

Functional Improvement of Canola Protein Bioplastic through Chemical Modification of Protein and Nanomaterial Integration

By

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Abstract

The food packaging industry is now shifting towards biopolymer-based materials from petroleum-based materials due to environmental concerns. Canola meal is the major byproduct of the canola oil milling industry and is limited in reusability. The low cost and the inability to consume it highlighted the vast potential of canola protein (CP) for developing plant protein-based food packaging materials. However, compared with synthetic polymers, plant-based materials exhibited reduced film-forming properties due to their organic nature. Ammonium persulfate (APS) is identified as a promising crosslinking agent for canola protein modification. Nanocrystalline cellulose (NCC) and graphite oxide (GO) are nanomaterials that can be incorporated in the protein matrix to enhance the film properties to avoid the above-mentioned limitations in CP films. Polyvinyl acetate (PVA) is a synthetic polymer often incorporated into plant-protein biopolymer systems to improve film-forming properties. Furthermore, solvent casting is a lab-scale method used to assess the preliminary behaviour of biopolymers, which requires longer drying times. In contrast, extrusion moulding is the commercial-scale method used in the manufacture of packaging materials. The extrusion method is preferred due to continuous production, zero waste, improved film properties, and low unit production cost. The objective of the first study was to modify the CP using APS at different addition levels (0, 1, 3, 5, 7, 9% w/w of protein) and to develop CP-based films by solvent casting with nanomaterials. The second study aimed to assess the effects of extrusion moulding on CP blends with APS modification, nanomaterial reinforcement, and the compatibility of CP with the PVA polymer. In both studies, the mechanical, barrier, thermal, and structural properties of the developed films were evaluated. The APS 5% addition level was identified as the optimal condition for APS modification of CP-based films using the solvent-casting method. The modification has improved the tensile, water-barrier, and thermal properties of the film by factors of $\times 1.33$, $\times 1.2$, and $\times 1.5$ compared to the neat film, respectively. The highest tensile strength (7.03 ± 0.39 MPa) was identified in modified NCC-added films. At the same time, the lowest water vapour permeability (WVP) (0.016 ± 0.0012 mm/m² kPa h) was observed in GO-added films, indicating the reinforcement potential of the nanomaterials. The extrusion moulding method improved the tensile properties of the neat film from 1.63 ± 0.17 to 2.90 ± 0.05 MPa, compared to the solvent casting method. The addition of modified NCC increased tensile properties by 150%, while the addition of GO reduced WVP by 500%. PVA-added films improved light transparency, while GO addition completely blocked it, making them suitable for translucent and opaque food packaging materials, respectively. Overall, APS modification improved the

CP-based film properties, and the extrusion-moulding method showed immense potential in CP-based film manufacturing. This research helps to ensure environmental sustainability through a circular economic approach for the byproducts of the canola oil industry.

Keywords: Chemical modification, Ammonium persulfate, Canola protein, Extrusion-moulding method, Sustainable Food packaging

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Dedication

This thesis is dedicated
to my mother and father,
Ganga Kulatunga and Prof. Chandana Abeysinghe
For their unconditional love and caring
to my loving wife,
Gayathri Subasinghe
for her trust, love support.

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List of abbreviations

APS-Ammonium persulfate

CNF-Cellulose nanofibers

CP-Canola protein

CPI- Canola protein isolate

DSC-Differential scanning calorimetry

DMA-Dynamic mechanical analysis

EB -Elongation at break

FTIR- Fourier-transformed infrared spectroscopy

GO-Graphite oxide

NCC-Nanocrystalline cellulose

OP- Oxygen permeability

PDCAAS- Protein digestibility corrected amino acid score

PVA-Polyvinyl acetate

SEM- Scanning electron microscopy

SPI- Soy protein isolate

TEMPO- 2,2,6,6- Tetramethylpiperidine-1-oxyl

TGA- Thermogravimetric analysis

TMNCC- TEMPO modified nanocrystalline cellulose

TS- Tensile strength

WVP-Water Vapour Permeability

XPS- X-ray photoelectron spectroscopy

XRD- X-ray diffraction

YM -Young's modulus

Chapter 1 : Introduction

1.1. Introduction

The global population is growing rapidly. The world population is expected to grow to 8.5 billion by 2030 and reach approximately 9.7 billion by 2050 (Rahman et al., 2024). This population growth will intensify competition for resources and food insecurity. Additionally, improper food packaging will contribute to significant food wastage, while synthetic packaging materials will continue to harm the environment through pollution. Consequently, plant-based and biodegradable food packaging materials offer a promising solution to address these challenges. The future of food packaging is progressively shifting towards biodegradable materials to promote environmental sustainability.

Canola protein is a major by-product of the canola oil industry, which generates nearly 6 million metric tons of canola meal annually in Canada alone (Oliveira & Yu, 2023). Developing value-added applications for canola meal can enhance carbon cycling in the environment, offering sustainable economic benefits for canola growers and processing industries and ultimately improving their quality of life. Canola meal is currently widely used as a source of animal feed in the dairy and cattle industries, limiting its use for human consumption (Canola Council of Canada, 2025; Dao & Moss, 2025). Canola meal contains 38-40% canola protein, which is not effectively utilized in human consumption due to its unfavourable functional properties (Bandara & Wu, 2018). Therefore, there is significant potential for canola protein in the development of biodegradable plant-based bioplastics. Canola-based bioplastics can reduce greenhouse gas emissions and protect soil health, as these polymers are biodegradable (Thakur et al., 2018). The degradation of plant-based materials releases nutrients, such as nitrogen from proteins in bioplastic films, back into the environment, where plants can reabsorb them. However, canola protein-based films prepared in previous studies exhibit limitations, such as poor mechanical and barrier properties due to the presence of hydrophilic amino acid residues in the canola protein matrix (Dissanayake et al., 2022). Protein crosslinking is one of the promising protein modification methods that can induce protein-protein interactions using a crosslinking agent (Bandara & Wu, 2018). Chemical crosslinking is important because it forms irreversible covalent networks that significantly enhance the mechanical strength, thermal stability, and moisture resistance of the canola protein matrix (Calva-Estrada et al., 2019). Ammonium persulfate (APS) has emerged as a promising chemical crosslinking agent with substantial potential for inducing cohesive interactions in plant protein-based bioplastic film

preparation (Bandara & Wu, 2018). APS is widely recognized in polymer chemistry as an effective oxidizing agent (Mascheroni et al., 2016). In addition, APS functions as a free-radical initiator in various grafting reactions (Ibrahim et al., 2016). However, reported studies on APS-mediated modification of canola protein are limited. Bandara & Wu (2018) demonstrated that APS alone can enhance the adhesion strength of canola protein-based adhesives by acting as a crosslinking agent. Despite its significant potential, the chemical modification of canola protein-based films using APS as a chemical crosslinking agent has not yet been extensively explored.

Furthermore, incorporating nanomaterials is a widely recognized strategy for reinforcing protein matrices. Among these, cellulose nanomaterials have demonstrated considerable promise, and their performance can be enhanced further through chemical modifications (Dissanayake et al., 2022). 2,2,6,6-Tetramethylpiperidine-1-oxyl (TEMPO)-mediated oxidation was identified as a promising nanoreinforcing material for canola protein-based packaging films, enhancing the overall surface polarity of Nanocrystalline cellulose (NCC) (Dissanayake et al., 2022). Similarly, graphite oxide (GO) has attracted attention as a cost-effective nanomaterial that offers many of the barrier and functional benefits of graphite while being more practical for large-scale processing (Chairez-Jimenez et al., 2024). The 1st study in this thesis aims to investigate the reinforcement of chemically modified canola protein-based films with nanocellulose and graphite oxide, presenting a novel approach to advancing the field of biodegradable food packaging materials.

Extrusion moulding is a scalable and industry-compatible method for producing protein-based food packaging films, offering clear advantages over traditional solvent casting. Unlike solvent casting, which requires long drying times and is difficult to scale up, extrusion uses heat and shear to continuously form films with better additive dispersion and more controlled processing conditions. This makes extrusion a practical and efficient approach for developing commercial bioplastic films. Polyvinyl acetate (PVA) is a synthetic thermoplastic polymer commonly used in adhesives, coatings, and film-forming applications due to its strong bonding ability and flexibility (Emblem, 2012). Its processability and compatibility with other polymers make it useful in developing composite materials. The development of food packaging materials for canola protein using extrusion moulding methods has not yet been studied.

Despite the growing interest in plant-based biodegradable films, significant challenges remain in translating canola protein into functional, industrially viable packaging materials. Limited research has explored the combined effects of chemical crosslinking, nanomaterial reinforcement, and scalable processing techniques such as extrusion moulding. Moreover, most

studies focus on solvent-cast films, which are not readily adaptable to commercial production. This thesis addresses these gaps by investigating the development of chemically modified canola protein films reinforced with TEMPO-oxidized nanocellulose and graphite oxide, produced via extrusion moulding. This work aims to advance the design of sustainable, high-performance food packaging materials and provide insights that can facilitate their large-scale adoption.

1.2. Hypothesis

1.2.1. Hypothesis - Study 1

Chemical modification of Canola protein with ammonium persulfate (APS) will enhance the mechanical and barrier properties of canola protein-based food packaging films.

1.2.2. Hypothesis - Study 2

The extrusion moulding method will translate the lab-scale-optimized solvent-casting conditions to improve the mechanical and barrier properties of canola protein-based packaging films.

1.3. Objectives

1.3.1. Objectives - Study 1

- To chemically modify Canola protein via crosslinking by APS modification.
- To prepare the canola protein films using the solvent casting method
- To reinforce the modified canola protein using TEMPO-modified NCC and modified GO.
- To characterize the prepared canola protein films in terms of mechanical, water barrier, and thermal properties

1.3.2. Objectives - Study 2

- To chemically modify the canola protein with APS using the reactive extrusion method.
- To identify the effects of different nanomaterial additives (TEMPO-modified NCC and GO) on the canola protein polymer blend during extrusion.
- To identify the effect of polymer blending with different addition levels of PVA and canola protein.
- To characterize the prepared canola protein films in terms of mechanical, water barrier, and thermal properties, and identify the best conditions for future applications.

Chapter 2 : Literature Review

2.1. Canola Industry

Rapeseed (*B. oleracea* and *B. rapa*) was introduced to Canada in 1943 as an industrial source of vegetable oil (Craig, 1971). In the beginning, rapeseed oil was deemed unfit for human consumption due to its high levels of glucosinolates and erucic acid. However, after a series of research, Canadian breeders developed low-glucosinolate and low-erucic acid cultivars, which were named “Canola” (*Brassica napus*) (Craig, 1971; So & Duncan, 2021). Canola, also known as rapeseed in some countries, is the world's second-largest oilseed crop, following soy. Canola oil is rich in polyunsaturated fatty acids and is considered one of the primary cooking oils in Canada and globally (Chang & Nickerson, 2014). Canola meal is the main by-product (~60%) of the canola oil milling industry, and it produces 51 million tons of canola meal worldwide each year (Dhakal & Acharya, 2025). Canola meal has been identified as a low-value byproduct, which reported Canadian dollars of 345.70 per metric ton (Canola Council of Canada, 2025; Dhakal & Acharya, 2025). Canola meal is a protein-rich source containing around 38-40% canola protein, which is currently used as an animal feed source, limiting its usage for human consumption (Bandara & Wu, 2018; Dao & Moss, 2025). Alkaline extraction was identified as the easiest, most common, and economically viable protein extraction method for canola protein extraction. Previous studies reported different protein contents in alkaline-extracted canola meal protein isolates, such as 41.68% (Patel et al., 2016) and 45.68% (Patel et al., 2011).

2.1.1. Canola protein

Canola protein is classified into four main categories based on the extraction method: water-soluble albumins, salt-soluble globulins, alcohol (ethanol)-soluble prolamins, and non-soluble fractions such as glutelin (Aider & Barbana, 2011). Krishnan & Coe (2001) further classified salt-soluble globulins into subcategories (7-8S and 11-12S) based on their sedimentation coefficients. Canola contains two primary storage proteins: cruciferin and napin. Cruciferin, a 12S globulin, has a neutral isoelectric pH ($pI = 7.2$), while napin, a 2S protein, exhibits a basic/alkaline isoelectric pH ($pI = 11$) (Dissanayake et al., 2022). Canola meal, the main industrial by-product of the canola oil industry, is typically produced through a two-step organic solvent extraction process, primarily using hexane. This process involves heating the meal to remove residual solvents, which adversely affects the remaining proteins. For example, Aider & Barbana (2011) noted a slight reduction in lysine content following processing. As a

result of these industrial steps, most salt-soluble globulins become denatured. Therefore, alkaline extraction has been identified as a suitable method for isolating canola protein, as it minimizes the denaturation of salt-soluble globulins (Bandara & Wu, 2018).

Canola protein has a well-balanced amino acid profile rich with sulphur-containing amino acids such as cysteine (Campbell et al., 2016). Table 2.1 shows the reported amino acid profiles of the canola protein isolates and their comparison with those of soy protein isolates. Fleddermann et al. (2013) studied the protein digestibility-corrected amino acid score (PDCAAS) values of both canola and soy proteins and obtained values of 0.86 and 0.84, respectively. Although canola protein has a high protein digestibility value, its applications for human consumption are limited due to several anti-nutritional factors. Canola protein have glucocinolates (Li et al., 2012). Glucocinolates, such as nitriles, thiocyanates, isothiocyanates, and epithionitriles, have unpleasant flavors and can cause toxic conditions (Zhang et al., 2020). Therefore, the application of canola meal is currently limited to animal feed formulations in the dairy cattle industry and aquaculture (Wanasundara et al., 2016).

Table 2.1. Comparisons of different amino acid profiles of canola and soy protein isolates

Amino acid	Canola meal (Wanasundara et al., 2016)	Canola meal protein (Ivanova et al., 2017)	CPI (Chmielewska et al., 2021)	CPI (Dhakal & Acharya, 2025)	Soy protein isolate (Fleddermann et al., 2013)
Non-polar/Hydrophobic amino acids					
Glycine	4.92	25.21(+Arg)	4.83	5.53	4.04
Alanine	4.36	4.45	4.17	5.10	4.23
Valine	4.97	4.79	4.69	4.43	4.16
Leucine	6.19	6.88	6.84	6.72	7.68
Isoleucine	3.47	3.66	3.52	3.42	4.04
Methionin	1.94	1.47	2.06	1.76	1.17
Proline	5.97	-	6.57	7.32	5.21
Phenylalanine	4.06	3.24	3.65	3.62	4.99
Tryptophan	1.33	-	1.36	-	-
Polar/Hydrophilic amino acids					
Tyrosine	2.50	-	1.96	1.69	3.73

Histidine	3.39	-	3.05	6.97	2.44
Lysine	5.92	5.54	6.08	4.78	6.43
Aspartate	7.25	5.15	5.68	8.20	11.60
Glutamine	18.14	22.34	22.40	25.00	19.40
Cystine	2.29	-	3.51	2.43	1.13
Arginine	6.62	25.21(+Gly)	6.32	5.53	7.36
Serine	4.00	4.30	3.91	3.66	5.36
Threonine	4.27	3.71	3.73	3.76	3.98

Values were given as mean values of the percentage or g of AA/100g of protein of the respective amino acids present in the protein source.

2.1.2. Applications of Canola protein

Due to ongoing challenges associated with food-related applications of canola protein, including functional limitations and antinutritional factors, researchers have increasingly investigated non-food uses in adhesives, bioplastics, and delivery systems, where its intrinsic functional properties, such as solubility, gelation, adhesion, and cohesion, play a critical role in performance (Bandara et al., 2018). In the field of bio-adhesives, canola meal and its protein fractions have been formulated into wood adhesives with enhanced bonding strength and improved mechanical properties through chemical modification and crosslinking, offering a renewable alternative to formaldehyde-based resins for medium-density fiberboards and particleboards (Tayo et al., 2022). Recent studies have further optimized adhesive formulations using crosslinkers such as sodium nitrite and sodium bisulphate, resulting in particleboard bonding performance that meets or exceeds conventional urea-formaldehyde adhesives (Tayo et al., 2025). In addition to adhesives, canola protein is actively researched as a biobased thermoplastic component for biodegradable plastics and composites. Its functional groups facilitate polymer blending and network formation, enabling the development of sustainable thermoplastic materials with potential utility in technical applications beyond packaging alone (Chairez-Jimenez et al., 2024; Dissanayake et al., 2022; Liu et al., 2025). Furthermore, the potential for canola protein to act as an encapsulating matrix in delivery systems has been highlighted by research demonstrating its ability to interact with bioactive compounds within three-dimensional protein networks, suggesting promise for controlled release technologies in biomedical and agricultural contexts (Bandara et al., 2018; Bandara & Wu, 2018). Collectively, these studies illustrate that canola protein's functional versatility can be harnessed for

renewable adhesives, biodegradable plastics, and advanced delivery systems, driving interest in high-value industrial applications that leverage readily available canola meal resources.

2.2. Bio-based materials as food packaging materials

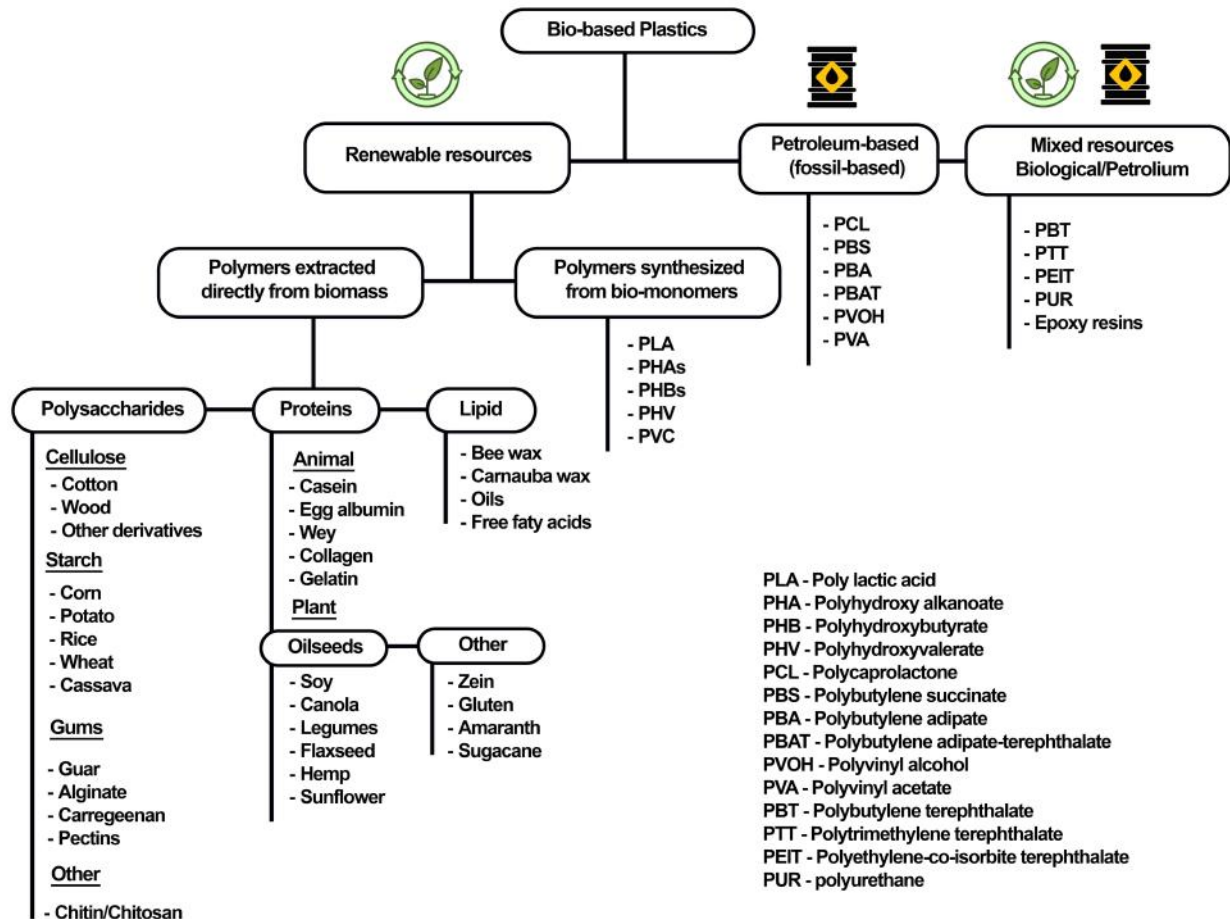


Figure 2.1. Classification of bio-based plastic materials. Adopted based on the information from Donkor et al., (2023) and Siracusa & Blanco, (2020).

