Bio-based Materials from Crambe and Carinata Industrial Oilseed Meals

Compression Moulded and Extruded Oilseed Meal Plastics

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Cover: Sketch of *Crambe abyssinica* (left) and *Brassica carinata* (right) (not to same scale)
(W. R. Newson)

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Abstract

Protein-based plastics are considered as a new route for valorisation of oilseed meal from the industrial oil crops *Crambe abyssinia* (crambe) and *Brassica carinata* (carinata) as they cannot be used for animal feed or human food. To convert oilseed meals into protein-based plastic films compression moulding was used with varying processing temperature, chemical additives and protein extraction conditions. Twin screw extrusion was utilized to make films from blends of crambe meal and wheat gluten with urea as a combination denaturant and plasticizer. Tensile properties were examined and related to protein solubility and protein molecular weight (MW) distribution to reveal the underlying effects of different processing conditions.

Varying the plasticizer content (glycerol) in crambe and carinata meal based plastics resulted in a variety of tensile responses with protein MW distributions (HPLC) indicating that the tensile changes were due to plasticization effects. Forming oilseed meal films at temperatures between 100 and 180 ^{o}C indicated a minimum in solubility between 130 and 140 ^{o}C, corresponding to the highest Young's modulus and maximum stress. From a range of additives to crambe and carinata meal films, NaOH and NH_4OH had the most positive effect on strain at maximum stress, especially at the lowest dose of NaOH (1.4%) which also resulted in the lowest protein solubility. Processing crambe meal/WG/glycerol/urea blends with co-rotating twin screw extrusion produced continuous protein-based plastic films within a limited temperature and composition window. Proteins were extracted from crambe meal under a variety of conditions with concentrates from alkali extraction/isoelectric precipitation showing the lowest protein solubility after heating. The relationship between processing, MW distribution and film properties is complex. Maximum protein aggregation resulted in improved properties in some cases, while in others the properties are controlled by mechanisms other than protein aggregation.

Keywords: bio-based materials, protein-based plastics, *Crambe abyssinica*, *Brassica carinata*, industrial oilseed meal, protein aggregation, protein-protein interaction, compression moulding, extrusion.

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Dedication

To Sharon for her unwavering and *very* long lasting support....

One who doesn't know and doesn't know that he doesn't know -He will be eternally lost in his hopeless oblivion!
One who doesn't know, but knows that he doesn't know -His limping mule will eventually get him home.
One who knows, but doesn't know that he knows -He is fast asleep, so you should wake him up!
One who knows and knows that he knows -His horse of wisdom will reach the skies.

Ibn Yamin Faryumadi (Persian-Tajik Poet, 1286-1368)

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List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I William R. Newson, Ramune Kuktaite, Mikael S. Hedenqvist, Mikael Gällstedt, Eva Johansson (2013). Oilseed Meal Based Plastics from Plasticized, Hot Pressed *Crambe abyssinica* and *Brassica carinata* Residuals. *Journal of the American Oil Chemists' Society*, vol 90 (8), pp. 1229-1237.

II William R. Newson, Ramune Kuktaite, Mikael S. Hedenqvist, Mikael Gällstedt, Eva Johansson (2014). Effect of Additives on the Tensile Performance and Protein Solubility of Industrial Oilseed Residual Based Plastics. *Journal of Agricultural and Food Chemistry*, vol 62 (28), pp. 6707-6715.

III Hannah Rasel, Therese Johansson, Mikael Gällstedt, William Newson, Eva Johansson, Mikael Hedenqvist (2016). Development of bioplastics based on agricultural side-stream products: Film extrusion of *Crambe abyssinica*/wheat gluten blends for packaging purposes. *Journal of Applied Polymer Science,* vol 133 (2), 42442.

IV William R. Newson, Maria Luisa Prieto-Linde, Ramune Kuktaite, Mikael S. Hedenqvist, Mikael Gällstedt, Eva Johansson. The effect of heating on the solubility and molecular weight distribution of *Crambe abyssinica* protein concentrates produced from multiple routes (manuscript).

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The contribution of William Newson to the papers included in this thesis was as follows:

I Planned and performed experimental work and analysis, wrote the manuscript.

II Planned and performed experimental work and analysis, wrote the manuscript.

III Consulted on experimental work and analysis, participated in manuscript writing with co-authors.

IV Planned and performed experimental work and analysis, wrote the manuscript.

Related articles by William Newson that do not appear in this thesis:

A. Johansson, E., Malik, A.H., Hussain, A., Rasheed, F., Newson, W., Plivelic, T.S., Hedenqvist, M., Gällstedt, M. & Kuktaite, R. (2013). Wheat gluten polymer structures: The impact of genotype, environment and processing on their functionality in various applications. *Cereal Chemistry*, vol 90 (4), pp. 367-376.

B. Rasheed, F., Newson, W.R., Plivelic, T.S., Kuktaite, R., Hedenqvist, M.S., Gällstedt, M. & Johansson, E. (2014). Structural architecture and solubility of native and modified gliadin and glutenin proteins: non-crystalline molecular and atomic organization. *RSC Advances*, vol 4 (4), pp. 2051-2060.

C. Muneer, F., Johansson, E., Hedenqvist, M.S., Gällstedt, M. & Newson, W.R. (2014). Preparation, Properties, Protein Cross-Linking and Biodegradability of Plasticizer-Solvent Free Hemp Fibre Reinforced Wheat Gluten, Glutenin, and Gliadin Composites. *BioResources*, vol 9 (3), pp. 5246-5261.

D. Rasheed, F., Newson, W.R., Plivelic, T.S., Kuktaite, R., Hedenqvist, M.S., Gällstedt, M. & Johansson, E. (2015). Macromolecular changes and nano-structural arrangements in gliadin and glutenin films upon chemical modification: Relation to functionality. *International Journal of Biological Macromolecules*, vol 79, pp. 151-159.

E. Newson, W.R., Rasheed, F., Kuktaite, R., Hedenqvist, M.S., Gällstedt, M., Plivelic, T.S. & Johansson, E. (2015). Commercial potato protein concentrate as a novel source for thermoformed bio-based plastic films with unusual polymerisation and tensile properties. *RSC Advances*, vol 5 (41), pp. 32217-32226.

Abbreviations

AA	Amino acid residue
AH	Ammonium hydroxide, NH_4OH
Anon.	Anonymous
As	Ascorbic acid
BP	Benzoyl peroxide
CA	Citric acid
carinata	*Brassica carinata*
crambe	*Crambe abyssinica*
db	Dry basis
DTT	Dithiothreitol
E	Young's modulus
HPLC	High performance liquid chromatography
JF	Jeffamine EDR 176
max	Maximum
MW	Molecular weight
MWCO	Molecular weight cut off, in Daltons
OTR	Oxygen transmission rate
PA11	Polyamide 11
PBS	Polybutylene succinate
PLA	Polylactic acid
pph	Parts per hundred
press	Pressure
RCF	Relative centrifugal force
RH	Relative humidity
RP-HPLC	Reversed phase-high performance liquid chromatography
RT	Room temperature
SA	Salicylic acid
SB	Sodium bisulphite
SDS	Sodium dodecyl sulphate

SE-HPLC	Size exclusion-high performance liquid chromatography
SEM	Scanning electron microscopy
T	Temperature
U	Urea
WG	Wheat gluten

1 Introduction

Whether or not we realize it, we are surrounded by protein-based materials. The leather of our shoes, the wool and silk in our clothes, the feathers in our pillows and duvets are all predominantly made of proteins. As you read this, the light is passing through the lenses of your eyes which are predominantly made of protein (Augusteyn & Stevens, 1998). Some naturally occurring proteins are structured to form materials that are impressive, even by the standards of synthetically manufactured polymers – insect silks show high degrees of strength and resilience (Omenetto & Kaplan, 2010) while squid ring teeth show high toughness in an isotropic protein material (Ding *et al.*, 2014). Proteins figure prominently in natural composite materials, such as bones and shells, in which they provide toughness to a brittle matrix (Meyers *et al.*, 2008).

Natural protein-based materials have been utilized by European humans in the form of woven animal fibres, such as wool, for more than 3500 years (Kovačević & Car, 2014). The working of animal protein in the form of horn is ancient as well, with the worshipful company of horners being one of the earliest Livery Companies of London, predating its first recorded mention in 1284 (Rosedale, 1912). Since 1943 the worshipful company of horners has incorporated the plastics industry as well, acknowledging the modern equivalent of the ancient art of manufacturing in natural protein-based horn (Anon., 2014b).

If proteins make up such a variety of useful natural products, why are they missing from the common modern engineered materials? Proteins are complex molecules constructed of 21 amino acids, the order of these amino acid residues (AA) in the protein determines the primary structure of the molecule (Figure 1). Each amino acid has different characteristics such as size, charge, and hydrophobicity. Some AA have the ability to form hydrogen or ionic bonds or covalent bridges, these properties determine how the protein molecule behaves in a given situation. These characteristics result in the folded

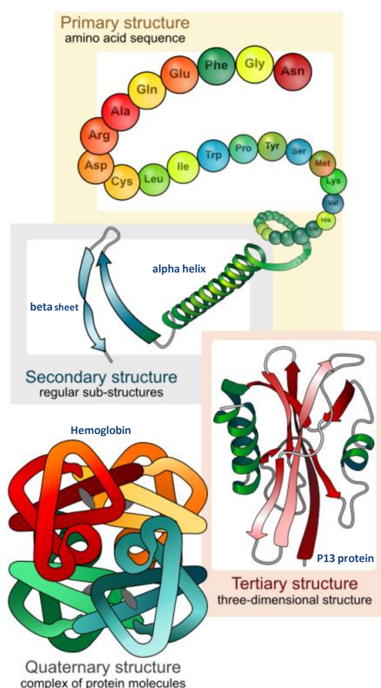

Figure 1. Hierarchical structure of proteins (LadyofHats, 2008)

configuration of the proteins that determines its local secondary structure such as α-helices or β-sheets (Figure 1).

The secondary structural elements of the protein interact to form tertiary structure, in which one or more protein molecules interact to form larger structures, sometimes stabilized by disulphide bonds. These assemblies can further interact to form quaternary structures composed of many protein subunits, each with their own primary, secondary and tertiary structure (Figure 1). The behaviour of proteins can be further affected by the environment around them, *e.g.* the pH alters the charge of the AA, urea (U) disrupts hydrogen bonds between AA or the application of heat can result in unfolding, all of which impact the structure.

