A COMPARATIVE STUDY OF CAMELINA, CANOLA AND HEMP SEED PROCESSING AND PRODUCTS

by

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A thesis submitted in conformity with the requirements for the degree of Master of Applied Science in the Department of Chemical Engineering and Applied Chemistry

University of Toronto

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ABSTRACT

Processes for the production of protein isolates from *Camelina sativa* and *Cannabis sativa* were developed by modifying the procedure used for *Brassica napus*. Due to the high concentration of mucilage in camelina, a water-to-seed ratio of 30 had to be used instead of the conventional ratio of 18. A rapid mucilage extraction process using hot, 55°C water was developed.

The final products were compared to the isolates made from Estonian rapeseed flour and canola. Recovery of the isolates was the highest from the Estonian rapeseed (67%), followed by hemp (65%), canola (29%) and camelina (22%). The hemp PPI had the highest protein concentration, 97%, and favourable colour, texture and flavour.

Camelina SPI and mucilage absorbed water and oil completely. Viscosity measurements of dried and redissolved mucilage showed the highest values at natural pH and the viscosity increased rapidly above 1% solids concentration.
Acknowledgements

I would like to express my sincere gratefulness to Professor Trass and Professor Diosady for the opportunity to work on this project, and for their guidance, advice and support during these years. I also want to thank Rein Otson Memorial Fellowship, whose financial support made my staying and working at University of Toronto possible.

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Those that I wish to thank foremost are my family. To my mother Marelli, my husband Aivar and my sons Märt, Mikk and Martin, thank you for your love and patience and your great support.

Finally, I would like to thank my Estonian friends in Canada, Eevi, Tiiu, Linda, Anne, Piret, Valdeko, Ilmar, Heiki and my mates in the Filiae Patriae Sorority and Toronto Estonian Academic Mixed Choir for making my staying in Toronto enjoyable and colourful.
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1.0 INTRODUCTION

Oilseed crops are grown throughout the world and the relatively high protein content (18-49%) of different solvent-extracted oilseed meals make them suitable sources of food grade vegetable protein. The main function of dietary protein is to supply amino acids for the growth and maintenance of body tissue. Digestion disassembles proteins into their basic building blocks: amino acids. The balance of amino acids found within the seed of oilseed crops compares favourably with that required for human nutrition. Given the increasing protein demand for protein due to population growth, oilseeds offer an alternative as a source of protein that is renewable and not animal-based. However, the difficulty with oilseed crops is the presence of several antinutritional and flavour components. These components include glucosinolates and their toxic breakdown products, phenolics and phytates which hinder the bioavailability of amino acids, proteins, and minerals (Naczk et al., 1998). These components are also responsible for the dark colour and bitter flavour of the products, so they must be removed or the amount substantially lowered before the oilseed meal can be considered as a protein source for human nutrition.

Consequently, a successful protein isolate is free of antinutritional substances, has a nutritious balance in terms of amino acid composition, is light in colour, and bland in flavour. In addition, to be commercially viable in the food industry, the protein isolates must also have good functional properties, such as the ability to absorb water or oil. Several methods have been studied in order to develop industrially relevant processes for producing human grade protein isolates from crops. Tzeng et al. (1990) successfully developed a process for canola which yields three products: a precipitated protein isolate, a soluble protein isolate, and a meal residue. The protein isolates are high in protein, light in colour, bland in taste, free of glucosinolates, and low in phytates. This procedure has since been adapted and
modified for different canola seed varieties as well as yellow and oriental mustard. The procedures involve oil extraction, alkaline extraction of protein, membrane processes for purification, isoelectric precipitation and drying.

The first objective of current research is to develop the procedures for obtaining precipitated and soluble protein isolates from Camelina sativa and Cannabis sativa and evaluation of these products. Working with camelina and hemp, the differences between precipitation profiles due to the dissimilar isoelectric points of different types of proteins must be taken into consideration. Hence, current work determined the influence of pH on protein extractability and precipitability of two novel oilseeds to get the optimal pH values for the extraction and precipitation and then using the membrane system for concentration and purification of protein isolates. Although Camelina sativa is a member of the Brassica family, it has one significant difference from the others. That is the high content of polysaccharides or mucilage, which makes it similar to flax, Linum usitatissimum and complicates the aqueous processing. However, several authors have emphasized the importance of plant based polymers in the pharmaceutical industry as well as this in use as suspending agents, thickeners and stabilizers in the food industry. Therefore, the second objective of this study is to develop the procedure for separating the layer of polysaccharides from whole intact camelina seeds, prior to the production of protein isolates, and investigation of the material. The third objective of current research is the comparison of camelina and hemp isolates to those from Estonian rapeseed and canola.
2.0 LITERATURE REVIEW

2.1 Oilseeds

Oilseeds store their oil reserves in oil bodies which consist of a triacylglycerol core surrounded by a phospholipid layer and an outer shell of strongly bound oleosin protein (Figure 1). The phospholipids enable the association of the hydrophobic oil and the hydrophilic protein. The oleosin protein layer stabilizes the oil bodies during periods of drought and avoids coalescence of the oil bodies (Huang, 1992).

![Oil Bodies in Brassica Seed. (Huang, 1992)](image)

The oilseeds are one of the more common sources of non-meat based protein on the market today; therefore, they can be used for the production of protein-rich food products.

2.1.1 Canola

Rapeseed is the collective term for the species Brassica napus and Brassica rapa, long cultivated in northern climates (Appelqvist 1971). In 1978, the Western
Canadian Oilseed Crushers Association registered rapeseed varieties with the name "canola" for marketing reasons. Canola contains less than 2% erucic acid in its fatty acid profile and the solid component contains less than 30 µM of any mixture of glucosinolates per gram of air-dry, oil-free solid (Canola Council of Canada, 2014). In the current study canola is used as a comparative material, as it has been previously used for production of protein isolates.

2.1.2 Camelina sativa

Camelina (Camelina sativa (L.) Crantz) is a relict oilseed crop of the Brassicaceae family with centres of origin in southeastern Europe and southwestern Asia. C. sativa was cultivated in Europe as an important oilseed crop for many centuries before being displaced by higher-yielding crops such as canola (Brassica napus) and wheat. Similarly to rapeseed and mustard, it is grown for the seed, for the production of oil. The main peculiarity of camelina is the higher content of omega-3 fatty acids compared to the other conventional oilseeds. Currently, Camelina sativa is cultivated on a small scale in Canada, the United States, Slovenia, and Italy. Current acreage devoted to camelina in Canada is approximately 50,000 acres. The Camelina Association of Canada projects Canada could have 1 to 3 million acres planted in the future. Several factors encourage the spread of camelina in Canada. Camelina has several agrotechnical benefits: cultivation of the crop is simple and environmentally friendly; application of pesticides/herbicides is not needed; the plant is adaptable to marginal soils, showing good productivity, and may be a suitable candidate for biofuel production in marginal environments (Zubr, 2010). Camelina, a drought resistant seed is adapted to cooler Northern climates. Camelina is an early maturing crop. The seeds are a pale yellowish brown color and are quite small. It is a Fall or Spring- planted annual oil
crop species. It was reported that camelina seeds contain polysaccharides, which show good water-binding capacity and are capable of aiding seed germination in dry environments (Grady and Tandive, 2010).

In general, camelina contains 29.9% to 38.3% oil, 23% to 30% protein, 27% carbohydrates, 6.6% ash and 5% moisture depending on the variety and variations of soil composition and environment (Budin et al., 1995).

### 2.1.3 *Cannabis sativa*

Botanically, hemp is classified as *Cannabis sativa* L. (Cannabaceae). Hemp grows in a range of soils, but preferably in well-drained, non-acidic soil, rich in nitrogen. Hemp can be grown without fungicides/herbicides and pesticides, it absorbs carbon dioxide five times more efficiently than the same acreage of forest and it matures in three to four months (Agriculture and Agri-Food Canada, 2013).

The breeding in Europe of industrial hemp varieties with a low THC (tetrahydrocannabinol) content, less than 0.3%, allowed the commercial production of hemp (*Cannabis sativa* L.) in Canada in 1998 after about 60 years (House et al., 2010). In 2013 there were 66,671 acres licensed for cultivation of hemp from coast to coast.

Unlike flax, wheat, corn, canola, and other major cultivated species, hemp was originally grown primarily for its fibre. However, it has been also used in food products. Hemp seeds are of high value with approximately 25–35% lipids, 20–25% proteins, 20–30% carbohydrate, 10–15% insoluble fibers and numerous natural source minerals (Oomah et al., 2002).

#### 2.1.3.1 Finola

In the current study the hemp cultivar Finola is used in experiments. Finola is an
especially well adapted cultivar for seed production in northern climes (Callaway 2004).

As an industrial source of vegetable nutrition, both hempseed and hempseed meal are rich sources of protein and polyunsaturated oils, in addition to considerable amounts of vitamins and useful minerals (Table 1).

<table>
<thead>
<tr>
<th></th>
<th>Whole seed</th>
<th>Pressed Seed meal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oil (%)</td>
<td>35,5</td>
<td>11,1</td>
</tr>
<tr>
<td>Protein</td>
<td>24,8</td>
<td>33,5</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>27,6</td>
<td>42,6</td>
</tr>
<tr>
<td>Moisture</td>
<td>6,5</td>
<td>5,6</td>
</tr>
<tr>
<td>Ash</td>
<td>5,6</td>
<td>7,2</td>
</tr>
<tr>
<td>Energy (kJ/100g)</td>
<td>2200</td>
<td>1700</td>
</tr>
<tr>
<td>Total dietary fiber (%)</td>
<td>27,6</td>
<td>42,6</td>
</tr>
<tr>
<td>Digestible fiber</td>
<td>5,4</td>
<td>16,4</td>
</tr>
<tr>
<td>Non-digestible fiber</td>
<td>22,2</td>
<td>26,2</td>
</tr>
</tbody>
</table>

Table 1: Typical Nutritional Content (%) of Finola (Callaway, 2004)

Finola produces more seed than any other hemp variety to date, over 2000 kg of seed per hectare under good agronomic conditions. The protein content and amino acid profile of Finola is not significantly different from other varieties of hemp, but the fatty acid profile has considerably more γ-linolenic acid (GLA) and stearidonic acid – an Ω-3 fatty acid (SDA) - than other varieties (Kriese et al., 2004).

2.2 Components in Oilseeds

2.2.1 Oil

*Camelina sativa* and *Cannabis sativa* with their large content of oil (30-38%) have gained renewed interest for their potential for biodiesel production. Camelina oil contains up to 90% unsaturated fatty acid, of which approximately 38 % is α-linolenic acid (omega-3), which is lower than flaxseed (55 %) but exceeds hemp (*Cannabis sativa*).
Earlier, an optimal Ω6/Ω3 ratio in oil was considered to be somewhere between 5:1 and 10:1 (WHO & FAO, 1995), which is similar to the ratio found in soybean oil (about 7:1). Recently suggested optimal Ω6/Ω3 balance would be somewhere between 2:1 and 3:1 (Simopoulos et al. 2008), which reflects the ratio found in the traditional Japanese and Mediterranean diets, where the incidence of coronary heart disease has been historically low. The Ω6/Ω3 ratio in most commercial hempseed oils is typically near 2.5:1 (Table 2) (Callaway et al., 2004; Kriese et al., 2004). The same rationale has been applied to rapeseed oil (Brassica napus), which has a Ω6/Ω3 ratio of about 2:1 (Callaway, 2004). Novel considerations suggest fortifying food with omega-3 fatty acid supplements. For that flax (Linum usitatissimum) and camelina oils can be used. Flax and camelina oil consist of approximately 55% and 38% linolenic fatty acids, respectively, which makes them richer than most fish oils in omega-3 fatty acids (Bartram, 2002). Camelina oil has been investigated as a sustainable lipid source to fully replace fish oil in diets for farmed Atlantic salmon, rainbow trout, and Atlantic cod (Hixson et al., 2014). The high levels of omega-3 fatty acids, vitamin E (110mg/100 g) and antioxidants such as tocopherols have been the focus of potential health benefits of camelina oil.

The most abundant fatty acids in camelina, canola and hemp are oleic (18.7 %, 60.6 % and 11.5 %); linoleic (16.0 %, 19.1 % and 58.5 %); linolenic (38.1%, 10.6 % and 22 %), respectively. Camelina sativa contains also high amount of gadoleic acid (11.6%). Linolenic and linoleic fatty acids are known as essential fatty acids (EFAs), because humans cannot produce them themselves, and must obtain them in their diet. Rich content of linoleic and linolenic acids, in the seed oils of Camelina sativa and Cannabis sativa makes them superior over other vegetable oils also in terms of proportions of polyunsaturated fatty acids (PUFAs).

The natural dark colour of hempseed oil is from chlorophyll within the mature seed,
which can hasten auto-oxidation of oil that is exposed to light. The oil from hemp seeds polymerizes through the double bonds, called “drying”, to become solid on exposure to air, similar to flax oil and, therefore, it is sometimes used in the manufacture of oil-based paints and in plastics. In contrast, the high stability of Camelina sativa oil was confirmed by the experiment of Jaskiewicz and Sagan (2003). Neither a three-month storage nor extrusion had any significant effect on the fatty acids in the Camelina sativa seeds.

<table>
<thead>
<tr>
<th></th>
<th>C16:0</th>
<th>C18:0</th>
<th>C18:1</th>
<th>C18:2</th>
<th>C18:3</th>
<th>C20:0</th>
<th>C20:1</th>
<th>C22:0</th>
<th>C22:1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Seed</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Camelina sativa</td>
<td>5.3</td>
<td>3.0</td>
<td>18.7</td>
<td>16.0</td>
<td>38.1</td>
<td>1.4</td>
<td>11.6</td>
<td>3.4</td>
<td>2.5</td>
</tr>
<tr>
<td>Canola</td>
<td>3.9</td>
<td>1.9</td>
<td>60.6</td>
<td>19.1</td>
<td>10.6</td>
<td>2</td>
<td>1.4</td>
<td>0.4</td>
<td>0.1</td>
</tr>
<tr>
<td>Cannabis sativa</td>
<td>5.4</td>
<td>1.6</td>
<td>11.5</td>
<td>58.5</td>
<td>22</td>
<td>0.8</td>
<td>0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sinapis alba</td>
<td>2.7</td>
<td>1.0</td>
<td>23.6</td>
<td>9.5</td>
<td>10.8</td>
<td>0.7</td>
<td>10.5</td>
<td>0.5</td>
<td>36.6</td>
</tr>
</tbody>
</table>

Table 2: Fatty Acid Composition of Oilseeds

Percentage of total fatty acids including: palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2) (omega-6), linolenic (18:3) (omega-3), arachidic (C20:0), gadoleic (C20:1), behenic (C22:0), erucic (C22:1) Data compiled from publications: Fatty Acid Composition and Seed Meal Characteristics of Brassica and Allied Genera, Binay Singh et al., 2014; Fatty acid composition and oxidation stability of hemp (Cannabis sativa L.) seed oil extracted by supercritical carbon dioxide, C. Da Porto et al., 2012; and Quality of Western Canadian Canola 2002, D. R DeClercq and J. K. Daun, 2002

Health Canada has approved camelina oil as a food in Canada (Saskatchewan Ministry of Agriculture, 2012). With almond-like flavour and aroma, camelina oil can be used as a cooking oil, in salad dressings, and in spreads and margarines.
Hempseed oil is primarily used as a food oil and dietary supplement. While camelina oil is able to withstand frying temperatures, it is not recommended it to be heated for prolonged periods of time. However, hempseed oil has a relatively low smoke point and is not suitable for frying (Yen et al., 1997).

### 2.2.2 Proteins

Different sources of protein are composed of different combinations of amino acids. Some of these amino acids can be synthesized by living organisms from other nitrogenous material. Other amino acids essential to the diet cannot be synthesized \textit{in vivo} but must be ingested as such. Isoleucine, Leucine, Lysine, Methionine, Phenylalanine, Threonine, Tryptophan and Valine (with Histidine for infants) are known as the essential amino acids and must all be provided by the food eaten.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Histidine</td>
<td>15</td>
<td>28</td>
<td>31</td>
<td>24</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>30</td>
<td>39</td>
<td>43</td>
<td>38</td>
</tr>
<tr>
<td>Leucine</td>
<td>59</td>
<td>69</td>
<td>71</td>
<td>64</td>
</tr>
<tr>
<td>Lysine</td>
<td>45</td>
<td>41</td>
<td>56</td>
<td>49</td>
</tr>
<tr>
<td>Methionine + Cysteine</td>
<td>22</td>
<td>40</td>
<td>44</td>
<td>52</td>
</tr>
<tr>
<td>Phenylalanine + Tyrosine</td>
<td>30</td>
<td>81</td>
<td>71</td>
<td>63</td>
</tr>
<tr>
<td>Threonine</td>
<td>23</td>
<td>35</td>
<td>44</td>
<td>37</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>6</td>
<td>8</td>
<td>13</td>
<td>12</td>
</tr>
<tr>
<td>Valine</td>
<td>39</td>
<td>51</td>
<td>55</td>
<td>52</td>
</tr>
</tbody>
</table>

Table 3: Literature Values of Amino Acid Contents in Hemp, Rapeseed and Camelina in mg/g protein.