Worldwide plastic use exceeds 300 billion kg each year. Polyesters and polyolefin-based plastics account for 45% of food packaging (Nguyen et al., 2018). These petroleum-based synthetic plastics have adverse environmental effects due to their longer environmental retention times (Aquila et al., 2024). Bio-based plastics are man-made or man-processed organic macromolecules derived from biological resources for plastic and fibre applications. They may be biodegradable or non-biodegradable, and in some cases compostable (Nguyen et al., 2018). The biodegradability of the packaging materials depends on their molecular structure rather than the origin of the monomer source (Siracusa & Blanco, 2020). Food

packaging materials are generally categorized into three main types: flexible films, rigid plastics, and semi-rigid plastic packaging items. As illustrated in Figure 2.1, bio-based plastic materials can be classified into three major categories (Donkor et al., 2023; Siracusa & Blanco, 2020). According to the authors, the first group includes plastics derived from renewable biological resources, many of which are biodegradable, such as starch-based plastics, cellulose derivatives, proteins, lignin, chitosan, polylactic acid (PLA), and polyhydroxyalkanoates (PHAs), including PHB, PHV, and their copolymers; this group also encompasses conventional polymers like PVC, PE, PP, PET, and polyamides when their monomers are sourced from biomass. The second group consists of bio-plastics produced from petroleum resources that are fully biodegradable, including polycaprolactone (PCL), polybutylene succinate (PBS), polybutylene adipate (PBA), polybutylene adipate-terephthalate (PBAT), and polyvinyl alcohol (PVOH). The third group comprises bio-plastics synthesized from a combination of biologically derived and petroleum-based monomers, such as polybutylene terephthalate (PBT), polytrimethylene terephthalate (PTT), polyethylene-co-isosorbide terephthalate (PEIT), polyurethane (PUR), and epoxy resins.

Various materials such as starch, cellulose, pectin, carrageenan, alginate, glycogen, chitin, bacterial cellulose, hydrogels, proteins, gelatin, galactomannans, polylactic acid (PLA), and their blends have demonstrated significant potential for the development of food packaging materials (Ahmed, 2018). Owing to their biodegradability, renewability, and environmental friendliness, plant-based materials are gaining prominence in the food packaging industry. It is possible to develop food packaging materials entirely from plant-based materials (Dissanayake et al., 2023), while some researchers have mixed different polymers and studied their synergistic effects (Tadele et al., 2023).

2.3. Plant proteins as food packaging materials

Among these materials, proteins play a particularly vital role in biodegradable food packaging due to their natural decomposition capability, abundant availability, and exceptional functional properties. Proteins are composed of twenty different amino acids with diverse functionalities. Globular proteins, in particular, can unfold and participate in crosslinking reactions, which is advantageous for the development of biopolymer-based food packaging materials (Schmid & Müller, 2019). In the meantime, many industrial byproducts from the oil milling industry are wasted due to limited reusability. Most byproducts are primarily used in animal feed formulation rather than for human consumption (Višnjevec et al., 2021). There is significant potential, and attention has been directed toward the protein extracted from the by-products.

Beyond availability, plant proteins are uniquely suited for food packaging applications because their chemical structure enables tunable material properties through processing and formulation. The presence of multiple functional groups, such as amino, carboxyl, hydroxyl, and sulfhydryl groups, allows plant proteins to form cohesive networks via hydrogen bonding, electrostatic interactions, hydrophobic associations, and covalent crosslinking, which are critical for film formation and mechanical integrity (Boutureira & Bernardes, 2015; Calva-Estrada et al., 2019). These interactions can be manipulated by thermal treatment, pH adjustment, plasticization, or chemical modification to tailor tensile strength, flexibility, and barrier performance according to packaging requirements. From a sustainability perspective, plant proteins offer a renewable, biodegradable, and low-carbon alternative to petroleum-based polymers, aligning with global efforts to reduce plastic waste and dependency on fossil resources (Wanasundara et al., 2016). Furthermore, the valorization of protein-rich agricultural by-products into packaging materials supports circular economy principles by converting low-value residues into high-value functional materials, thereby enhancing both environmental and economic sustainability of food systems (Višnjevec et al., 2021). Table 2.2 summarizes plant protein-based materials derived from oilseed industry byproducts, along with their respective advantages and limitations.

Table 2.2. Different plant protein materials and their advantages and limitations

Protein Type	Packaging type	Package Method	Advantages	Limitations	Reference
Gelatin	Thin films	Solvent casting method	Tensile strength and elongation at break have increased. Needle-less electrospinning technology has used. Edible packaging	Moisture barrier properties have decreased	(Karim et al., 2020)

Wheat gluten Protein	Thin films	Solvent casting method (water- ethanol mix solvent)	Films were aged at 50% of RH, 25C for 6 months of period. Good oxygen barrier properties	Poor water barrier properties	(Mojumdar et al., 2011)
Amaranth protein	Ultrathin films	spin-coating nano- layering	Higher hydrophobic behaviour than cast films Higher transparency in films		(López- Rubio et al., 2020)
Sugarcane protein	Rigid type	Extrusion- based 3D printing	Good moisture barrier properties	Printing defects	(Nida et al., 2021)
Pea protein	Rigid type	Injection molding	TS of the pea protein-based molds has higher compared to that of soy protein-based bioplastics.		(Perez- Puyana et al., 2016)
Soy protein	Rigid type	Extrusion- based 3D printing			(Dey, Rahman, Gupta, et al., 2023)
Zein protein	Thin films	Extrusion molding			(Tadele et al., 2023)

Canola Protein	Thin films	Solvent casting method	TEMPO-modified NCC-reinforced canola protein films.	Moisture barrier properties have decreased	(Dissanayake et al., 2022)
			High tensile strength, elongation at break properties		
Refined Canola Protein	Thin films	Solvent casting method	Enhanced water resistance, tensile strength, and elongation at break properties due to refining process	The refining process did have an impact for thermal properties	(Manamperi et al., 2010)

2.4. Different strategies for the modification of Plant protein-based food packaging materials

Previous studies on canola protein-based films have reported limitations in mechanical strength and barrier properties, which restrict their practical application in food packaging. For an ideal protein-based film, the material should exhibit high tensile strength, elasticity, and low permeability to gases and moisture to effectively protect food products. To address these limitations, several strategies have been explored, including plasticization, chemical and enzymatic cross-linking, nanotechnology approaches, and the development of composite films (Figure 2.2). While current protein films still fall short of the performance of conventional synthetic polymers, these techniques have demonstrated promising improvements in both mechanical and barrier functionalities (Calva-Estrada et al., 2019). Among these strategies, nanocomposite films represent a rapidly growing area of research, as they allow precise control over the film's microstructure and functional properties. By incorporating nanoparticles such as nanocellulose, nanoclays, or metallic oxides, it is possible to enhance tensile strength, flexibility, and water or gas barrier performance simultaneously.

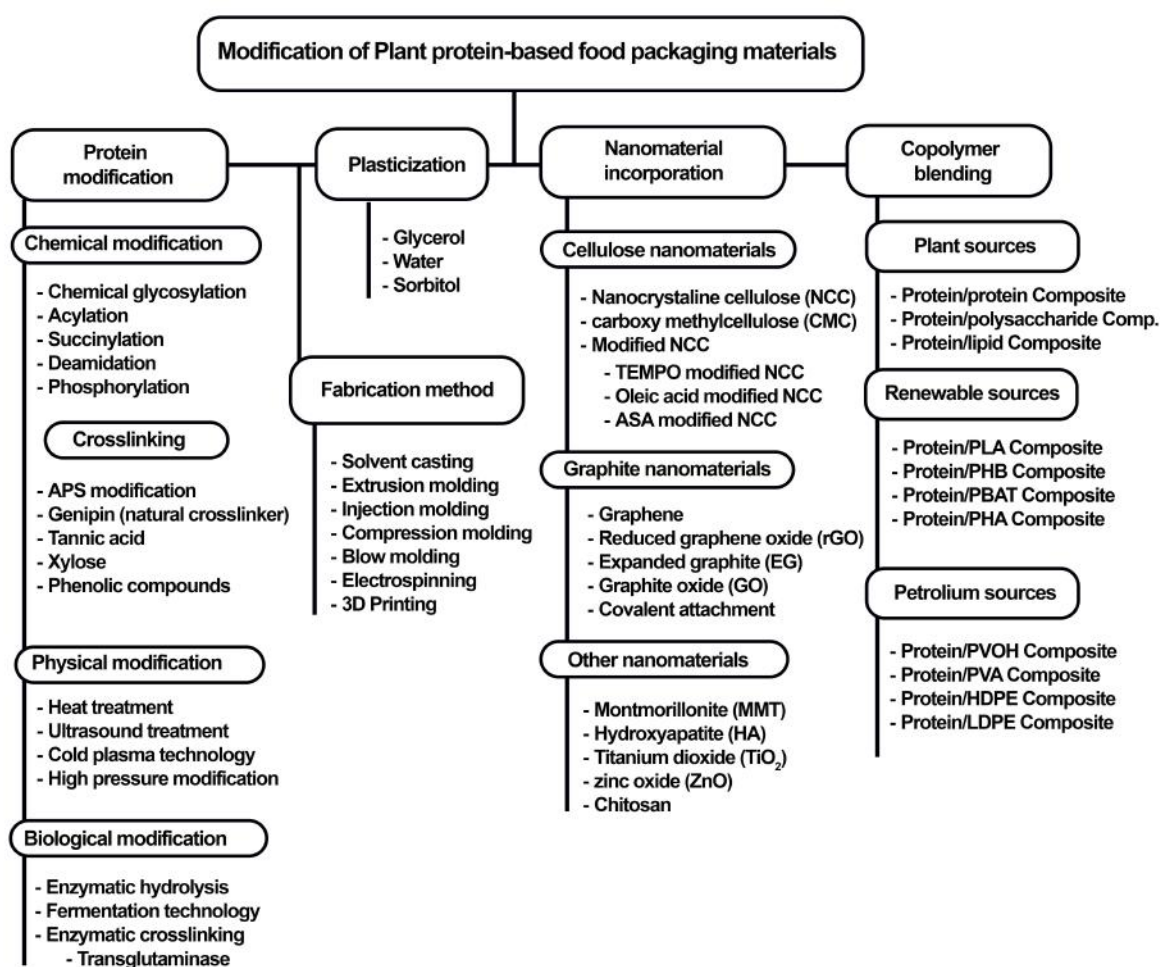


Figure 2.2. Classification of modification methods for plant protein-based food packaging materials

2.4.1. Protein Modification

Physical, chemical, and enzymatic modifications can be employed to enhance the material properties of protein-based films. According to Benbettaïeb et al. (2016), chemical modification is more effective than simply blending proteins with additional polymers, such as nanomaterials. Various chemical crosslinkers have been explored for modifying proteins, including 1,4-butanediol diglycidyl ether, glyceraldehyde, glyoxal, formaldehyde (Benbettaïeb et al., 2016), genipin (Chang & Nickerson, 2014), and ammonium persulfate (Bandara & Wu, 2018), Xylose (Rebezov et al., 2023), phenolic compounds, Tanic acids (Zhang et al., 2023). Benbettaïeb et al. (2016) reported that glyceraldehyde and glyoxal provide superior water barrier properties compared to formaldehyde when used as crosslinkers. However, one of the

primary challenges with chemical crosslinkers is their potential toxicity, which raises concerns about their use in food packaging. To address this issue, genipin, a natural crosslinker derived from the fruit of *Gardenia jasminoides Ellis*, has attracted attention for its significantly lower toxicity. In fact, genipin is up to 10,000 times safer than conventional synthetic crosslinkers (Chang & Nickerson, 2014). Akhila et al. (2023) used CaCl_2 as an ionic chemical crosslinking agent on flaxseed mucilage films due to its non-toxicity and promising film properties. They mention the swelling index was reduced by 3 times due to the crosslinking effect.

2.4.1.1. Ammonium persulfate (APS) Modification of proteins

Ammonium persulfate (APS) is a chemical crosslinker commonly used to crosslink agar gels in gel electrophoresis. It is recognized as an excellent oxidizing agent, a reliable bleaching agent, and an effective food preservative, highlighting its relevance in food packaging applications. Furthermore, APS is generally accepted as a safe (GRASS) compound, which is safe to use in food packaging materials (Mascheroni et al., 2016; Shi et al., 2017). The ionic behaviour of APS persulfate ions in an ionic medium is explained by Kolthoff & Miller (1951) and Fancy & Kodadek (1999), who reported protein-protein interactions arising from crosslinking reactions. According to Bandara & Wu (2018), APS functions as a free radical generator and a highly efficient crosslinking agent. As illustrated in Figure 2.3, it facilitates the formation of crosslinks between tyrosine-tyrosine residues and tyrosine-histidine residues in canola protein isolates. Furthermore, comparative FTIR spectral analysis of APS-modified and unmodified canola protein isolates revealed significant differences. When APS was added to the protein isolate at specific weight ratios (APS/protein, w/w), a distinct reduction in peak intensity was observed. Specifically, the reduction of hydroxyl (OH) groups on tyrosine and Histidine residues resulted in a characteristic decrease in the peak at 1518 and 1440 cm^{-1} , respectively. Additionally, a noticeable colour change was observed upon introduction of APS into the reaction medium, further supporting its role in modifying canola protein isolates (Bandara & Wu, 2018). The application of APS in protein modification for the preparation of food packaging films has not yet been studied, which may have significant potential.

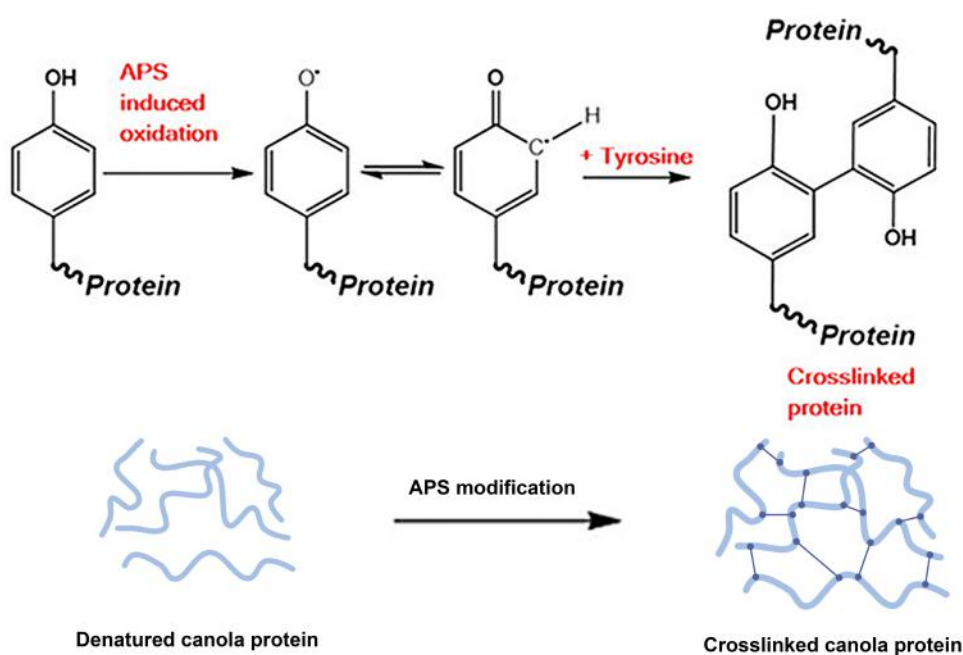


Figure 2.3. Graphical illustration of APS modification. Modified from Bandara & Wu (2018).

2.4.2. Nanomaterials

When addressing the limitations of conventional petroleum-based plastic food packaging materials, plant-protein-based materials often exhibit low mechanical properties (particularly tensile strength) and low thermal stability due to weak interactions within the protein matrix. To overcome these challenges, incorporating strong additives is necessary to enhance the films' strength and durability. Nanomaterials provide a promising solution. Examples of effective nanomaterials include nanocellulose, nanoclays, nanostarch, nanochitosan, and various other nanoparticles. Beyond improving physical properties, the inclusion of nanomaterials in protein films can also enhance functional attributes such as antimicrobial activity and extended shelf life, further optimizing their suitability for food packaging applications (Ahmed, 2018).

2.4.2.1. Cellulose Nanomaterials

Among all the nanomaterials, Nanocrystalline cellulose (NCC) plays a vital role in film formation. NCC is frequently used with film-forming polymers and has greater potential to reinforce polymers and improve mechanical, thermal, and barrier properties (Azeredo et al.,

2017). NCC has a unique ability to spread throughout the film-forming solution and bind with protein residues. López-Rubio et al. (2020) reported that the above 10% w/w NCC addition levels significantly ($p < 0.05$) increased the hydrophobicity and reduced the water solubility in Amaranth protein packaging films. The tensile strength increased from 0.5 MPa to 5.3 MPa when 20% NCC was added to amaranth protein films (López-Rubio et al., 2020). Similarly, Dissanayake et al. (2022) reported that tensile strength increased from 3 MPa to 8.3 MPa when 5% NCC was added to canola protein films. The authors reported that interactions between free protein residual ends with NCC are stronger than protein-protein interactions, underscoring the importance of nanomaterials as reinforcement in protein-based films.

The incorporation of nanomaterials has emerged as an effective means of reinforcing protein-based films. However, the properties of these films can be further enhanced by chemically modifying the nanomaterials. Several cellulose modification methods are available for nanocellulose, including esterification, etherification, oxidation, silylation, and polymer grafting. Among these, acetylation, TEMPO modification, silylation, and alkenyl succinic anhydride (ASA) modification are up-and-coming methods under the category of hydroxyl substitution (Habibi et al., 2010). modified hydrophobic cellulose nanofibers with ASA and used them as a reinforcement material in high-density polyethylene, reporting a 70% increase in tensile properties. Acetylation reactions can occur via homogeneous or heterogeneous mechanisms, typically on the surface of nanocrystalline cellulose (NCC). One notable example is Fischer's esterification process, which involves the reaction between an alcohol and an acid in the presence of an acid catalyst. According to Habibi et al. (2010), the single-step Fischer esterification process can be represented by the chemical equation in Equation (2.1).