The behaviour of the protein, at all these levels, is under the influence of the local environment which determines how the protein will behave. In naturally occurring protein materials, such as spider dragline silk, each step in silk formation is carefully controlled by the organism. The same high performance structure of natural silk has proven difficult to reproduce technologically (Brown *et al.*, 2015). The control of all protein structural levels stands as a challenge to developing new materials that can approach the performance of biologically produced protein materials.

Proteins have been used as feedstock for materials in the past, such as textile fibres or solid objects such as buttons (Ralston & Osswald, 2008). Indeed, in 1926 55% of the world's buttons were manufactured from casein protein (Ralston & Osswald, 2008). In order to improve these protein-based materials, treatments with chemicals, such as formaldehyde, were used to induce AA cross linking (Boyer, 1940), These treatments are viewed unfavourably today due to their toxicity (Álvarez-Chávez *et al.*, 2012). Manufactured protein-based products were replaced with petrochemical polymers after World War II, once the performance of petrochemicals were superior at a lower cost (Ralston & Osswald, 2008).

The rise of the petroleum industry that led to the end of early protein-based materials has brought with it new challenges. Petroleum-based plastics are largely not biodegradable, leading to disposal issues on a global scale. Their widespread and persistent presence in oceans affects seabirds, turtles and other life with its ubiquitous presence (Gall & Thompson, 2015; Wilcox *et al.*, 2015; Mrosovsky *et al.*, 2009). The widespread presence of micro plastics in the environment also acts to concentrate toxins (Hirai *et al.*, 2011), threatening the food chain. Protein-based plastics do not suffer from the same disposal issues as they are biodegradable. In addition to disposal issues, the production of green house gases during manufacturing and incineration of petroleum-based plastics is also cause for concern (Harding *et al.*, 2007). Along with these environmental concerns are economic ones related to the security of the petroleum supply and questions of long term petroleum sustainability that encourage us to explore alternatives.

Among the alternatives to petroleum-based polymers are those produced from the microbial conversion of agricultural products. Examples of such a conversion is turning starch into monomers for the manufacturing polylactic acid (PLA), and polybutylene succinate (PBS) or though the dehydration of alcohols in the case of bio polyethylene (Reddy *et al.*, 2013). Alternative polymers can also be synthesized from the chemical conversion of plant oils into monomers, such as polyamide 11 (PA11) (Mutlu & Meier, 2010).

Bio-based polymers synthesized from monomers allow for control over the chemical structure as in traditional petroleum-based polymers, which is a technological advantage. The energy and process steps required for the production of purified monomers may put them at a sustainability disadvantage compared to the direct use of agricultural products. Starches have also been extensively explored for use as bio-based materials (Zhang *et al.*, 2014) and have the advantage of simple, well developed commercial techniques for their bulk purification from agricultural products without microbial conversion.

All of the aforementioned alternatives to petroleum-based polymers rely on the primary agricultural product derived from the plants involved; be it plant oils, starch used directly, or starch fed to microbes. When agricultural resources are used for non food uses this generates some concern regarding food security, the so called “food vs. fuel” debate (Graham-Rowe, 2011), in our case “food vs. plastic”. In terms of both “food vs. fuel” and within the concept of bio-refineries, the use of lower value agricultural side streams is desirable, since the primary products such as starch and oil already have a ready market and multiple uses. Plant proteins from residuals appear to be an attractive alternative for bio-based materials as they do not have as many ready uses and take few process steps to produce compared with monomers from

fermentation (e.g. PLA and PBS) or through chemical reactions (PA 11). Proteins are already macromolecules and also incorporate readily reactive AAs, which could be advantageous in forming plastic replacement materials. Disadvantages of using proteins from residuals are the structural complexity of proteins and the nature of the plant AA sequence, which leaves us with the challenge of converting non-structural plant proteins into materials with useful properties.

Regarding the "food vs. fuel" debate, current food production requires plastics in its production and distribution. The continued use of petroleum-based plastics for these applications is unsustainable as it is a finite resource, with a reserve estimated to be 52.5 years (Anon., 2015). What will take the place of these materials in this not so distant future? The development and adoption cycle of new plastics when large changes in the production value chain occurs takes up to 24 years (Musso, 2009). The development of new materials from non-petroleum sources is now an imperative. There are many possible alternatives to be explored that are of plant origin and require competition for land use with food. In considering the entire system of food production, food loss accounts for roughly one third of production (Gustavsson *et al.*, 2011). Currently, petroleum-based plastics play a critical role in producing and delivering these products throughout the value chain. Finding sustainable replacements for these materials will ensure the future high levels of production and the integrity of the value chain which, in my opinion, justifies considering some part of agricultural land being dedicated to their production. Only further analysis of proposed solutions can resolve the balance between these competing forces, but first we must develop the technology to the point that reasonable comparisons can be made.

1.1 Industrial oilseeds

The use of oilseeds is one approach to address some of the issues with the petroleum-based economy, offering a sustainable bio-based alternative to petrochemicals in the form of plant oils. Using edible plant oils to replace petroleum oil is possible in many applications, but the conflict with food uses in terms of the market cost and oil quality will remain. Industrial oilseeds offer a platform in which the interaction with food production is minimized in both the product consumption and genetic sense; by choosing industrial oilseed crops that do not easily cross with common food crops more latitude is allowed in the composition of the seed (Carlsson, 2009). When industrial crops are employed seeds can include potentially toxic components and perhaps confront a reduced regulatory regime regarding genetic modification without issues of

possible contamination of the food supply or food crop gene pool (Carlsson *et al.*, 2014).

After the extraction of oils from *Brassica* oilseeds a substantial amount of seed meal is produced, typically 55-60% of the originally harvested seed (Wanasundara, 2011). In the case of industrial oil crops these de-oiled meals can be unfit for human or animal consumption due to the presence of antinutritional compounds and the regulatory regime these uses require. Therefore some use for these meals other than food or feed is sought. Since these de-oiled meals contain seed storage proteins, the use of these proteins for bio-based materials is an attractive avenue for study. Finding a use for these residuals of industrial oilseed processing could help replace products of the unsustainable petrochemical industry and improve the economic case for industrial oilseeds.

Of the possible industrial oilseeds available, *Crambe abyssinica* (crambe) and *Brassica carinata* (carinata) offer some valuable agronomic traits. Both grow well in temperate zones with crambe exhibiting good drought tolerance (Oplinger *et al.*, 1991) and carinata offering both excellent heat and drought tolerance (Rakow & Getinet, 1998). Both crops are unlikely to cross with commonly cultivated food crops (Carlsson, 2009) freeing plant breeders from concerns of gene pool contamination. Crambe has an established record as an industrial oil crop with acceptable yields and a high content of erucic acid in its oil, which is already a valuable industrial commodity (Endres & Schatz, 2010). In carinata, various oil qualities have already been developed (Taylor *et al.*, 2010) and in both crambe and carinata molecular techniques have been developed for their genetic manipulation (Li *et al.*, 2013; Taylor *et al.*, 2010).

Crambe and carinata are known to contain mainly 12S cruciferin and 2S napin, the same main storage proteins as are found in other *Brassica* oilseeds (Wanasundara, 2011). Although the exact sequence and structure of AAs of these proteins in crambe and carinata are not known, there is an expectation that they will be similar within the family (Wanasundara, 2011). This suggests that the related *Brassica napus* storage proteins can be used to indicate the expected protein properties in crambe and carinata. The globulin of *Brassica napus*, cruciferin, has a molecular weight (MW) of *ca.* 300 kDa and an isoelectic point of *ca.* 7.3 (Schwenke *et al.*, 1983). Cruciferin is a hexamer with each of the 6 units having a heavier α subunit (acidic, 30 kDa) and lighter β subunit (basic, 20 kDa) that are connected with a disulphide bridge (Dalgalarrondo *et al.*, 1986). The hexameric assembly of curciferin is held together through ionic forces as it dissociates reversibly into dimers at low ionic strength (Schwenke *et al.*, 1983). Cruciferin further dissociates to 2-3S units in 4M U under acidic conditions (Schwenke *et al.*, 1983). Cruciferin

originates from multiple precursors resulting in amino acid sequence variations within its subunits (Wanasundara, 2011).

Napin, the 2S albumin storage protein of *Brassica* seeds, is a dimer with a MW of *ca.*14 kDa held together with two disulfide bridges (Ericson *et al.*, 1986; Lönnerdal & Janson, 1972) and an isoelectric point above 11 (Lönnerdal & Janson, 1972). Within the *Brassica,* napins show a variety of MW making comparisons between *Brassica napus*, crambe and carinata less likely to be accurate in this specific regard, while all are basic, disulfide bonded dimers (Byczyñska & Barciszewski, 1999).

1.2 Plant proteins as a source for bio-based materials

Plant proteins from a variety of sources have been converted into bio-based plastics, including proteins from maize (Reddy *et al.*, 2009; Selling & Sessa, 2007), wheat (Reddy *et al.*, 2009; Gällstedt *et al.*, 2004), soy (Mo & Sun, 2002; Zhang *et al.*, 2001), cottonseed (Marquié, 2001), sunflower (Rouilly *et al.*, 2006b) and rapeseed (Johansson *et al.*, 2012; Baganz *et al.*, 1999). Unlike biologically produced proteins that have a structural role, such as horn or hair, these proteins need to be processed in some way to modify them to have the properties of useful structural materials in the desired shape.

The processes used to manufacture protein-based plastics are required to fulfil many roles. Processing is required to modify the protein conformation in such a way as to form a continuous solid that has adequate physical properties to be functionally useful. Various approaches have been tried, among them film casting (Chang & Nickerson, 2013), hot compression moulding (Johansson *et al.*, 2012), extrusion (Rouilly *et al.*, 2006a), injection moulding (Baganz *et al.*, 1999), and fibre spinning (Gillberg, 1979). Each of these processes has their analogue in current industrial practice and as such are viable routes to the commercial production of oilseed meal bio-based materials.

Although the various processing methods take different routes to producing bio-based materials, they share similar underlying processes. First, the proteins present in the feedstock are denatured. That is to say, their conformation is modified compared to the original conformation when extracted from the plant. In the most commonly used laboratory process, film casting, the protein is first dissolved in a suitable solvent. This leads to denaturation of the proteins, depending on the solution conditions (Brandenburg *et al.*, 1993). Heat is sometimes applied in the solution state promoting further denaturation and resulting in improved final properties (Roy *et al.*, 1999). In non-solution thermal processing, methods such as compression moulding, extrusion and injection moulding, heat is the main agent of denaturation (Bier *et al.*, 2014). In

these thermal methods plasticizers, such as glycerol, can be applied in order to lower both the glass transition temperature for enhanced processability and lower the start of denaturation (Bier *et al.*, 2014). Additives, such as U, can also contribute to denaturation as well as plasticization (Türe *et al.*, 2011)

During processing, protein-protein interactions in the form of AA cross links need to occur in addition to denaturation in order to improve the physical properties of the final product. In some proteins, such as wheat gluten (WG), there are ready pathways to increase protein-protein interactions through disulphide bridges (Gällstedt *et al.*, 2004). In most systems AA reactions are promoted by heat, changing the reaction pH, the use of enzymes, denaturants, reducing agents or chemical cross linkers. The application of heat promotes many AA interactions, especially in high pH conditions. These reactions have been reviewed by Friedman in the context of food processing (Friedman, 1999) and have been studied within the context of protein-based plastics of WG (Rombouts *et al.*, 2013; Lagrain *et al.*, 2011; Rombouts *et al.*, 2011; Rombouts *et al.*, 2010).

