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**Rapeseed proteins for technical applications – extraction, isolation and modification**

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*“The obstacle on the path becomes the way.”*

*- Marcus Aurelius*

## Preliminary Remarks

The work presented in this doctoral thesis was carried out at the Fraunhofer Institute for Process Engineering and Packaging (IVV) in Freising, Germany. The present thesis forms a compilation of papers published in international peer-reviewed journals. All scientific contributions to journals or congresses which resulted from the period of carrying out this thesis are listed below.

### Full papers

Fetzer, A., Herfellner, T., Stäbler, A., Menner, M., Eisner, P., 2018. Influence of process conditions during aqueous protein extraction upon yield from pre-pressed and cold-pressed rapeseed press cake. Industrial Crops and Products 112, 236-246.

Fetzer, A., Herfellner, T., Eisner, P., 2019. Rapeseed protein concentrates for non-food applications prepared from pre-pressed and cold-pressed press cake via acidic precipitation and ultrafiltration. Industrial Crops and Products 132, 396-406.

Fetzer, A., Hintermayr, C., Schmid, M., Stäbler, A., Eisner, P., 2020. Effect of Acylation of Rapeseed Proteins with Lauroyl and Oleoyl Chloride on Solubility and Film-Forming Properties. Waste and Biomass Valorization.

Fetzer, A., Müller, K., Schmid, M., Eisner, P., 2020. Rapeseed proteins for technical applications: Processing, isolation, modification and functional properties – A review. Industrial Crops and Products 158, 112986.

### Oral presentations

Fetzer, A., Menner, M., Herfellner, T., Eisner, P., 2016. Influence of various process conditions in protein extraction from rapeseed press cake on protein yield and functionality. 12$^{th}$ International Conference on Renewable Resources and Biorefineries. Ghent, Belgium. May 30$^{th}$ - June 1$^{st}$ 2016.

Fetzer, A., Herfellner, T., Eisner, P., 2018. Isolation and modification of side-stream rapeseed proteins from the oil industry as a renewable ingredient for technical applications. 14$^{th}$ International Conference on Renewable Resources and Biorefineries. Ghent, Belgium. May 30$^{th}$ - June 1$^{st}$ 2018.

**Poster presentations**

Fetzer, A., Herfellner, T., Eisner, P., 2018. Isolation and modification of side-stream rapeseed proteins from the oil industry as a renewable ingredient for technical applications. 14th International Conference on Renewable Resources and Biorefineries. Ghent, Belgium. May 30th - June 1st 2018.

Fetzer, A., Herfellner, T., Eisner, P. 2019. Rapeseed proteins for the chemical industry: Extraction, isolation and modification. 15th International Rapeseed Congress. Berlin, Germany. June 16th - 19th 2019.

# CONTENTS

# General Introduction

## 1 Rapeseed – an ongoing Success Story (?)

Rapeseed is now the third most abundant oil crop worldwide after palm and soya, with an annual production of 72.4 million metric tons globally and 20.0 million metric tons in the EU (2018/2019; USDA (2020a)). Due to its ability to grow on a wide variety of soils and in different climate zones, the crop is cultivated on every continent (Figure 1), with Canada, China, and India accounting for more than 60% of global production (USDA, 2020b).

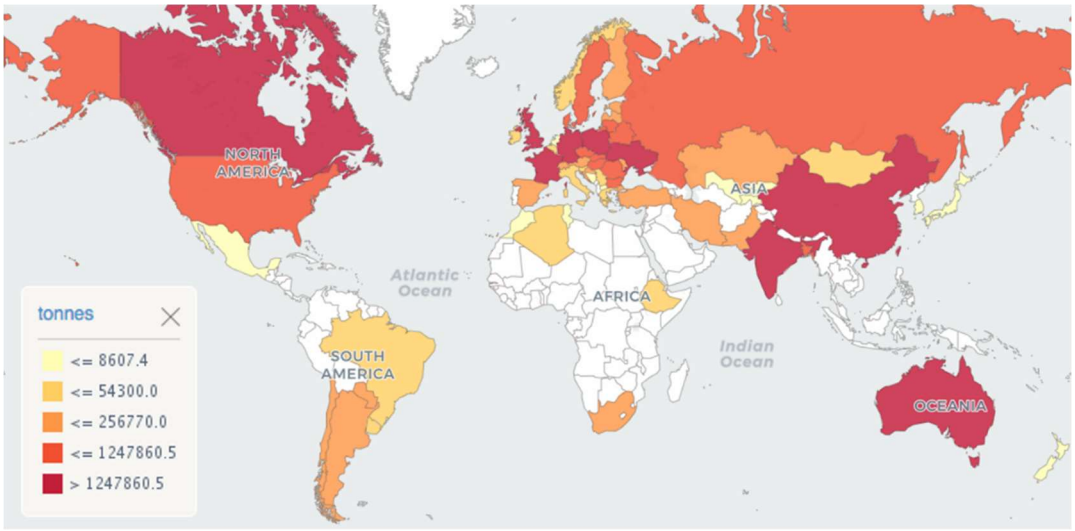


***Figure 1.*** *Annual production quantities of rapeseed by country; 10-year average 2009-2018 (FAO, 2020).*

Today, rapeseed is primarily cultivated for its high oil content, which is used both for food and non-food applications, including the production of biodiesel. However, rapeseed oil has not always had the high-quality standards it is known for today. In fact, it took quite a long journey to make its way to the top three of oil crops.

Rapeseed belongs to the family of Brassicaceae (or Cruciferae), commonly known as the mustard or cabbage family. The origin of the different brassica species is illustrated by the “triangle of U” (Figure 2), named after the Korean-Japanese scientist who first described their relationship (Nagaharu, 1935). The three species *B. nigra*, *B. oleracea* and *B. rapa* form the corners of the triangle as the parental generation for *B. carinata*, *B. juncea* and *B. napus*, which originated by natural hybridization.

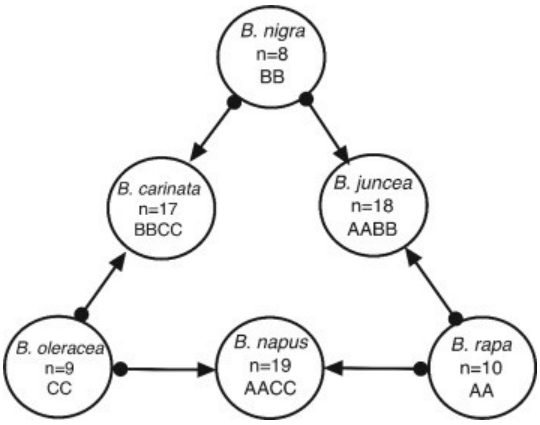


***Figure 2.*** *The „triangle of U" displays the genetic relationship between the different Brassica species (Daun, 2011; Nagaharu, 1935).*

The earliest cultivation of brassica species may date back as much as 7000 years, as is evidenced by carbon dating from the Banpo excavation in Xian, China (Daun, 2011). First mentions of the cultivation of mustard crops were found around 1500 BC and 1122 BC in Sanskrit and Chinese writings, respectively. The plants were probably used as potherbs and vegetables, and their seeds were used as spices and condiments. The latter may have led to the migration of some brassica species around the world. In Europe, descriptions for medicinal use of mustard are found starting from around 500 BC in writings of Pythagoras and Hippocrates (Daun, 2011).

Utilization of brassica plants as an oil crop presumably gained importance much later than their original use as potherbs, vegetables, or spices (Daun, 2011). Initially, rapeseed oil was predominantly used for soap-making and as a lamp oil, given to its slow-burning and relatively odorless properties (Appelqvist and Ohlson, 1972). While in China, Japan and the Indian subcontinent, rapeseed oil had a long history of use as a cooking oil, food utilization in Europe was only relevant for the poor population or in times of shortage of other oils (Kimber and McGregor, 1995). In Germany, cultivation of rapeseed was enforced during World War II due to the lack of imports. In the early 1970s, important research was carried out regarding rapeseed oil quality during processing and the analysis of its composition. Plant breeding efforts led to the generation of species low in erucic acid ("Null-Raps") and glucosinolates ("Doppelnull-Raps"), the main causes for bitterness and pungency. At the same time, breeding efforts in Canada resulted in the birth of canola ("**Can**ada-**O**il-**L**ow-**A**cid"), which should soon become the country's most abundant oil crop (Canola Council of Canada, 2015; Wanasundara et al., 2017). With its improved taste and physiologically highly valuable fatty acid profile, rapeseed oil from new varieties made its way to the third most abundant plant oil world-wide that it is today. With the turn of the century, the emerging production of biodiesel from rapeseed oil further supported the ongoing increase in rapeseed cultivation. In the EU, rapeseed oil still

remains as the predominant source for the production of biodiesel, with the share on the feedstock market peaking at 72% in 2008 (Flach et al., 2019).

Apart from the utilization of rapeseed oil, rapeseed press cake and meal are valuable resources emerging as residual materials during defatting. Due to a high protein content, these materials can be used as animal feed. However, due to the presence of remaining antinutritive components, such as phytic acids and glucosinolates, dosages within the feed mixture are often limited (Nega and Woldes, 2018). Similar problems arise for the production of protein isolates as a nutritional and functional ingredient for human food use. While protocols for the protein isolation from rapeseed press cakes and meals have been established for more than 50 years, the presence of off-flavors in rapeseed protein isolates remains a major hurdle for the commercialization in the food sector (Hald et al., 2019; Linnemann and Dijkstra, 2002; Matheis and Granvogl, 2016; Sghaier et al., 2016). In addition, the identification of rapeseed proteins as human allergens may limit food application (Puumalainen et al., 2006; Puumalainen et al., 2015; Rahman et al., 2020). As an alternative, the interesting functional properties of rapeseed proteins, such as surface active and film-forming properties, may be exploited for utilization in the chemical sector. The potential of rapeseed proteins as a bio-based ingredient in products such as adhesives, coatings, detergents, lubricants and polymers formed the basis of both the research project “TeFuProt” (Technofunktionelle Proteine; EJ (2014)), and the writing of the present doctoral thesis.

While the story of rapeseed has been a story of great success in the last century, this may not apply in particular for the last few years. Due to extreme weather conditions, the increased appearance of pests and diseases along with increased restrictions in regard to the use of pesticides and fertilizers in some countries, farmers around the world were struggling with a significant amount of crop failure (UFOP, 2019). In consequence, the global production of rapeseed dropped from 75.0 million metric tons in 2017/2018 to 68.2 million metric tons in 2019/2020 ((USDA, 2020a)). In face of decreasing crop yields, the importance of valorization of both rapeseed oil and protein in the future was emphasized as one important goal for the rapeseed industry during the 15$^{th}$ International Rapeseed Congress in 2019 (UFOP, 2019). With the imminent threat of reoccurring crop shortages, the importance of using rapeseed’s full potential as not only an oil crop, but in addition, as a source for highly valuable protein is now more relevant than ever.

## 2 Composition of Rapeseed

Rapeseed or canola can refer to any of the species *B. napus*, *B. rapa*, or *B. juncea*. However, the predominant species grown around the world is *B. napus* , which is known as rape, oil rape, oilseed rape, swede rape, Argentine rape (Canada) or canola (Canada) (Daun, 2011; Wanasundara et al., 2016). The chemical composition of rapeseed is displayed in Figure 3,

along with the compositions of press cake and meal, which result from defatting by screw pressing and solvent extraction, respectively.

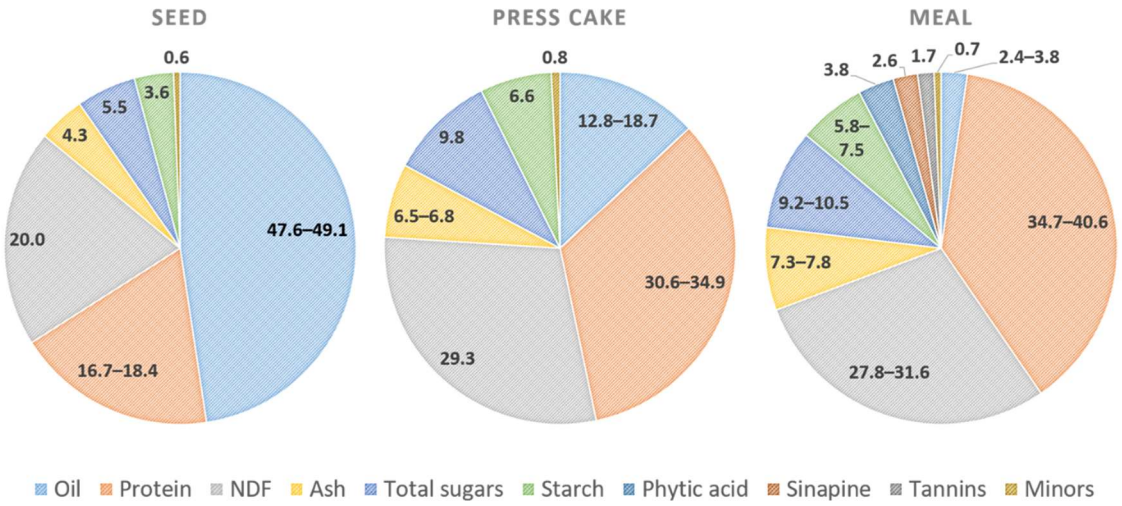


***Figure 3.*** *Chemical composition (dry basis) of rapeseed seed, press cake and meal; data obtained from (Fetzer et al., 2018; INRA CIRAD AFZ, 2020a, b, c; Wanasundara et al., 2017); protein contents calculated with N × 5.7; NDF = neutral detergent fiber; minors include phytic acid, sinapine, tannins (for seed and press cake), phenolic compounds, and glucosinolates.*

After cleaning and conditioning, the seeds of approximately 2–3 mm diameter are first ruptured using roller mills, followed by a cooking step (80–95°C) to inactivate the enzyme myrosinase, which is responsible for the undesired hydrolysis of glucosinolates (Unger, 2011). Additionally, the cooking step facilitates further processing due to the coalescence and beginning separation of oil and protein bodies. Separation of oil is achieved in a first step by screw pressing to remove 80%-90% of the oil. Subsequently, the residual press cake is objected to solvent extraction using isohexane to remove the remaining oil fraction. After removal of the solvent by a so called desolventizer toaster at 107–110 °C and drying, rapeseed meal is received as a side-product of the process (Unger, 2011). Rapeseed oil is received after distillation, degumming and additional refining steps.

### 2.1 Oil

Rapeseed oil presents the primary value fraction of the crop. Modern varieties used for the production of food-grade oil show a high content of unsaturated fatty acids, with a relative fatty acid composition of approximately 62% oleic (18:1), 19% linoleic (18:2) and 10% linolenic (18:3) acid (Barthet and Daun, 2011). Erucic acid in rapeseed oil has been the focus of intensive breeding efforts in the 1970s due to toxicity concerns. Today, the content of erucic acid is reduced to less than 2% in food-grade oil. However, varieties with contents of around 50% in the oil are still of relevance for the chemical industry, where they are used for the manufacture of lubricants, surfactants, plasticizers and coatings (Barthet and Daun, 2011;

Hammond, 2011; Mariliia et al., 2000). Moreover, rapeseed oil of technical grade presents the largest feedstock for biodiesel production in the EU (Flach et al., 2019).

### 2.2 Proteins

The main fraction of rapeseed protein is made up by storage proteins, which constitute up to 80% of the total seed protein content (Mieth et al., 1983). The two main protein families making up for rapeseed storage proteins are cruciferin, a 12S globulin, and napin, a 1.7–2S albumin (Höglund et al., 1992). Due to their different molecular weights, isoelectric points and solubility profiles, cruciferin and napin can be separated by aqueous extraction followed by precipitation and ultrafiltration. The amino acid composition of purified isolates and SDS-PAGE profiles are given in Figure 4.

**A**

| Amino acid | Albumins isolate | Globulins isolate |
|---|---|---|
| Asx | 5.1 | 9.5 |
| Glx | 30.4 | 20.2 |
| Ser | 4.1 | 4.4 |
| His | 4.7 | 5.1 |
| Gly | 1.9 | 1.7 |
| Thr | 4.4 | 4.7 |
| Ala | 3.4 | 3.5 |
| Arg | 8.6 | 9.8 |
| Tyr | 3.7 | 4.5 |
| Cys | 0.1 | 0.0 |
| Val | 4.3 | 3.3 |
| Met | 0.5 (0.6[a]) | 1.2 (1.2[a]) |
| Phe | 3.7 (7.4[b]) | 5.7 (10.2[b]) |
| Ile | 4.3 | 5.3 |
| Leu | 8.5 | 9.1 |
| Lys | 6.2 | 4.7 |
| Pro | 6.4 | 6.8 |
| Trp | n.d. | n.d. |

Asx: aspartic acid + asparagine; Glx: glutamic acid + glutamine. n.d.: Not determined.
[a] Met + Cys
[b] Phe + Tyr.

**B**

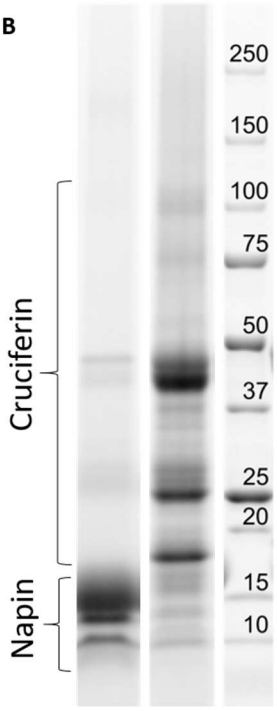


***Figure 4. A:*** *Amino acid composition of purified albumin (napin) and globulin (cruciferin) isolates (adapted from Chabanon et al. (2007) with permission);* ***B:*** *SDS-PAGE (non-reducing) of protein fractions obtained after aqueous extraction and isolation by precipitation and ultrafiltration (adapted from Fetzer et al. (2020)).*

Another major protein class found in the seed is formed by oil body proteins – predominantly oleosins – which are structural protein assisting in the formation of oil bodies (Jolivet et al., 2009; Wanasundara et al., 2016). Contents of oleosins were reported with 2%–8% in the seed (Huang, 1992); however, due to their lipophilic nature, they are generally not reported as constituents of press cake or meals. Nevertheless, oleosins (18–25 kDa) gained some interest in recent decades (Chen et al., 2019b; Jolivet et al., 2009; Tzen, 2012; Wanasundara et al., 2016). Minor protein fractions in rapeseed are presented by lipid transfer proteins, protease

inhibitors, $Ca^{2+}$-dependent-calmodulin binding proteins, thionins and dehydrins (Tan et al., 2011; Wanasundara, 2011).

### *2.2.1 Cruciferin*

Cruciferin is a high molecular weight (300–310 kDa) neutral globulin, which is composed of six subunits, as displayed in Figure 5 (Schwenke et al., 1983; Tan et al., 2011). The subunits are formed by heterodimers, each of which being formed by a heavy alpha-chain (~30 kDa) and a light beta-chain (~20 kDa) linked by a disulfide bond (Schwenke et al., 1983; Wanasundara, 2011). The protein complex is highly susceptible to changes in pH and ionic strength and can undergo reversible and irreversible dissociation. The hexameric 12S complex exists above an ionic strength of 0.5, and undergoes reversible dissociation into 7S timers below that level (Schwenke et al., 1983). Irreversible dissociation and denaturation of cruciferin is induced by acidic conditions or the presence of 4 M urea (Wanasundara, 2011). Additionally, dissociation into monomers can happen at higher concentrations of urea (8 M) together with the presence of sodium dodecyl sulfate (SDS).

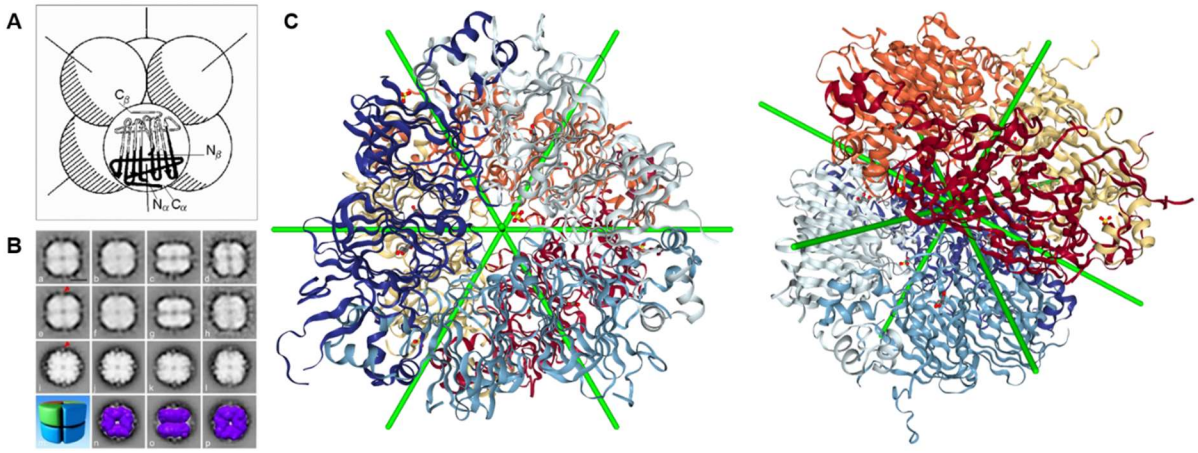


***Figure 5.*** *Structure of the hexameric cruciferin complex;* ***A:*** *model of the subunit arrangement with hypothetical arrangement of α- and β-chains (adapted from Plietz and Damaschun (1986));* ***B:*** *structural analysis by single particle electron microscopy (adapted from Nietzel et al. (2013) with permission);* ***C:*** *crystal structure of procruciferin (PDB ID 3KGL) at two different viewing angles; images created using NGL viewer (Rose et al., 2018; Tandang-Silvas et al., 2010).*

### *2.2.2 Napin*

Napin is a basic albumin with a molecular weight of 12.5–14.5 kDa, which is formed by two subunits (8.4–10 kDa, 3.8–4.5 kDa) linked by two disulfide bonds (Rico et al., 1996; Tan et al., 2011). Additionally, two intra-chain disulfide bonds are present in the large chain (Rico et al., 1996). In contrast to cruciferin, napin exhibits high stability across a wide range of pH values due to a high content of alpha-helical structure and stabilization by disulfide bonds (Schmidt et al., 2004). The schematic structure of napin showing the two intertwined peptide chains is shown in Figure 6.

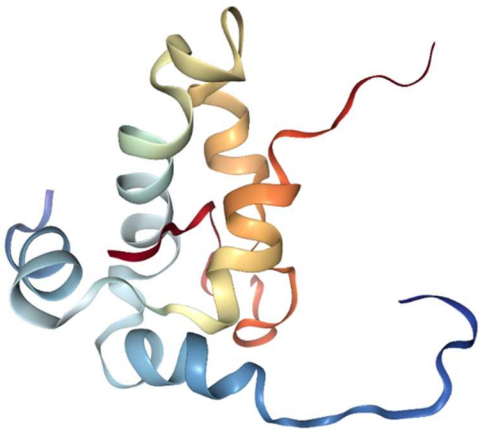

***Figure 6.*** *Structure of napin (PDB ID 1PNB) as obtained from NMR analysis and calculation; image created using NGL viewer (Rico et al., 1996; Rose et al., 2018).*

### 2.3 Carbohydrates

Carbohydrates present another major fraction in the seed of the rapeseed plant. Cellulose and hemicellulose account for the largest portion of this fraction, with a content of around 15%–20% in the seed (Carré et al., 2016; INRA CIRAD AFZ, 2020c). Other polysaccharides in rapeseed include starch and pectins (INRA CIRAD AFZ, 2020c; Jeong et al., 2014). In addition with lignin, polysaccharides have been negatively linked with protein availability in animal feed due to their association (Wanasundara, 2011). This is also a main reason of low protein extractability from conventionally prepared meals, as high temperatures during desolventizing can induce covalent binding of proteins and amino acids to form Maillard reaction products (Fetzer et al., 2018; Mosenthin et al., 2016). Carbohydrates are predominantly found in the seed hull of rapeseed, such that these problems could be reduced by using dehulling technologies (Carré et al., 2016). Due to the high costs associated with rapeseed dehulling, this step is however only carried out for the production of some premium oils (Teutoburger Ölmühle, 2017).

### 2.4 Phytates

Phytates are salts of phytic acid (inositol hexakisphosphate), an organic compound used for the storage of phosphorus in plant seeds. While they only present a minor fraction of the seed, phytates can be concentrated up to 3.3% in defatted meals (Wanasundara et al., 2017). This is particularly problematic, as phytic acid is considered as an antinutrient due to its strong binding of essential dietary minerals, such as calcium, iron and zinc (Wanasundara, 2011). Likewise, phytic acid can undergo association with proteins and can cause precipitation due to its strong ionic nature, especially at pH values below their isoelectric points. Consequently, care has to be taken in choosing the right pH regime for protein extraction to reduce the

interaction with phytic acid. Due to its antimicrobial properties, phytic acid is listed as a food preservative (E391).

### 2.5 Phenolic compounds

Lignin is the major contributor to polyphenolic compounds in rapeseed, with reported contents of 6.4%–9.9% (Carré et al., 2016; INRA CIRAD AFZ, 2020c). Similarly to polysaccharides, lignin contributes to the binding of proteins, such that protein extractability and digestibility are reduced (Wanasundara, 2011). Extractable phenolic compound in rapeseed include free phenolic acids, as well as their esters, and condensed tannins. Sinapine, the cholin ester of sinapic acid, is the major extractable phenolic compound, making up for a content of 80% (Dabrowski and Sosulski, 1984). These phenolic compounds are co-extracted with proteins, especially in alkaline environment, due to strong ionic and hydrophobic bonding (Wanasundara, 2011). Phenolic compounds largely contribute to the dark brownish color and bitter and astringent taste of rapeseed meal and protein preparations extracted therefrom. Moreover, the influence of phenolic binding to physicochemical and functional properties of rapeseed proteins was described (Schwenke and Dąbrowski, 1990; Spencer et al., 1988). As for other polyphenols, antioxidant properties of rapeseed phenols were demonstrated in various studies (Szydłowska-Czerniak et al., 2010; Szydłowska-Czerniak and Tułodziecka, 2014; Vuorela, 2005).

### 2.6 Glucosinolates

Glucosinolates are secondary plant metabolites found in the *Brassicaceae* family, most likely as natural protection against pests and diseases (Bischoff, 2016). The sulphur-containing compounds are responsible for the pungent taste of *Brassicaceae* plants, including cabbage, mustard and horseradish. Recent studies demonstrated beneficial aspects of glucosinolates, including regulation of inflammation, antioxidant, and antimicrobial properties. However, high intake of glucosinolates has long been associated with reduced feed intake and growth as well as antinutritive effects (Barthet and Daun, 2011; Bischoff, 2016). For rapeseed, intensive breeding efforts to reduce the content of glucosinolates presented a key step towards the broad acceptance of rapeseed oil for food utilization (Barthet and Daun, 2011). Today, contents of glucosinolates are greatly reduced. For canola varieties, <30 µmol/g are allowed in defatted meals (Barthet and Daun, 2011). However, residual contents of glucosinolates can still be a limiting factor for feed and food utilization of meal and protein preparations obtained therefrom, due to remaining negative effects on taste and nutritive properties.

## 3 PROTEINS IN TECHNICAL APPLICATIONS

Today, proteins from agricultural feedstock are predominantly used in the feed or food sector, due to their nutritional value and their functionality, including foaming, emulsifying and gelation properties. Utilization of proteins for non-food purposes, such as for adhesives or paints, has long been of high relevance for human civilizations. However, with the rise of the petroleum era in the 20$^{th}$ century, nearly all of those applications have been replaced due to better performance and cheaper production of synthetic alternatives (Frihart and Lorenz, 2018). Today, as concerns about diminishing fossil resources and environmental burdens are rising, the search for bio-based products that meet modern requirements has begun. Due to their multitude of functional groups and properties, proteins could offer an especially promising solution.

The utilization of proteins for non-food purposes dates back to long before humans knew of their existence. Ancient Egyptian drawings document the use of animal glues, which were prepared by boiling of bones and hides, for wood bonding (Frihart and Lorenz, 2018). Similarly, the use of blood as an adhesive and milk as a binder for paint is documented in early history. Today, it is possible to connect these functionalities to the proteins contained in these resources. The protein source and its structure plays an important role in these applications. Collagen, which is the main component of animal glue, is a fibrillary structural protein consisting predominantly of the amino acids glycine, proline, and hydroxyproline. By formation of a triple-helical structure and intense polar interactions between the fibrils, collagen provides great strength and flexibility (Frihart and Lorenz, 2018). In contrast to collagen, milk proteins and plant proteins, such as from soy, which was historically also widely used as an adhesive, contain globular storage proteins. The interaction of globules within a colloidal system has also been described to be of great importance for adhesive strength (Frihart and Lorenz, 2018). Protein adhesives based on soy are still of relevance in wood bonding. However, due to their comparatively low water stability, this is mostly limited to interior applications. Bonding strength and water resistance can be improved by addition of cross-linkers, such as formaldehyde. This was also used for the manufacture of galalith – a synthetic polymer produced from casein and formaldehyde – which was developed already in 1897 (Krätz, 2004). Chemical cross-linking using formaldehyde was also studied more recently for rapeseed protein-based adhesives (Yang et al., 2014). As formaldehyde is “known to be a human carcinogen” (National Toxicology Program, 2011), however, these technologies do not form a green alternative for fully synthetic products. Additional reports of rapeseed protein-based adhesives in the last decade include the use of polymerization using acrylates (Wang et al., 2014), and the addition of nanomaterials to improve adhesive properties (Bandara et al., 2017a, b). Moreover, good water stability was reported for rapeseed protein preparations obtained from acidic precipitation when using a curing temperature of 190 °C (Li et al., 2012). In this regard, a simple

cooking procedure of (non-rapeseed) proteins and sugars has been reported recently to receive high binding strength on metal and wood substrates (Román and Wilker, 2019).

Another field of extensive research is the production of protein-based films for food-coating or packaging applications. Compared to other bio-based materials, such as polysaccharides or lipids, proteins demonstrate great oxygen-barrier properties and have been reported to show better mechanical properties (Chen et al., 2019a). However, due to their poor water vapor barrier, protein modification or embedding in a multilayer material needs to be performed (Zink et al., 2016). Similar limitations were also found for rapeseed protein-based films and packagings (Shi and Dumont, 2014; Zhang et al., 2018).

Moreover, plant proteins can be found as emulsifiers, surfactants, foaming agents, or antioxidants in various products (Guéguen and Popineau, 1998). While some scientific publications exist describing the mentioned applications for rapeseed proteins (Rivera et al., 2015; Sánchez-Vioque et al., 2001), no such products could be identified on the market.

In the growing bioeconomy of the 21$^{st}$ century, proteins may again play an important role as alternatives for petrochemical ingredients in products such as adhesives, coatings, detergents or polymers. In this regard, rapeseed is one promising resource, which is however still not extensively studied for applications other than in the food sector. As certain limitations arise for the latter due to the presence of antinutritive components, the utilization in the chemical industry presents a promising alternative for protein valorization. In light of their high availability, rapeseed proteins from residual materials of the oilseed industry might form an important building block towards a bio-based future.

## 4 Aim of the Thesis

The present doctoral thesis aims to improve knowledge regarding the potential of rapeseed proteins as bio-based and biodegradable ingredients for the chemical industry. A review of rapeseed protein extraction, isolation, modification and application in the chemical industry is given as an introduction (Chapter I). In the initial study, different residual rapeseed raw materials were evaluated regarding their potential for aqueous protein extraction, with a detailed comparison of extraction conditions (Chapter II). Protein extraction and isolation conditions were subsequently tested for pre-pressed meal (PPM) and cold-pressed meal (CPM) in regard to resulting protein yield and functionality (Chapter III). Finally, chemical protein modification via acylation was used to improve functional properties relevant for utilization in the chemical industry (Chapter IV).

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# MATERIALS AND METHODS

This section contains a summary of all materials and methods used in the present work. For a more detailed description of the particular method, the reader may consult the corresponding section of the published journal paper.

## 1 RAW MATERIALS AND CHEMICALS

All rapeseed raw materials used in the present work were of 00 type. Full-fat hulled seeds and hulled, cold-pressed press cake (CPC), both reduced in seed coat content, were provided by Teutoburger Ölmühle GmbH (Ibbenbüren, Germany). Pre-pressed press cake (PPC) and solvent-defatted, desolventized/ toasted meal (TM) were provided by Bunge Deutschland GmbH (Mannheim, Germany). Full-pressed press cake (FPC) was provided by Saria A/S GmbH & Co. KG (Selm, Germany). Press cakes were further defatted as stated in the section "Preparation of Rapeseed Raw Material" to obtain the corresponding defatted meals. All rapeseed raw materials and samples were stored at 14 °C–20 °C.

Isohexane was obtained from Biesterfeld AG (Hamburg, Germany). Protease A-01 (Subtilisin, EC 3.4.21.62) was purchased from ASA Spezialenzyme GmbH (Wolfenbüttel, Germany). Phytase Quantum Blue 5G was obtained from AB Enzymes GmbH (Darmstadt, Germany). Lauroyl chloride and olyeol chloride were obtained from Sigma-Aldrich Chemie GmbH (Munich, Germany). All other chemicals were of analytical grade and obtained from Th. Geyer GmbH & Co. KG (Renningen, Germany), if not stated otherwise.

## 2 PREPARATION OF RAW MATERIALS

CPC and PPC were defatted with isohexane in a percolator of a volume of 1.5 $m^3$ (e&e Verfahrenstechnik GmbH, Warendorf, Germany) and flash desolventized with superheated isohexane (400–500 mbar) prior to steam desolventizing at a maximum product temperature of 60 °C to obtain cold-pressed meal (CPM) and pre-pressed meal (PPM), respectively. FPC was defatted in a Soxleth apparatus (volume 2 L) with isohexane for 24 h at a maximum product temperature of 60 °C and dried in a fume hood at room temperature to obtain full-pressed meal (FPM). Full-fat dehulled rapeseeds were defatted in a Soxleth apparatus (volume 2 L) with isohexane for 48 h three times at a maximum product temperature of 60 °C to obtain non-pressed meal (NPM). Defatted meals were ground to pass through a 1-mm screen.

### 3 Chemical Composition of Rapeseed Samples

The chemical composition (dry matter, protein, ash, fat, cellulose, hemicellulose, lignin and phytic acid) of rapeseed samples was analyzed in duplicate. Protein extracts and other liquid fractions were analyzed for dry matter and protein content in duplicate.

Dry matter and ash content were determined using a thermo-gravimetrical system (TGA 601, Leco Corporation, St. Joseph, MI, USA) at 105 °C and 950 °C, respectively. Protein content was measured by the Dumas combustion method on a TruMac N system (Leco Corporation, St. Joseph, MI, USA) by applying a conversion factor of 5.7 (Association of Official Analytical Chemists (AOAC), 1990b). Fat content was determined according to the method of Caviezel, DGF K-I 2c (00) (Deutsche Gesellschaft für Fettwissenschaft e. V. Münster, 2004). Contents of cellulose, hemicellulose and lignin were determined using Fibretherm (Gerhardt GmbH & Co. KG, Königswinter, Germany) according to Van Soest et al. (1991) and the Association of Official Analytical Chemists (AOAC) (1990a). Quantification of phytic acid was performed using an IonPac AS11 column (Thermo Fisher Scientific, Waltham, USA) in a HPAEC system with conductivity detection, as described by Fritsch et al. (2015). Amino acid analysis was performed by LUFA-ITL GmbH, Kiel, Germany according to VO(EG) 152/2009, III (EU, 2009). Free fatty acids in the ethanol washing fractions of modified samples were determined by gas chromatography with flame ionization detector (GC-FID) as described by (Mahmoud et al., 2018).

### 4 Thermal Behavior of Proteins

The thermal behavior of rapeseed proteins was analyzed by differential-scanning calorimetry (DSC). 50 mg of the rapeseed sample were extracted at pH 5.7–5.8 using 1 mL of 0.1 M NaCl solution in a Thermomixer (1,400 rpm, 30 °C, 45 min; Eppendorf AG, Hamburg, Germany). After centrifugation (13,400 rpm, 5 min), the supernatant was removed and concentrated by compressed air flow to a protein concentration of approximately 20 mg/mL. Approximately 20 mg of the concentrated protein extract were weighed into DSC pans (T Zero, TA Instruments, DE, USA), and thermograms were measured on a DSC-Q 2000 (TA Instruments). An empty DSC pan was used as a reference. The samples were heated at a rate of 2 K/min and cooled at a rate of 4 K/min in two cycles from 40 °C to 120 °C. Peak temperatures and related enthalpies were calculated automatically. The experiments were performed in duplicate.

### 5 Protein Solubility

The protein solubility (PS) was determined in duplicate within the range of pH 3–12, following the method of (Morr et al., 1985). For each measurement, 1.5 g of rapeseed sample (ground to < 1 mm) were suspended in 50 mL of 0.1 M NaCl solution. The pH was adjusted using 0.1 M NaOH or 0.1 M HCl. After stirring for 1 h at room temperature, the non-dissolved fraction

was separated by centrifugation (20,000 × g, 15 min, 15 °C) and the resulting supernatant was filtered (Whatman No. 1 filter paper). The protein content of the supernatant was determined according to Dumas following the method given in the section “Chemical Composition of Rapeseed Samples.” Protein solubility was calculated using Equation 1:

$$[1]\ \mathrm{PS}\ [\%] = \frac{\text{initial volume [mL]} \times \text{protein content in supernatant [mg/mL]}}{\text{sample mass [mg]} \times \text{protein content [\%dm]} \times \text{dry matter [\%]}} \times 100$$

## 6 Protein Extraction

Generally, protein extraction was carried out as follows: Rapeseed meal (RM) was added to deionized water with adjusted concentrations of NaCl. The mixture was stirred and the pH was adjusted by addition of NaOH before the start of the extraction time. After the extraction time was completed, the mixture was subjected to centrifugation and the weight of the supernatant was determined. For subsequent protein isolation processes, the supernatant was poured over a 140 µm sieve and used for further experiments. The protein extraction yield (PEY) was calculated using Equation 2:

$$[2]\ \mathrm{PEY}\ [\%] = \frac{\text{mass (extract) [g]} \times \text{protein content (extract) [–]}}{\text{mass (meal) [g]} \times \text{protein content (meal) [–]}} \times 100\ [\%]$$

## 7 Protein Isolation

*Ultrafiltration*

Ultrafiltration was carried out on a Microza polysulfone ultrafiltration module SIP-1023 (Pall Corporation, Port Washington, NY, USA) with a molecular weight cutoff of 6000 Da and a membrane area of 0.19 $m^2$. Ultrafiltration was performed in batch mode at 50 °C and a membrane pressure of 1.5 bar generated with a built-in peristaltic pump, with recirculation of the retentate and a concentration factor of 4. During operation, back-pressure of 4 bar was applied every 1–2 h to reduce membrane fouling. After reduction of the retentate volume to approx. 2 L, diafiltration was carried out using 6 × 1.0 L of demineralized water. The retentate was adjusted to pH 7.0, freeze-dried and prepared using a mortar and pestle to obtain the rapeseed protein concentrate (RPC).

*Acidic precipitation/ ultrafiltration*

The protein extract was cooled to 10 °C and stirred at 400 rpm, while the pH was adjusted quickly to pH 4.0 using 1 M HCl. The mixture was stirred for 15 min, centrifuged, and poured over a 140 µm sieve to separate the supernatant from the precipitate. The precipitate was resuspended in an equal amount of demineralized water and adjusted to pH 7.0. After freeze-

drying, the RPC was prepared using a mortar and pestle. The supernatant was further used for ultrafiltration as stated above.

The protein isolation yield (PIY) was calculated using Equation 3:

$$[3]\ \mathrm{PIY}\ [\%] = \frac{\text{mass (prec.) [g]} \times \text{protein content (prec.) [−]} + \text{mass (ret.) [g]} \times \text{protein content (ret.) [−]}}{\text{mass (meal) [g]} \times \text{protein content (meal) [−]}} \times 100\ [\%]$$

## 8 Protein Modification

Chemical protein modification was carried out on a RPC prepared by ultrafiltration at a small pilote scale (see Chapter IV). For the modification process, 100 g of RPC were dispersed in 900 g of demineralized water and stirred at 40 °C. The pH was adjusted to pH 9.5 before dropwise addition of the fatty acid chloride (FAC). The pH was controlled during addition of FAC and maintained at pH 9.5 for 3 h. The dispersion was cooled to 30 °C, neutralized, and dialyzed against demineralized water at 1 °C for 48 h with a molecular weight cutoff of 3.5 kDa (Spectra/Por® 3, Spectrum LifeSciences, LLC, Rancho Dominguez, CA, USA). The demineralized water was changed twice a day. The dialyzed samples were lyophilized, homogenized by mortar and pestle and washed with ethanol to remove non-reacted free fatty acids. After air-drying under a fume hood, the modified RPCs were used for further analysis. A control sample was prepared and treated as described, except for the addition of FAC to the protein dispersion. All samples were prepared in duplicate.

Calculation of the degree of modification (DM) of modified samples was performed by determination of free α-amino groups using o-phtaldialdehyde (OPA). The method was carried out according to Nielsen et al., using N,N-dimethyl-2-mercaptoethylammonium chloride as the thiol component (Frister et al., 1988; Nielsen et al., 2001). Protein samples were analyzed in triplicate using serine as a standard.

## 9 Molecular Weight Distribution of Protein Samples

The molecular weight distribution of protein samples was determined by SDS-PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis) using Laemmli sample buffer at non-reducing conditions (Laemmli, 1970). The samples were mixed with Tris-HCl buffer to a final concentration of 63 mM Tris-HCl (pH 6.8), 2% SDS, 10% v/v Glycerol and 0.01% bromophenol blue. Electrophoretic separation of proteins was performed by loading 50 µg of protein on pre-cast 4-20% CriterionTM TGX Stain-FreeTM gels (Bio-Rad Laboratories, Hercules, CA, USA). As a molecular weight marker, Precision Plus Protein™ Unstained Standard (10–250 kDa, Bio-Rad Laboratories) was loaded onto the gel. The gel running conditions for electrophoretic separation were 200 V, 60 mA, 100 W and room temperature. Visualization and documentation of the protein bands was performed using a Gel DocTM EZ Imager system and Image Lab software (Bio-Rad Laboratories).

# 10 FUNCTIONAL PROPERTIES

## 10.1 Foaming Properties

The foaming activity (FA, %) was determined according to Phillips et al. (1987), with minor modifications. An aqueous solution (5% w/w) of the protein sample was whipped using a Hobart N50 mixer (Hobart GmbH, Offenburg, Germany) at level 3 for 8 min. The FA was calculated as the relation of the foam volume before and after whipping. The foam density (FD, g/L) was determined by measuring the weight of a specified foam volume. The foam stability (FS, %) was measured as the percentage of remaining foam volume in a 250 mL measuring flask after 60 min.

## 10.2 Emulsifying Capacity

The emulsifying capacity (EC) was determined in duplicate as described by (Wäsche et al., 2001). An aqueous solution (1% w/w) of the protein sample was treated in an IKA LR-A 1000 laboratory reactor (IKA-Werke GmbH & Co. KG, Staufen, Germany) equipped with a stirrer (100 rpm) and an ULTRA-TURRAX® (11,000 rpm). Commercial corn oil was added by a titration system (Titrino 702 SM, Metrohm GmbH & Co. KG, Hertisau, Switzerland) at a constant rate of 10 mL/min while keeping temperature at 20–25 °C. The phase inversion of the emulsion was detected by monitoring the conductivity of the emulsion by a conductivity meter LF 521 with electrode KLE 1/T (Wissenschaftlich-technische Werkstätten GmbH, Weilheim, Germany). The volume of oil added until phase inversion occured was used to calculate the EC (mL oil per g sample).