Habibi et al. (2010) demonstrated that acetic anhydride and acetic acid could significantly improve the mechanical properties of cellulose nanofibers (CNF). The acetylation process reduces nanocellulose's hydrophilicity, thereby enhancing its compatibility with hydrophobic polymers or matrices. This modification is particularly advantageous for applications such as composites, coatings, and films. By decreasing the hydrogen bonding capacity of nanocellulose, acetic acid modification prevents agglomeration and ensures uniform dispersion within the matrix. Jamaluddin et al. (2021) explored the development of polylactic acid (PLA) based films incorporating modified CNF. The acetylation of CNF was performed using acetic anhydride, propionic anhydride, and butyric anhydride. Among these, propionic anhydride-

modified CNF exhibited the highest acetylation rate, achieving a 31.4% conversion of CNF into modified CNF. These findings highlight the potential of acetylation to enhance the performance of nanocellulose-reinforced polymer systems.

2.4.2.2. TEMPO-modified nanocrystalline cellulose

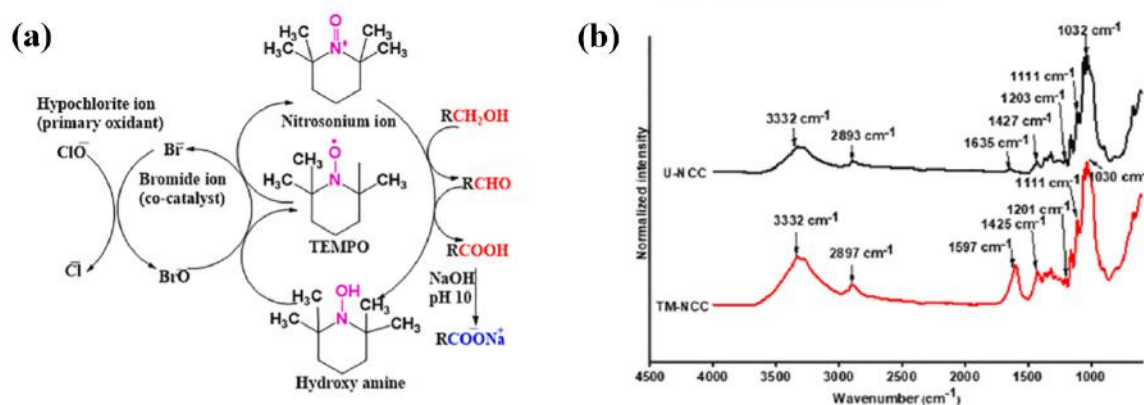


Figure 2.4. (a) Mechanism of TEMPO modification pathway (b) FTIR spectral diagram of unmodified NCC (U-NCC) and TEMPO modified NCC (TM-NCC), reprinted with the kind permission of Dissanayake et al. (2022).

TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl) modification is another promising chemical method for enhancing nanocellulose properties. According to Habibi et al. (2010) TEMPO oxidation converts the free hydroxymethyl groups on the nanocrystalline cellulose (NCC) surface into carboxylic acid groups. Dissanayake et al. (2022) provided further insights into this reaction mechanism, illustrating (Figure 2.4) that hypochlorite ions (ClO^-) act as the primary oxidant, while bromide ions (Br^-) serve as a co-catalyst, which is regenerated at the end of the reaction. Based on conductometric titration, Dissanayake et al. (2022) reported an oxidation degree of 19.61%. Similarly, Buffa et al. (2016) observed a slightly higher degree of oxidation (26.90%) in cellulose nanocrystals after TEMPO modification. These findings confirm that TEMPO modification effectively converts a portion of free hydroxyl (OH) groups into carboxylic acid (COOH) groups, thereby improving the dispersion of NCC within the film-forming matrix. Consequently, this enhanced dispersion significantly increases the tensile properties of the cast films.

2.4.2.3. Graphite-based nanomaterial

Graphite is made out of a pure carbon lattice, which is highly conductive. Graphite itself could also be used as a reinforcement material by converting it to expanded graphite via probe-tip sonication (Chow et al., 2023). A single plane of graphite is called graphene, which has higher

tensile strength than graphite. Han et al. (2017) reported a 27.7% improvement in the tensile properties of graphene-added soy protein-based films. Furthermore, Mansoori et al. (2019) reported that the addition of graphene oxide increased the tensile properties from 252 to 449 MPa in corn starch/soy protein films. Preparation of graphene from raw graphite is a costly process that is limited for large-scale production in food packaging development. Graphite oxide is an oxidized form of graphite (Figure 2.5) where oxygen-containing functional groups (epoxy, hydroxyl, carbonyl, carboxyl) are introduced between and on the basal planes of graphite layers (Hou et al., 2020). Compared to graphene, graphite oxide is less conductive and less mechanically strong, but because of its low-cost raw material, it could be a promising candidate for improving barrier properties (Bandara et al., 2017). Chairez-Jimenez et al. (2024) studied the addition of graphite oxide to canola protein films to identify the optimal conditions. They reported a 550% reduction in oxygen permeability upon adding 3% (w/w) graphite oxide to canola protein films.

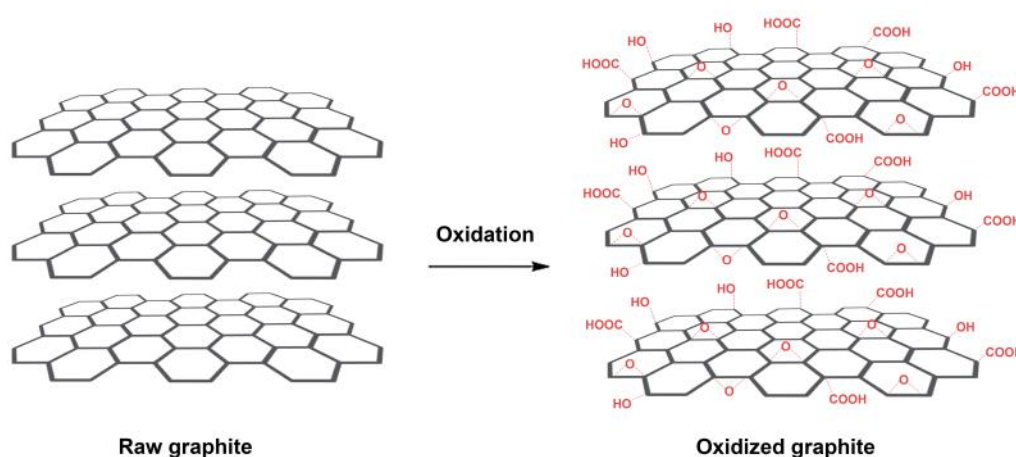


Figure 2.5. Graphical illustration of graphite oxide modification

2.4.3. Synthetic polymers

Synthetic polymers are widely used in food packaging because of their durability, flexibility, cost-effectiveness, and strong barrier properties. Common materials include polyethylene (PE), available as LDPE and HDPE, which provides moisture resistance and is used in films, bags, and bottles. Polypropylene (PP) offers a high melting point and transparency, making it suitable for microwave-safe containers, yogurt cups, and caps. Polyethylene terephthalate (PET) is preferred for beverage bottles and thermoformed trays due to its excellent barrier properties to oxygen and moisture. Polystyrene (PS) is used for rigid containers and foam trays, while

polyvinyl chloride (PVC) is applied in cling films and blister packaging. However, concerns exist regarding its additives and environmental impact. Additional high-barrier materials, such as ethylene vinyl alcohol (EVOH) and nylon (polyamide, PA), are employed in multilayer and vacuum packaging to enhance gas-barrier properties. Collectively, these synthetic polymers dominate conventional food packaging because they offer strong protection, ease of processing, and functional versatility (Hou et al., 2020; Hummers & Offeman, 1958).

2.4.3.1. Polyvinyl acetate (PVA)

Polyvinyl acetate (PVA) is a widely used, high-viscosity, amorphous synthetic polymer produced by free-radical polymerization of vinyl acetate monomers (Cheng et al., 2025). It is a thermoplastic, non-toxic polymer known for its strong film-forming ability, adhesion, and flexibility. PVA contains polar acetate groups that facilitate hydrogen bonding, thereby enhancing its compatibility with various natural biopolymers, including plant proteins and polysaccharides. Due to its moderate glass transition temperature and good processability, PVA is commonly used in adhesives, coatings, packaging materials, and biodegradable composite systems (Emblem, 2012). In biopolymer research, PVA has greater potential for blending with plant proteins to enhance flexibility, reduce brittleness, and improve the mechanical and barrier properties of the resulting films (Kaboarani & Riedl, 2015).

Polyvinyl alcohol (PVOH) is produced by hydrolysis of the PVA polymer, thereby improving its water solubility, and is used in a wide range of food packaging applications. PVOH has good oxygen barrier properties. However, the introduced OH groups make it susceptible to water-barrier properties. Therefore, sandwich-type polymer blending is recommended for PVOH-based packaging materials. Contrastingly, as the precursor of PVOH, PVA exhibits good water barrier properties but weaker oxygen barrier properties (Emblem, 2012). Several polymer blends were reported in the literature using PVA with other polymers. Blending of PVA with PLA/PCL blend (Cheng et al., 2023), PHBH (Cheng et al., 2025), PBS (Li et al., 2021), PLA (Li et al., 2022), PBAT (Zhang et al., 2022) and PHBH/PLA blend (Li et al., 2025). The addition of PVA increased the viscoelasticity of the biodegradable PLA films (Li et al., 2022). Its versatility and functional characteristics make PVA an essential component in the development of sustainable, bio-based packaging materials. Canola protein PVOH biocomposite was used by Patel et al. (2016) to assess the biodegradability of the films, and they reported that no environmental pollutants were generated. The materials are well-suited for producing disposable single-use spoons, cups, and lightweight food-service utensils.

2.5. Techniques used for food packaging materials from industrial by-products

2.5.1. Plasticizers

Plasticizers can be defined in several ways based on their root of applications. According to the handbook of plasticizers published by Wypych (2023) plasticizers are low-molecular-weight materials added to improve the flexibility, workability, and elongation of packaging materials. As shown in Table 2.3, glycerol, sorbitol, and water were identified as the frequently used plasticizers in protein-based polymer blending and fabrication techniques. When protein-protein crosslinking occurs, the resulting film can become fragile and brittle due to hydrogen bond formation. To address this issue, plasticizers are often incorporated to improve flexibility. Various plasticizers have been employed in the preparation of solvent-cast films, including glycerol, sorbitol, propylene glycol, and triethanolamine (Ullsten et al., 2016). (C. Chang & Nickerson, 2014a) investigated the effects of different plasticizers, glycerol, sorbitol, and propylene glycol 400 (PEG400), on canola protein-based food packaging films in the solvent casting method. Among the tested plasticizers, sorbitol demonstrated the highest tensile strength and elastic behaviour, followed by PEG₄₀₀ and glycerol. Although glycerol is one of the most commonly used plasticizers in protein-based films, it exhibited the highest water vapour permeability, which adversely impacts the film's barrier properties. On the other hand, increasing sorbitol content can enhance tensile strength but also tends to produce brittle films. Conversely, glycerol contributes to more uniform and flexible films. Thus, a combination of sorbitol and glycerol holds promise for achieving optimal mechanical and barrier properties in future film formulations (Chang & Nickerson, 2014). Patel et al. (2011) investigated the biodegradation of soy and canola protein-based composite films with PEG400 as the plasticizer, using the compression moulding method. The authors reported that the highest biodegradability (62%) was obtained with a 60% addition of PEG400, and when the plasticizer amount was reduced to 50%, the percentage of microbial biodegradation decreased to 46%. The biodegradation rate depends on the composition of the film-forming materials and the degree of covalent crosslinking within the protein films (Patel et al., 2011). The amount of plasticization varies with the fabrication technique. The addition of a flexible, soluble plasticizer is essential in the solvent-casting method, depending on the solvent used, whereas extrusion methods can work with a wide range of plasticizers (Ochoa-Yepes et al., 2019). Jiménez-Rosado et al. (2022) used both glycerol and water to develop soy protein-based injection moulded bioplastics. They reported higher thermomechanical resistance at elevated temperatures with glycerol, while water could only reach 115 °C. Castro-Criado et al. (2025)

studied the effects of glycerol and water as plasticizers on extruded bioplastics to evaluate the controllability of iron release. They mention that using 100% water as the plasticizer facilitates the porous structure of the extrudate, increasing the amount of iron released, whereas 100% glycerol does the opposite. Therefore, the authors suggested that a 1:1 ratio of glycerol and water as plasticizers was the optimal condition for controlled iron-releasing bioplastics. According to the literature reviewed in Table 2.3, the incorporation of byproducts into commercially available polymer blends often used water as the plasticizer, whereas protein-based blends used glycerol to enhance flexibility and polymer properties. The most common plasticizer used in the biobased fabrication methods is glycerol. Glycerol was identified as an environmentally friendly, non-toxic, and highly biodegradable material. Glycerol reported a high LD50 value (12,600 mg/Kg - oral mouse dosage) with a boiling point of 290 °C, which aligns with the thermal processing parameters (Silva & Ferreira, 2012). Glycerol favours the hygroscopic characteristics of the protein-glycerol bioplastics (Jerez et al., 2007). This limitation has to be addressed in the packaging films.

Table 2.3. Different bio-based materials, fabrication methods, and their advantages and limitations

Coproduct	Fabrication Technique	Type of packaging can form	Plasticizer	Key findings	Type of packaging systems	Reference
Flaxseed mucilage + pectin	Solvent Casting	Thin films	Glycerol	UV blocking and biodegradability. TiO ₂ is crosslinked with CaCl ₂ .	Biodegradable	(Akhila et al., 2023)
Faba bean, pea, lupin, lentil, and soy	Solvent Casting	Thin films	Glycerol	Different plasticizer levels (50%, 25%, 0%). Pea protein films showed the best potential over 5 different legume varieties.	N/A	(Hopkins et al., 2019)
Canola meal protein	Solvent Casting	Thin films	Glycerol, Sorbitol	The effect of the type of plasticizer was analyzed. Glycerol increased the flexibility, while sorbitol increased the tensile properties.	N/A	(Chang & Nickerson, 2014)
Canola meal protein	Solvent Casting	Thin films	Glycerol	Cinnamaldehyde was used as an antimicrobial agent TEMPO modified NCC as a reinforcement additive	Active packaging	(Dissanayake et al., 2022)

Canola meal protein	Solvent Casting	Thin films	Glycerol	Graphite oxide added as a barrier enhancement agent OP was reduced by up to 665%.	Active packaging	(Chairez-Jimenez et al., 2024)
Lentil protein + Cassava starch	Extrusion and Solvent casting	Thin films	Glycerol	Thermostable up to 240 °C & biodegradable within 5 weeks. Extruded films were better than cast films.	Biodegradable	(Ochoa-Yepes et al., 2019)
Hemp fiber + Jute fiber + PHB	Extrusion + Injection molding	Sheets	Water	Color was darkened due to heigh heat. TS was 17.9 MPa in the hemp fiber-added composite.	N/A	(Gunning et al., 2014)
Soy protein	Extrusion + Injection molding	Sheets	Glycerol & Water	Films made by injection molding were better than those made by extrusion. ZnSO ₄ was incorporated as a fertilizer to be released during composting after use.	Compostable	(Jiménez-Rosado et al., 2022)
Sunflower head waste + LDPE	Extrusion	Films	Water	NCC fillers derived from sunflower head waste as a reinforcement material for LDPE-based extruded plastic films. TS of 20 MPa and EB of 250%, which is perfect for food packaging.	Compostable	(Melikoğlu et al., 2023)

Modified zein protein + PBAT composite	Extrusion molulding	Films	Glycerol	OP was reduced by 59%, and the blend's water barrier property improved by 68%.		(Tadele et al., 2023)
Soybean hull fiber +TPC composite	Extrusion + 3D printing	Composite filament	N/A	Addition of soy bean hull (5%) increased YM from 44-84 MPa and redused the porosity of the fillaments. Higher compatibility with industrial polymers in extruded 3D printing.	N/A	(Balla et al., 2021)
Soy protein	Extrusion	Bioplastic	Glycerol +/- water	Cintroled iron released at 90 °C processing temperature and plasticier blend at 1:1 glycerol/water ratio.	Biodegradable	(Castro-Criado et al., 2025)
Canola meal protein + Soy protein	Compression molding	Sheets	PEG ₄₀₀	62% biodegradation within 52 days. The biodegradation rate depends on the composition of the film-forming materials and the degree of covalent crosslinking within the protein films.	Biodegradable	(Patel et al., 2011)
Canola meal protein + PVOH + CaCO ₃	Compression molding	Sheets	Glycerol	TS of 22.64 MPa and EB of 10.69 %. Suitable for preparing lightweight cups and spoons.	Biodegradable.	(Patel et al., 2016)

Soybean hulls and soya protein isolate	Injection molding	Bioplastic cups	Glycerol	Cups were morphologically very similar to polyethylene cups	Compostable	(Rahman et al., 2023)
Pea protein	Injection molding	Rectangular-shaped blocks	Glycerol	TS of pea protein-based molds is higher than that of soy protein-based bioplastics.	Compostable	(Perez-Puyana et al., 2016)
Lentil flour and polyethylene oxide	Electrospinning	Electrospun nanofibers	Water	Gallic acid was encapsulated to enhance the oxidative stability. Ideal for encapsulating heat-sensitive bioactive compounds.	Active packaging	(Aydogdu et al., 2019)
Black chickpea (BCP) peel anthocyanins into a BCP protein	Electrospinning	Electrospun films	Water	Black chickpea peel anthocyanins were act as an antioxidative agent inside the films to reduce the lipid oxidation.	Active packaging Smart intelligent packaging	(Amjadi et al., 2024) +
Soybean Protein + Soy hulls	3D printing	Biodegradable pots.	N/A	Suitable for the preparation of both rigid compostable trays for food packaging and for planting.	Compostable	(Rahman et al., 2023)
Soybean hull fiber +TPC composite	3D printing	Multilayer coupons	N/A	Diluted acid hydrolysed fibers increased the fiber matrix by redusing	N/A	(Balla et al., 2020)

				the pore sizes, eincreasing the YM and toughness.		
				Print defects were observed in this method.		
Hydrolysed sunflower meal	Fermentation in static tray bioreactors	Biofilms	Crude Glycerol as a carbon source	Bio-based films enhanced with bacterial nanocellulose networks. Showed 64.5% higher TS and 131% higher EB.	Biodegradable	(Efthymiou et al., 2022)
Pea protein + acetylated cassava starch	Blown-film extrusion	Edible films	Glycerol & distilled water	The sachets provided effective protection for soybean and olive oils stored under varying humidity conditions over 3 months.	Edible packaging	(Huntrakul et al., 2020)
Chickpea flour + chickpea hull	Surface coating method	Edible coating	N/A	Chickpea hull extract as an antioxidant for sunflower seed coating to extend shelf life. A consumer preference test (86%) confirmed that the texture of the sunflower seeds was improved; participants were willing to purchase the product.	Edible packaging	(Camiletti et al., 2023)

Soyabean protein hydrolysates	Surface coating method	Edible coating	N/A	Edible double layered films for vegan active packaging of tofu product. First layer was an extract from an algae while the second layer was soyabean protein hydrolysates from the bran of the soybeans.	Edible packaging	(Tkaczewska et al., 2023)
Butterfly pea blue petals	pH Indicator	Biosensor	N/A	Anthocyanins present showed high stability over pH, temperature and light. High potential in biosensors in smart food packaging.	Smart Packaging	(Escher et al., 2020)

TS=Tensile strength

YM=Young's modulus

EB=Elongation at break

OP=Oxygen permeability

WVP=Water vapour permeability

Each fabrication method varies in terms of mechanical performance, scalability, cost, and suitability for specific types of biopolymers and applications.