Increasing the pH in the reaction environment effects the state of the AAs through protonation of the ε-amino group of lysine making it more reactive (Friedman, 1999). The formation of new cross links can also occur through disulphide bridge rearrangement from intra-protein to inter-protein forms upon heating and β-elimination reactions involving non-cystine AAs (Lagrain *et al.*, 2011)(Rombouts *et al.*, 2013; Rombouts *et al.*, 2010). The variety of new bonds possible from a range of AAs suggests that improving the properties of oilseed meal-based materials could be possible without resorting to direct chemical cross linking.

When increased cross linking is desired there are multiple chemical options. The most common of which is the chemical cross linking of proteins with aldehydes, long known in the fixation of proteins in tissues and for leather tanning (Kiernan, 2000). Although effective, this method is not desirable for both environmental and occupational health and safety reasons due to its possible toxicity (Álvarez-Chávez *et al.*, 2012). A more environmentally attractive approach exists in the form of enzymatic cross linking (Jiang *et al.*, 2007), which is compatible with food uses of cross linked proteins.

The application of polycarboxylic acids has been shown to cross link proteins in the presence of sodium hypophosphite or under alkali conditions (Reddy *et al.*, 2012; Reddy *et al.*, 2009). Although citric acid (CA) cross linking has been demonstrated with gliadin at relatively mild conditions (Xu *et al.*, 2015), previous studies with the maize protein zein failed to induce cross linking (Selling & Sessa, 2007).

Increased protein-protein interaction may not always be desirable. Cross linking of AAs at the wrong time can be detrimental to processability and can be suppressed by additives that reduce disulphide bonds (*e.g.* sodium bisulphite (SB)) or oxygen scavengers, such as salicylic acid (SA) (Rouilly *et al.*, 2006b; Ullsten *et al.*, 2006). There are also opportunities for multiple effects, such as applying U, which both promotes denaturation and reduces cross linking through the carbamylation of lysine (Rombouts *et al.*, 2013; Türe *et al.*, 2011). Phytochemicals present in oilseeds may also affect protein behaviour. Phenolics in *Brassica* protein extracts are known to interfere with protein-protein interactions, negatively affecting the thermal gelation of cruciferin (Rubino *et al.*, 1996).

2 Objectives

The main objective of this work is the valorisation of industrial oilseed residuals. As food and feed applications are not available for these protein containing residuals, bio-based protein plastics have been selected as a route to valorisation. In order to be compatible with plastics industry practice, thermal processes for conversion of oilseed residuals were explored. The effects of various processing variables were evaluated with regard to application-related metrics; *i.e.* tensile mechanical properties and gas permeability. The effect of processing on the proteins was probed by determining changes in their solubility, protein MW profile using size exclusion and reversed phase high performance liquid chromatography (SE- and RP-HPLC) to be related to changes in protein-based plastic performance.

- Determine the effect of heat and plasticizers on the tensile properties and protein MW profile of plastics from de-oiled crambe and carinata oilseed meal.
- Use chemical additives to modify the reaction environment of plasticised crambe and carinata oilseed meals during thermal processing, thereby affecting the tensile properties and protein MW profile.
- Produce extruded films from blends of WG and de-oiled crambe meal and examine the effect of processing conditions, crambe:WG ratio, U content and glycerol level on film properties with respect to packaging applications.
- Evaluate the effect of protein fractionation on thermally processed films from de-oiled crambe meal; describe the MW profile of extracted protein concentrates and identify fractions that aggregate in response to thermal processing.

3 Methodology

3.1 Raw materials

Crambe seeds (*Crambe abyssinica*) (processed with pod intact) were obtained from the Plant Research Institute (Wageningen, Netherlands) and carinata seeds (*Brassica carinata*) from Agriculture Canada (Saskatoon, Canada). The petroleum distillate method of Appelqvist (Appelqvist, 1967) was used to produce oilseed meals of crambe (11.1 ± 0.02% dry basis (db) water) and carinata (10.1 ± 0.06% db water), resulting in a fine powder for compression moulding (Paper I, II, IV). For extrusion processing, rolled, hexane extracted crambe meal was ball milled to a fine powder (Paper III). Wheat gluten powder was supplied by Lantmännen Reppe AB, Sweden (77.7% (w/w) protein, 8.1% (w/w) starch, and 1.34% (w/w) fat) as reported by manufacturer (Paper III). Ball-milled crambe and WG powder were conditioned for a minimum of 48 h at room temperature (RT) and 23% relative humidity (RH) before further processing (Paper III).

Nitrogen content of the de-oiled meals was determined using combustion nitrogen analysis with a nitrogen to protein conversion factor of NX6.25 (Wanasundara *et al.*, 2012).

3.1.1 Protein concentrate production

Proteins were extracted from de-oiled crambe meal using three main schemes (Paper IV), for specific extraction conditions see Figure **2**. Both precipitates and supernatants were lyophilised to produce concentrates. In Scheme A meal was first water extracted and the supernatant lyophilised (concentrate A1). The residual was re-extracted with 3.5% NaCl with this supernatant dialyzed (2000 Da molecular weight cut off (MWCO)) against deionized water (Millipore) to remove salts and lyophilised (A2). The residuals were again extracted with

0.1N NaOH, the supernatant neutralized to pH 7 with the supernatant dialyzed then lyophilised (A3) and the precipitate lyophilised (A4).

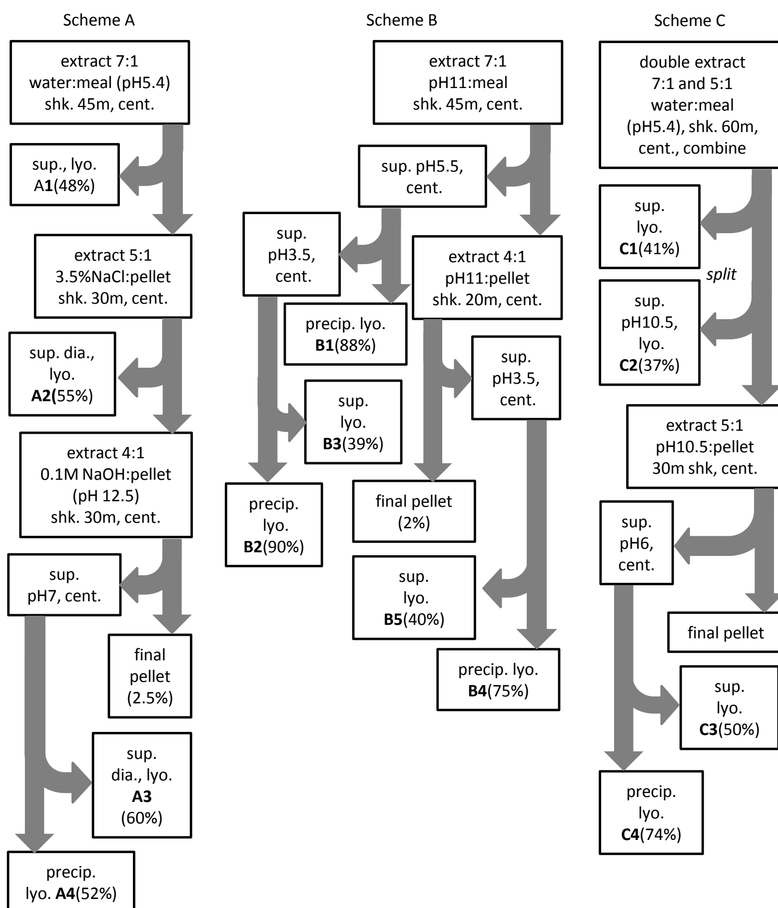

Figure 2. Extraction schemes for the production of crambe protein concentrates. sup.- supernatant, cent.- centrifuge 20 min at 5000 RCF, precip.- precipitate, dia.- dialyze (2000 MWCO), %- % protein content in lyophilised concentrate (combustion nitrogen analysis). Values denoted as (pHX) are a result of process conditions, all other pH values are control targets. All extraction solvent volumes (extract X:Y) are volume (ml):mass (g) based on the original mass of de-oiled crambe meal.

In Scheme B, de-oiled crambe meal was extracted at pH 11, the supernatant adjusted to pH 5.5 and the precipitate recovered (B1). The resulting supernatant was adjusted to pH 3.5 and the subsequent precipitate recovered (B2) and the supernatant concentrated by lyophilisation (B3). The pellet was again extracted at pH 11, adjusted to pH 3.5, centrifuged with the precipitate

and supernatant lyophilised (B4, B5 respectively). On adjusting to pH 3.5 for the production of B4 there was no observable precipitation at pH 5.5.

In Scheme C, the meal was doubly extracted with deionised water, combining the supernatants. The combined supernatant was then split and half directly lyophilised (C1) while the other half was adjusted to pH 10.5 before lyophilisation (C2). The pellet was re-extracted at pH 10.5 to recover remaining proteins, the supernatant adjusted to pH 6 then centrifuged where the supernatant and precipitate were lyophilised (C3, C4).

3.1.2 Protein-based plastics production

Compression moulding

Oilseed meal or protein concentrate was mixed with glycerol or glycerol/additives by hand using a mortar and pestle (2-5 min) (Paper I, II, IV). When additives were used they were pre-dissolved in glycerol if possible or premixed dry with the protein powder (Paper II). For comparison to extruded WG/crambe, U was pre-dissolved in glycerol (60 ^{o}C) and the raw materials were mixed in a kitchen type mixer (Paper III). The mixed material was placed in an aluminium frame (0.5 mm thick) to control final thickness using polyethylene terephthalate release films and placed in heated hydraulic press. For post extrusion compression moulding, extruded films were cut into squares and pressed in a 0.5 mm thick frame (Paper III).