As shown in Table 3, the proteins of the oilseeds hemp (\textit{Cannabis sativa}), rapeseed \textit{Brassica napus}, and \textit{Camelina sativa} contain all essential amino acids and possess
a well-balanced amino acid composition with respect to human requirements. The percentage of essential amino acids in camelina is slightly lower than in canola protein (42%) (Li et al., 2011). Lysine is the first limiting amino acid in all hemp products and grains.

According to “Report of the Joint FAO/WHO Expert Consultation”, the gold standard for measuring protein quality, since 1990, is the Protein Digestibility Corrected Amino Acid Score (PDCAAS) and by this criterion soy protein is the nutritional equivalent of meat, eggs, and casein for human growth and health (Schaafsma, 2000).

<table>
<thead>
<tr>
<th>protein source</th>
<th>PDCAAS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>100</td>
</tr>
<tr>
<td>egg white</td>
<td>100</td>
</tr>
<tr>
<td>whey (milk protein)</td>
<td>100</td>
</tr>
<tr>
<td>soy protein isolate</td>
<td>100</td>
</tr>
<tr>
<td>Mycoprotein</td>
<td>99</td>
</tr>
<tr>
<td>Beef</td>
<td>92</td>
</tr>
<tr>
<td>rapeseed protein isolate</td>
<td>83</td>
</tr>
<tr>
<td>Chickpeas</td>
<td>78</td>
</tr>
<tr>
<td>Fruit</td>
<td>76</td>
</tr>
<tr>
<td>Vegetables</td>
<td>73</td>
</tr>
<tr>
<td>other legumes</td>
<td>70</td>
</tr>
<tr>
<td>dehulled hemp seed</td>
<td>61</td>
</tr>
<tr>
<td>hemp seed</td>
<td>51</td>
</tr>
<tr>
<td>hemp seed meal</td>
<td>48</td>
</tr>
<tr>
<td>whole wheat</td>
<td>40</td>
</tr>
<tr>
<td>Almond</td>
<td>23</td>
</tr>
</tbody>
</table>

Table 4: Protein Digestibility Corrected Amino Acid Score (PDCAAS) Values of Some Sources of Protein

Removal of the hull fraction improves protein digestibility and the resultant PDCAAS value. The current results provide evidence that hemp and rapeseed proteins have a PDCAAS equal to or greater than certain grains, nuts, and some pulses (House et al., 2010). Unfortunately, the value of PDCAAS for camelina was not calculated at the time of writing this thesis.

The proteins from rapeseed and camelina are essentially equivalent in quality to soy.
The meal of camelina is high in protein (38-43%) similar to other crucifer oilseeds and a good feed protein source. The predominant proteins of camelina are seed storage proteins and oil body proteins (OBP), which could be useful in protein-based bioproduct development. The average content of crude protein in camelina meal is 457g/kg dry matter which is higher than that of canola 367 (Slominski, 2015) and slightly lower than the value (499) reported for soybean meal. Camelina proteins are characterized by high content of glutamate, aspartate, leucine, arginine, and phenylalanine but low content of methionine, histidine, and tyrosine. Nutritionally, camelina proteins contain approximately 40% essential amino acids that cannot be synthesized by human and many farm animals, and approximately 60% nonessential amino acids that can be produced in humans and animals. Among the amino acids, alanine, methionine, phenylalanine, isoleucine, and leucine belong to the hydrophobic group and account for 26.3% to 28.4% of camelina protein fractions. Hydrophobic properties of camelina proteins are comparable to canola protein (26%) but lower than soy protein (37%) (Li et al., 2016).

Hemp seeds contain approximately 20–25% proteins and the two main proteins in hemp seeds are edestin and albumin (Callaway, 2004). Although the hemp protein isolate (HPI) shows much poorer functional properties, especially protein solubility, HPI hasn’t been shown to be an allergen like soybean protein isolate. Moreover, the proportion of essential amino acids to the total amino acids for HPI is significantly higher than that of soy protein isolates. These results suggest that the protein isolates from hempseed are much more nutritional in amino acid nutrition and easily digestible than soy protein isolates, and can be utilized as a good source of protein nutrition for human consumption (Wang et al., 2009).

Rapeseed has been more thoroughly studied than camelina and has been shown to have over 45 different proteins: 20 weakly acidic, approximately 20 neutral, and 5 basic. Most of the proteins are in the range 120-150 kDa with ~5% in the 50-75 kDa range.
region. Rapeseed proteins are known to have structural, catalytic and storage functions. The major storage proteins in rapeseed are the 12S globulin (cruciferin) and the 2S albumin (napin), making up more than 70% of total rapeseed proteins. The 2S proteins have been identified as potential allergens.

### 2.2.3 Mucilage

The epidermal cells of the seed coat of certain species accumulate polysaccharides during seed development. The formation of seed mucilage, termed myxospermy, has been noted for seeds from a range of plant species belonging to at least 100 families (Western, 2012). Mucilage production can vary between seeds produced by the same plant (North et al., 2014). The polysaccharides have high water-binding capacity so that when hydrated on imbibition, they expand, fragmenting the outer distal cell wall of the epidermal cells and encapsulating the seed with viscous, sticky mucilage. The cellulosic mucilage of Brassicaceae seeds is divided into an outer diffuse layer and an inner adherent layer (Western, 2012).

Mucilage, a class of polysaccharides, are high molecular weight (200 kDa and more) biopolymers which commonly occur in higher plants (Ebrahimzadeh, 2000) and this class of natural products has received much attention since it is of great importance in industry and medicine. Polysaccharides, among other polymers, are frequently used in drug formulations as binding agents, viscosity increasing agents, coating agents or as active ingredients (Vanlaeke et al., 1989) and in food industries as suspending agents, thickeners and stabilizers (Cottrell and Baird, 1980). The studies have shown that the water-soluble yellow mustard mucilage exhibits strong antioxidant properties, compared to pectin and xanthan gum (Wu et al., 2016). The proportionally high content of mucilage, crude fibre and lignin indicates that Camelina sativa meal, when incorporated in food, can exert positive effects on gastrointestinal
processes. A long term human consumption of bread with added *Camelina sativa*

Figure 2 Natural Variation in Seed Coat and Mucilage Characteristic (North et al., 2014). Camelinae tribe: (A, E, I, M) Arabidopsis thaliana; (B, F, J, N) Arabidopsis lyrata; (C, G, K, O) Camelina sativa; and Linaceae family (D, H, L, P) Linum strictum ssp. strictum. Images in (E–H) and (M–P) are magnifications of regions in (A–D) and (I–L), respectively. M, mucilage.

meal confirmed that beneficial role of those ingredients in digestion (Zubr 2010).

The study of Torti et al. (2016) demonstrates that phosphate alternatives, such as
plant-based polysaccharides, are capable of helping retain water in shrimp under commercially relevant conditions. Additionally, there are some polysaccharides that will likely not change the way the shrimp tastes, feels, or looks to the consumer. Moreover, most polysaccharides used at the inclusion levels are less expensive than customarily used phosphates.

<table>
<thead>
<tr>
<th>Camelina gum</th>
<th>Other gums</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flax seed</td>
<td>Yellow mustard</td>
</tr>
<tr>
<td>Total protein (%)</td>
<td>12.3</td>
</tr>
<tr>
<td>Total sugar (%)</td>
<td>75.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Relative monosaccharide composition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylose</td>
</tr>
<tr>
<td>Galactose</td>
</tr>
<tr>
<td>Glucose</td>
</tr>
<tr>
<td>Rhamnose</td>
</tr>
<tr>
<td>Fucose</td>
</tr>
<tr>
<td>Arabinose</td>
</tr>
<tr>
<td>Mannose</td>
</tr>
</tbody>
</table>

Table 5 Comparison of analytical data of camelina gum and other gums. Cui (2000)

The highest amount of polysaccharides among oilseeds is contained in *Linum usitatissimum* (8%) and *Camelina sativa* (6.4-7.5%), followed by *Sinapis alba* (5%). Table 5 demonstrates the composition of camelina compared to the other gums. The mucilage content of canola and rapeseeds grown in Canada is marginal (0.6% and 2.8%), depending on variety and growing location. However, the interfacial tension is responsible for the formation of a stable salad dressing incorporating canola mucilage as emulsifier (Daun, 2015).

Camelina seed coat epidermal cells have a unique morphology dominated by the presence of an intracellular volcano-shaped structure known as the columella and thin radial cell walls (Fig.2). Moreover, camelina has a large and dense halo of cellulose staining at the periphery of the adherent mucilage (Fig. 2K, O), at the same time Linaceae, *Linum strictum*, shows cellulose staining (Fig. 2L, P) without rays (North et al., 2014).
2.2.4 Antinutritional Components

The hinderance to full utilization of Brassicaceae, including *Brassica napus* and *Camelina sativa*, is the high level of phytates, phenolics and the glucosinolates (Naczk et al., 1998). These natural chemicals most likely contribute to plant defence against pests and diseases. In addition, the high amount of indigestible fibre, mainly in the hull, makes it problematic for food use. These toxic and antinutritional substances must be removed, or substantially lowered, before the oilseed meal can be considered as a protein ingredient for human consumption.

Although the presence of certain antinutrients may limit their conversion into edible-grade products and utilization in human nutrition as they influence protein digestibility, organoleptic properties, and bioavailability of macro- and microelements, in recent decades an increasing trend of reutilization of plant by-products in food, cosmetic, and pharmaceutical industries has been observed. Moreover, apart from the well-known harmful effects of antinutrients, certain health-promoting and disease-preventing properties also have been attributed to them. A preventive impact of phytic acid, phenolics, saponins, protease inhibitors, phytoestrogens, and lignans on different diseases has been demonstrated (Jonnalagadda, 2011). So, the best solution would be the isolation of proteins and further separate treatment of antinutritionals, in order to get value-added products from both.

### 2.2.4.1 Glucosinolates

The glucosinolates, the natural components of pungent plants from Brassicaceae family, belong to the class of organic compounds glucosides. The glucosinolate composition of any Brassica seed is a combination of different glucosinolates, with one type as a main form.
The pungency of Brassicaceae plants is due to breakdown products (isothiocyanates, thiocyanates and nitriles) produced from glucosinolates when the plant material is chewed, cut or otherwise damaged. The presence of glucosinolate degradation products can result in unwanted colour, odour and taste.

However, the lower level of glucosinolates content has been reported to have positive effect on health. Glucosinolates level of 0.61µmol/g in broccoli can be linked to reduced cancer risk (Song and Thornalley, 2007) inducing detoxification enzymes, inhibiting enzyme activation, modifying steroid hormone metabolism, and generally improving the host-defense system.

The specific glucosinolates present in the seed vary according to plant species (Table 6). Camelina sativa contains relatively small amount of (18.6 µmol/g) and three different types of glucosinolates (Matthäus & Angelini, 2005).

<table>
<thead>
<tr>
<th>Seed</th>
<th>Main Glucosinolate</th>
<th>Minor Glucosinolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Brassica napus)</td>
<td>3-butenyl-4-pentenyl-</td>
<td>4-methyl thiobutyl-, 5-methyl thiopentyl, 4-methylsulfinylbutyl-, 5-ethylsulfinylpentyl-, 2-hydroxy-3-butenyl-, 2-hydroxy-4-pentenyl-, 2-phenylthyl-</td>
</tr>
<tr>
<td>Camelina sativa</td>
<td>10-methylsulfinyldecyl-</td>
<td>9-methylsulfinylnonyl-, 11-methylsulfinylandecyl-</td>
</tr>
</tbody>
</table>

Table 6 Glucosinolates Found in Rapeseed and Camelina Seed
These glucosinolates are glucocamelinin (10-methylsulfinyldecyl-Gls ), which is about 60% of the total glucosinolates, while 9-methylsulfinylnonyl- and 11-methylsulfinyl-undecyl- Gls come to about 30% and 10%, respectively (Matthäus & Angelini, 2005). The content of glucosinolates found in camelina is comparable to this amount found in rapeseed.

2.2.4.2 Phenolics

Phenolic acids and tannins represent a wide and diverse group of secondary plant products, which can be found in a wide range of plant species. Phenolic compounds are divided based on their chemical structures into non-flavonoid and flavonoid compounds. Phenolic compounds consist of at least one aromatic ring, at least one hydroxyl group, and several side branches.

![Figure 4 Chemical Structure of Sinapic Acid](image)

Many phenolic compounds play an active role in plant reproduction, growth and protection.

Most commonly, the association of the phenolic compounds with carbohydrates and lipids imparts antioxidant properties to these major food components. However, the main cause of bitter taste and dark colour in oilseed meal and extracted protein products are quinones that form on oxidation, as well as phenolic acid esters, mainly sinapate esters making them less palatable. The presence of sinapine in the
feed of hens at certain levels results in a fishy odour or taste in eggs (Russo, 2012). Nevertheless, the primary anti-nutritional behaviour of phenolics is their ability to form complexes with essential amino acids which prevents the amino acids from being assimilated by the body. The chemical nature of phenolic compounds allows them to interact with other food components through hydrogen bonding, covalent bonding, hydrophobic interactions and ionic bonding (Xu, 2000). Moreover, the association of phenolic compounds with proteins may affect functional properties (e.g. gelling properties, emulsification, and water holding capacity) and biological properties of the protein. Condensed tannins act as antinutrient compounds of plant origin because they precipitate proteins, inhibit digestive enzyme and decrease the utilization of vitamins and minerals (Amarowicz, 2010).

*Brassica napus* and *Camelina sativa* are potential rich sources of naturally occurring phenolic compounds such as sinapic acid and chlorogenic acid derivatives. Although there is limited information of polyphenol compounds in defatted hemp seed cake extract, three types of compounds, caffeic acid, quercetin and luteolin in defatted hemp seed cake, were detected. Quercetin appeared to have the highest concentration, followed by luteolin, while caffeic acid had the lowest concentration in defatted hemp seed cake extract (Teh et al., 2014). Rapeseed contains more phenolic compounds than most of the other oilseeds (Naszk et al., 1998). The predominant phenolic compounds in seeds of oilseed rape are sinapate esters 99%, with sinapoylcholine (sinapine) being the most prominent one, followed by sinapoylglycose (Amarowicz et al., 2010). The concentration of phenolic esters in rapeseed has been reported to be about 30 times higher than in soybean. The total content of phenolic acids in rapeseed meals is up to 1840mg/100g sample (Nazck et al, 1998). The sinapine content that ranged from 158 mg/100g to 293mg/100g in *Camelina sativa* can be considered low in comparison with other members of Brassicaceae (Matthäus, 1997). The condensed tannin content in
different camelina genotypes is quite high, ranging from 150 to 300 mg/100g of seed. A unique membrane-based process has been developed including the pretreatment of canola extract with low concentration of NaCl to reduce phenolic acids in the products by 80% and tannins by 90% (Xu, 2000).

2.2.4.3 Phytates

Although, the phytates are not toxic, when these compounds are consumed by animals or humans, they pose some health concerns. Due to the six phosphate groups, which act as twelve acid groups, the chelating power of phytic acid is very high. Phytic acid forms complexes with minerals and proteins, renders them insoluble and biologically unavailable.

![Chemical Structure of Phytic Acid](image)

**Figure 5 Chemical Structure of Phytic Acid**

*Camelina sativa, Brassica napus* and especially *Cannabis sativa* are characterized by a relatively high phytic acid content. Canola meal contains between 5% and 6% phytates, levels much higher than typical oilseeds. In camelina the content of phytic acid is approximately 2.5%, which is higher than the content of phytic acid in soybeans, 0.5% (Kwanyuen & Burton, 2005). The high level of phytic acid in hempseed meal, 6.3% -7.5%, is a major concern when hemp seed meal is fed to
monogastric animals lacking of the phytase enzyme (Russo and Reggiani, 2015), as it could cause important nutritionally deficiencies, especially for iron and zinc.

2.3 Production of Protein Isolates

To use oilseed protein as a source of food-grade material, the content of antinutritional substances must be substantially lowered and the proteins isolated. Therefore, the development of proper seed protein purification technology has been an ongoing challenge.