## 10.3 Color analysis

Photographs of rapeseed samples were taken on a DigiEye system (VeriVide Limited, Leicester, UK) equipped with a Nikon D90 camera (Nikon, Tokyo, Japan). Color measurement was performed in the CIELAB color space, using duplicate samples of RPCs (DigiEye software, VeriVide Limited).

## 10.4 Film-forming properties

*Cast film preparation*

Cast films of RPCs were prepared according to (Chang and Nickerson, 2015) with minor modifications. 3.0 g (11.25 g) of RPC were mixed with 30 mL (120 mL) of demineralized water and the pH was adjusted to pH 3 to induce protein denaturation. The mixture was stirred at room temperature for 60 min at 300 rpm, before the addition of 0.45–0.90 g (2.81 g) of glycerol. The mixture was adjusted to a total weight of 40 g (150 g) at pH 3 using demineralized water

and 1 M HCl and stirring was continued for 10 min. The mixture was degassed in an ultrasound bath at 35 kHz for 15 min, stirred at 50 °C and 300 rpm and 13.5 g (33 g) were cast in a petri dish with a diameter of 85 mm (120 mm × 120 mm). The films were dried at 23 °C and RH = 50% for at least 48 h and were removed from the petri dish after 14 days for textural comparison. The films were dried at 23 °C and 50% RH for 14 days to equilibrium moisture content. The rapeseed protein films were then peeled off the petri dishes for textural comparison and analysis of mechanical and barrier properties.

*Film thickness*

The film thickness was determined using a Precision Thickness Gauge (Hanatek Instruments, St. Leonards-on-Sea, UK) as previously described by Schmid (2013).The measurements were performed with a five-fold determination at five different positions at 23 °C and 50% RH. The arithmetic average of the film thickness was used for the calculation of the standardized oxygen permeability (OP) and mechanical film properties.

*Surface energy*

The surface energy of the films was measured by the sessile drop method as previously described by (Schmid, 2013), using the contact angle measuring system G2 (Krüss GmbH, Stephanskirchen/Rosenheim, Germany). The contact angle of three testing liquids (water, diiodomethane, ethylene glycol) was measured at five different positions on the film surface of five replicates at 22.4 °C and 42% RH. The results were reported as the arithmetic average ± standard deviation. According to Young's equation, the surface energy was calculated using Equation 4:

[4] $\sigma_s = \gamma_{sl} + \sigma_l \times \cos\theta$

where $\sigma_s$ is the surface tension of the solid (film) in mN/m, $\sigma_l$ is the surface tension of the liquid in mN/m, $\gamma_{sl}$ is the interfacial tension between the solid and the liquid in mN/m and cos Θ is the contact angle between the surface tension of the liquid and the interfacial tension between the liquid and solid in angular degree. The disperse and polar fractions of the surface energy were calculated according to the method by Owens, Wendt, Rabel and Kaelble (Kaelble, 1970; Owens and Wendt, 1969; Rabel, 1971). All rapeseed protein-based films were water soluble, such that the measurement had to be performed directly after drop application rather than at equilibrium. The calculated values were thus not classified as absolute surface energy values and were only used for comparison of the samples in the present study.

*Tensile testing*

The tensile properties of the films were measured on a universal compression-tension testing machine Zwick 1445 (ZwickRoell GmbH & Co. KG, Ulm, Germany) at 23 °C and 50% RH in accordance to the standard DIN 527-1. The cast films were cut into strips (15 mm × 70 mm) and the thickness of the film strips were measured. The film strip was clamped in the loading frame using pneumatic grips and an initial gauge length of 50 mm. The samples were subjected to an applied force with a load cell of 50 N and were stretched with a testing speed of 100 mm/min. For each sample, a five-fold determination was performed and the tensile strength (TS; MPa) and elongation at break (%) were reported as the arithmetic average ± standard deviation.

*Oxygen permeability*

The oxygen permeability (OP) was measured according to the standard DIN 53380-3 at 23 °C and 50% RH on a Mocon Twin instrument (Mocon Inc., Minneapolis, USA) as previously described (Deutsches Institut für Normung e.V., 1998; Schmid, 2013). The OP values (Q) were converted to a thickness (d) of 100 µm (Q100), according to Equation 5, and were reported in the unit [$cm^3 \cdot m^{-2} \cdot d^{-1} \cdot bar^{-1}$].

[5] $Q_{100} = Q \times \frac{d}{100}$

*Light transmission*

The light transmission of the films was measured in duplicate on a spectrophotometer TMQ (Carl Zeiss GmbH, Oberkochen, Germany) at wavelengths of 350 nm, 430 nm, 450–750 nm (intervals of 25 nm), and 800–1000 nm (intervals of 50 nm).

## 11 Statistical Analysis

All data are given as mean values ± standard deviation of at least two measurements (n = 2). Significant differences were statistically analyzed by one-way analysis of variance using Tukey's test ($p < 0.05$). For the protein modification study (Chapter IV), elimination of outliers was performed by applying Grubb's test and the data was tested for normal distribution according to Shapiro-Wilk. Statistical analysis was performed using the software OriginPro (OriginLab Corporation, Northampton, USA).

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# CHAPTER I – Rapeseed Proteins for Technical Applications: Processing, Isolation, Modification and Functional Properties – A Review

To serve as a thorough introduction to the present doctoral thesis, the available literature on rapeseed processing, protein extraction, isolation, functionality, modification, and application in the chemical industry was reviewed. Protocols for the isolation of proteins from residual materials after rapeseed defatting have been described for more than 50 years – almost exclusively for utilization in the food sector. However, the presence of antinutritive components in rapeseed protein preparations presents a major obstacle for their commercialization until today. Additionally, protein extraction yields are greatly limited due to harsh process conditions during defatting. Alternative defatting methods, including the utilization of cold-pressing, alternative solvents, microwave and ultrasound, and solvent-free methods, are presented. While the benefits on rapeseed oil and protein quality of these methods are discussed in some studies, their industrial application is not feasible at present. Protein recovery is preferentially performed by means of protein isolation, using precipitation or ultrafiltration. Most protocols apply strong alkaline extraction conditions, due to higher extractability, however, with the possibility of proteolysis occurring at extreme values. Protein preparations show different pH-dependent solubility profiles, determined by the isolation procedure used. Good foaming and emulsifying properties were reported across the literature. In addition, the reported gelation and film-forming properties are of particular interest for the application in the chemical industry in products such as adhesives, paints and polymers. Protein modification by physical, chemical or biochemical methods was also demonstrated as a valuable tool to improve functional properties in a range of studies. Finally, some application studies for adhesives, packaging, detergents and cosmetics are available to demonstrate the high potential of rapeseed proteins for utilization in technical products.

A. Fetzer designed the structure of the article, performed the literature research, and wrote the manuscript. K. Müller and P. Eisner contributed to the contents of the manuscript, predominantly for the sections referring to rapeseed processing, protein extraction, isolation, functionality, and modification. M. Schmid contributed to the contents of the manuscript, predominantly for the sections referring to protein functionality and modification.

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# Rapeseed proteins for technical applications: Processing, isolation, modification and functional properties – A review

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ABSTRACT

Plant proteins offer an interesting resource for the growing bio-economy of the 21st century, as they present a renewable resource with a variety of functional properties, such as foaming, emulsification and film-forming. Rapeseed proteins can be isolated as high-value components from residual materials of oilseed processing. Feed and food use, though currently being the primary area of research, is limited due to residual anti-nutritive components. Thus, the application of rapeseed protein preparations in the technical (non-food) industry, as alternatives to petro-based chemicals, is of great interest. The present review gives an overview of rapeseed processing with an emphasis on alternative defatting methods. Protein extraction and isolation studies are compared with a focus on raw material properties, processes, and the resulting protein yields. Functional properties and methods for their modification are discussed. Finally, examples for the application of rapeseed proteins in the technical industry are presented.

## 1. Introduction

In the growing bio-economy of the 21st century, bio-based materials and additives are becoming an ever greater point of interest, due to the increasing health and environmental concerns associated with petro-based chemicals. Plant proteins are a ubiquitous natural resource with versatile functional properties, such as foaming, emulsification, and film-forming (Gofferje et al., 2015; Reichert et al., 2020; Schäfer et al., 2018). Protein-based films are an interesting and well-investigated alternative to commonly used petroleum-based materials (Coltelli et al., 2016; Zink et al., 2016). Rapeseed proteins are of particular interest, as they can be extracted from residual materials from the rapeseed defatting process, thus offering a sustainable potential for generating new added value.

Rapeseed is the third-most abundant oil crop worldwide, with an average seed oil content of around 44 % (INRA CIRAD AFZ, 2020; Stępień et al., 2017; USDA Foreign Agricultural Service, 2020; Wanasundara et al., 2017). Residual press cakes and meals are currently used as animal feed, although they display certain limitations due to residual anti-nutritive components, such as phytates and glucosinolates (von der Haar et al., 2014). Similar challenges are evident in terms of their utilization as a food ingredient, where protein extraction and isolation protocols have been established for more than 50 years (Tranchino et al., 1983). The presence of bitter and astringent off-flavors still presents a major hurdle to commercialization in the food sector (Hald et al., 2019; Linnemann and Dijkstra, 2002; Naczk et al., 1998), however, a number of patents have been filed to achieve this (Barker et al., 2002; Pickardt et al., 2010; Ruf et al., 2008; Segall et al., 2006; Shi and Smolders, 2017; Shi et al., 2016; Sohling et al., 2012; Wnukowski and Kozlowska, 2015).

Rapeseed proteins are predominantly composed of the two main storage proteins of the seed: cruciferin, a 12S globulin of 300–310 kDa, and napin, a 1.7–2S albumin of 12.5–14.5 kDa (Tan et al., 2011). The high-molecular weight cruciferin is composed of six50 kDa subunits, which are each formed by heterodimers of ~30 kDa (heavy α-chain) and ~20 kDa (light β-chain) linked by a disulfide bond (Dalgalarrondo et al., 1986). Due to its multimeric quaternary structure, cruciferin shows a high level of association and dissociation depending on pH and ionic strength (Schwenke et al., 1983). Napin is formed by two subunits (~10 kDa, ~4.5 kDa), which are linked by two disulfide bonds (Gehrig et al., 1996). Minor proteins found in the seed include oil body proteins, lipid transfer proteins and protease inhibitors (Wanasundara, 2011). Among these, oleosins (18–25 kDa), which form the predominant oil body proteins in rapeseed, gained some interest in recent decades (Chen et al., 2019; Jolivet et al., 2009; Tzen, 2012; Wanasundara et al., 2016).

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The use of rapeseed proteins in technical products is not limited by secondary plant metabolites, as these do not necessarily interfere with the functionality. The utilization of proteins in products such as plastics, coatings and glues was very common until well into the 20th century. However, with the rise of the petroleum age and the progress in synthetic chemistry, petro-based chemicals subsequently replaced natural products, with the exception of only a few special applications, such as labeling or musical instrument adhesives (Brockmann et al., 2009; Platanianaki et al., 2017). With growing concerns with regard to diminishing oil resources and environmental pollution, the demand for bio-based and biodegradable materials is increasing (Reichert et al., 2020). Rapeseed proteins offer a promising solution for technical products such as polymers, coatings, adhesives, detergents and lubricants. In particular, studies investigating the application potential as adhesives, edible films, packaging and surfactants have been published recent decades (Bandara et al., 2017a, b; Chang and Nickerson, 2014, 2015; He et al., 2019; Jang et al., 2011b; Sánchez-Vioque et al., 2001; Shin et al., 2011; Zhang et al., 2018).

The present review covers all aspects of rapeseed processing relevant for the application of rapeseed proteins in the technical (non-food) industry. As such, this article provides an extensive overview of the most recent advances in seed processing comparing conventional defatting with cold-pressing and other emerging technologies. Protein extraction and isolation methods of rapeseed proteins are discussed in detail, with a focus on protein yields and protein contents of isolated preparations. The characteristics and functional properties of rapeseed protein preparations are reviewed, as are methods for protein modification. Finally, recent studies on rapeseed protein application in the technical industry are presented.

## 2. Rapeseed processing

The demand for vegetable oil has been the economic driver for rapeseed cultivation for decades. Defatting procedures have been optimized over the years in order to maximize yields, minimize costs and improve oil quality. However, protein quality can be negatively affected by conventional rapeseed processing, due to the harsh conditions involved (Mosenthin et al., 2016). Alternative defatting methods using milder conditions have also been studied, and are discussed towards the end of this chapter. A schematic representation of conventional processing and cold-press-procesing is shown in Fig. 1.

### 2.1. *Conventional defatting*

After harvesting, the seeds are cleaned, pre-conditioned to ensure optimal moisture content, and then flaked by roller mills to physically rupture the seed coat and cell walls in order to release oil bodies from the cell structure (Unger, 2011). The flaked seeds are heated to 85–95 °C for 30–40 min, predominantly to inactivate the enzyme myrosinase, which hydrolyses glucosinolates into non-desired sulfur-containing compounds. This heating step also inactivates other enzymes, such as lipases, and leads to the agglomeration of oil and protein bodies, which simplifies further processing.

Due to its high oil content of up to 50 % in the seed, rapeseed defatting is not suitable for direct solvent extraction (Unger, 2011). Conventionally, oil extraction from rapeseed is achieved by screw-pressing, either by expeller pressing (full-pressing) or by pre-pressing of the seeds, followed by solvent extraction. Oil contents in the resulting press cakes are in the range of 5 %–10 % (full-press) or 15 %–18 % (pre-press) (Kemper, 2005). In the latter case, the

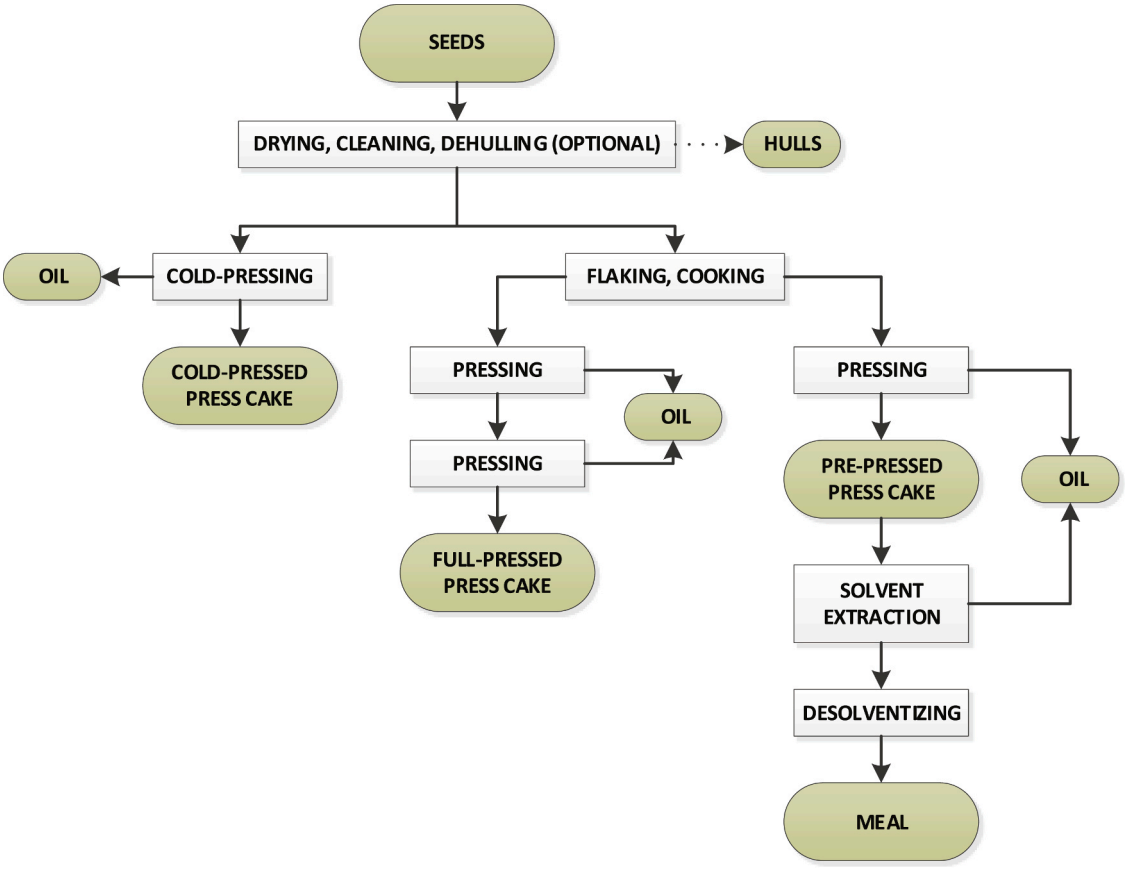


**Fig. 1.** Schematic representation of rapeseed processing steps: cold-pressing, full-pressing, and pre-pressing/ solvent extraction.

pre-pressed press cake is further defatted by solvent-extraction using isohexane, which is a mixture of 2-methylpentane, 3-methylpentane, *n*-hexane, and minor amounts of other solvents, with a boiling point of 63–67 °C (Unger, 2011). Oil content of the resulting meal is generally reduced to < 2 %. Subsequent solvent removal is conventionally achieved in a desolventizer vessel by the so-called toasting process at a maximum product temperature of 107–110 °C, so as to reach residual solvent contents of < 250 ppm.

### 2.2. Cold-pressing

The protein quality of press cakes or meals resulting from conventional processing can be greatly diminished due to strong shear forces and temperatures of up to 140 °C (Fetzer et al., 2018). Cold-pressing of rapeseed is one alternative that is used to produce high-quality oil, while reducing protein denaturation during the process. Temperatures during cold-pressing are as low as 40 °C, which can be achieved via the external cooling of the press (Kraljić et al., 2013; Rass and Schein, 2010). The residual oil content of cold-pressed press cake is in the range of 15 %–18 % (Fetzer et al., 2018). Subsequent defatting can be achieved by solvent extraction followed by flash desolventation at a maximal product temperature of 60 °C.

### 2.3. Alternative solvent defatting

Another technology that has attracted increasing interest over recent decades is the use of supercritical $CO_2$ as a solvent for the extraction of oil or other metabolites from oilseeds. The process works under mild temperatures as low as around 30 °C and pressures of around 6 MPa (Gaber et al., 2018; Manamperi et al., 2015; Sahena et al., 2009). Studies describing the use of supercritical $CO_2$ for rapeseed oil extraction were reviewed recently by Gaber et al. (2018), with a focus on oil yield and quality. One critical factor in accessing oil quality is oxidative stability, which is largely dependent on the presence of anti-oxidative phospholipids and sterols, and pro-oxidative free fatty acids. Generally, supercritical $CO_2$ extraction leads to oils with lower oxidative stability compared to hexane-extracted oil, presumably due to a lower co-extraction of antioxidants (Uquiche et al., 2012). Przybylski et al. (1998) showed an increase in oxidative stability with increasing volumes of supercritical $CO_2$ used for the extraction. The authors explained the effect with a high content of polyunsaturated fatty acids in the first fraction, which is balanced by increased phospholipid and tocopherol contents in subsequent fractions. In their study, they also reported a high oil-extraction yield of 90 %, which was in line with results obtained by Boutin and Badens (2009), who reported a yield of 89.7 % under optimal conditions. In summary, extraction with supercritical $CO_2$ could be an alternative to hexane extraction in the future, as $CO_2$ is ubiquitous, safe and can be easily removed by decompression, to provide solvent-free oil and press cake. However, improvements in regard to oil yield and oxidative stability are mandatory, if an economically feasible process for the industry is to be offered.

Furthermore, concerns about the toxicity of hexane have led to attempts to find other alternative solvents. In this regard, oil extraction was tested by a lecithin-based microemulsion system using a mixture of lecithin:propanol:water of 2:1:3 (Radi et al., 2013). While the authors claimed to have achieved an increase in oil quality compared to hexane extraction, the extraction yield was reduced to 82.5 % compared to 95 %–99 %. Higher yields of up to 92.8 % were claimed in a recent study, by employing alcohol-assisted aqueous extraction (Tian et al., 2019). The authors also reported lower acid and peroxide value, lower content of trans-fatty acids and higher tocopherol content of the oil compared to commercial oil. A high quality crude oil, which was free from fatty acids, phospholipids, and non-lipid compounds, was also obtained from ethanol extraction at 95 °C and 340–350 kPa, followed by cold miscella separation (Citeau et al., 2018). In a further study, a high economic feasibility of this type of process was claimed based on the laboratory results (Carré et al., 2018). However, the need for further experiments at a pilot-scale was concluded in order to validate the results and solve major issues, such as the demand for solvent regeneration. The development of a rapeseed-defatting process using mechanical cell disruption and ethanol extraction at a maximum temperature of 65 °C is currently being investigated in the German research project "EthaNa" (Pufky-Heinrich et al., 2019). The process aims to both enhance meal quality and provide additional value to the rapeseed industry by isolating minor components, such as sinapine and polyphenols.

### 2.4. Microwave-/ ultrasound-assisted defatting

The solvent-based extraction of oilseeds can be assisted by microwave or ultrasound treatment. Both technologies were shown to potentially increase oil yield and reduce solvent utilization as well as extraction time (Gaber et al., 2018). In addition, enhanced oil quality and oxidative stability were reported in a number of studies. (Azadmard-Damirchi et al., 2010, 2011; Jalili et al., 2018; Ramos et al., 2017; Sanchez et al., 2017, 2018). The effects were attributed to the increased co-extraction of antioxidants, including phytosterols, tocopherols, canolol, and other phenolic compounds. It shall be mentioned that the use of solvents other than hexane was also addressed in some of the studies. In this regard, ultrasound-assisted extraction of rapeseed flakes using isopropanol showed higher oil recovery yields compared to a hexane extraction without ultrasound (Perrier et al., 2017). In the same study, ethanol was not found to be a suitable solvent due to low yields. Similar limitations in regard to oil yield were found for ultrasound-assisted aqueous extraction for rapeseed press cake (Gaber et al., 2019). While the extracted oil met the quality standards, only 50 % of the yield achieved with hexane could be obtained. In another study, oils with a higher oxidative stability were obtained from ultrasound-assisted extractions using a mixture of isopropanol and hexane compared to using hexane alone (Jalili et al., 2018). Moreover, the positive influence of microwave pretreatment of seeds on oil yields and oxidative stability obtained through cold-pressing was reported (Azadmard-Damirchi et al., 2010). In addition to the benefits reported for oil extraction in the listed studies, a positive influence on the quality of residual press cakes and meals can be expected. Due to the possible reduction of extraction time and temperature, higher protein yields than for conventional residues should be obtained from these materials. In summary, microwave- and ultrasound-assisted processes offer a great potential for the development of alternatives to conventional hexane defatting, due to competitive oil yields and possible benefits on oil and meal quality. However, as the processes are currently only available at lab-scale, their economic feasibility has yet to be demonstrated for industrial application.

### 2.5. Solvent-free defatting

The solvent-free defatting of rapeseed was studied by Wäsche (2002) using wet crushing followed by aqueous extraction. For the combined process of isolating both oil and protein, the author reported maximum yields of 72 % and 88 %, respectively, using high-pressure homogenization for cell disruption. Additionally, the solvent-free extraction of oil can be performed by enzyme-assisted aqueous extraction. The method has been tested for rapeseed by a few authors using different enzyme combinations and conditions. It was possible to extract up to 90 % of free oil using a combination of cellulase, pectinase and β-glucanase (Zhang et al., 2007). However, an elaborate protocol of centrifugation, freezing and thawing cycles had to be employed for demulsification of the extraction mixture. In another study, a single centrifugation step after enzymatic treatment delivered up to 60 % of the yield achieved by solvent extraction (Latif et al., 2008). While the development of the solvent-free process is far from being achieved at an industrial scale, all studies emphasized the benefit of the co-extraction of rapeseed proteins (Latif et al., 2008; Wäsche, 2002; Zhang et al., 2007).

## 3. Protein recovery

Protein recovery from seeds or defatted meals is achieved either via isolation or concentration procedures. Depending on the method, cruciferins and napins are obtained in various ratios and purities. Isolation is carried out via the aqueous extraction of proteins followed by purification methods, such as precipitation or ultrafiltration (Aider and Barbana, 2011; Tan et al., 2011; Wanasundara, 2011). Protein concentration is achieved by increasing the protein content of the meal through the removal of non-protein compounds, generally using aqueous mixtures of organic solvents (von der Haar et al., 2014; Wanasundara, 2011). Care should be taken when using the terms "protein isolates" and "protein concentrates" as these are commonly not defined by the production process, but rather by their protein content (Codex Alimentarius, 2019). Methods for rapeseed protein recovery have been reported numerously in recent decades, mainly focusing on potential food applications. Literature reviews by various authors, summarizing these studies, are available (Aider and Barbana, 2011; Tan et al., 2011; Wanasundara, 2011), and have been up-dated more recently (Wanasundara et al., 2016, 2017). The present review gives an overview of different protein recovery methods (Fig. 2), with detailed protocols presented and discussed for protein isolation processes (Table 1). As such data are rarely available for protein concentration studies, they are presented in less detail. Moreover, recent studies describing the recovery of oleosins as a minor protein fraction are not further discussed within this review (Ntone et al., 2020; Östbring et al., 2020; Romero-Guzmán et al., 2020).

### 3.1. Protein isolation

Protein isolation is achieved starting from defatted meal, via an initial aqueous extraction step. Depending on the type of meal used, pH value, salt concentration, and temperature, protein extracts of varying compositions are obtained. For further protein isolation from the extract, different procedures, which are described in the following, are applied.

#### 3.1.1. Extraction

A number of studies investigated the influence of process conditions of rapeseed defatting on the resulting protein quality. High temperatures during pressing and solvent extraction are described as being the main cause for the reduction in protein quality. The effect is especially pronounced for conventional "toasted meal", where protein solubility is reduced due to excessive heat-induced Maillard reactions and protein cross-linking (Mosenthin et al., 2016; Salazar-Villanea et al., 2016). The presence of bound amino acids, predominantly of lysin, has been linked to reduced protein solubility (Jensen et al., 1995; Pastuszewska et al., 1998). Thus, rapeseed residues processed with high temperatures have a reduced protein solubility and thus show a lower economic feasibility of subsequent protein extraction (Fetzer et al., 2019). Additionally, a lower degree of protein denaturation as resulting from mild defatting, such as by liquid $CO_2$-extraction, enables the production of isolates with more versatile functional properties (Manamperi et al., 2015). An overview of protein solubility for meals obtained from various defatting procedures is given in Fig. 3. High protein solubility constitutes a critical factor for subsequent protein extraction and isolation. Rapeseed protein extraction was thus carried out at an alkaline pH in most studies (Table 1), due to a high protein solubility. Extraction protocols at pH 2–3 have also been described for the selective extraction of the napin fraction (Nioi et al., 2012; Perera et al., 2016). However, these studies were carried out only on a small scale for the structural analysis of purified napin, and were thus not included in the following detailed discussion. Nonetheless, it shall be mentioned that acidic extraction has been described in the patent literature as well for the separation of napin and cruciferin (Wanasundara and McIntosh, 2008).

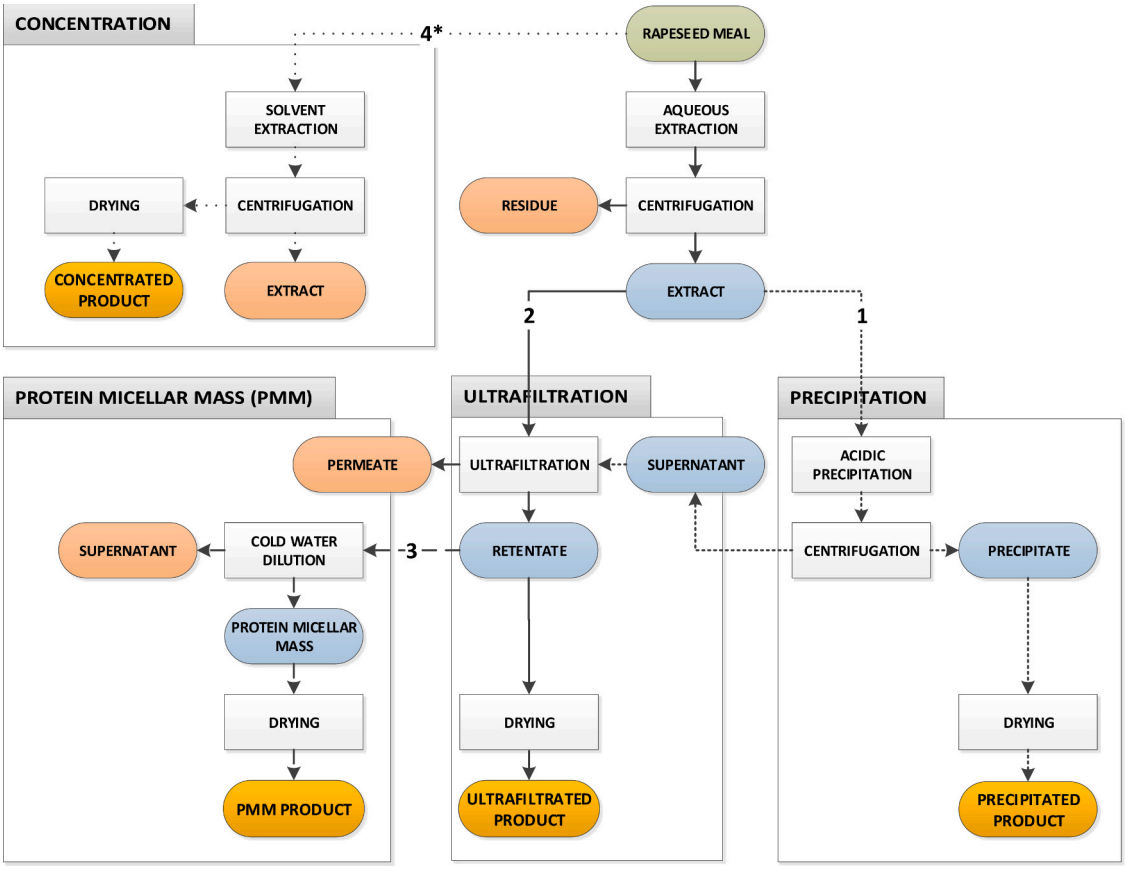


**Fig. 2.** Processes for rapeseed protein recovery from residual defatted meal; *for protein concentration, seed or press cake may also be used as a starting material.

**Table 1**
Comparison of studies reporting rapeseed protein isolation: overview of raw materials, process conditions, protein yields and protein contents (calculated with N × 5.7) of obtained preparations; UF = ultrafiltration, AP = acidic precipitation, CF = concentration factor, DV = diafiltration volume, n/a = not available.

| Raw material | Process conditions | Extraction yields[a] [% w/w] | Isolation yields[a] [% w/w] | | Protein contents of preparations (N × 5.7) [% dry basis] | | Reference |
|---|---|---|---|---|---|---|---|
| i) cold-pressed press cake, ground<br>ii) industrial hot-pressed press cake, ground<br>iii) industrial defatted meal (hexane), ground | 1) pH 10.5, 1:9, 3 h, 4 °C<br>2) 5000 × g, 4 °C, 30 min<br>3) pH 5.0 (citric acid)<br>4) heating: 80 °C, 4 min*<br>5) cooling to 25 °C in cold water bath (5.5 min)*<br>6) 5000 × g, 4 °C, 30 min<br>7) no drying<br>*steps 3)-5) optional | n/a | i) AP:<br>AP (heated):<br>ii)AP:<br>AP (heated):<br>iii)AP:<br>AP (heated): | 45<br>34<br>26<br>23<br>5<br>8 | i) AP:<br>AP (heated):<br>ii)AP:<br>AP (heated):<br>iii)AP:<br>AP (heated): | 59.3<br>40.1<br>72.0<br>55.6<br>65.7<br>80.3 | Östbring et al. (2019a) |
| cold-pressed press cake (5 varieties, 1 blend), ground | 1) pH 10.5, 1:9, 3 h, 4 °C<br>2) 5000 × g, 4 °C, 30 min<br>3) pH 5.0 (citric acid)<br>4) heating: 80 °C, 4 min*<br>5) cooling to RT in cold water bath*<br>6) 5000 × g, 4 °C, 30 min<br>7) no drying<br>*steps 3)-5) optional | n/a | AP:<br>AP (heated): | 29–41<br>51–54 | AP:<br>AP (heated): | 52.0–63.8<br>37.4–49.2 | Östbring et al. (2019b) |
| industrial defatted meal (hexane 60–65 °C), ground < 0.315 mm | 0) washed w/ EtOH:$H_2O$ (3:1) (4 × at s:l = 1:3, 30 min, RT)<br>1) pH 12, 1:19, 60 min, 40 °C<br>2) centrifugation: n/a<br>3) pH 4.5 (1 M HCl)<br>4) 1800 × g, 15 min<br>5) supernatant collected<br>6) freeze-dried | n/a | n/a | | AP:<br>supernatant: | 79.2<br>26.3 | Kalaydzhiev et al. (2019) |
| i) industrial cold-pressed cake, hexane-defatted (≤ 60 °C), ground < 1 mm<br>ii) industrial pre-pressed cake, hexane-defatted (≤ 60 °C), ground < 1 mm | 1) pH 5.7–7, 1:11, 60 min, RT, 0.25 M NaCl<br>2) 3300 rpm, 20 °C, 10 min*<br>3) pH 4 (1 M HCl), 10 °C, 15min*<br>4) 3300 rpm, 20 °C, 15 min*<br>5) 6 kDa, 0.19 $m^2$, 50 °C, CF = 4, DV = 3 (6 × 1 L)<br>6) freeze-dried<br>*steps 3)–5) were not performed for preparations obtained by direct UF | i) 45.1–54.5<br>ii) 28.3–35.9 | i) UF:<br>AP/UF:<br>ii) UF:<br>AP/UF: | 40.6<br>38.2 (13.7/24.5)<br>26.0<br>24.2 (7.9/16.3) | i) UF:<br>AP/UF:<br>ii)UF:<br>AP/UF: | 78.0–83.9<br>83.6–84.0/78.4–84.4<br>77.7–77.9<br>87.1–88.0/75.3–79.9 | Fetzer et al. (2019) |
| industrial defatted meal (hexane 60–65 °C), ground < 0.315 mm | 0) washed w/ EtOH:$H_2O$ (3:1) (4 × at s:l = 1:3, 30 min, RT)<br>1) pH 12, 1:12.33, 75 min, 40 °C<br>2) (separation parameters n/a)<br>3) pH 4.5 (HCl)<br>4) 1800 × g, 15 min, washed w/ $H_2O$ pH 4.5 (3 × 6 volumes)<br>5) no UF, supernatant and all washing liquids combined<br>6) freeze-dried | n/a | n/a | | AP:<br>supernatant: | 86.9<br>28.8 | Ivanova et al. (2017) |
| industrial hexane-defatted meal, ground < 0.5 mm | 0) washed (pH4)<br>1) pH 12.5, 1:10, 60 min, RT<br>2) 15,000 × g, 5 °C, 20 min, filtrated (Whatman No. 1)<br>3) pH 4/4.5 (0.1 M HCl, gradually within 15 min)<br>4) 15,000 × g, 5 °C, 20 min, washed w/ $H_2O$ (pH 4, 3 × 150 mL)<br>5) 10 kDa, 0.2 $m^2$, pH 4, CF = 10/20, DV = 5/10<br>6) freeze-dried | n/a | 51 (38.6/12.4) | | AP:<br>UF: | 83.2<br>74.7 | Akbari and Wu (2015) |
| industrial press cake, ground < 0.25 mm, Soxhlet-defatted (hexane:diethyl ether 1:1) | 0) washed w/ acidified MeOH:acetone (1:1)<br>(s:l = 1:20, 2 h, 25 °C); 10,000 rpm, 4 °C,<br>20 min, dried at 35 °C, 24 h, 0.02 MPa | n/a | 40–50 | | n/a | | Das Purkayastha et al. (2015) |

(*continued on next page*)

**Table 1** (*continued*)

| Raw material | Process conditions | Extraction yields[a] [% w/w] | Isolation yields[a] [% w/w] | Protein contents of preparations (N × 5.7) [% dry basis] | | Reference |
|---|---|---|---|---|---|---|
| | 1) pH 11, 1:10–1:30, 1–5 h, 25 °C, 0–0.2 M NaCl, 0–0.4 % $Na_2SO_3$<br>2) 10,000 rpm, 4 °C, 20 min<br>3) $(NH_4)_2SO_4$ to 85 % saturation, ice bath, 3 h<br>4) pH 7, dialyzed, freeze-dried | | | | | |
| ground seed, Soxhlet-defatted (hexane), ground < 0.25 mm | 1) pH 8, 1:10, 60 min, RT, 1 M NaCl, 0.05 M phosphate buffer<br>2) 10,000 rpm, 5 °C, 20 min<br>3) $(NH_4)_2SO_4$ to 85 % saturation, 3 h, RT, stored in refrigerator o/n<br>4) 10,000 rpm, 20 min, redissolved in 0.5 M NaCl<br>5) freeze-dried, stored at -70 °C | 50.1 | n/a | n/a | | Wu and Muir (2008) |
| industrial defatted meal, ground < 1 mm | 0) washed w/ EtOH:$H_2O$ (65:35) (4 × s:l = 1:3; 2000 × g, 10 min), air-dried<br>1) pH 14, 1:19, 60 min, RT<br>2) 4000 × g, 10 min<br>3) pH 4.5 (HCl), 30 min, precipitate dialyzed (12 kDa), 5 °C, 2 × 24 h<br>4) 4000 × g, 10 min<br>5) supernatant dialyzed (12 kDa), 5 °C, 2 × 24 h<br>6) freeze-dried | | 30 (20/10) | AP:<br>supernatant: | 83.4<br>82.8 | Chabanon et al. (2007) |
| defatted meal | 1) pH 12, 1:35, 85 min, 48 °C, ultrasound treatment (875 W)<br>2) pH 4.5 | 84.0 | 52.2 (52.2/-) | 68.2 | | Ma et al. (2007) |
| i) ground whole seed, Soxhlet defatted (hexane)<br>ii) ground dehulled seed, Soxhlet defatted (hexane) | 1) pH 12, 1:18, 30 min, RT<br>2) 5000 rpm, 15 min, filtered<br>3) pH 3.5–7.5 (6 M HCl)<br>4) 5000 rpm, 20 min, filtered, washed ($H_2O$, pH 3.5–7.5, s:l = 1:10)<br>5) 10 kDa, 0,1 $m^2$ (supernatant and washing liquids, CF = 10, DV = 5)*<br>6) oven-dried o/n<br>*UF preparation was obtained from a separate process with addition of 15 % $CaCl_2$ (by weight of starting meal) before AP | i) 45.4–64.7<br>ii) 61.0–68.2 | i) 46.5–56.5 (26.4–30.7/20.1–25.8)<br>ii) 59.9–67.2 (32.2–45.1/18.4–27.3) | i) AP:<br>UF:<br>ii) AP:<br>UF: | 76.2–77.8<br>83.0–86.2<br>74.1–78.6<br>85.6–89.4 | Ghodsvali et al. (2005) |
| ground seed, Soxhlet defatted (hexane), ground < 0.4 mm | 1) pH 13, 1:10, 20 min, 23 °C<br>2) 10,000 × g, 8 °C, 30 min, filtered<br>3) pH 4 (0.1 M HCl, gradually)<br>4) 10,000 × g, 8 °C, 30 min, washed ($H_2O$, s:l = 1:200) = APPI<br>or:<br>3′) pH 6 (dilute HCl), addition of $CaCl_2$ to 1 M, stirred 20 min<br>4′) 10,000 × g, 8 °C, 30 min, washed ($H_2O$, s:l = 1:200) = CPPI<br>5) freeze-dried | n/a | n/a | APPI:<br>CPPI: | 76.8–81.5[b]<br>73.2–82.8[b] | Aluko and McIntosh (2001) |
| industrial hexane-defatted meal | 1) pH 13, 1:19, 60 min, RT<br>2) 3000 × g, 5–10 °C, 20 min<br>3) pH 3.5 ($CH_3COOH$)<br>4) 3000 × g, 5–10 °C, 20 min, washed ($H_2O$, 3×)<br>5) freeze-dried | 99.6 | 87.5 | n/a | | Klockeman et al. (1997) |
| defatted meal | 1) pH 5.5–6.5, 60 min, 22 °C, 0.1 M NaCl/0.1 M $NaH_2PO_4$ or 0.01 M NaCl/ 0.01 M $NaH_2PO_4$<br>2) 16,300 × g, 30 min, filtered, concentration of supernatant<br>3) dilution (15×) w/ distilled $H_2O$, 4 °C, 16 h<br>4) 16,300 × g, 30 min<br>5) freeze-dried | n/a | n/a | 71.6[2] | | Ismond and Welsh (1992) |
| i) hexane-extracted meal (no pressing)<br>ii) industrial meal<br>iii) MeOH/$NH_3$/hexane (no pressing) | 1) pH 11–12, 1:18, 0.5–2 h, RT, 0 %/1 % $Na_2SO_3$<br>2) centrifuged, filtered; meal washed pH 11–12, 1:6 (2×)<br>3) 0–0.15 M $CaCl_2$, pH 3.5 (6 M HCl)<br>4) separation, precipitate washed pH 3.5–4, 1:10 | i) 85.4<br>ii) 38.1<br>iii) 54.8 | i) 75.4 (42.8/32.6)<br>ii) 33 (22/11)<br>iii) 45.7–46.8 (9.9–25.8/21.0–35.8) | i) AP:<br>UF:<br>ii) AP:<br>UF: | 79.7<br>87.6<br>75.3<br>78.6 | Tzeng et al. (1990) |

(*continued on next page*)

**Table 1** (*continued*)

| Raw material | Process conditions | Extraction yields[a] [% w/w] | Isolation yields[a] [% w/w] | Protein contents of preparations (N × 5.7) [% dry basis] | Reference |
|---|---|---|---|---|---|
| iv) dehulled (no pressing) | 5) 10 kDa, 0.05 $m^2$ (CF = 10, DV = 5) | iv) 60.5 | iv) 49.4 (14/35.4) | iii) AP: 83.0–91.7 | |
| v) dehulled + MeOH/$NH_3$/hexane (no pressing) | 6) freeze-dried | v) 47.4 | v) 42.2 (19.5/22.7) | UF: 82.8–94.0 | |
| | | | | iv) AP: 88.7 | |
| | | | | UF: 93.5 | |
| | | | | v) AP: 89.2 | |
| | | | | UF: 94.5 | |

[a] as % of protein in raw material; calculated with N × 5.7.
[b] moisture basis.

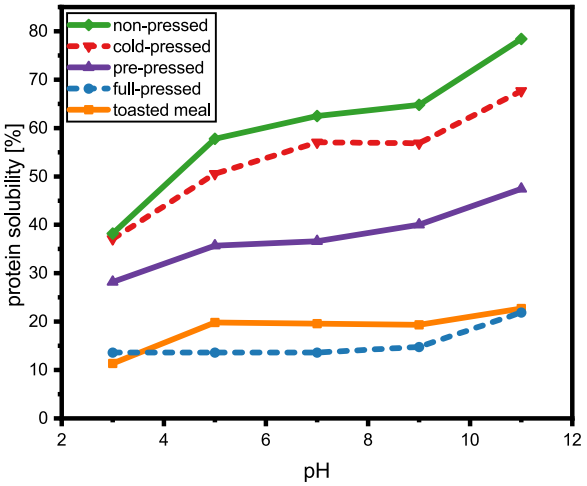


**Fig. 3.** Protein solubility (in 0.1 M NaCl, according to Morr et al. (1985)) of different rapeseed residual materials after defatting; adapted from Fetzer et al. (2018) with permission.

