Article

Novel protein-rich materials from rapeseed meal with enhanced mechanical behavior

Sara Aquilia , Luca Rosi , Michele Pinna , Sabrina Bianchi , Walter Giurlani , Marco Bonechi , Francesco Ciardelli * , Anna Maria Papini * , Claudia Bello

Posted Date: 27 June 2024

doi: 10.20944/preprints202406.1872.v1

Keywords: Rapeseed meal-based material; Protein-based biopolymers; Renewable biomaterials.

Preprints.org (www.preprints.org) | NOT PEER-REVIEWED | Posted: 27 June 2024 doi:10.20944/preprints202406.1872.v1

Article

Novel Protein-Rich Materials from Rapeseed Meal with Enhanced Mechanical Behavior

Sara Aquilia [1,2,3], Luca Rosi [2], Michele Pinna [3], Sabrina Bianchi [3], Walter Giurlani [2], Marco Bonechi [2], Francesco Ciardelli [3,*], Anna Maria Papini [1,2,*] and Claudia Bello [1,2,]

[1] Interdepartmental Research Unit of Peptide and Protein Chemistry and Biology, University of Florence, Via della Lastruccia 13, I-50019 Sesto Fiorentino, Italy
[2] Department of Chemistry "Ugo Schiff", University of Florence, Via della Lastruccia 13, I-50019 Sesto Fiorentino, Italy
[3] Spin-PET S.r.l., Viale R. Piaggio 32, I-56025 Pontedera, Italy
* Correspondence: annamaria.papini@unifi.it; ciardelli@spinpet

Abstract: In recent years there has been an increasing interest in developing novel materials based on natural biopolymers as a renewable alternative of petroleum-based plastics. The availability of proteins derived from agricultural by-products, along with their favourable properties, has fostered a renewed interest in protein-based materials, promoting research in innovative technologies. In this study we propose the use of rapeseed protein-rich meal as main ingredient for the preparation of novel sustainable materials combining excellent environmental properties such as biodegradability and renewable origin. Application as sustainable products in the present high-tech society needs modification of basic native properties of these natural compounds. The original route proposed in this paper consist in the preparation via compression moulding of flexible biomaterials stabilized by crosslinkers/chain extenders. Investigation of the effects of different denaturing and disulfide bond reducing agents, crosslinkers, and preparation conditions on the material mechanical behaviour demonstrated that the novel materials have appreciable strength and stiffness. The results show the potential of utilizing full meal from vegetable by-products to prepare protein-based materials with granted ecofriendly characteristics and mechanical properties adequate to specific structural applications.

Keywords: rapeseed meal-based material; protein-based biopolymers; renewable biomaterials

1. Introduction

Polymeric organic materials are extensively used across various sectors in modern society. The remarkable properties and versatility of polymers, coupled with their cost-effectiveness, have resulted in their widespread usage. Nonetheless, the unregulated disposal of the huge amount of plastic waste generated has given rise to one of the most pressing environmental challenges of our era [1–3], which concerns mainly with packaging and low performances, short live products.

Consequently, there is a growing interest in the development of environmentally friendly and biodegradable materials from renewable resources. This effort aims to replace petroleum-based materials in sustainable applications, which raise significant concerns regarding pollution and sustainability [4]. However, at present, bioplastics and in general bioderived organic materials constitute less than one percent of the annual production of plastic, which exceeds 390 million tonnes[5]. Transition to a circular economy model is also necessary for the development of innovative strategies to optimize resources utilization and eliminating the concept of waste throughout the entire supply chain[6–9]. Bio related plastics and organic materials derived from coproducts or waste materials offer the advantage of exploiting pre-existing, and often underutilized, resources. Furthermore, their utilization serves to mitigate disposal costs and to reduce environmental impacts [10].

Proteins from agri-food industry are a good alternative to petrochemical-based polymers. Rapeseed is a crucial oilseed crop, ranking second only to soybean, with Canada and United States being the top producers, while Europe accounts for almost 25% of the global production [11,12]. Rapeseed is manly employed for oil production in food and feed industry as well as for biodiesel preparation. The residual product from oil extraction is the rapeseed meal, also called canola meal, which is collected as a by-product after oil pressing and subsequent solvent extraction [12]. Depending on the growth conditions, harvest methods, and processing techniques, the protein content in rapeseed meals may vary from 35% to 40%[13]. Cruciferin (12S globulin) and napin (2S napin) represent the 60% and the 20% of the total rapeseed crude protein respectively. Cruciferin is a high molecular weight protein formed by six subunits each containing a heavy α-chain (30 KDa) and a light β-chain (20 KDa) linked by both disulfide bonds and non-covalent interactions. Napin is instead composed of one small (4-6 kDa) and one large (10-12 kDa) polypeptide chain that are held together by disulfide bonds[14,15].

Although rapeseed meal possesses significant nutritional potential and contains value-added compounds[16,17], its application in the food and feed sectors is limited by the presence of antinutritional compounds, such as glucosinolates, erucic acid, phytates, and phenols, which negatively affect the palatability and digestibility of the meal[12]. In recent years, because of its high storage protein content and low cost, exploitation of canola meal for the production of cosmetics and of bioplastic materials has emerged[13]. Rapeseed proteins offer indeed a promising ecofriendly alternative for the production of technical products such as polymers, coatings, adhesives, detergents and lubricants[18]. The development of a fully natural and potentially biodegradable protein-based material with acceptable mechanical properties could efficiently decrease societal request of fossil-based polymers in several applications[19].

However, application of proteins, including proteins from rapeseeds, as bio-based polymers is limited by certain drawbacks, including slow biodegradability, limited mechanical properties, and high water permeability[7,20–22]. Additives, such as plasticizers, are commonly integrated into the biopolymers to improve these limitations. Plasticizers break inter- and intra-molecular hydrogen bonds, resulting in enhanced mobility of polymer chains, greater spacing between polymer molecules, and a reduction in the proportion of crystalline regions compared to amorphous regions. Glycerol and water are common plasticizers in protein-based materials, because of their low molecular mass and ability to interact with polar residues[23–25]. Additionally, crosslinkers are used to enhance the mechanical strength, the thermal and chemical stability and to decrease the swelling properties of polymeric and biopolymeric materials[26,27]. Nevertheless, high crosslinking might lead to a decrease in biodegradability of the material. Therefore, using eco-compatible crosslinkers and optimizing the amount of crosslinker in the material enable exploitation of its favourable effects while minimizing the drawbacks.

We describe herein an original route for the preparation of flexible biomaterials based on raw, protein-rich rapeseed meal stabilized by modulated crosslinking with selected epoxides, using compression moulding. To the best of our knowledge, the development of commercially suitable industrial products from raw rapeseed meal is very limited and the studies involving rapeseed biomass are mainly focused on protein isolate-based bioplastics and on wet casting techniques[28–30]. Moreover, we investigate the effects of different denaturing and disulfide bond reducing agents, cross-linkers, and preparation conditions on the mechanical behaviour. Thermal properties were also studied through TGA, thus showing the potential of our approach to prepare novel biocompatible materials from vegetable waste.