Through the decades the different methods for isolation of the protein from undesirable components have been experimented with. The technologies include leaching (Sosulski et al., 1972); using multi-phase solvent extraction systems to reduce the content of glucosinolates (Naczk et al., 1985) or utilization of organic solvents, such as ethanol, methanol and acetone (Mawson et al., 1995) and enzymes like pectinase, protease and hemicellulase (Jensen et al., 1990). Also applying the heat treatment for removing antinutritional substances has been reported (Jensen et al., 1990).

A majority of studies were based on proteins extracted from the meal using alkaline solution, presumably due to its high nitrogen yield. Alkaline extraction using sodium hydroxide solution followed by precipitation with dilute acid is the most typical procedure used in preparation of canola protein isolates (Aluko et al., 2001). Protein content of isolates prepared by alkaline extraction is mostly in the range of 70 to 90% (Aluko et al., 2001; Ghodsvali et al., 2005), although isolates with protein recovery more than 90% have also been reported (Tzeng et al., 1988). The reported procedures, however, have had slight differences in the pH of extraction, type of acid and pH for protein precipitation.
Generally, the alkaline solution was first added to the defatted canola meal and stirred or shaken to solubilize the proteins. The mixture was then centrifuged and the pH of the supernatant adjusted by dilute acid to precipitate the proteins. Precipitated proteins were then separated by centrifugation and the precipitate was freeze-dried. High pH (11 and 12) is necessary to obtain high nitrogen extraction yield and high protein extraction rate from canola meal. To increase the yield, multistage extraction has been employed to achieve extractability >90%. The 4-stage countercurrent extraction process (Blaicher et al., 1983) for protein extraction from hexane defatted meal delivered an overall extractability of 94%, the highest ever reported. An alkaline extract of canola protein, however, had an unappetizing dark brown color. The addition of 2% sodium sulphite reduced significantly the color of the final product and didn’t affect the quality of the protein in terms of amino acid composition (Keshavarz et al., 1977). The heated commercial meal was compared to laboratory-prepared canola meal by Gillberg & Tönnell, (1976) and Tzeng et al., (1988).

Low nitrogen extractability in industrially-processed meal (58.1%) compared to high nitrogen extractability in laboratory-prepared canola meal was generated by protein denaturation during heating (Xu, 1999).

The adjustment of the pH of the extract’s supernatant to the isoelectric point is normally carried out by using dilute acid solutions. Adjustment of pH to 3.5 by using acetic acid was suggested by Klockeman et al. (1997) or hydrochloric acid suggested by Tzeng et al. (1990). In some studies (El Nockrashy et al., 1977) more than one isoelectric point was reported. Addition of CaCl₂ prior to (Tzeng et al., 1990; Ghodsvali et al., 2005) or after (Aluko et al., 2001 and 2005) the pH adjustment for isoelectric protein precipitation has been reported to produce low phytate Ca-precipitated protein isolates. In this case the percentage of soluble protein also increased to approximately 80% of the total...
<table>
<thead>
<tr>
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<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkali extraction</td>
<td>10(w/v) of solution of 0.1 M NaOH, 20 min stirred at 23°C, CaCl₂ added</td>
<td>10(w/v) of solution of 0.2% NaOH, 1h, pH10,11,12 twice</td>
<td>5% (w/v) extraction with 0.4%(w/v) NaOH, orbital shaker 180 to 200 rpm, 60min</td>
<td>5% NaOH, R= 18, pH9.5, 10, 10.5, 11, 11.5, 12</td>
<td>1.0%(w/v aqueous SHMP pH9, (or NaOH pH 11), R=18, 30 min</td>
<td>NaOH solution R= 18, pH 11 or 12 1% Na₂SO₃ added</td>
</tr>
<tr>
<td>1st centrifugation</td>
<td>10000 ×g, 30 min, 8°C</td>
<td>8000 rpm, 25 min</td>
<td>3000 ×g, 20 min, 5 to 10°C</td>
<td>5000 rpm, 15 min</td>
<td>4080 ×g, 10 min, 5°C</td>
<td>4080 ×g, 10 min, 5°C</td>
</tr>
<tr>
<td>Filtration</td>
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<td>no</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>pH adjustment by acid</td>
<td>pH 4.0, by 0.1 M HCl</td>
<td>pH 2.5 to 6.0 in 0.5 increments by 0.5 N HCl</td>
<td>pH 3.5, by acetic acid</td>
<td>pH 3.5 to 7.5 in 0.5 increments by 6 N HCl</td>
<td>pH 3.5, by 6 N HCl solution</td>
<td>pH 3.5, by 6 N HCl solution</td>
</tr>
<tr>
<td>2nd centrifugation</td>
<td>10000 ×g, 30 min, 8°C</td>
<td>8000 rpm, 25 min</td>
<td>3000 ×g, 20 min, 5 to 10°C</td>
<td>5000 rpm, 20 min</td>
<td>Centrifuged, but no details given</td>
<td>Centrifuged, but no details given</td>
</tr>
<tr>
<td>Washing</td>
<td>Washed (200 volumes of Milli-Q water) to remove salt</td>
<td>Precipitates were mixed</td>
<td>Precipitate was washed by distilled deionized water</td>
<td>Filtration, washed with distilled water, R= 10, repeated twice filtration,</td>
<td>Filtration, washed (10 volumes of acidic water pH 3.5), shaking, 2 h</td>
<td>Filtration, washed (10 vol. of acidic water, pH 3.5)</td>
</tr>
<tr>
<td></td>
<td>Centrifuged 10000 ×g, 30 min, 8°C</td>
<td></td>
<td></td>
<td>Centrifuged 3000 ×g, 20 min 5 to 10 °C, repeated 3 times</td>
<td>Centrifuged, repeated 3 times</td>
<td></td>
</tr>
<tr>
<td>Drying method</td>
<td>Freeze-dried</td>
<td>Freeze-dried</td>
<td>Freeze-dried</td>
<td>Freeze-dried</td>
<td>Freeze-dried</td>
<td>Freeze-dried</td>
</tr>
<tr>
<td>Collecting SPI</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

**Table 7 Summary of Alkaline Extraction Procedures for Canola Meal Proteins (Tan et al., 2011).**

SHMP = sodium hexametaphosphate. R = solvent to meal ratio. CF = concentration factor, for example, 10, meaning 100 g of protein solution was concentrated to 10 g by ultrafiltration. DV = diavolume, for example, 5 meaning 100 g of sample was diafiltered with 500 g water. SPI = soluble protein isolate (Tan et al., 2011).
yield but at the same time, precipitated protein decreased to about 20%.
This high yield is due to the “salting in” effect (Tzeng et al., 1990).
The conditions for extraction depend largely upon the proteins of the particular
species and varieties. However, characteristics of the protein fractions, such as
nitrogen yield, molecular weight profile, isoelectric point, solubility, and thermal
properties were found to be largely related to the extraction methods (Table 7).

2.3.1 Removal of Mucilage

Due to the high viscosity of solutions of mucilaginous herbs, the aqueous processes
are hindered; therefore, removing the mucilage beforehand is necessary. Moreover,
as described in 2.2.3, the recovered mucilage can be used as a product on its own.
The previous studies have demonstrated that the mucilage occurs in the seed coat
and is readily extracted with hot and less readily with cold water (Paynel, 2013).
In different studies the mucilage has been removed from the seeds by water
extraction of whole intact seed or by extraction from the hulls by Cui et al. (2000).
Balke (2006) in his research with yellow mustard developed the technique for
mucilage removal on 1 L and 5 L scales. In 2-stage extraction he used a magnetic
stirrer or a small propeller stirrer to mix the seed at a water-to-seed ratio of
8 for 3 hours. He experimented with different temperatures and achieved the best
results with 45°C. After mixing, he drained the seed using a hand sieve and collected
the liquid. For additional separation, he briefly washed the seed and then used a
basket centrifuge. The mucilage was recovered from the extract via precipitation into
with at least twice the volume of ethanol used.
2.3.2 Oil Extraction

The quality of the seed meal is especially influenced by the oil processing conditions. The oldest method of oil production from seeds and fruits is cold or hot pressing. A certain level of heating during processing is needed for ensuring inactivation of enzymes, such as myrosinase, lipase and lipoxygenase, to avoid the formation of antinutritional compounds during oil extraction and to use the defatted meal. Heat treatment improves the malleability of the seed, allows increasing oil yield, thus raising the economic return of the processing. However, the intense pressure and heat can damage the seed protein. Thus, the process of oil extraction may significantly affect the further production of protein products due to potential thermal denaturation of the proteins (Johnson & Lusas, 1983).

To avoid the decrease of extractability and functionality, alternative extraction procedures, based on different solvents, such as hexane, acetone, ethyl and isopropyl alcohol (IPA), or aqueous processing are used. Although hexane extraction allows very thorough recovery of oil from seeds, IPA is the most promising alternative solvent due to its low toxicity, acceptable cost and excellent triglyceride solubility. However, even for high purity of polar solvent IPA, the oil recovery via distillation is 50%-90% more energy intensive than for hexane due to IPA`s higher boiling temperature and latent heat (Johnson & Lusas, 1983).

The aqueous processing requires wet grinding, elevated temperature and an enzymatic treatment to break down the cells. However, the primary limitation in wet grinding is the dispersion of the oil phase into small droplets – the smaller the droplet size, the more stable the emulsion formed (Balke, 2006).

So far, hexane is the main solvent in industry and also on laboratory scale. In industry, oil extraction is normally carried out in a counter-current fashion to maximize the recovery and at the same time to minimize the solvent use. Then
follows the seed separation from the oil/hexane miscella, which will be desolventized in order to eliminate any traces of the solvent for health reasons, as well as to maximize hexane recovery. Finally the meal is toasted to enhance the digestibility. Unfortunately, toasting limits the extractability and functionality of the protein; therefore, in some cases enzymatic treatment of the meal is applied to improve the solubility and functionality (Marsman et al., 1997).

On laboratory scale, the removal of fat from the seed is usually carried out using hexane as a solvent and the Soxhlet apparatus (Tzeng et al., 1988) for 24 hours, followed by drying the defatted meal under vacuum in an oven at 40°C (Tzeng et al., 1990) or at room temperature in the fume hood (Ghodsvali et al., 2005). In some cases the dried and defatted meal has been ground to pass through 40-mesh (Aluko et al., 2001) or 60-mesh screens in order to assure thorough interaction of the meal with chemicals during the protein extraction process. Tzeng et al., (1990) reported about the method for preparing defatted meals, where the canola seeds were ground to a slurry using an orbital mill in the presence of hexane.

### 2.3.3 Membrane Processing

The utilization of the proteins recovered via isoelectric precipitation is limited due to the presence of undesirable substances which are somewhat toxic and can affect the flavour and colour of recovered protein. Antinutritional components interact with the proteins through ionic and hydrophobic bonding causing associations. Since proteins are large molecules with molecular weights >5 kDa, membrane technology has been used as an effective method for their recovery. In addition to the separation of solutes of different molecular weights, its other advantages include mild operating conditions, low energy consumption and neither pH nor phase
changes are required.

A membrane is a selective barrier that allows the passage of certain constituents and retains other constituents found in the liquid (Cheryan, 1998). Membranes can be classified based on the material used: biological (animal or plant origin) or synthetic (polymeric or ceramics). Membranes are made of different materials, such as modified cellulose, polymers, porous metals, and ceramic materials. Membrane processing can be driven by pressure, chemical, or electric potential differences. Food processing applications are usually pressure driven since that enables attainment of desirable permeate fluxes for large-scale operations. The combined use of ultra- and diafiltration can minimize the impurities in the system and produce macromolecular materials such as proteins of high purity and concentration.

2.3.3.1 Ultrafiltration

An efficient method for reduction of the level of the antinutritional compounds is an ultrafiltration (UF) process. In UF the mass of high molecular weight (MW) compounds remains constant, its concentration increases, while the concentration of low MW compounds remains constant and mass decreases. The mathematical model of membrane processes is based on the assumption that the probability of a particle passing through the membrane is highest for solutes with 0% rejection. Rejection (R) at any point in the process is defined as

\[ R = 1 - \frac{C_P}{C_R} \]

where \( C_P \) is the solute concentration in the permeate and \( C_R \) is the solute concentration in the retentate (Cheryan, 1998). If the solute permeates freely through the membrane, \( C_P = C_R \) and \( R = 0 \).
As the permeable species and solvent pass through the membrane, the volume of the feed will be reduced. The volume concentration factor is given by the following equation

\[ CF = \frac{V_0}{V_R} \]

where \( V_0 \) is the initial feed volume and \( V_R \) is the retentate volume.

### 2.3.3.2 Diafiltration

Diafiltration (DF) refers to the process of adding solvent to the retentate and continuing the elimination of membrane-permeating species along with the solvent. In DF the mass and concentration of high MW compounds remain constant, while the mass and concentration of low MW compounds decrease. Diafiltration can be conducted under either one of two modes: discontinuous or continuous. Continuous DF involves adding water at the appropriate pH and temperature to the feed tank at the same rate as the flux, while discontinuous DF involves volume reduction, followed by redilution and reultrafiltration steps (Cheryan, 1998). The extent of removal permeable solutes is expressed by diavolume DV, the ratio of a solvent
added during the diafiltration step to the initial volume.

\[ DV = \frac{V_p}{V_0}, \]

where \( V_p \) is the total volume of liquid permeated and \( V_0 \) is the feed or retentate volume.

### 2.4 Functional Properties

Functional properties of proteins are physical and chemical properties which have an influence on their behaviour in food systems, whether it is in their preparation, cooking, consumption or storage. The size, shape, amino acid composition and sequence, net charge, charge distribution, hydrophobicity, hydrophilicity, structural arrangements and molecular flexibility of proteins are intrinsic characteristics that define their functionality and interactions with other food ingredients (Kinsella and Melachouris, 1976).

Functional properties may be classified according to the mechanism of action on three main groups: properties related with hydration (absorption of water/oil, solubility, thickening, wettability); properties related with the protein structure and rheological characteristics (viscosity, elasticity, adhesiveness, aggregation and gellying); and properties related with the protein surface (emulsifying and foaming activities, formation of protein-lipid films, whippability) (Kinsella, 1979).

These properties vary with pH, temperature, protein concentration, protein fraction, prior treatment, ionic strength and dielectric constant of the medium as well as other treatments such as interactions with other macromolecules in the medium, processing treatments and modifications, physical, chemical and enzymatic methods (Kinsella, 1979).
Canola protein products usually have both high water absorption and oil absorption, thus leading to good emulsification capacity for almost all products (Ghodsvali et al., 2005), except the soluble protein isolate, which shows a poor emulsion activity despite a high fat adsorption (Xu and Diosady, 1994). The mechanism of fat adsorption relies mostly on the physical entrapment of fat by capillary attraction. In the case of solvent-treated meals for glucosinolate removal, increase in fat adsorption was observed, which may be a result of the unfolding of protein by the solvent to expose hydrophobic groups on the surface of protein molecules.

Foaming property of all canola protein products is better than that of their soy counterparts. The quickly growing demand for alternatives to soy proteins is providing an added impetus to the commercialization of canola proteins, encouraged by the dramatic rise in the price of soybeans in the last decade (Thiyam-Holländer et al., 2012).
3.0 MATERIALS AND METHODS

3.1 Starting Materials

One of the starting materials for the process of production of protein isolates was *Camelina sativa* seed obtained from Terramax Corporation, Saskatchewan, Canada. The seed was of Siberian origin, grown in Saskatchewan.

The other material was the seed of *Cannabis sativa* obtained from Estonia, grown in the centre of Estonia. Finola is the official denomination for the Finnish variety of hemp, which was used in the current study.

The comparative materials for this study were: 1) canola seed from Saskatchewan, Canada and 2) rapeseed 00 (double zero) flour, with low content of erucic acid and low content of glucosinolates, grown in Estonia and defatted in Baltimere Invest Corporation, Estonia.

The origins and other useful aspects of canola, *Camelina sativa* and Finola are described in the Literature Review section. The composition of the defatted ground camelina, hemp and canola seed and meal is presented in section 4.0.