2.5.2. Solvent casting

Solvent casting is one of the most accessible and widely used techniques for producing thin biopolymer films, particularly at the laboratory scale. In this method, biopolymers such as soy protein, pea starch, or lentil flour derivatives are dissolved in an appropriate solvent, often water or an alcohol-water mixture, along with plasticizers and functional additives. The solution is then cast onto a flat surface and dried under controlled conditions to remove the solvent, leaving a uniform film. Hopkins et al. (2019) studied five legume (soy, lupin, pea, lentil, and faba bean) protein-based solvent-casted films to characterize their film properties. The authors stated that the highest and lowest tensile strengths (TS) were observed in faba bean films and lupin protein films, respectively. However, the most promising and suitable coproduct was pea protein-based thin films due to their relatively high TS and elongation at break (EB), and their low water vapour permeability (WVP). Plasticizers are substances added to polymer matrices to improve flexibility and reduce brittleness, often using less volatile liquids or solids (Mishra & Duan, 2024). Their type and concentration significantly influence the mechanical and barrier properties of biopolymer films. Chang & Nickerson (2014) compared glycerol, sorbitol, and PEG 400 as plasticizers for canola protein-based films, finding that glycerol and PEG (liquids) enhance elasticity, while solid sorbitol reduces water vapour permeability (WVP). Glycerol is the most extensively studied due to its ability to form thin films from various plant co-products. Hopkins et al. (2019) found that a 50% glycerol-to-protein ratio yielded optimal tensile and water barrier properties in legume protein films. Similar findings have been reported in canola meal protein (Chairez-Jimenez et al., 2024; Dissanayake et al., 2022b) and cardoon seed oil cake proteins (Mirpoor et al., 2024). Some studies have reduced glycerol to 35% to improve material efficiency, such as with chia seeds (Muñoz-Tebar et al., 2021). This technique is particularly well-suited for hydrophilic polymers and has been used successfully to create edible coatings (Camiletti et al., 2023) and biodegradable films (Akhila et al., 2023) with moderate mechanical properties and barrier performance. Chairez-Jimenez et al. (2024) sought to reinforce solvent-cast canola protein-based films by adding graphite oxide to improve their water and oxygen barrier properties. The study improved the

oxygen barrier properties by 665% compared to neat protein films, demonstrating the potential of the solvent-casting method for future research.

The solvent-casting method is a preliminary, lab-scale method used to assess a biopolymer's compatibility before scale-up for pilot or large-scale production. According to Ghosal et al. (2018), a less-ordered crystalline structure was observed in the thermal properties due to longer solvent evaporation times in the solvent-casting method. Furthermore, rapid solvent evaporation could reduce crystallinity and impart greater amorphous character to flaxseed mucilage films (Akhila et al., 2023). This behaviour was observed in most solvent-cased oilseed proteins, where nanomaterials were incorporated to increase material crystallinity and enhance the tensile properties of the packaging material (Abookleesh et al., 2024; Dissanayake et al., 2023). The time required for solvent evaporation also varies with climatic conditions unless otherwise controlled; supplying environmental conditions is not economically viable. Plasticization plays an important role in this method. Once the solvent (usually water) evaporates, the polymer matrix is susceptible to brittleness, cracking, and breaking. Film properties vary with the amount and type of plasticizer, with higher levels increasing flexibility and reducing water barrier properties (Chang & Nickerson, 2014; Hopkins et al., 2019). Whereas the other methods could perform regardless of the level of plasticization (Jerez et al., 2007). The limitations of this method include longer drying times, concerns about solvent recovery, low water-barrier properties, and challenges with scalability for industrial production. In terms of industrial scalability, this method performs worse than other methods, such as electrospun films (Ghosal et al., 2018) and extruded thin films (Ochoa-Yepes et al., 2019).

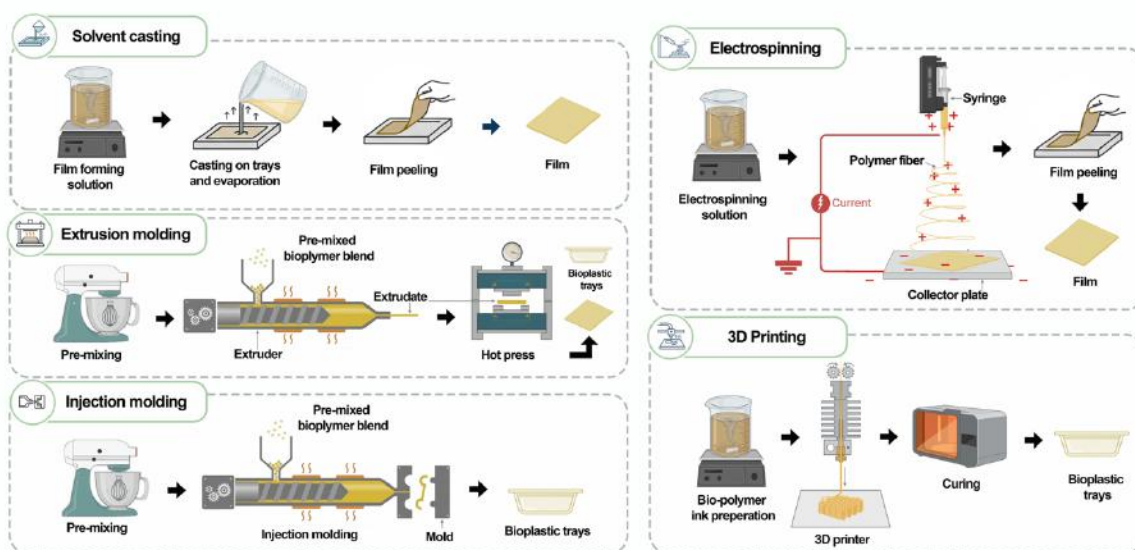


Figure 2.6. Different fabrication methods in food packaging material development

2.5.3. Extrusion

Extrusion is a scalable, industrially relevant process for fabricating films, sheets, and trays from thermoplastic biopolymers. Extrusion moulding involves melting or thermoplasticizing the polymer under heat and shear and forcing it through a die to produce a continuous film or sheet. The process relies on polymer flow behavior, thermal stability, and shear-induced molecular orientation to achieve uniform film formation (Pommet et al., 2003). Bioplastics derived from defatted soy flour, pea starch, or other coproduct blends are heated and pressurized as they are forced through a die to form continuous films. The technique allows for high throughput, uniform thickness, and the integration of functional additives, including antioxidants or antimicrobial agents from seed husks or meal extracts. Melikoğlu et al. (2023) investigated the effect of nanocrystalline cellulose (NCC) fillers derived from sunflower head waste as a reinforcement material for LDPE-based extruded plastic films. According to the results, the NCC content should be below 15 wt.% to obtain improved film properties, such as 20 MPa of TS and 250% of EB, which are perfect for food packaging. The authors noted that higher filler levels could affect exfoliation due to the nanocomposite's non-homogeneous nature. Extrusion is advantageous due to its compatibility with existing plastic processing infrastructure and its ability to improve the barrier properties of biopolymer films. Gunning et al. (2014) produced hemp fibre from hemp

waste for extruded films and obtained a TS of 17.9 MPa. However, challenges persist in processing heat-sensitive biopolymers and maintaining consistent film quality (Prabha et al., 2021). Ochoa-Yepes et al. (2019) studied the effect of lentil protein on starch films prepared by solvent casting and extrusion. Compared to the solvent-casting method, thermocompression can produce films with high toughness, while cast films are more flexible. Furthermore, starch-protein films prepared by casting showed some pinholes, whereas extruded films were homogeneous, indicating the high potential of the extrusion moulding method for industrial film preparation. As this method is continuous in production, maintaining a homogeneous film composition is very important. The extrusion method has the ability to pre-determine the degradation temperature point. Processing the biopolymer materials below that maximum point would allow the material to reach its full thermoelastic potential as a thermoplastic material (Castro-Criado et al., 2025). Extrusion technology can increase the flexural strength of blend materials, especially those containing industrial materials and byproducts, through high-temperature processing parameters. Rahman et al. (2023) reported a PLA and soy fibre and protein-induced polymer blend with higher mechanical properties, including flexural strength, in extruded filaments. As extrusion is already established in the food and polymer industries, it offers a technologically mature platform for upcycling oilseed industry residues into biodegradable, circular-economy packaging solutions (Granados-Carrera et al., 2025)

2.5.4. Compression molding

Compression moulding is primarily used to produce rigid packaging items, such as trays, clamshell containers, and biobased cutlery. Compression moulding is based on placing a pre-measured polymer mass between heated platens and applying pressure to induce flow and consolidation into a film. Film formation occurs through heat-assisted polymer softening and pressure-driven chain rearrangement, resulting in dense and uniform structures (Harper, 2006). In this method, a pre-measured amount of biopolymer blend, often reinforced with fibre residues or fillers (e.g., from flaxseed husks or soy hulls), is placed in a mould cavity and compressed under heat. The resulting products have high structural integrity and are suitable for replacing plastic or polystyrene (PS) in single-use applications. Versino et al. (2021) developed a composite bio-foam using sunflower oil press cake, incorporated with cassava starch and glycerol, via the thermocompression method. Researchers were trying to find a solution to replace the PS foams, which are widely used in food packaging and the transportation industry. The processing conditions were at 170 °C for 3.5 mins

under a hydraulic press with 100 bars. The density of the commercial PS was 0.03 g/cm³, while bio-foam had a minimum density of 0.15 g/cm³. Furthermore, the water solubility of the PS was 0.1%, whereas it was 5.1% in the biofoam. These higher densities would devalue the quality of the bio foam, even though it is helping the circular bioeconomic process. Another group of researchers developed bioplastic sheets using 41% canola protein, 41% polyvinyl alcohol (PVA), and 18% glycerol as the plasticizer (Patel et al., 2016). A 60% CaCO₃ filler was incorporated, resulting in a TS of 22.64 MPa and an EB of 10.69%. The authors suggested that these composites are suitable for the preparation of lightweight cups and spoons, offering a zero-waste alternative to the food industry.

2.5.5. Injection molding

Injection moulding enables the production of complex three-dimensional shapes, such as containers, cups, and lids, from thermoplastic biopolymers. Injection moulding involves injecting a molten polymer into a closed mould cavity, where it cools and solidifies into a predefined shape. The principle relies on rapid filling, controlled cooling, and pressure to ensure complete mould replication and structural integrity (Rosato et al., 2000). Agricultural coproducts can be formulated into granules or composites and melted into mould cavities under pressure. This technique enables precision fabrication and mass production, especially when using blends with thermoplastic starch, protein isolates, or PLA derived from agro sources. Rahman et al. (2023) used soybean hulls and soya protein isolate to produce bioplastic cups. These cups are morphologically very similar to polyethylene cups. Pea protein is another sustainable material for the preparation of bioplastics. (Perez-Puyana et al., 2016) moulded (60 X 10 X 1) mm³ rectangular-shaped blocks using pea protein and glycerol. The authors mentioned that the TS of pea protein-based moulds is higher than that of soy protein-based bioplastics. But, according to the literature, TS and EB are 30% and 40%, respectively, in pea protein bioplastics compared with commercial LDPE. Jiménez-Rosado et al. (2022) conducted a study to compare the injection moulding and extrusion methods for soy protein bioplastics. In this study, both water and glycerol were used as plasticizers, and injection moulding produced higher mechanical properties than extrusion. Even though the extrusion method did not improve the properties as expected, the authors suggested that it would be the next step towards the commercialization of the product in the packaging industry.

2.5.6. Blow Molding

Blow moulding is a key industrial route for converting oilseed industry by-products (soy, canola, sunflower hulls/fibers, and protein fractions) into high-value, hollow food-packaging items (bottles, jars, plant pots, and containers). Blow moulding is based on inflating a heated polymer tube or parison inside a mould using pressurized air to form thin-walled films or containers. Film thickness and orientation are controlled by melt strength, air pressure, and cooling rate during expansion (Harper, 2006). Its importance lies in very high throughput, low per-unit cost for hollow geometries, and direct compatibility with polylactic acid (PLA) and PLA-based biocomposite pellets produced from oilseed fillers. Therefore, the waste streams can be up-cycled into durable, compostable packaging while preserving established manufacturing infrastructure (Ilyas et al., 2021). However, successful use of oilseed by-products in blow moulding requires careful control of filler particle size and surface treatment, optimized melt rheology (to provide sufficient melt strength and stretchability during the parison/blow step), and often compatibilizers or polymer blends to avoid brittleness and poor dispersion. Rahman et al. (2023b) demonstrate feasible formulations and processing routes for PLA + soybean-hull-fibre composites and show how palletization and downstream moulding (injection/blow) can produce functional containers when composition and thermal/rheological processing are optimized. Huntrakul et al. (2020) developed transparent sachet pouches made from pea protein and acetylated cassava starch for storing soybean and olive oil via the blow-film extrusion method. The sachets provided effective protection stored under varying humidity conditions over 3-month period.

2.5.7. Electrospinning

Electrospinning is an advanced technique to create nanofibrous films with enhanced surface area, breathability, and functional properties. It involves applying a high-voltage electric field to a polymer solution, causing it to eject fine fibres that are collected on a grounded surface (Amjadi et al., 2024; Ghosal et al., 2018b). One of the major limitations of using industrial byproducts with advanced techniques like electrospinning is the compatibility of the polymer with the processing technology. Electrospun nanofibers were developed by Aydogdu et al. (2019) using lentil flour and polyethylene oxide (PEO) to encapsulate gallic acid, as the electrospinning method is ideal for encapsulating heat-sensitive bioactive compounds. The incorporation of PEO made the polymer blend more electrospinnable by reducing the strong repulsive forces between the protein materials.

These materials were used to pack walnuts in this study, and the results showed successful encapsulation, enhancing the oxidative stability of the active food packaging. Furthermore, as this method can form nano-level submicron fibres, it can effectively incorporate active ingredients such as gallic acid into packaging materials. Amjadi et al. (2024) successfully incorporated black chickpea (BCP) peel anthocyanins into a BCP protein film as a double-layered active packaging for seafood products. The authors noted that the high surface area improved the efficiency of the anthocyanin active agents. However, the main challenge of this method is the scalability and consistency of the double layer at the industrial level. According to Ghosal et al. (2018), the electrospinning method is better for preparing membrane films than the solvent-casting method. Because the electrospun membranes with an amorphous structure were soft and flexible without compromising the durability, while membranes made from the same solvent but with semi-crystalline regions were stiff and brittle. The authors suggested that the above behaviour is due to gaps between electrospun polymer strands, which weaken intermolecular cohesion and give the chains greater freedom to move. As a result, the material deforms more easily under applied stress, and once the stress is released, the polymer chains quickly return to their relaxed state because of the additional free volume. The electrospinning method produces ultrafine fibres with exceptionally high surface area, porosity, and tunable morphology. This allows the creation of lightweight, biodegradable nanofibrous mats with functional properties that conventional methods (casting, extrusion, compression moulding) cannot achieve (Mallavia & Falco, 2020). The thermal stability of the electrospinning method is achieved through proper, homogeneous mixing of the biopolymer blend. These properties make this method highly scalable compared to the solvent-casting method (Alsadat-Seyedbokaei et al., 2024).

2.5.8. 3D printing

3D printing is a highly customizable, emerging technique for producing biodegradable packaging on demand, especially for niche applications or short product runs, given its high cost. 3D printing fabricates films or structures through layer-by-layer deposition of polymeric materials based on a digital design (Panjabrao et al., 2025). Among several 3D printing methods, inkjet and extrusion-based 3D printing receive the most attention, offering high resolution and simultaneous printing. Using biopolymer-based inks derived from soy protein, chickpea flour, or flaxseed mucilage, customized containers and cushioning materials can be fabricated layer by layer (Thakur et al., 2025). The authors suggested that the fused filament fabrication (FFF) method has greater potential

for biopolymer-based thermoplastic 3D printing in packaging applications. Dey et al. (2023) used Soybean Protein and soy hulls to make 3D-printed compostable cups suitable for preparing both rigid compostable trays for food packaging using the FFF method. The gaps between the two consecutive layers were identified as a major limitation in maintaining cup consistency, resulting in low water retention. (Balla et al., 2020) studied the effect of 3D printing technology on soybean hull derivative fibres for food packaging materials. The authors observed some drawbacks with this method, including nozzle clogging due to wood particle aggregation, microscopic printing-induced defects in the printing materials, and increased porosity due to water and gas evaporation during printing. The authors suggested that increasing the processing temperature could address some of the shape-related issues. Solvent-cast 3D printing is another type of 3D printing in which solid polymer materials are mixed with a solvent within the printing machine, and the solvent evaporates after printing is complete. The major limitations of this method are the additional cost of solvent evaporation and the development of unsuitable mechanical and structural properties as a food packaging material due to solvent evaporation. Compared to the solvent casting method, this method has high customizability and faster processing time as advantages and is limited in mechanical characteristics (Witek-Krowiak et al., 2024). Despite their limitations, 3D printing techniques offer several advantages over other fabrication methods. Morales et al. (2021) reported the high potential and compatibility of combining synthetic and oilseed industry byproducts in a 3D-printed composite material formulation by mixing recycled polypropylene with cocoa bean shells. They mentioned that thermal processing should be done below 230 °C to avoid material degradation, and that it could be applicable at even higher temperatures with shorter processing times. Another advantage of this method is the ability to fabricate multilayer biocomposites with high efficiency. Li et al. (2022) reported a sandwich-type multilayered 3D-printed packaging material with antimicrobial intelligence, enabled by the proper dispersion of lemongrass essential oils. Authors further identified 3D printing as an efficient active food packaging system compared to the conventional solvent-casting method due to its multilayer compatibility. In summary, Solvent casting is suitable for small-scale edible and antimicrobial coatings due to its gentle processing and ability to incorporate bioactive compounds, but is limited by long drying times and low scalability. Extrusion moulding and compression moulding are industrially relevant, producing films, sheets, and biodegradable plates with higher scalability, though they require careful control of moisture, temperature, and protein properties. Injection moulding enables rigid

packaging but often needs protein blends or chemical modification to ensure melt processability. Electrospinning allows the creation of functional nanofibers for active packaging, while blow moulding and 3D printing offer options for bottles, containers, and custom designs, though both face challenges with protein melt strength and low mechanical performance. Overall, the choice of method depends on the desired product form, scalability, and functional requirements.

2.6. Extrusion moulding method in protein-nano composite blending

2.6.1. Extrusion moulding method

Premixing or pre-conditioning is the first critical step in extrusion–moulding–based preparation of plant protein films, to obtain a homogeneous, processable formulation. Plant proteins such as soy, canola, or pea protein are blended with suitable plasticizers (e.g., glycerol) to impart thermoplasticity and flexibility to the otherwise brittle protein matrix. Often, extrusion blend powder is kept overnight to equilibrate and properly hydrate with water molecules before extrusion. Water and plasticizers need time to interact with the protein to soften it. Proper hydration allows protein molecules to unfold and interact, making the material more processable and preventing it from becoming brittle or underplasticized during extrusion (Hernandez-Izquierdo et al., 2008). Optional additives such as nanomaterials, copolymers, or crosslinking agents may also be incorporated at this stage to tailor mechanical, barrier, or functional properties. Moisture content is carefully controlled during premixing, as it strongly influences protein plasticization, melt viscosity, and extrusion stability (Tadele et al., 2023). Uniform distribution of plasticizer and additives ensures consistent protein denaturation and flow behaviour during subsequent thermal processing (Hernandez-Izquierdo & Krochta, 2008; Y. Zhang et al., 2016).