### 3.1.2. *Precipitation/ ultrafiltration*

Protein isolation from extracts can be achieved by precipitation or ultrafiltration, or a combination of both. As proteins can have different isoelectric points with minimal solubility, changing the pH of the extract can lead to the precipitation of certain protein fractions. For rapeseed, precipitation predominantly of cruciferins can be achieved at an acidic pH, usually around pH 3.5–4.5 (Fig. 2, process path 1; Table 1). This simple method to obtain cruciferin-rich and napin-rich fractions was applied in a range of scientific studies (Table 1), and was described in various patents (Düring et al., 2015; Helling et al., 2007; Segall et al., 2006). Precipitation may also be assisted by the addition of $CaCl_2$ (Aluko and McIntosh, 2001; Ghodsvali et al., 2005; Tzeng et al., 1990). As an alternative, the separation of cruciferins from the extract by heat treatment was also described in the patent literature (Gosnell et al., 2008). Additionally, complete protein precipitation was described via ammonium sulfate precipitation, which is based on the "salting out" effect, as originally reported by Hofmeister (1888) (Das Purkayastha et al., 2015; Wu and Muir, 2008).

Another technology for protein purification is ultrafiltration. Here, a membrane with a specific molecular weight cut-off is used to remove small molecules, such as sugars and salts, while proteins are retained in the retentate (Fig. 2, process path 2). Ultrafiltration is generally used after precipitation to obtain napin-rich preparations, but can also be used directly after extraction to obtain mixed protein preparations (Fetzer et al., 2019).

An overview of raw material quality, isolation procedure, yields and protein contents (where reported) from studies investigating rapeseed protein isolation by precipitation and/ or ultrafiltration is given in Table 1. Most authors reported highly alkaline extraction conditions, which is beneficial for achievable yields. However, a high amount of lye utilization and the risk of protein hydrolysis has to be considered (Fetzer et al., 2018). The highest isolation yield found in the literature was reported as 87.5 % by Klockeman et al. (1997), using industrial defatted meal extracted at pH 13. This is specifically remarkable, as industrial defatted meal was used in this study, which usually shows only limited potential for efficient protein extraction (Fetzer et al., 2018). Tzeng et al. (1990) carried out a detailed study to compare rapeseed protein extraction from a variety of different raw material qualities. Using pH 11–12 for extraction, the highest isolated yield was achieved from Soxhlet-defatted meal, at 75.4 %. Yields were significantly lower when using a solvent mixture of MeOH/$NH_3$/hexane for defatting, and/or when using dehulled raw material. The lowest yield was obtained from industrial meal (33 %). A similar trend in regard to isolation yield was reported in a recent study by Östbring et al. (2019a) for cold-pressed press cake (34 %–45 %),

industrial hot-pressed press cake (23 %–26 %), and industrial hexane-defatted meal (5 %–8 %). In another study by the authors, yields for cold-pressed cakes of different varieties could even be improved to 51 %–54 % (Östbring et al., 2019b). Ghodsvali et al. (2005) also studied the effect of dehulling on protein yields and found that isolated yields increased from 56.5 %–67.2 % upon dehulling of lab-scale, Soxhlet-defatted meal. Some others also studied the influence of pre-washing of defatted materials prior to protein extraction using various solvent mixtures in order to reduce the content of secondary plant metabolites, such as polyphenols. Chabanon et al. (2007) reported a protein isolation yield of 30 % for industrial defatted meal using a mixture of EtOH:$H_2O$ (65:35). A similar approach was employed more recently by Ivanova et al. (2017) and Kalaydzhiev et al. (2019) using a mixture of EtOH:$H_2O$ (3:1), however, no yields were reported. Das Purkayastha et al. (2015) used Soxhlet-defatted (hexane:diethyl ether 1:1), industrial press cake as a starting material. Pre-washing with acidified MeOH:acetone (1:1), followed by alkaline extraction and ammonium sulfate precipitation afforded a 50 % yield. Lastly, acidic pre-washing (pH 4) of industrial meal was tested, and gave a 51 % yield via alkaline extraction followed by acidic precipitation and ultrafiltration (Akbari and Wu, 2015). Unfortunately, the listed studies failed to report an internal comparison without the use of a pre-washing step, such that the effect of this washing step on protein yield is difficult to interpret. In order to reduce the protein denaturation associated with the reduction of some functional properties, strong acidic or alkaline conditions should be avoided during the extraction. One study working under milder conditions was reported by Wu and Muir (2008). Using phosphate buffer at pH 8 as the extraction medium, an extraction yield of 50.1 % from lab-scale, Soxhlet-defatted meal was achieved. Unfortunately, no isolation yields were reported. In a study originating from our own labs, pH 5.7–7 was chosen as a mild extraction environment (Fetzer et al., 2019). Protein yields were highly dependent on the raw material used and were much higher for cold-pressed defatted meal (38.2 %–40.6 %) as compared to pre-pressed defatted meal (24.2 %–26.0 %).

The protein content of rapeseed preparations via isolation ranged between 37.4 % and 94.0 % in the reviewed studies, with the majority of values reported being at around of 80 %. Though most studies reported protein content using the traditional factor of 6.25, all values were recalculated for this review using the more accurate factor of 5.7 (Mariotti et al., 2008). The protein content of preparations obtained through precipitation can be increased by deploying additional washing steps or dialysis. For ultrafiltrated preparations, protein content can be increased by using high concentration factors (CF) and large diafiltration volumes (DV). High protein content is demanded especially for food applications, due to the nutritive value and the unwanted off-flavors which can be caused by secondary plant metabolites. For applications in the technical industry, protein content is less critical, as non-protein substances may not necessarily have a bad impact on functionality. In general, functionalities such as solubility, emulsifying and foaming properties were found to be more dependent on the isolation process and type of proteins isolated than on protein content (Aluko and McIntosh, 2001; Fetzer et al., 2019).

In summary, a comparison of the listed studies is somewhat difficult due to the utilization of raw materials of different origins that were obtained under different processing conditions. However, some conclusions can be drawn. Processing during defatting is the main limitation factor for achievable protein extraction yields, which in turn determine isolation yields. For an economic protein recovery process, a mild defatting method is thus essential. Furthermore, an increase in the extraction pH generally increases protein yields, with the drawback of possibly causing protein hydrolysis and an increase in phenol oxidation and cross-linking (Fetzer et al., 2019; Naczk et al., 1998). Extraction pH thus has to be carefully chosen based on a balance of yield and protein quality. Lastly, while the isolation strategy (precipitation vs. ultrafiltration) does not significantly influence yield, it does have an impact on protein content and can determine functionality (Akbari and Wu, 2015; Chabanon et al., 2007; Fetzer et al., 2019; Ghodsvali et al., 2005; Tzeng et al., 1990).

#### 3.1.3. *Protein micellar mass method*

The protein micellar mass method was developed in order to reduce protein denaturation and decrease the content of antinutritive components, including glucosinolates, phytic acid and phenolic compounds, in the protein isolate (Ismond and Welsh, 1992; Murray et al., 1981). Protein micellization is achieved via the cold-water dilution of a salt-containing protein extract (Fig. 2, process path 3), such that no extreme pH values are employed (Arntfield and Murray, 1981). Using phosphate-buffered solutions at pH 5.5–6.5, an average protein content of 71.6 % and reduced contents of phytic acid and phenols were reported (Table 1). The process was adapted by Burcon Nutrascience Corporation, with claimed protein contents of at least 91.2 % (N × 5.7) (Barker et al., 2002; Segall et al., 2009).

### 3.2. *Protein concentration*

Rapeseed protein concentration is achieved via the removal of secondary plant metabolites with organic solvents or aqueous mixtures thereof (Fig. 2, process path 4). The process has been used in attempts of simultaneous oil and protein recovery, although with significantly lower oil yields compared to commercial processes (Frische et al., 1996; Natsch, 2006). Using protein concentration, protein contents of 55 %–60 % (N × 5.7) can be achieved (Barbin et al., 2011). Rapeseed proteins prepared using solvent extraction have been reported as showing limited techno-functional properties caused by denaturation, such as reduced solubility and emulsion stability (Barbin et al., 2011; Natsch, 2006; von der Haar et al., 2014). These properties could be improved to some extent by physical modification using thermal or high-pressure treatment (Barbin et al., 2011; Natsch, 2006). For the stated reasons, organic solvent-assisted processes are generally of low interest for the production of rapeseed protein preparations. However, solvent-extracted meals may be used for subsequent protein isolation procedures to obtain protein isolates with reduced contents of carbohydrates, glucosinolates and polyphenols (Chabanon et al., 2007; Das Purkayastha et al., 2015; Ivanova et al., 2017; Kalaydzhiev et al., 2019).

Moreover, dry fractionation techniques were reported for the concentration of rapeseed proteins as an economically friendly, solvent-free process. One method to increase the protein content of the meal and remove bitter components of the seed coat is the dehulling of rapeseed, which has been described in patents (Nyenhuis, 2011; Palyi, 1971; Rass and Schein, 2010). However, the oil loss caused by the separation of the seed coat was described as a major issue for the industrial use of dehulling processes (Carré et al., 2014). More recently, the ultrafine milling of rapeseed meal followed by fractionation using air classification or electrostatic separation was reported (Laguna et al., 2018). Protein concentration was achieved to a significantly higher level through electrostatic separation compared to air classification, with protein contents of 53.8 % and 44.7 % (N × 5.7), respectively. However, the obtained recovery yield for electrostatic separation was very low (9.2 %), compared to 40.7 % when using air classification. Similar results for air classification were obtained by Rempel et al. (2020) with a protein concentration of 40.4 % and 35 %–50 % recovery yield. In another study using different milling techniques followed by electrostatic separation, protein contents of 36.5 %–39.9 % and recovery yields of 45.8 %–58.8 % were reported (Kdidi et al., 2019). In summary, dry fractionation may have great potential as a solvent-free and mild processing method for the production of rapeseed protein concentrates. However, major improvements in regard to the obtained protein content and recovery are required for industrial application to be viable.

## 4. Protein functionality

The functional properties of rapeseed preparations are strongly dependent on the recovery method, resulting in different protein

contents, compositions and dissociation/ denaturation states. The relevant functional properties of rapeseed protein preparations for application in the chemical sector are reviewed in detail in the following. The majority of these properties, including emulsifying, foaming, and gelation, was studied exclusively in food systems, and detailed reviews on this topic can be found (Aider and Barbana, 2011; Tan et al., 2011; Wanasundara, 2011; Wanasundara et al., 2016). As these data are often equally relevant to technical applications, they are included in the present review, with the addition of new studies published in recent years.

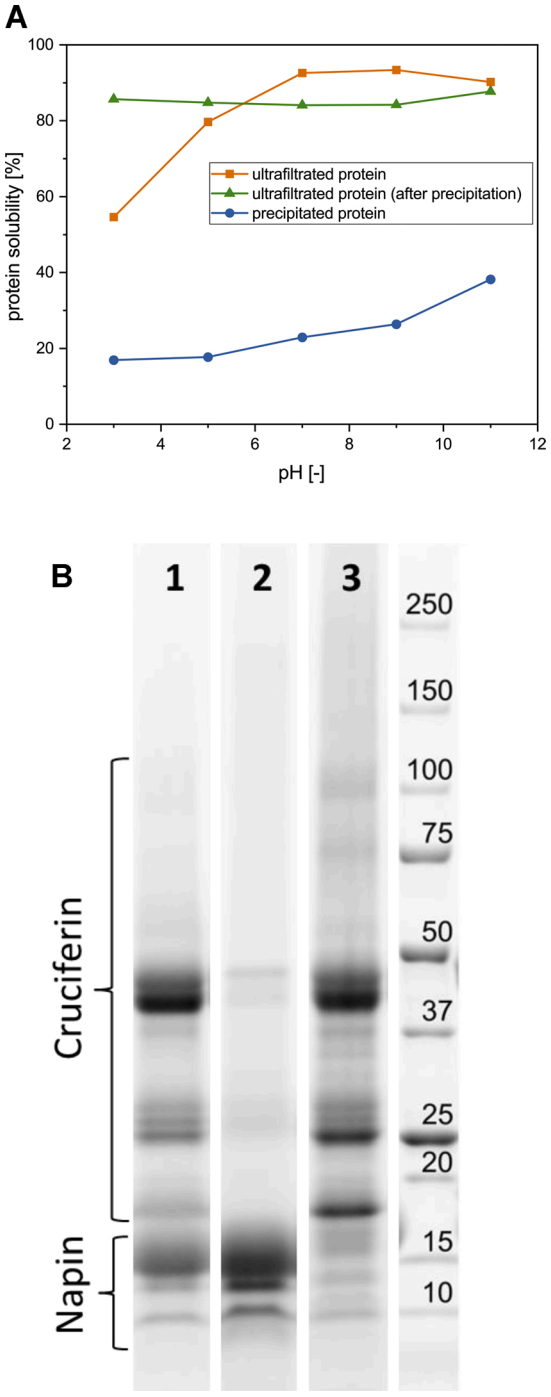


**Fig. 4.** A: Protein solubility (in 0.1 M NaCl, according to Morr et al. (1985)) of rapeseed protein preparations isolated via different strategies; B: SDS-PAGE (non-reducing conditions) of rapeseed protein preparations: 1: ultrafiltrated protein, 2: ultrafiltrated protein (after precipitation), 3: acidic precipitated protein, right lane: molecular weight standard [kDa] (adapted from Fetzer et al. (2019) with permission).

### 4.1. Solubility

Rapeseed protein preparations show distinct solubility profiles in aqueous media depending on their composition and isolation process. Generally, protein solubility is determined according to the method of Morr et al. (1985), but care has to be taken when comparing values from different labs. Both cruciferin and napin exhibit good solubility properties in alkaline media, which is why protein extraction from meals is generally carried out at a high pH. However, as cruciferin and napin differ greatly in their solubility properties across the pH spectrum, protein composition is the most crucial factor regarding protein solubility (Fetzer et al., 2019). While cruciferin is precipitated at an acidic pH and can undergo dissociation and denaturation, napin shows good solubility across the full pH spectrum. Thus, ultrafiltrated preparations generally show higher solubility compared to precipitated preparations, due to higher napin content. Fig. 4 displays the distinct solubility profiles as a function of the pH for protein preparations obtained by ultrafiltration or combined acidic precipitation and ultrafiltration (Fetzer et al., 2019). Good solubility across the full pH spectrum (> 80 %) is shown by samples obtained through ultrafiltration of the supernatant after precipitation. As visible from the SDS-PAGE analysis, this is due a high content of napin, which remains in the soluble supernatant after precipitation (Chabanon et al., 2007; Fetzer et al., 2019). Samples obtained from direct ultrafiltration after extraction show great solubility properties in the neutral to alkaline range, although a distinct drop of solubility can be observed in the acidic pH range. This is due to the precipitation of cruciferins under these conditions, while napin fractions remain soluble. Preparations obtained via acidic precipitation show low solubility across the full pH spectrum, which is caused by the partly irreversible denaturation of proteins upon acidic precipitation (Wanasundara, 2011).

### 4.2. Emulsifying

Emulsions are relevant in a range of technical applications, such as emulsion-based adhesives, coatings, cleaning products or lubricants. Emulsifying properties are determined using various methods in the literature. Generally, three terms can be differentiated when describing emulsifying properties: the emulsion activity index (EAI), emulsifying capacity (EC), and emulsion stability (ES). The EAI describes the interfacial area that can be covered by the protein as a surfactant, and is measured by turbidity (T) of the emulsion (Pearce and Kinsella, 1978). It is defined as $EAI = 2\,T/\Phi C$, where $\Phi$ is the volume fraction of the dispersed phase and C is the weight of protein per unit volume of aqueous phase before emulsion formation. The EC is defined, in a more practical manner, as the volume of oil emulsified per gram of protein sample (Yoshie-Stark, 2008). The ES is measured as the percentage of emulsified volume remaining from the initial volume after 30 min (Aluko and McIntosh, 2001). Given the various methods available to describe emulsifying properties of proteins in the literature, care has to be taken when comparing values reported from different studies.

Generally, rapeseed proteins have been reported to show great emulsifying properties, which were superior to other protein sources such as soy or flaxseed in some studies (Khattab and Arntfield, 2009). Values reported have been shown to depend largely on the raw material used for extraction and the isolation method used. Aluko and McIntosh (2001) found higher EAI values for acidic precipitated samples (pH 4) compared to calcium precipitated samples (pH 6), although with the opposite trend in regard to ES (30 min). A detailed study comparing emulsifying properties of cruciferins, napins and a mixed isolate was carried out by Wu and Muir (2008). Cruciferin showed a drastically higher specific surface area of emulsions than both the napin sample and the mixed isolate. At the same time, the particle size was lowest for

cruciferin emulsions, explaining their high stability in comparison to napin-containing samples. A similar trend was reported by Akbari and Wu (2015) who reported rapid destabilization of napin-based emulsions (5 min). In contrast, both napin-rich and mixed samples isolated by ultrafiltration were reported to show high EC in a study originating from our labs (Fetzer et al., 2019). Here, cruciferin-rich samples from acidic precipitation (pH 4) showed a significant reduction in EC, presumably due their limited solubility. The effect of pH and NaCl concentration on emulsifying properties was studied for cruciferin and napin isolates (Cheung et al., 2015, 2013). Values reported for EAI and ESI were in a similar range in both studies, with better emulsifying properties reported at pH 3 and pH 5 compared to pH 7, and more stable emulsions being formed at low salt concentration. Similar trends in regard to pH and ionic strength were also found in a recent study by (Wu et al., 2020). Interestingly, the authors reported a positive correlation between the α-helix content and emulsion stability, indicating a possible mechanism for napin-stabilized emulsions.

### 4.3. Foaming

Foam formation can be of relevance in various industrial sectors, such as building (insulants), cleaning (cleaning agents) and cosmetics (shampoo etc.). Foaming properties of proteins are described by the foam capacity (FC), or foam activity (FA), the foam stability (FS) and foam density (FD) in the literature. The terms FC and FA are used to describe the volume expansion of a protein solution upon whipping at defined speed and for a defined time. FS is measured as the resultant foam volume compared to the initial volume after a defined time. FD is measured and given in g/mL foam volume. Foaming properties have been studied predominantly for application in food systems; however, their relevance can be transferred to a range of technical products, such as foamed adhesives or polymers, and detergents.

Rapeseed proteins have been reported to show great foaming properties that equal or even surpass those of soybean or flaxseed proteins when comparing meals (Ghodsvali et al., 2005; Khattab and Arntfield, 2009). Protein concentrates and isolates prepared by ultrafiltration have been reported to show high foaming capacity, regardless of their protein composition. Napin-rich concentrates showed even higher emulsifying capacity than mixed samples, which is possibly due to a higher solubility (Akbari and Wu, 2015; Fetzer et al., 2019). Good foaming properties for the acid-soluble protein fraction without purification by ultrafiltration were also reported across the range of pH 2–10 (Ivanova et al., 2018). In the study, the addition of NaCl (0.03–0.25 M) did not affect foam capacity, but did reduce foam stability, presumably due to the induced weakening of protein-protein interactions. In contrast, isolation methods using protein precipitation led to the reduction of foaming properties, presumably due to their reduced solubility (Fetzer et al., 2019). Aluko and McIntosh (2001) reported better foam capacity and stability of acid-precipitated proteins compared to calcium-precipitated proteins. Chabanon et al. (2007) also reported better foaming capacity for napin compared to cruciferin.

### 4.4. Gelation

The gel formation of globular proteins is a result of a heat-induced unfolding of the protein structure followed by an aggregation into a three-dimensional network (Arntfield, 1996). The stabilization of this network is provided by hydrogen bonding, hydrophobic interactions, and covalent cross-links, such as disulfide bonds (Hammann and Schmid, 2014). For rapeseed proteins, it was suggested that hydrophobic interactions played a major role in network formation, while hydrogen and disulfide bonds had a lower influence (Kim et al., 2016a). One indicator of the potential of proteins to form gels is often provided by the measurement of their least gelation concentration (Gill and Tung, 1978; Khattab and Arntfield, 2009). This is defined as the least protein concentration of a solution, at which the solution will not slip in a test-tube after heat-treatment and inverting. Protein gelation mechanisms and gel properties are generally characterized by differential scanning calorimetry (DSC), rheometry, and textural as well as microscopic analysis.

According to the literature, rapeseed proteins seem to show rather low gelation properties. This was reported by Sosulski et al. (1976) for meals, concentrates and isolates. Also, Khattab and Arntfield (2009) reported low gelation properties of rapeseed meals, with lower least gelation concentrations than soy or flaxseed meals. The ability of a protein to form a gel is related to its molecular weight, with larger proteins exhibiting a higher ability to stabilize the three-dimensional network (Oakenfull et al., 1997). Consequently, cruciferins were reported to form stronger gels than napins (Schwenke et al., 1998; Tan et al., 2014; Yang et al., 2014a). Gelation temperatures reported were in the range of 82–90 °C (Kim et al., 2016a, b). Stronger gels were reported consistently for higher temperatures and higher pH, presumably due to increased protein unfolding and resulting molecular interactions (Gill and Tung, 1978; Kim et al., 2016a, b; Leger and Arntfield, 1993; Tan et al., 2014; Yang et al., 2014a). Gel properties can be enhanced by the mixture of rapeseed proteins with proteins from different sources. These effects can even be synergistic, as was demonstrated in one study for mixtures with whey proteins (Ainis et al., 2018). Similarly, non-protein compounds can enhance the properties of rapeseed protein gels. As such, it was suggested that the presence of carbohydrates in rapeseed protein samples played a major role in network formation (Gill and Tung, 1978). Accordingly, the addition of κ-carrageenan increased covalent bonding for rapeseed proteins within the gel structure (Uruakpa and Arntfield, 2006). However, the negative effects of guar gum on gelation properties were reported in another study, indicating that the nature of the carbohydrate is of high importance (Uruakpa and Arntfield, 2005). The addition of phytic acid alone or in combination with calcium chloride also demonstrated the possibility of increasing network formation of rapeseed gels by ionic bonding, with a strong influence of the pH and resulting protein charge (Arntfield, 1996). In contrast, the presence of phenols was detrimental to gel properties, possibly due to interference of hydrophobic interactions (Arntfield, 1996; Rubino et al., 1996).

### 4.5. Film-forming

Film-forming properties are mandatory for a range of applications, including adhesives, coatings, paints, lacquers and polymers (Schmid and Müller, 2019). Rapeseed proteins have been studied in this regard by either solution-casting or extrusion of films. For solution-casting, the protein is formulated with water and a plasticizer and then subjected to a denaturation step, before the mixture is poured into a petri-dish and dried. The resulting film is removed from the petri dish and is generally measured for its mechanical and barrier properties, which can vary depending on protein type and concentration, plasticizer content, and denaturation method. Properties discussed in this review are tensile strength (TS), elongation at break, elastic modulus (EM), water vapor permeation (WVP) and oxygen permeability (OP). Values obtained from different studies and film formulations are listed in Table 2.

Chang and Nickerson (2015) tested the influence of protein and glycerol concentration on film properties. TS was in the range of 1.2–6.6 MPa, and correlated positively with protein concentration, and negatively with glycerol concentration. The TS values from other studies were in a comparable range, with similar trends regarding the concentration of plasticizer (Jang et al., 2011b; Shi and Dumont, 2014a). In another study, the authors tested the influence of the plasticizer type and concentration, and found higher TS values for sorbitol (10.4 MPa) than for PEG-400 (5.8 MPa) and glycerol (1.8 MPa) (Chang and Nickerson, 2014). With the addition of genipin as a cross-linker, the TS increased by 21 %, 68 % and 140 % for sorbitol, PEG-400 and glycerol, respectively. Shi and Dumont (2014a) reported a slight increase in TS through the SDS-denaturation of rapeseed protein prior to film formulation, while the use of stearic acid as a co-plasticizer had a negative influence on TS.

**Table 2**
Studies reporting physical properties of rapeseed protein-based films; values may be approximate as they were taken from graphic presentations; RH = relative humidity.

| Formulation | Method (setting conditions) | Tensile strength (TS) [MPa] | Elongation at break [%] | Elastic modulus (EM) [MPa] | Water vapor permeability (WVP)/ Oxygen permeability (OP) [gmm/m$^2$hkPa]/ [cm$^3$/(m$^2$dbar)] | Reference |
|---|---|---|---|---|---|---|
| 7.5 % protein, 1.87 % glycerol | Casting (23 °C, 50 % RH, 14 days) | 0.6 | 151 | – | 14.9 (OP) | Fetzer et al. (2020) |
| 7.5 % protein (lauroyl-modified), 1.87 % glycerol | | 1.3/2.4 | 104/9.5 | – | 18.8–36.3 (OP) | |
| 7.5 % protein (oleoyl-modified), 1.87 % glycerol | | 1.2/2.1 | 121/43 | – | 17.0–38.6 (OP) | |
| 4 % protein | Casting (25 °C, 50 % RH, 48 h; conditioned at 54 % RH) | 3.4 | 84.2 | – | 0.8 (WVP) | He et al. (2019) |
| 4 % protein (succinyl-modified) | | 5.0–2.4 | 123.6–76.2 | – | 0.3–1.0 (WVP) | |
| 1 % protein, 0.4 % glycerol | Casting (25 °C, 55 % RH) | 1.5 | 8 | – | 0.65 (WVP) | Zhang et al. (2019) |
| 1 % protein, 1 % chitosan, 0.4 % glycerol | | 16.0 | 1 | | 0.5 (WVP) | |
| 1 % protein (hydrolyzed), 1 % chitosan, 0.4 % glycerol | | 16.0–23.5 | 3–41 | – | 0.3–0.4 (WVP) | |
| 2.67 % protein, 1.13 % glycerol | Casting (23.8 °C, 50 % RH, 48 h), heat compression (90 °C, 10 min, 1.5 MPa) | 5.2 (55 % RH) 1.1 (98 % RH) | 130 (55 % RH) 160 (98 % RH) | – | – | Li et al. (2017) |
| 2.67 % protein, 1.13 % glycerol, 0.0053–0.0267 % 1,4-butanediol diglycidyl ether | | 7.1–9.6 (55 % RH) 2.9–7.2 (98 % RH) | 90–65 (55 % RH) 90–60 (98 % RH) | – | – | |
| 35 % protein (liquid $CO_2$-extracted meal), 15 % glycerol, 40 % synth. co-polyester, 2 % PVP, 1 % $ZnSO_4$ | Extrusion (95–141 °C) | 9.5 | 15 | 350 | – | Manamperi et al. (2015) |
| 35 % protein (industrially defatted meal), 15 % glycerol, 40 % synth. co-polyester, 2 % PVP, 1 % $ZnSO_4$ | | 10 | 6.5 | 500 | – | |
| 35 % protein (laboratory screw-pressed, hexane-extracted meal), 15 % glycerol, 40 % synth. co-polyester, 2 % PVP, 1 % $ZnSO_4$ | | 13 | 4 | 700 | – | |
| 5 % protein, 1.5–2.5 % glycerol | Casting (21–23 °C, 54 % RH, 48 h) | 1.2–4.3 | 5.4–10.2 | 0.3–1.45 | 0.7–1.2 (WVP) | Chang and Nickerson (2015) |
| 7.5 % protein, 2.25–3.75 % glycerol | | 2.3–6.6 | 6.0–8.2 | 0.5–1.75 | 0.95–1.5 (WVP) | |
| 5 % protein, 2.5 % glycerol | Casting (21–23 °C, 54 % RH, 48 h) | 1.2 | 10.2 | 0.2 | 1.2 (WVP) | Chang and Nickerson (2014) |
| 5 % protein, 2.5 % sorbitol | | 10.0 | 3.9 | 3.3 | 0.5 (WVP) | |
| 5 % protein, 2.5 % PEG-400 | | 5.2 | 7.2 | 1.4 | 0.5 (WVP) | |
| 5% protein, 2.5 % glycerol, 1 % genipin | | 2.6 | 7.6 | 0.6 | 0.9 (WVP) | |
| 5 % protein, 2.5 % sorbitol, 1 % genipin | | 12.6 | 3.8 | 4.2 | 0.5 (WVP) | |
| 5 % protein, 2.5 % PEG-400, 1 % genipin | | 9.6 | 7.0 | 2.6 | 0.8 (WVP) | |
| 5 % protein, 1.25–2 % glycerol | Casting (25 °C, 30 % RH, 48 h) | 0.7–1.7 | 41.9–287.4 | – | – | Shi and Dumont (2014a) |
| 5 % protein, 1.25–2 % glycerol, 0.25–0.75 % stearic acid | | 0.2–2.2 | 8.8–128.9 | – | – | |
| 5 % protein, 1.25–2 % glycerol, 0.25–0.75 % SDS | | 0.9–3.8 | 24.6–551.4 | – | – | |
| 35 % protein, 15 % glycerol, 40 % synth. co-polyester, 2 % PVP, 1 % $ZnSO_4$ | Extrusion (95–141 °C) | 10 | 17.5 | 200 | – | Manamperi and Pryor (2012) |
| 35 % protein (SDS-denatured), 15 % glycerol, 40 % synth. co-polyester, 2 % PVP, 1 % $ZnSO_4$ | | 11.5–12.0 | 17–21 | 230–250 | – | |
| 35 % protein (SDBS-denatured), 15 % glycerol, 40 % synth. co-polyester, 2 % PVP, 1 % $ZnSO_4$ | | 13–14.5 | 19.5–21 | 250–290 | – | |
| 4 % protein, 1.5–2.0 % sorbitol, 0.5–1.0 % sucrose | Casting (25 °C, 48 h; conditioned at 50 % RH) | 1.4–3.7 | 11.6–20.6 | – | 9.1–9.5 (WVP) | Jang et al. (2011b) |
| 4 % protein, 2.0 % sorbitol, 0.5 % sucrose, 0.5–2.0 % polysorbate 20 | | 2.7–3.9 | 13.0–18.7 | – | 6.3–6.8 (WVP) | |
| 2–5 % protein, 2.0 % sorbitol, 0.5 % sucrose, 1.5 % polysorbate 20, 0.7 % *gelidium corneum* | | 5.2–7.3 | 38.6–54.5 | – | 6.5–9.5 (WVP) | |
| 2 % protein, 2.0 % sorbitol, 0.5 % sucrose, 1.5 % polysorbate 20, 0.5–1.5 % *gelidium corneum* | | 5.8–18.6 | 8.0–52.6 | – | 6.4–7.4 (WVP) | |
| 3–6 % protein, 2.0 % sorbitol, 0.5 % sucrose, 1.5 % polysorbate 20, 4 % gelatin | | 0.5–53.5 | 1.8–12.8 | – | 6.9–9.7 (WVP) | |
| 3 % protein, 2.0 % sorbitol, 0.5 % sucrose, 1.5 % polysorbate 20, 2–5 % gelatin | | 7.1–53.5 | 8.8–26.4 | | 6.4–7.2 (WVP) | |

(*continued on next page*)

**Table 2** (*continued*)

| Formulation | Method (setting conditions) | Tensile strength (TS) [MPa] | Elongation at break [%] | Elastic modulus (EM) [MPa] | Water vapor permeability (WVP)/ Oxygen permeability (OP) [gmm/m$^2$hkPa]/ [cm$^3$/(m$^2$dbar)] | Reference |
|---|---|---|---|---|---|---|
| 3 % protein, 3 % gelatin, 2 % sorbitol, 0.5 % sucrose, 1.5 % polysorbate 20, 0–1.5 % grapefruit seed extract | Casting (25 °C, 48 h; conditioned at 50 % RH) | 31.9–11.9 | 13.7–72.7 | | 7.2–10.3 (WVP) | Jang et al. (2011a) |
| 35 % protein, 15 % glycerol, 40 % synth. co-polyester, 2 % PVP, 1 % $ZnSO_4$ | Extrusion (95–141 °C) | ~6–14 | 1–18.5 | – | – | Manamperi et al. (2011) |
| 35 % protein (standard), 15 % glycerol, 40 % synth. co-polyester, 2 % PVP, 1 % $ZnSO_4$ | Extrusion (95–141 °C) | 10.3 | 17.5 | 192 | – | Manamperi et al. (2010) |
| 35 % protein (refined), 15 % glycerol, 40 % synth. co-polyester, 2 % PVP, 1 % $ZnSO_4$ | | 11.9 | 23.5 | 200 | – | |

In another study, the influence of different concentration of plasticizers and emulsifiers on the properties of edible rapeseed protein-based films was tested (Jang et al., 2011b). TS and elongation were in a similar range as in other studies, but were improved significantly by using rapeseed protein blends. As such, the addition of *gelidium corneum* (a red algae with a high agar content) and gelatin improved the physical properties, giving TS values of 18.6 MPa and 53.5 MPa, respectively. Zhang et al. (2019) reported an increase of TS from 1.5 MPa to 16 MPa by the addition of chitosan, and even higher values of up to 23.5 MPa when using hydrolyzed protein. Cast films treated by heat compression (90 °C, 1.5 MPa, 10 min) showed comparably high TS values and high elasticity, despite a low protein concentration of 2.67 % (Li et al., 2017). In addition, the cross-linking of rapeseed protein with 1,4-butanediol diglycidyl ether increased the TS with increasing concentration of the bisepoxide at relative humidities of 55 % and 98 %. Films prepared from a mixture of 3 % rapeseed protein and 3 % gelatin showed a comparably high TS of 31.9 % (Jang et al., 2011a). Upon addition of an antimicrobial grapefruit seed extract, a decrease in the TS and an increase in elongation was observed, presumably due to the interference of polyphenols with protein-protein interactions. Similarly, the WVP decreased with an increasing amount of grapefruit seed extract, which possibly resulted from an increased pore size of the film network. The WVP in the studies was in the range of 0.3–1.0 gmm/m$^2$hkPa (He et al., 2019), 0.3–0.65 gmm/m$^2$hkPa (Zhang et al., 2019), 0.5–1.5 gmm/m$^2$hkPa (Chang and Nickerson, 2014, 2015), and 6.3–10.3 gmm/m$^2$hkPa (Jang et al., 2011a, b), depending on the formulation. Chang and Nickerson (2015) reported an increase in the WVP with increasing protein concentration of films, indicating the hydrophilic nature of rapeseed proteins. The latter also explains why rapeseed protein-based films were found to be water-soluble (Fetzer et al., 2019). Oxygen permeability was reported only in one study with 14.9 cm$^3$/(m$^2$dbar) (Fetzer et al., 2020). Upon the chemical grafting of rapeseed proteins with lauroyl and oleoyl residues, OP increased to 36.3–38.6 cm$^3$/(m$^2$dbar).

Manamperi et al. studied plastics produced by the extrusion of rapeseed proteins from various origins (Manamperi et al., 2010, 2011, 2015; Manamperi and Pryor, 2012). In their experiments, formulations of 35 % protein, 15 % plasticizer (glycerol), 40 % synthetic co-polyester, 2 % compatibilizer (polyvinyl pyrrolidone (PVP)) and 1 % cross-linker ($ZnSO_4$) were used. Values for the TS and elongation were in the range of 6–14.5 MPa and 1 %–23.5 %, respectively, which were comparable to some studies using solution-casted films. However, in contrast to solution-cast studies, the EM was drastically improved by extrusion, with values of up to 700 MPa, which can be explained by the presence of high amounts of synthetic co-polyester in the formulations (Manamperi et al., 2015). In one study, the EM was increased by using sodium dodecyl sulfate (SDS) or sodium dodecylbenzenesulfonate (SDBS) for protein denaturation prior to film extrusion (Manamperi and Pryor, 2012). Moreover, the addition of SDS and SDBS led to a concentration-dependent increase in water absorption, which negatively affects the application possibilities of water-resistant plastics. More recently, the authors compared plastic properties in dependence of the defatting method using liquid $CO_2$-extracted meal, industrially defatted meal and laboratory screw-pressed and hexane-extracted meal for protein preparation (Manamperi et al., 2015). The authors concluded a higher general applicability of proteins prepared from liquid $CO_2$-extracted meal, due to lower denaturation levels and better solubility properties. Plastics from the meal showed higher elongation and toughness, however, the TS and EM were lower than for plastics prepared from proteins derived from industrially or laboratory-processed meals.

In summary, low mechanical properties of rapeseed protein-based cast films were reported in the literature. This is comparable to properties reported for other plant proteins (Shi and Dumont, 2014b; Zink et al., 2016). The processing of meals prior to protein extraction and during protein isolation does have an influence on film properties, due to the different denaturation levels of prepared proteins (Manamperi

et al., 2015). However, the main impact on resulting properties is seen in the film formulation and production procedure. As such, films prepared using cross-linkers under conditions where covalent bond-formation is induced lead to a significant increase in mechanical properties. In conclusion, adaption of the formulation and film-processing method is seen as a superior approach compared to protein modification before film-formation.

## 5. Protein modification

The modification of rapeseed proteins by physical, chemical or biochemical methods provides a valuable tool to alter their functional properties. The improvement of certain functional properties is often achieved at moderate modification degrees, while excessive treatments can lead to deterioration of protein functionality. Thus, the modification method and degree have to be carefully chosen in order to achieve the desired functional properties. Moreover, the economic and ecological aspects of protein modification have to be considered in regard to the intention of replacing petro-based chemicals.

### 5.1. Physical

#### 5.1.1. Heat

Plant proteins are highly susceptible to high temperatures, often leading to irreversible protein denaturation and aggregation. While high temperatures should be avoided during defatting due to decreased protein extractability (Mosenthin et al., 2016; Salazar-Villanea et al., 2016), thermal treatment can be used on isolated protein samples to modify their functional properties. Thermal treatment has been tested to improve deteriorated functional properties of rapeseed proteins obtained by alcoholic processes (Barbin et al., 2011). Positive effects were found in regard to water binding capacity and emulsion stability, while emulsion capacity and protein solubility decreased upon thermal treatment. Similarly, a reduction of protein solubility, along with the increased formation of protein aggregates was observed by He et al. (2014) with increasing temperature (60 °C, 80 °C, 100 °C). Surface hydrophobicity was increased, presumably due to the liberation of hydrophobic side chains, with the highest impact observed at 80 °C. Gelation properties (least gelation concentration, hardness) were improved and were also highest for the treatment at 80 °C. Napin was reported to be less heat resistant than cruciferin, however, with lower susceptibility to changes in ionic strength and pH (Folawiyo and Apenten, 1997). Salt concentration and pH – among other parameters – are known to have a great impact on the mechanism of heat-induced aggregation of globular proteins, thereby influencing functionality (Nicolai and Durand, 2013). However, little data is available for rapeseed proteins in this regard, such that further studies are demanded to enable the controlled heat-induced aggregation.

#### 5.1.2. High pressure

The emulsifying capacity and stability of rapeseed protein concentrates produced via alcoholic processing were improved by high-pressure homogenization (18 MPa), while protein solubility was reduced (Barbin et al., 2011). In another study, pressures of 200–600 MPa were applied to investigate the effect on surface hydrophobicity, thermal and gelation properties (He et al., 2014). The authors reported an increase in surface hydrophobicity, higher thermal stability and better gelation properties with increasing pressures. In particular, the least gelation concentration and gel hardness were significantly improved, and the effect was drastically higher in comparison to thermal treatment (60–100 °C). This could be explained by a significantly greater rise in surface hydrophobicity upon high-pressure treatment, which possibly contributed to gel network stabilization. In another study, rapeseed proteins subjected to high-pressure treatment (400 MPa) showed an increased encapsulation efficiency as wall materials for microcapsules (Wang et al., 2015). The increase was explained by a more stable secondary structure, which improved the mechanical stability of the microcapsules.

### 5.2. Chemical/ biochemical

#### 5.2.1. Acylation

Chemical acylation is the most studied modification method reported for rapeseed proteins. Acylation is achieved using reactive anhydrides or chlorides, which can react with nucleophilic groups within the protein, e.g. hydroxyl, sulfhydryl, or amino groups. Within those, the $\varepsilon$-amino group of lysyl residues is considered the most reactive nucleophile available. Thus, the degree of modification is generally determined by analysis of free amino groups before and after modification (Malabat et al., 2001).

Modification of free functional groups leads to a change in the charge distribution, often inducing the dissociation of protein subunits and the unfolding of the protein structure (Gruener and Ismond, 1997; Zink et al., 2016). For the rapeseed 11S cruciferin, a critical acylation degree of 60 %–70 % has been reported (Schwenke, 1990). Beyond this acylation degree, the hexameric protein was observed to completely dissociate into its subunits, leading to a dramatic alteration of functional properties (Gruener and Ismond, 1997). In the case of the succinylation of cruciferin, the introduction of negatively charged groups induced dissociation as well. Here, 7S and 3S components appeared at a succinylation degree of 19.4 % (Gueguen et al., 1990), while 3S was the predominant species at values above 66 % (Schwenke et al., 1998). Unfolding and increased surface charge were related to adsorption kinetics. The protein solubility of a precipitated cruciferin-rich isolate was increased by succinylation, while acetylation had little effect (Krause, 2002). In the same study, an increase in surface activity and foaming properties was found for acylation; however, acetylation led to reduced foam stability. The succinylation of up to 98 % and 83 % of free amino groups of cruciferin and napin, respectively, was reported by Schwenke (1990). In contrast to cruciferin, no structural changes were observed for napin even at high degrees of modification (Gerbanowski et al., 1999; Schwenke, 1990). This was explained by the proteins' remarkable stability due to stabilization through inter- and intrachain disulfide bonds. The solubility of modified napins was reduced in the acidic range, due to a shift in the isoelectric point caused by the blockage of basic amino groups. Foaming properties were reported to be unaffected by acetylation or succinylation (Schwenke, 1990), while positive effects on emulsion properties were reported for succinylation already at moderate degrees (Paulson and Tung, 1988). Malabat et al. (2001) studied rapeseed peptides before and after chemical modification and compared foaming and emulsifying properties using sodium dodecyl sulfate (SDS) as a reference. Upon the chemical grafting of peptides using *p*-toluenesulfonyl chloride, comparable foaming properties to SDS were achieved. In contrast, acylation using butyric anhydride decreased foaming properties in all cases. The formation of stable emulsions was neither observed for rapeseed peptides nor for their chemically modified derivatives. Gelation properties were also studied for acetylated and succinylated rapeseed proteins. While acetylation showed only moderate influence on gelation properties in one study (Schwenke et al., 1998), succinylation was reported to improve gelation properties, such as least gelling concentration, adhesiveness, water holding capacity and hardness (Paulson and Tung, 1989; Wang et al., 2018b). The effects were attributed to structural changes in the modified protein leading to increased hydrophobic interaction and hydrogen-bonding within the gel matrix. Similarly, the ability for succinylated rapeseed proteins to act as wall materials for microcapsules was increased (Wang et al., 2015). The authors reported increased stiffness for the modified proteins, which was related to an increased stability of the secondary structure, possibly induced by ionic forces and hydrogen bonding. The succinylation of rapeseed proteins was also studied in regard to film-forming properties (He et al., 2019). The authors reported increased TS and lower WVP only for the lowest modification degree, while the reversed effects were

observed at higher levels of succinylation (Table 2). While all modified samples showed similar secondary structure profiles, a significant increase in surface hydrophobicity only for the lowest succinylation degree may serve as an explanation for the observed effects.

