figure 19. Barriers to the establishment of a GM canola protein market.

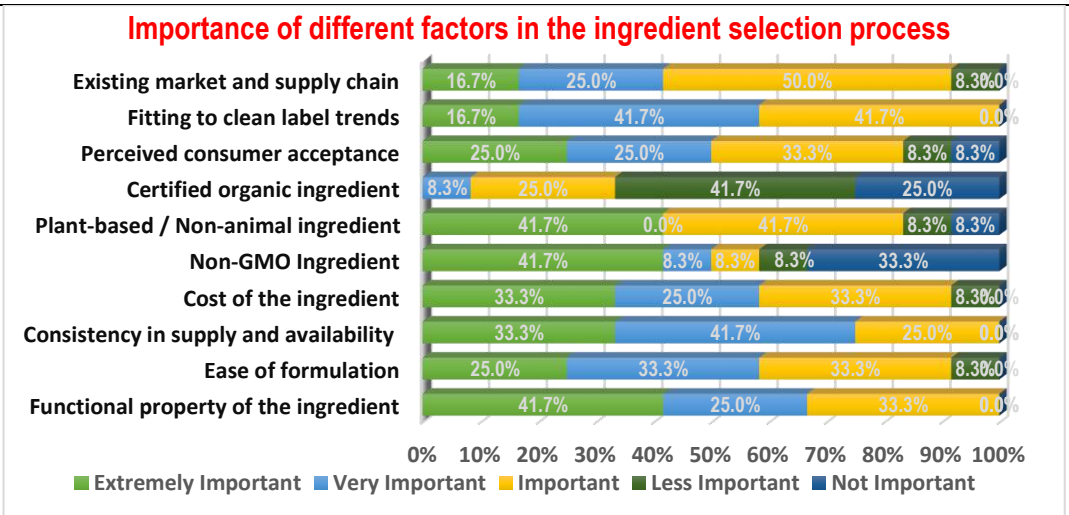

Figure 10. Importance of various factors in the ingredient selection process.

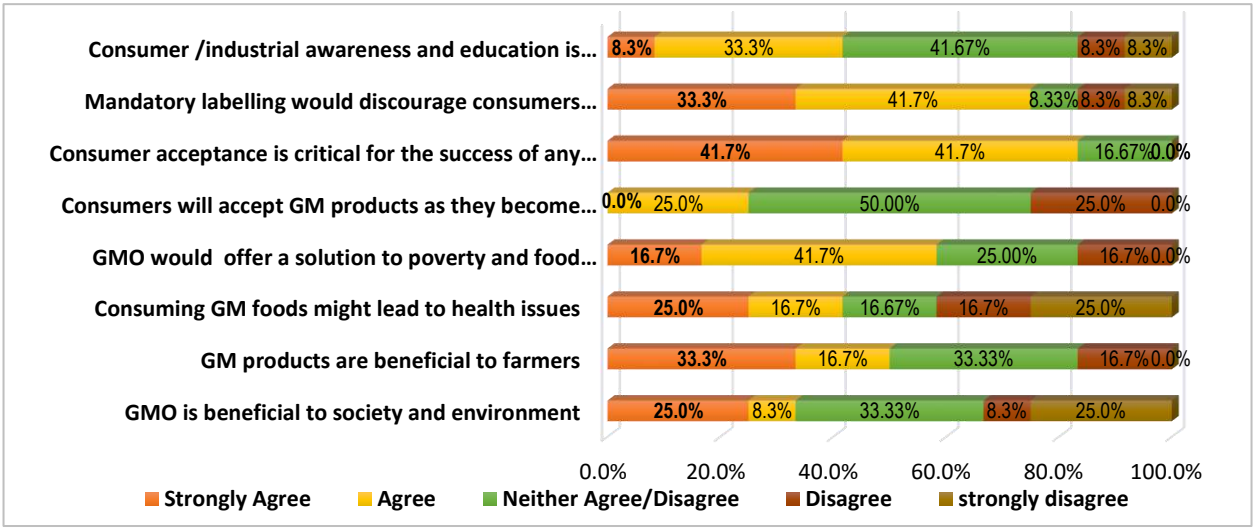

Figure 11. Opinion towards General GM related Questions.

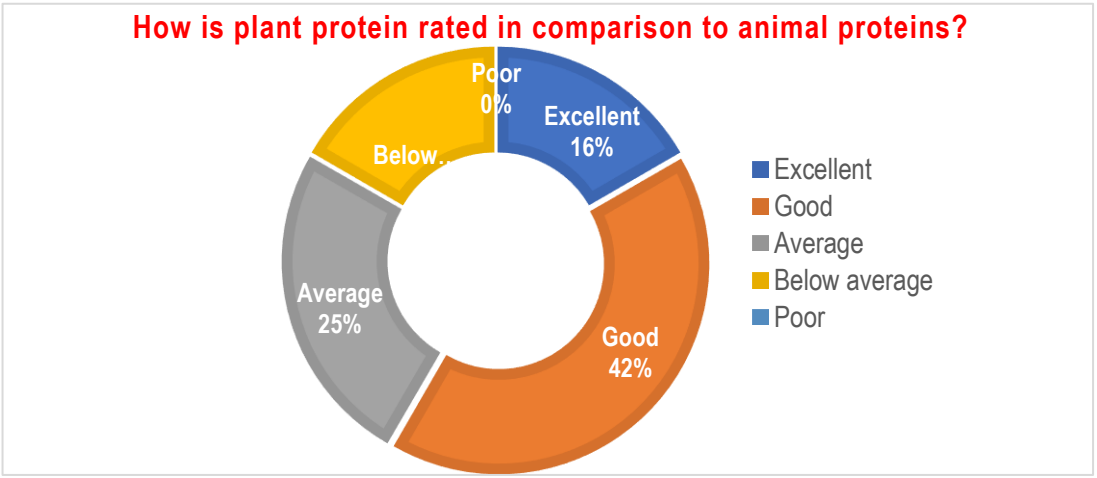

Figure 12. Rating of plant-protein in comparison to Animal proteins.

TABLES

Objective 1 (Wanasundara & Majumder)

Table 1. CCC treatment with ethanol and residual oil content of the resulting meal.

Input meal dry weight, g	Oil removal efficiency, %	Residual oil content of ethanol washed meal, g	Residual oil content after ethanol washing, %
1st extraction (0% water, 1:7.25 meal: solvent (w:v), 1.75 h, 25℃)			
46.5	91.2	0.6	1.2
46.3	92.5	0.5	1.0
46.3	90.4	0.6	1.3
46.3	90.7	0.6	1.3
46.2	91.8	0.5	1.1
46.2	89.0	0.7	1.5
2nd extraction (0% water, 1:4.5 meal: solvent (w:v), 1.75 h, 25℃)			
98.3	84.9	0.6	0.6
98.3	82.9	0.7	0.7
78.6	92.4	0.2	0.3

Table 2. Chemical composition (moisture, oil, protein content, total phenolic compound, and phytates) of N-CCC, E-CCM, and H-CCM.