Extrusion involves subjecting the premixed formulation to controlled heat, pressure, and shear within a single- or twin-screw extruder to convert it into a thermoplastic melt. During extrusion, plant proteins undergo unfolding and partial denaturation, allowing intermolecular interactions to reorganize in the presence of plasticizers and moisture. Process parameters, such as barrel temperature profile, screw speed, and residence time, are optimized to achieve adequate mixing and melt homogeneity while avoiding thermal degradation (Zhang et al., 2016). The extrudate can be shaped directly into films using flat dies or produced as pellets or sheets for downstream forming. Extrusion is particularly advantageous because it is continuous, scalable, and compatible

with reactive processing strategies to improve film strength and water resistance (Hernandez-Izquierdo & Krochta, 2008; Hurtado et al., 2024).

Compression moulding is commonly applied as a post-extrusion step to consolidate the extruded material into uniform films with controlled thickness and surface finish. Extruded pellets or strings are placed between heated platens and subjected to constant pressure and temperature sufficient to soften the protein matrix, eliminate voids, and enhance intermolecular bonding. This step can further promote crosslinking, structural rearrangement, and densification of the protein network, leading to improved mechanical integrity and reduced solubility compared with solvent-cast films. Controlled cooling under pressure helps stabilize the final film morphology and properties. Consequently, the combined extrusion–compression moulding approach is widely used for producing plant protein–based films with reproducible, industry-relevant performance (Ciannamea et al., 2014; Osorio-Ruiz et al., 2019).

2.6.2. Twin screw extruder

A single screw extruder consists of one central screw with varying diameters along the extruder barrel, often used for simple applications such as mixing, plasticization, and discharging of melt in film production. This processing method is very straightforward and mechanically reliable. (Delvar et al., 2025). As illustrated in Figure 2.7, the feed material is gravity fed, and the pressure gradient is not complex compared to twin screw extruders (Martin, 2016).

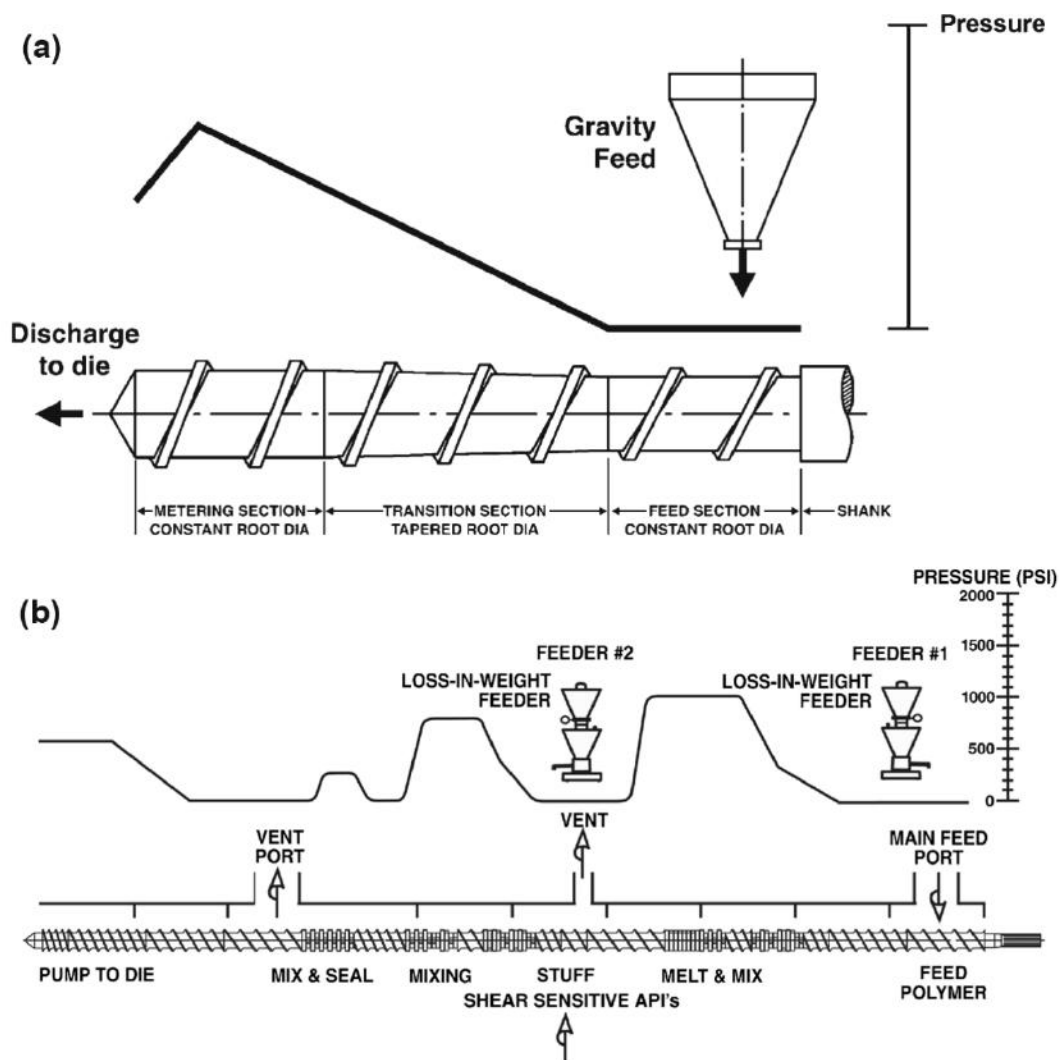


Figure 2.7. Cross-sectional illustrations of (a) single screw, (b) twin screw extruders with pressure gradients. (Martin, 2016).

Twin screw extruders have two parallel rotating screws in the barrel, with different rotating parts, which are often suitable for the processing of heat-sensitive polymers. Furthermore, in the polymer and packaging industry, these extruders are used for compounding different polymer materials, polymer filling, and are widely used for reinforcement applications (Delvar et al., 2025). As this contains two parallel screws, the rotation directions can be limited to two. Co-rotating screws rotate both screws in the same rotating direction, while counter-rotating screws rotate in opposite directions (Figure 2.8).

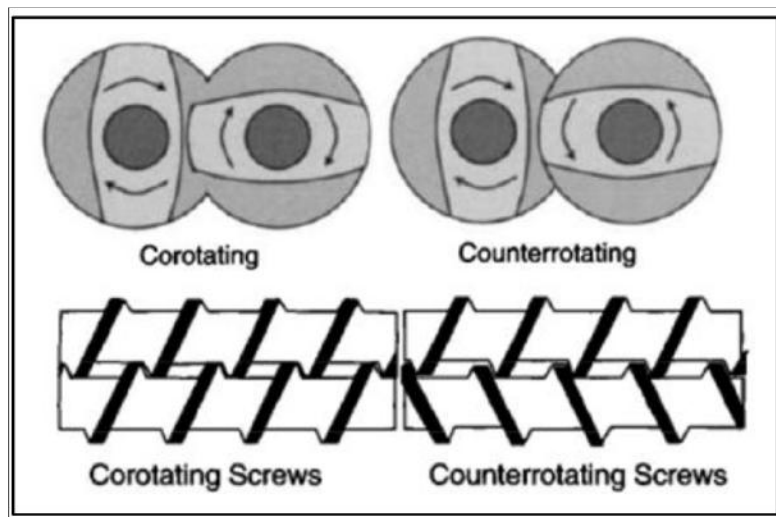


Figure 2.8. Illustration of screw rotations of single and twin screw extruders (Pitayachaval & Watcharamaisakul, 2019).

As illustrated in Figure 2.7, the twin screw extrusion has complex temperature and pressure gradients due to its internal structure (Martin, 2016). Furthermore, twin screws extruders are more suitable for dispersion of pigments and additives, polymerization reactions, and preparation of more homogenized polymer blends (Tsao et al., 1978). Padmanabhan (2008) reported the extruder processing zones as the heart of the twin screw extruder. The author studied on a 6-temperature zone profile, labelling the intake, melting, venting, mixing, vacuum and metering zones with specific objectives for each zones such as the material introduction to the extruder, heating and melting of the material with partial or total mixing, removal of volatile gasses and moisture, proper mixing, complete removal of volatiles and the building up the required pressure in the die, respectively. The Thermo Fisher process 11 twin screw extruder is one of the flagship benchtop extruders commonly used in biopolymer processing due to its 8 temperature profile, real-time customizability, continuous processing ability, and low ingredient usage. Jiménez-Rosado et al. (2022) used this twin screw extruder to process the zinc-incorporated soy protein-based bioplastics.

2.7. Properties of plant protein-based packaging films

Characterizing the mechanical, thermal, barrier, and structural properties of packaging films is essential to verify their effectiveness and suitability for food packaging applications. Among these properties, mechanical properties are paramount because they determine the material's stretchability and strength. Key mechanical parameters include tensile strength, Young's modulus, and elongation at break. For instance, Dissanayake et al. (2022) reported a significant increase in tensile strength when 5% nanocrystalline cellulose (NCC) was incorporated into canola protein films, from 3 MPa to 8.3 MPa. Li et al. (2017) studied the organic crosslinking effect by adding 1,4-Butanediol diglycidyl ether (BDDE) to canola protein films and reported that the modification enhanced tensile values by approximately 1.8×. (Akhila et al., 2023) used Genipin as a natural crosslinking agent and reported a 1.25× increase in the tensile strength, due to the protein modification. Furthermore, Akhila et al. (2023) used CaCl_2 as an ionic chemical crosslinking agent and reported a significant reduction in the elongation at break due to the anti-plasticization effect. According to the authors, the presence of Ca^{2+} facilitates ionic crosslinking, intensifying polymer–polymer interactions, thereby restricting chain mobility and enabling closer packing during elongation.

Barrier properties are another critical factor, as packaging materials must resist the transmission of water, moisture, air, and gases to extend shelf life and prevent fat oxidation by minimizing exposure to atmospheric oxygen. Key barrier property parameters include water vapour permeability, oxygen permeability, and water solubility. As illustrated in Figure 2.9, the incorporation of nanomaterials exfoliates inside the protein matrix properly and makes hydrogen bonds between both the nanomaterials and protein chains. This increases the tortuosity of the material, blocking available paths for moisture and gas molecule transmission. This is one of the reasons nano-reinforcement improves the barrier properties of protein films (Dissanayake et al., 2025). Furthermore, Chairez-Jimenez et al. (2024) reported that the incorporation of graphite oxide nanomaterials increased the oxygen barrier properties by 665% in canola protein-based solvent-casted films. As illustrated in Figure 2.5, the oxidation of graphite introduces new polar functional groups, such as COOH , OH , and ester groups, thereby increasing the polarity within the films. Due to the non-polar behaviour of O_2 molecules and the aforementioned toroidal nature of the nanoreinforced films, this may reduce the oxygen permeability of the canola protein-based films (Bandara et al., 2017; Chairez-Jimenez et al., 2024). Mojumdar et al. (2011) highlighted that wheat

gluten-based packaging films exhibit lower water-barrier properties than other materials, which poses a limitation for protein-based films. However, these films provide excellent oxygen barrier properties, making them advantageous for packaging high-fat foods by minimizing fat oxidation. Additionally, light and UV transmittance are sometimes assessed, particularly for applications where opaque films are preferred to reduce photodegradation of the packaged product. Akhila et al. (2023) reported the light transmittance and the UV blocking property of the CaCl_2 crosslinked flaxseed mucilage films with TiO_2 nano reinforcement. The UV-blocking property was not affected by the crosslinking. However, the addition of TiO_2 nanomaterials improved the clarity and the transparency of the films. The authors explained that this might be due to increased crystallinity of the nanomaterials in the films.

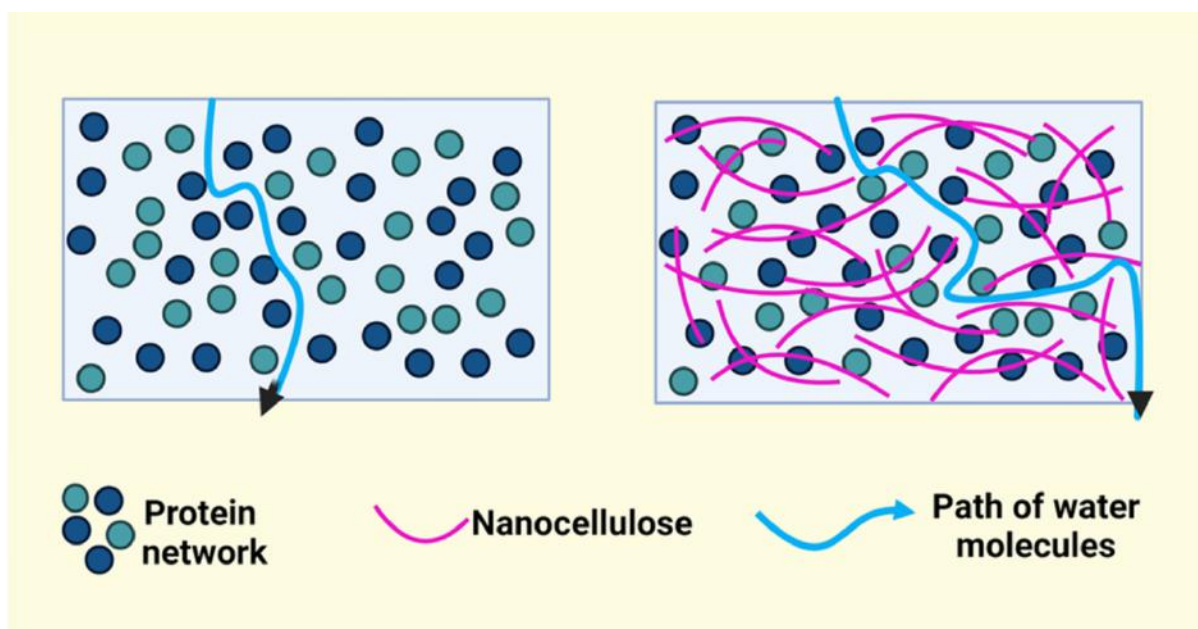


Figure 2.9. Increased water barrier properties due to the incorporation of nanomaterials in protein films. Reprinted with the kind permission of (Dissanayake et al., 2025).

Thermal properties are vital for industrial applications, as they help determine the film's stability and the protein's resistance to denaturation during modification and crosslinking processes. Commonly measured parameters include the glass transition temperature, melting temperature, and peak enthalpy. The melting temperature provides a general indication of the material's degradation point. Analytical techniques such as thermogravimetric analysis (TGA) and

differential scanning calorimetry (DSC) are typically used for such evaluations. Manamperi et al. (2010) reported that refined canola protein films exhibited a melting point of 111.5 °C, indicating greater thermal stability compared to soy protein, which has a melting temperature range of 70–90 °C.

Biodegradable plastics are gaining popularity for their eco-friendly nature and potential to reduce environmental plastic waste. Unlike traditional plastics, which can persist in the environment for centuries, biodegradable plastics are designed to decompose naturally through microbial activity. Bioplastic packaging is compostable, breaking down into natural components such as water, carbon dioxide, and biomass. The biodegradation process involves the growth of microorganisms, such as bacteria, fungi, and actinomycetes, which utilize starch as an energy source. These microorganisms secrete extracellular enzymes to degrade the polymer. Patel et al. (2016) explored the biodegradability of canola-based films through various approaches. For natural degradation to occur, the film must absorb moisture from the environment, and the surrounding conditions must be conducive to microbial growth. However, biodegradability tends to decrease when starch films are incorporated with nanomaterials, as these films exhibit enhanced water barrier properties compared to pure starch films. Additionally, treating films with antimicrobial agents, such as bacteriocins, can further hinder biodegradation by reducing microbial affinity for these materials (Ilyas et al., 2018). Patel et al. (2011) reported that various bacterial and fungal species, including *Rhodococcus* sp., *Enterobacter dissolvans*, *Bacillus subtilis*, and *Pseudomonas aeruginosa*, are responsible for the biodegradation of protein-based films within 2-9 months. They studied compression-moulded films using an 80:10:10 blend of soy protein, castor protein, and canola protein, with 60% PEG400 as the plasticizer. They reported the highest biodegradability of the films, highlighting the highest soluble protein concentration, especially in the presence of *Pseudomonas aeruginosa*. Furthermore, 62.06% of the highest degradation was observed in the above blend due to the microorganisms within 52 days. Contrastingly, increasing canola protein content from 10% to 20% and removing 10% of the plasticizer from the blend reduced the films' biodegradability from 62.06% to 51.52%. Therefore authors suggested that the plasticization is critical in the biodegradation of the protein films. Patel et al. (2016) studied the biological degradation of rapeseed protein-based packaging films in both lab-scale soil and on liquid media using 41% rape seed protein, 18% glycerol, and 41% PVOH. *Comamonas acidovorans* MTCC 3364, *Bacillus megaterium*, and the *Consortium* were used as microorganisms in the

biodegradation test. Authors reported that both *Comamonas acidovorans* and *B. megaterium* are responsible for 44% biodegradation on the 48th day under soil conditions, while the *Consortium* reduced up to 57% in soil and 74% in liquid conditions, indicating it as an optimal species for canola protein biodegradation. Furthermore, this study concluded that no pollutants were released to the environment and that the materials are suitable for the preparation of disposable single-use spoons, cups, and lightweight utensils. Palaniyappan et al. (2024) reported that 15% walnut shell/PLA cups made using the 3D printing method showed 6.4% and 19.1% biodegradation in 30 and 60 days, respectively. As depicted in Figure 2.10, the formation of covalent bonds during the crosslinked protein-based films increases the time for biodegradation due to the strong interactions in the film's matrix, limiting the microorganisms from breaking the linkages in the films (Patel et al., 2011). The addition of nanomaterials as reinforcement materials also acts similarly to a crosslinking effect, resulting in increased biodegradation time. Condés et al. (2015) studied the biodegradability of both native amaranth protein and maize nanocrystals added (6 wt% and 12 wt%) to amaranth protein-based films. They reported the weight loss of the native film after one week was 80% while 6 wt% and 12 wt% nanocrystals added films showed 18% and 24% weight loss in the films, respectively. However, the authors reported that after the 2nd week, the biodegradation of nanocellulose was induced, and complete biodegradation was observed after 3 weeks. The nanocellulose protected the protein matrix from degradation for a short period of time. Authors conclude that the nano-reinforced protein films are difficult to biodegrade in short periods of time and are suitable for a specific type of packaging application. Similarly, an increased biodegradation period (> 21 days) was reported by Akhila et al. (2023) in crosslinked flaxseed mucilage films by CaCl₂ as the crosslinking agent and TiO₂ as the nano reinforcement agent.