3.2 Mucilage Extraction from Camelina sativa seed

Whenever the seeds of *Camelina sativa* (1) and *Linum usitatissimum* (2) are immersed in water at any temperature, the viscosity of aqueous solutions increases instantly (Fig. 7). Although the aqueous solution of *Brassica napus* (4) doesn’t thicken significantly, there is no doubt that solutions 1, 2 and 4 are opaque. In contrast, the aqueous solution of *Cannabis sativa* (3) is entirely transparent. The reason for viscosity and opaqueness of the three solutions is the content of mucilaginous material in the outer layer of the seed hull. When water is added to flax seeds, they
form a heavy mucilage layer around the seed that makes them sink in water, while Camelina seeds float. Obviously, camelina seeds have a modified mucilage release, which reduces water mobility into internal seed tissues during imbibition causing buoyancy. Similar behaviour occurred in the case of fourteen natural Arabidopsis variants tested by Saez-Aguayo et al. (2014). Variations in the mucilage or soluble fibre content in different seeds account for a noted difference in drying process (Fig.8). *Linum usitatissimum* (A) and *Camelina sativa* (B), correlated with the content of mucilage 8% and 6.7% (Zubr, 2010), respectively, forming a crust-like substance during the drying process. Here the presence of polysaccharides plays a main role in crust formation. *Cannabis sativa* (C) and *Brassica napus* (D) seeds remain separately, although there are visible mucilaginous traces (Fig.8 D marked with arrows) around canola seeds. Unfortunately, the mucilage makes conducting aqueous processes complicated because solutions of mucilaginous herbs are highly viscous.
Whereas *Camelina sativa* contains a significant amount of mucilage (6.4-7.5%), it would be beneficial to isolate this material prior to conducting protein extraction. With this in mind, a process for the aqueous extraction of mucilage from *Linum usitatissimum* seed (Oomah & Mazza, 1998) was modified to *Camelina sativa* seed.
Camelina Seed 150g

H₂O 1500ml

Extraction at 55 °C for 3h agitating by a magnetic stirrer

Vigorous shaking on the 1 mm mesh

Centrifugation for 20 min at 6000rpm

2 x wash with 450g 55° C water

Wash with 150 g H₂O

Extract solution I

Extract solution II (not mixed with extract I for separate protein determination)

Seed

Oven Drying

Grinding

Defatting in Soxhlet

Analysis for solid and protein content and kinematic viscosity

Protein Analysis

**Figure 9 Process for Extraction of Camelina Mucilage**

*Camelina sativa* seed was agitated by a magnetic stirrer in an aqueous solution at 55 °C for 3 h. Subsequently, the seed solution was poured onto a 1 mm mesh
The seeds were washed twice with RO water (55 °C) with vigorous shaking in order to facilitate faster drainage and better washing. The seeds were washed with water at a seed-to-water ratio of 1:1 and centrifuged for 20 minutes at 6000 rpm for the final separation. The liquid portion was decanted from the seeds to a receiving flask. The samples from every extract solutions were analysed for solid content and kinematic viscosity. The protein content and colour of extract I and II was analyzed separately. The seed was dried in an oven overnight. The dried seeds were ground to a fine powder using a coffee grinder, and their oil content was determined by Soxhlet extraction with hexane. The oil content of mucilage-free ground camelina seed was 44%, which gave approximately 10% lower oil recovery than was obtained with mucilage. Finally, all the mucilage extract was freeze-dried. The extraction of the mucilage from the seeds yielded about 15 g freeze-dried mucilage per 150 g starting camelina seed.

### 3.3 Oil Extraction

In our laboratory the removal of fat from the ground seed is usually carried out using hexane as a solvent and the Soxhlet apparatus for 24 hours. In this study all of the batches, except the Estonian rapeseed flour and the last batch of mucilage-free *Camelina sativa* were defatted the same way. Every time approximately 3x 60g of ground seed was weighed into three thimbles, and approximately 100g of the defatted seed was recovered after 24 hours of defatting and desolventizing under the fume hood overnight. Being exposed to the boiling point of hexane, 68°C, for 24 hours can impair the quality of proteins; therefore, washing the ground seed with cold hexane could give the better quality of protein isolates. Therefore, the last batch of ~150g ground mucilage-free *Camelina sativa* was thoroughly washed with 3 litres
hexane on the vacuum filter and then dried overnight under the fume hood.

### 3.4 Protein Extraction and Precipitation

The efficiency of protein extraction is one of the essential conditions contributing to the product yield and, therefore, the extractability of *Camelina sativa* and *Cannabis sativa* proteins was investigated. To date, the best extractability has been achieved in an alkaline environment. So, the conditions for protein extraction were tested at pH levels 7 to 12.5. The extractability curve of *Camelina sativa* and *Cannabis sativa* protein was produced by conducting the process outlined in Figure 10.

In extractability experiments with *Camelina sativa* the protein solubility was determined by immersing 10 g portions of seed in aqueous NaOH at a solvent-to-seed ratio of 30 for 30 min with continuous stirring with a big magnetic stir bar on a hotplate stirrer. The camelina solution was very viscous at lower ratios. Therefore, the ratio of 30 was selected. In experiments with *Cannabis sativa* the solvent-to-seed ratio of 18 was applied, as the same ratio was previously used for canola by Tzeng et al. (1990). The pH of the solution was adjusted, using NaOH solution and pH meters VWR Scientific Model 8000 or perpHecT LogR meter model 310. The extract and solids were separated by centrifugation at 6000 rpm for 20 min. The liquid portion was decanted into a receiving flask. To achieve better protein extractability, the solids were washed twice with a water-to-seed ratio of 10 (camelina) and (at the ratio of) 6 (hemp) for 10 minutes using the stirring plate [and pH-meter] and adding NaOH if needed, to maintain the selected pH. The mixtures were then recentrifuged. Each time, the liquid was decanted into the same receiving flask and combined with the first portion. The combined liquid alkaline extract was then analysed for protein content. This procedure was repeated at pH values of 7, 9, 10, 10.5, 11, 11.5, 12, and 12.5. Each pH value was tested at least in duplicate. The extractability (%) was
Figure 10 Process for Protein Extractability Curve for *Camelina sativa* and *Cannabis sativa*
calculated by dividing the weight of protein in the extract by that in the 10g of starting material and multiplying by 100.

In order to optimize the process conditions for *Camelina sativa* and *Cannabis sativa*, also the precipitation curves were constructed. Protein extraction of 30 g of both defatted seeds was conducted at the preselected pH 11, according to Figure 11. The alkaline extract solution was divided into 120 to 200 g portions and one portion of at least 30g. This smaller portion was used for protein analysis (N x 6.25) in triplicate analysis of 10 g samples.

Each of the 120-200g portions were adjusted to different pH values, ranging from 2 to 7.5 using 3M phosphoric acid. For that the mixture was stirred by a magnetic stir bar and its pH was maintained by addition of H₃PO₄ for 30 minutes. The solid precipitate was separated from the liquid portion through centrifugation, followed by a single wash with 10g of H₂O. Unlike the extraction stages, the wash solution was shaken with the precipitate in the centrifuge tube, not stirred. The liquid portions were stored in plastic bottles in the refrigerator (4°C) until protein analysis was conducted.

For the protein analysis, the solution was allowed to reach room temperature and mixed to ensure uniform protein distribution. To achieve more uniform consistency for protein analysis, the samples of wet solid precipitates were put into aluminum foil packets and dried in the oven at 60°C overnight prior to protein analysis. However, the results of soluble protein analysis in triplicate were steadier.

All the procedures were repeated for each pH point between pH 2 and 7 in triplicate. The precipitability (%) was calculated by dividing the weight of protein in the precipitate by that in the extract and multiplying by 100. The percentage of soluble protein was calculated by dividing the grams of protein in the liquid by that in the extract and multiplying by 100.
Alkaline Extract at pH 11, with 30 g of DGC and 900 g of H₂O

Divide into 120 - 200 g portions and one 30 g portion

Protein analysis using 3x10g

Alkaline Extract pH 11
120 - 200 g

3M H₃PO₄

Protein Precipitation (pH 2 - 7)

Centrifugation

Liquid Add into

Solids
Wash with 10g H₂O

Centrifugation

Washed Solids

Oven Drying

Precipitated protein

Protein Analysis of Soluble Protein

Figure 11 Process for Protein Precipitation Curve
3.5 Ultra-and Diafiltration Equipment

Prior to isoelectric precipitation of proteins, the alkaline extract requires purification and concentration steps. Therefore, membrane systems are employed.

The portion of pH 11 extract solution was prepared according to the process described in the previous section. NaCl was added to the extract and the solution was heated at 55°C for 30 min. As soon as the solution was cooled down to 40°C, it was ready for membrane processes.

The equipment used for ultra- and diafiltration consists of:

1) SEPA CF II filtration system,
2) Hydracell diaphragm pump,
3) Baldor electric motor with frequency variator for controlling pump motor speed,
4) polyethersulfone membrane, with an effective membrane area of 140 cm².

The operating conditions for the SEPA CF II filtration system were the following:

1. Applied pressure of 40 psi (0.28MPa)

2. Motor frequency of 15 Hz

![Figure 12: Membrane System for Protein Purification](image)
The ultra- and diafiltration process proceeded according to the scheme shown in Figure 12. The heat- and salt-treated extract was passed through a 5 kDa polyethersulfone membrane at first in ultrafiltration mode to a concentration factor around 4, followed by diafiltration with an approximate diavolume of 3. The diawater was adjusted to the same pH 11 as the alkaline feed solution. The retentate from the membrane processing was subsequently precipitated at pH 5 to produce precipitated and soluble protein isolates. The soluble protein solution was again passed through the membrane [in ultrafiltration mode] for concentration and purification.

3.6 Methods of Analysis

Moisture Content
The moisture content was determined according to AACC Method 44-15A (AACC, 1976). Gravimetric moisture analysis was conducted on the starting material with at least 2 g portions in triplicate several times over the course of this work. For moisture analysis the samples were dried overnight at 105°C in the oven. The description of this method is given in Appendix A.1.

Oil
The oil content was determined of at least 10 g dry ground seed samples of every starting material (camelina, hemp and canola) by 24 hr of Soxhlet extraction. For every ~10g batch 300 ml of hexane was used as a solvent. Every time the analyzes were performed in triplicate. The exact method for determination of oil content based on the AOCS standard method Ba-38 (AOCS 1998) is given in Appendix A.2.
Crude Protein

The crude protein content of the starting materials and final products was determined using the Kjeldahl method. According to AACC Standard Method 46-12, 1976 (AACC 1995) the Büchi 425 digestor, Büchi 315 distillation units, a commercial catalyst (K_2SO_4, CuSO_4+5H_2O, TiO_2) and concentrated H_2SO_4 were used in these analyses. The content of nitrogen was determined by titration of ammonia distilled from the digested sample using 0.1N H_2SO_4 as a titrant. Protein content was calculated as (N x 6.25). All the protein determinations were performed at least in duplicate. The complete method is given in Appendix A.3.

Total Phenolic Compounds

The analytical method established by Xu and Diosady was used for the determination of total phenolic acid content with results expressed as sinapic acid equivalents per 100g sample. All the phenolic determinations were performed at least in duplicate. The procedure is presented in detail in Appendix A.4.

Colour Analysis

For colour analysis the two – step process was used:

1. Digital photographs of the samples were pre-processed in Image J software, an open scientific images analysis tool from US National Institutes of Health to obtain the RGB (red, green and blue) values.

2. Processing the data using EasyRGB software to retrieve Hunter L, a, b colour values from the sample photographs.

Details are given in Appendix A.5.
Viscosity

For viscosity measurements of mucilage solutions the Cannon-Fenske Routine Viscometer with universal size of 100 and approximate constant 0.015 was used. The viscosity of solutions was measured at different mucilage concentrations at a temperature ~25°C. Kinematic viscosity was calculated as

\[ v = t \times 0.015 \text{cSt/sec}, \]  
where \( t \) is the flow time in seconds.
4.0 RESULTS AND DISCUSSION

4.1 Composition of Camelina sativa, Cannabis sativa and Canola

Prior to use, all materials, except Estonian dehulled and defatted rapeseed 00 flour, were ground with a coffee grinder, defatted with hexane using a Soxhlet apparatus for 24 hours, and then dried overnight under the fume hood. However, the last batch of mucilage-free camelina was defatted in August 2016 without using a Soxhlet apparatus for 24 hours, but just washing the ground seed with hexane using a vacuum filter in order to avoid exposing the seed to high temperature for long hours. The previous batches of camelina were defatted from January to April, 2015. These batches were needed for initial protein determination and for the extraction and precipitation data. For the next trials and production of protein isolates the batches of camelina were defatted in October and December, 2015. The composition of the ground camelina seed is found in Table 8. The first batches of industrial hemp variety Finola were defatted in June, 2015. Defatted material was used for initial trials, such as oil, moisture and protein content. For phenolic analyzes and production of protein isolates the batches were defatted in November, 2015. The results of the analyzes are given in Table 9.

In the current study two rapeseed-based materials were used as comparative materials. The first of the two was canola seed obtained from Canada. The batches of ground canola seed were defatted in October and November, 2015. The dried material was used for determinations of composition and for production of isolates. The composition of canola is presented in Table 10.

The other comparative material was rapeseed 00 (double zero) flour, with low content (<2%) of erucic acid and low content of glucosinolates, similar to canola. The rapeseed 00 is grown in Estonia. It was defatted and dehulled in Baltimere Invest Corporation, Estonia. This material was analyzed for moisture, protein, phenolic
<table>
<thead>
<tr>
<th></th>
<th>GC</th>
<th>DGC</th>
<th>Data from Literature (Budin et al., 1995; Sampath, 2009, Toncea et al., 2013)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oil %</td>
<td>41.7 ± 2.7</td>
<td>29.9 to 38.3</td>
<td></td>
</tr>
<tr>
<td>Protein (N % x 6.25)</td>
<td>25.4 ± 0.2</td>
<td>36.6 ± 0.4</td>
<td>23 to 30; 36 in the meal</td>
</tr>
<tr>
<td>Moisture (%)</td>
<td>4.5 ± 0.1</td>
<td>8.0 ± 0.2</td>
<td>5</td>
</tr>
<tr>
<td>Phenolics (mg sinapic acid/100g sample)</td>
<td>222 ± 7.6</td>
<td>158</td>
<td></td>
</tr>
</tbody>
</table>

Table 8 Composition of Ground Camelina Seed GC Seed and Defatted Ground Camelina Meal DGC Compared to the Data from Literature

<table>
<thead>
<tr>
<th></th>
<th>GIH</th>
<th>DGIH</th>
<th>Data from Literature (Callaway, 2004)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oil %</td>
<td>40.4 ± 3.5</td>
<td></td>
<td>35-45</td>
</tr>
<tr>
<td>Protein (N % x 6.25)</td>
<td>24.4±0.3</td>
<td>35.4±1.5</td>
<td>24.8; 35 in the meal</td>
</tr>
<tr>
<td>Moisture (%)</td>
<td>6.6±0.1</td>
<td>9.6±0.04</td>
<td>6.5</td>
</tr>
<tr>
<td>Phenolics (mg sinapic acid/100g sample)</td>
<td>269 ± 5.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 9 Composition of Ground Hemp Seed GIH and Defatted Ground Hemp Meal DGIH (Finola) Compared to the Data from Literature

<table>
<thead>
<tr>
<th></th>
<th>GCan</th>
<th>DGCan</th>
<th>Data from Literature (Nazck, 1998; Slominski, 2015)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oil %</td>
<td>44.6 ± 0.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein (N% x 6.25)</td>
<td>21.2±0.4</td>
<td>37.8±1.01</td>
<td>18 to 20; 36.7 in the meal</td>
</tr>
<tr>
<td>Moisture %</td>
<td>7.1±0.1</td>
<td>5.5 ± 0.02</td>
<td>12</td>
</tr>
<tr>
<td>Phenolics (mg sinapic acid/100g sample)</td>
<td>1430 ± 38</td>
<td>1840</td>
<td></td>
</tr>
</tbody>
</table>

Table 10 Composition of Ground Canola Seed GCan and Defatted Ground Canola Meal DGCan Compared to the Data from Literature

<table>
<thead>
<tr>
<th></th>
<th>Estonian Rapeseed Flour</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (N% x 6.25)</td>
<td>47.3±0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moisture %</td>
<td>5.7±0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oil %</td>
<td>4.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenolics (mg sinapic acid/100g sample)</td>
<td>2159 ± 122</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 11 Composition of Estonian Rapeseed Flour
and oil content, and further used for the production of protein isolates. The results of the analyzes are found in Table 11.

The protein concentration, the moisture content and phenolic compounds in every defatted seed were measured in triplicate.

4.2 Protein Extraction Data

The previous investigations for determining the optimal conditions for protein extraction from oilseeds have been achieved in a strong alkaline environment (pH 10.5-12). However, the protein extractability data of every seed are different and needed separate research.

As shown in Figure 13 the extractability of camelina and hemp protein increases with increasing pH. This is consistent with the extractability data for the other oilseed crops investigated in our laboratory, such as canola and mustard. The reason of higher extractability in the case of canola (Tzeng et al., 1990) was the different defatting procedure, which involved grinding the batch of 1kg seed as a slurry with 2.5 L hexane in a Szego mill at a contact time of 1 min at a roller speed of 740 rpm. Then the meal was separated from the slurry by vacuum filtration and subsequently the meal was defatted with hexane using a 4-L solvent-capacity Soxhlet apparatus for 24 hours. The oil-free meal was dried in a vacuum oven at 40°C. The hemp and camelina meal were just defatted with Soxhlet for 24 hours.