Gerbanowski et al. (1999) compared aliphatic acylation (C2–C6) and aromatic sulfamidation for napin and cruciferin samples. As described earlier, napin structure showed remarkable conformational stability towards chemical modification, while cruciferin tended to unfolding already at low degrees of modification. Consequently, surface hydrophobicity was increased either by grafting of hydrophobic residues on the protein surface, or by liberation of hydrophobic residues upon unfolding. Acylation using longer hydrophobic side chains (C6–C16) was tested for rapeseed protein hydrolysate to generate surfactant-like peptides (Sánchez-Vioque et al., 2001). In reference to BSA (bovine serum albumin) and SDS (sodium dodecyl sulfate), C10- and C12-modified peptides showed good liquid volume incorporation and stability of foams, showing characteristics of both protein- and surfactant-type foams. Modification with shorter or longer acyl chlorides was reported to lack the ability to provide stable foam formation, presumably due to low hydrophobicity (C6, C8) or low solubility (C14, C16). In another study originating from our own labs, the modification of a napin-rich isolate using lauroyl (C12) and oleoyl (C18:1) was employed (Fetzer et al., 2020). The modified proteins showed increased hydrophobicity and formed films with increased tensile strength, with a positive correlation to their modification degree. This was possibly due to an increase in hydrophobic interactions within the film matrix.

#### 5.2.2. *Glycation/ phosphorylation*

Other chemical modifications reported for rapeseed proteins include glycation and phosphorylation. The phosphorylation of a cruciferin-rich isolate obtained by precipitation showed an overall increase in protein solubility, with values of up to 100 % above pH 7 (Krause, 2002). Additionally, positive effects on foaming properties were reported in the study. Foam capacity was increased to a higher level than with acetylation or succinylation, while foam stability was lower than for acetylation. In a previous study, the effect of phosphorylation of cruciferin on the protein structure was investigated (Schwenke et al., 2000). Increasing levels of phosphorylation induced protein unfolding and resulted in increased hydrophobicity, aggregation and cross-linking. While no functionalities were tested in the study, the authors suggested a possible stabilization effect of protein gels formed by phosphorylation-induced cross-linking. Glycation with dextrane of a precipitated isolate was compared to acylation in regard to the physicochemical and gelation properties (Wang et al., 2018b). Both modifications reduced the content of β-sheet in favor of α-helix, β-turn and random coil structure. Consequently, the surface hydrophobicity increased as a result of modification, presumably due to the liberation of hydrophobic residues, and the effect was higher for glycation. Thermal stability was increased for all modified samples. In contrast to succinylation, thermal stability increased with increasing glycation degrees. This effect was explained by the steric spacing of dextrane molecules, preventing protein aggregation by hydrophobic interactions. The limitation of hydrophobic interactions at increased glycation degrees could also explain the effects on gelation properties: While lower glycation degrees showed positive effects on gelation properties (i.e. least gelation concentration, hardness), the highest glycation level showed a negative effect.

#### 5.2.3. *Cross-linking*

The cross-linking of rapeseed proteins was studied with the aim of improving gelation properties. Protein cross-linking can be achieved by using transglutaminase, which catalyzes the formation of isopeptide bonds between the γ-carboxyl group of glutamine and the ε-amino group of lysine. Gelation properties following transglutaminase treatment were reported by Pinterits and Arntfield (2008). Higher gel elasticity and hardness were generally observed with increasing the concentration of protein and transglutaminase in the formulation. Treatment temperatures of 30–40 °C were recommended, while 60 °C showed far more detrimental effects on gel strength compared to 20 °C. Microstructural analysis revealed increased pore size of the gels at higher protein and transglutaminase concentrations. Additionally, wall thickness and smoothness between the pores increased especially with higher protein concentrations, explaining the positive effect on gel hardness. In a preceding study, the authors investigated the effect of limited proteolysis of rapeseed proteins with or without subsequent transglutaminase treatment on gelation properties (Pinterits and Arntfield, 2007). While proteolysis alone reduced the gel strength, the combination with transglutaminase resulted in gels with increased hardness compared to the non-hydrolyzed control. This most likely arose from an increased liberation of glutamine and lysine residues upon hydrolysis, which could participate in the formation of cross-links. Hydrolyzed rapeseed protein was also used in one study for chemical cross-linking with acrylic acid and bisacrylamide (Shi et al., 2014). The authors reported the formation of superabsorbent hydrogels, with a water uptake of up to 448 g/g hydrogel. Chemical cross-linking was also studied for rapeseed protein-based films (Li et al., 2017; Manamperi et al., 2010, 2011, 2015; Manamperi and Pryor, 2012), which is discussed in section 4.5 (Table 2).

#### 5.2.4. *Hydrolysis*

Protein hydrolysis can be achieved chemically or enzymatically. For rapeseed proteins, hydrolysis was studied predominantly using enzymatic hydrolysis. Protein solubility in water is always increased by hydrolysis due to the generation of small peptides and free amino acids (Chabanon et al., 2007; Vioque et al., 1999). This was used by various authors to increase protein extractability from rapeseed meals (Sari et al., 2013; Zhang et al., 2006, 2007). However, as subsequent protein purification of peptides from the extraction mixture cannot be achieved by means of precipitation or filtration, no functionalities were reported. Chabanon et al. (2007) showed an increase of solubility for a cruciferin isolate from 10 % to 79 % with an increasing degree of hydrolysis (DH), which is defined as the percentage of cleaved peptide bonds, as determined by the analysis of free amino-groups (Church et al., 1983; Nielsen et al., 2001). In contrast, the napin isolate showed a solubility $> 90$ %, and this could not be further improved by hydrolysis. Other functional properties are often altered in dependence of the DH, which is influenced by enzyme type, concentration and hydrolysis time. As such, emulsifying and foaming properties were increased by limited hydrolysis up to 5 % DH, while extended hydrolysis did not show further improvements, or even had the reverse effect (Alashi et al., 2011; Chabanon et al., 2007; Larre et al., 2006; Malabat et al., 2001). This is explained by the liberation of hydrophobic residues at limited DH, which increase the capacity for interfacial adsorption (Adler-Nissen and Olsen, 1979). At higher DHs, peptide size becomes too small to form stable networks at the interface, such that no stable emulsions or foams can be formed. In another study, detrimental effects on gelation properties were found even given limited proteolysis, due to the loss of high molecular weight fractions (Pinterits and Arntfield, 2007). Similarly, enzymatic hydrolysis reduced the capability of rapeseed proteins to act as wall materials for microcapsules (Wang et al., 2015). In contrast to these studies, beneficial effects on film-forming properties were reported for rapeseed hydrolysates mixed with chitosan (Zhang et al. (2019), Table 2). Here, the authors reported an increase in tensile strength at higher DHs (0 %–12 %), which was explained by the formation of denser networks in combination with the polysaccharide. In addition, increased antimicrobial properties were found for higher DHs in the same study.

## 6. Application in the technical industry

Most studies investigating the functionality and applicability of rapeseed proteins do so in regard to human nutrition. However, due to the hurdles presented by rapeseed proteins in regard to poor palatableness and antinutritive components, they offer a promising

alternative as a renewable ingredient for the technical industry. Few studies report the utilization of rapeseed proteins for technical products, however, there is a trend towards a more intensive investigation in this direction.

### 6.1. Adhesives

Rapeseed protein preparations obtained from acidic precipitation were tested as an adhesive for wood (Li et al., 2012). The authors observed good water-stability and a maximum shear strength of 3.97 MPa at a curing temperature of 190 °C. The addition of $NaHSO_3$ to the formulation generally reduced the adhesion strength. Protein-based adhesives were also synthesized by free radical polymerization to form cruciferin–poly(glycidyl methacrylate) conjugates (Wang et al., 2014). The grafting of polymer chains onto the protein showed a significant increase in adhesion strength under dry and wet conditions, which correlated positively with the grafting degree. As the polymer showed no adhesive properties by itself, the effects were clearly attributed to the covalently attached protein. The effect was explained by the partial unfolding of the protein structure to increase intermolecular interactions both within the protein and between the protein and the wood surface. Rapeseed protein-based adhesives were also studied for application in the wood industry in combination with nanomaterials. While adhesive properties and water resistance were found to be low for rapeseed protein by itself, significant improvements were achieved in particular with the addition of graphite oxide and nanocrystalline cellulose (Bandara et al., 2017a, b). One possible explanation given by the authors for the improvement was an increased exposure of functional groups induced by nanomaterial addition, thereby enhancing the surface interaction. Adhesion and cohesion properties were further improved by the moderate oxidation of graphite oxide, leading to increased interlayer spacing and resulting surface interactions (Bandara et al., 2017b). In another study, rapeseed meal hydrolyzed at acidic or alkaline conditions was tested in combination with phenol-formaldehyde prepolymers as an adhesive for medium-density fiberboard (Yang et al., 2014b). The authors reported better mechanical properties at limited hydrolytic treatment of rapeseed meal. While the adhesive properties met industrial standards to some extent, further adaption of phenol-formaldehyde content and pressing-time of the fiberboards was concluded in the study.

### 6.2. Packaging and plastics

The use of rapeseed proteins for the production of biodegradable packaging was reviewed recently by (Zhang et al., 2018). The studies described were compared in regard to their production methods (solution-casting or extrusion), and their mechanical, barrier and thermal properties, as well as the morphology of films. The authors conclude that rapeseed proteins show comparable mechanical and barrier properties to other plant protein sources. As such, they offer good gas barrier properties, while there is a demand to improve mechanical properties and the water-vapor barrier, in order to achieve successful application in the packaging industry. The production of protein-based films by thermal extrusion showed drastically higher values for the elastic modulus (EM) than could be achieved by solution-casting, thereby improving the application potential in the packaging sector (Manamperi et al., 2010, 2015; Manamperi and Pryor, 2012). Rapeseed meal was also studied without further purification for the preparation of bioplastics by injection molding (Delgado et al., 2018). The authors concluded good suitability of the material for bioplastic and biocomposite application.

### 6.3. Detergents and cosmetics

The application potential as surfactants for detergents or cosmetics was demonstrated for acylated rapeseed protein hydrolysates (Sánchez-Vioque et al., 2001). The authors recommended the grafting of C10 and C12 fatty acyl chains for foam properties that showed characteristics of both SDS- and BSA-type foams. Rivera et al. (2015) reported the generation of bioactive peptides from rapeseed proteins by enzymatic hydrolysis. The authors reported antioxidant, anti-wrinkle and anti-inflammatory properties of rapeseed peptides, which suggest the application in skin-care products. The best results were obtained from single-step, single-enzyme treatments and from extracts with higher levels of phenolic acids, possibly due to their synergistic activity. While non-hydrolyzed extracts showed anti-wrinkle and anti-inflammatory properties as well, their toxicity towards human cells prevent a potential application. In this context, it should also be mentioned that rapeseed proteins have been identified as allergens in humans, such that their use in cosmetics appears problematic (Puumalainen et al., 2006, 2015; Rahman et al., 2020). Similar concerns may also arise for the utilization of emulsifiers based on oleosins (Capuano et al., 2007; Östbring et al., 2020), which were identified as allergens in other plants (Ehlers et al., 2019; Schwager et al., 2017; Zuidmeer-Jongejan et al., 2014).

### 6.4. Others

For the sake of completeness, other applications that go beyond the scope of this review shall be mentioned. As such, the fabrication of rapeseed protein-based nanomaterials was described in the literature either as carrier systems for therapeutic use (Hong et al., 2017; Wang et al., 2019, 2018a), or as stabilizers for pickering emulsions (Wang et al., 2020). In addition, antifungal and antiviral properties were described for rapeseed proteins (Schmitt et al., 2018; Thery et al., 2019; Yust et al., 2004). The latter findings might be of interest for technical applications, as they suggest the potential development of protein-based biocides.

## 7. Conclusions

With rapeseed being the third-most abundant oil crop worldwide, a huge untapped value chain lies in the utilization of its residual proteins. Despite constant improvements in the reduction of antinutritive components, feed and food utilization remains limited. The promising alternative of using rapeseed proteins isolated from residual press cake and meal in the chemical sector is gaining more attention, as demonstrated by an increased number of studies published within this area. Interesting functional properties, such as film-forming and surface-active properties render rapeseed proteins an interesting material in a range of products of the technical industry. Recent studies demonstrated their potential application for adhesives, packaging, plastics and surfactants. However, one major limitation to the economic production of rapeseed protein isolates remains the low protein-extractability of conventional residual materials. Alternative defatting strategies through milder processing are thus an essential premise for the rapeseed biorefinery of the future. The successful introduction of rapeseed-protein-containing products into the market may thereby have the potential to change the status of rapeseed into a crop being valued for both its high oil and high protein content. Further efforts in research and development regarding rapeseed protein isolation, modification and application will hopefully support this cause, and thus achieve added value for the industry in the near future.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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# CHAPTER II – Influence of process conditions during aqueous protein extraction upon yield from pre-pressed and cold-pressed rapeseed press cake

The influence of process conditions upon protein extraction yield was investigated for pre-pressed meal (PPM) and cold-pressed meal (CPM). The two materials of different quality were selected from five different rapeseed raw materials (non-pressed, cold-pressed, pre-pressed, full-pressed and toasted meal) based on their pH-dependent solubility profile. The tested conditions were the solid to liquid (s:l) ratio, extraction time, temperature, pH value, concentration of sodium chloride, number of extraction cycles and the employment of protease.

The concentration of NaCl in the extraction medium was found to be the most critical factor. The highest protein extraction yields at a mild pH environment (pH 5.7–9) were 52.3% for CPM and 36.7% for PPM. Higher yields of up to 59.5% (CPM) and 43.9% (PPM) were obtained under strong-alkaline conditions. With the employment of Protease A-01, similar yields were achieved for both materials with up to around 60% and 80% for one-step and three-step-extractions, respectively. However, a significant occurrence of protein hydrolysis as was evident from SDS-PAGE analysis has to be considered under both high-alkaline and enzymatic conditions. In conclusion, the results demonstrate the potential of protein extraction from industrial waste streams and were used as a basis for further protein isolation studies.

A. Fetzer designed the study, performed the extraction experiments, interpreted the results and wrote the manuscript. T. Herfellner, A. Stäbler and P. Eisner contributed to the contents of the manuscript and the interpretation of the results. M. Menner contributed to the contents of the manuscript and managed the affiliated research project.

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# Influence of process conditions during aqueous protein extraction upon yield from pre-pressed and cold-pressed rapeseed press cake

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ABSTRACT

As rapeseed is the third most important plant oil source worldwide (after palm and soya), vast amounts of residual press cake containing high amounts of valuable protein are generated during industrial de-oiling processes. Because the utilization of rapeseed press cake in feed and food is limited due to antinutritional factors, such as glucosinolates, alternative applications of rapeseed proteins in the non-food area are being investigated to add value to the rapeseed industry. However, a major problem remaining in protein extraction from de-oiled rapeseed residues is low protein solubility, resulting from thermal protein denaturation during industrial pressing. The aim of this study was to enhance protein yields from aqueous protein extraction by investigating the influence of various extraction conditions. Two different rapeseed raw materials were examined: cold-pressed meal (CPM) and pre-pressed meal (PPM). Factors examined were the solid to liquid (s:l) ratio, extraction time, temperature, pH value, concentration of sodium chloride, number of extraction cycles and the employment of protease. Best yields at mild ambient conditions were 52.3% for CPM and 36.7% for PPM, with the NaCl concentration being the most critical factor among the studied parameters. Interestingly, a simple extraction at native pH (5.7–5.8) gave comparable yields to extractions at pH 7–9. Improved yields were obtained under strong-alkaline conditions and by the employment of Protease A-01 with the limitation of protein hydrolysis occurring under these conditions. The best protein-extraction yields obtained from enzyme-assisted processes for CPM and PPM were 59.5% and 60.6%, respectively, for one-step processes and 80.7% and 78.3%, respectively, for three-step processes. The results obtained contribute to improving the sustainability of protein utilization from industrial waste streams. Thus, they support the ongoing effort to add value to the rapeseed industry within a biobased economy.

## 1. Introduction

Rapeseed is the third most abundant oil plant worldwide (after palm and soya) and the most abundant oil plant in the European Union (EU). In 2014, the global annual production of rapeseed accounted for 73.8 Mt, with a share 24.3 Mt in the EU (Food and Agriculture Organization of the United Nations (FAO), 2017). In recent years, rapeseed oil has gained vast interest as a renewable source for the production of biodiesel (rapeseed oil methyl ester, RME), amounting to 70% of the total rapeseed-oil production in the EU in 2014/2015 (USDA Foreign Agricultural Service, 2016).

Rapeseed press cake (RPC) is the residual material left after defatting rapeseed by mechanical-extraction methods such as screw pressing. Conventionally, two different types of RPC are produced, depending on the residual fat content left after screw pressing. Full-pressed press cake (FPC) is obtained by complete mechanical oil extraction to a fat content of 5%–10% (Kemper, 2005). Pre-pressed press cake (PPC) is obtained by mechanical extraction to a lesser extent (15%–18% fat content). Pre-pressing is usually a pretreatment to prepare the seed material for subsequent solvent extraction (Kemper, 2005). In addition to these conventional processes, cold-pressing is sometimes applied for the production of niche-market native rapeseed oils (Leming and Lember, 2005). The residual material, cold-pressed press cake (CPC) has a residual fat content of approximately 15%–18%. If solvent extraction is applied to further extract oil from the press cake, rapeseed meal (RM) is obtained containing approximately 35%–40% protein (based on nitrogen content using a conversion factor of 5.7) and 1%–2% fat (Mosenthin et al., 2016). In 2014/2015, 14.6 Mt of RM were produced in the EU (USDA Foreign Agricultural Service, 2016).

Rapeseed protein is mainly composed of two globular storage

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proteins: napin (basic 1.7–2S albumin) and cruciferin (neutral 12S globulin) (Dalgalarrondo et al., 1986; Ericson et al., 1986; Höglund et al., 1992). The more stable napin (12–15 kDa) is composed by two subunits linked through a disulfide bond (Nioi et al., 2012). Cruciferin (~300 kDa) show distinct dissociation characteristics with six subunits (~50 kDa), each composed of heavy α-chain (~30 kDa) and a light β-chain (~20 kDa) that are linked by a disulfide bond (Dalgalarrondo et al., 1986; Wanasundara, 2011). Minor proteins found in rapeseed include oleosins, lipid transfer proteins and protease inhibitors (Wanasundara, 2011). Although RPC and RM are rich in physiologically valuable protein, their utilization as feed can be limited due to their high fiber content and residual antinutrients, in particular phytic acid, glucosinolates and phenolic compounds (Von Der Haar et al., 2014). Utilization of rapeseed protein as an ingredient in human food is of growing interest due to its techno-functional properties, such as foaming and emulsification capacity (Wanasundara, 2011). Consequently, rapeseed protein was authorized as a novel food ingredient in the EU in 2014 (EU, 2014). However, the application of rapeseed protein for human nutrition continues to be problematic. This is mainly due to the remaining glucosinolate content of and the association of phenolic acids and condensed tannins with proteins, which causes dark colors and a bitter taste in protein products (Linnemann and Dijkstra, 2002; Naczk et al., 1998).

The utilization of proteins in technical applications, such as paints and glues, has been a common practice in the early 20th century (Audic et al., 2003), but was displaced by the triumphant advance of petrochemistry. However, with increasing concerns regarding resource scarcity and the environmental burden associated with fossil raw materials, the concept of using renewables in technical applications is regaining attention (Barone and Schmidt, 2006). Therefore, the development of bio-based or biodegradable products is gaining increasing interest on both the company and consumer sides. With their great variety in functional groups, plant proteins and in particular rapeseed proteins offer a multitude of functionalities, such as foaming, emulsification and film-forming properties (Wanasundara, 2011). Film formation of rapeseed proteins by wet casting was studied by Chang and Nickerson (2015) for the production of biodegradable edible packaging. Li et al. (2017) reported the preparation of cross-linked rapeseed protein films with increased thermostability using bisepoxide and a combination of wet casting and heat compression. The preparation of rapeseed protein plastics by extrusion was demonstrated by Manamperi et al. (2015), who reported good elongation and toughness properties. Moreover, adhesive properties of rapeseed proteins were studied recently. In this regard, the potential use of nanomaterials, in particular graphite oxide and nanocrystalline cellulose, to improve adhesive and water resistance of rapeseed protein based wood-adhesive was demonstrated (Bandara et al., 2017a, 2017b). These recent studies emphasize the potential of rapeseed proteins as ingredients in the non-food sector. Exploiting the techno-functional properties of rapeseed proteins in non-food applications such as adhesives, coatings and polymers particularly poses a promising alternative to feed and food utilization, as minor non-protein impurities may be negligible in the final product.

One general limitation remaining for both food and non-food utilization of rapeseed protein from processing by-products is the limited protein solubility in RPC and RM (Mosenthin et al., 2016; Salazar-Villanea et al., 2016). Many oil extraction technologies such as conventional screw pressing, and subsequent solvent defatting and solvent removal involve high temperatures (Kemper, 2005). This causes protein denaturation and thus reduced quality, in particular reduced protein solubility, of the residual material for further applications (Mosenthin et al., 2016; Salazar-Villanea et al., 2016).

Various rapeseed-protein-extraction studies have been reported in the literature with great variations in the utilized raw-material quality and process conditions. Klockeman et al. (1997) reported an extraction yield of 99% from conventionally defatted RM by using 0.4% (w/v) NaOH as a highly alkaline extraction medium. Ismond and Welsh (1992) applied a method using different buffers at mild acidic pH, based on a method previously reported by Murray et al. (1981). However, both studies failed to report yields. Another study by Tzeng et al. (1988b) reported rapeseed-protein extractability of more than 80% at pH ≥ 7 with the addition of sodium hexametaphosphate, using mildly defatted seeds as a non-denatured raw material. More recently, several authors have studied various methods in more detail to improve protein extractability from rapeseed-residual materials. Gerzhova et al. (2016) studied the influence of pH (10–12), s:l ratio, and salt addition on protein extractability from conventional RM and obtained an optimal yield of 58%. Das Purkayastha and Mahanta (2014) applied a response-surface-methodology approach to optimize conditions and reported yields in the range of 35%–46% at pH 11. Furthermore, the employment of various types of enzymes to increase protein yield was studied. Employment of carbohydrate-hydrolyzing enzymes was reported to obtain protein yields for rapeseed materials of various qualities of 50%–80% (Rommi et al., 2014; Sari et al., 2013). Combined utilization of carbohydrate-hydrolyzing enzyme and protease afforded yields of 80% and 80%–83% for cold-pressed rapeseed material, and directly extracted dehulled seeds, respectively (Niu et al., 2012; Zhang et al., 2006). Protease treatment was also studied in combination with the application of ultrasound and was found to improve protein yield by 64% compared to experiments without ultrasonic treatment (absolute yields not reported; Wang et al. (2016)). Lastly, utilization of phytase was tested, resulting in a protein-extraction yield of 72% at pH 12.5 (Rodrigues et al., 2016).

In summary, there are some limitations when comparing methods for rapeseed-protein extraction. First, the raw materials employed differ greatly in quality from conventionally defatted materials to laboratory-scale, mildly treated materials. Second, strongly alkaline extraction is often reported. This leads to improved yields but can lead to protein hydrolysis and thus alter functionality. Finally, some authors failed to report protein yields, preventing an economic evaluation of their extraction methods.

The goal of this study was to overcome these limitations by comparing various process parameters for protein extraction from rapeseed raw materials of different qualities. In particular, the potential of different rapeseed-residual materials (CPC, PPC, FPC and conventional RM) for protein extraction was investigated. Two different RM qualities, cold-pressed meal (CPM) and pre-pressed meal (PPM), were obtained from the appropriate press cakes by mild defatting and compared in detail in terms of protein extractability. Protein-extraction yields were investigated in dependence of solid to liquid (s:l) ratio, extraction time, temperature, pH value, concentration of sodium chloride and number of extraction cycles. Moreover, employment of protease was studied as a tool for enhancing protein extractability.

## 2. Materials and methods

### 2.1. Raw materials and chemicals

*Rapeseed (Brassica napus* L.*) raw materials*

All rapeseed raw materials were of 00 type. Full-fat hulled seeds and hulled, cold-pressed press cake (CPC), both reduced in seed coat content, were provided by Teutoburger Ölmühle GmbH (Ibbenbüren, Germany). Pre-pressed press cake (PPC) and solvent-defatted, desolventized/toasted meal (TM) were provided by Bunge Deutschland GmbH (Mannheim, Germany). Full-pressed press cake (FPC) was provided by Saria A/S GmbH & Co. KG (Selm, Germany). Temperatures during pressing typically did not exceed 40 °C (cold-pressed) and were in the range of 105 °C–140 °C (pre-pressed) or 100 °C–125 °C (full-pressed). For the production of TM, temperatures during solvent extraction typically were in the range of 55 °C–60 °C and temperatures during desolventizing/toasting typically did not exceed 107 °C– 110 °C. All press cakes were further defatted as stated in the section “Preparation of Rapeseed Raw Material” to obtain the corresponding

RMs. All rapeseed raw materials were stored at 14 °C–20 °C.

*Chemicals*

isohexane was obtained from Biesterfeld AG (Hamburg, Germany). Protease A-01 (Subtilisin, EC 3.4.21.62) was purchased from ASA Spezialenzyme GmbH (Wolfenbüttel, Germany). All other chemicals were of analytical grade and obtained from Th. Geyer GmbH & Co. KG (Renningen, Germany), if not stated otherwise.

### 2.2. Preparation of rapeseed raw material

CPC and PPC were defatted with isohexane in a percolator of a volume of 1.5 $m^3$ (e&e Verfahrenstechnik GmbH, Warendorf, Germany) and flash desolventized with isohexane (400–500 mbar) prior to steam desolventizing at a maximum product temperature of 60 °C to obtain cold-pressed meal (CPM) and pre-pressed meal (PPM), respectively. FPC was defatted in a Soxleth apparatus (volume 2 L) with isohexane for 24 h at a maximum product temperature of 60 °C and dried in a fume hood at room temperature to obtain full-pressed meal (FPM). Full-fat dehulled rapeseeds were defatted in a Soxleth apparatus (volume 2 L) with isohexane for 48 h three times at a maximum product temperature of 60 °C to obtain non-pressed meal (NPM). RMs were ground to pass through a 1-mm screen prior to solubility and extraction studies.

### 2.3. Analysis of the chemical composition of rapeseed raw material and protein extracts

The chemical composition (dry matter, protein, ash, fat, cellulose, hemicellulose, lignin and phytic acid) of RPCs was analyzed in duplicate. Protein extracts were analyzed for dry matter and protein content in duplicate.

Dry matter and ash content were determined using a thermo-gravimetrical system (TGA 601, Leco Corporation, St. Joseph, MI, USA) at 105 °C and 950 °C, respectively. Protein content was measured by the Dumas combustion method on a TruMac N system (Leco Corporation, St. Joseph, MI, USA) by applying a conversion factor of 5.7 (Association of Official Analytical Chemists (AOAC), 1990b). Fat content was determined according to the method of Caviezel, DGF K-I 2c (00), (Deutsche Gesellschaft für Fettwissenschaften and Münster, 2004). Contents of cellulose, hemicellulose and lignin were determined using Fibretherm (Gerhardt GmbH & Co. KG, Königswinter, Germany) according to Van Soest et al. (1991) and the Association of Official Analytical Chemists (AOAC) (1990a). Quantification of phytic acid was performed using an IonPac AS11 column (Thermo Fisher Scientific, Waltham, USA) in a HPAEC system with conductivity detection, as described by Fritsch et al. (2015). Amino acid analysis was performed by LUFA-ITL GmbH, Kiel, Germany according to VO(EG) 152/2009, III (EU, 2009).

### 2.4. Thermal behavior of proteins from RMs

The thermal behavior of rapeseed proteins was investigated by differential-scanning calorimetry (DSC). 50 mg of rapeseed raw material were extracted at native pH of 5.7–5.8 using 1 mL of 0.1 M NaCl solution in a Thermomixer (1400 rpm, 30 °C, 45 min; Eppendorf AG, Hamburg, Germany). After centrifugation (13,400 rpm, 5 min), the supernatant was concentrated by compressed air flow to a protein concentration of approximately 20 mg/mL. Approximately 20 mg of extract was weighed into DSC pans (T Zero, TA Instruments, DE, USA), and thermograms were measured with a DSC-Q 2000 (TA Instruments). An empty DSC pan was used as a reference. Samples were heated at a rate of 2 K/min and cooled with 4 K/min in two cycles from 40 °C to 120 °C. Peak temperatures and related enthalpies were calculated automatically. The experiments were performed in duplicate.

### 2.5. Analysis of protein solubility from RMs

Protein solubility was determined in duplicate within the range of pH 3 to pH 12 following the method of Morr et al. (1985). For each measurement, 1.5 g of RM (ground to 1 mm) were suspended in a total volume of 50 mL of deionized water containing 0.1 M NaCl. The pH was adjusted to the appropriate value using 0.1 M NaOH or 0.1 M HCl. After stirring for 1 h at room temperature, non-dissolved fractions of the samples were separated by centrifugation (20,000*g*, 15 min, 15 °C) and the resulting supernatants were filtered (Whatman No. 1 filter paper). The protein content of the supernatant was determined according to Dumas following the method given in the section "Analysis of the Chemical Composition of *Rapeseed* Raw Material and Protein Extracts." Protein solubility was calculated using Formula (1):

$$\text{Protein Solubility}[\%] = \frac{\text{initial volume [mL]} \times \text{protein content in supernatant [mg/mL]}}{\text{sample mass [mg]} \times \text{protein content [\%dm]} \times \text{dry matter [\%]}} \times 100 \quad (1)$$

### 2.6. Aqueous protein extraction

Aqueous protein extraction was carried out as follows: 25 g of RM (CPM or PPM) were added to various amounts of deionized water with adjusted concentrations of NaCl. The mixture was stirred at 300 rpm in a 400-mL beaker in a water bath at various temperatures by using magnetic stirring. Adjustment of pH was conducted using 1 M or 3 M NaOH before the start of the extraction time. In experiments using Protease A-01, 1% ($w/w_{Protein}$) of the enzyme formulation was added before starting extraction time. For experiments comprising several consecutive extraction steps, the residue from centrifugation was re-dispersed in 275 mL of an aqueous solution of 0.25 M NaCl. After various extraction times, the mixture was subjected to centrifugation (3300 × *g*, 10 min) and the weight of the supernatant was determined. The dry matter and protein contents of the supernatant were determined as described in section "Analysis of the Chemical Composition of Rapeseed Raw Material and Protein Extracts." Experiments were performed in duplicate applying a one-factor-at-a-time model. The tested parameters are listed in Table 1. Protein-extraction yield was calculated according to Formula (2):

$$\text{Protein} - \text{extraction yield}[\%] = \frac{\text{mass (extract) [g]} \times \text{protein content (extract) [\%dm]}}{\text{mass (sample) [g]} \times \text{protein content (sample) [\%dm]}} \times 100 \quad (2)$$

### 2.7. Analysis of the molecular weight (MW) distribution of protein extracts (SDS-PAGE)

The MW distribution of protein extracts was determined by SDS-PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis) using Laemmli sample buffer (Laemmli, 1970) with adaption to non-reducing conditions. The samples were mixed with Tris-HCl buffer to a

**Table 1**
Tested parameters applied for protein extraction in this work.

| Tested parameter | | Tested values |
|---|---|---|
| s:l ratio | – | 1:9, 1:11, 1:14, 1:19 |
| $c_{NaCl}$ | [M] | 0, 0.1, 0.25, 0.5, 1.0 |
| Time | [min] | 15, 30, 60, 90, 120, 240 |
| Temperature | [°C] | 20, 30, 40, 50, 60, 70 |
| pH | – | 5.8/5.7, 7, 8, 9, 11, 12 |
| Extraction steps | – | 1, 2, 3 |
| Enzymatic treatment | – | -/Protease A-01 |

final concentration of 63 mM Tris-HCl (pH 6.8), 2% SDS, 10% v/v Glycerol and 0.01% bromophenol blue. Electrophoretic separation of proteins was performed by loading 50 µg of protein on pre-cast 4–20% Criterion™ TGX Stain-Free™ gels (Bio-Rad Laboratories, Hercules, CA, USA). As a molecular weight marker, Precision Plus Protein™ Unstained Standard (10–250 kDa, Bio-Rad Laboratories) was loaded onto the gel. Electrophoretic separation was carried out at 200 V, 60 mA, 100 W and room temperature. Visualization and documentation was performed using a Gel Doc™ EZ Imager system and Image Lab software (Bio-Rad Laboratories) for analysis of the protein distribution.

### 2.8. Statistical analysis

All data are given as mean values ± absolute deviation of at least two measurements ($n = 2$). Significant differences in protein-extraction yields were statistically analyzed by one-way analysis of variance using Tukey's test ($p < 0.05$).

## 3. Results and discussion

### 3.1. Characterization of rapeseed raw materials

#### 3.1.1. Chemical composition

The chemical composition of rapeseed raw materials is shown in Table 2. After defatting, all press cakes showed fat contents of less than 3%dm and similar ash contents of 7.2–7.4%dm. CPM showed the highest protein content (40.6%dm) and the lowest content of cellulose, hemicellulose and lignin due to partial removal of the seed coat during processing. Protein contents were the lowest for PPM and TM, which originated from the same processing plant, with values of 34.4%dm and 35.0%dm, respectively. FPM showed slightly higher protein content (37.2%dm) and the highest content of phytic acid (6.6%dm). Differences in contents of protein and phytic acid among the conventionally processed materials (PPM, FPM and TM) can be attributed to variations in growth conditions and seed origin (Tan et al., 2011). The contents of cellulose, hemicellulose, and lignin were comparable between PPM, FPM, and TM and were significantly lower for CPM due to partial removal of the seed coat.

NPM, which is not produced industrially, served as a reference material. As a result of partial removal of the seed coat, protein content of NPM was comparable to CPM, which originated from the same process plant. Contents of cellulose, hemicellulose and lignin were the lowest for NPM due to the smallest proportions of seed coat among the tested materials. NPM showed the highest oil content, as it was not subjected to screw pressing.

#### 3.1.2. Thermal stability of proteins from RMs

The thermal stability of rapeseed proteins from the different raw materials was determined by DSC measurement as an indicator for the degree of protein denaturation (Table 3). For all tested raw materials only one denaturation peak in the range of 97.8 °C–109.6 °C was observed, which was comparable to values reported for napins (Nioi et al., 2012; Wu and Muir, 2008). Enthalpy of denaturation decreased with increasing processing of raw materials from 0.38 J/g for NPM to 0.25 J/g for FPM and TM, however, observed differences were not significant ($p > 0.05$). This trend correlated with reduced protein solubility of raw materials (Fig. 1). Wu and Muir (2008) also reported endothermic peaks attributed to cruciferin at 84 °C for rapeseed protein isolate and at 91 °C for purified cruciferin. As none of these peaks could be observed in our measurements, a high level of cruciferin denaturation can be assumed for all tested materials.

**Table 3**
Thermal behavior of rapeseed raw materials, as obtained from DSC measurements; values within one row having the same superscript indicate no significant difference ($p > 0.05$); NPM: non-pressed meal, CPM: cold-pressed meal, PPM: pre-pressed meal, FPM: full-pressed meal, TM: toasted meal.

| | $T_m$ [°C] | Enthalpy [J/g] | Onset of Peak [°C] | End of Peak [°C] |
|---|---|---|---|---|
| NPM | 102.8 ± 0.2 [a] | 0.38 ± 0.00 [a] | 97.8 ± 1.2 [a] | 108.7 ± 0.5 [a] |
| CPM | 101.4 ± 1.4 [a] | 0.32 ± 0.06 [a] | 98.7 ± 0.8 [a] | 106.6 ± 0.1 [a] |
| PPM | 102.6 ± 0.5 [a] | 0.28 ± 0.02 [a] | 98.6 ± 0.6 [a] | 109.1 ± 0.2 [a] |
| FPM | 102.3 ± 0.2 [a] | 0.25 ± 0.02 [a] | 98.4 ± 0.5 [a] | 109.6 ± 0.8 [a] |
| TM | 102.7 ± 0.4 [a] | 0.25 ± 0.05 [a] | 99.0 ± 0.0 [a] | 107.4 ± 0.2 [a] |

#### 3.1.3. Protein solubility

Protein solubility of rapeseed raw materials was determined as a function of pH in the range from pH 3 to pH 12. The data presented in Fig. 1 show a large dependence of protein solubility upon the processing conditions applied during oil extraction (see section "2.1 Raw Materials and Chemicals"). All raw materials showed a similar trend in the pH spectrum, with minimum protein solubility in the acidic range (pH 3–4), medium protein solubility in the neutral to mild alkaline range, and a sharp increase in protein solubility in the strong alkaline range (pH 11–12). The latter can be attributed to the increasing effects of protein hydrolysis occurring at high alkalinity, as visible from SDS-PAGE analysis (Fig. 2).

NPM, which served as a reference material, showed the highest protein solubility. The material was defatted solely by Soxhlet solvent extraction, without any screw-press processing. Protein solubility was > 60% at neutral pH, the lowest at pH 3 (38.2%), and the highest at pH 12 (82.9%). NPM showed a rather high amount of phytic acid with 5.3%dm, which is known to form insoluble protein complexes (Thompson, 1990). However, this did not seem to have a strong negative impact on protein solubility.

FPM showed the lowest protein solubility with values below 15% in the range of pH 3–9. In addition to protein denaturation due to high temperatures during pressing, protein solubility was possibly limited by a high content of phytic acid (6.6%dm). Accordingly, an overall low but slightly higher protein solubility was obtained for TM with a phytic acid content of 5.5%dm. Furthermore, TM showed a distinct dark brown color (Fig. 1), indicating a high proportion of Maillard reaction products, possibly formed during desolventizing/toasting. Advanced Maillard reaction products, such as glyoxal and methylglyoxal, can induce protein-cross-linking through reaction with lysyl, arginyl or tryptophanyl residues (Le et al., 2011). Le et al. (2011) demonstrated

**Table 2**
Chemical composition of the defatted rapeseed raw materials; NPM: non-pressed meal, CPM: cold-pressed meal, PPM: pre-pressed meal, FPM: full-pressed meal, TM: toasted meal.

| | | NPM | CPM | PPM | FPM | TM |
|---|---|---|---|---|---|---|
| dry matter (dm) | [%] | 94.3 ± 0.1 | 92.0 ± 0.1 | 94.2 ± 0.0 | 91.4 ± 0.0 | 89.8 ± 0.0 |
| protein | [%dm] | 39.3 ± 0.1 | 40.6 ± 0.0 | 34.4 ± 0.2 | 37.2 ± 0.1 | 35.0 ± 0.1 |
| ash | [%dm] | 7.5 ± 0.2 | 7.3 ± 0.0 | 7.2 ± 0.0 | 7.4 ± 0.0 | 7.4 ± 0.0 |
| fat | [%dm] | 4.9 ± 0.3 | 2.8 ± 0.1 | 2.3 ± 0.0 | 2.3 ± 0.1 | 3.6 ± 0.0 |
| phytic acid | [%dm] | 5.3 ± 0.1 | 4.0 ± 0.1 | 4.4 ± 0.0 | 6.6 ± 0.0 | 5.5 ± 0.1 |
| cellulose | [%dm] | 4.5 ± 0.2 | 6.8 ± 0.6 | 8.5 ± 1.3 | 10.2 ± 1.4 | 10.5 ± 2.1 |
| hemicellulose | [%dm] | 3.4 ± 0.2 | 3.9 ± 0.6 | 6.6 ± 1.7 | 4.5 ± 1.5 | 6.4 ± 1.0 |
| lignin | [%dm] | 8.7 ± 0.2 | 11.4 ± 0.1 | 16.1 ± 0.1 | 21.8 ± 0.8 | 19.1 ± 1.6 |

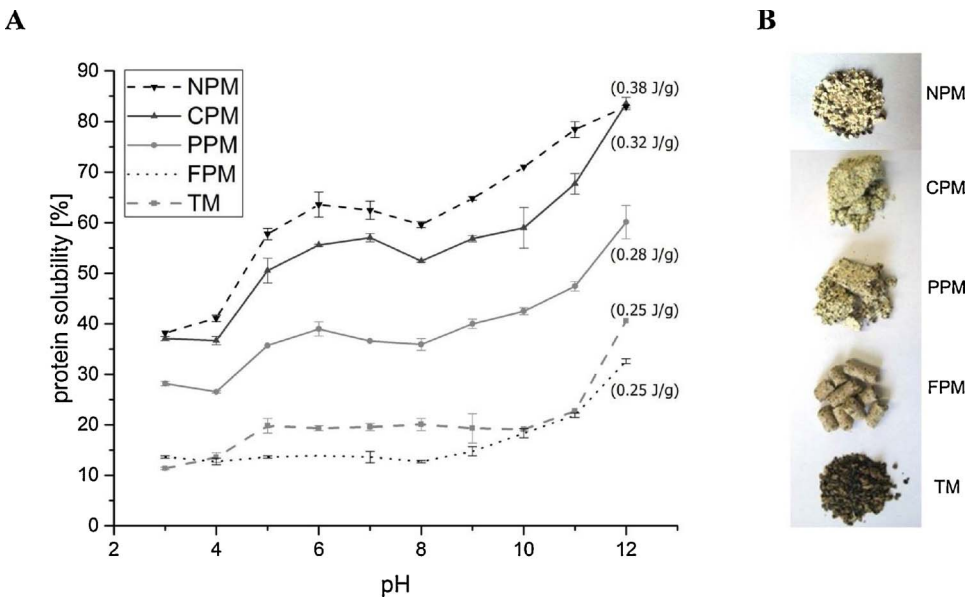


**Fig. 1.** A: pH-dependent protein solubility of rapeseed raw materials derived from different defatting processes (values in brackets indicate denaturation enthalpies obtained from DSC measurements (Table 3)); B: photographs of rapeseed raw materials; NPM: non-pressed meal, CPM: cold-pressed meal, PPM: pre-pressed meal, FPM: full-pressed meal, TM: toasted meal.

the negative impact of Maillard reactions upon protein solubility by using different milk powders. In addition, the presence of sinapic acid and other phenolic substances in rapeseed can influence protein solubility (Naczk et al., 1998). While the exact mechanism of interaction is still not known, phenolic substances can lead to protein precipitation and thereby limit protein accessibility (Alu'datt et al., 2013). With protein solubility below 20% in the range of pH 3 to pH 10, FPM and TM are unsuitable raw materials for an economic extraction process of functional protein and are therefore not further regarded for protein extraction experiments in this study.