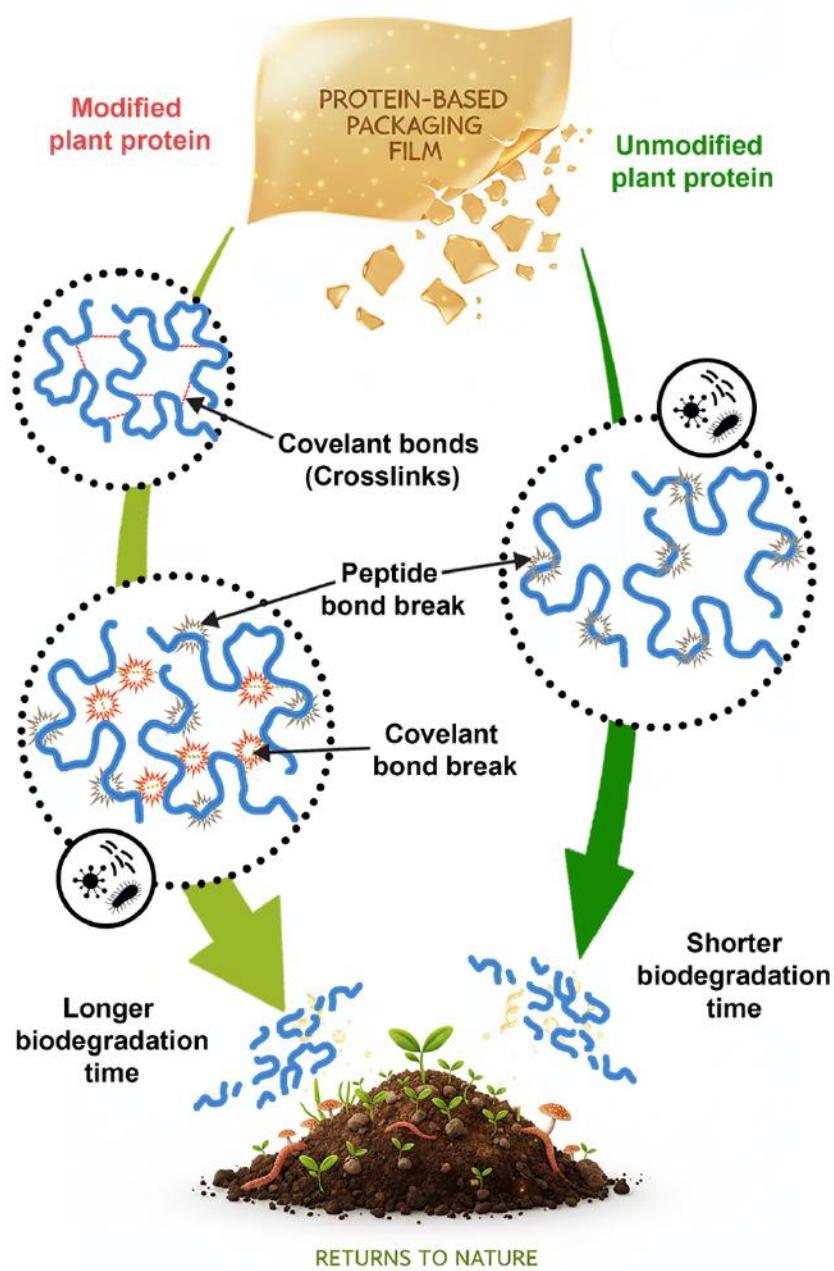


Figure 2.10. Graphical illustration of microbial biodegradation of modified and unmodified plant protein-based films

Ochoa-Yepes et al. (2019) conducted a comparative study on the biodegradation of lentil protein-cassava starch composite thin films prepared by extrusion and solvent casting. They reported that all films were completely degraded within 5 weeks, and it was difficult to distinguish differences in biodegradability due to the fabrication method.

2.8. Environmental and agricultural aspects of canola protein-based biodegradable plastic packaging.

Greenhouse gas emissions and environmental pollution are two critical challenges associated with traditional plastic industries, particularly in the food sector (e.g., single-use plastics and food wraps) and agriculture (e.g., bale wraps and mulching). As a result, there is a growing demand for bioproducts and biopolymers to address these environmental issues. Utilizing canola processing by-products to develop sustainable food packaging materials offers a promising solution to mitigate these challenges while contributing to the circular economy. These biobased products can help reduce greenhouse gas emissions and toxic volatile emissions (such as formaldehyde) while minimizing environmental harm (Evode et al., 2021). Biopolymers derived from agricultural by-products, including those from the canola processing industry, are gaining attention for their potential to address sustainability concerns. Dey et al. (2023) reported the potential of soy protein and soy hulls for 3D-printed bioplastic cups, both as food packaging materials and as compostable planting pots, highlighting the reusability of the byproducts. Similarly, Palaniyappan et al. (2024) reported on walnut-shell-incorporated PLA pots, and (Castro-Criado et al. (2025) studied the iron-releasing properties of soy-protein-based extruded bioplastics. Castro-Criado et al. (2025) used both glycerol and water as plasticizers for this and noted that mixing both improved the controllability of iron release into the water at the end. Similarly, Jiménez-Rosado et al. (2022) studied the behaviour of zinc released from soy protein-based extruded biopolymers. Furthermore, Rahman et al. (2023) developed soy protein and PLA-based injection-moulded plant containers to assess their nutrient-releasing ability and biodegradability. They identified the potential of plant-protein-based bio-composites for the development of biodegradable materials for agricultural applications. Currently, canola meal has limited value-added applications, primarily as an ingredient in ruminant animal feed. As mentioned above, canola protein-based single-use bioplastics can also produce biodegradable films, cups, pots, and sheets, with a high potential for a circular bioeconomic process.

2.9. Future aspects

The solvent casting method is the most commonly used technique for fabricating films in laboratory settings. However, extrusion moulding is the preferred method in the packaging industry for commercial production. Therefore, investigating extrusion moulding for canola protein-based packaging films is crucial to bridge the gap between laboratory-scale and industrial-scale applications. The use of mixed plasticizers can yield a synergistic effect on film formation, warranting further in-depth exploration to optimize film properties. Additionally, ammonium persulfate has shown promise as a chemical modifier to enhance the mechanical and barrier properties of canola-based films. However, the application of the APS crosslinking agent in other fabrication methods, such as extrusion moulding, injection moulding, and electrospinning, could be further examined to identify its fabrication potential for canola protein-based films. Notably, extrusion methods and 3D printing technology remain largely unexplored for producing canola protein-based food packaging materials. Furthermore, the potential of copolymer extrusion with available natural and synthetic polymers will enable assessment of the compatibility of canola protein with copolymer biocomposite fabrication. The injection moulding method is a promising technique for developing rigid bioplastics, which might have significant potential for developing canola protein-based rigid or semi-rigid food packaging materials and for modifying synthetic plastic-based single-use kitchen items, such as cutlery and coffee cups, in the future. The biodegradability and potential food toxicity of canola protein-based packaging films have not yet been adequately studied; they need further analysis. This represents a significant research gap and an exciting opportunity for innovation in this emerging field. Through this, the improvement of canola protein-based nano/bio composites could be realized, helping environmental sustainability while supporting the circular bioeconomic process.

Chapter 3 : Chemically Modified Canola Protein for Enhanced Material Properties in Canola Protein-Based Nanocomposites

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3.1. Abstract

The future of food packaging is moving toward biodegradable materials to promote environmental sustainability. Canola protein is a key component in the by-products of the canola oil industry. Limitations of previously developed canola protein-based biopolymer films include poor mechanical, structural, thermal, and barrier properties. In this study, a chemical modification approach was employed to enhance the canola protein, using ammonium persulfate (APS) to improve material properties through protein crosslinking and stronger cohesive interactions. Different concentrations of APS (1-9% w/w APS/protein) were used to modify canola protein isolate. The optimized protein (APS 5%) was mixed with nanomaterials [TEMPO-modified nanocrystalline cellulose (TMNCC) and Graphite oxide (GO)], and the solvent casting method was used to produce the films. Glycerol and sorbitol served as plasticizers. The film properties were characterized after fabrication. Optimized APS 5% modification significantly increases tensile strength, water vapour permeability (WVP), and the average enthalpy of the modified film by 1.33, 1.2, and 1.5 times, respectively, compared to the neat film. The addition of TMNCC increased the mechanical and thermal properties, with the highest tensile strength (7.03 ± 0.39 MPa), elongation at break (106.83 ± 20.58 %), and enthalpy (108.40 ± 3.75 J/g), while GO improved the barrier properties, with the lowest WVP (0.016 ± 0.0012 mm/m² kPa h). These findings highlight the potential of APS-modified canola protein films in advancing the reusability of

industrial by-products as valuable raw materials for sustainable plant biopolymer-based food packaging.

Keywords: Chemical modification, Ammonium persulfate, Canola protein, Food packaging, Sustainability

Graphical abstract



Author Contribution

Funds acquisition: Bandara N.; Conceptualization: Bandara N. and Abeysinghe V.; Methods: Abeysinghe V., Dissanayake T., Tadele D., and Bandara N; Analysis: Abeysinghe V.; Writing the first draft: Abeysinghe V.; Editing and reviewing: Bandara N., Mekonnen T. and Abeysinghe V.; Research Supervision: Mekonnen T. and Bandara N.; Student academic supervision: Bandara N.

3.2. Introduction

The growing global demand for sustainable materials has drawn significant attention to the development of biodegradable, bio-based food packaging materials. Though conventional petroleum-derived plastics are widely used for food packaging due to their durability, barrier properties, and low cost, they have raised substantial environmental concerns (Rajendran et al., 2025). Their resistance to degradation contributes to the accumulation of plastic waste in landfills and oceans, while their production is heavily dependent on non-renewable fossil fuels (Aquila et al., 2024). Recent machine learning models have predicted that uncontrolled single-use plastic consumption could generate 121 million tons of managed plastic waste by 2025 (Pottinger et al., 2024). Consequently, research has increasingly shifted toward renewable, biodegradable, and environmentally friendly alternatives that can reduce reliance on synthetic plastics while maintaining the critical functional requirements for food packaging (Dissanayake et al., 2022).

Among the promising candidates, plant protein-based biopolymers have emerged as attractive materials due to their biodegradability, film-forming ability, and versatility in functional modification. Proteins possess unique molecular structures, which enable them to form cohesive, flexible, and continuous films through intermolecular interactions such as hydrogen bonding, hydrophobic interactions, and disulfide linkages (Chairez-Jimenez et al., 2024).

Canola, also known as rapeseed (*Brassica napus*), is gaining particular attention as a raw material for biopolymer development. Canola is the world's 2nd-largest oilseed crop. Canada is one of the largest producers of canola seeds, primarily used for oil extraction, generating large amounts of protein-rich by-product often called “Canola-meal”. This low-value protein resource contains 37–40% w/w protein (C. Wang et al., 2014). Canola meal is currently used as an animal feed, especially in the dairy and aquaculture industries. Human consumption of this protein source is limited due to its unfavourable flavour profile and high glucosinolate content (Chairez-Jimenez et al., 2024; Višnjevec et al., 2021). However, extracted canola protein from the leftover meal provides a sustainable and cost-effective source for developing protein-based films (Dissanayake et al., 2022). Canola protein consists mainly of two storage proteins: cruciferin (~80%) and napin (~20%), as well as some oil body proteins, such as oleosins (~8%). Cruciferin (11S globulin) is a large hexameric protein with the potential for strong intermolecular bonding, such as hydrophobic and electrostatic disulfide interactions, while napin (2S albumin) is a smaller protein with good

solubility and film-forming ability. Oleosins are rarely extracted from defatted canola meal using alkaline extraction. Together, these proteins provide a suitable matrix for the development of biopolymer films (Wanasundara et al., 2016).

Previously made canola protein films exhibit limitations, including brittleness, poor mechanical stability, and poor thermal and barrier properties (Dissanayake et al., 2022). These shortcomings restrict their practical use in food packaging applications. To overcome these issues, researchers have incorporated several additives, such as reinforcement materials, water-barrier enhancers, and various types of protein crosslinkers. Chemical modification of protein chains has been studied as a promising way to reinforce canola protein films. The objective of chemical protein modification is to alter the protein's net charge by breaking or forming new bonds with reactive amino acid residues using chemical agents (Akharume et al., 2021). Several chemical crosslinking agents were used to modify the protein matrix, such as thiols (Woerdeman et al., 2004), carbodiimide (Tropini et al., 2004), formaldehyde (Benbettaïeb et al., 2016), tannic acids, bisepoxide (Li et al., 2017), and glycidyl methacrylate (Liu et al., 2025). Ammonium persulfate (APS) is an oxidizing agent in polymer chemistry, a bleaching agent, and a GRAS-approved food preservative widely used in the food industry (Mascheroni et al., 2016; H. Shi et al., 2017). Furthermore, APS has been used as a free radical initiator to graft materials (Ibrahim et al., 2016; Mascheroni et al., 2016). APS is widely used as a crosslinking agent in the preparation of polyacrylamide hydrogels, where it facilitates network formation by initiating polymerization reactions (Safronov & Terziyan, 2015). The available literature on canola protein modification using APS is limited. Bandara & Wu (2018) observed improved adhesion strength in canola protein-based adhesives when APS was used as a crosslinking agent, without any grafting compounds. Liu et al. (2025) used APS and glycidyl methacrylate to modify canola meal protein via compression moulding. This study highlighted the potential of APS alone to crosslink canola protein.

TEMPO [(2,2,6,6-Tetramethylpiperidin-1-yl)oxyl] oxidation can convert primary OH groups on crotaline nanocellulose (NCC) into COOH groups, thereby increasing the NCC surface's polarity. Dissanayake et al. (2022) reported that the TEMPO-modified NCC showed higher mechanical properties of reinforced canola protein films compared to neat films. The highest tensile strength value of 8.36 ± 0.85 MPa was identified in the 5% (w/w of protein) TM-NCC added films.

Graphite Oxide (GO) was identified as a promising nanomaterial, a cost-effective alternative to monolayer graphene, with good barrier properties. Chairez-Jimenez et al. (2024) reported a 5.65

$\times 10^{-3} \text{ cm}^3\text{m}^2/\text{day}/\text{Pa}$ of oxygen permeability in the 3% (w/w of protein) GO-embedded canola protein films, which was a 665% reduction compared to neat films.

A study on APS-modified canola protein-based packaging has not yet been conducted. We hypothesized that incorporating TEMPO-modified NCC and GO nanoparticles would further improve the functional properties of the chemically modified canola protein-based packaging films. Therefore, the objectives of this study were to develop chemically modified canola protein films at different APS concentrations (1, 2, 3, 4, 5, 7, and 9% w/w APS/protein) to identify the optimum APS concentration. Additionally, previously optimized conditions for TM-NCC (5% w/w protein) and GO (3% w/w protein) were tested to assess improvements in the functional properties of the packaging films.

3.3. Materials and methods

3.3.1. Materials

Defatted Canola meal pellets and native NCC were donated for research purposes by Richardson International (Lethbridge, AB, Canada) and InnoTech Alberta (Edmonton, AB, Canada), respectively. TEMPO (98.0% purity) was purchased from TCI America (PA, USA). Graphite ($\geq 99.5\%$ purity), Ammonium persulfate ($\geq 98\%$ purity), glycerol ($\geq 99.5\%$ purity), NaOH ($\geq 97.0\%$ purity), HCl (12N), H_2SO_4 ($\geq 95\%$ purity), and H_2O_2 ($\geq 30\%$ purity), and 3.5 kDa dialysis tubes (snakeskin) were obtained from Fisher Scientific (Ottawa, ON, Canada). Additionally, NaNO_3 ($\geq 97\%$ purity), NaBr (99.5% purity), KMnO_4 ($\geq 99\%$ purity), NaClO (14.5% available chlorine), along with anhydrous ethanol and glycerol ($\geq 99.5\%$ purity), were purchased from Sigma-Aldrich (Sigma Chemical Co, St. Louis, MO, USA). All chemicals employed were of analytical grade.

3.3.2. TEMPO modification of NCC

Surface oxidation of NCC was performed using a TEMPO/NaBr/NaClO system, with minor modifications based on protocols by Buffa et al. (2016) and Frascini et al. (2017). A dispersion containing 61.68 mmol of anhydrous glucose units was prepared by combining 10 g of NCC with 200 mL of Mili-Q water, followed by probe sonication in an ice bath for 30 min (using 15 s intervals every 30 s) at 60% amplitude, 20 kHz frequency, and 500 W output (Fisher Brand Model 505, Fisher Scientific, Connecticut, USA). Separately, TEMPO (107.06 mg, 0.69 mmol) and NaBr (1.27 g, 12.35 mmol) were dissolved in 200 mL of Mili-Q water under continuous stirring at 500 rpm. The NCC suspension was subsequently combined with these solutions, and

the mixture was stirred at 900 rpm. In an alternative beaker, a NaClO solution was prepared (molar ratio of NCC: NaClO = 1:2.5), and its pH was adjusted to 10.0 with 1 M HCl. This NaClO solution was gradually added to the NCC/TEMPO/NaBr mixture while maintaining the pH between 10.0 and 10.5 with 0.5 M NaOH. The reaction was terminated when the pH remained stable for 1 min without additional NaOH addition, typically after approximately 1.5 h. Subsequently, 20 mL of absolute ethanol was added to quench the reaction. The pH was then adjusted to 7.0 with 0.5 M HCl, and the suspension was stirred for an additional 15 min. Purification was achieved by dialysis against excess deionized water using 3.5 kDa membrane dialysis tubes for 5 days, with frequent water changes. Post-dialysis, the oxidized NCC was briefly sonicated (3 min, 30 s pulses with 15 s rests), freeze-dried, and stored at -20 °C until further utilization.

3.3.2.1. Degree of Oxidation (DO) of TEMPO-modified NCC

The degree of oxidation (DO), expressed as the percentage of primary -OH groups converted to -COOH groups, was determined using the conductometric titration approach described by Da Silva Perez et al. (2003). For this, a TMNCC suspension was prepared by dispersing approximately 30 mg of oven-dried TMNCC in 30 mL of 0.1 M HCl, followed by 3 min of sonication to ensure proper dispersion. The resulting suspension was titrated with 0.1 M NaOH, and conductivity (mS cm⁻¹) was monitored throughout the process using a conductivity meter (Model 06-662, Fisher Scientific, Pittsburgh, PA, USA). From the titration curve, the NaOH volume corresponding to the neutralization of -COOH groups was calculated as (V_f - V_i). This value was then applied in Equation (3.1) to determine the DO or -COOH group content formed.

$$DO = \frac{162 \times C \times (V_f - V_i)}{W - [36 \times C \times (V_2 - V_1)]} \quad (3.1)$$

Where, C = Concentration of NaOH (mol dm⁻³)

(V_f - V_i) = Volume of the NaOH

W = Weight of the oven-dried sample (g)

3.3.3. Preparation of graphite oxide

Graphite oxide (GO) was synthesized following the procedure of Hummers & Offeman (1958) with adjustments to the oxidation duration to generate materials with varying oxidation degrees and C:O ratios. Initially, 5 g of graphite was combined with 5 g of NaNO₃, after which 120 mL of

H₂SO₄ was slowly introduced while stirring in an ice bath at 3000 rpm. The mixture was stirred for 60 min. Subsequently, 15 g of KMnO₄ was incorporated while maintaining the reaction temperature at 35 ± 3 °C, and stirring continued for 60 min. After this stage, 92 mL of deionized water was added, and mixing proceeded for an additional 15 min. The reaction mixture was then neutralized using 80 mL of 3% H₂O₂ in deionized water at 60 °C.

Following neutralization, the products were cooled to room temperature and centrifuged at 10,000 rpm for 15 min at 4 °C. The resulting precipitates were washed three times with deionized water to eliminate residual reagents. Each washed precipitate was then subjected to probe sonication for 5 min at 20 kHz, 500 W, and 50% amplitude. Finally, the GO samples were freeze-dried and stored at –20 °C until further analysis.

3.3.3.1. Characterization and functional groups of GO

X-ray photoelectron spectroscopy (XPS) was employed to determine the elemental composition of GO samples, to evaluate the carbon-to-oxygen (C/O) ratio, and to monitor variations in surface functional groups as a function of oxidation time. Measurements for canola protein films were conducted using an Axis Ultra X-ray photoelectron spectrometer (Kratos Analytical Ltd., Manchester, UK) equipped with a monochromatic Al K-alpha X-ray source (186.6 eV). Survey scans were collected at a pass energy of 160 eV, while high-resolution spectra for C and O were obtained at 20 eV pass energy. A charge neutralizer was applied with a charge balance setting of 2.5 eV. Spectral data were processed and quantified using Casa XPS software, version 2.3.25PR1.0 (Casa Software Ltd.), to obtain the elemental composition and C/O ratios.