Similarly to the canola meal protein, the extractability of industrial hemp meal protein reaches its maximum at pH 11.5 and then starts to decrease. However, there is a steady increase in camelina meal protein recovery, from 37% at pH 7 to 70% at pH 12. The extractabilities for the defatted camelina and the industrial hemp meal proteins at pH 10 are equal within experimental error.
The highest extractabilities of camelina, canola and hemp meal proteins are 70%, 91% and 79%, respectively. However, the difference in extractability of camelina meal protein between pH 11 and 12 (65% and 70%, respectively) is minimal, whereas at pH 11, approximately 65% of the protein is soluble compared to 55% at pH 10, showing a more significant difference. Extracting proteins at pH 12 or beyond pH 12 requires much more highly corrosive NaOH than at pH 11 and can be harmful to the membranes. It is also impractical due to the denaturing of proteins and production of a toxic compound, lysinoalanine, N6-(DL-2-amino-2-carboxyethyl) – L-lysine, an unusual amino acid implicated as a renal toxic factor in rats (Pfaender, 1983). Keeping that in mind, pH 11 was chosen for further work with canola, camelina and hemp.
4.3 Protein Precipitation Data

The behavior of proteins is pH-dependent, and each protein has an isoelectric point, where the contributions from positive and negative charges cancel out to give the molecule no net charge. At this point proteins tend to coagulate, and precipitate. The isoelectric pH of rapeseed protein has been reported to be pH 3.5. However, the precipitation of protein was reported to be only 40-50% at the isoelectric point (Naczk et al., 1985). Some other authors have found that rapeseed protein shows a wider isoelectric point range or more than two isoelectric points, which complicates the complete precipitation of rapeseed proteins.

To determine the best conditions for recovering proteins from the two novel oilseeds, camelina and hemp, isoelectric precipitation needed to be performed. As can be seen Figure 14, the minimum solubility of camelina proteins occurs at pH 4.5, and that of industrial hemp proteins in pH 5-6 range. At pH 4.5, approximately 75% of the original soluble camelina protein is isoelectrically precipitated. Tzeng (1990) found that 42.8% of canola protein was precipitated at pH 3.5.

![Figure 14 % Soluble Protein of Alkali-extracted Camelina and Hemp Protein](image-url)
The hemp proteins precipitate over a much broader range of pH and there is a clear plateau at the pH range from 5 to 6.5, where the recovery of precipitated protein is 87%. Even at pH 4.5 and pH 7 more than 80% of proteins are precipitated. The differences between precipitation profiles of three crops are due to the dissimilar isoelectric points of different types of proteins found in each crop. As pH 5 requires a smaller amount of acid than pH 4.5, the pH of 5 has been selected to maintain the maximum level of precipitation during production of protein isolates.

4.4 Mucilage extraction Data

_Camelina sativa_ seeds are small, 0.8 to 1.4 mm long (Francis and Warwick, 2009). Therefore, dehulling would be problematic. The best recovery in the current study was achieved by mixing the seeds with warm water and subsequently washing the seeds twice on a screen. Further washing the seeds with warm water didn’t improve the mucilage recovery considerably. Similar processes have previously been used for the aqueous extraction of mucilage from flax seed (Oomah and Mazza, 1998) and _Brassica alba_ (Balke, 2006).

The previous studies (Cui et al., 2000, Balke, 2006) have demonstrated that temperature has a great effect on the mucilage recovery due to increased solubility of the heterogenous mix of polysaccharides comprising the mucilage. However, the results show that extraction levels off at temperatures above 60°C, supposedly due to the mucilage`s inherent viscosity (Balke, 2006). As temperatures above 60° C could denature the proteins and, therefore, decrease their quality, accordingly, in current mucilage extraction procedures the temperature was maintained at values of 55°- 60° C.

Extraction was carried out by adding the _Camelina sativa_ whole seed to water and
agitating it by a magnetic stirrer at a water - to - seed ratio of 10:1 for 3 h. As the study of Balke (2006) with yellow mustard mucilage demonstrated the sufficiency of approximately 1 hour for maximum mucilage extraction and water to seed ratio of 8:1, the next extraction was conducted within 1 hour, but still with the same ratio of 10. In a similar study with flaxseed the extraction time of 3 hours and water to seed ratio of 13:1 were used (Mazza and Oomah, 1998). Due to the great affinity of mucilage for the seed, long extraction periods have been reported in previous studies (Sharafabadi, 1990, and Cui et al., 2000).

Previous research has shown the significant effect of elevated pH on mucilage extraction, demonstrating that over the range from pH 4 to pH 9, mucilage recovery increases as pH is increased. However, it was discovered that higher pH values cause higher protein losses into the mucilage extract. The protein content in mucilage increases substantially also at very low pH (2-3), especially at high temperature. The increase in protein content in mucilage with decrease in pH can be explained by the easier solubilisation of proteins at lower pH region (Oomah and Mazza, 1998). The aim of the current study is to preserve the maximum amount of protein in the seeds for further production of protein isolates. Therefore, the native pH value ~6 of the suspension was used for mucilage extraction.

After mucilage extraction the oil content of mucilage- free ground camelina seed was 44%, which gives an approximately 10% lower oil recovery than was obtained with mucilage. It means that 10% of oil was separated with mucilage.

After extracting the mucilage, the extract was freeze-dried. The camelina mucilage yield after freeze-drying was 10% of 150 g starting material (wet basis): 6.3% (9.4 g) was recovered directly from the screen and 3.7% through centrifuging the seed with 1:1 additional wash water. In the case of the second extraction, the centrifugation was carried out without addition of water, in order to avoid an extra dilution of mucilage, which could complicate the drying. The protein content in camelina gum
(dry weight) influences the quality of the extracted mucilage, but also the yield and quality of the camelina meal. The protein concentration in the freeze-and spray-dried mucilage was 15% and in the centrifuged part the concentration was a little higher, 19%, which means that more proteins were dissolved in the mucilage extract during washing and centrifugation. Therefore, the next extraction was conducted with only one centrifuge step for separating the remaining mucilage extract and the seeds. This time the protein concentration in the freeze-dried mucilage was 10% and in the centrifuged one 12%.

4.4.1 Viscosity

After the extraction of mucilage, the viscosity of different dilutions of the camelina mucilage were measured. Every time the solid content of the extract solutions was determined by drying the wet samples overnight in the oven. Prior to measuring viscosities with a Cannon-Fenske Routine Viscometer (Size 100) the samples were cooled to 25º C. This particular viscometer can be used for the measuring the kinematic viscosity of Newtonian fluids at a viscosity range of 3-15 cSt, where 1 centistoke $= 1 \text{ mm}^2\cdot\text{s}^{-1} = 10^{-6}\text{ m}^2\cdot\text{s}^{-1}$.

Only the number of samples that could be processed within the same or next day was extracted each day. The kinematic viscosity of the extracts was calculated in centistokes (cSt) as the efflux time in seconds multiplied by the viscometer constant. The approximate constant of this viscometer is 0.015 cSt/sec. The equation for the kinematic viscosity is: $v = t \times c$,

where

$v =$ kinematic viscosity (cSt)

$t =$ efflux time (s)

$c =$ viscometer constant (cSt/s)
The results presented in Figure 15 show that pH affects the viscosity of the camelina mucilage showing the highest values in natural (pH~6) conditions compared to the highly acidic or alkaline environment.

![Figure 15 Kinematic Viscosity of Camelina Aqueous Solutions](image)

![Figure 16 Kinematic Viscosity of Camelina, Canola and Flax Aqueous Solutions.](image)
The results of the viscosities of camelina mucilage solutions were compared to those of canola and flax solutions. The results of trials with three oilseeds are shown in Figure 16. It is obvious that the mucilaginous extracts of flax and camelina achieve high viscosity already at low solid concentrations, compared to canola, which doesn`t show significant viscosity ascent. Camelina and flax mucilage extracts with higher solids concentrations were too viscous for this viscometer. The viscosity values of camelina protein extract increase according to the increase of solid content in the extract (Fig. 17).

![Graph](image_url)

Figure 17 Kinematic Viscosity of Camelina Protein Extract at pH 11

To ascertain the influence of freeze drying, recovered product was rehydrated and the viscosity trials at different mucilage concentrations were conducted. After freeze drying, the kinematic viscosity was much higher than it was before drying (Fig. 18), although the material was freeze dried less than a week after extraction. The viscosity of freeze-dried material was also compared to that of spray-dried material.
and the results showed similar viscosity values. Accordingly, both freeze drying and spray drying alter the structure of the product, although in freeze drying low temperature (-20 °C) and in spray drying high temperature (160 °C) is used. According to Li et al. (2016) camelina mucilage is strongly pseudoplastic, which means that all the given viscosity values are relative.

![Figure 18 Kinematic Viscosity of Freeze-and Spray-dried Mucilage Solutions Compared to the Viscosity Before Drying](image)

The pH of freeze- and spray-dried products is an important factor amongst physicochemical properties, which could provide good knowledge about the stability and physiological activity of it. Therefore, the pH of 1% w/v dispersion of the sample in water was determined using a Corning pH meter. The pH value of freeze-dried and spray-dried material was 6.5.

Scanning electron microscope (SEM) images done with SU 3500 using scattered electron mode, demonstrate the different shape and structure of differently dried mucilage (Fig.19). The spray-dried mucilage particles are spherical with crimped
surfaces, while the freeze-dried mucilage has a smooth sheet-like structure. For images the different magnifications were used: 2000 for a, 10,000 for b, 370 for c and 160 for d.

Figure 19 SEM Images of Spray-dried (a, b) and Freeze-dried (c, d) Camelina Mucilage
4.5 Production of protein isolates

As described in section 2.3, the content of antinutritional substances in oilseed proteins must be substantially lowered and the proteins isolated, before these proteins are considered for human nutrition.

As reported on the development of Tzeng, Diosady and Rubin (1990) the procedures for producing isolates from rapeseed and canola consisted of extraction of defatted meal at pH 10.5-12.5, isoelectric precipitation to recover proteins and ultrafiltration followed by diafiltration to concentrate and purify the remaining soluble proteins. This technology yielded high quality precipitated and soluble isolates, both of high (>85 %) protein content, low in phytates (<1%), and essentially free of glucosinolates (<2µmol/g). To reduce the phenolic content the process was further modified by Xu & Diosady (2002).

During 25 years, these membrane-based processes have been adapted to different species of rapeseed and mustard, all from the Brassica family (Fig.20).

![Figure 20 The Protein Content (%) in Isolates and Meal Residue of Different Oilseeds Recovered in our Laboratory through the Years.](image_url)
The initial trials demonstrated the possibility to produce protein isolates also from *Camelina sativa* and *Cannabis sativa*. Therefore, the process of Tzeng et al. (1990) as described in Figure 21 was followed with the afore-mentioned novel oilseeds. In addition, the comparative procedures with canola and rapeseed 00 flour were carried out. The defatting process enriches the protein content from around 35 to 52-55% in the defatted oilseed cakes (The et al., 2014). Therefore, prior to alkaline extraction of proteins, all the starting materials, except the Estonian rapeseed 00 flour, were defatted with hexane. The Estonian rapeseed flour had already been defatted in Estonia. The oil extraction is described in section 3.3. The major part of mucilage was also extracted from the camelina seed prior to grinding and defatting. The procedure for mucilage extraction is described in section 4.4.

The process for the production of protein isolates shown in Figure 21 started with an alkaline extraction stage at pH 11 for all investigated starting materials. The only difference was the water to seed ratio of 18 for hemp, rapeseed flour and canola, and the ratio of 30 for camelina. The higher water to seed ratio was used due to the high viscosity of camelina, which was significant even after mucilage extraction. The viscosity was caused by the remaining polysaccharides in the camelina seed material.

The ground seed was mixed with 0.1% of ascorbic acid to prevent oxidation of proteins. To extract the proteins, the pH of the solution was adjusted to 11 with 25% w/w NaOH with stirring for 30 minutes. After centrifugation at 6500 rpm for 20 minutes the solids were washed twice for 10 minutes, maintained at pH 11, to remove any extracted protein from the surfaces of the solid particles. To produce a sufficient amount of isolates for further trials, 60-200g of starting seed material was used and 2 to 5 liters of extract solution was prepared for filtration.

Meal residue and extract solution were separated through centrifugation for 20 min at 6500 rpm and 25°C. The crude protein content of every meal residue was analyzed.
after freeze drying.

The next processes, involving ultra- and diafiltration followed by isoelectric precipitation and purification of soluble protein, are described in the next section.
Figure 21 Process for Production of Protein Isolates
4.5.1 Membrane Processing of Protein Solutions and Isoelectric Precipitation

The extract solution was vacuum filtered with Whatman No. 41 in order to remove small particles before ultra- and diafiltration. To break the ionically bound phenolic-protein complexes, NaCl (0.05M) was added to the extract solution and it was heated between 55°C and 60°C for 30 min (Diosady et al., 2005). Prior to ultra-and diafiltration, the solution was cooled down to at least 40°C, in order not to damage the membrane cartridge. In current work, it was assumed that any problems with the content of glucosinolates and phytates can be solved by application of repeatedly tested membrane technology for use with protein extracts (Xu et al., 1998).

The previous investigations (Diosady, 2003, Marnoch, 2004, Hijar, 2013) have indicated that the extracts of oilseeds, such as canola and mustard, contain proteins with molecular weight smaller than 10 kDa. Therefore, in order to achieve the maximum protein recovery, the heat- and salt-treated solution was passed through the 5 kDa membrane. Thus, the components with molecular weight higher than 5kDa were concentrated into the retentate solution, while the lower molecular weight components passed through the membrane and were collected as the permeate solution. After membrane processing, the samples of ultra-and diafiltration permeate solutions were analyzed for solids and protein content.

The concentration factor (CF) of 4-5 for ultrafiltration, as well as diavolume (DV) of 3 for diafiltration have previously shown the best efficiency for purifying the protein extract of anti-nutritional substances (Marnoch, 2004, Hijar, 2013). Therefore, the initial plan was to use CF of 5 and DV of 3 for every investigated oilseeds in current work. Later the CF of 3.4 to 4.4 and a DV of 1.5 to 2.4 were used, to shorten the time when the proteins were exposed to highly alkaline sodium hydroxide. In the current study the significant darkening of canola precipitated protein isolate occurred due to the long period of being exposed to the alkaline environment. The dark colour of PPI
due to the long period (12 hours) for ultrafiltration and diafiltration was also claimed by Marnoch (2004).

The alkaline extracts of the second batches of hemp and mucilage-free camelina were analyzed for Kjeldahl nitrogen right before the membrane treatment, to determine the exact protein content of extracts proceeding to the membrane. Before the membrane treatment the protein concentration in the hemp extract was 33.1% (dry basis), and that of camelina extract was 32% (dry basis). After ultrafiltration of the protein solution, the protein content in the hemp permeate was approximately 6.2% (1.6 g), while the diafiltration permeate contained only 1.6% (0.4 g) of non-protein nitrogen of the starting material.

Analyzing the protein content in camelina ultrafiltration and diafiltration permeates, the approximate nitrogen losses into permeates were 1.2 g and 0.6 g, respectively, which is 3.6% and 1.7% of input. The nitrogen values in the permeates show the protein (smaller than 5kDa) losses through the membrane (Appendix 6).

The average flux during processing varied a lot, while the processing time of ultra- and diafiltration depended on the volume of the starting solution. Large volumes of extract, such as 4-5 liters, required longer periods for ultra- and diafiltration. This is due to the increase of feed stream viscosity or density and the rate of diffusion as solids levels increase causing an additional hydrodynamic resistance to the flow of permeate, which in turn lowers the flux (Cheryan, 1998).

The average permeate flux (J) is calculated by using the equation

\[ J = \frac{V_p}{At}, \]

where \( V_p \) is the volume of the collected permeate that passed through the membrane surface area (A) in the processing time (t) required to reach the desired concentration factor.

A major limiting factor in membrane technology is fouling of the membrane. Fouling demonstrates itself as a decline in flux with time of operation, when all the operating
parameters, such as pressure, flow rate, temperature, and feed concentration are kept constant (Cheryan, 1998). As almost all feed components foul the membranes to a certain extent, membrane fouling is inevitable. In the current study from start to finish of the ultra- and diafiltration stages the flux ranged from 39 L/hm$^2$ to 13.7 L/hm$^2$, depending on the volume of extract to be consecutively processed, the nature of the solute, and the number of runs (cleaning cycles) of the particular membrane. The best values of flux were achieved with brand new membranes.