Extrusion

For extrusion, the raw materials were mixed as in WG/crambe above (Paper III). The premix was either fed into the extruder as raw dough with a manual pusher to ensure feeding or was pre-pelletized by extrusion with a low barrel temperature profile. Pre-pelletized material was fed both by hand and volumetrically with a screw feeder. The extruder used was a 20 mm co-rotating twin (48:1 length:diameter, LTE20-48, Labtech Engineering) equipped with either a flat sheet die, 45 mm X 0.07 mm, or 2 strand die for pellet manufacture. At the die exit the extrudates were carried by an air cooling conveyor with fans (Paper III).

3.2 HPLC

3.2.1 SE-HPLC

A three step extraction procedure was used to determine the amount of soluble protein and its MW distribution by size exclusion HPLC (SE-HPLC) (Gällstedt *et al.*, 2004) (Paper I, II, IV). Samples were reduced in size (approx. 0.2 mm)

by hand cutting where necessary, weighed (16.5 mg) into 1.5 ml centrifuge tubes and 1.4 ml extraction buffer added (0.5% sodium dodecyl sulphate (SDS), 0.05 M NaH_2PO_4, pH 6.9). The samples were processed by serial extraction: 1) vortex 10 s, shake 5 min at 2000 revolutions per minute, 2) sonicate 30 s (amplitude 5 μm), 3) sonicate 30 s followed by cooling and sonication for 60 s (amplitude 5 μm). Each extraction step was followed by centrifugation at 16000 RCF for 30 min, decanting the supernatant into HPLC vials and re-extracting the residual with buffer in the next step.

Chromatographic separation was performed with a 20 μl sample injection under a isocratic mobile phase flow of 0.2 ml min^{-1} (Waters 2690 Separations Module, 50/50 water/acetonitrile, 0.1% trifluoracetic acid) through a prefilter (SecurityGuard GFC 4000, Phenomenex) and main column (Biosep-SEC-S 4000 300 X 4.5, Phenomenex). Chromatograms were extracted at 210 nm (Waters 996 Photodiode Array Detector) and integrated into two arbitrary fractions denoted as high and low MW with the intervals chosen based on the specific situation, approximately 8 to 18.5 min and 18.5 to 30 min, respectively. Integrated areas were normalized to the total extractable protein for the source material and adjusted for protein dilution from glycerol or additives. Overlapping peaks from additives (e.g. SA, Paper II) were removed manually where necessary. All samples were evaluated in triplicate (Paper I, II, IV).

3.2.2 RP-HPLC

Serial extraction was carried out on 100 mg samples using 6 extraction steps with 1 ml for each extraction, separated by reversed phase HPLC (RP-HPLC) as in Rasheed *et al.* (Rasheed *et al.*, 2015) (Paper IV). Extraction steps were as follows: 1) 70% ethanol, RT, 2) 50% propanol, RT, 3) 50% propanol, 60 ^{o}C 30 min, 4) 0.5% SDS, 50% propanol, 60 ^{o}C 30 min, 5) 1% dithiothreitol (DTT), 50% propanol, 60°C 30 min, 6) 1% DTT + 1% SDS, 6 M U 100 ^{o}C (oven) for 5 min. These steps were designed to perform the following functions: 1,2) disrupt weak non-covalent bonds; 3) thermal unfolding assisting in solubilising weakly bound aggregates; 4) disrupt inter-molecular hydrogen bonds and denature structure; 5) reduce disulphide bonds and assist in unfolding, 6) inter- and intra-molecular hydrogen bonds disrupted with disulfide bonds reduced for maximum denaturation and solubility. After the 6 serial extraction steps remaining insoluble proteins are considered cross linked with nonreducible covalent bonds.

Separation was carried out with a linear mobile phase gradient of 28-72% (acetonitrile in water, 0.1% trifluoracetic acid, Waters 2690 Separations Module) over 40 min and a flow of 0.8 ml min^{-1} with a C8 pre-column (5 μm,

2 cm X 4.0 mm, Discovery bio wide, Supelco) and main C8 column (5 µm, 250mm X 4.6mm, Discovery bio wide, Supelco) with a 50 µl injection volume. Absorbance was measured at 210 nm (Waters 996 Photodiode Array Detector) and integrated over the entire elution. For comparisons between samples, normalization was carried out as in SE-HPLC (Paper I, II, IV). Extractions were carried out in triplicate.

3.3 Tensile testing

Tensile specimens were manufactured to ISO 37 type 3 (die cut) and conditioned for a minimum of 48 h at 23 ^{o}C and 50% RH before testing. Specimens were tensile tested at 10 mm min^{-1} on a universal testing machine (Instron 5566 (Paper I, II, IV) or Zwick 7010 (Paper III)) under the same conditions as during the conditioning period.

3.4 Immersion

Five replicates were punched (5 mm diameter disks) from oilseed meal films and lyophilised, weighed and immersed in water for 24 h at 4 ^{o}C to prevent microbial growth. After immersion, sample disks were blotted to remove surface water and weighed, re-lyophilised and again weighed. The water absorption and mass loss during immersion was calculated on a dry basis. Drying was carried out by lyophilisation before immersion not with heat as in section 3.7 to avoid thermally induced changes to protein structure (Paper II).

3.5 Oxygen permeability

The oxygen transmission rate (OTR) was measured according to ASTM D3885, 50% RH, 23 ^{o}C (Mocon OX-Tran Twin). Each sample was covered on both sides by aluminium foil exposing 5 cm^2 to oxygen. Specimen diffusion cells were initially purged with nitrogen to measure background oxygen leakage. One side of the film was exposed to flowing oxygen at atmospheric pressure (Paper III).

3.6 Scanning electron microscopy

Sample surface and fracture cross sections of extruded and compression moulded samples were examined by scanning electron microscopy (SEM; field emission Hitachi S-4800 and Hitachi TM3000). Samples were coated with a conductive palladium–platinum layer prior to imaging (Paper III).

3.7 Density measurements and moisture content

Density was measured by the Archimedes principle, weighed in air and n-hexane. The moisture content was obtained according to standard ASTM D664. After drying at 105 ^{o}C for 24 h and cooling in a desiccator containing silica gel for 1 h at room temperature, the final mass was determined (Paper III).

4 Results and discussion

4.1 Processing effects on protein molecular weight profile

In protein-based materials, protein-protein interactions determine the material properties. In naturally occurring protein materials, like silk, these interactions occur as a consequence of many factors; the primary, secondary and tertiary structure and AA bonds such as disulphide bonds (Buehler *et al.*, 2008) and those promoted by enzymes such as peroxidase (Bailey, 1991). In oilseed storage proteins the natural protein structure does not promote good material properties. During processing the conditions to form structures that improve material properties of the protein must be provided. Oilseed meals present another challenge as they consist of considerable material other than protein. As protein is the largest proportion of the oilseed meal, the materials produced are viewed in terms of changes to the protein MW profile as a result of processing.

Of the processing options available, the application of heat is simple to perform, creates no residues for evaporation or disposal, and fits into existing commercial plastics practice. Heat is known to result in protein denaturation, the promotion of new AA bonds and the rearrangement of existing bonds (Rombouts *et al.*, 2013; Rombouts *et al.*, 2010; Friedman, 1999). On the other hand, increased temperatures can also result in protein breakdown (Pommet *et al.*, 2004), setting an upper processing temperature range. In examining changes in MW profile there are two aspects, the soluble proteins and the proteins that have become insoluble during heating by forming a cross linked network. The MW of the soluble proteins can be revealed through SE-HPLC, the amount of proteins that have become insoluble can be calculated as well.

4.1.1 Processing of Industrial oilseed meals

In the studied oilseed meals the minimum of protein solubility appears at about 140 °C, indicating the maximum extent of aggregation, with increasing solubility thereafter (Figure 3a). This is not surprising as the storage proteins both meal sources mainly consist of cruciferins and napins which are highly conserved between brassica species (Wanasundara, 2011). This similarity in the protein makeup leads to their similar behaviour. These changes are not distributed evenly across the MW spectrum, with the high MW fraction aggregating to a greater extent than low MW fraction in the temperature range of 100-140 °C (Figure 3b, c). Above 140 °C the gains in solubility are mainly in the low MW range, suggesting protein fragmentation by thermal breakdown (Figure 3b, c) (Pommet *et al.*, 2004) (Paper I).

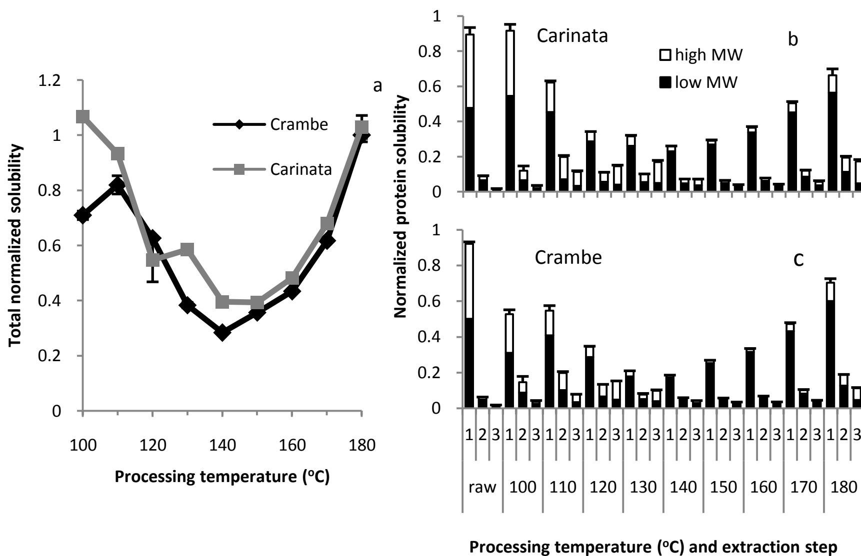

Figure 3. Changes in solubility and MW distribution of crambe and carinata oilseed meal plasticized with 30% glycerol on heating from 100 to 180 °C: a) total soluble proteins, summed from three extraction steps, b, c) solubility for each extraction step (1-3) divided into high MW and low MW fractions. Reproduced from Paper I with kind permission from Springer Science and Business Media.

Plasticizers, such as glycerol (Rasheed *et al.*, 2014; Athamneh *et al.*, 2008) or water (Mohammed *et al.*, 2000), have been observed to affect the aggregation of proteins on heating. The presence of glycerol results in variations in the protein solubility of thermally processed proteins, although the effect depends on the source meal and temperature (Figure 4). As with the effect of temperature, plasticizers affect high MW and low MW fractions

differently (Paper I). At 100 ^{o}C crambe showed lower solubility at the highest glycerol level, while carinata was unaffected by glycerol level. At 130 ^{o}C both oilseed meal types show increased high MW extractable proteins vs. lower temperatures, in carinata this increase resulted in a higher total solubility. The differences in behaviour indicate that there are changes in protein-protein interaction driven by plasticization. The distribution of glycerol between protein and non-protein components may be playing a role, where in crambe at 100 ^{o}C and 30% glycerol protein mobility and thus reactivity is enhanced compared to lower glycerol levels. At 160 ^{o}C there was little difference in both MW ranges with glycerol content**.** As temperature is increased the proteins have more thermal mobility making the effect of glycerol as a plasticizer less important.