For CPM and PPM, protein solubility was significantly higher compared to FPM and TM. The decrease in protein solubility by 15–20 percentage points from PPM to TM demonstrates the high negative effect of conventional solvent defatting and desolventizing/toasting on protein quality (Salazar-Villanea et al., 2016). Reduced protein solubility possibly originates from excessive protein cross-linking induced through Maillard reactions and thermal denaturation. Recently, Mosenthin et al. (2016) demonstrated the negative effect of increasing residence time in a desolventizer/toaster upon protein solubility. The negative impacts under dry toasting conditions were even more pronounced than those under wet toasting conditions, which can be explained by an increased formation of Maillard reaction products at lower moisture levels. This is in accordance with previous studies reporting a negative correlation between bound amino acids (in particular lysine) and protein solubility (Jensen et al., 1995; Pastuszewska et al., 1998). In comparison to FPM, the protein solubility for PPM increased by 14–27 percentage points in the range of pH 3–12. This result indicates a strong negative effect of residence time in a conventional oil press, which is generally lower for PPM (Kemper, 2005). In comparison to PPM, protein solubility in CPM was higher by approximately 10–20 percentage points. This strongly indicates the positive effect on protein quality by lower temperatures during pressing. In summary, the results clearly indicate the strong influence of defatting conditions upon protein solubility as a consequence of denaturation effects and reduced protein quality. The low protein solubility of rapeseed-residual materials as a result of overheating is a well-known problem and has been reported previously by others (Anderson-Hafermann et al., 1993; Anwar and Clandinin, 1971; Mosenthin et al.,

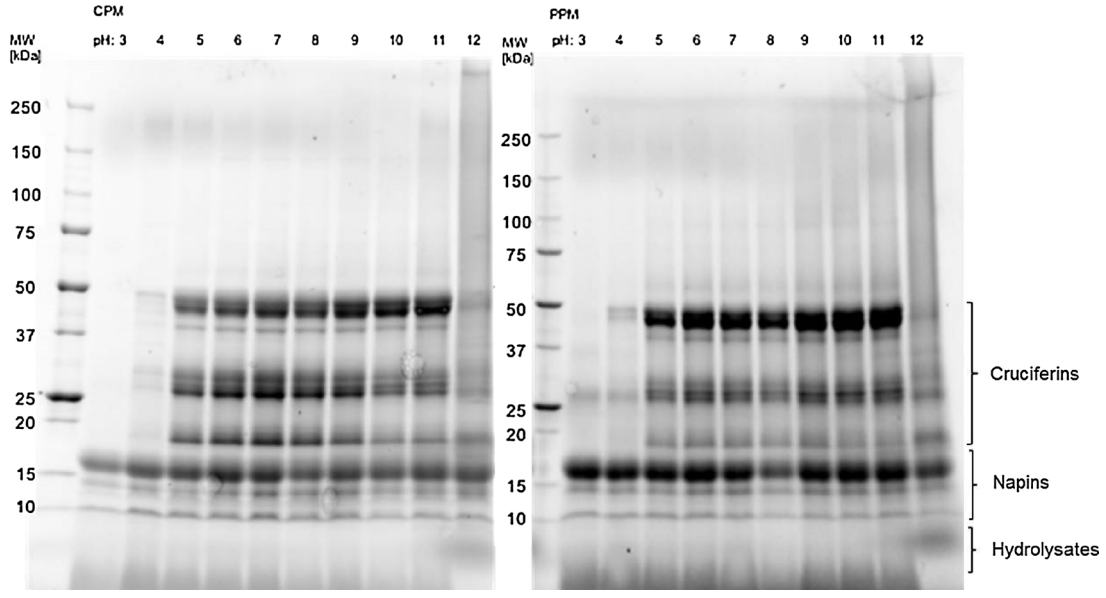


**Fig. 2.** SDS-PAGE (non-reducing conditions) of rapeseed soluble protein fractions from CPM (left) and PPM (right) extracted at different pHs.

**Table 4**
MW distribution (band percentage within each lane) of rapeseed soluble protein fractions at different pHs; *SUM cru: sum of cruciferin fractions (≥18 kDa), **SUM nap: sum of napin fractions (10–16 kDa); protein fractions were attributed to MW based on literature (Nietzel et al., 2013; Wanasundara, 2011).

| MW [kDa] | pH 3 | | pH 4 | | pH 5 | | pH 6 | | pH 7 | | pH 8 | | pH 9 | | pH 10 | | pH 11 | | pH 12 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CPM | PPM | CPM | PPM | CPM | PPM | CPM | PPM | CPM | PPM | CPM | PPM | CPM | PPM | CPM | PPM | CPM | PPM | CPM | PPM |
| 45–48 | – | – | 4.5 | 10.6 | 12.1 | 18.6 | 12.2 | 22.4 | 12.3 | 20.6 | 14.0 | 19.4 | 17.3 | 19.5 | 20.2 | 20.1 | 20.5 | 17.1 | 8.9 | 4.1 |
| 43–45 | – | – | – | – | 12.5 | 17.9 | 11.4 | 10.7 | 12.0 | 8.5 | 13.3 | 14.3 | 14.5 | 22.1 | 19.7 | 25.6 | 12.4 | 23.9 | – | – |
| 38–40 | – | – | – | – | 3.2 | 3.4 | 2.7 | 4.0 | 2.9 | 4.2 | 3.1 | 4.3 | 3.4 | 3.7 | 3.5 | 5.1 | 2.8 | 3.8 | 0.5 | 1.7 |
| 28–30 | – | – | 4.4 | – | 11.0 | 6.6 | 13.1 | 7.4 | 13.0 | 8.1 | 13.6 | 8.5 | 11.2 | 5.8 | 9.9 | 6.1 | 8.1 | 7.1 | 8.3 | 6.4 |
| 27–28 | – | – | – | – | 8.4 | 9.1 | 5.7 | 7.4 | 7.5 | 7.1 | 6.0 | 7.5 | 6.5 | 6.3 | 7.5 | 5.0 | 6.1 | 6.5 | – | – |
| 26–27 | – | 10.1 | – | 8.7 | 8.2 | 3.6 | 11.0 | 6.5 | 9.8 | 7.3 | 9.7 | 6.8 | 7.9 | 5.8 | 6.5 | 5.6 | 7.6 | 5.3 | 9.4 | 12.7 |
| 18–19 | – | – | – | – | 12.4 | 3.4 | 12.5 | 5.0 | 12.8 | 6.0 | 12.3 | 6.4 | 9.3 | 4.5 | 6.3 | 1.8 | 10.2 | 3.8 | 17.7 | 11.0 |
| 15–16 | 74.2 | 74.3 | 64.4 | 66.7 | 22.7 | 30.7 | 21.6 | 29.5 | 18.8 | 29.9 | 16.2 | 26.2 | 18.2 | 26.5 | 19.5 | 25.6 | 24.1 | 27.0 | 33.0 | 31.4 |
| 13–14 | 16.9 | 9.8 | 15.4 | 8.6 | 5.0 | 3.3 | 5.8 | 4.0 | 6.3 | 5.3 | 7.4 | 4.4 | 7.2 | 3.8 | 4.1 | 3.5 | 5.3 | 3.9 | 8.6 | 4.5 |
| 10–11 | 9.0 | 5.8 | 11.3 | 5.4 | 4.5 | 3.3 | 4.1 | 3.1 | 4.6 | 3.0 | 4.4 | 2.2 | 4.5 | 2.0 | 2.8 | 1.8 | 2.8 | 1.6 | 3.2 | 1.2 |
| < 10 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | 10.4 | 26.9 |
| SUM cru* | 0.0 | 10.1 | 8.9 | 19.3 | 67.8 | 62.6 | 68.5 | 63.3 | 70.3 | 61.8 | 72.0 | 67.2 | 70.1 | 67.7 | 73.6 | 69.2 | 67.8 | 67.5 | 44.8 | 35.9 |
| SUM nap** | 100.0 | 89.9 | 91.1 | 80.7 | 32.2 | 37.4 | 31.5 | 36.7 | 29.7 | 38.2 | 28.0 | 32.8 | 29.9 | 32.3 | 26.4 | 30.8 | 32.2 | 32.5 | 44.8 | 37.1 |
| ratio cru:nap | 0.0 | 0.1 | 0.1 | 0.2 | 2.1 | 1.7 | 2.2 | 1.7 | 2.4 | 1.6 | 2.6 | 2.0 | 2.3 | 2.1 | 2.8 | 2.2 | 2.1 | 2.1 | 1.0 | 1.0 |

2016; Pastuszewska et al., 1998; Radwan and Lu, 1976).

### 3.1.4. Protein and amino acid distribution in RMs

Protein and amino acid distribution was analyzed for CPM and PPM, which were used for further protein extraction studies in this work. Soluble protein fractions obtained in dependence of pH are shown in Fig. 2. The relative distributions within each fraction are given in Table 4. All protein fractions apparent from SDS-PAGE analysis were attributed to either cruciferin-related or napin-related proteins, based on MWs reported in the literature (Nietzel et al., 2013; Wanasundara, 2011). The possible contribution of minor protein fractions, such as oleosins, was neglected, as differentiation of napins and cruciferins or their breakdown products was not possible. At strong acidic conditions, only napins (10–16 kDa) were present in the extract, with minor fractions of cruciferins (≥18 kDa) appearing at pH 4. In the range of pH 5–8, the ratio of cruciferins to napins varied from 2.1:1 to 2.6:1 (CPM) and from 1.6:1 to 2.0:1 (PPM). Similarly, cruciferins were the predominant protein fraction in extracts obtained in the range of pH 9–11. These ratios are in accordance with values stated in the literature, which report proportions of 20–40% (napin) and 50–60% (cruciferin) on total rapeseed protein (Höglund et al., 1992; Von Der Haar et al., 2014). Interestingly, the ratio of intact heterodimers of cruciferin (42–48 kDa) to dissociated α- and β-chains (18–30 kDa) is higher for CPM compared to PPM in the range of pH 5–9. This can be explained by a higher degree of disulfide bond cleavage of cruciferin heterodimers for PPM, which is likely caused by increased thermal processing. For extracts obtained at pH 12, major effects of proteolysis were observed. Cruciferin bands were degraded to a large extent, while napin bands were only partly affected. As protein functionality can be significantly altered or lost through hydrolysis, strong alkaline conditions are thus often inapplicable for extraction processes.

The amino-acid compositions of CPM and PPM, which were used for protein-extraction studies in the present work, are given in Table 5. Amino-acid compositions were comparable to database values and did not show significant differences among the two materials, indicating similar protein distributions in both materials. Hence, differences in protein extraction yields originating from different protein distributions can be ruled out.

**Table 5**
Amino-acid composition of CPM and PPM (reference: RM, solvent extracted; data obtained from Feedipedia Animal Feed Resources Information System (2016)).

| | CPM [%protein] | PPM [%protein] | reference (RM) [%protein] |
|---|---|---|---|
| Lys | 6.3 | 6.2 | 5.5 |
| Met | 2.1 | 2.2 | 2.0 |
| Cys | 2.6 | 2.6 | 2.3 |
| Asp | 8.2 | 8.3 | 7.2 |
| Thr | 4.8 | 5.0 | 4.3 |
| Ser | 4.7 | 5.0 | 4.4 |
| Glu | 18.6 | 18.1 | 16.8 |
| Pro | 6.5 | 7.0 | 6.0 |
| Gly | 5.5 | 5.7 | 5.0 |
| Ala | 4.7 | 4.9 | 4.3 |
| Val | 5.3 | 5.3 | 5.1 |
| Ile | 4.1 | 4.2 | 4.0 |
| Leu | 7.6 | 7.6 | 6.8 |
| Tyr | 3.2 | 3.0 | 2.9 |
| Phe | 4.6 | 4.3 | 3.9 |
| His | 3.0 | 2.8 | 2.6 |
| Arg | 6.5 | 6.5 | 6.0 |
| Trp | 1.4 | 1.5 | 1.2 |

## 3.2. Influence of process conditions upon protein-extraction yield

### 3.2.1. Solid-to-Liquid ratio (s:l)

The solid-to-liquid ratio was varied from 1:9 to 1:19, while keeping the other extraction parameters constant (T = 30 °C, t = 60 min, pH = 7.0, no addition of NaCl). Protein extractability under these conditions was comparatively low, with maximum yields of 32.8% and 16.9% for CPM and PPM, respectively, obtained at an s:l ratio of 1:19 (Fig. 3A). Gerzhova et al. (2016) studied protein extractability from conventionally defatted canola meal at pH 10, 11 and 12 and generally found a slight increase in protein-extraction yield with increasing meal concentration from 5%–15%. Other authors commonly report extraction protocols with meal concentrations 5%–10% (Aluko and McIntosh, 2001; Ghodsvali et al., 2005; Klockeman et al., 1997; Tzeng et al., 1988a, 1988b). In our studies, differences in protein-extraction yields were not significant within each raw material tested ($p > 0.05$), suggesting the use of the highest meal concentration in order to limit water usage during the process. However, at an s:l ratio of 1:9, the elevated viscosity of the extraction mixture hindered proper mixing during protein extraction. The effects can be explained by the high water-binding capacity of rapeseed raw materials originating from high fiber content. Selecting an s:l ratio of 1:11 for further studies ensured proper mixing during the extraction process while keeping water-usage low. These conditions were also found most suitable for further up-scaling of the extraction process to a pilot plant (data not shown).

### 3.2.2. NaCl concentration

NaCl was added in concentrations ranging from 0.1 M to 1.0 M to the extraction medium. Protein extraction was carried out at T = 30 °C, t = 60 min, pH = 7 and s:l = 1:11. Compared to an extraction without

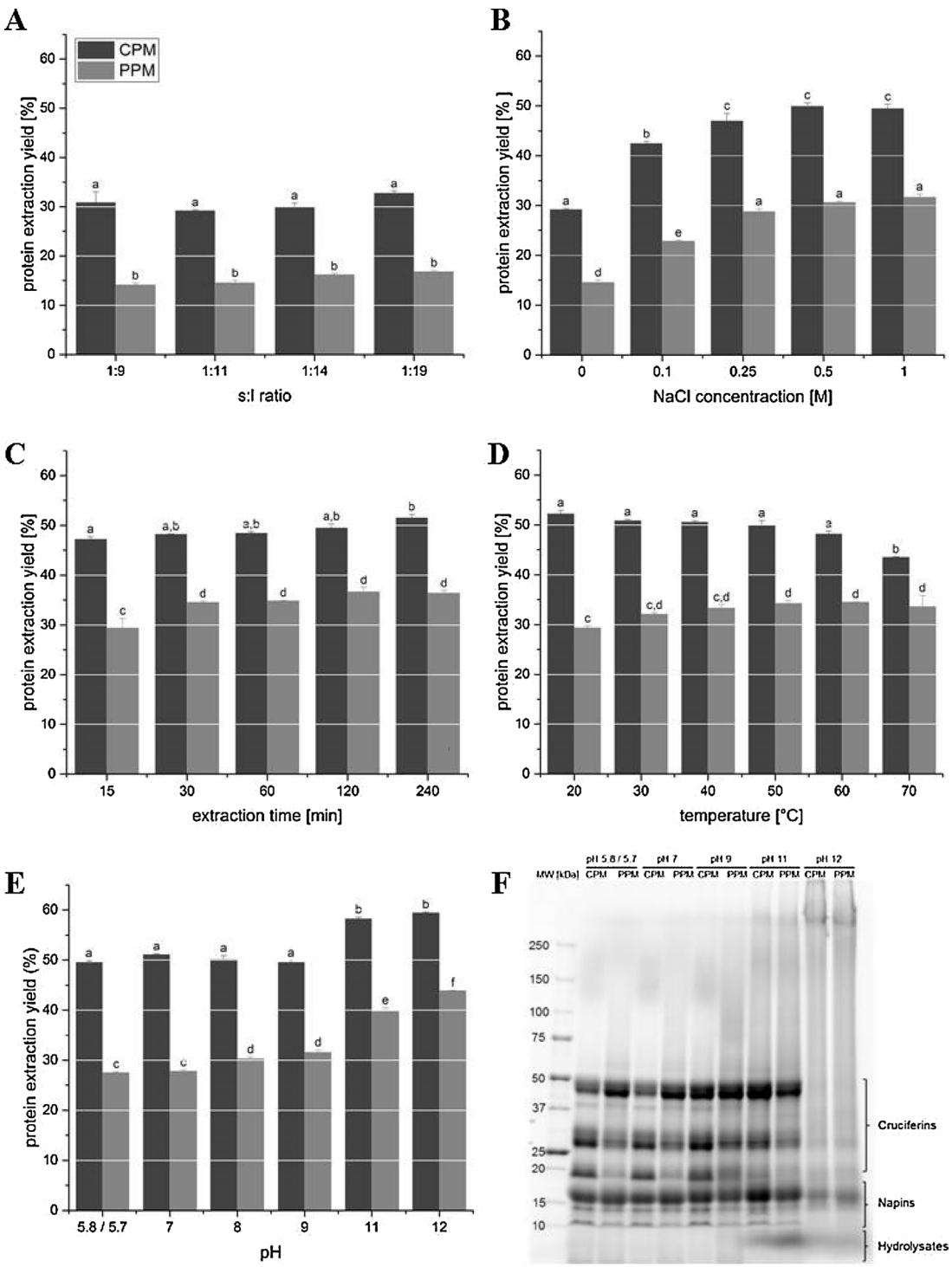


**Fig. 3.** A-E: Screening of the protein-extraction yield obtained under various conditions (one-factor-at-a-time); dark gray columns: CPM; light gray columns: PPM; conditions: A: T = 30 °C, t = 60 min, pH = 7.0; B: T = 30 °C, t = 60 min, pH = 7.0, s:l = 1:11; C: T = 30 °C, pH = 7.0, s:l = 1:11, $c_{NaCl}$ = 0.25 M; D: t = 60 min, pH = 7.0, s:l = 1:11, $c_{NaCl}$ = 0.25 M; E: T = 20 °C, t = 60 min, s:l = 1:11, $c_{NaCl}$ = 0.25 M; F: SDS-PAGE (non-reducing conditions) of extracted samples at different pH values.

addition of NaCl at an s:l ratio of 1:11, the protein-extraction yield was improved by approximately 50% at the lowest NaCl concentration of 0.1 M for both raw materials (Fig. 3B). This is in accordance with a high portion of salt-soluble globulins (cruciferins) present in rapeseed (Von Der Haar et al., 2014). A further increase in ionic strength to 0.25 M further improved protein-extraction yields significantly ($p < 0.05$) to 47.1% (CPM) and 28.9% (PPM). Similar positive effects upon rapeseed-protein extractability by salting-in have been described in early studies (Bhatty et al., 1968; Smith et al., 1959). However, minor effects at pH 11 and negative effects at pH 12 are also reported (Gerzhova et al., 2016; Klockeman et al., 1997). Klockeman et al. (1997) studied protein extractability from pH 2–12 and found major improvements by adding 0.1 M NaCl, while further adjustment to 1.0 M NaCl had smaller impacts. This is in agreement with our findings, as a further increase in salt concentration above 0.25 M did not lead to significant improvements of extraction yields ($p < 0.05$). Thus, a concentration of 0.25 M NaCl in the extraction medium was chosen for further experiments.

### 3.2.3. Extraction time

Extraction time was varied from 15 to 240 min, with the other parameters kept at T = 30 °C, pH = 7.0, s:l = 1:11 and $c_{NaCl}$ = 0.25 M. Extraction time had little to no influence upon protein-extraction yield (Fig. 3C). For CPM, a significant difference ($p < 0.05$) in the protein-extraction yield was only observed between the shortest (15 min) and longest (240 min) extraction times. Compared to an extraction time of 30 min, an increase in extraction time did not lead to a significant increase in protein yield for the tested range ($p < 0.05$). For PPM however a slight but statistically significant improved yield was achieved for an extraction time of 30 min compared to 15 min. The results indicate that wettability of the raw material by the extraction medium was fast and only marginally affected by differences in the fiber contents of both raw materials (Table 2). Accordingly, most authors report extraction times of 30–60 min (Gerzhova et al., 2016; Ghodsvali et al., 2005; Klockeman et al., 1997; Tzeng et al., 1988b). To ensure proper mixing, 60 min was chosen as the extraction time for further experiments. However, it should be noted that the results obtained suggest the possibility of a more time-efficient process design where applicable. In particular, in a multi-step process, extraction steps may be replaced by short washing steps to reduce time and resources during the process. Alternatively, a continuous extraction method using either countercurrent- or cross-flow-extraction techniques seams feasible.

### 3.2.4. Temperature

The effect of temperature upon protein-extraction yield was investigated in the range of 20 °C to 70 °C, while maintaining the other parameters at t = 60 min, pH = 7.0, s:l = 1:11, and $c_{NaCl}$ = 0.25 M NaCl. Interestingly, in the case of CPM, the best yield of 52.3% was achieved at room temperature (20 °C, (Fig. 3D)). At higher temperatures, the extraction yield was subsequently lowered, with a significant decrease to 43.6% at 70 °C ($p < 0.05$). This was partly owed to reduced extract volumes obtained at higher temperatures, due to elevated water binding of CPM with increasing temperature (data not shown). This effect was not observed in significance for PPM. In contrast to CPM, PPM showed an opposing trend, with protein-extraction yields slightly increasing with increasing temperatures. Protein-extraction yield was improved to 34.3% at 50 °C compared to 29.5% at room temperature ($p < 0.05$). An optimum yield of 34.6% was obtained at 60 °C; however, yields did not differ significantly within the range of 30–70 °C ($p < 0.05$). These results can be explained with regard to exposure to high temperatures of proteins from PPM during screw pressing. Proteins from PPM showed no further denaturation at higher temperatures during extraction studies. Hence, an improvement of protein solubility through thermodynamic effects could be seen from the temperature-dependent extraction profile. In the case of CPM, this effect was not visible, as it was negligible compared to the negative effects on protein solubility due to protein denaturation. Other authors have commonly reported extraction protocols at room temperature. (Aluko and McIntosh, 2001; Gerzhova et al., 2016; Ghodsvali et al., 2005; Klockeman et al., 1997; Tzeng et al., 1988b) Further experiments in this study were also carried out at room temperature, as the obtained yields for higher temperatures were not improved significantly or only marginally for CPM and PPM, respectively ($p < 0.05$).

### 3.2.5. Extraction pH

Extraction pH was screened both in an environment from mildly acidic “native” rapeseed pH of 5.8 (CPM) or 5.7 (PPM) to pH 9, and in the strong-alkaline region at pH 11 and 12. Extraction parameters were kept at T = 20 °C, t = 60 min, s:l = 1:1,1 and $c_{NaCl}$ = 0.25 M NaCl. The results shown in Fig. 3E were comparable to those from protein-solubility studies (Fig. 1). Adjusting the pH to neutral or mild-alkaline conditions had no effect upon protein-extraction yield in the case of CPM, and showed only a slight increase of 4% for PPM ($p < 0.05$). MW distributions of protein extracts from pH 5.8/5.7, pH 7, and pH 9 were similar, as shown by SDS-PAGE analysis (Fig. 3F). Thus, under mild ambient conditions, protein extraction can be carried out at native pH and 0.25 M NaCl with no need for any pH adjustment during the extraction process. At strong-alkaline pH values of 11 and 12, protein-extraction yield showed an increase by 9–12 percentage points compared to the best results obtained under mild ambient conditions. For CPM, yields at pH 11 and pH 12 were similar at 58.3% and 59.5%, respectively. For PPM, yields differed significantly, with 39.8% at pH 11 and 43.9% at pH 12 ($p < 0.05$). Most authors have studied rapeseed-protein extraction in strongly alkaline environments (pH 10–12), with maximum yields varying from 46 to 79%, depending on extraction conditions and pre-processing of the raw material employed (Aluko and McIntosh, 2001; Gerzhova et al., 2016; Ghodsvali et al., 2005; Rodrigues et al., 2016; Rommi et al., 2015a, 2015b; Thiel et al., 2015). Klockeman et al. (1997) screened protein solubility from pH 2–12 and reported a maximum solubility of 92.9% at pH 10 and the presence of $CaCl_2$ at an ionic strength of 0.2 M. Although the highest yields are obtained under strong-alkaline conditions, a significant amount of protein hydrolysis has to be considered. SDS-PAGE analysis showed distinct differences in the protein distributions of extracts at pH 11 and pH 12 (Fig. 3F). At pH 11, high amounts of cruciferin and napin fractions are still visible; however, a distinct band at the gel front indicates significant protein hydrolysis. At pH 12, cruciferin fractions were hydrolyzed completely and only minor fractions of napin could be detected, indicating severe effects of protein hydrolysis. Thus, for processes working under strong alkaline conditions, pH 11 is the recommended extraction pH, as a significant portion of the extracted proteins seem to be intact.

### 3.2.6. Multiple extraction steps

Multiple-step protein extraction was studied employing different pH values while maintaining the other parameters at T = 20 °C, t = 60 min (per step), s:l = 1:11 and $c_{NaCl}$ = 0.25 M. The data given in Fig. 4 show a comparison of the protein-extraction yields obtained from one-step (pH 12), two-step (step 1 at pH 5.8 (CPM) or pH 5.7 (PPM); step 2 at pH 12) and three-step (step 1 at pH 5.8 (CPM) or pH 5.7 (PPM); step 2 at pH 7; step 3 at pH 12) protein extractions. These conditions were selected to investigate the effect on the total protein-extraction yield of one or two pre-extraction steps at mild ambient conditions before highly alkaline conditions were applied. pH 12 was selected for one-step extraction and the last process in multiple-step extraction as it resulted in maximum yield.

Protein-extraction yield was improved for CPM and PPM by 13.0%

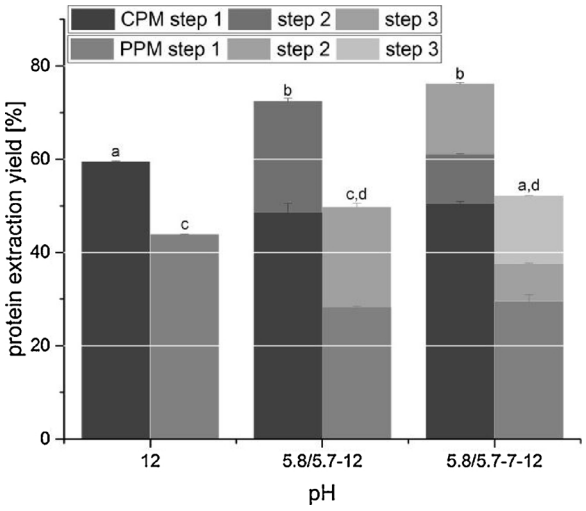


**Fig. 4.** Comparison of protein-extraction yields obtained from one-step (pH 12, data copied from Fig. 3), two-step (pH 5.8/5.7-12), and three-step (pH 5.8/5.7-7-12) extractions (left columns: CPM, right columns: PPM).

and 5.9%, respectively, by adding an extraction step at native pH (no pH adjustment) prior to pH 12 extraction. The addition of another extraction step at pH 7 in between only had a marginal effect without statistical significance ($p < 0.05$). A choice of two extraction steps thus seems economically feasible for rapeseed-protein extraction, especially when different qualities of protein products (i.e., native and hydrolyzed) can be utilized. A study by Tan et al. (2011) compared a one-step alkaline extraction to a multi-step procedure referring to the classical method by Osborne and Mendel (1914) on various rapeseed raw materials. For pre-toasted meal, an improved protein recovery of 67.8% was reported for the Osborne-type method, as compared to 47.2% for one-step alkaline extraction. However, the method, which sequentially obtains albumins, globulins, glutelins and a minor fraction of prolamins, is laborious and thus unsuitable as an economic process.

### 3.2.7. Enzymatic treatment

Utilization of various classes of enzymes is among the most prominent methods employed in the literature to improve protein extraction efficiency from rapeseed raw materials (Niu et al., 2012; Rodrigues et al., 2016; Rommi et al., 2014; Sari et al., 2013; Wang et al., 2016; Zhang et al., 2006). In the present study, Protease A-01 (Subtilisin, EC 3.4.21.62) was employed to investigate the effect of the enzyme in liberating protein fragments from the meal matrix through one-step and multi-step processes upon the protein-extraction yield. Protease A-01 (1% wt/$wt_{Protein}$ dosage) was found to be the most efficient enzyme among a range of commercially available protease formulations tested to improve protein-extraction yields (data not shown). At optimal enzyme conditions (pH 9, 50 °C), protein-extraction yield was improved by 9.9 and 29.0 percentage points for CPM and PPM, respectively, compared to a single extraction at pH 9 without enzyme dosage (see Fig. 5A). The total protein-extraction yield for a three-step process (pH 5.8/5.7-9-12) increased by 12.8 and 30.0 percentage points for CPM and PPM, respectively. Here, the dosage of Protease A-01 was applied in the second extraction step conducted at pH 9.

Both the enzyme-assisted one-step and three-step processes showed a drastically increased yield when PPM was used as a raw material. The total protein-extraction yields were 60.6% (one-step) and 78.3% (three-step). These values were approximately equal to those obtained for CPM (59.5% and 80.7%, respectively). The results indicate that the effect of protease treatment to liberate proteins from insoluble protein aggregates is especially pronounced for PPM. This is most likely due to the high ratio of denatured protein in this raw material compared to CPM. Interestingly, enzyme activity was not hindered by the elevated fiber content present in PPM compared to CPM (Table 2). The fiber fraction may possibly limit protease accessibility to the denatured proteins and thus reduce the efficacy of enzymatic treatment. However, this effect was not observed. SDS-PAGE for samples from one-step experiments showed the influence of protease treatment upon the protein fractions obtained (Fig. 5B). Cruciferin fractions were degraded by Protease A-01 to a large extent. As hydrolyzed fractions appeared predominantly at an MW range close to that of napins (10–16 kDa), the degree of napin hydrolysis could not be determined. In summary, the employment of proteases gives an especially useful tool to improve protein-extraction yields from low-quality rapeseed raw materials and can be embedded in a multi-step extraction process to optimize the overall yield. Protein yields for PPM did surpass those obtained by extraction at high alkalinity (pH 12). However, subsequent protein-isolation strategies have to be adapted for protein hydrolysates, as classic ultrafiltration or precipitation techniques can pose difficulties for lower MW fractions.

## 4. Conclusion

The present study demonstrated the strong negative impact of high temperatures during conventional processing of rapeseed upon protein solubility in solid by-products. The protein-extraction yield for CPM was significantly higher compared to that for PPM under all tested conditions in the mild ambient range (pH 5.7–9), with maximum yields of 52.3% for CPM and 36.7% for PPM. Interestingly, a simple extraction at native pH (5.7–5.8) gave comparable yields to neutral to mild-alkaline extractions (pH 7–9), thus offering a more economic process design. Protein-extraction yields were significantly improved under strong alkaline conditions as well as with the employment of Protease A-01, with the limitation of protein hydrolysis occurring in both processes. Under strong-alkaline conditions (pH 11 and 12), yields were increased up to 59.5% for CPM and 43.9% for PPM. The best protein-

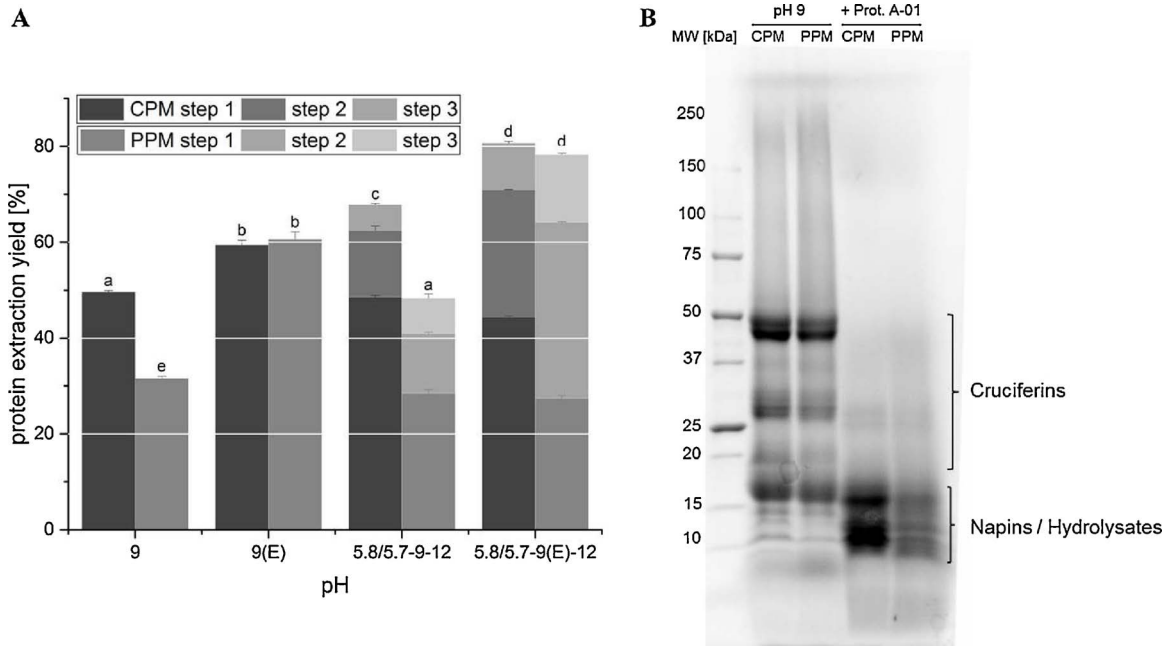


**Fig. 5.** A: Comparison of extraction yields obtained without or with an enzyme dosage of 1% Protease A-01 for one-step (pH 9) and three-step (pH 5.8/5.7-9-12) extractions (left columns: CPM, right columns: PPM); B: SDS-PAGE (non-reducing conditions) of extracted samples from one-step experiments: 1: standard, 2: CPM pH 9, 3: PPM pH 9, 4: CPM pH 9(E), 5: PPM pH 9(E).

extraction yields obtained from enzyme-assisted processes for CPM and PPM were 59.5% and 60.6%, respectively, for the one-step condition and 80.7% and 78.3%, respectively, for the three-step condition. As differences in protein-extraction yield were comparable between CPM and PPM ($p < 0.05$), the described enzyme-assisted method proves to be particularly valuable for rapeseed raw material derived from conventional processing. In conclusion, the present study demonstrates that the process conditions for rapeseed-protein extraction have to be adapted depending upon the quality of the raw material. Further studies on protein isolation from CPM and PPM and the resulting techno-functionality of protein preparations are the subject of current research. The results will provide further important insights into an added-value chain for rapeseed proteins, in particular, as a bio-based alternative to current petro-based constituents of technical products.

## Conflicts of interest

None.

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# CHAPTER III – Rapeseed protein concentrates for non-food applications prepared from pre-pressed and cold-pressed press cake via acidic precipitation and ultrafiltration

Protein isolation was investigated for pre-pressed meal (PPM) and cold-pressed meal (CPM) with regard to yield and functionality. Using optimal extraction conditions determined in the previous study, the protein concentrates were prepared by acidic precipitation and/ or ultrafiltration.

Protein isolation was carried out via aqueous extraction at pH 5.7–7.0 followed by i) ultrafiltration or ii) a combination of acidic precipitation and ultrafiltration. Protein isolation yields were 60%–90% higher for CPM (36.5%–40.6%) compared to PPM (19.4%–26.0%), which was mainly attributed to higher extraction yields obtained from mild-processed CPM. Protein contents of the obtained rapeseed protein concentrates (RPCs) were 75.3%–87.1% (N × 5.7). In general, RPCs obtained by ultrafiltration showed better solubility properties and higher values for emulsifying capacity and foaming activity compared to precipitated RPCs. In detail, emulsifying capacity and foaming activity were 688–768 mL/g and 1834%–2834%, respectively, for ultrafiltrated RPCs, and 410–445 mL/g and 888%–938%, respectively, for precipitated RPCs. In addition, great film-forming properties of all RPCs were demonstrated in cast-film experiments. In conclusion, rapeseed proteins offer a great functional potential as renewable ingredients in technical applications. While the raw-material quality has a great impact on achievable isolation yields, no such influence could be found for protein functionality.

A. Fetzer designed the study, performed the experiments, interpreted the results and wrote the manuscript. T. Herfellner managed the affiliated research project, contributed to the contents of the manuscript and the interpretation of the results. P. Eisner contributed to the contents of the manuscript and the interpretation of the results.

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# Rapeseed protein concentrates for non-food applications prepared from pre-pressed and cold-pressed press cake via acidic precipitation and ultrafiltration

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ABSTRACT

Rapeseed protein concentrates (RPCs), prepared from residual press-cakes, show excellent techno-functional properties, such as emulsification and foaming. However, food application is hindered by residual contents of anti-nutritive components. Limited research has been invested in studying RPCs as a potential ingredient in non-food applications. The present study reports RPC preparation from cold-pressed rapeseed meal (CPM) and pre-pressed rapeseed meal (PPM) as a potential ingredient for the chemical industry. Protein isolation was achieved after extraction at pH 5.7–7.0 via i) ultrafiltration or ii) a combination of acidic precipitation followed by ultrafiltration. Protein isolation yields obtained from CPM (36.5%–40.6%) were 60%–90% higher compared to PPM (19.4%–26.0%), highlighting the positive effect of low-temperature processing during defatting. Protein contents of RPCs were 75.3%–87.1% and were highest for preparations obtained through precipitation. RPCs obtained through ultrafiltration showed good solubility properties and very high values for emulsifying capacity (688–768 mL/g) as well as foaming activity (1834%–2834%). In contrast, precipitated RPCs showed lower functional values (410–445 mL/g, 888%–938%, respectively). All RPCs had excellent film-forming properties in cast-film experiments. Thus, RPCs are promising ingredients for industrial non-food applications such as adhesives, detergents, paints, varnishes and biodegradable polymers.

## 1. Introduction

Rapeseed is the third most abundant oil crop worldwide and the second most abundant oil crop in Europe, with annual production quantities of 71.3 million metric tons and 20.5 million metric tons, respectively (2016/2017; USDA (2018)). Rapeseed meal is produced as a side-stream material of the oil extraction in annual quantities of 39.8 million metric tons (world) and 13.9 million metric tons (EU-28) (USDA, 2018)). As feed utilization of rapeseed meal is still limited due to anti-nutritive components, such as glucosinolates and phytates, an alternative utilization of this residual material is of high interest to the industry. Protein extraction from defatted meal has been shown to depend highly on process conditions during oil extraction. While protein extractability from conventional meals obtained under harsh conditions is limited, milder processing of rapeseeds would allow the utilization of both oil and protein fractions. However, we have not been able to trace any detailed study that compares conventional and mild-processed meals both in regard to protein yield and functionality.

Rapeseed protein is mainly comprised of two globular storage proteins: the neutral 12S globulin (cruciferin) and the 1.7–2S albumin (napin) (Perera et al., 2016; Wanasundara, 2011). Conventionally, rapeseed proteins are extracted from defatted meals in aqueous media using either alkaline extraction or salt extraction (Tan et al., 2011). Many studies reported extractions under strong alkaline conditions (pH > 10) to increase the protein yield (Akbari and Wu, 2015; Aluko and McIntosh, 2001; Chabanon et al., 2007; Das Purkayastha et al., 2015; Ghodsvali et al., 2005; Klockeman et al., 1997; Ma et al., 2007; Tzeng et al., 1990). However, a significant degree of protein hydrolysis has to be considered under these conditions, leading to altered functionality of obtained rapeseed protein concentrates (RPCs) (Fetzer et al., 2018). Therefore, protein extraction ideally should be carried out under mild ambient conditions, which is performed in the present study, in order to obtain native and highly functional proteins.

Rapeseed protein isolation is commonly achieved by either acidic precipitation and/ or ultrafiltration (Tan et al., 2011). Using precipitation, a separation into a cruciferin-rich fraction in the precipitate

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and a napin-rich fraction in the supernatant can be achieved (Akbari and Wu, 2015; Chabanon et al., 2007). Using these isolation strategies, protein contents of approx. 74%–88% (based on nitrogen content using a nitrogen conversion factor of 5.7) were obtained in previous studies from hexane-defatted meals (Akbari and Wu, 2015; Aluko and McIntosh, 2001; Chabanon et al., 2007; Ghodsvali et al., 2005; Klockeman et al., 1997; Tzeng et al., 1990). Protein contents of > 90% have been reported by Tzeng et al. (1990) for non-pressed meal extracted with mixtures of methanol, ammonia and hexane.

In addition, the protein micellar mass method has been developed for the production of RPCs with reduced contents of anti-nutritive and toxic components, including phytic acid, phenols and glucosinolates (Ismond and Welsh, 1992; Murray et al., 1981). Protein contents reported for RPCs obtained by the micellar method in more recent studies were 79.3%–88.5% (Cheung et al., 2014; Yang et al., 2014).

Recent studies have demonstrated the techno-functional potential of RPCs, predominantly as a functional ingredient in food products (Aider and Barbana, 2011; Tan et al., 2011). Functional properties of RPCs include water and oil binding capacity (Ghodsvali et al., 2005), good emulsification as well as foaming properties (Akbari and Wu, 2015; Aluko and McIntosh, 2001; Chabanon et al., 2007; Wu and Muir, 2008), and gelling or film-forming properties (Chang and Nickerson, 2015; Léger and Arntfield, 1993; Yang et al., 2014). However, the use of rapeseed proteins in foods is often not accepted due to remaining components leading to a bitter and astringent taste of products (Linnemann and Dijkstra, 2002; Naczk et al., 1998).

More recently, the utilization of RPCs in the chemical sector has gained more interest. Good film-forming properties of RPCs were demonstrated by wet casting using various plasticizers for the production of edible films (Chang and Nickerson, 2014, 2015; Shi and Dumont, 2014). Moreover, cross-linking of rapeseed protein films was studied by wet casting followed by heat compression using bisepoxide for increased thermostability of resulting films (Li et al., 2017). The production of rapeseed protein-based plastics has also been studied recently. In this regard, the preparation of rapeseed protein-based plastics by extrusion was reported, investigating the influence on raw material quality, protein isolation procedure and protein modification on mechanical properties of resulting plastics (Manamperi et al., 2015, 2010; Manamperi and Pryor, 2012). Moreover, the potential application of RPCs as biodegradable packaging has been reviewed recently (Zhang et al., 2018). The listed studies demonstrate the high potential of rapeseed proteins in the chemical industry as an alternative to petrol-based ingredients.

In the present study, two different rapeseed press cakes (cold-pressed and conventional pre-pressed) were used for protein isolation using mild protein extraction (pH 5.7–7) followed by ultrafiltration or a combination of acidic precipitation and ultrafiltration. The isolated RPCs were analyzed for protein yield and techno-functional properties with an aim of end use in non-food applications.

## 2. Materials and methods

### 2.1. Raw materials and chemicals

Rapeseed raw materials used in this study were of 00 type (indicating low contents of erucic acid and glucosinolates). Hulled, cold-pressed rapeseed press cake (CPC) with reduced seed coat content was provided by Teutoburger Ölmühle GmbH (Ibbenbüren, Germany). Pre-pressed rapeseed press cake (PPC) was provided by Bunge Deutschland GmbH (Mannheim, Germany). Temperatures during pressing typically did not exceed 40 °C for CPC and were in the range of 105–140 °C for PPC. Both press cakes were defatted as stated in the section “Preparation of Rapeseed Raw Material” and stored at 14–20 °C. Isohexane was obtained from Biesterfeld AG (Hamburg, Germany). All other chemicals were of analytical grade and obtained from Th. Geyer GmbH & Co. KG (Renningen, Germany), if not stated otherwise.

### 2.2. Preparation of rapeseed raw materials

CPC and PPC were defatted with isohexane in a percolator of a volume of 1.5 $m^3$ (e&e Verfahrenstechnik GmbH, Warendorf, Germany) and flash desolventized with superheated isohexane at 400–500 mbar prior to steam desolventizing at a maximum product temperature of 60 °C. The obtained cold-pressed rapeseed meal (CPM) and prepressed rapeseed meal (PPM) were ground to pass through a 1-mm screen prior to protein isolation studies.