To avoid the effects of fouling, the membrane system was meticulously cleaned at the end of the each day of filtration. The cleaning procedure started with draining the remaining solution from the system, prior to flushing it with 1.5L of distilled water. Subsequently 1L of enzymatic detergent solution (10 g/L) was recycled through the system for ~1 hour. Then 60 L of reverse osmosis water was used to rinse the system completely. However, it is reported that the frequent cleaning regimes and cleaning agents will decrease membrane lifetime significantly (Cheryan, 1998). Unfortunately, every washing step also causes some losses of extract solution. Therefore, the best way to use the membrane system, would be to conduct the ultra- and diafiltration continuously on the same day, also because of the shorter period for proteins to be exposed to highly alkaline (pH11) environment (Table 12).

<table>
<thead>
<tr>
<th>Starting material</th>
<th>The number of runs of the membrane, when started</th>
<th>The number of days in alkaline environment</th>
<th>UF time (hours)</th>
<th>The Average Flux (L/m$^2$h)</th>
<th>DF time (hours)</th>
<th>The Average Flux (L/m$^2$h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemp</td>
<td>1</td>
<td>2</td>
<td>7</td>
<td>39</td>
<td>7</td>
<td>23.8</td>
</tr>
<tr>
<td>Camelina</td>
<td>4</td>
<td>2</td>
<td>11</td>
<td>21.4</td>
<td>6</td>
<td>18.5</td>
</tr>
<tr>
<td>Canola</td>
<td>8</td>
<td>3</td>
<td>12</td>
<td>13.7</td>
<td>10</td>
<td>8.6</td>
</tr>
<tr>
<td>Rapeseed flour</td>
<td>1</td>
<td>2</td>
<td>8</td>
<td>25.9</td>
<td>9</td>
<td>22.7</td>
</tr>
</tbody>
</table>

Table 12 The Data of Membrane Processing
The main disadvantage of polysulfone and polyethersulphone membranes is their hydrophobic nature, which leads to an apparent tendency to interact strongly with a variety of solutes, making it prone to fouling in comparison to the more hydrophilic polymers such as cellulose and regenerated cellulose (Cheryan, 1998). In addition, as the working area of the membrane used in our laboratory is only 140 cm$^2$ and the fouling is controlled by pore blocking as well as pore constriction (Xue, 2014), the membranes need to be changed at least after every eighth working stage. Thus, working with high volumes is impractical with this kind of membranes. Moreover, during the long processing periods, it was noted that the pH of the extract decreased significantly causing the precipitation of some proteins, which in turn plugged the membrane, resulting in prolonging of the processing time. To prevent this problem the pH needed to be observed constantly and readjusted as needed. Therefore, the diawater also was adjusted to pH 11 to prevent the decrease of pH during the diafiltration procedure. This alkaline diawater was continuously added to the retentate, maintaining the volume of feed, in order to clean the protein concentrate. Unfortunately, sitting overnight under the fume hood caused the decrease of pH and the colour change of the extract showed that the precipitation of proteins will occur soon. While hemp has a wide variety of proteins, it has a broad spectrum (Fig.14) of isoelectric points. Thus, some of the hemp proteins (~10%), with isoelectric points are at pH $> 7$, precipitated overnight. These proteins can plug the membrane during membrane processes, resulting in the extremely long processing time. Therefore, the pH of all the extracts needed readjusting to 11 and filtering prior to using the membrane system. This, in turn, decreased the yield, as some of the precipitated proteins remained on the filter paper, as well as some losses of the extract solution occurred during these additional steps. Therefore, the losses of hemp proteins during processing the first run were the highest (46.8%), compared to rapeseed flour and
camelina, 20.8% and 3.3%, respectively. Although the pH decreased also in case of extracts of the other starting materials, fortunately, the precipitation wasn`t observed yet in these cases.

After the concentration and purification of the protein extract, 3 M phosphoric acid was added in a drop-wise manner to the extract solution under continuous stirring for recovering precipitated protein isolates. During the preliminary studies, the pH value of 5 was considered to be in the range of the highest precipitation of hemp and camelina proteins. After the precipitation, the supernatant was separated from the precipitated protein through centrifugation at 6500 rpm for 20 minutes. The precipitate was washed with reverse osmosis water, and after centrifugation it was frozen at -20°C. The frozen precipitated protein isolate was subsequently freeze-dried. The soluble protein solution and the wash water were combined to further concentrate and purify by ultrafiltration. After purification, the soluble protein isolate was frozen and then freeze-dried.

To avoid the precipitation overnight, the extract solutions of the second runs (hemp and camelina) were put into the fridge between the days of ultrafiltration. In case of the second run with hemp, the losses were much smaller (15%) as the extract solution was kept in the fridge and, therefore, the additional steps of readjusting the pH and filtering were avoided. The losses decreased also due to the more careful draining and rinsing of the remaining extract solution from the tubing of the membrane system. The recovery of the precipitated protein was almost double the previous value and the protein concentration in the hemp PPI was the same 96% - 97%.

In the case of camelina, the procedure started with mucilage extraction. This time the mucilage extraction was much faster, compared to the previous 3 hours, only one hour of stirring the seed at water - to - seed ratio of 10 at 55°C. In the previous experiment the seed had been thoroughly washed with a bucket of water, to
eliminate most of the mucilage. This time the extra washing steps were excluded, to avoid protein losses into wash water. After extraction of mucilage, the seed was dried in the oven at 60°C and then ground. Subsequently, the ground seed was defatted, using the quick defatting procedure with hexane described in section 3.3, instead of extracting oil with the Soxhlet apparatus for 24 hours. After defatting the seed was dried overnight. The protein extraction was carried out at water - to - seed ratio of 30 as, despite of the mucilage extraction, the solution still remained very viscous and the extraction of proteins was carried out as described in section 3.4. Then the preparation and processes for ultra- and diafiltration followed. The procedures are described at the beginning of this section. This time the recovery of the major part of SPI succeeded, as it was collected thoroughly from the tubing. This was one of the reasons, why the protein recovery into SPI was much higher, increasing from 0.9% to 11.4%. The other reason for much a higher yield is the higher protein solubility of differently treated (mucilage and oil extraction) starting material. In the last case only 29.5% protein remained in the meal residue, compared to the previous protein concentration of 40%. The twice as high protein concentration in the SPI can also be explained with the differently treated starting material and the lower (10-12%) protein concentration in the mucilage, compared to the previous (16-19%), using the rapid mucilage extraction procedure. The overall losses of mucilage-free camelina proteins were 14.1%, with 3.8% of that in the mucilage. However, the protein concentration in the PPI was lower in the second batch. The explanation for that is the different composition of the previous PPI. The previous protein concentration was determined using the mixture of PPI-s, which had different starting materials: only a quarter of mucilage-free and three quarters with mucilage. Thus, the materials, obtained from the two experiments are not comparable. However, the conclusions of procedures are still relevant.
4.5.2 Product Properties/Composition

4.5.2.1 Taste and Composition of Final Products

The precipitated protein isolates (PPI) of camelina, canola and Estonian rapeseed 00 were slightly salty, and hemp PPI was bland in taste. The soluble protein isolates made of camelina, hemp, canola and rapeseed flour had likewise salty taste. It was obvious that some of the added sodium chloride remained in the isolates. According to previous studies (Marnoch, 2004; Kappak, 2008 and Hijar, 2013) the diawater had the same salt concentration as the extract (0.05M). To remove the salty taste, in the second batches of hemp and camelina, salt was not added to the diawater. Thus, the isolates of the second batches had a bland taste.

<table>
<thead>
<tr>
<th></th>
<th>Canadian Canola</th>
<th>Kappak Finnish Rapeseed</th>
<th>Eigi Estonian Rapeseed</th>
<th>Estonian Rapeseed Flour</th>
<th>Camelina</th>
<th>Hemp I / II</th>
<th>Marnoch Mustard</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPI</td>
<td>6.6</td>
<td>3.9</td>
<td>9.4</td>
<td>18.7</td>
<td>5.2</td>
<td>12.6/20.6</td>
<td>21.9</td>
</tr>
<tr>
<td>SPI</td>
<td>10</td>
<td>4.9</td>
<td>2.2</td>
<td>17.4</td>
<td>8.7</td>
<td>2.1/3.3</td>
<td>2.8</td>
</tr>
<tr>
<td>MR</td>
<td>60.2</td>
<td>78.1</td>
<td>70.6</td>
<td>29.3</td>
<td>69</td>
<td>45.9/59.1</td>
<td>38.4</td>
</tr>
</tbody>
</table>

Table 13 Solids Recovery % of the Final Products of the Different Seed Materials

<table>
<thead>
<tr>
<th></th>
<th>Canadian Canola</th>
<th>Kappak Finnish Rapeseed</th>
<th>Eigi Estonian Rapeseed</th>
<th>Estonian Rapeseed Flour</th>
<th>Camelina</th>
<th>Hemp I / II</th>
<th>Marnoch Mustard</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPI</td>
<td>13.6</td>
<td>9.15</td>
<td>20.1</td>
<td>35.3</td>
<td>10.7</td>
<td>34.5/60.9</td>
<td>44.8</td>
</tr>
<tr>
<td>SPI</td>
<td>15.2</td>
<td>8.6</td>
<td>2.87</td>
<td>31.3</td>
<td>11.4</td>
<td>2.7/3.9</td>
<td>3.6</td>
</tr>
<tr>
<td>MR</td>
<td>53.3</td>
<td>79.5</td>
<td>67.62</td>
<td>8.6</td>
<td>63.8</td>
<td>14.4/20.2</td>
<td>28.3</td>
</tr>
</tbody>
</table>

Table 14 Protein Recovery% of the Final Products of the Different Seed Materials
Table 15 Protein Concentration% of the Final Products of the Different Seed Materials

<table>
<thead>
<tr>
<th>Starting Material</th>
<th>Canadian Canola</th>
<th>Kappak Finnish Rapeseed</th>
<th>Eigi Estonian Rapeseed</th>
<th>Estonian Rapeseed Flour</th>
<th>Camelina</th>
<th>Hemp</th>
<th>Marnoch Oriental Mustard</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPI</td>
<td>78</td>
<td>92</td>
<td>84.8</td>
<td>87</td>
<td>67</td>
<td>97</td>
<td>95</td>
</tr>
<tr>
<td>SPI</td>
<td>58</td>
<td>69.6</td>
<td>46</td>
<td>83</td>
<td>42</td>
<td>45</td>
<td>72</td>
</tr>
<tr>
<td>MR</td>
<td>33.5</td>
<td>40.5</td>
<td>38.3</td>
<td>13.6</td>
<td>41</td>
<td>11</td>
<td>36</td>
</tr>
</tbody>
</table>

The solids recovery into precipitated protein isolate and soluble protein isolate was the highest for those obtained from Estonian rapeseed flour, 18.7% and 17.4%, respectively (Table 13). The same applied to protein recovered in Estonian rapeseed flour PPI and SPI, 35.3% and 31.3%, respectively (Table 14).

However, the solids and protein recovery into the II batch of hemp PPI were even higher (Tables 13, 14). This was the first time ever to use the membrane systems to produce protein isolates from hemp. The precipitated protein isolate of hemp had the highest protein concentration (97%) among the final products recovered in the current study, which was comparable to that previously achieved with oriental mustard (95%) (Table 15). The previous study of Teh et al. (2013) obtained a similar protein concentration (94.6%) employing alkali extraction (without membrane systems), but supposedly the current study was the first to produce the soluble protein isolate (SPI) of hemp. Although the protein recovery into the first batch of hemp PPI was comparable (34.5%) to that obtained from Estonian rapeseed flour, the protein recovery from hemp SPI was much lower (3.9%). This correlated to the small amount of hemp SPI recovered, as well as the lower protein concentration in hemp SPI (45%), compared to the SPI obtained from the Estonian rapeseed flour (83%) (Table 15). The protein recovery into canola PPI and SPI was...
comparable to mucilage-free camelina (Table 14).

The Estonian rapeseed flour had the lowest percentage of solids remaining in the less valuable product, meal residue, since the starting material didn’t contain any hulls. On the contrary, camelina, which contained both the hulls and the mucilage, had the highest percentage of meal residue among the seeds investigated in the current study. However, the previously investigated Finnish and Estonian rapeseed had even higher percent of meal residue (Table 13). The crude protein concentration of hemp and camelina meal residue were 11% and 41%, respectively. For the comparative starting materials, canola seed and Estonian rapeseed flour, the protein values in meal residue were 33.5% and 13.6%, respectively.

The higher the solids recovery into meal residue was, the higher part of protein was recovered into less valuable meal residue (Table 14). Therefore, dehulling (i.e. Estonian rapeseed flour) would enable the higher solids and protein recovery into the protein isolates.

While 4.77% of oil was present in the Estonian rapeseed flour, 3.57% was found in the precipitated isolate and the remaining oil could be lost during membrane processing or into meal residue. The oil content in permeates or meal residue wasn’t analyzed (Table 16).

The previous studies show that the cold - defatted meals have a higher nitrogen extractability than the heat treated, commercially, or Soxhlet defatted meals. The lower nitrogen extractability can be attributed to protein denaturation due to the exposure to hexane boiling temperature (68°C) for 24 hours. Gillberg and Törnell, (1976) demonstrated that heat treatment at 90°C from 0 to 20 min. reduced the nitrogen extractability at pH11 from 94% to 87% and boiling at 100°C for 4 min. reduced it to 43%. This explains the higher protein recovery in the case of Estonian rapeseed flour, which was cold defatted.
<table>
<thead>
<tr>
<th>Protein (Nx6.25)%db</th>
<th>PPI</th>
<th>SPI</th>
<th>MR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starting material</td>
<td>47.3</td>
<td>86.9</td>
<td>82.4</td>
</tr>
<tr>
<td>Moisture (%)</td>
<td>5.7</td>
<td>3</td>
<td>5.1</td>
</tr>
<tr>
<td>Oil (%)</td>
<td>4.8</td>
<td>3.6</td>
<td>NA</td>
</tr>
<tr>
<td>*Phenolics</td>
<td>2159</td>
<td>70.5</td>
<td>67.5</td>
</tr>
</tbody>
</table>

Table 16 Composition of Starting Material and Final Products of Estonian Rapeseed

<table>
<thead>
<tr>
<th>Protein (Nx6.25)%db</th>
<th>PPI</th>
<th>SPI</th>
<th>MR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starting material</td>
<td>37.8</td>
<td>78</td>
<td>58.2</td>
</tr>
<tr>
<td>Moisture (%)</td>
<td>5.5</td>
<td>4.2</td>
<td>6.5</td>
</tr>
<tr>
<td>*Phenolics</td>
<td>1430</td>
<td>118</td>
<td>177</td>
</tr>
</tbody>
</table>

Table 17 Composition of Starting Material and Final Products of Canola

<table>
<thead>
<tr>
<th>Protein (Nx6.25)%db</th>
<th>PPI</th>
<th>SPI</th>
<th>MR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starting material</td>
<td>36.6</td>
<td>67</td>
<td>41.9</td>
</tr>
<tr>
<td>Moisture (%)</td>
<td>8.0</td>
<td>0.9</td>
<td>3.7</td>
</tr>
<tr>
<td>*Phenolics</td>
<td>222</td>
<td>91</td>
<td>89</td>
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</table>

Table 18 Composition of Starting Material and Final Products of Camelina

<table>
<thead>
<tr>
<th>Protein (Nx6.25)%db</th>
<th>PPI</th>
<th>SPI</th>
<th>MR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starting material</td>
<td>35.4</td>
<td>97.2</td>
<td>45.1</td>
</tr>
<tr>
<td>Moisture (%)</td>
<td>9.6</td>
<td>8.2</td>
<td>8</td>
</tr>
<tr>
<td>*Phenolics</td>
<td>269</td>
<td>24</td>
<td>NA</td>
</tr>
</tbody>
</table>

Table 19 Composition of Starting Material and Final Products of Hemp

* Phenolics – the results given in mg sinapic acid equivalents/100g sample

NA – not analyzed
Phenolic substances are the main obstacle to incorporating the proteins of oilseeds into food. Kappak (2004) showed that approximately 94% of total phenolic compounds in the starting material (Finnish rapeseed) are extracted into the alkaline solution. The addition of sodium chloride and further heat treatment at 55-60°C for 30 min. breaks the bonds between protein molecules and phenolics, reducing the amount of sinapic acid bound to proteins (Xu and Diosady, 2000). The ultra- and diafiltration lower the phenolics content substantially. The phenolics content in permeates was not analyzed, but the final products of hemp and canola contained approximately 4-9% of phenolics compared to that in the starting materials (Tables 16-19). Although only approximately 60% of phenolic compounds were removed from camelina isolates, they contained the comparable amount of phenolics, as the starting value was already much lower than in canola and rapeseed. Phenolic analysis results are average in triplicates in the case of the starting materials. While there was a lack of material, the average of two replicates was taken into account in the case of the final products. The phenolic content was not analyzed in meal residue, as the value of meal residue is much lower and here the phenolic content is not as important as in the isolates.