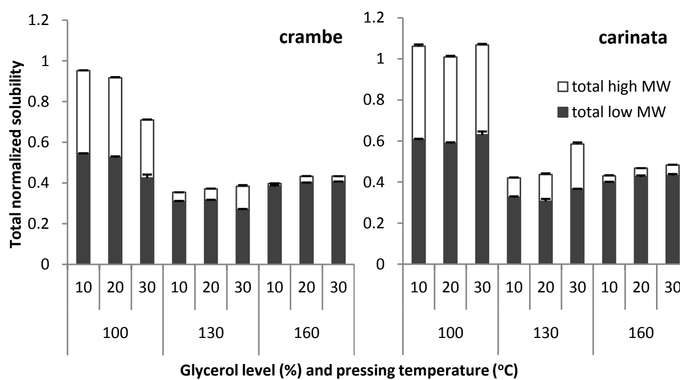

Figure 4. Solubility of hot pressed oilseed meals as affected by temperature and glycerol level. Sum of three serial extractions. Error bars denote one standard deviation. Adapted from Paper I with kind permission from Springer Science and Business Media.

Plasticizers have been observed to affect the aggregation and rearrangement of proteins on heating, such as glycerol (Rasheed *et al.*, 2014; Athamneh *et al.*, 2008) or water (Mohammed *et al.*, 2000). In our system it appears that, in some cases, the glycerol level is important and in others less so (Figure 4, Paper I). This may be due to the presence of discrete events of protein denaturation at certain glycerol/temperature combinations. In the crambe case at 100 ^{o}C one of these transitions may be between 20 and 30% glycerol, in both meals it appears there is a transition between 20 and 30% glycerol at 130 ^{o}C. At 160 ^{o}C all glycerol levels and meals behave similarly, possibly because thermal effects dominate at higher temperatures. The presence of the plasticizer glycerol may allow protein rearrangement at lower temperatures and affect the thermal

aggregation response. Further study with a finer scale of glycerol level and temperature is needed to resolve these effects.

4.1.2 Processing effects in crambe protein concentrates

To change the final protein profile resulting from processing protein-based materials one can also modify the starting profile rather than the processing conditions (Paper IV). By solubilising de-oiled crambe meal in different aqueous solutions such as pure water, 3% NaCl, or pH11 (NaOH) (Figure 2), solutions with differing MW profiles can be obtained (Figure 5). These solutions can be lyophilised whole to recover the protein in solution, membrane processed (e.g. dialysis) (Kroll *et al.*, 1991) or isoelectrically precipitated to concentrate a fraction of the protein (Massoura *et al.*, 1998). The combination of different extraction and recovery procedures results in protein concentrates of various MW distributions, which when processed with heat and plasticizers affects the final MW distribution and level of unextractable proteins (Paper IV).

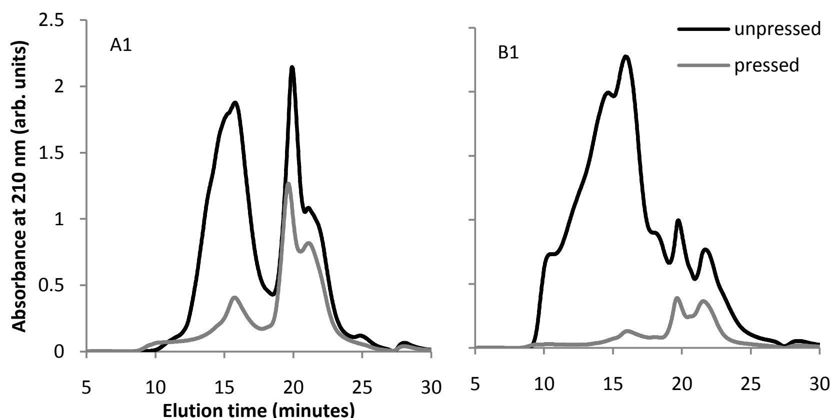

Figure 5. Size exclusion chromatogram of concentrated crambe protein before and after processing (130 °C, 15% glycerol, 5 minutes): A1) water extract - lyophilisation, B1) pH 11 extraction – pH 5.5 precipitation. Absorbance adjusted for protein content. All chromatograms are the sum of three serial extractions. Arb. units – arbitraty units.

The extraction and concentration of protein from crambe meal affects the types of protein-protein interactions that occur during protein processing (Paper IV). Through the use of selected solvents on the pressed films, specific types of protein-protein interactions can be disrupted, such as DTT disrupting disulphide bonds (Rasheed *et al.*, 2014). Through this selective disruption the effect of procedures used to extract protein from the meal on the resulting

protein behaviour can be revealed (Figure 6). Proteins produced by direct lyophilisation of supernatants (Figure 2, A1 and A3, B3, B5, C1, C3) result in protein-protein interactions that are relatively easily disrupted, with high solubility in 70% ethanol that disrupts weak intermolecular bonds (Figure 6) (Rasheed *et al.*, 2014). Successive extraction of the pressed film samples using increasingly disruptive aqueous solutions results in a high level of total extraction (Figure 6, Paper IV).

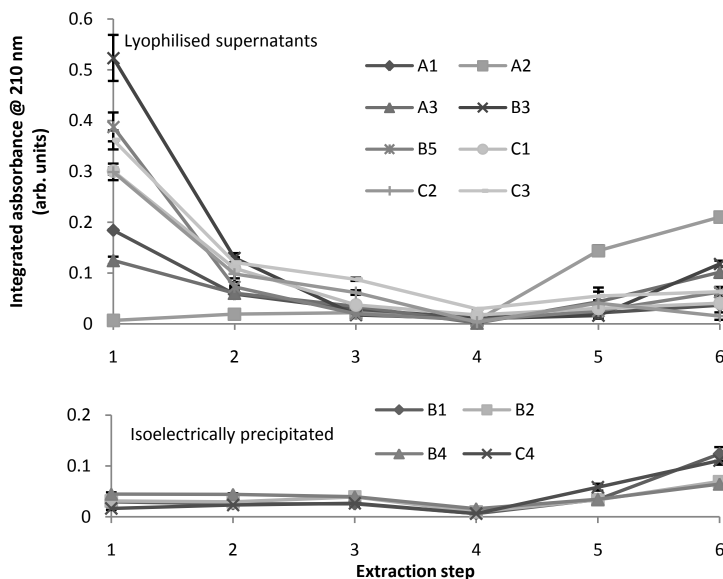

Figure 6. Total integrated absorbance from RP-HPLC chromatograms for hot pressed crambe protein concentrates (130 °C, 15% glycerol 5 minutes) extracted in different solvents: 1) 70% ethanol, RT, 2) 50% propanol, RT, 3) 50% propanol, 60 °C 30 min, 4) 0.5% SDS, 50% propanol, 60 °C 30 min, 5) 1% DTT, 50% propanol, 60 °C 30 min, 6) 1% DTT + 1% SDS, 6 M U 100°C (oven) for 5 min. Absorbance normalized for protein content. Error bars denote one standard deviation. Arb. units – arbitraty units

When proteins are isoelectrically precipitated (Figure 2, B1, B2, B4, C4), protein concentrates of higher purity are produced with their MW distribution shifted to the high MW end (Figure 5, B1). When hot pressed (130 °C, 15% glycerol) these protein concentrates show low solubility in SDS buffer, with the greatest decreases in the high MW end of the protein distribution (Figure 5, B1). Furthermore, the isoelectrically precipitated protein concentrates are resistant to alcoholic extraction, with DTT or DTT + U in the solvent extractability increases (Figure 6). Despite DTT and DTT + U treatment the isoelectric precipitates still have a large amount of unextractable protein

compared with most of the lyophilised supernatants (Paper IV). Resistance to extraction when DTT and DTT + U is present indicates the existence of covalent cross links other than the disulphide type (Rasheed *et al.*, 2014).

This may lead one to believe that a high MW-rich protein distribution leads to a high degree of protein aggregation, but the behaviour of fraction A2 indicates an alternative hypothesis (Figure 6). Although A2 has a MW distribution and purity similar to the other lyophilised supernatants (such as A1 and A3 (Paper IV)), it has been dialyzed (2000 MWCO) removing low MW (non-storage protein) components. On hot pressing (130 ^{o}C, 15% glycerol) the SDS extractable protein in A2 drops substantially across the entire MW range in contrast to A1 and A3, despite their similar MW distributions (Paper IV). A2 also shows low extractability in solutions without DTT. With DTT and DTT + U there is some increase in extractability for A2 indicating a degree of reducible disulphide bonding and hydrogen bonded aggregation (Figure 6) (Paper IV). Despite the increases in solubility of A2 with DTT and DTT + U, a large amount of protein remains unextractable, similar to the isoelectrically precipitated fractions (B1, B2, B4, C4). The behaviour of A2 suggests that the aggregation response observed may not only be a consequence of the protein profile, but of low MW substances (<2000 MWCO) that interfere with aggregation (Paper IV).

4.2 Impact of additives on protein profile and processing behaviour

Hot compression moulding of unmodified crambe and carinata industrial oilseed meals (Figure 3, 4) indicates that there may be room to improve properties by modifying the processing environment; even the best conditions resulted in over 20% protein solubility (Paper I). In order to affect the outcome of hot compression moulding a series of additives were studied in order to assess their effect (Table 1). The additives chosen are fairly benign from an industrial health and safety point of view and can be renewably sourced. An exception to this is Jeffamine™ (JF) which may be toxic but was chosen to provide thermally stable and reactive amine sites compared to U.

Additives that raise pH (NaOH and ammonium hydroxide (AH)) were found to have the largest effect on crambe and carinata meal-based materials (Figure 7, Paper II). Solubility decreased with lower additions of NaOH (1.5, 3 parts per hundred parts of 70:30 meal:glycerol (pph)) and at all levels of AH. At higher levels of NaOH (4.5 pph) an increase in both high MW and low MW in the extractable proteins compared to the control indicated protein breakdown at our pressing conditions (130 ^{o}C, 30% glycerol). Basic conditions are known

to promote protein-protein cross linking such as isopeptide and lanthionine cross links (Rombouts *et al.*, 2013; Rombouts *et al.*, 2010; Friedman, 1999). Sugars that are also present in the meals (Pedroche *et al.*, 2004; Steg *et al.*, 1994) could form Maillard type cross links, are also promoted by a basic environment (Singh, 1991). In AH these effects do not change appreciably with dose, perhaps due to the mechanism being saturated at the initial dose and AH not being a strong enough base to result in degradation at high doses.