Parameter	N-CCC	E-CCM	H-CCM
Moisture content (%)	7.7 ± 0.0^{a}	1.8 ± 0.1^{c}	6.8 ± 0.1^{b}
Oil content (%)	13.7 ± 0.7^{d}	0.5 ± 0.2^{e}	0.2 ± 0.0^{e}
N-based protein content (%)	31.1 ± 0.4^{i}	40.6 ± 0.2^{g}	35.4 ± 1.6^{h}
Total phenolic content (TPC) (mg/g)	1.4 ± 0.0^{jk}	1.3 ± 0.1^{k}	1.4 ± 0.0^{j}
Phytates (g/100g)	2.5 ± 0.1^{m}	3.0 ± 0.1^{m}	2.5 ± 0.1^{m}

All values are on a dry weight basis (dwb). Means ± Standard deviation (SD); mean values presented in the column following the same superscript are not significantly different ($p<0.05$).

Table 3. The dry matter and protein recovered in each fraction compared to the starting meal (N-CCC, E-CCM, and H-CCM) and the protein content of each fraction.

Meal Fraction	N-CCC	E-CCM	H-CCM
Dry matter recovered from meal, % (w/w)			
NPI	4.4 ± 0.5^{a}	4.6 ± 0.8^{a}	4.2 ± 0.4^{a}
CPC	10.2 ± 0.8^{d}	10.6 ± 1.4^{d}	13.3 ± 0.8^{d}
IPF	2.7 ± 0.0^{g}	2.8 ± 0.2^{g}	2.4 ± 0.2^{g}
SSF	13.5 ± 5.3^{j}	13.9 ± 4.7^{j}	11.3 ± 3.5^{j}
SCF	12.1 ± 0.2^{m}	12.6 ± 0.9^{m}	9.3 ± 0.2^{n}
Percentage of protein recovered from meal (w/w)			
NPI	20.1 ± 3.0^{a}	17.2 ± 2.2^{a}	17.1 ± 2.6^{a}
CPC	19.8 ± 5.0^{d}	30.7 ± 5.6^{d}	33.2 ± 5.6^{d}
IPF	3.2 ± 0.8^{g}	3.2 ± 0.2^{g}	2.9 ± 0.2^{g}
SSF	9.8 ± 3.3^{j}	9.0 ± 3.9^{j}	7.1 ± 2.3^{j}

SCF	12.1±0.2[m]	11.4±0.1[m]	9.6±1.2[n]
Protein content, %			
NPI	97.9±2.7[a]	96.2±0.6[a]	96.7±4.3[a]
CPC	55.3±1.9[d]	74.2±1.2[e]	59.2±7.4[de]
IPF	25.2±4.6[g]	29.7±1.8[g]	28.0±0.0[g]
SSF	15.9±0.0[j]	16.3±0.8[k]	15.0±0.2[j]
SCF	21.4±1.2[m]	23.1±0.6[m]	24.7±2.2[m]

All values are Mean ± Standard deviation (SD); For each parameter of protein fraction, the mean values presented in the column following the same superscript are not significantly different (p>0.05).

Table 4. Percentage of oil content of the fractions from N-CCC (Fractions from E-CCM and H-CCM oil content was 0%).

Meal fraction/ Component	Oil content[1], %
$NPI_{N\text{-}CCC}$	2.2 ± 0.7
$CPC_{N\text{-}CCC}$	26.1 ± 1.7
$IPF_{N\text{-}CCC}$	32.7 ± 3.4
$SSF_{N\text{-}CCC}$	0.4 ±0.1
$SCF_{N\text{-}CCC}$	12.4 ± 1.1

[1]*Mean ± SD.*

Table 5. Total phenolic content and phytate level of NPIs, CPCs, IPFs, and SCFs.

Meal fraction/ component	N-CCC	E-CCM	H-CCM
	Total Phenolic content (TPC) (mg/g)		
NPI	12.5 ± 0.5[a]	0.8 ± 0.0[b]	0.8 ± 0.0[b]
CPC	0.4 ± 0.0[d]	0.4 ± 0.0[d]	0.5 ± 0.0[e]
IPF	-	-	-
SCF	0.7 ± 0.0[g]	0.8 ± 0.0[g]	0.8 ± 0.0[h]
	Phytates (g/100g)		
NPI	0.5± 0.1[j]	1.8 ± 1.2[j]	1.2 ± 1.0[j]
CPC	1.9 ± 0.2[m]	2.8 ± 0.2[n]	2.0 ± 0.2[m]
IPF	15.3 ± 1.3[p]	16.0 ± 4.5[p]	19.3 ± 2.5[p]
SCF	-	-	-

All values are on dry weight (dw) basis. Means ± Standard deviation (SD); mean values presented in the column following the same superscript are not significantly different (p<0.05).

Table 6. Solubility, surface hydrophobicity, and zeta potential of NPI and CPC obtained from N-CCC, E-CCM, and H-CCM.

Meal & Protein fraction	Solubility, %			Surface Hydrophobicity			Zeta potential, mV		
	pH 4.0	pH 7.4	pH 9.5	pH 4.0	pH 7.4	pH 9.5	pH 4.0	pH 7.4	pH 9.5
NPI									
$NPI_{N\text{-}CCC}$	80.1	100.4	99.4	20.6	13.3	39.9	-3.6	-4.7	-8.9
$NPI_{E\text{-}CCM}$	68.0	98.7	103.4	22.7	10.6	35.5	-11.0	-5.3	-7.1

NPI_{H-CCM}	72.8	101.2	103.7	13.4	13.8	37.7	-6.6	-5.0	-7.4
CPC									
CPC_{N-CCC}	13.9	58.9	43.5	78.1	13.1	67.0	-6.3	-5.2	-33.4
CPC_{E-CCM}	13.4	47.4	26.4	30.4	24.9	76.8	-5.1	-5.9	-25.3
CPC_{H-CCM}	21.8	63.6	37.9	34.3	15.1	100.4	-16.1	-5.0	-24.4

Table 7. Oil holding capacity and water holding capacity of NPI, CPC, and commercial SPI.

Protein fraction	Oil holding capacity, g/g protein	Water holding capacity, g/g protein
	NPI	
NPI_{N-CCC}	1.7 ± 0.1^{c}	NA
NPI_{E-CCM}	2.9 ± 0.0^{a}	NA
NPI_{H-CCM}	1.6 ± 0.1^{b}	NA
	CPC	
CPC_{N-CCC}	1.5 ± 0.0^{r}	0.9 ± 0.1^{b}
CPC_{E-CCM}	1.7 ± 0.1^{q}	1.0 ± 0.0^{b}
CPC_{H-CCM}	2.3 ± 0.1^{p}	1.0 ± 0.0^{b}
	Reference (commercial protein)	
SPI	1.3 ± 0.0^{d}	2.2 ± 0.0^{a}

All values are on dry weight (dwb) basis. Means ± Standard deviation (SD); mean values presented. Values for each protein fraction from different meals following the same superscript are not significantly different (p>0.05). NA= Not available.

Table 8. Thermal denaturation properties of NPI and CPC.