### 2.3. Chemical composition of rapeseed raw materials and preparations

Dry matter and ash content were determined using a thermo-gravimetrical system (TGA 601, Leco Corporation, St. Joseph, MI, USA) at 105 °C and 550 °C, respectively. Protein content was measured by the Dumas combustion method on a TruMac N system (Leco Corporation) by applying a nitrogen conversion factor of 5.7 (Association of Official Analytical Chemists (Association of Official Analytical Chemists (Association of Official Analytical Chemists (AOAC), 1990). Quantification of phytic acid was performed using an IonPac AS11 column (Thermo Fisher Scientific, Waltham, USA) in a HPAEC system with conductivity detection, according to Fritsch et al. (2015) with the following modifications: 100 mg of sample were treated with 2 mL of 3 M HCl in an Eppendorf ThermoMixer® (Eppendorf AG, Hamburg, Germany) at 99 °C and 1400 rpm for 10 min. After centrifugation in an Eppendorf MiniSpin® (Eppendorf AG) at 13,400 rpm for 2 min, the supernatant was separated and diluted to a volume of 5 mL. After filtration (0.22 µm), dilutions of 1:40 were used for measurement of phytic acid content. The chemical composition (dry matter, protein, ash, phytic acid) was analyzed in duplicate for all samples.

### 2.4. Functional properties of rapeseed preparations

#### 2.4.1. Protein Solubility (PS)

The PS was determined in duplicate within the range of pH 3 to pH 11 following the method of Morr et al. (1985). For each measurement, 1.5 g of sample (ground to < 1 mm) were suspended in a total volume of 50 mL of deionized water containing 0.1 M NaCl and adjusted to appropriate pH using 0.1 M – 1,0 M NaOH or 0.1 M – 1,0 M HCl, respectively. After stirring for 1 h at room temperature, non-dissolved fractions of the samples were separated by centrifugation (20,000 × *g*, 15 min, 15 °C) and the resulting supernatants were filtered (Whatman No. 1 filter paper). The protein content of the supernatant was determined according to Dumas following the method given in the section “Analysis of Chemical Composition of Rapeseed Raw Materials and Preparations”. PS was calculated using Formula 1:

$$PS\ [\%] = \frac{initial\ volume\ [mL] \times protein\ content\ in\ supernatant\ [mg/mL]}{sample\ mass\ [mg] \times protein\ content\ [-]} \times 100[\%] \quad (1)$$

#### 2.4.2. Foaming properties

Foaming activity (FA) was determined according to Phillips et al. (1987). Aqueous protein solution samples (5% w/w) were whipped using a Hobart N50 mixer (Hobart GmbH, Offenburg, Germany) at level 3 for 8 min. The FA (%) was calculated as the relation of the foam volume before and after whipping. The foam density (FD, g/L) was determined by measuring the weight of a specified foam volume. The foam stability (FS, %) was measured as the remaining percentage of foam volume in a 250 mL measuring flask after 60 min.

#### 2.4.3. Emulsifying capacity (EC)

The EC was determined in duplicate as described by Wäsche et al. (2001). Aqueous protein solution samples of 1% (w/w) were treated in an IKA LR-A 1000 laboratory reactor (IKA-Werke GmbH & Co. KG, Staufen, Germany) equipped with a stirrer (100 rpm) and an ULTRA-

TURRAX® (11,000 rpm). Commercial corn oil was added by a titration system (Titrino 702 SM, Metrohm GmbH & Co. KG, Hertisau, Switzerland) at a constant rate of 10 mL/min while keeping temperature at 20–25 °C. Phase inversion of the emulsion was determined by monitoring the conductivity of the emulsion by a conductivity meter LF 521 with electrode KLE 1/T (Wissenschaftlich-technische Werkstätten GmbH, Weilheim, Germany). The volume of oil needed for phase inversion was used to calculate the EC (mL oil per g sample).

#### 2.4.4. Color analysis (CIELAB color space)

Photographs of rapeseed preparations were taken on a DigiEye system (VeriVide Limited, Leicester, UK) equipped with a Nikon D90 camera (Nikon, Tokyo, Japan). Color measurement was performed in the CIELAB color space, using the RPCs prepared in duplicate (DigiEye software, VeriVide Limited).

#### 2.4.5. Film-forming properties

Cast films of RPCs were prepared according to the method by Chang and Nickerson (2015) with minor modifications. 3,0 g of RPC were mixed with 30 mL of demineralized $H_2O$ in a 100 mL glass bottle was adjusted to pH 3 using 1 M HCl to induce protein denaturation. The mixture was stirred at room temperature for 60 min at 300 rpm, before 0.45–0.90 g of glycerol were added. The mixture was adjusted to a total weight of 40 g at pH 3 using demineralized $H_2O$ and 1 M HCl and stirred for 10 min. The mixture was degassed in an ultrasound bath at 35 kHz for 15 min, stirred at 50 °C and 300 rpm and 13.5 g were cast in a petri dish (85 mm diameter). The films were dried at 20 °C and RH = 50% for at least 48 h and were removed from the petri dish after 14 days for textural comparison.

### 2.5. Protein isolation

#### 2.5.1. Protein extraction

Protein extraction from rapeseed meals was carried out as follows: To 11.0 kg of demineralized water, 160.7 g of NaCl were added and the solution was stirred in a food-grade polyethylene container using mechanical stirring. Subsequently, 1.0 kg of meal (CPM or PPM) was added in small portions. For extractions at neutral pH (pH 7), the pH was adjusted during addition of the meal using 3 M NaOH and was monitored and adjusted during the extraction. For extractions at native pH (pH 5.7–5.8) the pH was not adjusted. After complete addition of the meal, the extraction mixture was stirred at 400 rpm for 60 min at room temperature (20 °C). The extraction mixture was centrifuged (3300 × *g*, 10 min, 20 °C) and poured over a 140 µm sieve to separate the supernatant (extract) from the residue (raffinate). The extract was further used in protein isolation experiments.

#### 2.5.2. Ultrafiltration (process UF)

Ultrafiltration was carried out on a Microza polysulfone ultrafiltration module SIP-1023 (Pall Corporation, Port Washington, NY, USA) with a molecular weight cutoff of 6000 Da and a membrane area of 0.19 $m^2$. Ultrafiltration was performed in batch mode at 50 °C and a membrane pressure of 1.5 bar generated with a built-in peristaltic pump, with recirculation of the retentate and a concentration factor of 4. During operation, back-pressure of 4 bar was applied every 1–2 h to reduce membrane fouling. After reduction of the retentate volume to approx. 2 L, diafiltration was carried out using 6 × 1.0 L of demineralized water. The retentate was adjusted to pH 7.0 during diafiltration where necessary using 3 M NaOH, freeze-dried and prepared using a mortar and pestle to obtain the RPC.

#### 2.5.3. Acidic precipitation/ ultrafiltration (process AP/UF)

Experiments with combined protein isolation through acidic precipitation and ultrafiltration were carried out as follows: The protein extract was cooled to 10 °C and stirred at 400 rpm, while the pH was adjusted quickly to pH 4.0 using 1 M HCl. The mixture was stirred for 15 min, centrifuged (3300 × *g*, 15 min, 20 °C) and poured over a 140 µm sieve to separate the supernatant from the precipitate. The precipitate was resuspended in an equal amount of demineralized water and adjusted to pH 7.0. After freeze-drying, the RPC was prepared using a mortar and pestle. The supernatant was further used for ultrafiltration as stated in the section “Ultrafiltration”.

Each RPC was isolated in duplicate. Protein extraction yield (PEY), protein precipitation yield (PPY), protein ultrafiltration yield (PUY) and protein isolation yield (PIY) were calculated according to the following formulas:

$$PEY\ [\%] = \frac{mass\ (extract)\ [g]\ \times\ protein\ content\ (extract)\ [-]}{mass\ (meal)\ [g]\ \times\ protein\ content\ (meal)\ [-]} \times 100[\%] \quad (2)$$

$$PPY\ [\%] = \frac{mass\ (precipitate)\ [g]\ \times\ protein\ content\ (precipitate)\ [-]}{mass\ (extract)\ [g]\ \times\ protein\ content\ (extract)\ [-]} \times 100\ [\%] \quad (3)$$

$$PUY\ [\%] = \frac{mass\ (retentate)\ [g]\ \times\ protein\ content\ (retentate)\ [-]}{mass\ (extract\ OR\ supernatant)\ [g]\ \times\ protein\ content\ (extract\ OR\ supernatant)\ [-]} \times 100\ [\%] \quad (4)$$

$$PIY\ [\%] = \frac{mass\ (prec.)\ [g]\ \times\ protein\ content\ (prec.)\ [-]\ +\ mass\ (ret.)\ [g]\ \times\ protein\ content\ (ret.)\ [-]}{mass\ (meal)\ [g]\ \times\ protein\ content\ (meal)\ [-]} \times 100\ [\%] \quad (5)$$

### 2.6. Molecular weight distribution (SDS-PAGE)

Molecular weight distribution of RPCs was determined by SDS-PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis) according to Laemmli, using non-reducing sample buffer (Laemmli, 1970). Sample masses equaling 50 mg of protein were weighed in and treated with 1 mL of non-reducing sample buffer in a ThermoMixer® (Eppendorf AG, Hamburg, Germany) at 30 °C and 1400 rpm for 15 min. The samples were centrifuged in an Eppendorf MiniSpin® (Eppendorf AG) at 13,300 rpm for 2 min. The supernatant was diluted 1:10 in sample buffer and 10 µL were loaded on a pre-cast 4–20% CriterionTM TGX Stain-FreeTM gel (Bio-Rad Laboratories, Hercules, CA, USA) at room temperature, applying 200 V, 60 mA and 100 W as running conditions. 10 µL of Precision Plus Protein™ Unstained Protein Standard (Bio-Rad Laboratories) were used as a MW standard. Documentation was carried out using a Gel DocTM EZ Imager system and Image Lab software (Bio-Rad Laboratories).

### 2.7. Statistical analysis

All data are given as mean values ± absolute deviation of at least two measurements (n = 2). Significant differences were statistically analyzed by one-way analysis of variance using Tukey’s test ($p < 0.05$).

## 3. Results and discussion

### 3.1. Characterization of rapeseed raw materials

The chemical composition of the rapeseed meals used in this work is presented in Table 1. CPM and PPM were used in a previous study to determine optimal protein extraction conditions (Fetzer et al., 2018).

**Table 1**
Chemical composition of rapeseed raw materials used for protein isolation studies (data obtained from Fetzer et al. (2018)).

| | | CPM | PPM |
|---|---|---|---|
| **dry matter (dm)** | [%] | 92.0 ± 0.1 | 94.2 ± 0.0 |
| **protein** | [%dm] | 40.6 ± 0.0 | 34.4 ± 0.2 |
| **ash** | [%dm] | 7.3 ± 0.0 | 7.2 ± 0.0 |
| **fat** | [%dm] | 2.8 ± 0.1 | 2.3 ± 0.0 |
| **phytic acid** | [%dm] | 4.0 ± 0.1 | 4.4 ± 0.0 |
| **cellulose** | [%dm] | 6.8 ± 0.6 | 8.5 ± 1.3 |
| **hemicellulose** | [%dm] | 3.9 ± 0.6 | 6.6 ± 1.7 |
| **lignin** | [%dm] | 11.4 ± 0.1 | 16.1 ± 0.1 |

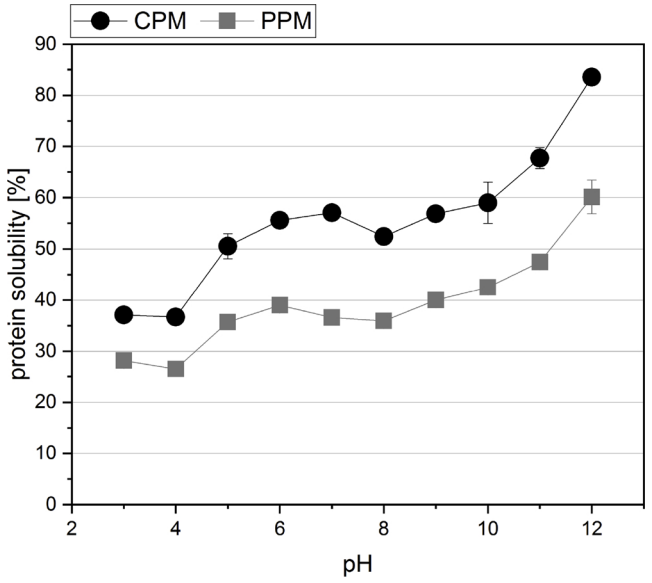



**Fig. 1.** Protein solubility of raw materials used for this study (data obtained from Fetzer et al. (2018), figure adapted and modified).

The protein content for CPM was significantly higher as compared to PPM, due to hulling and partial reduction of the seed coat content. Due to this, cellulose, hemicellulose and lignin contents were significantly lower for CPM as compared to PPM. Contents of ash, fat and phytic acid were similar for both materials.

Due to different process conditions during defatting, the PS differed largely between the two raw materials used in this study (Fig. 1). CPM showed a PS of ˜10–20 percentage points higher as compared to PPM in the range of pH 3–12. The difference can be attributed to protein denaturation effects, induced Maillard-reactions and reactions with phenolic substances, which occur at higher pressures and temperatures during the conventional pressing process (Le et al., 2011; Naczk et al., 1998). For CPM, which was only heated to 40 °C during pressing, these effects occurred to a lesser extent, resulting in a PS of > 50% around the neutral pH range. At strong acidic conditions (pH 3–4) both raw materials showed a distinct drop of the PS. This can be attributed to protein precipitation, predominantly of cruciferin, which are not soluble at pH 3–4 (Fetzer et al., 2018). Consequently, pH 4 was chosen for protein isolation through precipitation in the present study. The PS at pH > 10 showed a sharp increase for both materials, caused by increasing effects of protein hydrolysis (Fetzer et al., 2018). Thus, protein extraction and isolation at these conditions are not favored in order to obtain intact rapeseed proteins.

### 3.2. Protein yields

The parameters for protein extraction from CPM and PPM to optimize protein extraction yields were determined in a previous study

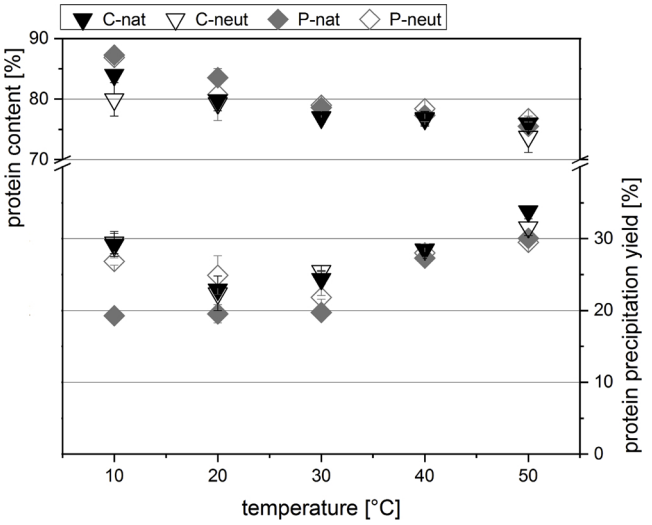



**Fig. 2.** Protein contents (left axis) and protein precipitation yields (right axis) obtained at different precipitation temperatures; C: cold-pressed; P: pre-pressed; nat: native pH (5.7–5.8); neut: neutral pH (7).

(Fetzer et al., 2018). Native rapeseed pH (pH 5.7–5.8) and neutral pH (pH 7.0) were chosen for comparison of protein isolation yields and functionalities in the present study.

#### 3.2.1. Protein precipitation yields

Acidic protein precipitation was studied in more detail in order to obtain optimized conditions. As PS showed a minimum both for CPM and PPM at pH 4 (Fig. 1), this condition was chosen for acidic precipitation. The dependence of protein concentration in the extract before precipitation was tested, but found to be not significant (data not shown). Additionally, the influence of temperature on acidic protein precipitation was studied in the range of 10–50 °C (Fig. 2). Protein precipitation yield was relatively low in the ambient temperature range (20–30 °C) and showed an increase both at high and low temperatures. In contrast to heat denaturation of proteins, the phenomenon of cold denaturation, which is found for some proteins, is not fully understood. Currently, it is explained by the increase of nonpolar group hydration, leading to the weakening of hydrophobic interactions and exposure of buried nonpolar side chains to the protein surface (Gulevsky and Relina, 2013). Interestingly, the protein content of the precipitates also increased with decreasing precipitation temperature. This suggests lower interactions of proteins with other substances at decreased temperature. At 10 °C and 20 °C, all RPCs showed protein contents of ≥ 80% with a maximum of 87.3% at 10 °C. In contrast, the protein content for precipitation temperatures of ≥ 30 °C was < 80% and decreased to 73.8–76.8% at 50 °C.

In addition, the PS at pH 7 of the RPCs at 10 °C, 20 °C and 50 °C was compared to gain insight into protein functionality (Fig. 3). Functional properties, such as emulsifying and foaming properties, are described to be dependent on the PS (Aider and Barbana, 2011; Tan et al., 2011), and therefore, better functional properties were expected for precipitates obtained at 10 °C. The PS was low for all precipitates, however, especially low for precipitates obtained at 50 °C, possibly due to an increased level of irreversible protein denaturation. Thus, higher precipitation temperatures negatively influence protein functionality of RPCs.

Based on the above results, a precipitation temperature of 10 °C was chosen for the scaling-up of the process. At this temperature, precipitation yields were only slightly lower than at 50 °C (with the exception of P-nat), while protein contents of RPCs were significantly higher. Additionally, the PS at pH 7 from precipitates at 10 °C was higher compared to precipitates at 20 °C and 50 °C. Thus, a

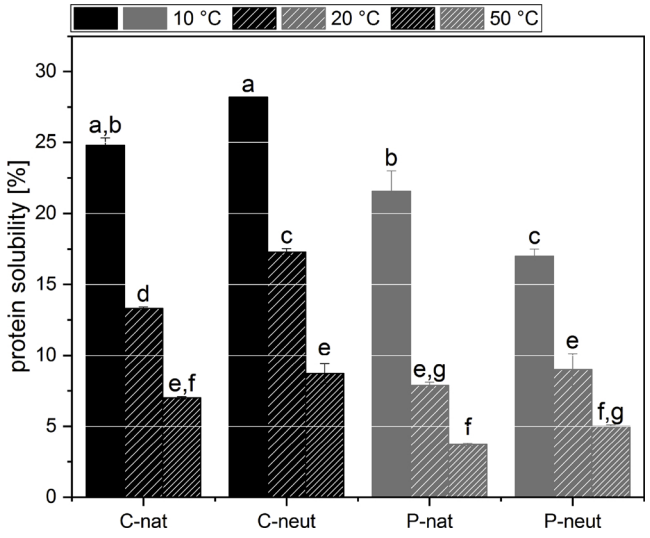



**Fig. 3.** Protein solubility at pH 7 for RPCs precipitated at 10 °C, 20 °C and 50 °C; P: pre-pressed; C: cold-pressed; nat: native pH (5.7–5.8); neut: neutral pH (7).

precipitation temperature of 10 °C gave the best overall results in terms of precipitation yield, protein content and functionality. In contrast to these findings, previous studies commonly reported precipitation at room temperature (Ghodsvali et al., 2005; Tzeng et al., 1990). As an exception, Aluko et al. (2005) and Klockeman et al. (1997) reported centrifugation temperatures of 8 °C and 5–10 °C, respectively, prior to precipitation, however, neither temperatures during precipitation nor precipitation yields were reported.

### 3.2.2. Protein isolation yields

Two protein preparation processes were compared in this study: ultrafiltration (UF) and combined acidic precipitation followed by ultrafiltration (AP/UF). A schematic overview is given in Fig. 4.

Protein yield of the obtained fractions was determined to evaluate the process efficiency (Table 2). According to the solubility profiles of rapeseed meals (Fig. 1), protein yield obtained from CPM was significantly higher than from PPM for all isolation processes ($p > 0.05$). Protein extraction yield (PEY) was in the range of 45.1%–49.0% for CPM and 28.3%–32.8% for PPM, respectively and was slightly higher (without significance) for extractions at pH 7. However, total protein isolation yield (PIY) from UF processes was comparable or higher than for processes at pH 5.7–5.8 due to lower degrees of protein loss into the permeate. The latter was similarly found for the AP/UF process. However, due to a lower precipitation yield (PPY) at pH 5.7–5.8, the isolated yield was lower than for processes at pH 7.

The PIY obtained from PPM was 19.4%–26.0%, which was lower than the values reported for commercially hexane defatted meal under high-alkaline conditions in other studies (values adapted to a nitrogen conversion factor of 5.7). Tzeng et al. (1990) reported an isolated yield of 33% at an extraction pH of 11. A higher yield (52.2%) from pressed material was achieved by Ma et al. (2007) using an ultrasound-assisted process at pH 12. Das Purkayastha et al. (2015) reported a process at pH 11 using ammonium sulfate precipitation, with an experimental yield of 203 mg/g dry meal (protein content of meal not reported) under optimal conditions. These values were achieved at high alkalinity and were thus higher than PIYs obtained from CPM at pH 5.7–7 in this study (36.5%–40.6%).

PIYs were drastically improved in previous studies by the utilization of non-pressed, solvent-defatted meals. Tzeng et al. (1990) reported yields ranging from 42.2%–75.4% at pH 11–12. Similarly, high yields were reported by Ghodsvali et al. (2005) for whole-seed meal (46.5%–56.5%) and dehulled meal (59.9%–67.2%) at pH 12. Using hexane-defatted, air-dried commercial canola meal, Akbari and Wu (2015) reported an isolated yield of 51% at pH 12.5 (after acidic washing at pH 4).

In summary, the findings highlight the strong negative effect of rapeseed screw-pressing on resulting protein extractability. Limited protein solubility of meals at pH 5.7–7.0 was responsible for comparably low yields obtained in this study. However, the isolated protein yield from CPM was significantly higher compared to PPM, demonstrating the positive effect of cold-pressing on resulting protein quality.

### 3.3. Chemical composition

Chemical composition of rapeseed RPCs differed in dependence of starting material and isolation process used (Table 3). For the UF process, protein contents ranged from approx. 78% (PPM) to approx. 78%–84% (CPM). For comparison of RPCs with the literature, reported values were adapted with the nitrogen-to-protein conversion factor of 5.7 (compared to 6.25), which was used in this study.

The protein contents of RPCs were comparable to values reported in previous studies. Interestingly, APPs with protein contents of 83.6%–88.0% were higher than in most studies (73.2%–82.8%), indicating the positive effect of protein precipitation at 10 °C (Aluko and McIntosh, 2001; Ghodsvali et al., 2005; Ivanova et al., 2017; Von Der Haar et al., 2014). Higher protein contents for precipitated proteins were reported by Tzeng et al. (1990) with values up to 91.7% by precipitation at pH 3.5 and an additional washing step of the precipitate. The latter could be applied for the process in this study in order to reduce the ash-content and further increase the protein content. Contents of phytic acid were significantly higher in APPs than in RPCs obtained through ultrafiltration, which is in agreement with previous studies (Akbari and Wu, 2015; Ghodsvali et al., 2005; Tzeng et al., 1990). This can be explained by the co-precipitation of phytates and proteins at low pH, which has been described in various studies, predominantly for soy proteins (Frida and Munir, 1989; Hill and Tyler, 1954; Pontoppidan et al., 2007; Schwenke et al., 1979). Contents of phytic acid were higher for APPs obtained under neutral pH extraction, possibly due to increased formation of protein-phytate complexes at neutral pH before the rapid pH-shift for precipitation.

Protein contents of UFPs and SNPs were 77.7%–83.9% and 75.3%–84.4%, respectively, which were generally lower than those obtained for APPs in this study. Low protein contents were partly caused by high residual ash contents of > 6%. Ash content should be significantly reduced by using a higher diafiltration volume upon up-scaling of the process. Tzeng et al. (1990) reported protein contents for SNP-type preparations of 78.6% from commercial canola meal and up to 94.5% from non-pressed and dehulled meal obtained through extraction with a mixture of methanol/ammonia/hexane (values adapted to a nitrogen conversion factor of 5.7). The latter demonstrates the positive impact of phenol reduction on resulting protein content. High values for SNPs were also obtained by (Ghodsvali et al., 2005), with 83.0%–86.2% and 85.6%–89.4% for whole seed and dehulled, non-pressed meals, respectively.

In summary, protein contents of RPCs were comparable to values reported in the literature. Protein precipitation at 10 °C showed positive impacts on protein contents, which were highest for APP-Ps. Protein contents for SNP-type preparations were lower than in some previous reports, which possibly derived from lower contents of polyphenols in the non-pressed, solvent defatted materials used in these studies (Ghodsvali et al., 2005; Tzeng et al., 1990). The pH value of the process did not seem to have a significant influence on the resulting protein content, except for SNPs, where protein contents were higher at neutral extraction conditions. All preparations showed relatively high ash contents, which can be expected to decrease by additional washing steps or increased diafiltration volume upon up-scaling of the processes. Levels of phytic acid were lower for UFPs and SNPs than for APPs, demonstrating the positive effect of ultrafiltration on phytate reduction. Levels of phytic acid were generally lower for RPCs obtained from processes at native extraction pH.

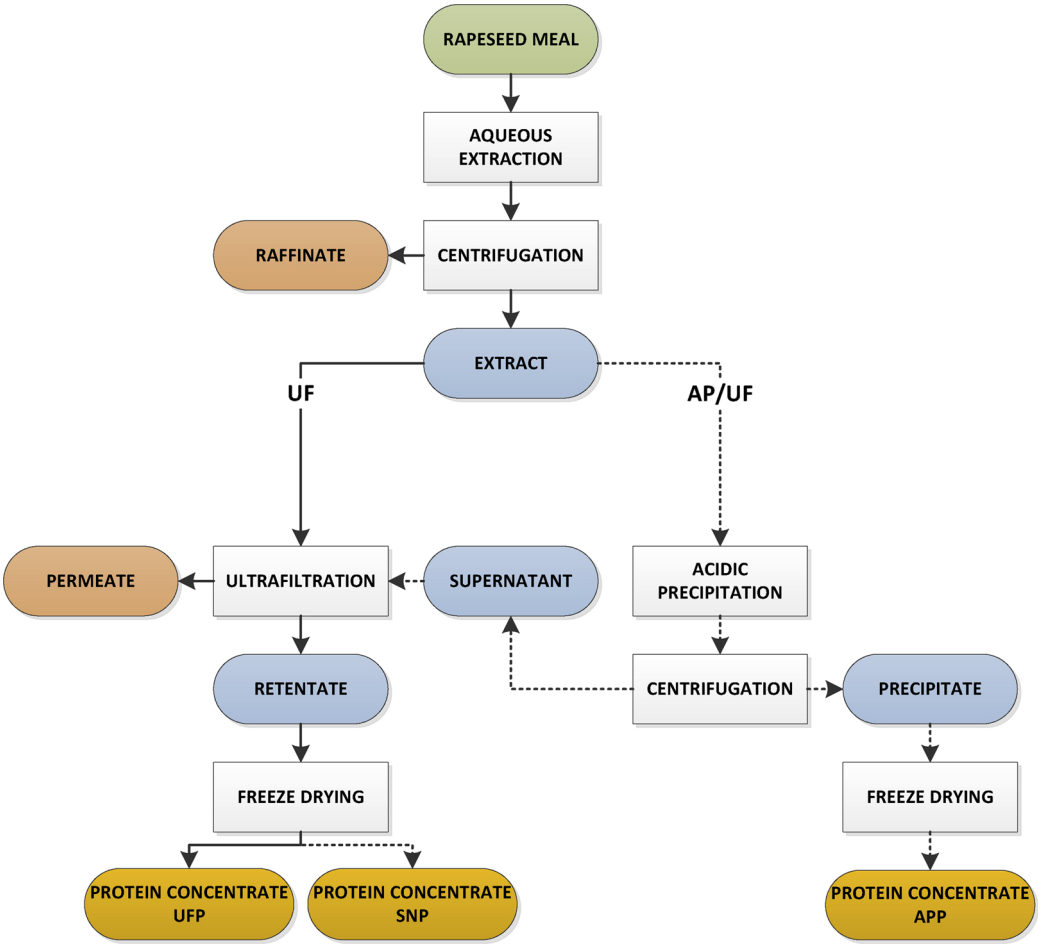



**Fig. 4.** Process scheme for protein preparation processes used in this study; UF: ultrafiltration process; AP/UF: combined process with acidic precipitation followed by ultrafiltration; UFP: ultrafiltration protein; SNP: supernatant protein; APP: acidic precipitation protein.

*3.3.1. Protein distribution (SDS-PAGE)*

SDS-PAGE of RPCs revealed the different distributions of soluble protein fractions obtained from the isolation processes (Fig. 5, Table 4). Cruciferin fractions were mainly observed in the range of 38–45 kDa and 18–30 kDa, according to intact heterodimers and dissociated α and β chains, respectively (Nietzel et al., 2013; Wanasundara, 2011). Napin fractions were visible at ≤16 kDa, with the majority appearing at 15–16 kDa as attributed to the napin dipeptide with an intact disulfide linkage (Perera et al., 2016). Additionally, bands at the lower end of the gel were visible to various excess, which could be attributed either to hydrolyzed protein fractions or the small napin subunit with a reported MW of approx. 4.5 kDa (Wanasundara, 2011).

For the UF processes, mixtures of cruciferin (MW ≥ 18 kDa) and napin (MW ≤ 16 kDa) with minor differences in distribution depending on the employed raw material and extraction pH were obtained. For UFPs, cruciferin contents very slightly higher for CPM (69.7%–72.5%) than for PPM (66.5%–67.6%).

In case of the combined AP/UF process, a separation of the two main protein fractions from rapeseed could be achieved. APPs showed cruciferin contents of 84.0%–84.8% (CPM) and 87.0%–88.0% (PPM), while SNPs were high in napin content varying between 90.3%–93.7% (CPM) and 80.5%–81.2% (PPM). To a minor extent, 74 kDa and 95 kDa were visible for APPs, which were not present in any other RPCs. The bands could possibly be attributed to dimeric complexes of cruciferin heterodimers. Additionally, heterodimers of 55 kDa were visible for APP-Ps (Nietzel et al., 2013).

**Table 2**
Protein distribution in fractions obtained during protein extraction and isolation as percentage of starting material (meal); PEY (protein extraction yield), PIY (protein isolation yield): yields as percentage of protein present in the starting material; PPY (protein precipitation yield), PUY (protein ultrafiltration yield): yields as percentage of protein present in extract or supernatant; values within one row having the same superscript indicate no significant difference ($p > 0.05$).

| process | conditions | PEY [%] | precipitate [%] | supernatant [%] | PPY [%] | retentate [%] | permeate [%] | PUY [%] | PIY [%] |
|---|---|---|---|---|---|---|---|---|---|
| UF | C-nat | 49.0 ± 3.4$^{a,c}$ | – | – | – | 40.4 ± 1.8$^{a}$ | 3.6 ± 0.3$^{a,b}$ | 82.6 ± 2.1$^{a}$ | 40.4 ± 1.8$^{a}$ |
| | C-neut | 54.5 ± 0.3$^{a}$ | – | – | – | 40.6 ± 0.0$^{a}$ | 4.7 ± 0.5$^{a}$ | 74.5 ± 0.4$^{a}$ | 40.6 ± 0.0$^{a}$ |
| | P-nat | 32.8 ± 0.9$^{b}$ | – | – | – | 26.0 ± 1.1$^{b}$ | 2.6 ± 0.1$^{b}$ | 79.1 ± 1.0$^{a}$ | 26.0 ± 1.1$^{b,c,d}$ |
| | P-neut | 35.9 ± 0.1$^{b}$ | – | – | – | 23.2 ± 3.0$^{b}$ | 2.9 ± 0.2$^{a,b}$ | 64.6 ± 8.1$^{a}$ | 23.2 ± 3.0$^{b,c,d}$ |
| AP/UF | C-nat | 45.1 ± 2.7$^{c}$ | 12.2 ± 0.9$^{a,b}$ | 31.6 ± 1.3$^{a,b}$ | 27.1 ± 0.4$^{a}$ | 24.3 ± 2.2$^{b}$ | 2.3 ± 0.7$^{b}$ | 77.3 ± 10.0$^{a}$ | 36.5 ± 1.3$^{b}$ |
| | C-neut | 49.8 ± 0.0$^{a,c}$ | 13.7 ± 1.8$^{a}$ | 34.5 ± 2.7$^{a}$ | 27.5 ± 3.6$^{a}$ | 24.5 ± 0.9$^{b}$ | 3.1 ± 0.1$^{a,b}$ | 71.4 ± 3.1$^{a}$ | 38.2 ± 0.9$^{c}$ |
| | P-nat | 28.3 ± 0.5$^{b}$ | 6.5 ± 0.5$^{b}$ | 21.2 ± 1.6$^{b}$ | 22.9 ± 1.3$^{a}$ | 12.9 ± 0.5$^{c}$ | 1.8 ± 0.3$^{b}$ | 61.6 ± 7.2$^{a}$ | 19.4 ± 1.0$^{d}$ |
| | P-neut | 30.2 ± 1.4$^{b}$ | 7.9 ± 0.1$^{a,b}$ | 21.5 ± 2.2$^{b}$ | 26.2 ± 1.6$^{a}$ | 16.3 ± 0.2$^{b,c}$ | 2.0 ± 0.0$^{b}$ | 76.8 ± 8.6$^{a}$ | 24.2 ± 0.3$^{d}$ |

**Table 3**
Chemical composition of rapeseed preparations; UFP: ultrafiltration protein; APP: acidic precipitation protein; SNP: supernatant protein; C: cold-pressed; P: pre-pressed; nat: native pH (5.7–5.8); neut: neutral pH (7); values within one row having the same superscript indicate no significant difference ($p > 0.05$).

| process | preparation | dm [%] | protein [%dm] | ash [%dm] | phytic acid [%dm] |
|---|---|---|---|---|---|
| UF | UFP-C-nat | 96.6 ± 0.2$^{a}$ | 78.0 ± 2.5$^{a,d}$ | 7.5 ± 0.8$^{a}$ | 1.4 ± 0.0$^{a,b,d}$ |
| | UFP-C-neut | 93.8 ± 2.0$^{a}$ | 83.9 ± 1.6$^{a,b,d}$ | 5.9 ± 0.7$^{a}$ | 1.1 ± 0.2$^{a,b,d}$ |
| | UFP-P-nat | 95.6 ± 1.7$^{a}$ | 77.9 ± 1.4$^{a,d}$ | 7.8 ± 0.5$^{a}$ | 0.6 ± 0.1$^{a}$ |
| | UFP-P-neut | 96.5 ± 0.8$^{a}$ | 77.7 ± 3.7$^{a,d}$ | 6.2 ± 0.3$^{a}$ | 1.0 ± 0.1$^{a,b}$ |
| AP/UF | APP-C-nat | 93.5 ± 1.2$^{a}$ | 84.0 ± 0.5$^{a,b,d}$ | 6.3 ± 0.3$^{a}$ | 1.6 ± 0.0$^{b,d}$ |
| | APP-C-neut | 96.9 ± 1.0$^{a}$ | 83.6 ± 1.4$^{a,b,d}$ | 6.0 ± 1.2$^{a}$ | 2.8 ± 0.2$^{c}$ |
| | APP-P-nat | 95.4 ± 0.7$^{a}$ | 88.0 ± 0.5$^{b}$ | 4.3 ± 0.4$^{a}$ | 1.8 ± 0.1$^{d}$ |
| | APP-P-neut | 96.2 ± 0.1$^{a}$ | 87.1 ± 1.2$^{b,c}$ | 6.1 ± 1.7$^{a}$ | 3.3 ± 0.1$^{c}$ |
| | SNP-C-nat | 92.7 ± 0.8$^{a}$ | 78.4 ± 3.3$^{a,c,d}$ | 6.6 ± 0.8$^{a}$ | 0.8 ± 0.3$^{a,b}$ |
| | SNP-C-neut | 95.3 ± 2.7$^{a}$ | 84.4 ± 1.0$^{a,b,c}$ | 4.4 ± 0.2$^{a}$ | 0.8 ± 0.2$^{a}$ |
| | SNP-P-nat | 92.9 ± 0.7$^{a}$ | 75.3 ± 1.3$^{d}$ | 6.8 ± 0.0$^{a}$ | 0.9 ± 0.0$^{a,b}$ |
| | SNP-P-neut | 91.9 ± 0.0$^{a}$ | 79.9 ± 0.3$^{a,b,c,d}$ | 4.3 ± 0.0$^{a}$ | 0.9 ± 0.1$^{a,b}$ |

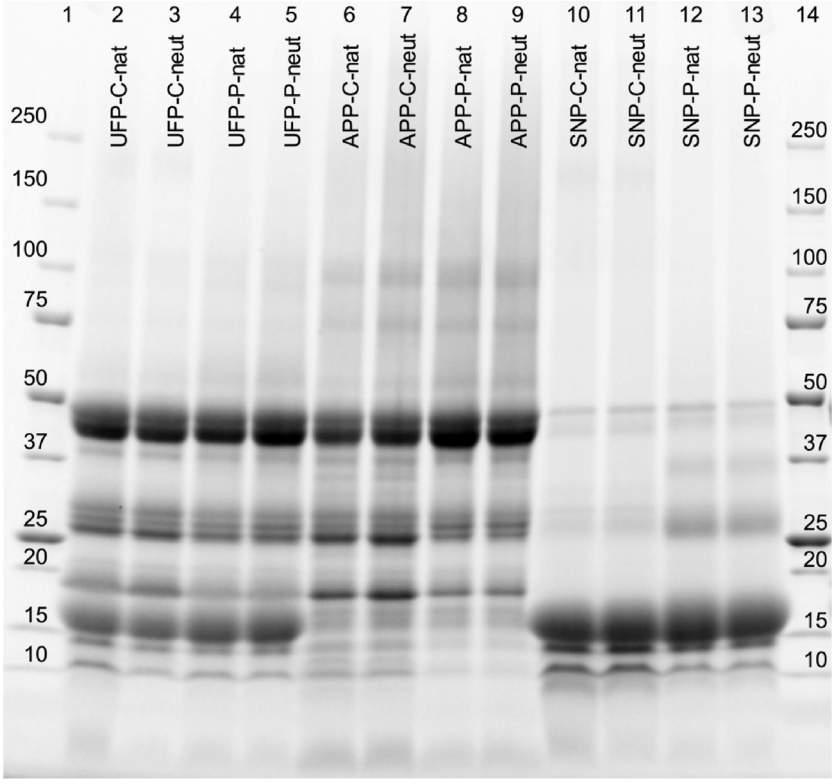



**Fig. 5.** SDS-PAGE of RPCs (non-reducing conditions); lanes 1 and 14: protein standard (Bio-Rad Precision Plus); UFP: ultrafiltration protein; APP: acidic precipitation protein; SNP: supernatant protein; C: cold-pressed; P: pre-pressed; nat: native pH (5.7–5.8); neut: neutral pH (7).

### 3.3.2. Color analysis (L*a*b)

All RPCs showed yellow to brownish color, probably due to the presence of polyphenols (Fig. 6). Lightness ($L^*$) was in the range of 49.3–57.2 and did not show a clear correlation with extraction pH. While $L^*$ was generally higher for native extraction conditions in case of CPM, RPCs obtained from PPM showed a contrary trend with higher $L^*$ values at neutral extraction conditions. Values for $a^*$ and $b^*$ were generally higher for RPC-Ps than for RPC-Cs, indicating stronger coloring of magenta and yellow of these preparations.

In general, strong coloring induced by polyphenols and their oxidation products must be rated as hindering for applications in the chemical industry. Especially in fields where colorless coatings or solutions are required, further process steps to absorb or degrade color active components have to be implemented. However, depending on the final concentration of RPCs in the formulation, minor colorings of the final product may often be negligible. Additionally, possible applications in the packaging sector as a light or UV-barrier might be of interest.

## 3.4. Functional properties

### 3.4.1. Protein solubility (PS)

The PS of RPCs was determined in 0.1 M NaCl solution in the range of pH 3–11. The three different protein types (UFP, APP, SNP) showed distinct solubility profiles (Fig. 7).

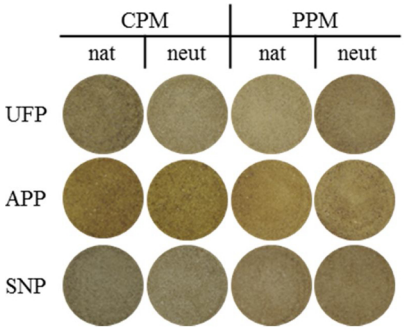
For UFPs, high (> 80%) to very high solubility (> 90%) was achieved in the range of pH 7–11. The PS of UFP-C-nat was lowest, possibly due to a slightly higher presence of low-soluble cruciferin fractions (Table 4). In the acidic pH range, the solubility of UFPs dropped to approx. 45%–65% (pH 3), which can be explained by the presence of low-soluble cruciferin fractions (Aider and Barbana, 2011; Perera et al., 2016).

**Table 4**
MW distribution (band percentage within each lane) of RPCs as deduced from SDS-PAGE analysis (Fig. 5); SUM cru: sum of cruciferin fractions (≥18 kDa), SUM nap: sum of napin fractions (≤16 kDa).

| MW [kDa] | band percentage [%] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | UFP-C-nat | UFP-C-neut | UFP-P-nat | UFP-P-neut | APP-C-nat | APP-C-neut | APP-P-nat | APP-P-neut | SNP-C-nat | SNP-C-neut | SNP-P-nat | SNP-P-neut |
| **95** | – | – | – | – | 2.2 | 2.5 | 3.0 | 3.3 | – | – | – | – |
| **74** | – | – | – | – | 1.3 | 2.2 | 1.1 | 1.4 | – | – | – | – |
| **55** | – | – | – | – | – | – | 2.7 | 2.2 | – | – | – | – |
| **45** | 22.0 | 20.7 | 22.7 | 20.8 | 15.8 | 16.5 | 24.6 | 23.3 | 4.9 | 2.1 | 2.6 | 1.9 |
| **42** | 18.0 | 19.0 | 17.7 | 20.7 | 23.8 | 19.4 | 29.7 | 27.2 | – | – | – | – |
| **38** | 1.8 | 2.2 | 1.9 | 3.1 | 1.5 | 2.2 | 3.3 | 4.3 | – | – | – | – |
| **34** | – | – | – | – | 0.5 | 0.9 | – | – | – | – | 2.4 | 2.5 |
| **28** | 5.2 | 5.8 | 4.3 | 6.0 | 6.7 | 6.8 | 6.1 | 5.7 | – | – | – | – |
| **27** | 4.5 | 3.6 | 3.4 | 2.9 | 5.1 | 3.6 | 4.1 | 5.0 | 4.8 | 4.2 | 14.4 | 14.4 |
| **26** | 8.3 | 8.9 | 8.3 | 6.7 | 13.4 | 13.3 | 5.3 | 4.9 | – | – | – | – |
| **18** | 10.0 | 12.2 | 8.3 | 7.5 | 14.5 | 16.6 | 9.0 | 9.7 | – | – | – | – |
| **16** | – | – | – | – | 3.2 | 3.6 | 3.1 | 2.7 | – | – | – | – |
| **15** | 20.7 | 20.9 | 26.0 | 25.9 | 2.5 | 2.9 | 3.1 | 5.0 | 62.5 | 57.7 | 65.0 | 66.8 |
| **13** | 4.4 | 3.8 | 2.6 | 2.7 | 1.8 | 2.5 | 1.2 | 1.2 | 12.2 | 16.7 | 5.3 | 5.1 |
| **11** | – | – | – | – | 0.9 | 1.1 | – | – | – | – | – | – |
| **10** | 4.3 | 2.4 | 3.2 | 2.5 | 2.7 | 2.3 | 1.2 | 1.1 | 9.8 | 13.3 | 9.4 | 8.7 |
| **< 10** | – | – | – | – | – | – | – | – | 3.7 | 5.1 | – | – |
| **< < 10** | 1.0 | 0.3 | 1.6 | 1.3 | 4.1 | 3.6 | 2.7 | 2.9 | 2.1 | 0.9 | 0.9 | 0.7 |
| **SUM cru** | 69.7 | 72.5 | 66.5 | 67.6 | 84.8 | 84.0 | 88.8 | 87.0 | 9.7 | 6.3 | 19.5 | 18.8 |
| **SUM nap** | 30.3 | 27.5 | 33.5 | 32.4 | 15.2 | 16.0 | 11.2 | 13.0 | 90.3 | 93.7 | 80.5 | 81.2 |
| **cru:nap** | 2.3 | 2.6 | 2.0 | 2.1 | 5.6 | 5.3 | 7.9 | 6.7 | 0.1 | 0.1 | 0.2 | 0.2 |

For the AP/UF process, the two types of RPCs (APP and SNP) showed greatly differing solubility profiles. APPs showed a low solubility, which increased with increasing pH from < 23% (pH 3) to 31%–43% (pH 11). Low PS for rapeseed proteins obtained by acidic precipitation is caused by the presence of predominantly low-soluble cruciferin (Aider and Barbana, 2011). Additionally, denaturation effects caused by the pH-shift are reported for rapeseed proteins, leading to a decrease in solubility (Wanasundara, 2011). Interestingly, the solubility from precipitates at 10 °C in the present work was higher compared to values reported for APPs obtained under room temperature (Chabanon et al., 2007). Using lower precipitation temperatures presumably leads to a lower degree of protein denaturation, thus yielding RPCs with higher solubility. APP-Cs showed a higher solubility than APP-Ps, possibly due to a higher napin content (Table 4). A correlation of phytic acid contents (Table 3) with the PS could not be observed.