Table 1. Additives for modifying hot compression molded crambe and carinata oilseed meal with their proposed action.

Type	Additive	Abbreviation	Proposed Action
Base	NaOH	NaOH	Promote protein-protein interactions
	NH_4OH	AH	Promote protein-protein interactions
Acid/Base	Citric acid/NaOH	CA/NaOH	Cross linking
Acid	Salicylic acid	SA	Retard protein-protein interactions
	Citric acid	CA	Cross linking
	Ascorbic acid	As	Improve protein behaviour
Reactive	Jeffamine EDR 176™	JF	Provide reactive amine sites
	Benzoyl peroxide	BP	Thermally decomposing oxidant
Denaturant	Sodium dodecyl sulphate	SDS	Allow protein rearrangement
	Urea	U	Allow protein rearrangement
Reductant	Sodium bisulphite	SB	Allow protein rearrangement

In aqueous protein extracts from crambe the modification of pH to 10.5 (NaOH) in solution after extraction (C2, Paper IV) resulted an increase in the high MW fraction compared with the unmodified extract (C1, Paper IV). Despite this shift of MW in the concentrate with increased pH, the outcome after thermal processing was not affected by the pH modification (hot pressed C1 vs. hot pressed C2, Paper IV). This indicates that the starting composition, which is identical in C1 and C2, plays a larger part than aggregation before pressing induced in C2. This may also be due to a lower level of alkali present during pressing of C2 compared to the oilseed meals of Paper II.

Combinations of CA and NaOH were added to the oilseed meals in order to examine the possibility of CA cross linking as observed in other protein systems (Xu *et al.*, 2015; Reddy *et al.*, 2009). At a constant level of CA (3 pph) and varying NaOH (1.5, 3, 4.5 pph) the lowest level of NaOH addition resulted in high solubility for crambe, as in the case of CA alone (Paper II supplemental data). Higher levels of NaOH resulted in small changes in solubility and MW profile, indicating a simple pH effect (no CA cross linking) (Figure 7). A constant CA/NaOH ratio (2:1) resulted in only slight changes in solubility and

MW profile, with the exception of the highest level in crambe (9/4.5 pph) where the extractable high MW fraction was reduced (Figure 7). In the case of CA cross linking one would expect a dose dependant response and a marked departure from the control, which was absent in our case.

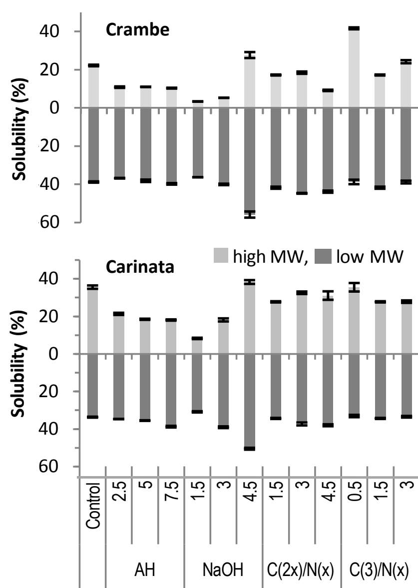

Figure 7. Protein solubility of hot compression moulded crambe and carinata oilseed meals (30% glycerol, 130 ^{o}C, 10 minutes), divided into high MW and low MW fractions by SE-HPLC. Solubility is expressed as a % of the total solubility of unpressed raw meal. Error bars denote one standard deviation. Adapted with permission from Paper II, Copyright 2014 American Chemical Society.

Recent work has highlighted the importance of deprotonating the CA for successful protein cross linking (Xu *et al.*, 2015) and it is unknown if this condition has been met during pressing oilseed meals as a plasticized solid. The use of CA alone has been shown to fail in cross linking of zein protein (Selling & Sessa, 2007) and did not result in any improvement in the MW profile at any level used, showing higher protein solubility than the raw unprocessed meal in both MW fractions (Paper II supplemental information). The additives surveyed not represented in Figure 7 were found to have only a small effect on the protein behaviour (As, SDS, SA) or resulted in an increase in protein solubility (U, SB, CA) (Paper II supplemental information).

In examining the samples with reduced solubility due to the series of additives, the bulk of the decreases come from the high MW end of the MW

range demonstrating a higher tendency of the high MW protein fraction to form aggregates compared to the low MW fraction (Figure 7). A similar trend was seen for purified proteins when small molecules were not removed by dialysis (section 4.1).

Previous work has shown that extrusion of crambe and carinata oilseed meals is difficult to achieve (Johansson, 2010) but processability is improved for crambe by the incorporation of WG and U (Henne, 2011). As an additive for modifying extrusion processing U levels of 15% were required for the production of acceptable films, 10% U could be extruded but not at acceptable quality for further testing (60:40 crambe:WG, 25.5% glycerol, Paper III). Under extrusion conditions U is likely to dissociate into ammonium and cyanate reacting with lysine and cystine groups of the proteins (Rombouts *et al.*, 2013). This effectively blocks cross linking reactions with these groups in addition to contributing to denaturing the proteins (Türe *et al.*, 2011). The effectiveness of this approach in the 60% crambe, 40% WG + 15% U system is in contrast to compression moulding of crambe meal without WG where 7.5 pph U resulted in films that were of poor quality (Paper II supplementary data).

4.3 Functional property relationships between processing and protein profile

4.3.1 Mechanical properties: strength stiffness and elongation

In crambe and carinata meals compression moulded with plasticizer, the combination of plasticizer level (10, 20 and 30% glycerol) and temperature (100, 130, 160 ^{o}C) results in a broad range of mechanical properties (Figure 8) (Paper I). The general change in properties at each temperature is as expected from plasticized protein systems in the literature; higher plasticizer levels lead to lower Young's modulus (E) and maximum stress values with a higher strain at maximum stress (Johansson *et al.*, 2012; Cho *et al.*, 2010; Rouilly *et al.*, 2006a; Zhang *et al.*, 2001). Similar systematic changes are not seen in the MW distribution (Figure 4), where changes do not follow glycerol level. This suggests that plasticization is responsible for the mechanical property changes with glycerol level, not changes in MW profile.

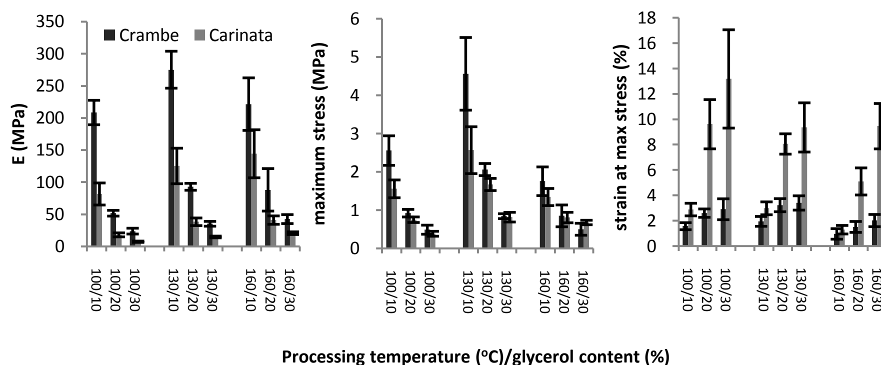

Figure 8. Effect of glycerol and temperature on mechanical properties of compression moulded crambe and carinata meal (10 minutes). Error bars denote one standard deviation. Adapted from Paper I with kind permission from Springer Science and Business Media.

At a constant glycerol level (30%) the effect of temperature on mechanical properties of hot compression moulded crambe and carinata meals showed 2 regimes, with a change between 130 and 140 °C (Figure 9). Young's modulus and maximum stress initially increase with temperature to 130 °C, then decreasing or staying constant thereafter. Maximum stress peaks at 130 °C for both meals. Strain at maximum stress shows the opposite behaviour in carinata with an initial decrease then slight increase after 140 °C, while crambe demonstrated little effect of processing temperature on strain at maximum stress. This transition is mirrored in the changes in total protein solubility, with a minimum at approximately 140 °C for both meals (Figure 3a).

The difference in response between the measured mechanical properties indicates their underlying relationship with the protein network. As E results from a rule of mixtures response of the phases present, an increase in protein aggregation increases E of the protein phase and thus the overall E. Maximum stress in our case appeared to coincide with the initiation of fracture (tearing), which is resisted by a more extensive network (Paper I). In the case of strain at maximum stress, carinata behaved in line with the changes in total protein solubility (Figure 3). Strain at maximum stress in crambe showed little change with temperature despite the changes in MW distribution, indicating that the failure mode was not dominated by the network in this case. It is suggested that failure in crambe is controlled by crack nucleation around seed pod particles (Paper I).

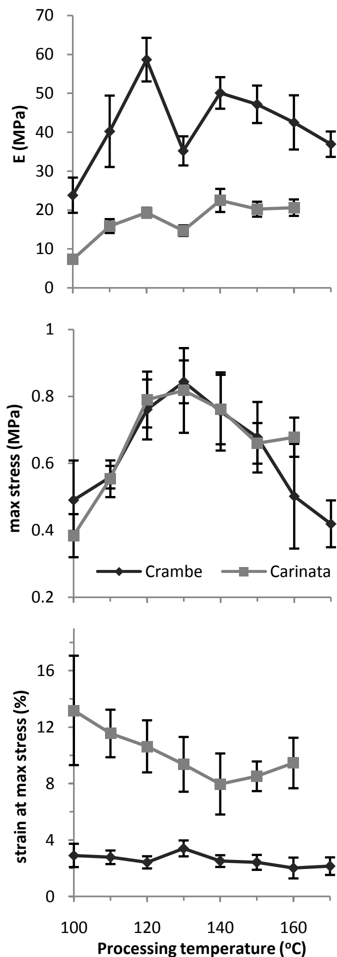

Figure 9. Effect of processing temperature on mechanical properties of compression moulded crambe and carinata meal. Error bars denote one standard deviation. Adapted from Paper I with kind permission from Springer Science and Business Media.