Fraction/ Ingredient	Denaturation temperature, TD (℃)	Enthalpy (J/g)	Onset of peak (℃)	End of peak (℃)
		NPI		
NPI_{N-CCC}	100.7 ± 0.2^{b}	4.8 ± 1.0^{bc}	87.8-91.5	107-124.5
NPI_{E-CCM}	100.9 ± 0.4^{ab}	8.2 ± 1.5^{a}	86.8-92.0	113-124
NPI_{H-CCM}	101.2 ± 0.1^{a}	6.3 ± 0.3^{ab}	86.3-93.0	110-123.7
		CPC		
CPC_{N-CCC}	NA	NA	NA	NA
CPC_{E-CCM}	NA	NA	NA	NA
CPC_{H-CCM}	NA	NA	NA	NA
		Reference (commercial protein)		
WPI	75.86 ± 0.03^{c}	3.47 ± 0.04^{c}	71-71.54	84-86.8

All values are on dry weight (dw) basis. Means ± Standard deviation (SD); mean values presented in the column following the same superscript are not significantly different (p>0.05). NA= Not available.

Table 9. Percentage of in vitro protein digestibility of NPI, CPC and casein.

Fractions	In vitro protein digestibility, %

	Without heat treatment	With heat treatment
	NPI	
NPI_{N-CCC}	142.13 ± 2.49[a]	180.75 ± 5.02[d]
NPI_{E-CCM}	119.70 ± 1.17[b]	141.67 ± 3.55[f]
NPI_{H-CCM}	144.80 ± 4.28[a]	164.28 ± 1.92[e]
	CPC	
CPC_{N-CCC}	103.13 ± 2.47[i]	95.72 ± 3.56[l]
CPC_{E-CCM}	123.38 ± 0.83[g]	114.35 ± 1.20[j]
CPC_{H-CCM}	109.70 ± 4.85[h]	103.03 ± 2.94[k]
Reference sample		
Casein	94.57 ± 4.37	NA

Values expressed as Means ± Standard deviation (SD); mean values presented in the column following the same superscript are not significantly different (p>0.05). NA= Not available.

Objective 2 (Nickerson & C. Li)

Table 10. The pH value , degree of protein hydrolysis (%DH), crude protein, lipid, ash, phytic acid and total phenolics content of fermented and unfermented control hexane-extracted (HE) and cold-pressed (CCC) canola meals.

Meal / Organism	0 h	24 h	48 h	72 h
		pH		
CCC – *A. niger* NRRL 334	5.9 ± 0.1[a]	6.1 ± 0.3[a]	7.0 ± 0.1[b]	6.9 ± 0.2[b]
CCC – *A. oryzae* NRRL 5590	5.9 ± 0.1[a]	6.6 ± 0.2[a]	8.2 ± 0.1[a]	8.2 ± 0.1[b]
HE – *A. niger* NRRL 334	6.0 ± 0.2[a]	6.8 ± 0.3[b]	8.1 ± 0.2[c]	8.2 ± 0.1[c]
HE– *A. oryzae* NRRL 5590	6.0 ± 0.2[a]	6.6 ± 0.2[b]	8.2 ± 0.1[c]	8.2 ± 0.1[d]
		DH (%, d.b.)		
CCC – *A. niger* NRRL 334	0	30.1 ± 1.2[a]	45.0 ± 1.0[b]	46.4 ± 0.7[b]
CCC – *A. oryzae* NRRL 5590	0	28.3 ± 1.0[a]	36.9 ± 1.0[b]	45.1 ± 0.8[c]
HE – *A. niger* NRRL 334	0	23.4 ± 0.9[a]	41.9 ± 1.0[b]	52.3 ± 2.5[c]
HE– *A. oryzae* NRRL 5590	0	15.8 ± 1.7[a]	43.7 ± 0.1[b]	45.7 ± 0.4[b]
		Crude protein (%, d.b.)		
CCC – *A. niger* NRRL 334	33.9 ± 0.7[a]	34.2 ± 1.0[a]	36.5 ± 3.2[a]	38.4 ± 3.3[b]
CCC – *A. oryzae* NRRL 5590	33.9 ± 0.7[a]	34.3 ± 0.7[a]	36.4 ± 2.1[b]	36.1 ± 2.4[b]
HE – *A. niger* NRRL 334	39.7 ± 0.1[a]	40.3 ± 0.2[ab]	41.5 ± 0.4[b]	41.5 ± 1.0[b]
HE– *A. oryzae* NRRL 5590	39.7 ± 0.1[a]	40.6 ± 2.0[a]	41.1 ± 1.8[a]	40.7 ± 1.2[a]
		Crude lipids (%, d.b.)		
CCC – *A. niger* NRRL 334	12.3 ± 0.2[a]	12.7 ± 0.3[a]	10.7 ± 0.7[b]	9.5 ± 0.4[c]
CCC – *A. oryzae* NRRL 5590	12.6 ± 0.2[a]	11.1 ± 2.0[ab]	10.3 ± 1.8[b]	8.9 ± 2.1[c]
HE – *A. niger* NRRL 334	2.4 ± 0.4[a]	1.6 ± 0.2[b]	1.4 ± 0.2[b]	0.9 ± 0.1[c]
HE– *A. oryzae* NRRL 5590	2.5 ± 0.2[a]	2.2 ± 0.2[a]	1.2 ± 0.4[b]	0.9 ± 0.1[b]
		Ash (%, d.b.)		
CCC – *A. niger* NRRL 334	5.9 ± 0.1[a]	6.6 ± 0.4[a]	7.0 ± 0.4[a]	7.5 ± 0.8[b]
CCC – *A. oryzae* NRRL 5590	5.9 ± 0.2[a]	6.6 ± 0.2[ab]	7.2 ± 0.2[bc]	7.4 ± 0.3[c]
HE – *A. niger* NRRL 334	7.9 ± 0.2[a]	8.7 ± 0.4[a]	9.6 ± 0.6[a]	9.9 ± 0.5[b]
HE– *A. oryzae* NRRL 5590	7.8 ± 0.9[a]	8.4 ± 0.5[a]	9.7 ± 0.2[b]	10.0 ± 0.3[b]
		Phytic acid content, PAC (%, d.b.)		
CCC – *A. niger NRRL 334*	5.9 ± 0.1[a]	2.8 ± 0.3[b]	1.1 ± 0.9[c]	0.9 ± 0.7[d]
CCC – *A. oryzae NRRL 5590*	5.9 ± 0.2[a]	2.4 ± 0.2[b]	1.9 ± 0.1[c]	1.6 ± 0.1[d]
HE – *A. niger NRRL 334*	5.9 ± 0.2[a]	2.4 ± 0.3[b]	1.9 ± 0.3[c]	1.5 ± 0.8[d]

HE – *A. oryzae NRRL 5590*	5.8 ± 0.1[a]	1.9 ± 0.3[b]	1.9 ± 0.1[b]	1.3 ± 0.5[c]
Total phenolic content, TPC (mg GAE/ g DM, d,b.)				
CCC – *A. niger NRRL 334*	2.7 ± 0.1[a]	1.9 ± 0.1[b]	1.3 ± 0.1[c]	1.0 ± 0.2[d]
CCC – *A. oryzae NRRL 5590*	2.7 ± 0.1[a]	1.3 ± 0.3[b]	1.2 ± 0.1[c]	1.0 ± 0.1[d]
HE – *A. niger NRRL 334*	3.1 ± 0.0[a]	1.3 ± 0.1[b]	0.7 ± 0.1[c]	0.6 ± 0.0[d]
HE– *A. oryzae NRRL 5590*	3.1 ± 0.0[a]	2.0 ± 0.1[b]	1.2 ± 0.1[c]	1.0 ± 0.0[d]

Data are mean ± one standard deviation (n=3). Significant difference exists between data with different letters as a function of time (p<0.05). Fermentation time (h).