2. Materials and Methods

2.1. Materials

The rapeseed meal (RM) was generously supplied by Italcol S.p.A. (Castelfiorentino, Italy). Rennet casein was provided from Fontana Enzo s.r.l..Glycerol, poly(ethylene glycol) diglycidyl ether, neopentyl glycol diglycidyl ether, epoxidized soybean oil, sodium sulfite (Na_2SO_3), sodium dodecyl sulfate (SDS), urea, guanidinium chloride, and anhydrous KBr were purchased from Sigma-Aldrich (St. Louis, USA). They were of analytical or reagent grade and were employed without additional purification.

2.2. Rapeseed Meal-Based (RM) Material Preparation

The rapeseed meal was dry-milled at 1800 rpm for 15 minutes at room temperature. The resulting powder was placed in an oven overnight at 50°C to remove moisture. Next, 40g of rapeseed meal (53% w/w total) was mixed with 10g of a protein-binding agent (Rennet casein, 13 wt/wt%), 20g of glycerol (27% w/w), 5g of distilled water (7% w/w), and 2.5g of Poly(ethylene glycol) diglycidyl ether (2x mol lysine-based) using a blender blade for 15 minutes to obtain a homogeneous blend. Glycerol was used as plasticizer, and Poly(ethylene glycol) diglycidyl ether was employed as a cross-linker. Subsequently, the mixture was heated to 150°C for 15 minutes and pressed using a flat-bed press (CAMPANA model: PRESSA PM20/200) at 150°C for 20 minutes at 250 Bar (mould dimensions: 8 cm x 16 cm x 1.5 mm). The resulting material (RM, Table 1) was then cooled to room temperature and stored in a desiccator with 75% humidity control.

Table 1. Rapeseed meal-based (RM) specimens formulation.

Sample	Formulation			
	Rapeseed meal/Compatibilizer	**Glycerol**	**Water solution**	**Epoxy-PEG**
RM-R0	100:0	27%	Distilled water 7%	-
RM	04:01	27%	Distilled water 7%	2x mol
RM-R1	04:01	27%	(Na_2SO_3 10%) 7%	2x mol
RM-R2	04:01	27%	(Na_2SO_3 20%) 7%	2x mol
RM-D1	04:01	27%	(UREA 2M) 7%	2x mol
RM-D2	04:01	27%	(Guanidine 1M) 7%	2x mol
RM-D3	04:01	27%	(SDS 3%) 7%	2x mol
RM-R1D2(7%)	04:01	27%	(Na_2SO_3 10% + Guanidine 1M) 7%	2x mol
RM-R1D2(14%)	04:01	27%	(Na_2SO_3 10% + Guanidine 1M) 14%	2x mol
RM-R2D2(7%)	04:01	27%	(Na_2SO_3 20% + Guanidine 1M) 7%	2x mol
RM-R2D2(14%)	04:01	27%	(Na_2SO_3 20% + Guanidine 1M) 14%	2x mol

Modified rapeseed meal specimens (see Table 1 for detailed composition) were prepared following the above described procedure except for the substitution of the distilled water with an aqueous solution of Na_2SO_3 (10% w/w), Na_2SO_3 (20% w/w), Urea (2M), Guanidine (1M), or SDS (3%w/w).

2.3. Mechanical Properties

After pressing, the rapeseed meal specimens were kept at 25°C and 75% RH for 24 hours, and then cut into three or four standard dumbbell-shaped moulds, each measuring 60 mm (length) x 5 mm (width) x 1.3 mm (thickness). The mechanical properties were evaluated using a Shimadzu instrument (Model AGS-X 5 KN) following the Standard Test Method for Tensile Properties of Plastics (ASTM D638-91). The testing speed was set at 5 mm/min. Tensile strength, elongation at break (%), and elastic modulus were determined based on measurements taken from at least three replicates.

2.4. Fourier-Transform Infrared Spectroscopy

Fourier-Transform Infrared Spectroscopy (FT-IR) analysis of the specimens was conducted in transmittance mode using a Jasco FT/IR-4200 instrument. Prior to analysis, the materials were dried in an oven at 60°C for 48 hours. Subsequently, the dried samples were milled into powder using a mortar and pestle, and blended with anhydrous KBr in a 1:50 ratio. The resulting mixture was then pressed to form transparent pellets. Spectra were collected over the range of 400 to 4000 cm-1 with 32 scans at a resolution of 4 cm-1. The obtained spectra were further analysed using Origin Pro 2021.

doi:10.20944/preprints202406.1872.v1

2.5. Thermogravimetric Analysis

The thermal stability of the protein-based materials was assessed via thermogravimetric analysis (TGA) conducted utilizing a Perkin Elmer 4000 instrument. The samples were subjected to controlled heating under a continuous flow of nitrogen (30 mL/min) from room temperature to 815 °C at a programmed ramping rate of 10 °C/min.

2.6. Scanning Electron Microscopy

Scanning electron microscopy (SEM) analysis were performed with a variable pressure Hitachi SU3800 (Hitachi High-Tech, Tokyo, Japan) equipped with a Ultim Max 40 silicon drift EDS detector and AZtecLive software (Oxford Instruments NanoAnalysis, Abingdon, United Kingdom). The measurements were performed at various magnifications with an accelerating voltage of 15 kV. Before the SEM analysis the samples were metallised using a SC7640 Polaron Sputter Coater with a SC510-314B gold/palladium target (Quorum Technologies, Laughton, United Kingdom). Both the surface and the section of the samples were analysed. For sectioning we opted for a cryofracture: the samples were immersed for approximately one minute in liquid nitrogen to ensure that all the material reached the same temperature and then it was split in two using two forceps while keeping the polymer immersed in liquid nitrogen.

3. Results and Discussion

Preliminary tests to assess the behaviour of canola meal in hot pressing were carried out and optimized temperature (150°C) and pressure (250 bar) conditions were determined. Afterwards the influence of the method used for blend preparation and of the addition of plasticizers and cross-linkers were evaluated. The best results were obtained using a blender blade as mixing method, water and glycerol as plasticizers, and poly(ethylene glycol) diglycidyl ether (Epoxy-PEG) as cross-linker.

Glycerol, an environmentally friendly, effective plasticizer for biopolymer-based materials,[31] in particular those derived from proteins[32], was used in the same quantity as in applications reported in the literature[33–35]. Epoxy-PEG, a hydrophilic epoxydic compound which can also be obtained from natural resources, was used in amount based on the moles of Lys (2.86% of the full amino acid content) [36] present in the meal proteins. The epoxy groups exhibit high reactivity with the amine and carboxyl functional groups present in the protein's side chains. Notably, even at 70°C, the amine and epoxy groups demonstrate a significant reaction rate[19]. Further significant improvements were obtained adding Rennet casein[37,38] in a 4:1 ratio canola meal/casein to the preparation.