The composition of starting materials and final products is shown in Tables 16 to 19. The mass balances in Appendix A.6 show also the approximate protein contents of the permeates. Marnoch (2004) has claimed that 10 to 15% of the total nitrogen in the seed is the non-protein nitrogen, however using the 1kDa membrane could recover (<5 kDa) proteins into the isolates. The unrecovered part of solids also represents several analytical and transfer losses between glassware and centrifuge tubes, and also filtration. A remarkable part of material outflow occurred during washing of the membrane, as by the “Instruction Manual” of membrane, 70 ml can remain in the tubing.
4.5.2.2 Water and Oil Absorption Properties

Besides nutritional value, the quality of food proteins is often determined by their functional properties, among others water and oil absorption, which affect their behaviour during food processing and in end products. These characteristics can surmount the importance of nutritional value, in case the proteins are targeted to function as performance ingredients in foods. The functionality of rapeseed protein fractions is greatly affected by the processes used for oil and protein extraction, which influence the folding state of protein and the composition of protein extracts (Manamperi et al., 2011).

In the current study, water and oil absorption properties of hemp and camelina were investigated, comparing the results to those of Estonian rapeseed. The water-binding properties of a protein determine its degree of interaction with water. Sosulski et al. (1976) reported that rapeseed protein products produced by different extraction methods had high water absorption capacity. However, in this study it was found that the WAC value for the Estonian rapeseed PPI was low, which could be explained by the presence of oil (3.77%) in the isolates (Table 16). The physical-chemical environment (oil) influences significantly the water binding properties of proteins, and might also suggest low availability of polar amino acids, which are the primary sites for water interaction with proteins (Sathe et al. 1982). This value is comparable to those reported by Hijar (2013) for PPI (131%) produced from yellow mustard flour, which contained a low amount of oil (7.7%), but it is also lower than the highest value obtained with the camelina PPI. Estonian rapeseed SPI showed low WAC values, presumably because of some oil residue (Table 20).

In the trial with hemp SPI most of the soluble isolate dissolved in the added portion of water, and only approximately 20% of initial material remained in the test tube. So there was no weight gain for the remaining part of sample, and the previously used
calculation gave a negative WAC value. In the case of water-soluble material, the procedure should be focused on the dissolved part, which contains all the water and most of soluble material. For example, 100% camelina SPI absorbed the entire water making solution. Similarly, 80% of hemp SPI absorbed the entire water.

The mechanism of oil absorption, according to Kinsella (1976), relies mostly on the physical entrapment of oil by capillary attraction. However, in the case of rapeseed isolates, the increase of fat absorption may also be explained by the presence of a high concentration of hydrophobic groups on the surface of the protein molecules, which have a great affinity for oil. The hydrophobicity of proteins is likely to play a major role in fat absorption.

\[
\text{Sample} \quad \text{WAC} = \frac{W_f - W_i}{W_i} \times 100\% \quad \text{Reference}
\]

<table>
<thead>
<tr>
<th>Sample</th>
<th>WAC = \frac{W_f - W_i}{W_i} \times 100%</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemp PPI</td>
<td>141%</td>
<td>Current study</td>
</tr>
<tr>
<td>Estonian Rapeseed PPI</td>
<td>138%</td>
<td>Current study</td>
</tr>
<tr>
<td>Camelina PPI</td>
<td>158%</td>
<td>Current study</td>
</tr>
<tr>
<td>Spanish Rapeseed PPI</td>
<td>131%</td>
<td>Vioque et al. (2000)</td>
</tr>
<tr>
<td>Soybean Supro 670</td>
<td>241%</td>
<td>Liadakis et al. (1998)</td>
</tr>
<tr>
<td>Chinese Rapeseed PPI</td>
<td>219%</td>
<td>Xu et al. (1994)</td>
</tr>
<tr>
<td>Hemp SPI</td>
<td>80% absorbed in water</td>
<td>Current study</td>
</tr>
<tr>
<td>Rapeseed SPI</td>
<td>99%</td>
<td>Current study</td>
</tr>
<tr>
<td>Camelina SPI</td>
<td>solution</td>
<td>Current study</td>
</tr>
<tr>
<td>Camelina freeze-dried mucilage</td>
<td>solution</td>
<td>Current study</td>
</tr>
</tbody>
</table>

Table 20 Water Absorption Capacity

<table>
<thead>
<tr>
<th>Sample</th>
<th>OAC = \frac{W_f - W_i}{W_i} \times 100%</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemp PPI</td>
<td>104%</td>
<td>Current study</td>
</tr>
<tr>
<td>Rapeseed PPI</td>
<td>77%</td>
<td>Current study</td>
</tr>
<tr>
<td>Camelina PPI</td>
<td>107%</td>
<td>Current study</td>
</tr>
<tr>
<td>Spanish Rapeseed</td>
<td>66%</td>
<td>Vioque et al. (2000)</td>
</tr>
<tr>
<td>Chinese Rapeseed PPI</td>
<td>256%</td>
<td>Xu et al. (1994)</td>
</tr>
<tr>
<td>Soybean Supro 670</td>
<td>210%</td>
<td>Liadakis et al. (1998)</td>
</tr>
<tr>
<td>Hemp SPI</td>
<td>130%</td>
<td>Current study</td>
</tr>
<tr>
<td>Rapeseed SPI</td>
<td>98%</td>
<td>Current study</td>
</tr>
<tr>
<td>Camelina SPI</td>
<td>solution</td>
<td>Current study</td>
</tr>
<tr>
<td>Camelina freeze-dried mucilage</td>
<td>solution</td>
<td>Current study</td>
</tr>
</tbody>
</table>

Table 21 Oil Absorption Capacity
The oil absorption capacities (OAC) of hemp and camelina PPI were very similar, but the absorption value of rapeseed PPI was lower than all of the isolates that were compared (Table 21). The comparative OAC values suggest that these proteins have similar surface hydrophobicity, since OAC can reflect the ability of the hydrophobic groups of proteins to interact with the lipids. The OAC of rapeseed SPI was significantly lower than that of the hemp soluble protein isolate, but it was higher than that of rapeseed PPI (Table 21). Camelina SPI absorbed oil completely.

In order to know more about the functional properties of camelina freeze-dried mucilage, the WAC and OAC were investigated. The results demonstrate that the water holding capacity and oil holding capacity of camelina mucilage is similar to camelina SPI, becoming solutions in both water and oil, so that they cannot be separated by centrifugation. The same behaviour appeared in previous work with xanthan gum and guar gum, which have been used as hydrocolloids in the food industry to enhance viscosity and stability in many food products (Singer et al., 2011).

4.5.2.3 Colour Analysis of Final Products

For colour analysis the samples were ground, put into Petri dishes and digitally photographed (Fig.21). The photos were pre-processed in Image J software, to obtain the RGB (red, green and blue) values. The data were processed using EasyRGB software to retrieve Hunter $L, a, b$ colour values from the photographs. The Hunter System was designed by Hunter (1942) for the measurement of opaque surface colours. When Hunter $a$ attribute is positive, the colour has redness; when negative, greenness. Similarly, when $b$ is positive the colour has yellowness; when negative, blueness. The third Hunter attribute was $L$ for lightness. The maximum for $L$ is 100, which would be a perfect reflecting diffuser. The minimum of $L$
would be zero, which would be black.

Soy PPI  
Hemp PPI  
Camelina PPI  
Canola PPI (A)  
Canola PPI (B)  
Canola PPI (C)  
Rapeseed PPI  
Hemp SPI  
Rapeseed SPI  
Canola SPI(A)  
Camelina SPI  
Spray-dried camelina mucilage
Freeze-dried camelina mucilage  
Dried directly after washing out on the 1 mm mesh  
Dried after washing out and centrifuging for the final separation of mucilage solution and the seeds

Figure 22 Samples of Final Products for Colour Analysis
A more exact description of the method is given in Appendix A.5.

According to Table 22 the soy PPI has the highest $L$ value of 97.8 among all 11 isolates presented, thus it is the lightest and the best example to achieve. The $L$ value of hemp PPI, 89.8 is comparable to soy PPI due to the lightness, as well as the negative $a$ value of -6.2 and -2.0, respectively, indicating the greenness probably caused by the colour of chlorophyll. However, all the other isolates have positive $a$ values, which show the redness, although rapeseed PPI and rapeseed SPI have very low $a$ value of 0.4 and 0.5, respectively.

Estonian rapeseed PPI and SPI have the highest lightness $L$ values among the other canola isolates. It could be caused by the absence of the hull in the starting material, which usually contains a remarkable amount of phenolic compounds and other dark colour components. At the same time rapeseed isolates have rather high $b$ values, which are comparable to these values of the other lighter canola and camelina isolates – all from the Brassica family.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>$L$</th>
<th>$a$</th>
<th>$b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soy PPI</td>
<td>97.8</td>
<td>-6.2</td>
<td>12.9</td>
</tr>
<tr>
<td>*Hemp PPI</td>
<td>89.8</td>
<td>-2.0</td>
<td>18.8</td>
</tr>
<tr>
<td>*Rapeseed PPI</td>
<td>80.4</td>
<td>0.4</td>
<td>31.2</td>
</tr>
<tr>
<td>*Camelina PPI</td>
<td>67.7</td>
<td>12.1</td>
<td>32.6</td>
</tr>
<tr>
<td>*Canola PPI (A)</td>
<td>30.1</td>
<td>14.5</td>
<td>15.2</td>
</tr>
<tr>
<td>Canola PPI (B)</td>
<td>33.4</td>
<td>10.2</td>
<td>16.9</td>
</tr>
<tr>
<td>Canola PPI (C)</td>
<td>78.1</td>
<td>7.8</td>
<td>29.7</td>
</tr>
<tr>
<td>*Hemp SPI</td>
<td>83.0</td>
<td>2.2</td>
<td>29.7</td>
</tr>
<tr>
<td>*Rapeseed SPI</td>
<td>82.1</td>
<td>0.5</td>
<td>29.8</td>
</tr>
<tr>
<td>*Camelina SPI</td>
<td>68.6</td>
<td>9.6</td>
<td>32.0</td>
</tr>
<tr>
<td>*Canola SPI (A)</td>
<td>73.8</td>
<td>5.9</td>
<td>30.2</td>
</tr>
</tbody>
</table>

**Table 22 Colour Analysis of Protein Isolates**

Hunter $L$, $a$, $b$ values of soy, hemp, camelina, and different canola isolates.  
*Protein isolates obtained during current study.

Canola PPI (A) has the lowest $L$ value of 30.1, which is comparable to the $L$ value of canola PPI (B), 33.4. It is visually seen that these isolates are the darkest. The
same isolates show also the highest $a$ values of 14.5 and 10.2, respectively, however camelina PPI and SPI $a$ values are comparable, 12.1 and 9.6, respectively.

There are several possible explanations of the darkness and redness of these isolates. The main reason is too long period of time for membrane processes, which allowed the colour components to bind more to the proteins and, therefore, be not removed during ultrafiltration, but rather concentrated, producing a darker isolate at the end.

In the current study the longest filtration procedures were conducted with canola (A) and (B) solutions, both more than 20 hours and 2-3 nights sitting under the fume hood. These processes resulted in the darkest isolates. The long period was caused by the large batches of starting material, with resultant fouling and the need to change the membrane.

This was the first try to use *Camelina sativa* for production of protein isolates. The high values of $L$, $a$ and $b$ of both PPI and SPI can be caused by the specific colour of camelina, which is inherent to this crop.

Although the one purpose of membrane processing is to remove the colour components from isolates, Marnoch (2004) in her study observed that the protein products were lighter in colour without membrane processing. A significant darkening of the extract was observed during the heating (Marnoch, 2004), which is used in order to improve the astringent taste of the final products, dissociating phenolic-protein bonding.

In the current study darkening was observed during the freezing in the freezer, which could be caused by the slowness of this procedure. It means that the wet precipitate and soluble protein isolate are exposed to pollutants for a longer period of time and the colour forming reactions proceed before the compounds are completely frozen. Here rapid freezing with liquid nitrogen could help.
Among the dried mucilage samples $L$ value shows the highest level of 93.0; therefore, the spray-dried sample is the lightest (Table 23). The $L$ value of centrifuged sample is the lowest; however, the difference between $L$ values of A and B samples are marginal compared to the C, the spray-dried sample. Consequently, the lightness of the sample is influenced by the drying process.

<table>
<thead>
<tr>
<th>Mucilage</th>
<th>$L$</th>
<th>$a$</th>
<th>$b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>84.5</td>
<td>-0.1</td>
<td>28.1</td>
</tr>
<tr>
<td>B</td>
<td>81.9</td>
<td>4.0</td>
<td>33.1</td>
</tr>
<tr>
<td>C</td>
<td>93.9</td>
<td>-6.2</td>
<td>18.0</td>
</tr>
</tbody>
</table>

Table 23 Colour Analysis of Freeze-and Spray-dried Mucilage

Hunter $L, a, b$ values of following mucilage samples: A-freeze-dried directly after washing out on the 1 mm mesh; B-freeze-dried after centrifuging for the final separation of remaining mucilage solution from the seeds; C-spray-dried directly after washing out.

The values of $a$ indicate that the samples which were dried immediately after washing out on the mesh, were greenish, especially the freeze-dried one with $a$ value of –6.2 compared to the freeze-dried sample, with $a$ value of -0.1. The greenness can be explained by the colour of chlorophyll.

The centrifuged sample showed a positive $a$ value, which means the redness, which probably could be caused by colour components of the seeds dissolved in the mucilage solution during centrifugation. The yellow tonality $b$ is inherent in all dried seed products.

The data of the three parameters evaluated show that the colour of the camelina mucilage powder dried immediately after washing is greenish yellow with high lightness. The centrifuged sample is rather light orange.
5.0 CONCLUSIONS

- A process for obtaining precipitated and soluble isolates from camelina and hemp seed meal was established. The best conditions for protein extraction for both oilseeds was at pH 11, and for isoelectric precipitation at pH 5. However hemp proteins may be precipitated at higher pH, e.g. 6, requiring less acid. Due to the high content of mucilage in camelina, it must be removed prior to aqueous processing and a higher water-to-seed ratio must be used for protein extraction.

- The experiments demonstrated that the solubility of hemp and camelina proteins was 79% and 65% of starting material at pH 11, respectively. Estonian rapeseed had the best recovery into the two isolates, followed by hemp, canola and camelina. Hemp PPI had the highest protein concentration, 97%. Other protein concentrations in PPI were between 73 and 87 percent, with values in SPI ranging from 42% to 83%.

- Hemp PPI had the lowest content of phenolics, 24mg sinapic acid per 100g sample, followed by Estonian rapeseed PPI and SPI and Camelina PPI and SPI. The highest content of phenolics was in canola PPI and SPI, 118 mg and 177mg sinapic acid per 100g sample, respectively.

- Camelina PPI had the highest water and oil absorption capacity (WAC) values, followed by hemp isolates. The isolates made of Estonian rapeseed flour had
the lowest WAC and OAC values among all investigated isolates. Camelina SPI absorbed both water and oil completely.

- The colour of the SPI-s was lighter than that of the PPI-s. The lightest among the PPI-s was hemp PPI, followed by rapeseed and camelina PPIs and finally canola PPI being very dark as it was exposed to high alkali for a long time.

- Extraction of the mucilage from the whole camelina seed can be achieved through mixing the seed with 10 parts of 55°C water for one hour and separation by screening and centrifugation. Up to 10% of the seed mass may be recovered as mucilage within an hour.

- The viscosity measurements of dried and redissolved mucilage showed the highest values at natural pH and the viscosity increased rapidly above 1% solids concentration in aqueous solution.

- Camelina mucilage forms solutions with both water and oil. Similarly to the xanthan gum and guar gum, it can be used as hydrocolloid in food industry to enhance viscosity and stability in many food products.