Table 11. Protein content (%, d.b.) of alkaline extraction-isoelectric precipitation (AE-IP) and salt extraction-dialysis (SE) protein products prepared from fermented and unfermented (control) cold-pressed canola cake(CCC) and hexane-extracted (HE) canola meals.

Protein isolation process	Cold-pressed cake (CCC)				Hexane-extracted (HE) meal		
	Unfermented		Fermented		Unfermented	Fermented	
	No defatting	Defatted	*A. niger*	*A. oryzae*	Control	*A. niger*	*A. oryzae*
AEIP	68.9 ± 0.3[c]	81.6 ± 1.1[b]	62.7 ± 0.2[d]	57.9 ± 0.2[f]	86.5 ± 0.2[a]	56.5 ± 0.2[g]	58.5 ± 0.2[e]
SE	99.6 ± 1.0[a]	96.5 ± 1.3[d]	93.3 ± 2.2[f]	99.0 ± 1.2[b]	97.7 ± 3.3[c]	95.9 ± 2.3[e]	95.8 ± 4.2[e]

** Defatted CCC control: canola protein isolates extracted from defatted cold-press meal using AE-IP or SE Data was reported as mean ± one standard deviation across each extraction process (n=3). Significant difference exists between data with different letters in a row (p<0.05).*

Table 12. Protein (nitrogen-based) solubility (%, d.b.) of protein products obtained from alkaline extraction-isoelectric precipitation (AE-IP) and salt extraction-dialysis (SE) from fermented and unfermented (control) canola meals.

Protein product	AE-IP product (solubility, %)			Salt-extracted product (solubility, %)		
	pH 3	pH 5	pH 7	pH 3	pH 5	pH 7
CCC control	88.9 ± 3.7[a]	53.5 ± 2.6[a]	27.7 ± 2.2[d]	94.0 ± 0.4[a]	37.7 ± 0.2[a]	51.8 ± 1.1[bc]
DCCC control	76.2 ± 3.6[cd]	36.9 ± 2.1[b]	37.8 ± 2.4[c]	97.1 ± 0.5[a]	37.3 ± 0.8[a]	49.3 ± 0.8[c]
CCC *A. niger*	25.9 ± 1.9[f]	5.9 ± 0.9[d]	47.5 ± 2.9[b]	21.3 ± 1.6[e]	4.5 ± 0.2[d]	37.2 ± 0.1[e]
CCC *A. oryzae*	72.7 ± 1.2[d]	7.5 ± 0.5[cd]	25.6 ± 0.9[d]	83.4 ± 0.2[b]	6.6 ± 0.4[c]	90.7 ± 3.8[a]
HE control	81.4 ± 0.9[b]	2.0 ± 0.9[e]	70.0 ± 1.4[a]	94.6 ± 1.7[a]	29.4 ± 2.3[b]	43.2 ± 1.1[d]
HE *A. niger*	10.1 ± 1.0[g]	11.8 ± 1.0[c]	70.0 ± 1.1[a]	43.8 ± 0.9[d]	5.9 ± 0.4[c]	48.5 ± 1.6[c]
HE *A. oryzae*	53.0 ± 1.1[e]	5.8 ± 0.4[d]	38.5 ± 3.2[c]	49.8 ± 0.7[c]	6.5 ± 0.3[c]	55.6 ± 2.7[b]

** DCCC: defatted unfermented cold-press canola cake*. Data was reported as mean ± one standard deviation (n=3). Significant difference exists between data with different letters across each pH (p<0.05).

Table 13. Water/oil holding capacity (WHC) (g/g) of protein products obtained from alkaline extraction-isoelectric precipitation (AE-IP, 6a) and salt extraction-dialysis (SE, 6b) from fermented and unfermented (control) canola meals.

Property	Cold-pressed cake				Hexane-extracted meal		
	Unfermented		Fermented		Unfermented	Fermented	
	No defatting	Defatted	*A. niger*	*A. oryzae*	Control	*A. niger*	*A. oryzae*
AE-IP protein products							
WHC	2.1 ± 0.0[d]	2.7 ± 0.1[c]	3.1 ± 0.1[ab]	3.0 ± 0.0[b]	1.8 ± 0.0[e]	2.9 ± 0.1[b]	3.2 ± 0.0[a]
OHC	2.9 ± 0.0[b]	2.4 ± 0.1[c]	2.8 ± 0.1[b]	2.8 ± 0.1[b]	2.3 ± 0.1[c]	3.1 ± 0.1[ab]	3.3 ± 0.1[a]
SE protein products							
WHC	1.1 ± 0.1[c]	2.1 ± 0.1[b]	1.3 ± 0.1[c]	1.4 ± 0.1[c]	1.3 ± 0.1[c]	2.3 ± 0.1[b]	2.7 ± 0.0[a]
OHC	3.2 ± 0.1[d]	3.9 ± 0.1[c]	4.6 ± 0.1[a]	4.7 ± 0.0[a]	2.4 ± 0.1[e]	4.5 ± 0.0[b]	3.9 ± 0.1[c]

**AE-IP: alkaline extraction-isoelectric precipitation; SE: salt extraction-dialysis. Defatted CCC control: canola protein isolates extracted from defatted cold-press meal using AE-IP or SE. Data was reported as mean ± one standard deviation (n=3). Significant difference exists between data with different letters in a row (p<0.05).*

Table 14 (a, b). Emulsifying activity index (EAI, m^2/g) and stability index (ESI, min) of protein products obtained from alkaline extraction-isoelectric precipitation (AE-IP, a) and salt extraction-dialysis (SE, b) of fermented and unfermented (control) canola meals.