SNPs obtained from the AP/UF processes showed a PS of 80%–90% across the full range of pH 3–11. This is beneficial for a range of application fields, including the cleaning industry, where both acidic as well as alkaline cleaning agents are used. As low-soluble cruciferin fractions were precipitated in the previous step, no drop of the PS within the acidic pH range was observed. However, the PS at pH 7–11 was generally lower compared to values obtained for UFPs, indicating a




| RPC | *L** | *a** | *b** |
|---|---|---|---|
| **UFP-C-nat** | 51.6 ± 1.5[a] | 6.6 ± 0.0[a,f,g] | 22.3 ± 0.1[a,d] |
| **UFP-C-neut** | 57.2 ± 0.8[a] | 6.8 ± 0.5[a,f,g] | 23.4 ± 0.1[a,e] |
| **UFP-P-nat** | 56.4 ± 5.8[a] | 8.7 ± 0.9[a,b,g] | 25.1 ± 1.3[a,c] |
| **UFP-P-neut** | 49.7 ± 2.5[a] | 9.2 ± 0.0[a,b,d] | 22.3 ± 1.1[a,d] |
| **APP-C-nat** | 49.3 ± 1.9[a] | 11.5 ± 0.5[b,c,d] | 33.2 ± 1.8[b] |
| **APP-C-neut** | 53.3 ± 3.1[a] | 11.0 ±0.6[b,d] | 36.1 ± 2.1[b] |
| **APP-P-nat** | 55.0 ± 0.4[a] | 14.7 ±0.8[c,e] | 34.0 ± 1.2[b] |
| **APP-P-neut** | 51.4 ± 4.9[a] | 12.5 ±1.3[d,e] | 30.8 ± 1.9[b,c] |
| **SNP-C-nat** | 49.3 ± 0.0[a] | 4.5 ±0.5[f] | 16.8 ± 0.0[d] |
| **SNP-C-neut** | 51.9 ± 2.2[a] | 5.3 ±0.3[f,g] | 18.5 ± 0.8[d,e] |
| **SNP-P-nat** | 53.4 ± 0.9[a] | 8.9 ±0.3[a,b] | 22.6 ± 0.4[a,d] |
| **SNP-P-neut** | 51.3 ± 0.1[a] | 8.6 ± 0.1[a,b,g] | 22.5 ± 0.1[a,d] |

**Fig. 6.** Left: photographs of RPCs taken on a DigiEye system; right: color measurements of RPCs in the CIELAB color space; values within one row having the same superscript indicate no significant difference ($p > 0.05$).

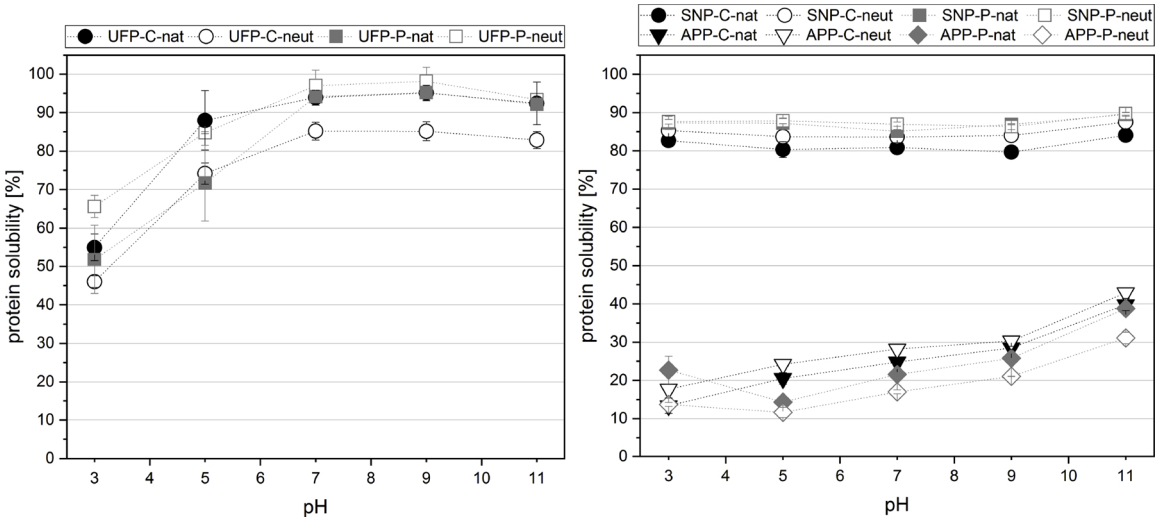



**Fig. 7.** PS of RPCs in 0.1 M NaCl solution; UFP: ultrafiltration protein; APP: acidic precipitation protein; SNP: supernatant protein; C: cold-pressed; P: pre-pressed; nat: native pH (5.7–5.8); neut: neutral pH (7).

**Table 5**
Foaming activity (FA), foam stability (FS) and foam density (FD) for RPCs; UFP: ultrafiltration protein; APP: acidic precipitation protein; SNP: supernatant protein; C: cold-pressed; P: pre-pressed; nat: native pH (5.7–5.8); neut: neutral pH (7); values within one row having the same superscript indicate no significant difference ($p > 0.05$).

| process | preparation | foaming activity (FA) [%] | foam stability (FS) [%] | foam density (FD) [g/L] |
|---|---|---|---|---|
| UF | UFP-C-nat | 2834 ± 159$^{a,b}$ | 97 ± 1$^{a,b}$ | 33 ± 1$^{a}$ |
| | UFP-C-neut | 2193 ± 344$^{a,b}$ | 95 ± 3$^{a,b}$ | 43 ± 7$^{a}$ |
| | UFP-P-nat | 1834 ± 45$^{b}$ | 97 ± 0$^{a,b}$ | 52 ± 1$^{a}$ |
| | UFP-P-neut | 1842 ± 67$^{b}$ | 92 ± 1$^{a,b}$ | 49 ± 1$^{a}$ |
| AP/UF | APP-C-nat | 938 ± 81$^{d}$ | 93 ± 2$^{a,b}$ | 152 ± 17$^{b}$ |
| | APP-C-neut | 905 ± 17$^{d}$ | 86 ± 4$^{a}$ | 175 ± 8$^{b}$ |
| | APP-P-nat | 1034 ± 24$^{d}$ | 93 ± 1$^{a,b}$ | 94 ± 1$^{c}$ |
| | APP-P-neut | 842 ± 0$^{d}$ | 92 ± 0$^{a,b}$ | 158 ± 7$^{b}$ |
| | SNP-C-nat | 2713 ± 53$^{a,c}$ | 94 ± 5$^{a,b}$ | 33 ± 4$^{a}$ |
| | SNP-C-neut | 2876 ± 36$^{c}$ | 99 ± 1$^{b}$ | 34 ± 2$^{a}$ |
| | SNP-P-nat | 2040 ± 23$^{b}$ | 95 ± 1$^{a,b}$ | 47 ± 1$^{a}$ |
| | SNP-P-neut | 2387 ± 24$^{a,b,c}$ | 92 ± 2$^{a,b}$ | 34 ± 1$^{a}$ |

small effect of protein denaturation by the precipitation step also for SNPs. The PS for SNP-Cs was lower than for SNP-Ps, despite a higher napin content by approx. 10 percentage points (Table 4). Hence, globulin fractions remaining in the supernatant after the precipitation step seem to have good solubility properties.

### 3.4.2. Foaming properties

All RPCs obtained through ultrafiltration showed a high foaming activity (FA) of > 1800% (Table 5). The FA of UFP-Cs (2193%–2834%) was higher than for UFP-Ps (1834%–1842%). Extraction pH seemed to impact the FA only for UFP-Cs, with values of 2193% for an extraction at pH 7 compared to 2834% at native conditions (pH 5.7–5.8). The FA for SNPs was on the same level (CPM: 2713%–2876%) or slightly higher (PPM: 2040%–2387%) as compared to UFPs. The foam stability (FS) was ≥ 92% for all ultrafiltrated proteins. The foam density (FD) correlated with the FA and was in the range of 33–52 g/L.

The FA for APPs was 900%–981% and did not show great differences depending on raw material or extraction pH. The FD (152–175 g/L) was significantly higher as compared to UFPs and SNPs as a consequence of a lower FA. The FS (86%–93%) was slightly lower as

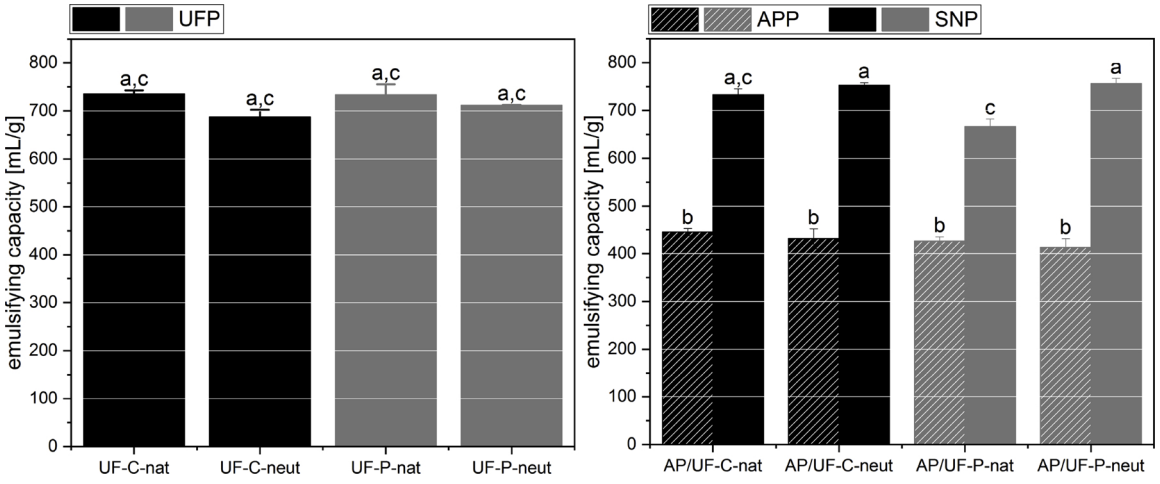



**Fig. 8.** Emulsifying capacity of RPCs; UFP: ultrafiltration protein; APP: acidic precipitation protein; SNP: supernatant protein; C: cold-pressed; P: pre-pressed; nat: native pH (5.7–5.8); neut: neutral pH (7); same superscripts of columns indicate no significant differences ($p > 0.05$).

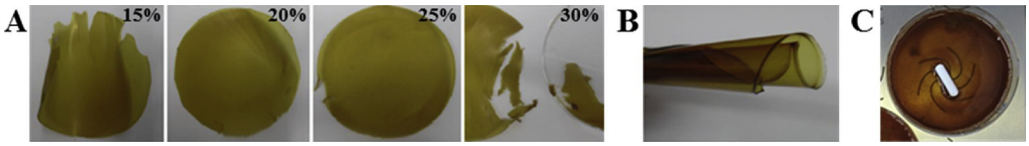



**Fig. 9.** A: Cast films obtained using various amounts of glycerol as plasticizer (% of RPC); B: rolled cast film made with 20% glycerol based on the RPC content; C: dissolved RPC cast film in water (after 5 min).

compared to ultrafiltrated preparations.

Differences in analytical methods to determine foaming properties make it difficult to compare the results between the studies. In general, the FA increased with napin content of the RPC. This observation is in agreement with studies reporting better foaming properties for napin than for cruciferin (Akbari and Wu, 2015; Chabanon et al., 2007). This might be caused by a high degree of a-helical structures in napin (Perera et al., 2016), which possibly accumulate at the interfacial surface and assist the formation of stable foams. In addition, the PS is an essential parameter contributing to foaming properties (Aluko and McIntosh, 2001). Thus, good FA can be observed for all napin-rich RPCs, which showed good solubility at pH 7. In contrast, low-soluble APPs showed comparably low FA.

### 3.4.3. Emulsifying capacity (EC)

UFPs showed a high EC of 688–735 mL/g with no significant differences depending on raw material or extraction pH (Fig. 8). Similar values of 666–756 mL/g were obtained for SNPs. In contrast, the EC for APPs was much lower (413–445 mL/g).

Comparison of emulsifying properties reported in the literature must be performed with care, as the applied methods and environments differ greatly. Cheung et al. (2014; 2015) reported a higher emulsion activity index for a cruciferin isolate compared to a napin isolate, however, the opposite trend was observed at 100 mM NaCl content. Low emulsion stabilities have been reported for napin due to the formation of emulsions with higher particle sizes compared to cruciferin (Akbari and Wu, 2015; Wu and Muir, 2008). In contrast, Krause and Schwenke (2001) reported higher emulsion activity index for napin and mixtures of cruciferin and napin as compared to cruciferin alone. This is in agreement with the present study, where EC was high for all high-soluble RPCs with high napin contents. In contrast, low-soluble APPs showed a much lower EC. However, values were still comparable or even higher compared to traditional animal-based protein-emulsifiers, such as whey protein (˜300 mL/g) and egg yolk protein (˜500–600 mL/g) (Zayas, 1997). In summary, all RPCs can be considered as potential emulsifiers for technical products such as emulsion-based lubricants, coatings or paints.

### 3.4.4. Film-forming properties (cast films)

All RPCs showed good film-forming properties in cast-film experiments using glycerol as plasticizer. Optimal glycerol concentrations to obtain films with even structure and good flexibility were 20%–25% glycerol based on the RPC content. Films with lower or higher glycerol concentrations were either brittle and broke upon removal from the petri dishes or showed pasty texture, respectively (Fig. 9).

The films formed with 20% and 25% (based on the RPC content) were flexible and stretchable, and all films were of brown color, originating from polyphenols and their oxidation products. These properties suggest applications in the packaging sector, possibly as packaging foils with a light or UV-barrier. However, all films were highly water-soluble, hindering potential applications as water-resistant coatings. As a consequence, improvement of water stability is essential for the application of rapeseed proteins in packaging, coatings, and polymers. Methods of protein modification to increase hydrophobicity and thereby water stability of rapeseed proteins are currently being investigated in our laboratories.

## 4. Conclusion

Protein isolation yields, protein contents, and techno-functional properties were similarly high under native and neutral extraction conditions. Thus, no need for pH adaption during rapeseed protein extraction is recommended. The solubility properties of rapeseed protein concentrates depended on the isolation process (ultrafiltration or combined acidic precipitation and ultrafiltration). Therefore, the protein isolation strategy has to be chosen in accordance to solubility demands of the application. All rapeseed protein concentrates showed good emulsifying and foaming properties. Emulsifying capacity and foaming activity were very high for all ultrafiltrated samples and much lower for acid precipitated proteins. All concentrates showed good film-forming properties in cast-film experiments, demonstrating potential applications for coatings or the packaging sector. However, low water-stability of films posed a problem, which is the subject of current research in our laboratories. The protein isolation yields were 60%–90% higher fold for cold-pressed meal as compared to pre-pressed meal, possibly due to increased thermal protein damage during conventional screw-pressing. This highlights the demand for a paradigm-shift in the oilseed industry in favor of milder processing during rapeseed defatting. The latter is mandatory to lever an added value to the industry through the utilization of side-stream rapeseed proteins as renewable ingredients in the chemical industry.

## Conflicts of interest

None.

## Acknowledgements

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# CHAPTER IV – Effect of Acylation of Rapeseed Proteins with Lauroyl and Oleoyl Chloride on Solubility and Film-Forming Properties

Rapeseed protein preparations obtained from residual materials were modified by chemical acylation in order to increase the water-stability of protein-based films.

The rapeseed protein concentrate (RPC) was chemically acylated at low and high modification degree using lauroly chloride and oleoyl chloride. The effect of acylation was investigated in regard to protein solubility and film properties of protein-based cast-films. The protein solubility in water (pH 7) was reduced from 100% for the non-modified RPC to < 15% for RPCs at high modification degree. Films obtained from modified rapeseed proteins showed an increase in tensile strength by factors of 3.5–4 for highly modified samples. The increased hydrophobicity of films obtained from modified rapeseed proteins was revealed by reduced surface energy and increased oxygen permeability, both effects correlating positively with the modification degree. In addition, the light transmission of films was reduced by modification. In conclusion, the results confirm the possibility to increase hydrophobicity of RPCs by means of chemical acylation. The modified protein samples demonstrate an increased potential for the utilization as an ingredient for technical products, such as packaging layers, coatings and adhesives.

A. Fetzer designed the study, interpreted the results and wrote the manuscript. C. Hintermayr performed the experiments, contributed to the contents of the manuscript and the interpretation of the results. T. Herfellner managed the affiliated research project, contributed to the contents of the manuscript and the interpretation of the results. M. Schmid, A. Stäbler and P. Eisner contributed to the contents of the manuscript and the interpretation of the results.

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ORIGINAL PAPER

# Effect of Acylation of Rapeseed Proteins with Lauroyl and Oleoyl Chloride on Solubility and Film-Forming Properties

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## Abstract

Rapeseed proteins show good film-forming properties, giving them a promising potential as bio-based ingredients for the technical industry, e. g. for films and coatings. However, their hydrophilicity often poses problems in regard to water-stability of coatings. Protein modification using fatty acids is known to reduce hydrophilicity, however, it has not been tested to improve film-forming properties of rapeseed proteins before. In the present study, a rapeseed protein concentrate (RPC) was acylated at low and high modification degree using lauroyl chloride and oleyol chloride. The protein solubility was determined and the modified RPCs were used for the preparation of cast-films to measure the changes of mechanical properties (tensile strength, elongation at break), surface energy, oxygen permeability and light transmission. The protein solubility in water was lowered from 100% for the non-modified RPC to < 15% for highly modified RPCs at pH 7. The tensile strength of films increased by factors of 3.5 and 4 for highly modified samples, respectively. Surface energy and oxygen permeability revealed an increase of hydrophobicity that correlated with the modification degree. The light transmission was reduced by modification. The results confirm the increased hydrophobicity of acylated RPCs and demonstrate the potential of modified rapeseed proteins as an ingredient for technical products, such as packaging layers, coatings and adhesives.

### Graphic Abstract


**Keywords** Rapeseed · Canola · Protein modification · Acylation · Film-forming properties · Coating

## Statement of Novelty

Rapeseed proteins can be isolated from side-stream products of the rapeseed oil industry and could potentially be valorized as bio-based additives in the technical industry. Despite their great functional properties, rapeseed proteins are not well studied for utilization in the non-food industry. In the present study, acylation of rapeseed proteins was carried out in order to increase their hydrophobicity. This is the first time acylation of rapeseed proteins with long hydrocarbon chains was carried out with the aim to enhance the film-forming properties, which are of high relevance to a

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range of technical applications, such as adhesives, coatings or polymers. The results clearly demonstrate the increase of hydrophobicity, thereby improving the application potential of rapeseed proteins as a renewable alternative to conventional additives.

## Introduction

Plant proteins from oilseed by-products are a promising alternative for petrol-based ingredients in technical applications. In particular, rapeseed proteins with their great functionalities such as foaming, emulsification and film-forming properties are an interesting functional ingredient, e.g. for paints, varnishes, glues and lubricants. Specifically, film-forming properties are of high relevance for such applications. However, as these products are often based on hydrophobic media, the polar nature of proteins can be problematic in regard to product formulation or water-stability [1]. Hence, methods to render rapeseed proteins more hydrophobic and thus to stabilize these formulations are needed.

Application of proteins in detergents, paints, glues and polymers is gaining increasing interest in the proclaimed bio-economy of the twenty-first century. Historically, proteins—predominantly from animal sources—have been an important raw material for adhesives, fire extinguishing foams or plastics [2, 3]. However, with the advent of the petroleum era, most of these products have been replaced by easier and cheaper methods to produce synthetic materials.

The main storage proteins in rapeseed are the 12S globulin cruciferin and the 1.7–2S albumin napin [4]. Due to their flexible structure comprising polar and non-polar residues, they are able to attach on interfaces, such as air/water or oil/water interfaces, to form and stabilize foams and emulsions [5]. Additionally, great film-forming properties of rapeseed proteins were demonstrated [6–8]. However, methods to increase the hydrophobicity of rapeseed proteins while preserving their functional properties are demanded to enhance the applicability in the non-food sector. A promising technique to achieve this is chemical derivatization of free functional groups in the protein with reagents bearing long hydrocarbon chains [9]. Thereby, polar residues, such as hydroxyl, sulfhydryl or amino groups can be converted to non-polar residues to increase the overall hydrophobicity.

Film-forming properties of rapeseed proteins were described in a number of studies. Several studies describe rapeseed protein-based film-formation by the solvent-cast method, using different protein concentrations, plasticizer types and concentrations [6, 8, 10, 11]. Mechanical and barrier properties of these films were reported to be low and comparable to other plant proteins [12]. Some improvements of physical properties were achieved using blends of rapeseed protein and *gelidium corneum* (a red algae with high agar content) or gelatin [11]. In another study, denaturation by sodium dodecyl sulfate (SDS) prior to film formation also showed some improvement in tensile strength of films [8]. Moreover, the addition of genipin as a natural cross-linker increased tensile strength of sorbitol-plasticized films [10].

Chemical acylation of rapeseed proteins has been reported in a number of previous studies, predominantly in the form of acetylation and succinylation [13–18]. Film properties were investigated only in one study using succinylated rapeseed proteins [13]. The authors reported enhanced barrier and mechanical properties as well as increased hydrophobicity at the lowest modification degree. To the best of the author's knowledge, no study was carried out using rapeseed proteins modified with long hydrocarbon chains for film-preparation.

In this work, we applied the acylation of rapeseed protein preparations by Schotten-Baumann-reaction with fatty acid chlorides (FACs) [19, 20]. Type and degree of modification were investigated and resulting functional properties were compared with the non-modified standard. In particular, the influence on water solubility was studied. Additionally, cast films of rapeseed protein preparations were characterized regarding their mechanical and barrier properties. The overall aim was to show the application potential of rapeseed proteins for technical products requiring film-forming properties such as glues, lubricants, paints and varnishes.

## Materials and Methods

### Raw Materials and Chemicals

Prepressed rapeseed press cake was provided by Bunge Deutschland GmbH, Mannheim, Germany. Rapeseed press cake was defatted with isohexane in a percolator (volume 1.5 $m^3$, e&e Verfahrenstechnik GmbH, Warendorf, Germany) and flash desolventized with isohexane (400–500 mbar) prior to steam desolventation to obtain defatted rapeseed meal. Isohexane was obtained from Biesterfeld AG (Hamburg, Germany). Lauroyl chloride and olyeol chloride were obtained from Sigma-Aldrich Chemie GmbH (Munich, Germany). Phytase Quantum Blue 5G was obtained from AB Enzymes GmbH (Darmstadt, Germany). All other chemicals were of analytical grade and obtained from Th. Geyer GmbH & Co. KG (Renningen, Germany), if not stated otherwise.

### Preparation of Rapeseed Protein Concentrate

The RPC was prepared on a pilot scale as follows: Rapeseed meal was added to a 0.25 M aqueous NaCl-solution at 30 °C with an s:l-ratio of 1:13. The mixture was adjusted to pH 9 using 3 M NaOH and stirred for 60 min, before separation

was carried out using a decanter (GEA Westfalia Separator Group GmbH, Oelde, Germany). The supernatant was adjusted to pH 4 with 3 M HCl at 30 °C and stirred for 30 min. Separation of the mixture was carried out using a disc separator (GEA Westfalia Separator Group GmbH). The sediment was not of further use in this study. The supernatant was adjusted to pH 5.5 with 3 M NaOH and 1.1 g of phytase (Quantum Blue 5G) were added. Ultrafiltration was carried out at 50 °C on a Microza polysulfone ultrafiltration module SLP-3053 (Pall Corporation, Port Washington, NY, USA) with a molecular weight cutoff of 10 kDa and a membrane area of 4.5 $m^2$. The neutralized retentate was spray-dried at a product inlet temperature of 180 °C and a product outlet temperature of 75–80 °C to obtain an RPC with high protein solubility. This was mandatory to ensure proper reactivity during further protein modification.

## Modification of RPC

100 g of RPC were dispersed in 900 g of demineralized water and stirred at 40 °C. The pH was adjusted to pH 9.5 using 1 M NaOH and lauroyl chloride or oleoyl chloride (15 or 120 mmol) were added dropwise. The pH was controlled during addition of fatty acid chloride (FAC) and maintained at pH 9.5. After 3 h, a stable pH indicated the end-point of the reaction (Fig. 1). The dispersion was cooled to 30 °C, neutralized using 1 M HCl and dialyzed against demineralized water at 1 °C for 48 h with a molecular weight cutoff of 3.5 kDa (Spectra/Por® 3, Spectrum LifeSciences, LLC, Rancho Dominguez, CA, USA). The demineralized water was changed twice a day. The dialyzed samples were lyophilized, homogenized by mortar and pestle and washed six times with ethanol (3 mL/g) for removal of non-reacted free fatty acids. After air-drying under a fume hood, the modified RPCs were used for further analysis. A control sample was prepared and treated the same way without the addition of FAC to the protein dispersion. All samples were prepared in duplicate.

## Chemical Composition of Rapeseed Samples

Chemical composition (dry matter, protein) of rapeseed protein samples was analyzed in duplicate. Dry matter was analyzed using a thermo-gravimetrical system (TGA 601, Leco Corporation, St. Joseph, MI, USA) at 105 °C and 950 °C, respectively. Protein content was measured by Dumas combustion method on a TruMac N system (Leco Corporation), using 5.7 as the conversion factor [21]. Free fatty acids in the ethanol washing fractions of modified samples were determined by gas chromatography with flame ionization detector (GC-FID) as described by Mahmoud et al. [22].

## Properties of Rapeseed Protein Samples

### Protein Solubility

The protein solubility (PS) was determined in duplicate following the method of Morr et al. [23], as previously described [24]. For each measurement 1.5 g of rapeseed protein concentrate were suspended in a total volume of 50 mL of a 0.1 M NaCl solution. The pH was adjusted using 0.1 M NaOH or 0.1 M HCl, respectively. After stirring for 1 h at room temperature, the samples were centrifuged (20,000×*g*, 15 min, 15 °C) and the supernatant was filtrated (Whatman No. 1 filter paper). Protein content of the supernatant was determined according to Dumas following the method given in section "Analysis of Chemical Composition of Rapeseed Preparations". Protein solubility was calculated using Eq. 1 and reported giving the mean value ± absolute deviation.

$$\text{Protein solubility } [\%] = \frac{\text{initial volume [mL]} \times \text{ protein content in supernatant } \left[\text{mg/mL}\right]}{\text{sample mass } \left[\text{mg}\right] \times \text{ protein content [\%dm]} \times \text{dry matter[\%]}} \times 100 \quad (1)$$

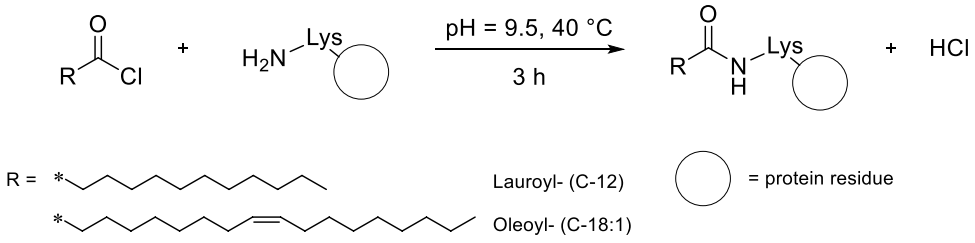


**Fig. 1** Reaction scheme for the modification of rapeseed proteins via Schotten-Baumann-reaction

## Film-Forming Properties

### Preparation of Cast Films

Cast films of RPCs were prepared as described by Chang and Nickerson [6] with minor modifications. 11.25 g of modified or non-modified RPC were mixed with 120 mL of demineralized water in a glass bottle and the pH was adjusted to pH 3 using 1 M HCl. The mixture was stirred at room temperature for 60 min at 300 rpm on a MIX 15 magnetic stirrer (2mag AG, Munich, Germany), before 2.81 g of glycerol were added. The total weight was adjusted to 150 g at pH 3 using demineralized water and 1 M HCl and stirring was continued for 10 min. The mixture was degassed in an ultrasound bath at 35 kHz and 50 °C for 15 min, stirred at 50 °C and 300 rpm and 33 g were cast in square petri dishes (120 mm × 120 mm). The cast film was dried at 23 °C and 50% RH for 14 days to equilibrium moisture content.

### Film Thickness

The rapeseed protein films were peeled off the petri dishes and the film thickness was determined using a Precision Thickness Gauge (Hanatek Instruments, St. Leonards-on-Sea, UK) as previously described by Schmid [25]. The measurements were performed at 23 °C and 50% RH with a five-fold determination at five different positions. The arithmetic average of the film thickness was used to calculate standardized oxygen permeability (OP) and mechanical film properties.

### Surface Energy

The surface energy of RPC-based films was measured by the sessile drop method as previously described [25], using the contact angle measuring system G2 (Krüss GmbH, Stephanskirchen/Rosenheim, Germany). The contact angle of three testing liquids (water, diiodomethane, ethylene glycol) was measured at 22.4 °C and 42% RH at five different positions on the film surface of five replicates. The results are given as the arithmetic average ± standard deviation. According to Young's equation, the surface energy was calculated as follows:

$$\sigma_s = \gamma_{sl} + \sigma_l \times \cos\theta \tag{2}$$

where $\sigma_s$ is the surface tension of the solid (film) in mN/m, $\sigma_l$ is the surface tension of the liquid in mN/m, $\gamma_{sl}$ is the interfacial tension between the solid and the liquid in mN/m and cos θ is the contact angle between the surface tension of the liquid and the interfacial tension between the liquid and solid in angular degree. The disperse and polar fractions of the surface energy were calculated according to the method by Owens et al. [26–28]. As the rapeseed films were water-soluble, the measurement had to be performed directly after drop application rather than at equilibrium. The calculated values can thus not be classified as absolute surface energy values and are only used for comparison of the samples in this study.

### Tensile Testing

Tensile properties of RPC films were measured on a universal compression-tension testing machine Zwick 1445 (ZwickRoell GmbH & Co. KG, Ulm, Germany) at 23 °C and 50% RH in accordance to the standard DIN 527-1. The cast films were cut into strips (15 mm × 70 mm) and the thicknesses of the film strips were measured. The film strip was clamped in the loading frame using pneumatic grips and an initial gauge length of 50 mm. The sample was subjected to an applied force using a load cell of 50 N and was stretched with a testing speed of 100 mm/min. For each sample, a five-fold determination was performed and the tensile strength (TS; MPa) and elongation at break (%) were reported as the arithmetic average ± standard deviation.

### Oxygen Permeability

The oxygen permeability (OP) was measured according to the standard DIN 53380-3 at 23 °C and 50% RH on a Mocon Twin instrument (Mocon Inc., Minneapolis, USA) as previously described [25, 29]. The OP values ($Q$) were converted to a thickness ($d$) of 100 µm ($Q_{100}$), according to Eq. 3, and are given in the unit [$cm^3\ m^{-2}\ d^{-1}\ bar^{-1}$].

$$Q_{100} = Q \times \frac{d}{100} \tag{3}$$

### Light Transmission

Light transmission of rapeseed protein films was measured with a spectrophotometer TMQ (Carl Zeiss GmbH, Oberkochen, Germany) in duplicate at wavelengths of 350 nm, 430 nm, 450–750 nm (intervals of 25 nm), and 800–1000 nm (intervals of 50 nm).

## Degree of Modification Using the *o*-Phtaldialdehyde Method

Calculation of the degree of modification (DM) was performed via determination of free α-amino groups with *o*-phtaldialdehyde (OPA) according to Nielsen et al. using *N*,*N*-dimethyl-2-mercaptoethylammonium chloride as the thiol component [30, 31]. Protein samples were analyzed in

triplicate using serine as a standard. Initially, the degree of hydrolysis (DH) was calculated using the Eqs. 4–6.

$$DH = \frac{h}{h_{tot}} \times 100\% \tag{4}$$

where $h_{tot}$ is the total number of peptide bonds per protein equivalent (adopted from soy protein with $h_{tot} = 7.8$ according to Adler-Nissen [32]) and h is the number of hydrolyzed bonds.

$$h = \frac{Serine - NH_2 - \beta}{\alpha} \frac{meqv}{g_{Protein}} \tag{5}$$

where Serine-$NH_2$ = meqv serine-$NH_2/g_{protein}$, $\alpha = 0.970$ and $\beta = 0.342$ (constants adopted from soy protein according to Adler-Nissen [32]).

$$Serine - NH_2 = \frac{OD_{sample} - OD_{blank}}{OD_{standard} - OD_{blank}} \times 0.9516 \frac{meqv}{L} \times \frac{0.1L \times 100}{Xg \times P} \tag{6}$$

where 0.1 L is the sample volume, X is the sample mass (in grams) and P is the protein content of the sample (in percent).

The DM was calculated from the DH using Eqs. 7 and 8.

$$Percentage\ of\ free\ amino\ groups = \frac{DH_{modified\ RPC}}{DH_{RPC}} \times 100\% \tag{7}$$

where $DH_{modified\ RPC}$ is the DH of the RPC after modification and $DH_{RPC}$ is the DH of the RPC.

$$DM = 100\% - percentage\ \ of\ free\ amino\ groups \tag{8}$$

Due to the inaccuracy of the method at low DH [30], no absolute values were used for the comparison of modified RPCs. Instead, the relative degree of modification (RDM) was used, which was calculated in relation to the sample with the highest DM (RPC-LH) according to Eq. 9:

$$RDM = \frac{DM_{modified\ RPC}}{DM_{RPC-LH}} \times 100\% \tag{9}$$

### Statistical Analysis

All data are given as mean values ± standard deviation of at least two measurements (n = 2). Outliers were eliminated after applying Grubb's test and the data was tested for normal distribution according to Shapiro–Wilk. Significant differences were statistically analyzed by one-way analysis of variance using Tukey's test ($p < 0.05$). Statistical analysis was performed using the software OriginPro (OriginLab Corporation, Northampton, USA).

## Results and Discussion

### Modification of Rapeseed Protein Concentrate

The RPC was chemically modified by the Schotten-Baumann-reaction using lauroyl chloride (LC) and oleoyl chloride (OC). A screening showed the relative degree of modification (RDM) in dependence of the amount of fatty acid chloride (FAC) used (Fig. 2). As expected, the RDM increased with increasing amounts of FAC. The RDM was in the range of 41.0–86.6% and 39.5–70.4% for LC and OC, respectively. Higher RDMs for lauroyl-modified samples were possibly due to lower sterical hindrance for the reaction with the protein residues, due to the smaller and less bulky chemical structure of LC in comparison to OC.

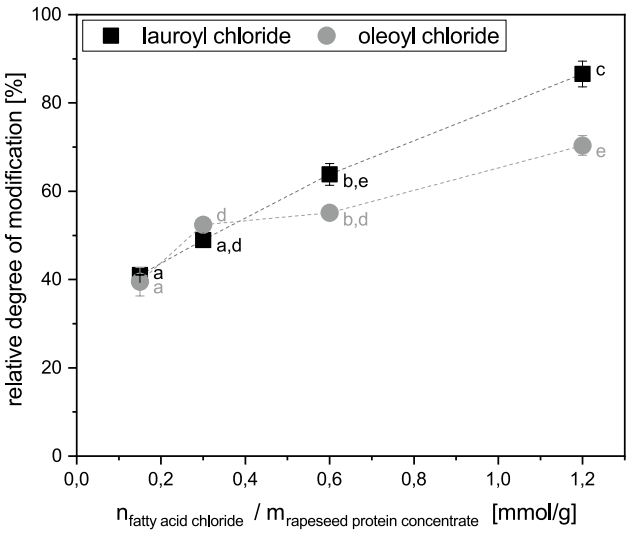


**Fig. 2** Screening for the degree of modification in dependence of the amount of fatty acid chloride (FAC) used in the reaction (mean values ± absolute deviation); data points having the same superscript indicate no significant difference ($p > 0.05$)

**Table 1** Relative degree of modification and composition of rapeseed protein concentrates (mean values ± absolute deviation): *LL* Lauric-Low, *LH* Lauric-High, *OL* Oleic-Low, *OH* Oleic-High; protein contents were calculated with a nitrogen-to-protein conversion factor of 5.7; values within one row having the same superscript indicate no significant difference ($p > 0.05$)

| | Relative degree of modification (RDM) (%) | Dry matter (dm) (%) | Protein content (% dm) |
|---|---|---|---|
| RPC | – | $94.9 \pm 0.0^{a}$ | $83.7 \pm 0.1^{a}$ |
| RPC-LL | $32.6 \pm 0.2^{a}$ | $94.4 \pm 0.1^{a}$ | $83.5 \pm 0.1^{a}$ |
| RPC-LH | $100.0 \pm 3.2^{b}$ | $95.3 \pm 0.0^{a}$ | $76.7 \pm 0.5^{b}$ |
| RPC-OL | $27.8 \pm 2.6^{a}$ | $94.4 \pm 0.0^{a}$ | $82.7 \pm 0.4^{a}$ |
| RPC-OH | $72.3 \pm 1.3^{c}$ | $94.6 \pm 0.4^{a}$ | $77.1 \pm 0.0^{b}$ |

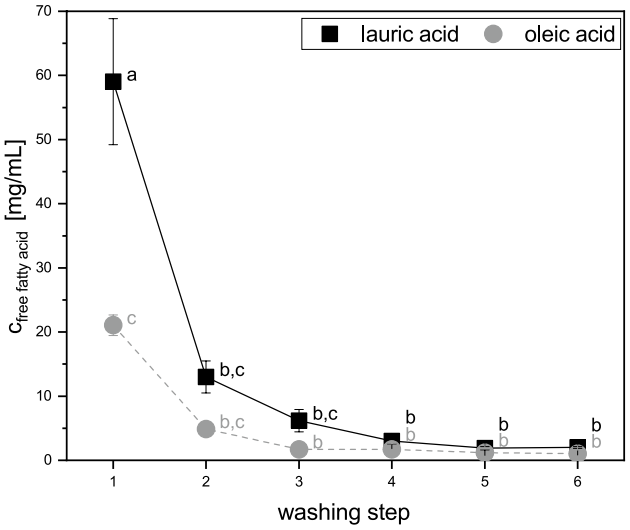


**Fig. 3** Concentration of free fatty acids in the ethanol washing fractions (mean values ± absolute deviation); data points having the same superscript indicate no significant difference ($p > 0.05$)

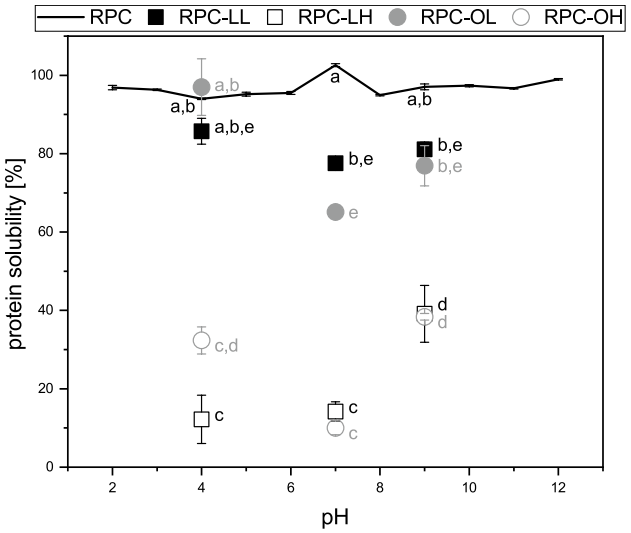


**Fig. 4** Protein solubility in 0.1 M NaCl of rapeseed protein concentrates (mean values ± absolute deviation); data points having the same superscript indicate no significant difference ($p > 0.05$)

Based upon the screening, the amount of FAC added to the reaction was chosen to yield one low-modified and one high-modified RPC for lauroyl and oleoyl chloride. Accordingly, an up-scale of the reaction was carried out using 0.15 and 1.2 $mmol_{FAC}/g_{RPC}$. The RDM for the low-modified species was determined to be 32.6% and 27.8% for LC and OC, respectively (Table 1). Lower RDMs compared to the screening could be explained by a higher degree of FAC that was hydrolyzed in the aqueous mixture due to a lower initial concentration upon dropwise addition of the reagent. In case of the high-modified species, the RDM of 72.3% was comparable to the screening for OC. However, for LC the RDM (100.0%) was higher compared to the screening.

To remove free fatty acids from the reaction product, dialysis of the reaction mixture was followed by ethanol washing of the lyophilized samples. The content of fatty acids in the washing fractions was analyzed for the high-modified samples RPC-LH and RPC-OH (Fig. 3). The concentration of fatty acids in the first fraction was 59.0 mg/mL and 21.1 mg/mL and dropped to 2.0 mg/mL and 1.0 mg/mL in the final fraction for RPC-LH and RPC-OH, respectively. In total, six washing steps were carried out for the purification of the modified RPCs. Statistical analysis revealed no significant differences in the concentration of free fatty acids after the third washing step ($p > 0.05$).

## Characterization of Rapeseed Protein Concentrates

### Chemical Composition

The chemical composition was determined for the non-modified and all modified RPCs (Table 1). The protein content of the non-modified RPC was 83.7% dm (N × 5.7). As expected, the protein content determined using the same conversion factor was reduced with increasing modification degree. The protein content was reduced marginally (0.2–1.0 percentage points) for the low-modified species RPC-Lauric-Low (-LL) and RPC-Oleic-Low (-OL). For the high-modified species, the protein content dropped to 76.7% and 77.1% for RPC-Lauric-High (-LH) and RPC-Oleic-High (-OH), respectively.