3.3.4. Canola protein extraction

Protein extraction from canola meal was carried out following the procedure of Manamperi et al. (2010) with minor adjustments. The defatted canola meal pellets were first milled twice using a Hosokawa milling system (Hosokawa Micron Powder Systems, Summit, NJ, USA) and then passed through 0.75 mm and 0.25 mm sieves to facilitate protein extraction. The ground material was then combined with distilled water at a 1:10 (w/v) ratio. The slurry pH was adjusted to 12.0 using 3 M NaOH, followed by stirring at 300 rpm for 1 hr at room temperature to facilitate protein solubilization. Non-protein material was removed via centrifugation (Beckman Coulter Avanti JXN-30 centrifuge, CA, USA; 10,000 g, 15 min, 4 °C), and the protein-rich supernatant was collected. The pH of the supernatant was then lowered to 4.0 to induce isoelectric protein

precipitation, and the precipitate was recovered by centrifugation as above. The resulting protein precipitate was freeze-dried in a semi-pilot plant freeze dryer (SP VirTis, Oakville, ON) and stored at -20°C until further use. Proximate composition of the extracted CPI, including protein (AOAC 990.03), fat (AOAC 920.39), and ash (AOAC 942.05) contents, was analyzed following AOAC methods.

3.3.5. Chemical modification of canola protein using APS

Modification of canola protein was carried out as described by Wang et al. (2014) with minor modifications. A weight of 3 g of freeze-dried CPI was added to 90 mL of deionized water, and the pH was adjusted to 7.0 using 1 M NaOH. Protein dispersions were transferred to a circulating water bath. All samples were purged with N_2 gas for 5 min prior to adding APS, and the water bath temperature was maintained at $30 \pm 2^{\circ}\text{C}$ during the experiment. The required amounts of APS were measured (0%, 1%, 3%, 5%, 7%, and 9% w/w APS/protein), added into the flask, and purging was done with N_2 gas for another 5 min. Flasks were sealed, stirred for 4 h (300 rpm, $30 \pm 2^{\circ}\text{C}$), and centrifuged (10 000 g, 15 min, 4°C) to collect the precipitate. The resulting APS-modified CPI samples were freeze-dried (SP VirTis, Oakville, ON) and stored at -20°C until further use. The average ash content of the modified CPI was determined according to the AOAC 942.05 method.

3.3.5.1. Particle size distribution and zeta potential of APS-modified CP

Particle size distribution of modified canola protein was analysed using static laser light diffraction (Mastersizer 2000; Malvern Instrument Ltd, Malvern, UK). Samples were dispersed separately in 0.01 mol/mL 7 pH phosphate buffer at 1 mg/mL and stirred for 2 h (300 rpm) at room temperature. For canola protein dispersion, the refractive index, absorption index, and dispersant refractive index were adjusted to 1.46, 0.10, and 1.33, respectively, and measurements were taken once a laser obscuration of 10–11% was reached. The particle size distribution was expressed as $D_v(0.1)$, $D_v(0.5)$, and $D_v(0.9)$. The zeta potential of the APS-modified protein samples was analyzed using the Litesizer 500 (Anton Paar Instruments, Austria). Samples were dispersed separately with Milli-Q water at a concentration of 1 mg/mL and stirred for 2 h (300 rpm) at room temperature. The dispersed proteins were transferred into an omega cuvette (Model no. 22528, Anton Paar, Austria), and their absorbance was measured via dynamic light scattering (DLS). The resulting data were collected and processed using Kalliope software (Anton Paar Instruments, Austria).

3.3.6. Fabrication of APS-modified canola protein films, reinforced with TMNCC and GO.

APS-modified CP films were prepared by solvent casting, adapted with minor modifications from the procedure outlined by Agarwal et al. (2020). To formulate the film-forming solution, 3.5 g of different APS modification levels (0%, 1%, 3%, 5%, 7%, and 9% of APS% content) of CPI (5% w/v, protein/ distilled water) was dispersed in 70 mL of distilled water, and the pH was adjusted to 10.0 using 0.5 M NaOH. This dispersion was stirred at 650 rpm for 30 min at room temperature, followed by heating in a water bath (VWR International, Cornelius, OR, USA) at 70 °C with agitation at 100 rpm for 20 min. After cooling to room temperature, glycerol was incorporated at a protein-to-plasticizer ratio of 1:0.5 (w/w). The resulting mixture was homogenized using a Fisher brand 850 homogenizer (Fisher Scientific, ON, Canada) at 14,000 rpm for 5 min.

When preparing the TEMPO-modified NCC and GO-reinforced APS-modified films, 20 mL of deionized water was separately mixed with 5% TMNCC (w/w TMNCC/protein) and 3% GO (w/w GO/protein), respectively. These dispersions were sonicated for 2 min at 60% amplitude, 20 kHz, and 500 W. At the same time, optimized APS modification conditions (APS 5%) were used to prepare the film-forming solution by dispersing 3.5 g of APS 5% CPI in 50 mL of distilled water, and the procedure was continued until the homogenization step. Sonicated nanomaterial (TMNCC and GO) suspensions were then separately added to the homogenized APS 5% CP solutions, followed by another final homogenization step at 14,000 rpm for 5 min to facilitate the exfoliation of nanomaterials through the protein matrix.

A final sonication step (5 min, 60% amplitude, 20 kHz, 500 W) was performed on all mixtures to ensure uniformity. These film-forming solutions were cast onto hexagonal polystyrene dishes at a volume of 32 mL. The films were air-dried for 48-72 h, carefully removed, and conditioned at 23 °C and 50% RH for an additional 48 h before further characterization.

3.3.7. Characterization of TEMPO NCC, GO, and APS modified canola protein films

3.3.7.1. Fourier transform infrared spectroscopy (FTIR)

The molecular structural changes in the APS-modified canola protein powder, APS-modified protein films, TMNCC powder, and nanomaterial-reinforced APS protein films were tested using using attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) (Invenio S, Bruker, Leipzig, Germany). Prior to each measurement, a background scan was conducted to reduce noise. Spectra of the films were collected over the wavenumber range of the 400–4000 cm⁻¹

with 120 scans per sample at a resolution of 4 cm⁻¹. Baseline correction was applied in the amide I region (1600–1700 cm⁻¹), and the spectra were normalized using Origin 2025 software (OriginLab Corporation, MA, USA). The second derivative of the amide I region was computed using the Savitzky–Golay method with a nine-point smoothing window, following Yang et al. (2015). Protein secondary structure components were determined through peak fitting with Gaussian functions. All FTIR measurements were performed in triplicate for each film replicate, averaged, and subjected to second derivative analysis.

3.3.7.2. X-ray diffraction (XRD)

X-ray diffraction (XRD) was used to assess the crystallinity of the APS-modified CP proteins, nanomaterials, and films, and to evaluate their exfoliation behaviour within CP films. Analyses were performed using a Siemens/Bruker diffractometer (D5000, BrukerNano Inc., Madison, WI, USA) equipped with a Cu X-ray source and operated in Bragg–Brentano reflection mode. Diffraction patterns were recorded over a 2 θ range of 5° to 50°, using a step size of 0.02° and a dwell time of 1 s per step. Background correction and data processing were carried out using Jade software (Materials Data and the International Centre for Diffraction Data, Newton, USA). The interlayer d-spacing values were calculated using the XpolderXTM software (XpolderXTM, Granada, Spain). Final data analysis and plotting were performed with OriginPro 2025 (OriginLab Corporation, Northampton, MA, USA).

3.3.7.3. Surface morphology analysis (SEM)

Surface morphology was examined using a field emission scanning electron microscope (FE-SEM; Quanta 650 FEG, FEI Company, OR, USA). Modified canola protein films were sectioned into 1 × 1 cm squares prior to analysis, and both surface and cross-sectional views were examined. To enhance conductivity, samples were sputter-coated with a metal layer composed of 60% gold (Au) and 40% palladium (Pd) under a nitrogen atmosphere (50 mTorr pressure, 45 mA current, for 60 s) using a Denton Vacuum Desk II coater (Denton Vacuum Inc., NJ, USA). SEM micrographs of the surface and cross-sectional morphologies were obtained at magnifications of ×2000 and ×300, respectively.

3.3.7.4. Mechanical properties

Mechanical properties of the films were evaluated in accordance with ASTM D882 (ASTM D882–18, 2018). Prior to testing, the canola protein films were conditioned at 23 °C and 50% relative

humidity for 48 hr. The impact of APS modification and nanomaterial reinforcement on the tensile strength (TS), elongation at break (EAB), and elastic modulus (EM) of films was assessed using an Instron 68TM-50 (Illinois Tool Works Inc., MA, USA) equipped with a 500 N load cell. Film specimens were prepared with dimensions of 50 mm (length) × 10 mm (width) × 0.25 mm (thickness) and tested at a crosshead speed of 5 mm/min. The force–displacement data were used to compute TS, EAB, and EM using Equations (3.2), (3.3), and (3.4), respectively. Each treatment was tested in 6 replicates, and the results were expressed as mean ± standard deviation (SD).

$$TS = \frac{\text{Maximum force of the film at break (N)}}{\text{Film length (m)} \times \text{Film width (m)}} \quad (3.2)$$

$$EB = \frac{\text{Film elongation (mm)}}{\text{Initial film length (mm)}} \times 100\%. \quad (3.3)$$

$$YM = \frac{\text{Film stress (MPa)}}{\text{Film strain (\%)}} \quad (3.4)$$

3.3.7.5. Water vapour permeability (WVP)

The WVP was measured using a gravimetric method adapted from the ASTM E96/E96M-16 standard (ASTM E96/E96M-16, 2016). Prior to testing, canola protein films were conditioned at 23 °C and 50% relative humidity for 48 h, as per the standards. Experiments were carried out in a controlled environmental chamber (Mettler HPP 260 Constant Climate Chamber, Germany) using disposable plastic cups (4.24 cm mouth diameter, 4.02 cm height). Each cup was filled with deionized water to approximately three-quarters of its capacity. Film samples were trimmed to fit the diameter of the cup opening and sealed with a water-resistant adhesive. The combined weight of the cup, water, and film was recorded, and the setup was placed in the chamber maintained at 23 °C and 50% RH for 4 h. After the exposure period, weight loss due to water vapor transmission was measured, and WVP was calculated using Equation (3.5).

$$WVP = \frac{\text{Weight change (g)} \times \text{Film thickness (mm)}}{\text{Area of the cup mouth (m}^2\text{)} \times \text{vapour pressure difference (kPa)} \times \text{time (h)}} \quad (3.5)$$

3.3.7.6. Water solubility

The water solubility of canola protein-based films was evaluated using a modified protocol based on the method described by González et al. (2019). Film samples were cut into 2 × 2 cm squares

and first dried in an oven at 105 °C for 24 h to determine their initial dry weight (W_i). The dried films were then immersed in 50 mL of deionized water in individual test tubes and incubated in a water bath at 25 °C with continuous agitation at 100 rpm for 24 h. After incubation, the remaining undissolved film was recovered and dried again at 105 °C for another 24 h to obtain the final dry weight (W_f). The water solubility of each film was then calculated as a percentage using Equation (3.6).

$$\text{Water solubility} = \frac{w_f - w_i}{w_i} \times 100\% \quad (3.6)$$

3.3.7.7. Water Contact angle (WCA)

The WCA measurements were performed to assess the surface hydrophobicity of the canola protein films. Film samples were cut into strips measuring 20 × 50 mm and analysed using a contact angle goniometer (Model 200–00-115, Rame-hart Inc., Mountain Lake, NJ, USA) equipped with DROP image Standard software. For each sample, the instrument took 10 contact angle readings on either the left or right side at 0.001 s intervals. The average of the valid angles was calculated and used for further analysis.

3.3.7.8. Thermal properties

The thermal behaviour of APS samples was characterized using a differential scanning calorimeter (DSC) (DSC 2500, TA Instruments, New Castle, DE, USA). Before analysis, the instrument was calibrated for both temperature and heat flow with an empty reference pan. About 10–15 mg of oven-dried samples was sealed in T-Zero hermetic aluminium pans. The differential heat flow of each sample was measured relative to the empty reference pan under a constant nitrogen purge. Samples were initially equilibrated at 0 °C for 10 minutes, then heated to 250 °C at 10 °C/min. Data acquisition and analysis were performed using TA Universal Analysis and OriginPro 2025 (OriginLab Corporation, Northampton, MA, USA).

3.3.7.9. Oxygen permeability (OP)

The OP of the canola protein films was evaluated using a custom-designed bubble flow rate apparatus. Films were carefully mounted in a dual-chamber cartridge, which served as a seal between the two compartments. One side of the cartridge was connected to a source of ultra-high-purity oxygen gas, while the other was connected to a bubble flow meter. A pressure gradient of 30 psi was maintained across the chambers, facilitating oxygen transmission through both the film

and the sealing interface. Bubble flow was monitored by recording the time required for the gas to displace a volume equivalent to 100 mL. The OP values, expressed in $\times 10^{-3} \text{ cm}^3 \text{ mm/m}^2 \text{ day Pa}$, were calculated using Equation (3.7).

$$OP = \frac{V}{t \times A} \times \frac{L}{P_2 - P_1} \quad (3.7)$$

where: $\frac{V}{t \times A}$ = Oxygen flux

L = Film thickness

($P_2 - P_1$) = Pressure difference across the film

3.3.8. Statistical analysis

All experimental treatments were performed in triplicate, and the results were expressed as mean $\pm$ standard deviation. Statistical significance was evaluated using one-way analysis of variance (ANOVA) at a confidence level of $p < 0.05$. Comparisons between means were conducted using the Tukey multiple comparison test. All analyses were performed using OriginPro 2025 (OriginLab Corporation, Northampton, MA, USA).

3.4. Results and Discussion

Proximate analysis of the extracted CPI revealed protein, fat, and ash content of $81.07 \pm 0.85\%$, $7.85 \pm 0.68\%$, and $2.47 \pm 0.02\%$ (DWB), respectively. These samples were used for the APS modification and subsequent film preparation. The average ash content of the modified CPI was $1.84 \pm 0.05\%$ (DWB).

3.4.1. Characterization of TEMPO modification of NCC

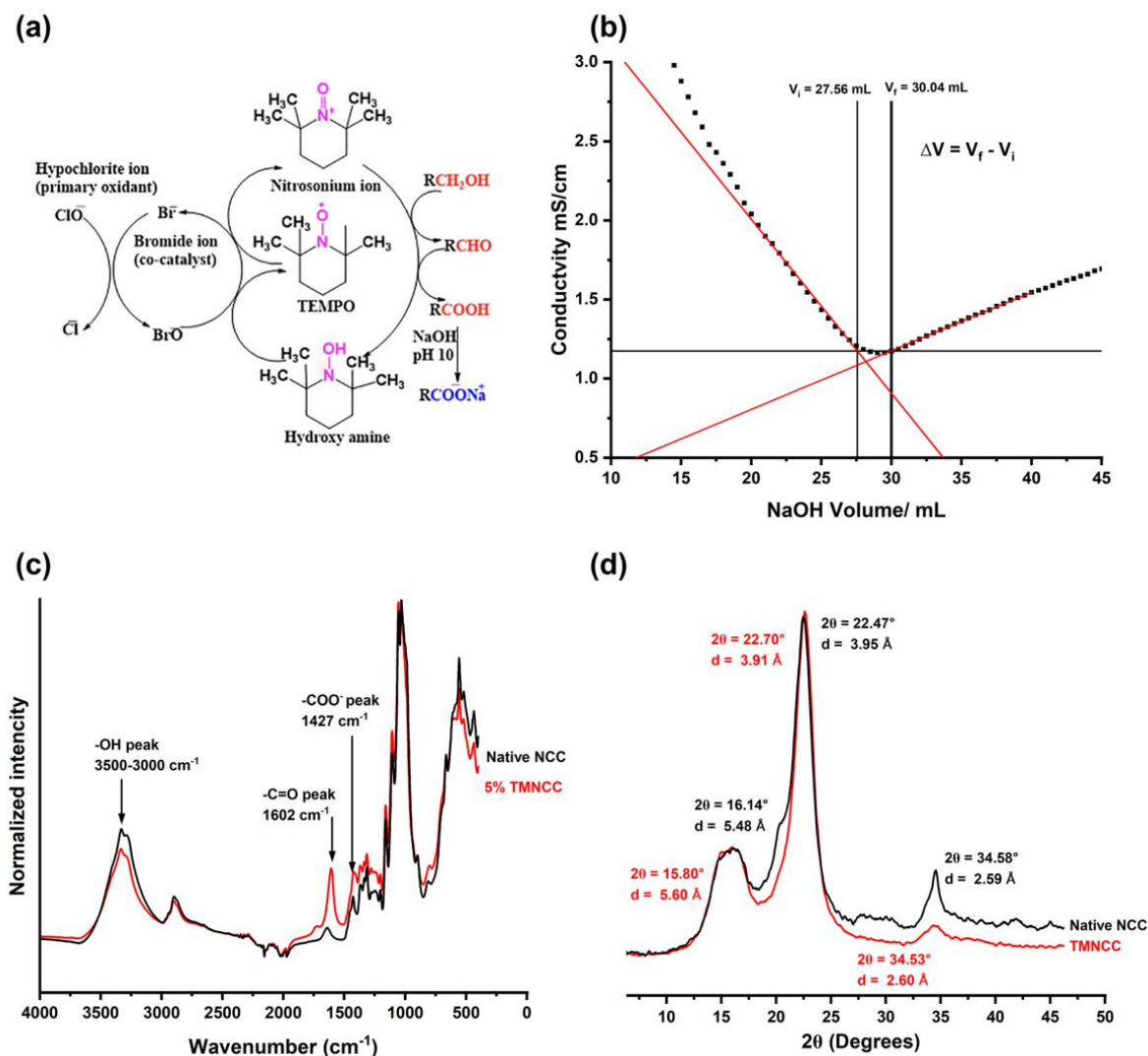


Figure 3.1. Characterization of TEMPO modified and native NCC powders (a) reaction mechanism of TEMPO modification (used with permission from Dissanayake et al. (2022)) (b) conductometric titration curve used to calculate the degree of oxidation of TMNCC (c) FTIR

spectrum of the TEMPO modified and native NCC (d) XRD spectra of the TEMPO modified and native NCC

Chemical modification of NCC using TEMPO converts primary -OH groups into -COOH by forming a nitrosonium intermediate (Buffa et al., 2016). NaClO and NaBr salts increase the reaction rate by acting as the primary and co-catalysts, respectively. TEMPO is regenerated through a hydroxylamine intermediate, and carboxylate ions are produced by consuming the free -OH groups on the surface of the NCC nanomaterial. Figure 3.1 (a) illustrates the reaction mechanism of the TEMPO-mediated oxidation of NCC (Dissanayake et al., 2022). To examine the effectiveness of the formed TEMPO-NCC, a strong acid-base conductometric titration was performed to quantify the degree of oxidation (DO) of the primary -OH groups on the surface of the NCC (Buffa et al., 2016). Figure 3.1 (b) illustrates the titration curve, and the region (V_F - V_i) is responsible for the generated carboxylate groups on the NCC surface. DO give the amount of TEMPO-modified NCC content compared to the initial unmodified NCC material. According to the results, $28.37 \pm 2.76\%$ of DO was observed in this study. A similar type of value was reported as 26.60% by Buffa et al. (2016) and 19.61% by Dissanayake et al. (2022). The DO obtained in this study is higher than that in previous studies. Variation in DO values could be due to differences in the source of the raw NCC, molar ratios, and initial -OH group content. Figure 3.1 (c) illustrates the FTIR comparison of TMNCC and native NCC. The broad peak around $3000\text{--}3500\text{ cm}^{-1}$ is the -OH stretching vibration due to free OH groups, which has been reduced during oxidation. Conversely, the narrow peak at 1602 cm^{-1} and 1427 cm^{-1} has been increased during the oxidation, which represents the C=O stretching vibration of the carbonyl groups and -COO^- stretching vibration of the carboxylate groups (Calderón-Vergara et al., 2020; Rozali et al., 2015). This confirmed the conversion of primary OH groups into COO^- ions. Figure 3.1 (d) illustrates the XRD patterns of the TMNCC and native NCC samples. Three main characteristic crystalline peaks for cellulose materials have been identified at glancing angles of 15.7° , 22.6° , and 35.19° , corresponding to the lattice planes 110, 200, and 004, respectively. (Bano & Negi, 2017; Trilokesh & Uppuluri, 2019). Modified NCC showed reduced crystallinity because of the low intensity peaks compared to the unmodified NCC. This could be due to the introduction of a new functional group, which disrupts the existing primary -OH groups. However, a reduction in glancing angles was observed due to the modification in TMNCC from 16.14° to 15.80° and 34.58° to 34.53° , corresponding to increases in the interlayer d-spacing values from 5.48 to 5.60 Å and 2.58 to 2.60

Å, respectively. This might be a reason to obtain a fluffy type of TMNCC material at the end of the modification. This nature facilitates easy penetration into the protein matrix and enhances the properties of TMNCC-incorporated protein films (Dissanayake et al., 2022).