AE-IP protein products

AE-IP Protein product	EAI, m^2/g			ESI, min		
	pH 3	pH 5	pH 7	pH 3	pH 5	pH 7
CCC control	11.4 ± 0.9^{cd}	4.1 ± 0.3^{a}	11.7 ± 1.5^{c}	4.5 ± 1.4^{ab}	1.4 ± 0.1^{ab}	1.2 ± 0.1^{b}
DCCC control	8.3 ± 0.6^{d}	5.0 ± 0.4^{a}	11.6 ± 1.7^{c}	1.5 ± 0.1^{ab}	1.6 ± 0.1^{ab}	1.4 ± 0.2^{b}
CCC *A. niger*	13.8 ± 0.7^{c}	2.1 ± 0.3^{bc}	25.8 ± 1.9^{a}	4.8 ± 2.9^{a}	1.1 ± 0.1^{b}	1.4 ± 0.2^{b}
CCC *A. oryzae*	21.1 ± 1.4^{a}	5.5 ± 0.1^{a}	27.6 ± 1.8^{a}	1.8 ± 0.3^{ab}	1.5 ± 0.1^{ab}	1.6 ± 0.1^{ab}
HE control	13.2 ± 1.6^{c}	3.0 ± 0.5^{b}	18.3 ± 1.6^{b}	3.1 ± 0.6^{ab}	2.2 ± 0.7^{a}	2.0 ± 0.3^{a}
HE *A. niger*	8.5 ± 0.8^{d}	1.3 ± 0.1^{c}	18.0 ± 1.9^{b}	1.6 ± 0.4^{ab}	1.3 ± 0.2^{b}	1.3 ± 0.2^{b}
HE *A. oryzae*	17.6 ± 1.9^{b}	4.8 ± 0.6^{a}	25.5 ± 2.5^{a}	1.2 ± 0.1^{b}	1.3 ± 0.1^{b}	1.2 ± 0.2^{b}
SE protein products						
CCC control	5.6 ± 1.1^{c}	5.8 ± 0.5^{bc}	12.9 ± 0.5^{b}	1.7 ± 0.2^{b}	1.2 ± 0.1^{d}	3.4 ± 0.3^{a}
DCCC control	11.0 ± 0.4^{a}	9.4 ± 0.2^{a}	14.5 ± 0.4^{a}	1.2 ± 0.0^{b}	1.2 ± 0.0^{d}	1.6 ± 0.1^{b}
CCC *A. niger*	8.5 ±0.5^{b}	1.8 ±0.2^{d}	12.2 ± 0.7^{b}	4.2 ± 1.0^{a}	2.8 ± 0.4^{a}	1.8 ± 0.2^{b}
CCC *A. oryzae*	9.7 ± 0.4^{ab}	4.6 ± 0.5^{c}	14.9 ± 0.3^{a}	1.2 ± 0.0^{b}	1.2 ± 0.1^{d}	1.7 ± 0.1^{b}
HE control	5.9 ± 0.5^{c}	6.8 ± 0.4^{b}	13.9 ± 0.7^{ab}	1.6 ± 0.2^{b}	2.4 ± 0.3^{ab}	4.3 ± 1.3^{a}
HE *A. niger*	8.5 ± 0.5^{b}	4.1 ± 0.6^{cd}	11.4 ± 0.4^{b}	1.3 ± 0.1^{b}	1.9 ± 0.1^{bc}	1.5 ± 0.1^{b}
HE *A. oryzae*	11.3 ± 0.9^{a}	3.0 ± 0.3^{d}	7.6 ± 0.8^{c}	1.2 ± 0.1^{b}	1.4 ± 0.2^{cd}	1.2 ± 0.0^{b}

**AE-IP: alkaline extraction-isoelectric precipitation; SE: salt extraction-dialysis; DCCC: CCC control prepared from defatted and unfermented cold-press meal using AE-IP or SE. Data was reported as mean ± one standard deviation (n=3). Significant difference exists between data with different letters across each pH (p<0.05).*

Table 15. Foaming capacity (%) and stability (%) (as a function of pH 3, 5 and 7) of alkaline extraction-isoelectric precipitation (AE-IP, a) and salt-extraction dialysis (SE, b) canola concentrates and isolates from different fermented and control canola meals.

AE-IP protein products

SE protein product	Foaming capacity (%)			Foam stability 30-min (%)		
	pH 3	pH 5	pH 7	pH 3	pH 5	pH 7
CCC control	224.4 ±20.4^{c}	241.1±16.4^{a}	165.0±5.2^{bc}	93.0±2.3^{a}	83.2± 5.5^{a}	68.0±1.4^{d}
DCCC control	306.7±20.0^{a}	131.1± 7.7^{b}	243.8±8.1^{a}	87.0±1.9^{b}	32.3±4.2^{b}	78.9± 0.6^{abc}
CCC *A. niger*	/	/	230.7±6.8^{a}	/	/	82.3±3.1^{ab}
CCC *A. oryzae*	228.9±3.8^{c}	/	180.0±3.3^{b}	33.0 ±1.1^{c}	/	84.6±4.0^{a}
HE control	244.4±10.2^{bc}	/	154.4±6.9^{cd}	17.3 ±1.6^{d}	/	75.2±.8^{bcd}
HE *A. niger*	/	/	244.2±12.4^{a}	/	/	72.0± 0^{cd}
HE *A. oryzae*	/	/	136.7± 8.8^{d}	/	/	79.6±3.0^{ab}
SE protein products						
SE protein product	Foaming capacity (%)			Foam stability 30-min (%)		
	pH 3	pH 5	pH 7	pH 3	pH 5	pH 7
CCC control	322.2±7.7^{a}	211.1±20.4^{b}	480.0±11.5^{a}	87.2±1.5^{a}	87.2±4.0^{a}	83.8±0.7^{ab}
DCCC control	246.7±17.6^{b}	222.2±7.7^{a}	464.4±20.4^{b}	84.2±0.4^{a}	87.4±4.2^{a}	89.0±2.0^{a}
CCC *A. niger*	196.5±6.6^{c}	/	191.1±13.9^{d}	54.6±0.1^{c}	/	88.4±1.2^{a}
CCC *A. oryzae*	175.6±10.2^{c}	/	153.3±8.8^{e}	51.0±1.1^{c}	/	89.0±4.3^{a}
HE control	266.7 ± 6.7^{b}	/	145.6±10.2^{e}	53.4 ± 4.9^{c}	/	77.2±5.4^{b}
HE *A. niger*	195.6 13.9^{c}	/	306.7±17.6^{c}	51.8±2.0^{c}	/	86.4±4.6^{ab}
HE *A. oryzae*	135.6±10.2^{d}	/	155.6±3.8^{de}	51.3±1.4^{d}	/	77.6±2.9^{ab}

**AE-IP: alkaline extraction-isoelectric precipitation; SE: salt extraction-dialysis. DCCC: unfermented defatted CCCI control prepared from defatted unfermented cold-press meal using AE-IP or SE. /: no obvious foams were found, or foams disappeared in 5 min. Data was reported as mean ± one standard deviation (n=3). Significant difference exists between data with different letters across each pH (p<0.05).*

Objective 3 (Mupondwa & X. Li)

Table 16. Plant capital cost for three production capacities.

	Annual Plant Capacity (Tonnes canola seed)		
	33000	***66000***	***132000***
Total Plant Direct Cost (TPDC)			
1. Equipment Purchase Cost	2,258,000	2,914,000	4,090,000
2. Installation	812,000	1,060,000	1,497,000
3. Process Piping	790,000	1,020,000	1,432,000
4. Instrumentation	903,000	1,166,000	1,636,000
5. Insulation	68,000	87,000	123,000
6. Electrical	226,000	291,000	409,000
7. Buildings	1,016,000	1,311,000	1,841,000
8. Yard Improvement	339,000	437,000	614,000
9. Auxiliary Facilities	452,000	583,000	818,000
TPDC	6,863,000	8,869,000	12,458,000
Total Plant Indirect Cost (TPIC)			
10. Engineering	1,716,000	2,217,000	3,115,000
11. Construction	2,402,000	3,104,000	4,360,000
TPIC	4,118,000	5,321,000	7,475,000
Total Plant Cost (TPC = TPDC+TPIC)			
TPC	10,980,000	14,191,000	19,933,000
Contractor's Fee & Contingency (CFC)			
12. Contractor's Fee	549,000	710,000	997,000
13. Contingency	1,098,000	1,419,000	1,993,000
CFC = 12+13	1,647,000	2,129,000	2,990,000
Direct Fixed Capital Cost (DFC = TPC+CFC)			
DFC	12,627,000	16,319,000	22,923,000
Working Capital	2,066,000	3,784,000	7,218,000
Start-up Cost	631,000	816,000	1,146,000
Total Investment	**15,324,000**	**20,919,000**	**31,287,000**

Table 17. Decrease in cost: Relationship between capital investment and plant capacity of the protein production plant.