Those preliminary tests demonstrated that raw canola meal can be used in heat pressing processes to produce a compact material with promising mechanical properties even if still not at the level of fossil plastic polymers.

3.1. Influence of Different Crosslinkers on the Tensile Properties of the Specimens

Starting from the results obtained from the preliminary tests, the effect of three different epoxydic crosslinkers on the properties of our material were evaluated. We thus compared the hydrophilic Epoxy-PEG with epoxidized soybean oil, a biobased hydrophobic crosslinking agent already used as stabilizer and plasticizer in PVC and bio-based materials[39,40] and neopentyl glycol diglycidyl ether. The latter is hydrophobic in nature, with a smaller steric size and lower molecular weight than the other two. This may allow for better diffusion within the blend, compared to the epoxidized soybean oil[41,42]. The Young’s modulus and the stress at fracture of samples RM-ES and RM-NE decreased substantially, as reported in Table 2. The particularly low elongation is probably primarily due to structural microfracture of the materials deriving from a not complete and uniform diffusion of the crosslinker in the protein-rich matrix and the heterogeneous chemical nature of the matrix itself. Moreover, only the material prepared using epoxidized soybean oil (RM-ES) and neopentyl glycol diglycidyl ether (RM-NE) showed an exudate on the surface already few hours after preparation, probably due to the migration of the non-reacted crosslinker.

doi:10.20944/preprints202406.1872.v1

Table 2. Tensile mechanical properties of blends with different crosslinkers.

Sample	Mechanical properties		
	Elastic modulus (N/mm^2)	stress at fracture (N/mm^2)	Elongation at fracture (%)
RM	172.23 ± 2.17	0.86 ± 0,24	0.83 ± 0.11
RM-ES	55.91 ± 9.93	0.38 ± 0,06	2.18 ± 0.25
RM-NE	60.94 ± 0.85	0.81 ± 0,02	4.70 ±0.56

3.2. Effects of Disulfide Bond Reducing Agents and Denaturant

Disulfide bond reducing or/and denaturing agents in different percentages were added to the blend to improve the mechanical properties of the material.

The tensile strength and Young's modulus increased with the addition of a 10%w/v aqueous solution of Na_2SO_3 (RM-R1, Table 3) when compared to the unmodified RM material. However, the material made with a 20% w/v aqueous solution of Na_2SO_3 showed comparable tensile strength to RM material, but with a higher elastic modulus and elongation at fracture (Figure 1 and Table 3). The mechanical properties of the material exhibited a strong correlation with their molecular structures and constituent components. Na_2SO_3 acts as a reducing agent for the disulfide (S-S) bonds within the proteins, thereby inducing protein denaturation. Certain functional groups, such as amino, carboxylic, and sulfhydryl groups, exhibit relatively high reactivity and can participate in reactions with both Epoxy-PEG and other functional groups, facilitating the formation of cross-links, particularly under the elevated temperatures of processing.

Table 3. Tensile mechanical properties of blends with different concentration of reducing agent (Na_2SO_3).

Sample	Mechanical properties		
	Elastic modulus (N/mm^2)	stress at fracture (N/mm^2)	Elongation at fracture (%)
RM	172.23 ± 2.17	0.86 ± 0,24	0.83 ± 0.11
RM-R1	234.82 ± 8.15	2.00 ± 0.37	1.24 ± 0.37
RM-R2	103.60 ± 8.66	1.13 ± 0.06	1.92 ±0.06

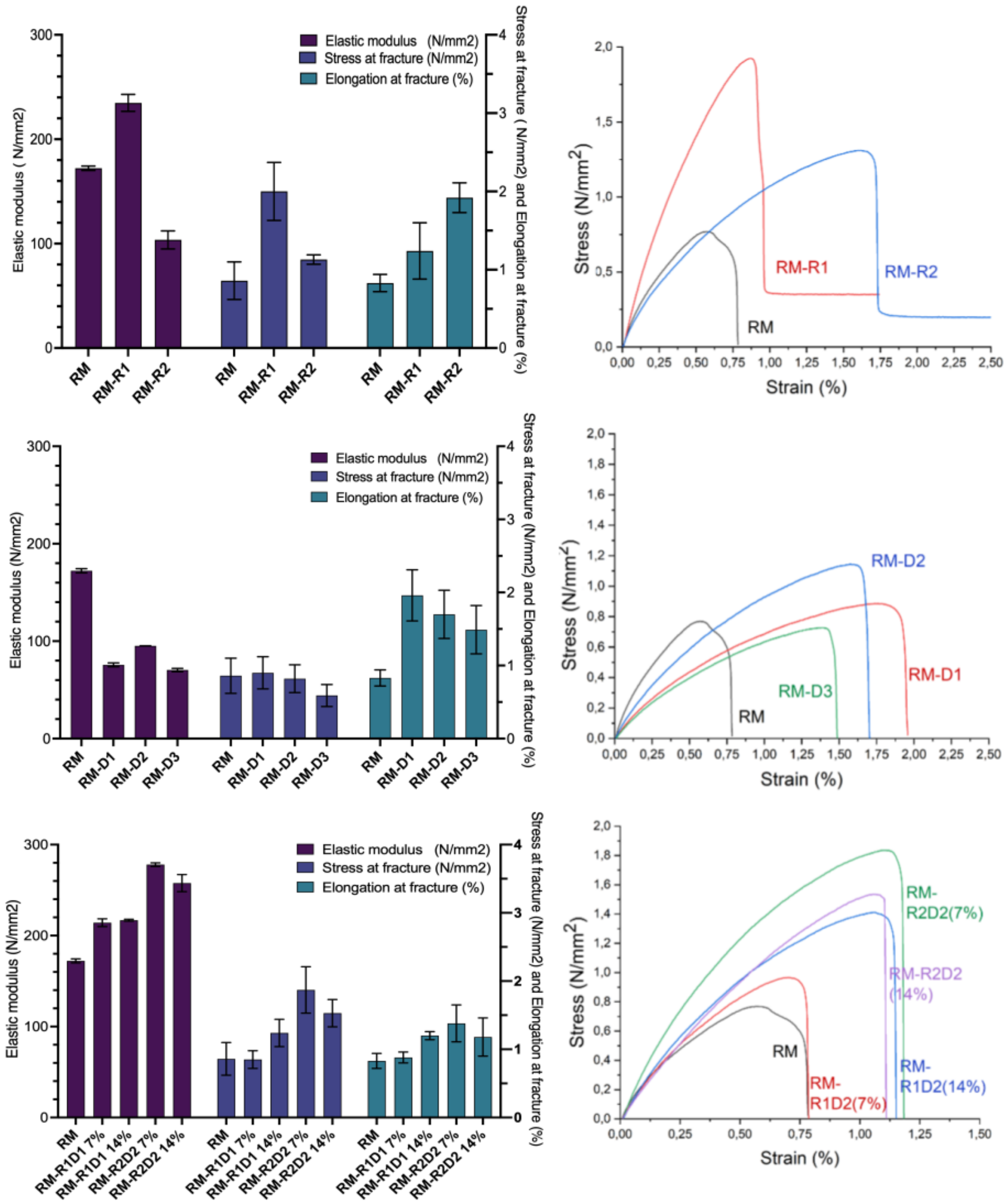

Figure 1. Histogram of tensile properties (left) and stress/strain diagram (right) of meal/casein/water/glycerol blends without or with addition of Na_2SO_3 and/or a denaturing agent.