- The long exposure to strong alkali, pH11, causes an undesirable dark colour of isolates. Therefore, the processes in alkaline environment should be completed as rapidly as possible.
6.0 RECOMMENDATIONS

- In order to avoid darkening the final products, the membrane procedures must be done as fast as possible and successively, using brand new membranes for every new material, or a larger membrane area.
- Dehulling the starting material should be considered prior to processing for all seeds.
- The previously used procedure for defatting seeds with the Soxhlet apparatus for 24 hours at hexane boiling point, 68°C, will impair the quality of proteins. The best way of defatting would be multistage washing of the material with hexane.
- Analysis of the carbohydrate composition in camelina mucilage, as well as the Molecular weight distribution of proteins should be conducted for both hemp and camelina isolates.
- Functionality tests, such as gelation, emulsifying and foaming properties should be conducted with all isolates and camelina mucilage.
- Glass transition temperature of camelina mucilage should be determined.
- Work should be done to find applications for camelina mucilage.
- To add value to permeates, treatment methods for the permeates need to be worked out in order to recover useful components and remove harmful substances.
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8.0 APPENDICES

A.1 Moisture Analysis (AACC Method 44-15A)

1. Weigh an aluminum foil tray and a sheet of foil with holes punched into it to be used as the cover, wt

2. Tare tray and weigh out samples of at least 2g in the aluminum foil tray.

3. Cover and reweigh, tray + sample, wb.

4. Put samples in oven at 105 °C for overnight to dry.

5. Remove the samples from the oven and place directly into a desiccator. Allow to cool to room temperature.

6. Reweigh the cooled samples, wa.

7. Moisture content (%) = \( \frac{wb-wa}{wb-wt} \times 100\% \)

A.2 Oil Extraction and Analysis (AOCS Method Ba-38)

1. Weigh a cellulose extraction thimble and weigh 50-60 g of ground oriental mustard in it. (Weight of the thimble and sample is the initial weight).

2. Place the thimble in the Soxhlet glassware and connect to condenser.

3. Add 300ml of hexane to a 500ml round flask, connect to unit, and put on heating mantle.

4. Start condenser water running until cool, then turn on heat to 70 °C. 5. Allow extraction to continue for a total of 24 hours.

6. Cool, remove thimble. Allow hexane to evaporate in the fume hood and weigh. (This is the final weight).

7. Recover hexane by rotavapour, and weigh remaining oil.

\[
\text{Oil content} = \left( \frac{\text{initial weight} - \text{final weight}}{\text{initial weight} - \text{weight of thimble}} \right) \times 100\%
\]
A.3 Protein Analysis (Kjeldahl Method)

Sample digestion

1. Preheat the Digestion unit (Buchi 425) to setting 4.

2. Weigh out each solid sample onto nitrogen free paper and fold to prevent spilling. Put each sample into a separate labeled digestion tube. Weigh liquid samples directly into digestion tube.

3. To each tube add: 4 Kjeldahl Tablets and 25 mL of conc. H₂SO₄.

4. Clamp the suction manifold onto the 4 digestion tubes ensuring that the clamps are solidly secured. Insert the suction tube into the end of the manifold and a tuft of glass wool into the other to allow air passage through the manifold. Turn on the aspirator attached to the suction tube and lift the manifold and digestion tube assembly out of the rack and place into the digester (manifold resting on the stands).

5. Heat on setting 4 for 20 minutes. Raise the temperature to setting 6 for 10 minutes or until the foam subsides and the air in the tube starts to show some mist. Then turn the setting up to 10 (full power) and digest for 35 minutes ensuring that the walls of the glass are clean and that the solution is colourless (or very pale yellow) for at least 5-10 minutes before taking off the heat.

6. Remove tubes from the digester and place in a rack with the suction continuing until the solution is cool. (Once the solution is sufficiently cool, the suction tube can be removed and the rack moved into a fume hood to finish cooling) The tubes should cool for at least 15 minutes.

Sample analysis

7. Warm up the distillation unit (Buchi 315). Turn on the unit, turn on the water (full) and close the stopcock leading from the heater to the drain. Place a tube filled with water into the distillation unit and a 500 mL flask under the condenser outlet.

8. After the lower section of the heater fills up to the bottom of the heater plates, close the drain valve on the front and open the steam valve allowing steam to bubble into the tube.

9. When about 350 mL of distillate has been collected, remove the flask and empty the water, replacing it with 200 mL of distilled water. Before replacing the flask under the condenser outlet, turn off the steam flow. When the water starts to be sucked back into the steam chamber, replace the water flask to allow clean water to be sucked through the system. Open the drain valve on the front once flushing is complete. Lower the digestion tube from the steam inlet straw so that the water in the
collection chamber flows out the drain. Once the collection/steam chamber has been emptied remove the digestion tube.

10. Prepare 4 Erlenmeyer flasks each with 50 ml 4% w/w Boric Acid solution and 4 drops of N-Point indicator, (two stage indicator - green for basic and red (pink) for acidic.

11. Place each digestion tube in the distillation unit, and a receiving flask under the condenser. Then add 32% NaOH solution to the digestion tube until the total volume of solution turns from a clear liquid to a dark cloudy liquid. Set the time of distillation to 5 min.

12. Titrate the boric acid solution to the same shade of pink as that in the blank using O.1ON H₂SO₄.

Calculate the nitrogen content using the following equation

\[
\text{% Nitrogen} = \frac{1.4007 \times N \times (V_1 - V_0)}{W},
\]

where \(V_1\) is the volume of titrant (ml) used for the sample, \(V_0\) is the volume of titrant (ml) used for the blank. \(N\) is the acid normality used for titration, \(W\) is the sample weight in grams.

13. Calculate the protein content considering that the average nitrogen (N) content of proteins is about 16% and 1/0.16 = 6.25, thus use the calculation

\[
\text{%P} = \text{%Nitrogen} \times 6.25,
\]

to convert the nitrogen content into protein content.

A.4 Total Phenolic Content in Meals(Xu, 2000)

Determination in meals

a) All samples are first hexane-defatted in a Soxhlet apparatus for 24 h and air-desolventized overnight.

b) Acetone extraction:

b.1) Place 2g of defatted mustard flour in a flat-bottom flask. Add 50mL of 60% acetone (acidified to pH 3 with trichloroacetic acid), and 3 boiling stones

b.2) Reflux for 30min. (30 min from once the mixture boils. Boiling should be controlled at all times)

b.3) Cool down the sample. Shake the cool liquid and pour it in a 50 ml centrifuge tube
b.4) Centrifuge for 10 min at 1800 \times g

b.5) Collect the supernatant in a separate flat-bottom flask.

b.6) Add 50mL of the acidified acetone to the precipitate (residue) and return it to its corresponding reflux flask. Repeat from b.2 twice. (the solid sample needs to be refluxed with acidified acetone and centrifuged a total of 3 times). Combine the corresponding supernatant.

b.7) Evaporate the acetone from the extract (supernatant) using a rotavapor at 50°C

c) Alkaline hydrolysis:

c.1) Place the acetone-free extract in an Erlenmeyer flask. Add 20mL of 4M NaOH and a magnetic stirrer. Treat the solution for 4h under a N₂ blanket with constant stirring.

c.2) The residual meal obtained after the acetone extraction is also treated with 20 mL of 4M NaOH under N₂ for 4h.

c.3) After 4h, acidify the mixtures described in c.1 and c.2 to pH 2 with concentrated HCl. For this, empty the hydrolysed mixture in a beaker (with magnetic stirrer). For the residual meal mixture (c.2); after acidification, centrifuge the mixture for 10 min at 1800 \times g.

c.4) Combine the supernatant of the residual meal with its corresponding acidified extract obtained earlier (c.1), and dilute the solution to 200 mL.

d) Ethyl acetate/diethyl ether extraction of phenolic acids:

d.1) Prepare a solution 1:1 (v/v) of ethyl acetate/diethyl ether (EA/DE). Measure the same volume of each reagent in separate volumetric flasks, then pour them together into a beaker with constant stirring.

d.2) Place 25 mL of the diluted acidified solution in a Mojonnier extraction flask, and add 50mL EA/DE solution. Mix for 30s, then let it settle.

d.3) Collect the top layer. Add 50mL EA/DE solution, and extract again for a total of 6 times.

d.4) Combine the corresponding organic extracts (top layers) in a beaker, and evaporate to dryness at room temperature under the fumehood.

e) Colorimetric assay:

e.1) Redissolve the dried organic extract in 50mL of MeOH. Make sure to recover all the crystals.
e.2) Dilute 5mL of the MeOH solution described in e.1) to 100mL with distilled water.

e.3) Place 7mL of the solution described in e.2 in a 10mL volumetric flask. Add 0.5mL of Folin-Denis reagent. Wait 3 min, then add 1mL saturated Na$_2$CO$_3$ and shake. Wait 1h.

e.4) Read the absorbance of the coloured solution at 725nm. Report the total phenolic content in mg sinapic acid equivalents/100 g of sample.

**A.5 Colour Analysis**

**PRE-PROCESSING USING IMAGE J - SOFTWARE**

1. Select the image file that you want to analyse (JPG, PNG, GIF, or BMP).

2. Save the image in BMP format for image analysis while saving from the microscope. The images are generally saved as JPG format cell phone or digital camera.

3. Go to Image J – File – Open

4. Select “colour picker” icon from the Image J pallet – this will give you coordinates for a point in the image in RGB coordinates.

5. Note down the RGB value for at least 25-50 data points on the image

6. Enter individual data value in the colour calculator and copy the transformed data in different colour value formats.

7. Analysis your data

**PROCESSING USING EasyRGB SOFTWARE**

1. The RGB data obtained from Image J software is entered into EasyRGB software to obtain colour values.

2. Go to EasyRGB – colour calculator

3. Select setting options to RGB 0-255, daylight illumination and observer 10°.

4. Enter the RGB values in Data 1,2,3

5. Click start to calculate the colour values. Use Hunter colour values.
   1) Select a point on the image using colour picker
   2) Insert the RGB value to colour calculator and get colour output
   3) Analyse the data
      - Hunter L,a,b colour space is organized in a cube form
      - Max. for L axis=100 (perfect reflecting diffuser)
      - Min. for L axis=0 (black colour)
• a and b axes have no specific numerical limits

The diagram representing this is as follows:

\[(+)a=\text{red} \quad (-)a=\text{green} \quad (+)b=\text{yellow} \quad (-)b=\text{blue}\]

![Diagram of L, a, b colour space as conceived by Richard S. Hunter](image)

Figure 23 L, a, b colour space as conceived by Richard S. Hunter

### A.6. Mass Balances

<table>
<thead>
<tr>
<th>Product</th>
<th>Dry Matter</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g</td>
<td>%</td>
</tr>
<tr>
<td>Defatted Ground Canola</td>
<td>85.1</td>
<td>100%</td>
</tr>
<tr>
<td>Meal Residue</td>
<td>51.2</td>
<td>60.2</td>
</tr>
<tr>
<td>Ultrafiltration Permeate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diafiltration permeate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precipitated Protein Isolate</td>
<td>5.6</td>
<td>6.6</td>
</tr>
<tr>
<td>Soluble Protein Isolate</td>
<td>8.5</td>
<td>10.0</td>
</tr>
<tr>
<td>Unrecovered</td>
<td>19.8</td>
<td>23.2</td>
</tr>
</tbody>
</table>

**Table 24 Mass Balance of Defatted Ground Canola**
<table>
<thead>
<tr>
<th>Product</th>
<th>Dry Matter</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g</td>
<td>%</td>
</tr>
<tr>
<td>Estonian Rapeseed Flour</td>
<td>141.5</td>
<td>100</td>
</tr>
<tr>
<td>Meal Residue</td>
<td>41.4</td>
<td>29.3</td>
</tr>
<tr>
<td>Ultrafiltration Permeate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dialfiltration Permeate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precipitated Protein Isolate</td>
<td>26.4</td>
<td>18.7</td>
</tr>
<tr>
<td>Soluble Protein Isolate</td>
<td>24.6</td>
<td>17.4</td>
</tr>
<tr>
<td>Unrecovered</td>
<td>49.1</td>
<td>34.6</td>
</tr>
</tbody>
</table>

**Table 25 Mass Balance of Estonian Rapeseed Flour**

<table>
<thead>
<tr>
<th>Product</th>
<th>Dry Matter</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g</td>
<td>%</td>
</tr>
<tr>
<td>Defatted Ground Hemp (I)</td>
<td>180.8</td>
<td>100%</td>
</tr>
<tr>
<td>Meal Residue</td>
<td>86.3</td>
<td>45.9</td>
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<tr>
<td>Ultrafiltration Permeate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dialfiltration Permeate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precipitated Protein Isolate</td>
<td>22.7</td>
<td>12.6</td>
</tr>
<tr>
<td>Soluble Protein Isolate</td>
<td>3.8</td>
<td>2.1</td>
</tr>
<tr>
<td>Unrecovered</td>
<td>68</td>
<td>39.4</td>
</tr>
</tbody>
</table>

**Table 26 Mass Balance of Defatted Ground Hemp (I)**

<table>
<thead>
<tr>
<th>Product</th>
<th>Dry Matter</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g</td>
<td>%</td>
</tr>
<tr>
<td>Defatted Ground Hemp (II)</td>
<td>78.0</td>
<td>100%</td>
</tr>
<tr>
<td>Meal Residue</td>
<td>46.1</td>
<td>59.1</td>
</tr>
<tr>
<td>Ultrafiltration Permeate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dialfiltration Permeate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precipitated Protein Isolate</td>
<td>16.1</td>
<td>20.6</td>
</tr>
<tr>
<td>Soluble Protein Isolate</td>
<td>2.6</td>
<td>3.3</td>
</tr>
<tr>
<td>Unrecovered</td>
<td>13.2</td>
<td>17</td>
</tr>
</tbody>
</table>

**Table 27 Mass Balance of Defatted Ground Hemp (II)**
<table>
<thead>
<tr>
<th>Product</th>
<th>Dry Matter</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g</td>
<td>%</td>
</tr>
<tr>
<td>Mucilage-free Defatted ground Camelina(II)</td>
<td>107.3</td>
<td>100</td>
</tr>
<tr>
<td>Mucilage</td>
<td>10.8</td>
<td>10.1</td>
</tr>
<tr>
<td>Meal Residue</td>
<td>74.1</td>
<td>69</td>
</tr>
<tr>
<td>Ultrafiltration Permeate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diaphragm Permeate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precipitated protein isolate</td>
<td>5.5</td>
<td>5.2</td>
</tr>
<tr>
<td>Soluble Protein Isolate</td>
<td>9.3</td>
<td>8.7</td>
</tr>
<tr>
<td>Unrecovered</td>
<td>7.6</td>
<td>7</td>
</tr>
</tbody>
</table>

**Table 29** Mass Balance of Mucilage-free Ground Camelina

<table>
<thead>
<tr>
<th>Product</th>
<th>Dry Matter</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g</td>
<td>%</td>
</tr>
<tr>
<td>Defatted ground Camelina</td>
<td>92</td>
<td>100</td>
</tr>
<tr>
<td>Meal Residue</td>
<td>59.3</td>
<td>64.6</td>
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<tr>
<td>Ultrafiltration Permeate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diaphragm Permeate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precipitated protein isolate</td>
<td>12.0</td>
<td>13.0</td>
</tr>
<tr>
<td>Soluble Protein Isolate</td>
<td>5.9</td>
<td>9.9</td>
</tr>
<tr>
<td>Unrecovered</td>
<td>22.8</td>
<td>12.5</td>
</tr>
</tbody>
</table>

**Table 30** Mass Balance of Defatted Ground Camelina
A.7. Flow diagrams

Figure 24 Process flow diagram for Estonian Rapeseed Flour
Figure 25 Process flow diagram for production of canola protein isolates
**Figure 26** Process flow diagram for production of hemp protein isolates

- **200.2g defatted ground hemp seed**
- **3599.8g H₂O**
- **20.2g NaOH**
- **washed twice with H₂O (802.1+783.6g)**
- **Meal residue 415.6g**
- **3.5% H₃PO₄ 29.7g**
- **2M H₂O 210.1g liquid**
- **Freeze drying of 56.5g solids**

1. **alkaline extraction 30 min at pH11**
2. **centrifugation 20 min 6500rpm**
3. **filtration of extract**
4. **add 11.6g NaCl**

- **Ultrafiltration CF=5**
  - **4958.1g extract solution**
  - **Permeate 3874.44g**
  - **Retentate 991.62g**

- **Diafiltration DV=2**
  - **Permeate 1983.3g**
  - **Retentate 991.62g**

- **Protein precipitation at pH=5**
  - **Precipitate 51.5g**
  - **Liquid 970.12g**
  - **Combine & wash with H₂O**

- **Ultrafiltration CF=4.1**
  - **1179.92g extract solution**
  - **Permeate 894.4g**
  - **Retentate 285.5g**

- **Centrifugation**
  - **Freeze drying**
  - **Precipitated Protein Isolate 23.7g**

**Losses 23.6g**
**Losses to wash water + filtration 86.94g**
Figure 27 Process flow diagram for production of mucilage-free camelina protein isolates
Figure 28 Process flow diagram for production of camelina protein isolates
Figure 29 Process flow diagram for production of hemp protein isolates (II)
Figure 30 Process flow diagram for production of mucilage-free camelina protein isolates