Total Capital Investment (CDN $)	Capacity (Tonnes)	Unit cost ($)	% Decrease in Unit Cost
15,324,000	33,000	464	
20,919,000	66,000	317	31.7%
31,287,000	132,000	237	48.9%

Table 18. Estimated NPV for the canola protein production plant.

	Canola Protein Price $ kg^{-1}	$0.45				$0.55				$0.65			
	Canola Seed Price $ kg^{-1}	$0.50	$0.55	$0.60	$0.65	$0.50	$0.55	$0.60	$0.65	$0.50	$0.55	$0.60	$0.65
Capacity tonnes y^{-1}	NPV ($'000)												
33000 tonnes	NPV $1,35 kg^{-1} oil price	(2,234)	(16,221)	(33,796)	(51,371)	(694)	(5,642)	(21,225)	(38,800)	12,719	2,429	(9,899)	26,229
	NPV $1,25 kg^{-1} oil price	(13,234)	(30,210)	(47,786)	(65,359)	(3,193)	(17,639)	(35,214)	(52,789)	4,780	(6,775)	(22,643)	(40,218)
	NPV $1,15 kg^{-1} oil price	(26,624)	(44,198)	(61,773)	(79,348)	(14,217)	(31,628)	(49,202)	(66,777)	(4,073)	(19,075)	(36,632)	(54,206)
66,000 tonnes	NPV $1,35 kg^{-1} oil price	34,780	13,274	(8,338)	(41,180)	50,103	28,596	7,962	(16,933)	65,425	43,918	22,412	1,432
	NPV $1,25 kg^{-1} oil price	17,728	(3,333)	(34,000)	(69,160)	33,051	11,544	(10,767)	(34,018)	48,373	26,867	6,133	(19,180)
	NPV $1,15 kg^{-1} oil price	11,153	(26,840)	(61,989)	(97,139)	15,999	(5,256)	(36,848)	(71,997)	31,321	10,833	(13,198)	(46,856)

Conclusions and Recommendations *(maximum 500 words)*

Highlight significant conclusions based on the findings of this project, with emphasis on the project objectives specified above. Provide recommendations for the application and adoption of the project findings.

Studies of this project show the possibilities existing for cold-pressed canola cake(CCC). Ethanol (without added water) is an alternative to hexane to reduce oil content of CCC producing a suitable material for protein product preparation. AAFC Brassica meal fractionation can successfully be applied to obtain two protein-rich and three fibre-derived fractions from CCC without de-oiling or with de-oiling using ethanol (E-CCM) or heaxane (H-CCM). Low-molecular weight , basic protein containing napin protein isolate (NPI) can be obtained without residual oil contamination however, some of the oil in CCC were distributed to other protein-rich fraction; canola protein concentrate (CPC). NPI and CPC show protein nutritional quality parameters and techno-functional properties owing to their composing proteins and non-protein compounds.

Solid-state fermentation using *Aspergillus niger* NRRL 334 or *Aspergillus oryzae* NRRL 5590 can be applied on both cold-pressed canola cake (CCC) and industrially hexane-extracted (HE) canola meal to modify the composition, especially 72 h of fermentation time can give a reduction residual fat of CCC, phytic acid and phenol compounds of both CCC and HE meals. An increase in crude protein content and meal protein hydrolysis happened to different extents due to the growth of fungi and the degree of effect depended on the fungal strain used. Meals of 72-hours of SSF gave protein products with differences in techno-functionalities than unfermented meals. Fungal strains and meal types had an effect on all functionalities to a varying degree. The differences between functionality of extracted proteins with alkaline extraction-isoelectric precipitation (AE-IP) and salt-extraction dialysis (SE) methods were mainly due to the complexity of canola protein fractions (different isoelectric points and molecular weights). SE method compared to AE-IP can produce canola protein products with better functionality such as protein solubility, foaming and emulsifying properties. Canola proteins extracted from cold-pressed meal showed better functionality than those from HE meal could mainly due to the alterations of proteins during commercial level hexane extraction. Modification of meal proteins due to the enzymes released during fungal growth, also the changes occur in non-protein components affect recovery of proteins from depending on the conditions used.

Techno-economic analysis of showed the inverse relationship between capital investment and plant capacity of the protein production plant. However, the unit capital cost decreases correspondingly, clearly demonstrating the concept of economies of scale which refer to cost advantages (decrease in overall plant costs) that a processing plant can derive from expanding its operating scale.

Canola meals for creating value-added protein products for suitable product markets require understanding of the acceptance level of the participant industries. The survey conducted to understand how willing the industries are to include GM canola proteins in their food products and what could be the barriers for that market. There is a greater level of acceptance by various industrial manufacturers towards plant protein in general. If canola proteins were to be extracted, produced, and exhibit exceptional functional properties compared to alternatives, and if the pricing also competitive with similar ingredients available; nearly half of the industries are willing to include canola proteins in their ingredient list. Even though there are industries that are reluctant to include canola protein considering it being GM, the survey results showed that almost 40-50% of the industries would consider canola proteins regardless of their GM status.

Follow-up Research

Please identify if there is a need to conduct further research. Detail any further research, development and/or communication needs arising from this project.

This research brought foundational level information to utilize and add value to cold-pressed GM canola cake. Further research on aligning canola protein products, either fractionated or bio-transformed with plant proteins in the market, demonstrating their nutritional and functional strength in consumer products haven't happened yet. These involves working with industry who is truly willing to take canola protein or meal -based products into market.

Further scientific investigations can be carried for detailed understanding of chemical- or bio-technological transformation of canola protein and fibre into new products with demonstrated functions.

Developing canola proteins with exceptional functional properties superior to available alternatives and increasing consumers awareness about the benefits of GM products in general would help address decreased consumer acceptance, identified as one of the major barriers by the industries.

Patents/ IP generated/ Commercialized Products

List any products developed from this research.

No patents or commercial products developed.

Sustainable Canadian Agricultural Partnership (Sustainable CAP) Performance Indicators

a) List of performance indicators for the entire lifespan of the project

Sustainable CAP Indicator	Total Number
Scientific publications from this project (List the publications under section b)	
• Published	2
• Accepted for publication	0
Highly Qualified Personnel (HQP) trained during this project	
• Master's students	3
• PhD students	0

• Post docs	0
Knowledge transfer products developed based on this project (presentations, brochures, factsheets, flyers, guides, extension articles, podcasts, videos)[1]	1

[1] Please only include the number of unique knowledge transfer products.

b) List of scientific journal articles published/accepted for publication from this project. Please ensure that each line includes the following: **Title, Author(s), Journal, Date Published or Accepted for Publication and Link to Article (if available).** *Add additional lines as needed*.