Modified RM materials RM-R1 and RM-R2, characterized by a high degree of denaturation, tend to develop a greater number of entanglements and crosslinks, resulting in enhanced mechanical strength. At higher concentrations, Na2SO3 primarily serves as a plasticizer, increasing the mobility of the protein chains. However, the elongation at fracture (Figure 1 and Table 3) remain low, thus suggesting that the mobility is limited to small domains inside the material surrounded and confined by densely crosslinked species. The use of TCEP (tris(2-carboxyethyl)phosphine) as an alternative reducing agent to sodium sulfite was not effective, as the tensile properties of the material worsened dramatically when a 10% w/v aqueous solution of TCEP (7% w/w) instead than 10% w/v Na2SO3 was added to the blend before heat pressing (Elastic modulus: 56.36 ± 5.64 N/mm2, stress at fracture: 0.55 ± 0.12 N/mm2).

SDS, urea, and guanidinium chloride, which are commonly employed denaturing agents for proteins, were utilized to investigate their influence on the mechanical properties of RM-based materials. The Young’s modulus of the obtained slabs decreased by two order of magnitude compared to unmodified RM material indicating the important role of the ordered structure on mechanical strength. However the stress at fracture remained relatively constant and small, suggesting that the weak points remain the same (Figure 1 and Table 4). Urea destabilizes globular proteins by establishing robust hydrogen bonds with water molecules that surround the protein. Simultaneously, it interferes with the hydrogen bonds within the protein structure, leading to the formation of partially unfolded protein conformations and flexible peptide chains. Similarly, the interaction with SDS alters both tertiary and quaternary protein structures by disrupting hydrophobic and electrostatic interactions within the protein without breaking covalent bonds. This results in the protein structure becoming partially unfolded and more flexible. The elongation remains relatively low due to fractures occurring in defective areas, which cause the material breaking at the interface between distinct domains.

Table 4. Tensile mechanical properties of blends containing denaturing agents (Urea, guanidinium chloride, Sodium Dodecyl Sulphate (SDS)).

Sample	**Mechanical properties**		
	Elastic modulus (N/mm^2)	**stress at fracture (N/mm^2)**	**Elongation at fracture (%)**
RM	172.23 ± 2.17	0.86 ± 0.24	0.83 ± 0.11
RM-D1	75.67 ± 2.00	0.90 ± 0.22	1.96 ± 0.35
RM-D2	95.17 ± 0.10	0.82 ± 0.19	1.70 ± 0.33
RM-D3	70.35 ± 1.77	0.59 ± 0.15	1.49 ± 0.33

The above results suggest that a higher degree of denaturation and, consequently, a greater number of cross-links are achieved when a reducing agent such as Na_2SO_3 acts upon covalent S-S bonds, as compared to the utilization of denaturing agents like urea, guanidinium chloride, and SDS.

As shown in Table 5, when both Na_2SO_3 and guanidinium chloride are employed in the production of RM-based materials, an increase in stress at fracture and in elastic modulus was observed. Denaturation exposes the hydrophobic residues that are buried within the core of folded protein molecules at the molecular surface. Moreover, these hydrophobic groups are capable of engaging in more extensive hydrophobic interactions among protein molecules during the compression molding process[41,43]. Young's modulus increases significanlty due to the formation of cross-links between the protein chains and the cross-linker (Epoxy-PEG) due to higher amount of reactive groups exposed by the denaturating agent. Moreover, the plasticizer effect of water was observed when RM-R2D2 7% and RM-R2D2 14% were compared, as evidenced by the reduction in Young's modulus and tensile stress. Indeed water has high dielectric constant and establishes strong interactions with the hydrophilic and charged groups of the proteins through the formation of hydrogen bonds[44].

Table 5. Tensile mechanical properties of blends containing sodium sulphite and guanidinium chloride in different percentages.

Sample	**Mechanical properties**		
	Elastic modulus (N/mm^2)	**stress at fracture (N/mm^2)**	**Elongation at fracture (%)**
RM-R1D2 7%	214.18 ± 4.27	0.85 ± 0.13	0.88 ± 0.08
RM-R1D2 14%	216.89 ± 0.98	1.24 ± 0.20	1.20 ± 0.06
RM-R2D2 7%	277.99 ± 1.95	1.87 ± 0.34	1.38 ± 0.27
RM-R2D2 14%	257.71 ± 9.40	1.53 ± 0.20	0.18 ± 0.28

3.2. Thermal Properties

To determine the effect of the different compositions on the thermostability of the material, we performed thermogravimetric analysis of all the samples. TGA and first-derivative curves (Figure S1 and Figure 2 respectively) show a weight loss profile for all materials similar to what observed for other protein-based materials[41]. The initial weight loss, between 30°C and ca 150°C, is particularly evident in samples RM and RM-R2 (Figure 2) and corresponds to loss of moisture. In particular, RM exhibits a higher amount of residual and eliminable moisture, which is therefore considered non-structural. In contrast, the other specimens did not display any water elimination, indicating their inability to absorb water due to increased reticulation or higher structural water content. Additionally, RM-D2 and RM-R2D2 samples, which have a greater reticulation degree, show an inversion of the area ratios between the first and second thermal degradation steps, reflecting a decline in quantity. The peak of the first degradation step (around 225°C) shifts slightly to a higher temperature, suggesting a marginal increase in stability. The second stage (ca 150°-250°C) can be attributed to loss of glycerol (bp 290°C). A third step weight loss from 250°C to ca 400°C is due to degradation of residual protein-rich material. RM-D1 also shows an extra stage (400°-500°) probably due to decomposition of urea with loss of ammonia and carbon dioxide.