The inclusion of chemical additives (Table 1) with the de-oiled crambe and carinata seed meals resulted in changes in their tensile behaviour; with those that raise pH, AH and NaOH, making the greatest positive impact (Figure 10, Paper II supplemental data). The largest changes in mechanical properties with NaOH were observed in maximum stress and strain at maximum stress, and occurred at the lowest dose. This corresponds to the lowest solubility for NaOH treated proteins (Figure 7). The largest change from AH addition occurred with the initial dose, having relatively small changes thereafter, similar to the changes in protein solubility (Figure 7). Despite changes in protein solubility that indicate a more extensive network (Figure 7), E decreased with dose for both AH and NaOH. This may reflect changes in secondary structure due to the increased pH. Even though the network is more extensive, changes in secondary structure may make the network more flexible resulting in a lower E value (Paper II).

In the literature pH adjustment has been mainly applied to cast protein films where increased pH improves E for soy protein isolate and strength for both WG and soy protein isolate (Gennadios *et al.*, 1993). In compression molded wheat proteins, AH has been shown to improve E values of WG, gliadin and glutenin while tensile strength increased for gliadin and elongation at break decreased for all (Rasheed *et al.*, 2015), contrary to our results here (Figure 10). Previous work has been done with relatively pure proteins such as WG (*ca.* 80% protein) and soy protein isolate (*ca.* 90% protein), while in our case there are non-protein components present

that can interact with the proteins. For some of the non-protein components these reactions are promoted by a high pH environment, such as phenolics (Tan *et al.*, 2011), sugars (Ajandouz *et al.*, 2008) and phytic acid (Wanasundara, 2011), leading to a different outcome compared to the relatively pure protein case.

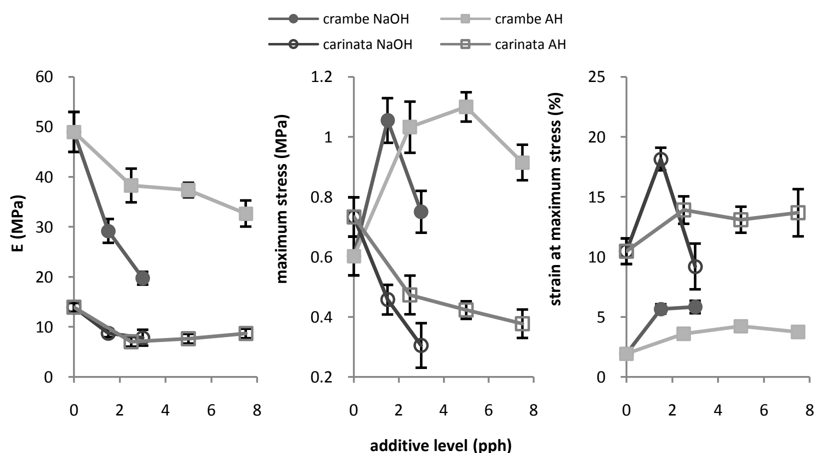

Figure 10. Effect of NaOH and AH on tensile properties. Additive level is parts of additive per hundred parts of oilseed meal:glycerol (70:30) mixture. All samples pressed at 130 ^{o}C, 10 minutes. Error bars denote one standard deviation. Adapted with permission from Paper II, Copyright 2014 American Chemical Society.

In the case of crambe meal/WG extrudates, the rheological demands of extrusion processing limit the process variables such as temperature, glycerol plasticizer or chemical additives to a small window (Türe *et al.*, 2011; Ullsten *et al.*, 2009; Pommet *et al.*, 2003). As the ability to adjust the conditions is limited, so is the ability to use them for modifying the final mechanical properties or MW distribution. For extruded film A of Paper III (60:40 crambe:WG, 25.5% glycerol, 15% U) the window for successful processing spanned die temperatures of 125 ^{o}C or 130 ^{o}C where the extrudates achieved similar tensile properties at either temperature (Table 2). By pre-pelleting composition A for improved feeding (strand extrusion, die temperature 85 ^{o}C) little impact was seen on the mechanical properties of film extruded from these pellets, the additional thermal history did not adversely affect the mechanical properties (Paper III).

A decrease of glycerol (composition C of Paper III, 60:40 crambe:WG, 20% glycerol, 16.1% U) required 130 ^{o}C die temperatures to achieve similar properties to composition A at the 130 ^{o}C or 125 ^{o}C die temperature. Reduced plasticizer content may be expected to increase E and maximum stress while

reducing strain at maximum stress. In this case these properties remained relatively constant. The additional temperature and lower plasticization may have resulted in protein degradation during extrusion, with the degradation products acting as plasticizers (Tuck *et al.*, 2014), thus modifying the mechanical properties. Increases in crambe content (70:30 and 80:20 crambe:WG, 25.5% glycerol, 15% U) resulted in decreases in mechanical properties compared to formula A, although they could be still extruded into films (Table 2, Paper III).

Examination of the morphology of the extruded films by SEM revealed internal and surface voids that could affect mechanical properties (density 1220 kg/m^3) (Figure 11). Hot compression moulding of extruded films was used to reduce these defects, with the possible effect of improving properties through thermally modifying the protein MW distribution. A second process, compression moulding at 110 ^oC with an aluminium frame to control thickness, increased density (1310 kg/m^3) and tensile properties with 2.7 times the E value, 2 times the maximum stress and little to no penalty in strain at maximum stress compared with the extruded film (Table 2). Re-pressing extruded films with no frame constraining the thickness resulted in higher densities (1330 kg/m^3) but inferior properties compared with those pressed with a constraining frame (Table 2). Deformation during re-pressing without a frame resulted in additional material flow compared to re-pressing with the frame that may have expanded the defects in the compression plane, leaving internal flaws that result in property degradation.

Table 2. *Tensile properties of selected crambe:WG extrudates and re-pressed extrudates (Paper III)*

Crambe:WG	Glycerol (%)	Urea (%)	Die T (^oC)	Re-press conditions T, press., time, constraint	E (MPa)	max. stress (MPa)	strain @ max. stress (%)
60:40	22.5	15	125	n/a	5.6(1.8)	0.5(0.1)	14(2)
60:40	22.5	15	130	n/a	5.1(0.6)	0.6(0.1)	19(2)
60:40	20	16.1	130	n/a	5.4(0.9)	0.7(0.1)	16(2)
60:40	22.5	15	125	110, 40.8, 10, frame	15(0.6)	1.1(0.2)	16(4)
60:40	22.5	15	125	120, 12.9, 5, no frame	8.6(1.5)	0.6(0.1)	10(1)

WG- wheat gluten, T-temperature (^oC), press. – pressure (MPa), time in minutes, max. – maximum. () denote one standard deviation.

In the case of concentrated crambe proteins (Paper IV), concentrates from dried supernatants (A1, A2 and A3, Figure 2) were not testable at high levels of glycerol, leading to 15% glycerol being used for all samples. In the high protein content precipitated proteins (B1, B2, B4 and C4, Figure 2) this low

level of glycerol led to brittle, glassy behaviour; characterized by a high E and low strain at maximum stress. Although there were a variety of different MW distributions in the materials made from precipitates, their behaviour is not expected to vary due to MW profile changes. In the glassy state it has been shown in WG based plastics that the mechanical properties no longer depend on the protein network dynamics (Bruyninckx et al., 2015) and this behaviour is suggested in precipitates B1, B2, B4 and C4 as well.

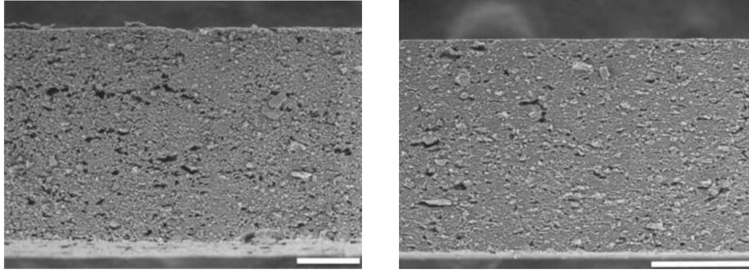

Figure 11. Cross sectional image of extruded film (60:40 crambe:WG, 25.5% glycerol, 15% U, left) and the same film re-pressed by compression moulding (110 ^{o}C, 20 min, right) demonstrating a reduction in voids. Bars represent 500 µm. Reproduced from Paper III with kind permission from Springer Science and Business Media.

4.3.2 Aqueous swelling

The effect of additives on the water absorption of crambe and carinata oilseed meals varied greatly, with NaOH having the greatest effect, with a water absorption of 73% for crambe and 126% for carinata (Figure 12, Paper II). Comparing results between the basic additives NaOH and AH, the differences may be a result of the evaporation losses of AH during pressing reducing the residual effect of AH on the pH of the immersed film. It has been shown that in cast cottonseed flour films, ammonia in the formulation does not affect the final pH (Marquie *et al.*, 1995) while for NaOH a residual effect is expected. Of the CA/NaOH treatments, little effect of treatment level on water absorption was shown (Paper II), perhaps as the effect of NaOH was neutralized by the CA content, mirroring their low effect on the MW distribution compared to the controls.

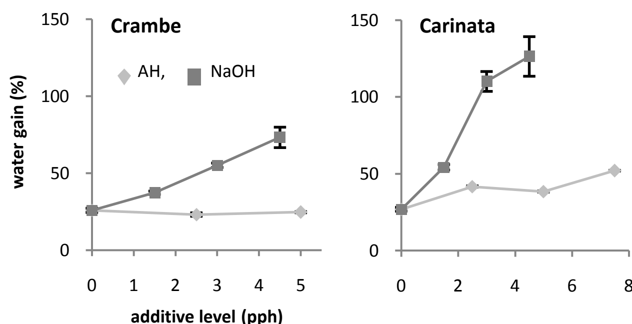

Figure 12. Effect of NaOH and AH on swelling in water. Additive level is parts of additive per hundred parts of oilseed meal:glycerol (70:30) mixture (pph). All samples pressed at 130 °C, 10 minutes. Error bars denote one standard deviation. Adapted with permission from Paper II, Copyright 2014 American Chemical Society.

Swelling in cross linked systems is expected to be controlled by cross link density, in which the macromolecular network responds to swelling with an equilibrium force stopping further solvent absorption (Van der Sman, 2012). In our case the water absorption increases with NaOH (Paper II) as the protein solubility initially decreases compared with the control, then increases (Figure 7). A reduced solubility implies a more compact network that should swell less (NaOH level 1.5 pph), but this is not the case. In expanding the network of a protein during swelling, the state of the segments between cross links play a role as well as the cross link density, local interactions such as secondary structure can prevent the segment from fully extending reducing swelling.