1. Li, C., Shi, D., Stone, A., Wang, Y., Wanasundara, J., Tanaka, T., Nickerson, M. 2023. Effect of canola meal fermentation on select antinutrients and protein digestibility of cold-pressed and hexane-extracted meals. Journal of the American Oil Chemists' Society. 100, 529-538. doi: 10.1002/aocs.12705
2. Li, C., Shi, D., Stone, A., Wang, Y., Wanasundara, J., Tanaka, T., Nickerson, M. 2023. Effect of canola meal fermentation and protein extraction method on the functional properties of resulting protein products. Journal of the American Oil Chemists' Society. 100, 437-448. doi: 10.1002/aocs.12701
3.
4.

Technology Transfer Activities

List any technology transfer activities. Include presentations to conferences, producer groups or articles published in magazines except scientific journals.

Presentations:

Wanasundara, J.P.D. 2023. Canola meal valorization opportunities. Canola Industry Week. December 05- 07, Calgary, Canada.

Majumder, D., Nickerson, M.T., Wanasundara, J.P.D. 2022. Generation and characterization of protein and co-products from cold-pressed canola meal. E-poster presented at Sustainable Protein Forum of AOCS (hybrid event). October 4-6.

Wanasundara, J.P.D. 2022. September 28-30th, 2022. Proteins of canola/rapeseed: Potential for food product systems. SEEDFOOD mini-conference. University of Copenhagen.

Wanasundara, J.P.D. 2021. Protein from canola/rapeseed –An overview. 2021. Annual meeting of Chinese section of American Oil Chemists' Society. November 5th. Virtual presentation.

Wanasundara, J.P.D. 2021. Protein from canola/rapeseed –An overview. 2021. Protein Science and Technology Forum. October 12-14. Virtual presentation.

Thesis:

Chenghao (Charles) Li. 2021. Masters degree thesis: Investigation of solid-state fermentation to enhance nutritional value of cold-pressed and hexane-extracted canola meal and functional properties of extracted canola protein. (University of Saskatchewan, Food and Bio-product Sciences, Co-supervised by M. T Nickerson and J.P.D. Wanasundara).

Dipika Majumder. 2024. Masters degree thesis: Generation and characterization of protein and co-products from cold-pressed canola meal. (University of Saskatchewan, Food and Bio-product Sciences, Co-supervised by J.P.D. Wanasundara and M. T Nickerson).

Ninu Kallingal Mohandas. 2024. Acceptance of GMO canola derived protein products by the food ingredients industry. MSc thesis under review by advisors (University of Saskatchewan, Chemical and Biological Engineering, Co-supervised by Edmund Mupondwa and Venkatesh Meda)

Contributions and Support

List any industry contributions or support received.

from Pleasant Valley Oil Mills (Clive, AB) and Bunge Canada (Harobe, MB) provided cold-pressed canola cake and industrially processed hexane extracted canola meals.

Acknowledgements

Include actions taken to acknowledge support by the Ministry of Agriculture, the Canadian Agriculture Partnership (for projects approved between 2017 and 2023) and the Sustainable Canadian Agriculture Partnership (for projects approved between 2023 and 2028).

Acknowledged Ministry of Agriculture for funding support in all publications and science communications.

Appendices

Identify any changes expected to industry contributions, in-kind support, collaborations or other resources.

Appendix I

Survey Questions

1) What region does your company conduct business? (Multiple selections)

☐Canada
☐US
☐Mexico and middle America region
☐South America
☐Europe
☐Asia
☐Africa
☐Australia
☐ Middle East
☐ Mediterranean
☐ Other regions, please specify _____

2) Does your organisation/company currently use any plant-based proteins as an ingredient in your manufactured products?
   a) Yes
   b) No

   If yes to question 2, then 2.a):

2.a) Could you please specify which plant-based proteins are you currently using?

If no to Question 2, then 2.b):

2.b) Would your company be interested in using plant-based proteins as a functional ingredient?

a) Yes, definitely.
b) Yes, Probably
c) Not Sure
d) Probably not
e) Definitely not

3) Does your organisation/company currently use any Genetically modified (GM) products as an ingredient in your manufactured products?

a) Yes
b) No
c) Not Sure

If no or Not Sure to Question 3, then 3.a)

3.a) Would your company be willing to use Genetically modified (GM) products as an ingredient in your manufactured products?

a) Yes, definitely.
b) Yes, probably.
c) Not sure
d) Probably not
e) Definitely not

4) Based on your insights, do you think consumers would pay a premium to purchase products with non-genetically modified canola proteins than GM canola proteins with similar nutritional properties?

a) Yes, definitely.
b) Yes, probably.
c) Not sure
d) Probably not
e) Definitely not

5) As a manufacturer, would your company pay a premium to purchase non-genetically modified canola protein ingredients than GM canola proteins with same nutritional value?

a) Yes, definitely.
b) Yes, probably.
c) Not sure
d) Probably not
e) Definitely not

6) In your opinion, what factors would your organisation consider in choosing genetically modified canola proteins as a functional ingredient in your products? (Please select all that apply)

☐ Quality of GM canola ingredient vs the alternative/conventional ingredients

☐ Price of GM canola ingredient relative to the alternatives
☐Existing or prospective legal regulations like mandatory labelling of GM products
☐ Consumer acceptance towards GM canola protein
☐ Environmental benefits while using GM canola proteins.
☐ Existing and predicted market opportunities for GM canola protein products
☐ Existing negative publicity in the media towards GM canola and products
☐ Would consider canola proteins only if they are non-genetically modified.
☐Not interested in using GM or non-GM canola protein as an ingredient
☐ Others (Please specify)
---

7) In relation to any barriers to the establishment of a GM canola protein market in the current plant protein industry, could you indicate what barriers you believe exist? (Mark all that apply)

☐Consumer acceptance of genetically modified foods
☐Lack of evidence on benefits of plant proteins and GM products
☐Additional associated costs in production and distribution of GM canola proteins
☐ Additional costs that might occur in segregation, in case of mandatory labelling.
☐Economic viability in producing GM canola protein products.
☐ Non availability of an established GM canola protein supply chain
☐ Lack of investment or equipment update for incorporating canola proteins.
☐ Lack of technical support /expertise to incorporate new GM canola proteins.
☐ Existing negative publicity in the media towards GM canola and products
☐ I don't think there are any barriers.
☐ Others (please specify) _____

8) When choosing any functional ingredient in your product development, how would you rate the importance of the following factors in the selection process? (Choose one option)

	Extremely Important	Very Important	Important	Less Important	Not Important
Functional property of the ingredient selected	☐	☐	☐	☐	☐
Ease of formulation in products	☐	☐	☐	☐	☐
Consistency in supply and availability of the ingredient	☐	☐	☐	☐	☐
Cost of the ingredient	☐	☐	☐	☐	☐
Non-GMO Ingredient	☐	☐	☐	☐	☐
Plant-based / non-animal ingredient	☐	☐	☐	☐	☐
Certified organic ingredient	☐	☐	☐	☐	☐
Perceived consumer acceptance	☐	☐	☐	☐	☐
Fitting to the current clean label trends	☐	☐	☐	☐	☐