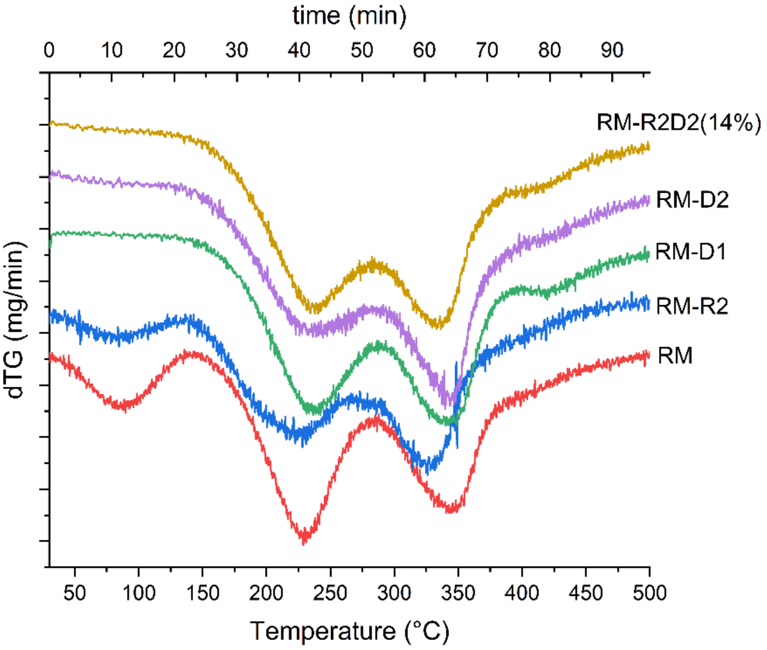

Figure 2. First-derivative curve of TGA of rapeseed meal protein-based materials RM-R0, RM, RM-R2, RM-D1, RM-D2 and RM-R2D2 14%.

A slight shift to higher temperatures in the weight loss of glycerol can be observed going from R0 to RM-R2D2 14%, except for RM-R2. That indicates an enhanced thermal stability of the material containing both the reducing and the denaturing agent (RM-R2D2 14%).

3.3. Protein Conformation Analysis

Untreated meal and the modified materials were analyzed by FT-IR spectroscopy. No substantial change can be observed when comparing the full spectra of the materials (Figure 4). To investigate eventual changes in the secondary structures of the protein components we compared the amide I band region (from ca 1590 to 1700 cm-1), which is mainly due to the stretching vibration mode of the carbonyl in the amide groups and it is representative of protein secondary structure (Figure 5). Only slight changes can be detected in the shapes of the band in the spectra of the modified materials, which can be attributed to the effect of the different component of the blend on protein structure (Figure 5A). Clear changes can be seen, instead, when comparing that same region of the spectrum of the unmodified rapeseed meal with the ones of the materials (Figure 5B). The material

R0, made with rapeseed meal, glycerol (27%) and water (7%), gives a peak whose shape indicates a change in the secondary structure composition and the appearance of new bands in the region 1700-1750 cm-1. Also in the case of RM-R and RM-D2R2 14% the band has a different shape compared to the one in the spectrum of the meal, suggesting a change in conformation of the proteins, despite the differences among the materials themselves are less evident.

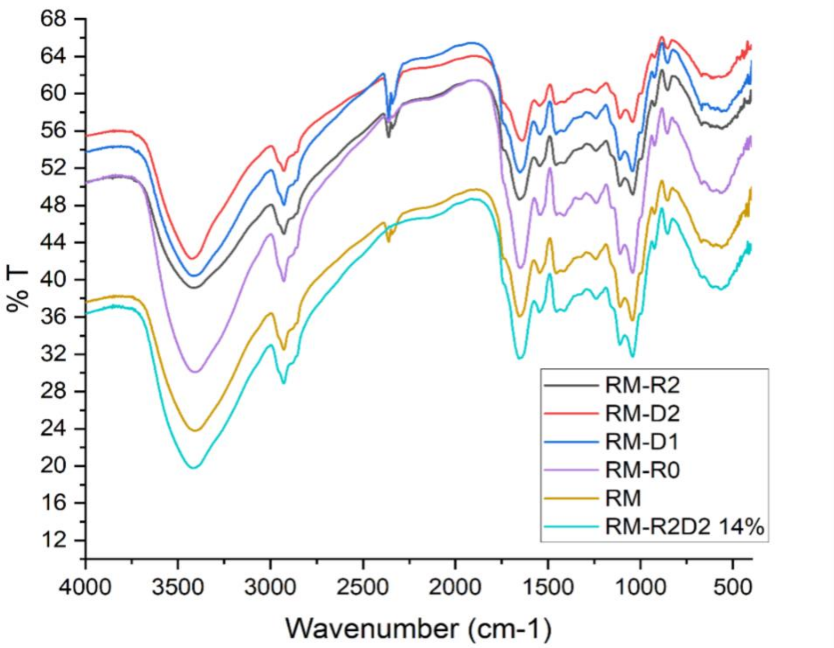

Figure 3. Overlapped full FT-IR spectra of rapeseed meal based materials.

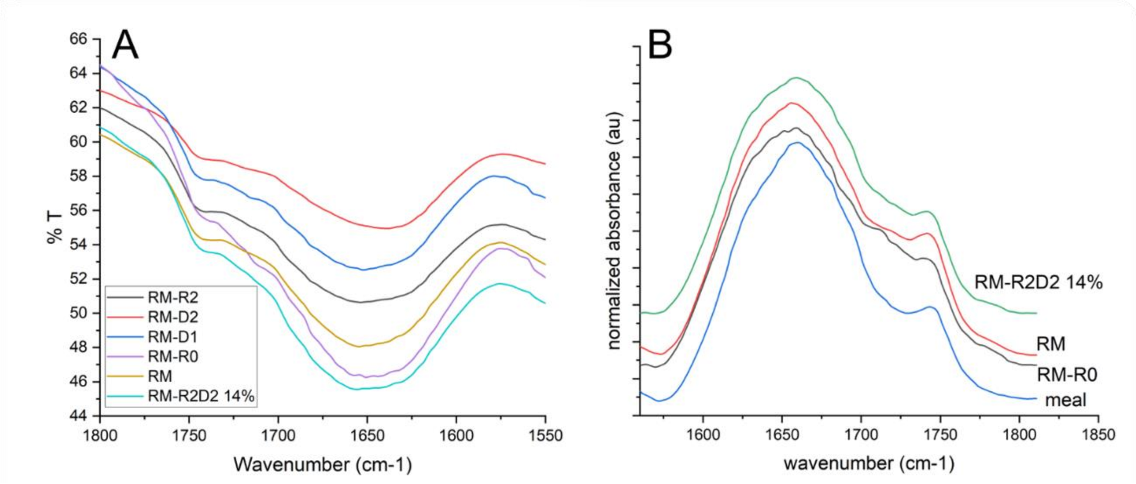

Figure 4. FT-IR spectra of amide I band of (A) RM-R0, RM, RM-R2, RM-D1, RM-D2 and RM-R2D2 14% materials. (B) Rapeseed meal and RM.R0, RM and RM-R2D2 14% materials (baseline corrected).