### Properties of Rapeseed Protein Concentrates

**Protein Solubility** To obtain good reactivity during the reaction, an RPC with good water solubility was chosen. The protein solubility of the RPC was > 90% in the range of pH 2–12 (Fig. 4), which was achieved by removal of the low-soluble protein fraction by a preceding precipitation step. For the modified RPCs the solubility was determined at pH 4, 7 and 9 to analyze the effects of acylation on water solubility in acidic, neutral and alkaline environment. The solubility was reduced for all modified RPCs with one exception. The effect was less pronounced for the low-modified species RPC-LL and RPC-OL, with reductions of 8–25% points and 0–37% points, respectively. For RPC-LH and RPC-OH, the protein solubility dropped to 12.2–39.1% and 10.0–38.4%, respectively. Reduction of solubility can be explained by fatty acid amidation of free amino groups (primarily the $\varepsilon$-amino group of exposed lysyl residues), and to some degree of free hydroxyl and sulfhydryl groups. Water solubility is reduced, as these groups are limited in the ability to form hydrogen bonds in aqueous environment after acylation. Additionally, refolding of the protein structure has to be considered, which can lead to the liberation

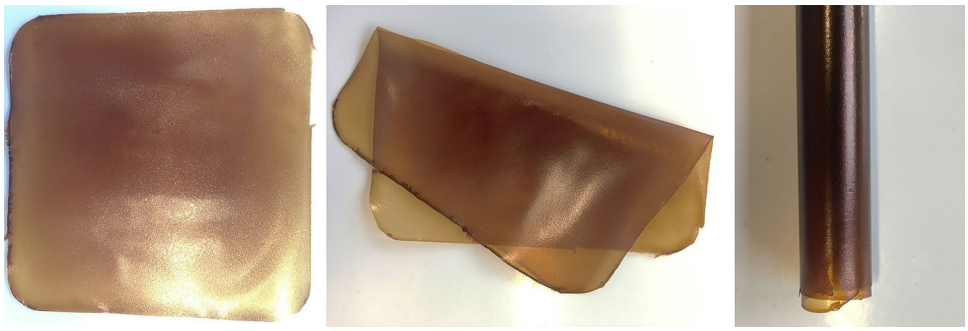

**Fig. 5** Photographs of rapeseed protein films after drying and removal of the petri dish

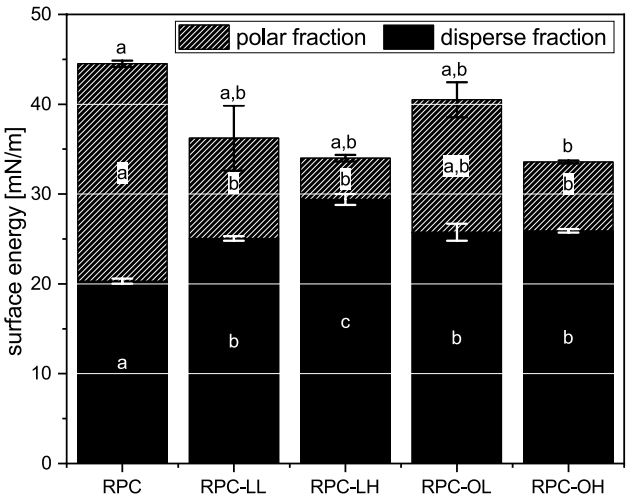


**Fig. 6** Surface energy of cast-films made from rapeseed protein concentrates (mean values ± absolute deviation); columns having the same superscript indicate no significant difference ($p > 0.05$); significance indexes are given for disperse fraction, polar fraction and surface energy separately

of buried hydrophobic amino acid residues. For the low-modified samples, the protein solubility was reduced only to a limited extent, with significant differences only at pH 7 ($p < 0.05$). However, the solubility was greatly reduced for high-modified samples. The solubility reduction was most pronounced at pH 7. This might be explained by a shift of the isoelectric point from the alkaline into the neutral region upon amidation of basic lysyl groups.

### Properties of Rapeseed Protein Concentrate Films

All RPCs showed good film-forming properties in cast-film experiments using glycerol as a plasticizer (25% on the RPC sample basis). After removal of the dried film from the petri-dish, all films appeared homogenous, without defects, flexible, and were of brown color. The appearance as well as foldability and rollability of films is displayed in Fig. 5.

**Surface Properties** Measurement of the surface energy showed the increased hydrophobicity of films prepared from acylated RPCs (Fig. 6). Films from the unmodified RPC showed a surface energy of 44.5 mN/m, with a polar fraction of 24.2 mN/m. For the modified RPCs, the surface energy decreased with increasing modification degree. This was caused by a decrease of the polar fraction, which was higher than the increase of the disperse fraction. The surface energy decreased to 36.2 mN/m (LL) and 34.0 mN/m (LH) for lauroyl-modifications and to 40.5 mN/m (OL) and 33.6 mN/m (OH) for oleoyl-modifications. The findings and the increase of disperse interactions caused by the introduction of hydrophobic side chains into the protein.

**Barrier Properties** All films (non-modified and modified) showed a low water-stability and thus water vapor permeability (WVP) could not be measured. WVP values were reported previously for cast films of rapeseed proteins prepared by a similar method as the one used in this study [6, 10]. This indicates that the nature of the rapeseed protein sample may be crucial for the resulting water stability of the obtained film. High water solubility of rapeseed protein films suggests possible applications such as a water-soluble packaging for dish-washer tabs. For the application as a packaging material or coating, improvement of water resistance must be achieved, e. g. by means of protein denaturation, such as through temperature treatment, or the inclusion of hydrophobic materials in the film. However, the introduction of lauroyl or oleoyl side chains in this study did not sufficiently improve the water stability of the films.

The oxygen permeability (OP) increased with increasing modification degree of the samples (Fig. 7), demonstrating the increase in hydrophobicity within the protein film layer, as oxygen is a nonpolar molecule. In addition, the covalently bound acyl residues can act as an internal plasticizer [33], thereby increasing gas diffusivity. For low-modified RPCs, the OP increased only marginally by 9–26% without significance ($p > 0.05$). The effect was greatly higher with significance ($p < 0.05$) for high-modified samples, with an increase of the OP by 143–159%. No studies were found reporting OP values for films made from rapeseed proteins such that

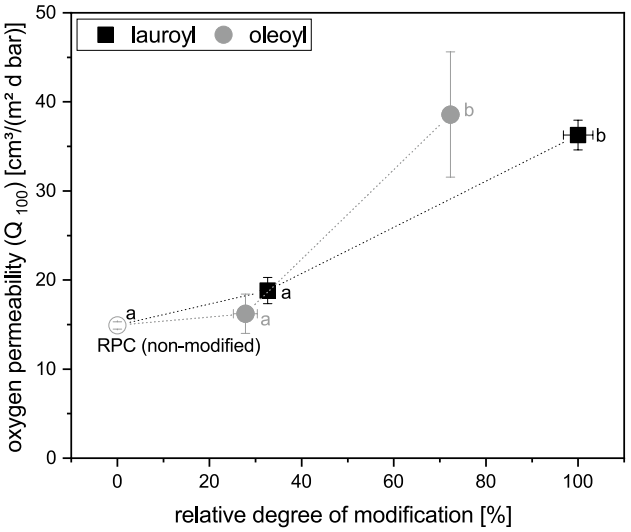


**Fig. 7** Oxygen permeability ($Q_{100}$) at 23 °C and 50% RH of films prepared from modified and non-modified rapeseed protein concentrates (mean values ± absolute deviation); data points having the same superscript indicate no significant difference ($p > 0.05$)

a comparison was not possible. However, the OP obtained for the non-modified RPC and low-modified RPCs was comparable for the value of 18.4 $cm^3\ m^{-2}\ d^{-1}\ bar^{-1}$ reported by Schmid et al. [34] for a whey protein based cast film.

**Mechanical Properties** Compared to other studies using rapeseed proteins, the tensile strength (TS) for films prepared from unmodified RPC was low with 0.6 MPa (Fig. 8). Modification of the RPC showed an increase of the TS with increasing modification degree, presumably due to an increase in non-polar interactions (van der Waals forces) within the film matrix. Low-modified samples showed values of 1.3 MPa (lauroyl) and 1.2 MPa (oleoyl). The TS of high-modified samples increased to 2.4 MPa (lauroyl) and 1.7 MPa (oleoyl). In accordance, the elongation at break was reduced with increasing degree of modification from 151% (non-modified RPC) to 104%/121% and 9.5%/43.3% for RPC-LL/-OL and RPC-LH/-OH, respectively (Fig. 8).

The modification of RPC showed an increase in the TS, however, all values determined were low in comparison to literature studies. Chang and Nickerson [6] reported a TS of 6.6 MPa for cast-films prepared with a similar method as in the present study using 7.5% rapeseed protein sample (based on the sample mass) and 30% glycerol. In a previous study by the authors, formulations of 5% protein and 50% glycerol, sorbitol or PEG-400 as a plasticizer were tested [10]. While glycerol showed the lowest TS (1.2 MPa), improvements were achieved using PEG-400 (5.2 MPa) or sorbitol (10.0 MPa) as a plasticizer. With the addition of genipin as a cross-linker, TS values increased to 2.6 MPa (glycerol), 9.6 MPa (PEG-400) and 12.6 MPa (sorbitol). Using a casting process at pH 11 and 70 °C, Shi and Dumont [8] measured a TS of 0.7–1.7 MPa for films prepared from 5% protein sample and 25–40% glycerol. Denaturation with SDS (5–15%) prior to film formation increased TS to 2.3–3.8 MPa, while the use of stearic acid (5–15%) had a lesser effect (1.5–2.2 MPa). In another study, the TS of non-modified rapeseed protein was ~ 5.3 MPa for films prepared using 2.6% protein sample and 42.5% glycerol by solvent-casting followed by heat-compression [35].

The studies listed above demonstrate the various methods available to increase the TS of protein films. While the effect

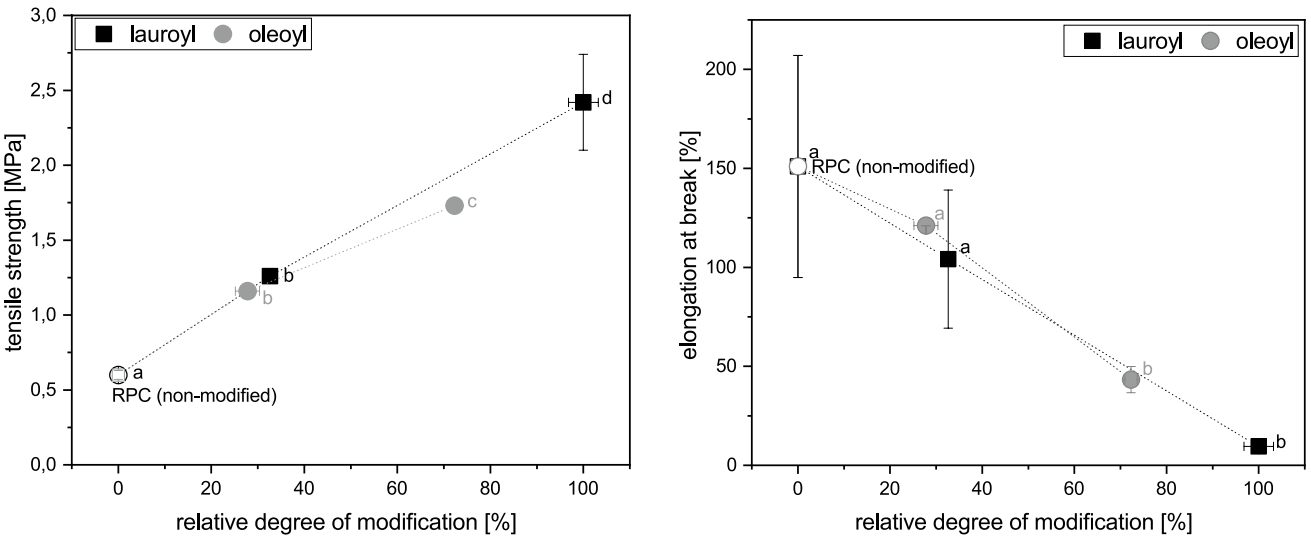


**Fig. 8** Tensile strength and elongation at break of films prepared from rapeseed protein concentrates (mean values ± absolute deviation); data points having the same superscript indicate no significant difference ($p > 0.05$)

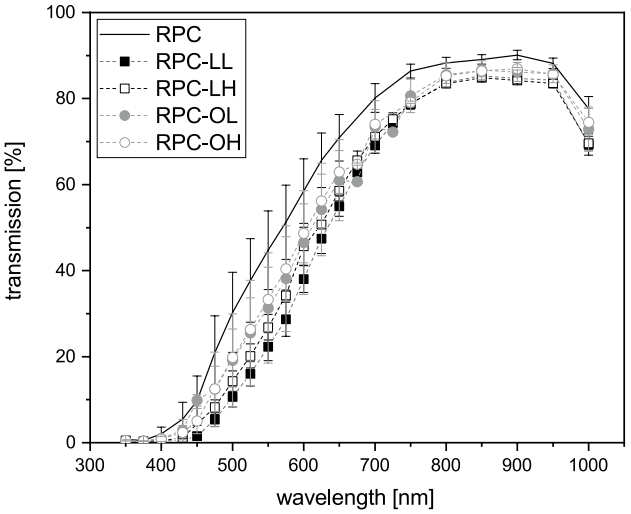


**Fig. 9** Light transmission of films prepared from rapeseed protein concentrates in the range of 350–1000 nm (mean values ± absolute deviation)

of protein acylation prior to film formation applied in the present study showed only limited effect, a combination of methods, such as chemical modification and heat treatment, may be the subject of further studies.

**Light Transmission** All films were of brown color and showed only limited transparency. Light transmission was measured at wavelengths of 350–1000 nm. All films showed a transmittance of < 10% below 450 nm and very low transmittance of ≤ 2% in the range of 350–400 nm (Fig. 9). Modification of the RPC reduced the light transmittance of the prepared films. The effect was stronger for lauroyl modifications as compared to oleoyl modifications. The RDM also had a minor effect such that, interestingly, films with lower modification degree showed slightly lower transmittance. The introduction of lipids into protein films has been related to increased opacity in a number of studies [36–40]. In one study, the fatty acid saturation degree correlated with opacity, indicating that saturated stearic acid increased opacity, whereas the effect of oleic acid and linoleic acid was only marginal [40]. The observation may be explained by a lower melting temperature of oleic and linoleic acid compared to stearic acid. Similarly, Jiménez et al. [41] studied the opacity of hydroxypropyl-methylcellulose films formulated with various fatty acids. Saturated fatty acids showed an increased ability to form lipid aggregates, which was thought to be the reason for higher opacity.

The low light transmittance of rapeseed protein films in general, and of films from modified RPCs in particular, suggests the utilization as a packaging material with good light protection. However, as the films were highly water-soluble and showed low barrier properties against oxygen in this study, the rapeseed protein film needs to be used in combination with other materials, e. g. in a multi-layered material. Alternatively, the natural color of the obtained rapeseed protein films might serve well in an application such as a coating for cardboard or wood.

## Conclusions

The reduction of hydrophilicity of rapeseed protein concentrates was successfully achieved by acylation with lauroyl and oleoyl chloride. The protein solubility in water at neutral pH was reduced from 100% to < 15% for highly modified samples. Contact angle measurement revealed the increased hydrophobicity of cast films from modified RPCs, however, the films still showed low water stability. The observed properties may serve well for the production of water-soluble foils, such as for dish-washer tabs. Films from modified protein samples showed an increase in tensile strength with increasing modification degree, while the elongation at break was reduced consequently. The light transmission of all films was < 10% below 450 nm and was further reduced by modification, suggesting a potential application as a light-protecting packaging layer. In summary, the application potential of rapeseed proteins—both modified and non-modified—as bio-based and biodegradable ingredients with great film-forming properties was demonstrated. Based on the results, suggested application fields are adhesives, coatings, paints, packaging layers, or detergents. Further studies need to be performed to test their applicability in more detail.

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## Compliance with Ethical Standards

**Conflict of interest** All authors declare that they have no conflict of interest.

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# General Conclusions

The extraction and valorization of rapeseed proteins from residual materials of the oilseed industry has been pursued for more than 50 years (Tranchino et al., 1983). Despite the great efforts invested in establishing extraction and isolation protocols, and the elucidation of protein structure and functional properties, the placement of a rapeseed protein isolate on the market has not been successfully achieved to date. This is mainly due to two reasons. Firstly, conventional rapeseed residual materials are processed at high temperatures to extract a maximum amount of oil from the seed (Kemper, 2005). This causes denaturation and covalent bonding of proteins to other contents of the seed, leading to low protein solubility (Mosenthin et al., 2016; Salazar-Villanea et al., 2016). In consequence, low protein yields are achieved during subsequent protein extraction, leading to a low economy of the process. Secondly, antinutritive components of the plant, including phytates, glucosinolates, and phenolic compounds, are difficult to separate from the produced protein isolates. Thus, bitter or pungent taste along with various off-flavors – often described as musty or fishy – remain a major disadvantage in comparison to other plant protein isolates (Linnemann and Dijkstra, 2002; Matheis and Granvogl, 2016; Sghaier et al., 2016). Despite recent progress in the determination of compounds responsible for these odors/ off-tastes, utilization in the food sector remains problematic (Hald et al., 2019; Matheis and Granvogl, 2019; Zhou et al., 2018).

The present doctoral thesis addressed the two major obstacles mentioned above by:

1) Investigation of protein extraction and isolation of different raw materials, such that recommendations can be drawn on the economic feasibility of rapeseed protein valorization from these materials.

2) Investigation of an alternative, non-food utilization of rapeseed proteins as functional ingredients in the technical industry, such that the presence of antinutritive components can be neglected.

Low protein extractability of conventional raw materials remains to be the fundamental limitation for the valorization of rapeseed proteins from residual materials of the oilseed industry. As demonstrated in the first study (**Chapter II**, Fetzer et al. (2018)), rapeseed meal originating from desolventizer (“toaster”) processing as well as full-pressed press cake (after solvent defatting at mild conditions) display very limited protein solubility and can thus not be used for subsequent protein extraction. In contrast, pre-pressed press cake and cold-pressed press cake show significantly higher protein solubility after solvent defatting at mild conditions. For these materials, protein extraction and isolation can be carried out at around neutral pH conditions, such that no need for the utilization of extreme pH values or protease treatment arises. Consequently, rapeseed proteins can be obtained in non-hydrolyzed form and in their

native state. Interestingly, pre-pressed cake, which is obtained during conventional processing before hexane-defatting, showed a significantly higher protein solubility compared to full-pressed cake or toasted meal. Thus, an adaption of the solvent-defatting process towards milder conditions could potentially be implemented in existing processing to obtain a defatted meal of higher quality. In consequence, the establishment of alternative methods – such as solvent defatting at low temperatures – at an industrial scale is seen as prerequisite for the successful valorization of rapeseed proteins in an economic manner. In recent decades, a range of studies reported the use of alternative defatting methods (Fetzer et al., 2020b). These include the use of supercritical $CO_2$ (Boutin and Badens, 2009; Gaber et al., 2018; Uquiche et al., 2012), alternative solvents (Radi et al., 2013; Tian et al., 2019), microwave- and ultrasound-assisted extraction (Azadmard-Damirchi et al., 2011; Gaber et al., 2019), and solvent-free processes (Latif et al., 2008; Wäsche, 2002; Zhang et al., 2007). Some authors reported oil yields of at least 90% and/ or beneficial effects on the oil quality. In particular, this was the case for methods using supercritical $CO_2$ (Boutin and Badens, 2009; Przybylski et al., 1998), alternative solvents (Tian et al., 2019), or solvent-free processes (Zhang et al., 2007). While these studies were carried out only on a laboratory-scale, the ability to compete with conventional defatting processes in terms of oil yield and quality was demonstrated. However, significant improvements have to be achieved especially regarding to process costs in order to form a true alternative for conventional defatting. The implementation of alternative defatting processes at an industrial scale, which enables both the valorization of the oil fraction as well as the protein fraction of the seed, is essential for a benefit of the rapeseed industry. Ultimately, the rapeseed biorefinery of the future may even go one step further and enable the valorization of secondary plant metabolites. Efforts to achieve this goal are currently being made in the research project “EthaNa” (Pufky-Heinrich et al., 2019). Using an ethanol-based extraction process of dehulled seeds, an increased quality of the residual meal along with the co-extraction of bioactive ingredients, including phospholipids, sinapine, and polyphenols, is pursued. Initial protein extraction studies from the residual meal obtained via this process showed high protein solubility, which was comparable to non-pressed, laboratory-defatted seeds using hexane (data not published).

The second major limitation for utilization of rapeseed proteins in the food sector is the presence of residual antinutritive components, including phytates, glucosinolates, and phenolic compounds. These remain in the final protein preparation and are known to cause bitter taste and various off-flavors (Linnemann and Dijkstra, 2002; Matheis and Granvogl, 2016; Sghaier et al., 2016). Due to this limitation, an alternative approach of using rapeseed proteins technical applications – where these limitations can be neglected – was investigated in the present doctoral thesis. Rapeseed proteins offer a promising potential as ingredients in technical products due to their great film-forming, emulsifying and foaming properties. These properties

are of high relevance to a range of products, including adhesives, cosmetics, detergents, lubricants, paints, and polymers. As shown in the second study of this thesis (**Chapter III**, Fetzer et al. (2019)), the raw material quality seems to have no significant influence on these properties. More precisely, rapeseed protein preparations obtained from pre-pressed meal and cold-pressed meal showed similar functionalities. However, the functional properties were strongly dependent on the protein composition and denaturation state, which are determined by the isolation strategy. As such, the protein solubility in water, as well as the emulsifying and foaming properties, were highest for ultrafiltrated, napin-rich preparations. In contrast, preparations obtained through acidic precipitation showed very low protein solubility, and reduced emulsifying and foaming properties. However, protein solubility, as well as protein content, increased with lower precipitation temperature. As a conclusion, a precipitation temperature of 10 °C can be recommended for this type of process, in order to increase the functional properties of obtained protein preparations. Interestingly, no significant differences were found in regard to protein yield or functional properties of any type of protein preparation when comparing the extraction at native pH (5.7–5.8) to neutral pH. Thus, protein extraction using this process can be performed without the need for pH adaption. The results obtained in the study demonstrate the possibility to isolate rapeseed protein preparations with great functional properties at mild conditions. This is in contrast to previous studies studying rapeseed protein isolation, which predominantly describe the use of highly alkaline ($pH \geq 11$) conditions (Akbari and Wu, 2015; Aluko and McIntosh, 2001; Chabanon et al., 2007; Das Purkayastha and Mahanta, 2014; Ghodsvali et al., 2005; Ivanova et al., 2017; Klockeman et al., 1997; Ma et al., 2007; Tzeng et al., 1990).

The great film-forming properties of all rapeseed preparations obtained in the second study, regardless of the isolation strategy, are of particular interest. These offer a promising application potential in a range of products, including adhesives, detergents, lubricants, paints and polymers. In recent decades, a number of studies reported the potential of various (plant) proteins for the production of bio-based films and coatings (Zink et al., 2016). Detailed studies for rapeseed proteins used in these application fields are available only to a limited extent (Li et al., 2012; Rivera et al., 2015; Sanchez-Vioque et al., 2001; Zhang et al., 2018). Thus, further research is mandatory to prove rapeseed proteins as valuable ingredients for these products.

The low water-stability of rapeseed protein-based films poses a limiting factor for its application potential in products such as adhesives and paints. Thus, chemical grafting of acyl-residues was tested as a method to increase the hydrophobicity of rapeseed proteins in the third study of this thesis (**Chapter IV**, Fetzer et al. (2020a)). As expected, the modified protein samples showed a decreased solubility in water, especially at high modification degrees. Moreover, the surface hydrophobicity of films was significantly increased; however, the films remained water-soluble. Higher water stabilities were reported for rapeseed proteins in studies using similar

procedure for cast-film production (Chang and Nickerson, 2014; Chang and Nickerson, 2015). This indicates that the isolation procedure of the rapeseed protein sample may be crucial for the resulting water stability of the obtained film. In our study, a protein sample with high solubility was chosen in order to increase the reactivity during chemical acylation. In conclusion, in areas where high water-stability is demanded, an adaption of either the isolation, modification or the film-forming procedure has to be performed in order to increase the level of intermolecular bond formation. Increased hydrophobicity of rapeseed proteins was also reported in a range of studies by various modification approaches. These include physical modification by heat or high pressure (He et al., 2014), chemical modification by acylation, glycation, phosphorylation, or sulfamidation (Gerbanowski et al., 1999; He et al., 2019; Schwenke et al., 2000; Wang et al., 2018), or enzymatic modification by limited proteolysis (Adler-Nissen and Olsen, 1979). Depending on the employed method, increased surface hydrophobicity is caused either by the introduction of hydrophobic residues into the protein, or by the liberation of buried hydrophobic residues from within the protein. As methods for protein modification can be laborious, the production of protein-based films with higher water stability can also be achieved by heat-denaturation or by addition of a cross-linking agent during the application procedure, as reported in recent years (Li et al., 2017; Zhang et al., 2019). In summary, further improvements in the development of rapeseed protein-based films with good barrier and mechanical properties are mandatory to offer a functional and economically feasible alternative to petrochemical ingredients in the future.

Application studies for rapeseed proteins are predominantly available for adhesives in the literature. One problem associated with all types of plant protein-based adhesives is their low water stability (Frihart and Lorenz, 2018). This was addressed by a few studies using different approaches. Li et al. (2012) reported good water stability for adhesives using high curing temperatures of 190 °C. Moreover, chemical cross-linking using free radical polymerization to form cruciferin–poly(glycidyl methacrylate) conjugates (Wang et al., 2014), or by using hydrolyzed rapeseed meal in combination with phenol-formaldehyde prepolymers was reported (Yang et al., 2014). In addition, good water stability of rapeseed protein-based adhesives for wood applications was tested in combination with nanomaterials. Here, significant improvements could be achieved with the addition of graphite oxide and nanocrystalline cellulose (Bandara et al., 2017a, b). In summary, the listed studies demonstrate various possibilities to enhance the properties of rapeseed protein-based adhesives.

In addition, modified rapeseed proteins were studied for detergents and cosmetics. Sanchez-Vioque et al. (2001) described good foaming properties for acylated rapeseed protein hydrolysates. Due to the high foaming activity and stability, the use as green surfactants in detergents or cosmetics was suggested by the authors. In another study, the generation of

bioactive peptides by enzymatic hydrolysis was demonstrated (Rivera et al., 2015). The authors reported antioxidant, anti-wrinkle and anti-inflammatory properties, thus suggesting the application in skin-care products.

While the investigation of specific applications was beyond the scope of the present thesis, the application of rapeseed protein preparations was tested in the affiliated research project “TeFuProt” (Technofunktionelle Proteine, EJ (2014)). With the project still being in progress during the writing of this thesis, it can be said that promising results were obtained in particular for the sectors polymers, detergents, and wood paints. The applicability of rapeseed proteins in these sectors can generally be attributed to their film-forming and surface-active properties. Further research efforts in these areas may lead to the commercialization of rapeseed protein-based products in the future.

In conclusion, the results of the present thesis demonstrate the great potential of rapeseed proteins for the application in technical products, in particular due to their great film-forming properties. Good functional properties were found for rapeseed protein preparations regardless of the quality of the raw material used for the extraction. However, low protein yields obtained from conventional materials remain a major obstacle for the commercial production of rapeseed protein preparations. Consequently, the optimization of mild defatting processes and their industrial implementation need to be achieved in the future. With the availability of rapeseed protein preparations at a competitive cost, their implementation into technical products to replace petro-based ingredients appears promising. Ultimately, the valorization of both the oil and the protein fraction of rapeseed is seen as an essential development in order to ensure its status as one of the planet’s most important crops.

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# Summary

Today, rapeseed is the third most abundant oil crop worldwide with the primary value lying in its high quality oil, which is used in the food and non-food sector. Proteins constitute the main fraction after oil extraction. Their utilization in the feed and food sector faces some limitations, as antinutritive components remain in the press cake and meal, as well as isolated protein preparations. As rapeseed proteins offer interesting functional properties, such as film-forming, foaming and emulsifying properties, their implementation into products of the chemical industry appears as a promising alternative. Thus, the present thesis aimed to improve knowledge regarding the potential of using rapeseed proteins as bio-based and biodegradable ingredients for the chemical industry.

As reviewed in the beginning of this thesis, the extraction of rapeseed proteins from residual materials of the oilseed industry has been studied extensively (Chapter I). The functionality of protein preparations, predominantly in the form of isolates, has been described mainly for the utilization in the food sector. However, functionalities such as foaming, emulsifying, gelation and film-forming properties are of high relevance for products of the chemical industry, including adhesives, paints, detergents or polymers. In the present thesis, the extraction, isolation and modification of rapeseed proteins was studied for a potential application in this regard. As rapeseed proteins for technical applications demonstrate a great potential as bio-based ingredients in the chemical sector, the present work of research aimed to increase knowledge in this promising, yet little studied field.

The quality of the raw material is essential for an economic extraction of rapeseed proteins from side-streams of the oilseed industry (Chapter II). Conventional defatting strategies for rapeseed are optimized for maximum oil yield, which is achieved by harsh conditions during screw-pressing, solvent defatting and solvent evaporation. As a result, protein solubility is greatly reduced due to denaturation and binding to the other components in the matrix. Protein solubility for full-pressed press cake and toasted meal were determined to be < 20% across a wide pH range, which is not suitable for an economic extraction of protein. Milder processing led to residual materials with significantly higher protein solubility. As such, pre-pressed press cake and cold-pressed press cake showed protein solubility of 37% and 57%, respectively, at pH 7. In comparison, a non-pressed reference of rapeseed defatted at laboratory scale showed a protein solubility of 63% at pH 7. When comparing extraction conditions, the most significant influence on protein extraction yields was found for the concentration of NaCl in the extraction medium. At 0.25 M NaCl, extraction yields for pre-pressed and cold-pressed meal were improved by 98% and 61%, respectively, compared to a salt-free extraction medium. Other parameters, including s:l-ratio, extraction time and temperature, had little to no significant

influence on protein extractability. Under mild conditions (pH 5.7–9), maximum yields of 37% and 52% were achieved for pre-pressed and cold-pressed meal, respectively. Yields were greatly improved by employment of high pH (11–12) or protease treatment, especially for multi-step processes. The highest protein extraction yields of around 80% were achieved for both raw materials by a three-step process under employment of protease. However, the limitation of protein degradation occurring under these conditions has to be considered, as this alters protein functionality and poses problems with subsequent isolation.

The significance of raw material quality was further studied in regard to protein isolation yields and resulting functional properties (Chapter III). Protein isolation yields were 19%–26% for pre-pressed meal and 37%–41% for cold-pressed meal, demonstrating the positive effect of mild processing during defatting. Protein isolation yields, protein content and functionality of obtained protein preparations were not significantly influenced by extraction pH (5.7–7). Hence, native pH (5.7–5.8) can be recommended for this type of process, as no NaOH utilization is required. Functional properties were comparable for preparations obtained from both raw materials. The pH-dependent protein solubility profile was influenced by the isolation strategy and resulting protein composition. Preparations obtained through acidic precipitation showed low solubility (< 45%) due to high contents of cruciferin, while napin-rich samples obtained from subsequent ultrafiltration showed high solubility (> 80%). Mixed preparations obtained by direct ultrafiltration showed good solubility above pH 7 (> 80%) and reduced solubility in the acidic region. Protein contents were 75.3%–87.1% (dry matter basis, N × 5.7) and were highest for preparations obtained through precipitation. Preparations obtained through ultrafiltration showed high values for emulsifying capacity (688–768 mL/g) as well as foaming activity (1834%–2834%). In contrast, precipitated samples showed lower functional values of 410–445 mL/g and 888%–938%, respectively. All preparations demonstrated excellent film-forming properties in cast-film experiments. In summary, the conclusions drawn from the first study concerning the importance of raw material quality on the economy of protein isolation were further supported. However, no significant influence of raw material quality on the resulting functionality of protein preparations was found. For the utilization of rapeseed proteins in the chemical industry, the choice of the right isolation strategy gives the opportunity to isolate rapeseed proteins with specific solubility profiles, as well as emulsifying and foaming properties.

In the final study, chemical acylation of rapeseed proteins was proved as an adequate tool for the modification of functional properties of rapeseed protein preparations (Chapter IV). Upon grafting of hydrophobic side chains in the form of lauroyl- and oleoyl-residues, the protein solubility in water was reduced from > 90% to < 40% for high modification degrees. The increased hydrophobicity of modified proteins was further verified by increased surface hydrophobicity along with an increase in oxygen permeability of protein-based cast films. Tensile strength of films was increased from 0.6 MPa for non-modified samples to 1.2–

2.4 MPa, with a positive correlation to their modification degree. As lauroyl-modified samples showed higher modification degrees, the effects were generally more pronounced for these samples. As all films remained water soluble, an application such as foils for dishwasher tabs is suggested. However, in areas where high water-stability is demanded, an adaption of the modification or the film-forming procedure to increase the level of intermolecular bond formation has to be performed.

In summary, the results of the present thesis demonstrate the great potential of using rapeseed proteins as green alternatives in products of the chemical industry. Rapeseed protein preparations show great functionality, including foaming, emulsifying, and film-forming properties, which can be of great use for products such as adhesives, cosmetics, detergents, paints, or polymers. However, low protein yields for isolation processes, which are caused by low protein solubility of conventional, highly processed press cakes and meals, present a major hurdle for the commercialization of rapeseed protein preparations. With the prospective implementation of milder defatting methods at an industrial scale to tackle this obstacle, rapeseed protein preparations appear as a promising alternative to current petrochemical ingredients.

# Zusammenfassung

Hochwertiges Rapsöl findet heute sowohl für Lebensmittel als auch den technischen Einsatz vielfach Anwendung, wodurch Raps weltweit den dritten Platz unter den am meisten verbreiteten Ölpflanzen einnimmt. Proteine bilden nach der Entölung die verbleibende Hauptfraktion. Da nach der Entölung antinutritive Bestandteile im Presskuchen und Schrot verbleiben, ist die Nutzung der Proteine als Futtermittel oder Lebensmittel in Form von Proteinisolaten begrenzt. Durch ihre interessanten funktionellen Eigenschaften, wie Filmbildung, Schaum- und Emulgiereigenschaften, erscheint die Nutzung von Rapsproteinen in Produkten der chemischen Industrie als vielversprechende Alternative. Die vorliegende Promotionsarbeit zielt darauf ab, den Wissensstand hinsichtlich des Potentials der Nutzung von Rapsproteinen als biobasierte und bioabbaubare Inhaltsstoffe in der chemischen Industrie zu erweitern.

Zur Extraktion von Rapsproteinen aus Reststoffen der Ölsaatenverarbeitung finden sich in der Literatur eine Vielzahl von Studien (Kapitel I). Die Funktionalität der Proteinpräparate, bei denen es sich vorwiegend um Proteinisolate handelt, wurde hierbei nahezu ausschließlich für die Anwendung im Lebensmittelbereich untersucht. Allerdings sind Eigenschaften wie Schäumen, Emulgieren, Gelieren und Filmbildung ebenso für Produkte der chemischen Industrie (z. B. Klebstoffe, Farben, Reinigungsmittel, Kunststoffe) von großer Bedeutung. In der vorliegenden Arbeit wurde die Extraktion, Isolation und Modifikation von Rapsproteinen für eine potentielle Anwendung in diesem Bereich untersucht. Da Rapsproteine ein enormes Potential für den Einsatz als biobasierte Inhaltsstoffe der chemischen Industrie aufweisen, zielte die vorliegende Forschungsarbeit darauf ab, den Wissensstand in diesem vielversprechenden – wenngleich bislang wenig beachteten – Gebiet zu erweitern.

Für die wirtschaftlich sinnvolle Extraktion von Rapsproteinen aus Seitenströmen der Ölsaatenverarbeitung ist die Qualität des verwendeten Rohmaterials ausschlaggebend (Kapitel II). Die konventionelle Rapsentölung zielt auf eine Maximierung der Ölausbeute ab, was mit harschen Bedingungen (hohe Temperaturen, Drücke) während der Pressung, Lösemittel-Entölung und Lösemittel-Rückgewinnung einhergeht. Im Ergebnis wird hierdurch die Proteinlöslichkeit aufgrund von Denaturierung und chemischen Vernetzung der Proteine mit anderen Bestandteilen drastisch reduziert. Die Proteinlöslichkeit für vollgepressten Presskuchen und „getoastetes“ Rapsschrot lag mit < 20% in einem breiten pH-Bereich zu niedrig für eine wirtschaftlich sinnvolle Extraktion der Proteine. Durch mildere Bedingungen während der Entölung wurden Rohstoffe mit signifikant höherer Proteinlöslichkeit erhalten. So zeigten vorgepresster und kaltgepresster Presskuchen bei pH 7 eine Löslichkeit von 37% bzw. 57%. Im Vergleich hierzu zeigte eine nicht gepresste Referenz an Rapssaat, die unter

Laborbedingungen mittels Lösemittel entölt wurde, eine Löslichkeit von 63% bei pH 7. Bei der Untersuchung unterschiedlicher Bedingungen zur Steigerung der Proteinextraktionsausbeute zeigte die Zugabe von NaCl zum Extraktionsmedium die höchste Signifikanz. Mit 0,25 M NaCl wurden die Extraktionsausbeuten für entfettete vorgepresste und kaltgepresste Rapssaat um 98% bzw. 61% im Vergleich zur Extraktion ohne Salzzugabe gesteigert. Andere Parameter, wie s:l-Verhältnis, Extraktionszeit und Extraktionstemperatur, zeigten wenig bis keinen Einfluss auf die Proteinextraktionsausbeute. Unter milden Bedingungen (pH 5,7–9) wurden maximale Ausbeuten von 37% (vorgepresst) bzw. 52% (kaltgepresst) erzielt. Unter stark alkalischen Bedingungen (pH 11–12), sowie durch Protease-Behandlung während der Extraktion zeigten sich deutlich gesteigerte Ausbeuten, insbesondere für mehrstufige Extraktionen. Die höchste Proteinextraktionsausbeute mit ca. 80% wurde für beide Rohstoffe durch Verwendung eines dreistufigen Prozesses mit Protease-Behandlung erzielt. Allerdings muss das Auftreten von Proteinhydrolyse unter diesen Bedingungen beachtet werden, was sich auf die Proteinfunktionalität auswirkt und Probleme bei einer anschließenden Isolierung der Proteine verursacht.

Die Bedeutung der Rohstoff-Qualität wurde darüber hinaus in Bezug auf Proteinisolationsausbeuten sowie die funktionellen Eigenschaften untersucht (Kapitel III). Die Proteinisolationsausbeuten lagen bei 19%–26% (vorgepresst) und 37%–41% (kaltgepresst), wodurch erneut der positive Einfluss von milden Bedingungen während der Entölung belegt wurde. Die Einstellung unterschiedlicher pH-Werte während der Extraktion (pH 5,7–7) zeigte keinen signifikanten Einfluss auf Proteinisolationsausbeuten, Proteinanteil und Funktionalität der erhaltenen Proteinpräparate. Die Verwendung des nativen pH-Wertes (pH 5,7–5,8) konnte somit als vorteilhaft für die Proteinisolierung bewertet werden, da hierbei eine Zugabe von NaOH entfällt. Die funktionellen Eigenschaften waren für beide verwendeten Rohstoffe vergleichbar. Das pH-abhängige Löslichkeitsprofil wurde durch die Isolationsstrategie und der daraus resultierenden Proteinzusammensetzung der Präparate bestimmt. Proteinpräparate, die durch saure Fällung gewonnen wurden, zeigte eine niedrige Proteinlöslichkeit (< 45%), die sich auf hohe Anteile an Cruciferin zurückführen ließ. Die Proteinlöslichkeit für Präparate, die durch anschließende Ultrafiltration gewonnen wurden, zeigten aufgrund hoher Napin-Anteile hingegen eine hohe Löslichkeit (> 80%). Für gemischte Präparate mit signifikanten Cruciferin- und Napin-Anteilen, die über eine direkte Ultrafiltration isoliert wurden, zeigte sich ein entsprechendes Löslichkeitsprofil: Während eine hohe Löslichkeit lediglich über pH 7 (> 80%) erzielt wurde, zeigte sich im sauren Bereich ein starker Löslichkeitsabfall. Der Proteingehalt lag bei 75,3%–87,1% (Trockensubstanz-Basis, N × 5.7) und lag für sauer gefällte Präparate am höchsten. Ultrafiltrierte Präparate zeigten hohe Emulgierkapazität (688–768 mL/g) sowie Schaumaktivität (1834%–2834%). Im Gegensatz dazu zeigten gefällte Präparate geringere Werte von 410–445 mL/g bzw. 888%–938%. Alle Präparate zeigten hervorragende

filmbildende Eigenschaften in Gießfilm-Versuchen. Zusammenfassend wurde die Wichtigkeit der Rohstoffqualität in Hinblick auf die Wirtschaftlichkeit der Proteinisolierung erneut untermauert. Allerdings konnte kein signifikanter Einfluss der Rohstoffqualität auf die resultierenden funktionellen Eigenschaften der Proteinpräparate gefunden werden. Zur Nutzung von Rapsproteinen in der chemischen Industrie, ergibt sich durch die Wahl der geeigneten Isolationsstrategie die Möglichkeit, Rapsproteine mit spezifischen Löslichkeitsprofilen, Emulgier- sowie Schaumeigenschaften zu gewinnen.

In der abschließenden Studie wurde die chemische Acylierung von Rapsproteinen als geeignetes Verfahren zur Modifikation der funktionellen Eigenschaften von Rapsproteinpräparaten demonstriert (Kapitel IV). Durch Aufpfropfen hydrophober Seitenketten in Form von Lauroyl- and Oleoyl-Resten, wurde die Proteinlöslichkeit in Wasser von > 90% bei hohen Modifikationsgraden auf < 40% reduziert. Die erhöhte Hydrophobizität der modifizierten Proteine wurde darüber hinaus durch einen Anstieg der Oberflächenhydrophobizität sowie eine erhöhte Sauerstoffdurchlässigkeit von proteinbasierten Gießfilmen bestätigt. Die Zugfestigkeit der Filme wurde durch die Modifikation von 0.6 MPa auf 1,2–2,4 MPa erhöht und zeigte eine positive Korrelation mit dem Modifikationsgrad. Da Lauroyl-modifizierte Proben höhere Modifikationsgrade aufwiesen, war der Effekt für diese Proben generell stärker. Aufgrund der verbleibenden Wasserlöslichkeit aller Proben, wurde eine Anwendung beispielsweise in der Form von Folien für Geschirrspüler-Tabs als aussichtsreich bewertet. Für Anwendungsbereiche, in denen höhere Wasserstabilität gefordert ist, sollte eine Anpassung der Modifikationsbedingungen oder des Filmbilde-Prozesses untersucht werden, um den Grad der intermolekularen Bindungen innerhalb der Filmmatrix zu erhöhen.

Zusammenfassend demonstrieren die Ergebnisse der vorliegenden Arbeit das enorme Potential der Nutzung von Rapsproteinen als „grüne“ Alternative in Produkten der chemischen Industrie. Rapsproteinpräparate zeigen durch ihre schaumbildenden, emulgierenden und filmbildenden Eigenschaften eine hervorragende Funktionalität, die in Produkten wie Klebstoffen, Kosmetika, Reinigungsmitteln, Farben oder Kunststoffen von hohem Nutzen sein können. Allerdings erweist sich die aufgrund der geringen Proteinlöslichkeit niedrige Proteinausbeute aus konventionellen Presskuchen und Schroten als maßgebliche Hürde für eine Kommerzialisierung der Rapsproteinpräparate. Durch die zukünftige Überführung milderer Entölungsmethoden in den industriellen Maßstab zur Behebung dieser Problematik erscheinen Rapsproteinen als eine vielversprechende Alternative zu derzeitigen fossil-basierten Inhaltsstoffen.