Existing established market and supply chain	☐	☐	☐	☐	☐

9) Please share your opinion on the following statements (Choose one option)

	Strongly Agree	Agree	Neither agree/ Disagree	Disagree	Strongly disagree
Genetically modified products are beneficial to society and environment	☐	☐	☐	☐	☐
GM products are beneficial to farmers	☐	☐	☐	☐	☐
Consuming GM foods might lead to health issues	☐	☐	☐	☐	☐
GM technology in food production would increase productivity and offer a solution to poverty and food problems around the world	☐	☐	☐	☐	☐
Consumers will accept GM products as they become more commonly available	☐	☐	☐	☐	☐
Consumer acceptance is critical for the success of any products of GM canola proteins.	☐	☐	☐	☐	☐
Mandatory labelling of GM foods would discourage consumers from purchasing GM products	☐	☐	☐	☐	☐
Consumer /industrial awareness and education regarding genetic modification is required for successful acceptance	☐	☐	☐	☐	☐

10) How would you rank plant-based proteins compared to animal-based proteins on a scale from excellent to poor? (Choose one)

Excellent	Good	Average	Below average	Poor
☐	☐	☐	☐	☐

11) What are your company's main products or services?

_____

12) What is the approximate annual revenue of your business?

a) < $1 million
b) <$5 million
c) $5-10 million
d) $10-50 million

e) $50-100 million
f) $100-500 million
g) >$500million

13) Is your company a Canadian-controlled corporation?

Yes ☐ No ☐

14) Do you have any other comments on GM canola protein products for use in food and beverage industry?

End of survey. Thank you for your time.

Consent Form

A survey on the acceptance of Genetically Modified (GM) canola protein as a functional ingredient by food manufactures

Purpose of the Research:

Canola is one of the Canadian leading crops. Canola proteins have a well-balanced amino acid profile and potential functional properties such as emulsifying, foaming, and gelling abilities. However, canola protein meal is currently mainly used in animal feed, not widely used in human food-grade protein products. The purpose of this research is to identify the barriers and challenges of promoting the application of Genetically Modified (GM) canola protein in food and beverage manufacturers. Through this survey, we are expecting to understand industrial opinions toward accepting GM food-grade canola proteins as a functional ingredient in their products.

Description of the Research:

You are invited to participate in an online questionnaire survey about the opinion and acceptance of Genetically Modified (GM) canola protein as a functional ingredient by food manufactures. As you may be aware, almost 95% of the canola grown in Canada is genetically modified (GM) whose genetic material has been modified in a way that does not occur naturally.

This questionnaire asks for your views about incorporating GM canola proteins as a functional ingredient in your food manufacturing process. You will be asked single and multiple answer multiple-choice questions, open and closed-end questions, and Likert scale questions (for example; starting at “Yes, definitely” scaling all the way down to “Definitely, not”) regarding to adoption of plant-based proteins and genetically modified (GM) canola proteins, and any barriers in market establishment from a business perspective. This survey will probably take about 5 to 10 minutes to finish.

We highly appreciate if you can fill the survey in two weeks. However, to maximum cover the industrial partner’s

thoughts, we may send you reminders and extend the survey for more weeks.

We highly appreciate your time and participation. Neither the respondents nor the companies will be identified in the results. There would not be any personal identifiable information in the collected raw survey data. Once the survey is completed, the survey link and the collected data will be destroyed from the online server. The raw data will be only retained in the Agriculture and Agri-food Canada's secured account for research purpose with minimum 5 years to maximum 25 years.

Your responses will not be used for any commercial purpose but only in the research findings and training in a Master student's program in University of Saskatchewan. The study is conducted through Agriculture and Agri-food Canada, sponsored by Agriculture Development Fund (ADF) - Saskatchewan Ministry of Agriculture and Food ADF. There are no conflicts of interest to declare related to this study.

Access to Research Information:

Kai Analytics and Survey Research Inc. (Vancouver, BC) will collect survey responses using a survey platform called Alchemer. The raw data will be encrypted and delivered to Agriculture and Agri-Food Canada for analysis and preparation of reports.

The data will be analyzed and maybe published in a scientific publication if significant findings are observed from this study. Raw data files will not be shared with other institutions and/or researchers for other purposes.

If you wish to be provided with the study results, please contact our researcher provided at the end of the survey. This will not be linked to questionnaires or data files in any way.

Potential Harm, Injuries, Discomforts, or Inconvenience:

There is no known harm associated with participation in this study.

Potential Benefits:

You will not benefit directly from participating in this study, but your data will be advantageous to the researchers to identify the status and challenges of the application and development of GM canola protein in food and beverage industries, which may bring potential benefits to Canadian farmers and economics in the future.

Confidentiality:

Confidentiality will be respected and no information that discloses the identity of the participant will be released or published without consent unless required by law.

You will be asked to enter a survey link which will not have any identifiable information disclosed. The survey link is created using platform of Alchemer that complies with personal information protection laws in Canada. The data collected will be encrypted and stored on Canadian soil. Once the survey is completed, the survey link and the collected data will be destroyed from the online server.

The information you will share with us if you participate in this study will be kept completely confidential to the full extent of the law. Although the data from this research project may be published and presented at conferences, the data will be reported in aggregate form so that it will not be possible to identify individuals.

Survey responses will not be linked to any email address, IP address, and personal or any business names.

Reimbursement:

We highly appreciate your time and participation, but there is not any payment, reimbursement, or gifts for filling out this survey.

Participation:

Your participation is completely voluntary; you can refuse to participate or can withdraw at any time.

Waiver of Rights:

Investigators are prohibited from seeking or obtaining waivers of participant's legal rights.

Contact:

If you have any questions about this study, please contact:
Dr. Sue Li - Research Associate, Agriculture and Agri-Food Canada
Email: xue.li@agr.gc.ca

If you have questions about your rights as a research participant, you may contact:
Nancy Ames
Chair, Agriculture and Agri-Food Canada, Human Research Ethics Committee
E-mail: **aafc.hrec-cerh.aac@agr.gc.ca**

Consent

By filling this form, I hereby consent to participate in this study. I agree that:

1. The study has been explained to me. **Yes** ☐ **No** ☐
2. All my questions were answered. **Yes** ☐ **No** ☐
3. Possible harm and discomforts and possible benefits (if any) of this study have been explained to me. **Yes** ☐ **No** ☐
4. I understand that I have the right not to participate and the right to stop at any time. **Yes** ☐ **No** ☐
5. I understand that I may refuse to participate without consequence. **Yes** ☐ **No** ☐
6. I have a choice of not answering any specific questions. **Yes** ☐ **No** ☐
7. I am free now, and in the future, to ask any questions about the study. **Yes** ☐ **No** ☐
8. I have been told that my personal information will be kept confidential. **Yes** ☐ **No** ☐
9. I understand that no information that would identify me will be released or printed without asking me first. **Yes** ☐ **No** ☐

By returning the questionnaire, you are giving consent to include your responses in the study:

Agree ☐ **Not agree** ☐

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