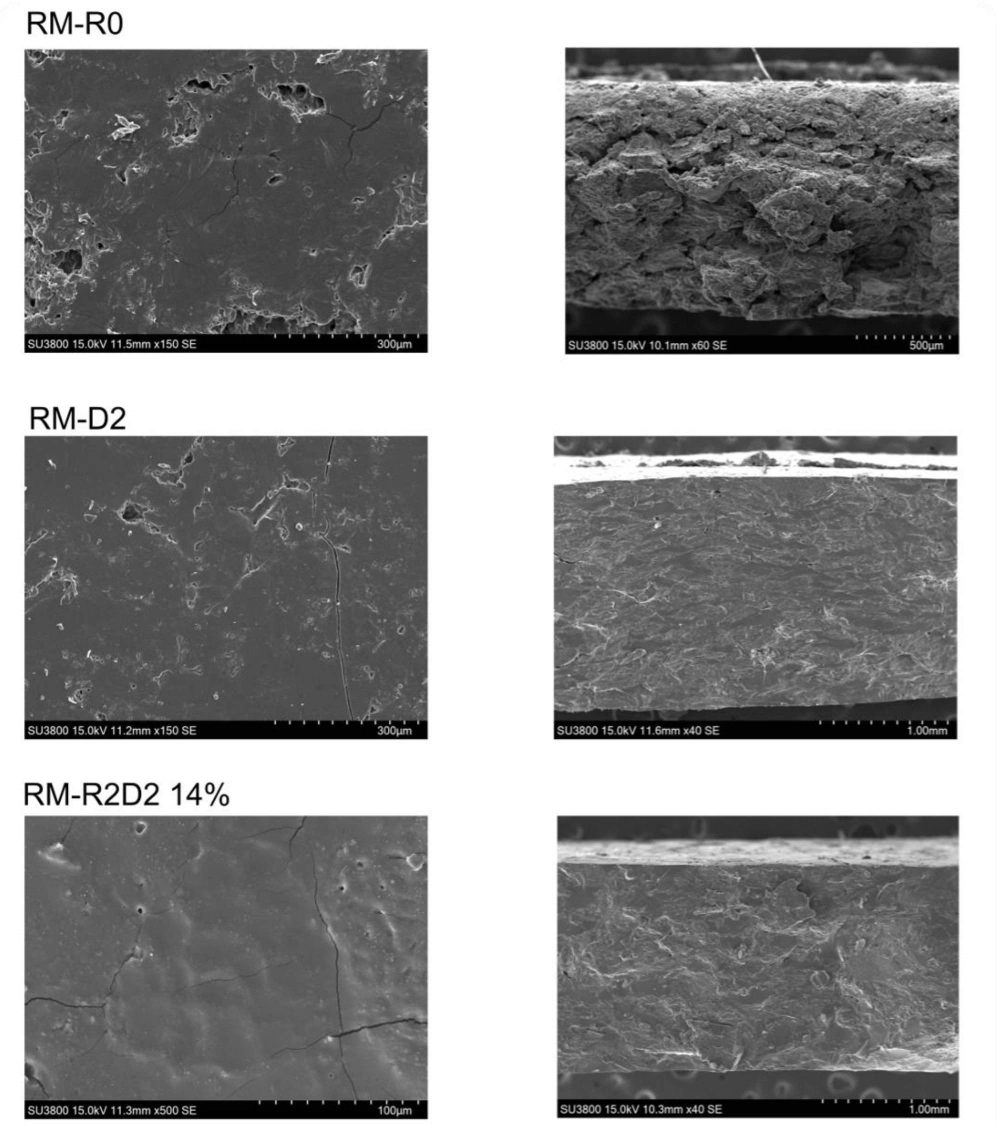

Figure 5. Scanning electron microscopy images of surface (left) and cryofracture cross-section (right) of specimens RM-R0, RM-D2 and RM-R2D2 14%.

3.4. Material Appearance

All the material obtained from compression moulding were brown, compact and flexible.

Scanning electron microscopy was used to investigate the morphology of the material in more detail. Already when the materials were fractured at room temperature it was possible to observe a clear change in their morphology comparing both the section and the interior of the single specimens to each other (Figures S2 and S3). Indeed, the presence of casein and crosslinker (comparison RM-R0/RM) gives a more compact aspect to the section of the material (Figure S2): it is still possible to distinguish layers in RM, but they are much less separated than in the case of R0. When 20% Na_2SO_3 is added (specimen RM-R2), the material appears more homogeneous. Different morphologies can be observed in the case of RM-D1 and RM-D2, probably because of the different effect of the denaturing agent used. Among all the specimens analysed RM-R2D2 14% is the one that looks as the most compact and uniform. Taking images at higher magnification (Figures S4-S7) it was possible to distinguish the cells of rapeseed: a well-defined cell wall was visible in RM-R0, while cells are more broken in the other specimens, which indicates that the high protein content in them went out and was more available for processing.

We also performed SEM measurements on cryogenic fractured RM-R0, RM-D2 and RM-R2D2 14% (Figure 6). In that case the differences in the sections are striking: while RM-R0 is clearly still a simply pressed material, RM-D2 and RM-R2D2 14% are homogeneous and compact materials.

4. Conclusions

In this study, a novel protein rich organic material with improved mechanical strength but limited elongation response has been developed. Raw rapeseed meal was processed with appropriate interactive additives by pression molding. The addition of Rennet casein increased the protein content of the material and clearly improved its mechanical resistance. Different reducing and denaturing agents were tested at various concentrations to induce protein denaturation and to understand the role of the molecular feature on the material ultimate properties. The polypeptide chain became so more flexible and, at the same time, led functional groups more accessible for crosslinking reaction. The best results were obtained when a 20% w/w aqueous solution of Na_2SO_3, acting both as disulfide bond reducing agent and as plasticizer, and 1M guanidinium chloride as denaturing agent were added to the blend (14% w/w total), with a significant increase in Young's modulus and very modest elongation at fracture. A change in protein structure was evidenced by the FT-IR analysis when comparing the amide I bands of the specimens with different composition to each other and to the one of rapeseed meal, indicating effective protein denaturation. The presence of the disulfide bond reducing and of the denaturing agents causes partial unfolding of the proteins and led to a major exposure of the protein's side chains. Thus, hydrophobic groups are capable of engaging in more extensive hydrophobic interactions among protein molecules and the reactive functional groups form more cross-links with the crosslinker. Moreover, TGA revealed improved thermal stability of the material containing both the reducing and the denaturing agent (RM-R2D2 14%), suggesting stronger interaction of glycerol with the other component of the material, particularly with the amino acid side chains of the denatured proteins. Taken together, the presented results demonstrate the potential of cross-linked, rapeseed protein-rich meal-based materials obtained by pression moulding as alternative to environmentally demanding polymeric material.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Figure S1: TG curves of specimens; Figure S2: SEM of the rapeseed meal-based materials (cross section, room temperature) at 500 or 300 μm, Figure S3: SEM of the rapeseed meal-based materials (longitudinal section, room temperature), Figure S4: SEM of the rapeseed meal-based materials (cross section, room temperature) at 100 μm, Figure S5: SEM of the rapeseed meal-based materials (longitudinal section, room temperature) RM-R0, RM and RM-R2, Figure S6: SEM of the rapeseed meal-based materials (longitudinal section, room temperature) D1 and D2, Figure S7: SEM of the rapeseed meal-based materials (longitudinal section, room temperature) RM-R2D2 14%.