In samples of both meals pressed at 100 °C to 170 °C (30% glycerol) there was little change in swelling across the entire range (data not shown) although there was a large change in the protein profile (Figure 3). This indicates that the effect of NaOH on protein denaturation may be responsible for the increased water absorption, not MW profile or protein network changes. Chemically denatured protein segments between cross links will be able to more fully extend resulting in a higher degree of swelling. A similar effect of U denaturation in protein gel networks results in increased swelling than without denaturation (Van Kleef *et al.*, 1978).

4.3.3 Oxygen permeability

Low oxygen permeability has been put forward as a useful functional property in protein-based materials (Miller & Krochta, 1997). In WG/crambe meal composites SEM investigation revealed the presence of voids in the film surface and interior (Figure 11). Despite these voids, an oxygen permeability of 36.1 cm^3·mm/(day·m^2·atm) was obtained (60:40 crambe:WG, 25.5% glycerol,

15% U), considerably higher than extruded WG films of 0.26 $cm^3 \cdot mm/(day \cdot m^2 \cdot atm)$ (26% glycerol, 15% U) (Türe *et al.*, 2011). Increasing the crambe:WG ratio to 80:20 resulted in a minor change in permeability to 38.7 $cm^3 \cdot mm/(day \cdot m^2 \cdot atm)$ indicating that the crambe content alone is not responsible for the poor oxygen permeability compared to a similarly processed WG/glycerol/U film. In order to decrease the oxygen permeability, hot re-pressing was used (Paper III). This decreased the void content, increasing the density from 1220 to 1310 kg/m^3 thereby decreasing the oxygen permeability to 17.1 $cm^3 \cdot mm/(day \cdot m^2 \cdot atm)$, still far above similarly processed WG/glycerol/U film. Despite the repressing and density increase, many voids still exist in the film that may be responsible for the poor performance (Figure 11).

5 Applied aspects

In order to utilize de-oiled crambe or carinata meals directly, they have been plasticized with glycerol (Paper I-IV), hot compression moulded (Paper I, II, IV) extruded (Paper III) and treated with additives (Paper II). Of the additives investigated NaOH was most effective in improving the mechanical properties, and is also approved for food use (E524) reducing restrictions on the application of the final product in food contact. Compression moulding is a convenient laboratory process that can directly form parts such as packaging trays (Figure 13) and similar processes exist in current plastics production. The response of compression moulded oilseed meals to water immersion (Paper II) indicates their possible use in applications where some water permeability or absorption is desired in combination with resistance to dissolution and biodegradeability, such as in containers for plant propagation (Rouilly *et al.*, 2006b).

Figure 13. Hot compression moulded tray from de-oiled crambe meal.

In order to successfully implement the use of crambe oilseed meal/WG based materials, it is a great advantage these potential feedstocks can fit into the existing plastics production infrastructure. Pre-extruding crambe/WG/U/glycerol materials to form pellets that can be subsequently fed

into process equipment by dosing equipment has been demonstrated without a reduction in properties (Paper III). The possibility of using these materials in existing processing facilities is more likely without the need to purchase specialized equipment for extruder feeding. It has been demonstrated that post-processing such as hot compression moulding of extruded crambe/WG/U/glycerol films improves their aesthetics and barrier properties (Paper III). Similar processing may be possible with the use of a heated multi-roll calendar or a hot belt press to provide the same benefits in continuous film production.

In order to improve the performance of protein-based materials derived from de-oiled crambe meals it will be necessary to extract proteins. In terms of process simplicity and cost, the process route of alkali dissolution followed by isoelectric precipitation is the most attractive (Paper IV, Scheme B) resulting in isolates of high purity (*ca.* 90%, Paper IV). Protein isolates manufactured by isoelectric precipitation showed extensive network formation that was resistant to disruption (Paper IV). This highly aggregated state is desired in applications such as food packaging where migration from packaging material into the contents is an issue.

6 Concluding remarks

The industrial oilseed meals crambe and carinata are a new supply of materials for non feed or food uses. Their protein content makes it possible to process them with heat and plasticizers to make bio-based plastic films. The variation in the reactivity of proteins to heat in different environments allows the tailoring of film properties through modification of the protein MW profile. Protein profile is only part of the total material response not all properties correspond to changes in the protein component. The main conclusions and key findings are as follows:

- Compression moulding of crambe and carinata meals resulted in a minimum solubility at 130 ^{o}C – 140 ^{o}C which corresponded with maxima in E and maximum stress (crambe and carinata) and minima in strain at maximum stress (carinata). Above this 140 ^{o}C solubility increased due to protein breakdown.
- The addition of low levels of NaOH (1.5 pph) in hot compression moulding increased protein aggregation resulting in increased strain at maximum stress (crambe and carinata), and maximum stress (crambe only), and while E was reduced. The reduction of E at higher protein aggregation indicates that protein secondary structure could be modified by the high pH to form a more flexible network, despite the level of aggregation. An alternate explanation is that non-protein components were modified by pH, thus reducing the overall E. AH had a similar effect to NaOH, but to a lower degree, possibly due to AH evaporation at pressing temperatures.
- Crambe meal/WG/U/glycerol blend films were successfully produced with co-rotating twin screw extrusion, but the processing and composition window was small. Internal porosity reduced OTR

performance, but it was improved with hot compression moulding as a post-process.

- Protein extraction procedures had an effect on the MW profile of the proteins recovered and also on their response to subsequent thermal processing. High MW fraction of the concentrated proteins preferentially aggregated, in some cases.
- Increasing protein solution pH before freeze drying shifted the MW profile of the proteins to higher MW's. After thermal treatment there was no effect on MW profile of the increased pH. This contrasts with cast protein films in which initial solution pH plays a strong role.
- Separation of non-protein components from proteins by isoelectric precipitation or dialysis increased protein aggregation on heating. This indicates that the fraction of protein in the concentrate is not the only important parameter for thermal aggregation - the exclusion of low MW components is also important.
- In the cases of extensive protein concentrate aggregation on heating, extraction with reducing and denaturing agents was required for substantial protein extraction, indicating that some of the aggregation was due to disulphide bonding and secondary structural effects. In the presence of DTT-U solubility was still not high, indicating the presence of non-disulfide covalent cross links.
- The insights into the behaviour of concentrated proteins point to procedures that produce highly aggregated films. With levels of plasticization customized for each concentrate these proteins promise to form interesting materials in the future.

7 Future directions

In order to utilize crambe and carinata de-oiled meals directly their properties need to be improved. Although the use of NaOH improved the properties of pressed de-oiled crambe and carinata seed meals (Paper II) lower doses of NaOH need to be investigated to further optimize the system. Performance enhancing additives such as formaldehyde, glutaraldehyde and glyoxal that have been successful in seed meals (Yue *et al.*, 2012) should be investigated with proper controls regarding their health and safety issues (Álvarez-Chávez *et al.*, 2012). Polyaldehydes from oxidised sucrose hold some promise as a more sustainable alternative to conventional aldehydes (Liu *et al.*, 2015) and may improve properties of these protein materials while maintaining "green" credentials.

There are further possibilities for composites using oilseed meals as components such as in crambe/WG extruded films (Paper III). The success in extruding these compounds under industrial-like conditions (screw extrusion with pre-compounded pellets) warrants further investigation of this method, mainly to expand the processing envelope. This could take the form of additional additives, such as SA (Ullsten *et al.*, 2009), but equipment development to control early cross linking during processing is also of interest. Low level additions of NaOH (<1.5%) are suggested as they are known to improve the behaviour of both the oilseed components (Paper II) and WG (Ullsten *et al.*, 2009).

The glucosinolates in the seed meals of crambe and carinata that make them unattractive as food or feed (Pedroche *et al.*, 2004; Carlson & Tookey, 1983) may be used to some advantage. The breakdown products of glucosinolates are known to be natural pesticides (Brown & Morra, 2005) and could be included in plastics for horticultural applications. For such functionality processing would have to be carried out in such a way as to preserve the function of the enzyme myrosinase, which is required for glucosinolate breakdown. This may

be carried out using the aliphatic polyester polycaprolactone in combination with oilseed meal as polycaprolactone has a melting point of about 60 ^{o}C (Anon., 2014a). The processing window will be small as myrosinase loses its activity at 75 ^{o}C as measured in the mustard *S. Alba* (Van Eylen *et al.*, 2008). Myorsinases from other brassicas are expected to be similar, setting an upper temperature limit for processing. Such products could have self fertilizing properties as the protein degrades (Schrader *et al.*, 2013) in addition to the pesticide effects of glucosinolate breakdown products.

Extracted crambe proteins demonstrate improved protein-protein interactions for some fractions (Paper IV) and offer the possibility of selecting protein fractions that offer improved properties in the future. Once target proteins are identified, genetic modification is a possible route to increasing their concentration in the seed. As crambe is already a target for genetic modification of its lipid profile (Li *et al.*, 2012), the same plants could have a modified protein profile, thus increasing their overall value. Another *Brassica*, *Camelina sativa*, has already been modified to greatly reduce the napin content in the seeds, replacing it with an increased level of cruciferin (Nguyen *et al.*, 2013). The genetic modification approach could be applied to suppress the production of certain storage proteins and replace them with proteins of interest, such as insect resilin for medical biomaterials (Qin *et al.*, 2011) which has already been successfully expressed in genetically modified *Camelina sativa*[1].

The theoretical underpinnings of protein-based materials for practical applications require further study. If the macromolecular theories such as entropic elasticity (Van der Sman, 2012; Van Kleef *et al.*, 1978) are to be applied to cross linked protein materials a better molecular/structural description of the system needs to be determined. Measurement of protein MW profile and overall solubility are not adequate to address this type of model as it provides no direct information on where cross links occur or on their chemical type. New methods need to be applied to examine the required parameters, such as distance between cross links and the position and extent of secondary structure along the protein chains within the network. Control over cross link position on the protein chain, avoiding dangling non-network chain ends, closed loops (intra-chain cross links), and the extent and position of intra and inter-chain secondary structure will need to be elucidated in order to maximize material performance.

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[1] Personal communication, Prof. Edgar B. Cahoon (University of Nebraska, Lincoln), August 21, 2015

By utilizing techniques from proteomics, the specifics of individual cross links can be established (Sinz, 2003) and using this data the reaction conditions may be tailored to control cross link type and position and thus protein-based material properties. Atomic force microscopy infra-red techniques now offer a lateral resolution of 10 nm with secondary structure identification and local E measurement (Ruggeri *et al.*, 2015) which is reaching the scale of protein aggregates. This offers the possibility of probing the fine scale of structural development compared to conventional Fourier transform infrared bulk structural determination which may also provide insight into the phase structure of protein-based materials. These techniques will open up new opportunities to define the structures resulting from processing; allowing researchers to identify desired structures and tailor processing conditions to obtain them, thereby improving material properties.

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