Author Contributions Conceptualization, F. C.; A. M.P., C.B. and L. R.; methodology, S.A., F. C.; A. M.P., C.B., and L. R..; validation, S.A., M.P. and S.B.; formal analysis, S.A., C.B. M.P.; investigation - materials preparation and characterization, S.A.; investigation - Surface Electron Microscopy (SEM), W. G.; .resources, F. C.; L.R. and A.M.P.; data curation, S.A., F. C.; A. M.P., C.B. and L. R.; writing—original draft preparation, S.A. and C.B.; writing—review and editing, S.A., F. C.; A. M.P., C.B., L. R., W. G. and M. B.; visualization, S. A. and C. B. W. G. and M. B.; supervision, F. C., A. M.P., C.B. and L. R.; project administration, F. C. and A. M. P. funding acquisition, F. C., A. M. P. , L.R. All authors have read and agreed to the published version of the manuscript.

Funding: S.A. is the recipient of the PhD fellowship PON Research and Innovation 2014–2020 (D.M 1061/2021) XXXVII Cycle in Chemical Sciences: "Development of macromolecular and cross-linked materials based on proteins/peptides from vegetable or animal sources", funded by MUR/EU-FSE.

Acknowledgments: This study was supported by funding from the 5th Seed Funding Program of EUniWell "Technology Tranfer of Peptides for the Well-being Economy (TTPep - Peptides for Well-being)". Sara Aquilia thanks MUR-Italy for her PhD fellowships funded by PON Ricerca e Innovazione 2014–2020. Italcol S.p.A. is kindly acknowledged for the generous gift of raw canola meal. Cassa di Risparmio di Firenze Foundation for the financial support (ENFORCE PROJECT, Ricercatori a Firenze, 2021).

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Hatti-Kaul, R.; Nilsson, L.J.; Zhang, B.; Rehnberg, N.; Lundmark, S. Designing Biobased Recyclable Polymers for Plastics. *Trends Biotechnol.* **2020**, 38, 50–67.
2. North, E.J.; Halden, R.U. Plastics and Environmental Health: The Road Ahead. *Reviews on Environmental Health* **2013**, 28, 1–8.
3. Hahladakis, J.N.; Velis, C.A.; Weber, R.; Iacovidou, E.; Purnell, P. An Overview of Chemical Additives Present in Plastics: Migration, Release, Fate and Environmental Impact during Their Use, Disposal and Recycling. *J. Hazard. Mater*. **2018**, *344*, 179–199.
4. Liu, D.; Zhang, L. Structure and Properties of Soy Protein Plastics Plasticized with Acetamide. *Macromol. Mater. Eng*. **2006**, *291*, 820–828.
5. Manger, C. Bioplastics Facts & Figures. *European Bioplastics e.V*. **2023**.
6. Ancuța, P.; Sonia, A. Oil Press-Cakes and Meals Valorization through Circular Economy Approaches: A Review. *Appl. Sci.* **2020,** *10*.
7. Álvarez-Castillo, E.; Felix, M.; Bengoechea, C.; Guerrero, A. Proteins from Agri-Food Industrial Biowastes or Co-Products and Their Applications as Green Materials. *Foods* **2021**.
8. Dziuba, R.; Kucharska, M.; Madej-Kiełbik, L.; Sulak, K.; Wiśniewska-Wrona, M. Biopolymers and Biomaterials for Special Applications within the Context of the Circular Economy. *Materials* **2021**, *14*.
9. Aguilar, A.; Twardowski, T.; Wohlgemuth, R. Bioeconomy for Sustainable Development. *Biotechnol. J.* **2019**, *14*.
10. Ryder, K.; Ali, M.A.; Billakanti, J.; Carne, A. Evaluation of Dairy Co-Product Containing Composite Solutions for the Formation of Bioplastic Films. *J. Polym. Environ*. **2020**, *28*, 725–736.
11. Production of Rapeseed by Main Producing Countries 2023/24 Available online: https://www.statista.com/statistics/263930/worldwide-production-of-rapeseed-by-country/ (accessed on 29 May 2024)
12. Di Lena, G.; Sanchez del Pulgar, J.; Lucarini, M.; Durazzo, A.; Ondrejíčková, P.; Oancea, F.; Frincu, R.-M.; Aguzzi, A.; Ferrari Nicoli, S.; Casini, I.; et al. Valorization Potentials of Rapeseed Meal in a Biorefinery Perspective: Focus on Nutritional and Bioactive Components. *Molecules* **2021**.
13. Raboanatahiry, N.; Li, H.; Yu, L.; Li, M. Rapeseed (Brassica Napus): Processing, Utilization, and Genetic Improvement. *Agronomy* **2021**, *11*.
14. Bhatty, R.S.; McKenzie, S.L.; Finlayson, A.J. The Proteins of Rapeseed (Brassica Napus L.) Soluble in Salt Solutions. *Can. J. Biochem*. **1968**, 46, 1191–1197.
15. Yang, C.; Wang, Y.; Vasanthan, T.; Chen, L. Impacts of pH and Heating Temperature on Formation Mechanisms and Properties of Thermally Induced Canola Protein Gels. *Food Hydrocoll*. **2014,** 40, 225–236.
16. Gallorini, R.; Aquilia, S.; Bello, C.; Ciardelli, F.; Pinna, M.; Papini, A.M.; Rosi, L. Pyrolysis of Spent Rapeseed Meal: A Circular Economy Example for Waste Valorization. *J. Anal. Appl. Pyrolysis* **2023**, *174*, 106138.
17. Wanasundara, J.P.D.; Kapel, R.; Albe-Slabi, S. Proteins from Canola/Rapeseed—Current Status. In *Sustainable Protein Sources*, 2nd ed.; Nadathur, S., Wanasundara, J.P.D., Scanlin, L., Eds.; Academic Press, 2024; pp. 285–309.
18. Fetzer, A.; Müller, K.; Schmid, M.; Eisner, P. Rapeseed Proteins for Technical Applications: Processing, Isolation, Modification and Functional Properties – A Review. *Ind. Crops Prod*. **2020**, *158*, 112986.
19. Li, S.; Ciardullo, K.; Donner, E.; Thompson, M.R.; Rempel, C.; Liu, Q. Reactive Processing Preparation of Sustainable Composites from Canola Meal Reinforced by Chemical Modification. *Eur. Polym. J.* **2018**, *102*, 187–194.
20. Álvarez-Castillo, E.; Bengoechea, C.; Felix, M.; Guerrero, A. Protein-Based Bioplastics from Biowastes: Sources, Processing, Properties and Applications. In *Bioplastics for Sustainable Development*; Kuddus, M., Roohi, Eds.; Springer Singapore: Singapore, 2021; pp. 137–176.
21. Mensitieri, G.; Di Maio, E.; Buonocore, G.G.; Nedi, I.; Oliviero, M.; Sansone, L.; Iannace, S. Processing and Shelf Life Issues of Selected Food Packaging Materials and Structures from Renewable Resources. *New Chall. Food Preserv*. **2011**, *22*, 72–80.
22. Newson, W.R.; Kuktaite, R.; Hedenqvist, M.S.; Gällstedt, M.; Johansson, E. Oilseed Meal Based Plastics from Plasticized, Hot Pressed Crambe Abyssinica and Brassica Carinata Residuals. *J. Am. Oil Chem. Soc*. **2013**, *90*, 1229–1237.
23. Felix, M.; Perez-Puyana, V.; Romero, A.; Guerrero, A. Development of Protein-Based Bioplastics Modified with Different Additives. *J. Appl. Polym. Sci.* **2017**, *134*, 45430.
24. Mekonnen, T.; Mussone, P.; Khalil, H.; Bressler, D. Progress in Bio-Based Plastics and Plasticizing Modifications. *J. Mater. Chem. A* **2013**, *1*, 13379–13398.
25. Krochta, J.M. Proteins as Raw Materials for Films and Coatings: Definitions, Current Status, and Opportunities. *Protein-Based Films Coat.* **2002**, *1*, 1–40.
26. Martinez, A.W.; Caves, J.M.; Ravi, S.; Li, W.; Chaikof, E.L. Effects of Crosslinking on the Mechanical Properties, Drug Release and Cytocompatibility of Protein Polymers. *Acta Biomater.* **2014**, *10*, 26–33.

27. Reddy, N.; Reddy, R.; Jiang, Q. Crosslinking Biopolymers for Biomedical Applications. *Trends Biotechnol*. **2015,** *33*, 362–369.
28. Zhang, Y.; Liu, Q.; Rempel, C. Processing and Characteristics of Canola Protein-Based Biodegradable Packaging: A Review. *Crit. Rev. Food Sci. Nutr.* **2018,** *58*, 475–485.
29. Jang, S.-A.; Lim, G.-O.; Song, K.B. Preparation and Mechanical Properties of Edible Rapeseed Protein Films. *J. Food Sci*. **2011**, *76*, C218–C223.
30. Li, S.; Donner, E.; Thompson, M.; Zhang, Y.; Rempel, C.; Liu, Q. Preparation and Characterization of Cross-Linked Canola Protein Isolate Films. *Eur. Polym. J.* **2017**, *89*, 419–430..
31. Ben, Z.Y.; Samsudin, H.; Yhaya, M.F. Glycerol: Its Properties, Polymer Synthesis, and Applications in Starch Based Films. *Eur. Polym. J.* **2022**, *175*, 111377.
32. N. Henrik Ullsten; Mikael Gällstedt; Mikael S. Hedenqvist Plasticizers for Protein-Based Materials. In *Viscoelastic and Viscoplastic Materials*; Mohamed Fathy El-Amin, Ed.; IntechOpen: Rijeka, 2016; p.
33. Bonnaillie, L.M.; Zhang, H.; Akkurt, S.; Yam, K.L.; Tomasula, P.M. Casein Films: The Effects of Formulation, Environmental Conditions and the Addition of Citric Pectin on the Structure and Mechanical Properties. *Polymers* **2014**, 6, 2018–2036.
34. Johansson, E.; Spencer, G.M.; Bettini, E.; Cho, S.-W.; Marttila, S.; Kuktaite, R.; Gällstedt, M.; Hedenqvist, M.S. Biobased Materials Production from Biodiesel Residuals of Rapeseed. *ISRN Mater. Sci.* **2012**, *2012*, 193541.
35. Lu, S.; Wang, X.; Lu, Q.; Zhang, X.; Kluge, J.A.; Uppal, N.; Omenetto, F.; Kaplan, D.L. Insoluble and Flexible Silk Films Containing Glycerol. *Biomacromolecules* **2010**, *11*, 143–150.
36. Zahari, I.; Ferawati, F.; Purhagen, J.K.; Rayner, M.; Ahlström, C.; Helstad, A.; Östbring, K. Development and Characterization of Extrudates Based on Rapeseed and Pea Protein Blends Using High-Moisture Extrusion Cooking. *Foods* **2021**, 10.
37. Audic, J.-L.; Chaufer, B.; Daufin, G. Non-Food Applications of Milk Components and Dairy Co-Products: A Review. *Lait* **2003**.
38. Wusigale; Liang, L.; Luo, Y. Casein and Pectin: Structures, Interactions, and Applications. *Trends Food Sci. Technol*. **2020**, 97, 391–403
39. W. He, G. Zhu, Y. Gao, H. Wu, Z. Fang, K. Guo. Combining Renewable Eleostearic Acid and Eugenol To Fabricate Sustainable Plasticizer and Its Effect of Plasticizing on PVC *Chem. Eng. J.* **2020**, *380*, 122532.
40. L. Song, W. Chi, Q. Zhang, J. Ren, B. Yang, F. Cong, Y. Li, W. Wang, X. Li, Y. Wang. Preparation of degradable chemically cross-linked polylactic acid films and its application on disposable straws *Int. J. Biol. Macromol.* **2023**, *253*, 127407.
41. He, W.; Zhu, G.; Gao, Y.; Wu, H.; Fang, Z.; Guo, K. Green Plasticizers Derived from Epoxidized Soybean Oil for Poly (Vinyl Chloride): Continuous Synthesis and Evaluation in PVC Films. *Chem. Eng. J.* **2020**, 380, 122532.
42. Song, L.; Chi, W.; Zhang, Q.; Ren, J.; Yang, B.; Cong, F.; Li, Y.; Wang, W.; Li, X.; Wang, Y. Improvement of Properties of Polylactic Acid/Polypropylene Carbonate Blends Using Epoxy Soybean Oil as an Efficient Compatibilizer. *Int. J. Biol. Macromol.* **2023**, 253, 127407.
43. Singha, S.; Mahmutovic, M.; Zamalloa, C.; Stragier, L.; Verstraete, W.; Svagan, A.J.; Das, O.; Hedenqvist, M.S. Novel Bioplastic from Single Cell Protein as a Potential Packaging Material. *ACS Sustain. Chem. Eng.* **2021**, 9, 6337–6346.