3.4.2. Characterization of GO modification

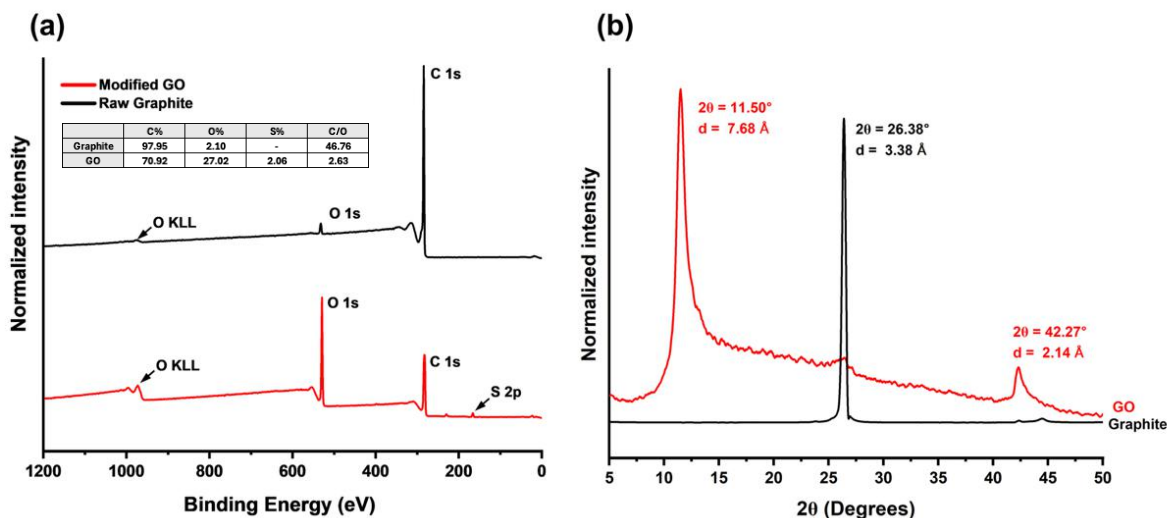


Figure 3.2. Characterization of Graphite oxide (GO) and raw graphite (a) high resolution C1 spectra of GO and raw graphite (b) XRD patterns of GO and raw graphite.

The objective of the graphite oxide modification is to introduce additional oxygen functional groups, which would ultimately enhance intramolecular interactions between -OH groups in protein chains/residues and the added GO nanomaterials (Mun et al., 2015). The oxygen content (C:O ratio) on the graphite surface was determined by XPS analysis. According to the XPS data shown in Figure 3.2(a), modified GO showed a C/O ratio of 2.63 ± 0.05 , whereas native graphite showed a C/O ratio of 46.76 ± 0.45 . The O% has significantly increased by x13 fold during the modification from 2.10 ± 0.02 % in the native state (graphite) to 27.02 ± 0.40 % in the modified state (GO). Residual sulphur content of 2.06 ± 0.03 % was identified, which is a residue of the H_2SO_4 used during the oxidation process (Hou et al., 2020). Furthermore, to assess the nanomaterial's crystallinity, an XRD analysis was performed. As shown in Figure 3.2(b), raw graphite exhibited a characteristic sharp peak at a glancing angle of 26.38° and a d-spacing of 3.37 Å . Hou et al. (2020) reported similar results for a raw graphite sample at a glancing angle of 26.31° and a d-spacing of 3.39 Å . The modification has drastically reduced this peak in the modified GO sample.

This indicates the reduced crystallinity due to the introduction of new oxygen functional groups. However, two new peaks were observed at 2θ values of 11.49° and 42.27° , with interlayer spacings of $7.68 \text{ \AA}$ and $2.14 \text{ \AA}$, respectively. These results align with the previously reported literature data on modified graphite oxide nanomaterials (Bandara et al., 2017; Chairez-Jimenez et al., 2024; Hou et al., 2020). Newly generated peaks may account for the increased strength of GO when used as an additive in canola protein films (Chairez-Jimenez et al., 2024).

3.4.3. Characterization of modified canola protein-based films

3.4.3.1. Structural properties of the APS-modified canola protein-based films

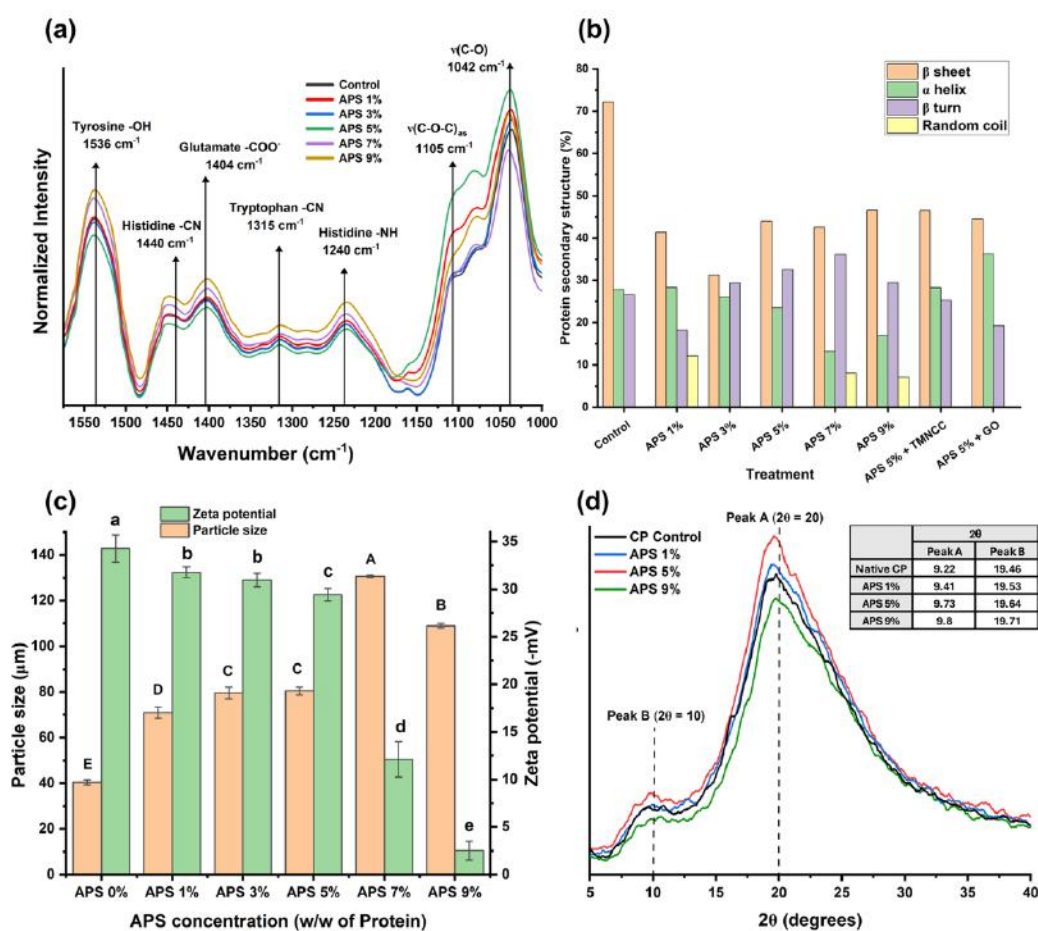


Figure 3.3. Structural properties of APS modification of CP (a) enlarged FTIR spectra showing the changes in amino acid residues due to the APS modification of CP (b) second derivative peaks of the amide I region of APS modified CP films (c) relative proportions of protein

secondary structures due to the APS modification of CP films (d) XRD patterns of the APS modified CP. All the values are illustrated as the average $\pm$ SD (n=3).

APS is a strong electron acceptor, generating free radical species that can trigger selective protein modification. The persulfate ion thermal decomposition in aqueous medium can occur as an uncatalyzed reaction (Equation 3.8) by breaking the asymmetrical O-O bond, resulting in two sulphate free radicals (Kolthoff & Miller, 1951; Mascheroni et al., 2016).



Generated sulphate radicals are strong one-electron oxidants that can abstract H⁺ ions from the side chains of redox-active functional groups in protein residues. Tyrosine (Try) -OH, Tryptophan (Trp) -CN, Histidine (His) -CN, Cystein (Cys) -SH are potential redox-active protein amino acid residues in canola protein (Bandara & Wu, 2018). Sulphate-free radicals convert the above amino acid into radical cations, Equation (3.9), which then undergo the radical-radical coupling also known as protein-protein crosslinking reactions, Equation (3.10) (Bandara & Wu, 2018; Fancy & Kodadek, 1999; Sato & Nakamura, 2013). These crosslinking modifications alter the physicochemical properties of the canola protein matrix.

Changes occurring in the canola protein matrix were examined using FTIR analysis Figure 3.3. Infrared absorptions of amino acid side chains play a fundamental role in protein stability and help determine the extent of modification (Barth, 2000). Characteristic canola protein peaks were identified in the amide I, II, and III regions at 1700-1600 cm⁻¹, 1500-1600 cm⁻¹, and 1350-1200 cm⁻¹, respectively (Pirestani et al., 2018). As illustrated in Figure 3.3(a), the dominant residual Try-OH peak appeared at 1518 cm⁻¹, and the peak intensities decreased with increasing APS content, except for the 7% and 9% APS treatments, which showed higher peak intensities. Similarly, the -CN vibrations of Trp (1315 cm⁻¹) and His (1440 cm⁻¹) followed a similar pattern, with the lowest intensity in APS 5% (w/w protein). Bandara & Wu (2018) reported that higher APS contents might overlap with other functional groups, as they are exposed during modification. Glutamate is one of the major amino acids in industrial canola meal (Li et al., 2012). Glutamate -COO⁻ vibration

showed a similar variation pattern at 1404 cm^{-1} (Barth, 2000). Masui et al. (2022) reported similar fluctuation of the FTIR peak intensity in glutamate COO^- vibrations.

Variations of the protein secondary structure due to the APS and nanomaterial modification were identified by the 2nd derivative peak fitting method in the amide-I peak. As depicted in Figure 3.4, the conformational changes in secondary structures of protein films in β sheets ($1622, 1636, 1671\text{ cm}^{-1}$), random coils ($1640\text{--}1648\text{ cm}^{-1}$), α helix (1650 cm^{-1}), and β turns ($1683, 1695\text{ cm}^{-1}$) with variations (Kong & Yu, 2007). The overall content of the β sheets was reduced, and β -turn content has been increased with the addition of APS. This may be due to increased interactions between APS-modified canola protein structures, which open the structures and expose more buried regions and functional groups. Furthermore, the helix percentage in the amide I region increased with the addition of TMNCC and further with GO incorporation compared to the APS 5% film. This might be due to the abundance of the surface functional groups (hydroxyl, carboxyl, and epoxide groups) of TMNCC and GO, which form strong hydrogen bonds with protein amide and carbonyl groups, stabilizing intra-chain interactions and promoting a more ordered α -helical structure (Bandara et al., 2017; Malik et al., 2020). A new peak corresponding to random coil structures appeared at 1643 cm^{-1} in higher APS concentrations, due to protein aggregation (Barth, 2000). Similar results were reported at 1641 cm^{-1} for APS-modified canola protein by Bandara & Wu (2018). Unordered random coil structures were not observed in both TMNCC and GO-added canola films. This observation matches the previously reported TMNCC (Dissanayake et al., 2022) and GO (Chairez-Jimenez et al., 2024) modified canola protein films.

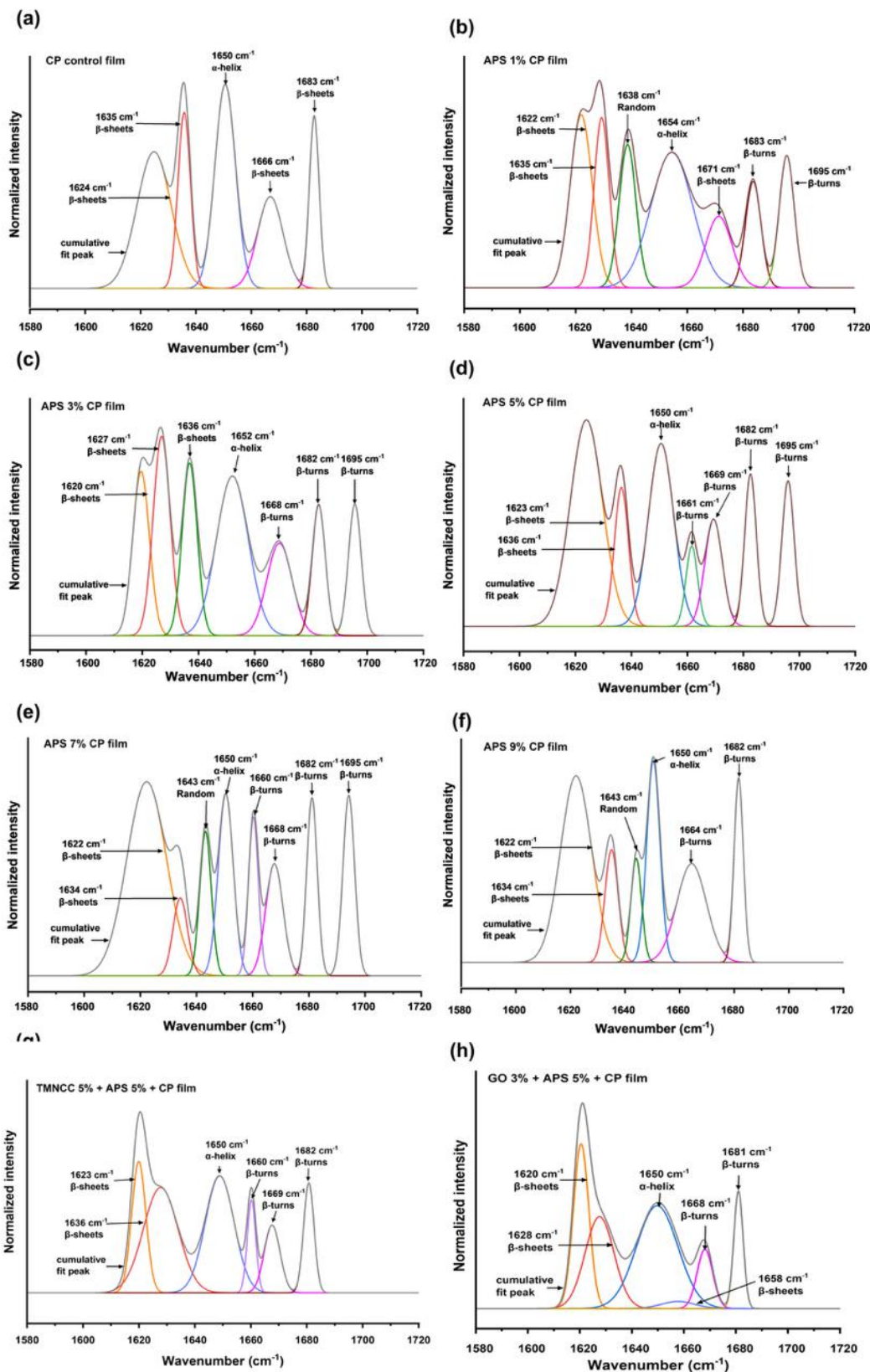


Figure 3.4. FTIR second derivative peaks of the amide I region of APS-modified CP films and nanomaterial added 5% APS-modified CP films

Zeta potential is an important electrokinetic parameter that measures the net surface charge of the slipping plane of the suspended protein particles. When the net charge is high, repulsive forces dominate, repelling the molecules and increasing particle stability. When the net charge is closer to zero, protein aggregation and flocculation will be facilitated (Gerzhova et al., 2016). Meanwhile, particle size corresponds to the protein molecules' hydrodynamic diameter. In this study, as illustrated in Figure 3.3 (c), the negative zeta potential of the protein solution was reduced with the addition of APS content. This might be due to a reduction in charged amino acid residues on protein surfaces resulting from the APS crosslinking effect, as depicted in Figure 3.3(a). The hydrodynamic diameter of the hydrated protein particles was increased with the APS addition level, as illustrated in Figure 3.3 (c). At high APS levels ($\geq 7\%$), the particle size increased by 300% compared to the unmodified protein sample (Schlangen et al., 2023). This might be due to protein aggregation and flocculation at high levels. FTIR and XRD results also confirm this behaviour. Similar variation in canola protein due to the APS modification was reported by Bandara & Wu, (2018).

X-ray diffraction (XRD) patterns reveal the crystalline nature of a material by measuring the intensities of scattered X-rays from its crystalline planes. The glancing angle (2θ) and the interlayer spacing (d-spacing) of the material can be calculated using Bragg's equation ($n\lambda = 2d \sin \theta$). Canola protein shows characteristic peaks at glancing angles of 20° (peak A) and 10° (peak B) (Dissanayake et al., 2022). Figure 3.3 (d) illustrates the variations of canola protein crystallinity due to the APS modification. The areas of both peaks increased compared to the neat protein in APS 1% and APS 5%, with the highest value in APS 9%, while APS 9% showed the lowest intensity. This is related to the crystallinity index and helps determine the strength of the modified protein material (Pirestani et al., 2018). The 2θ values of both A and B peaks have increased upon incorporation of APS, thereby reducing the interlayer d-spacing between the polymer molecules. However, APS 9% samples showed reduced crystallinity, indicating that the molecules packed more closely together than in other samples. This might be due to protein aggregation (Dissanayake et al., 2022). Initial optimization indicated that APS at 5% (w/w protein) is a suitable

APS content for canola protein modification in packaging film preparation. Reinforcement using TMNCC and GO was tested on the optimized 5% APS samples to characterize reinforced APS canola films.

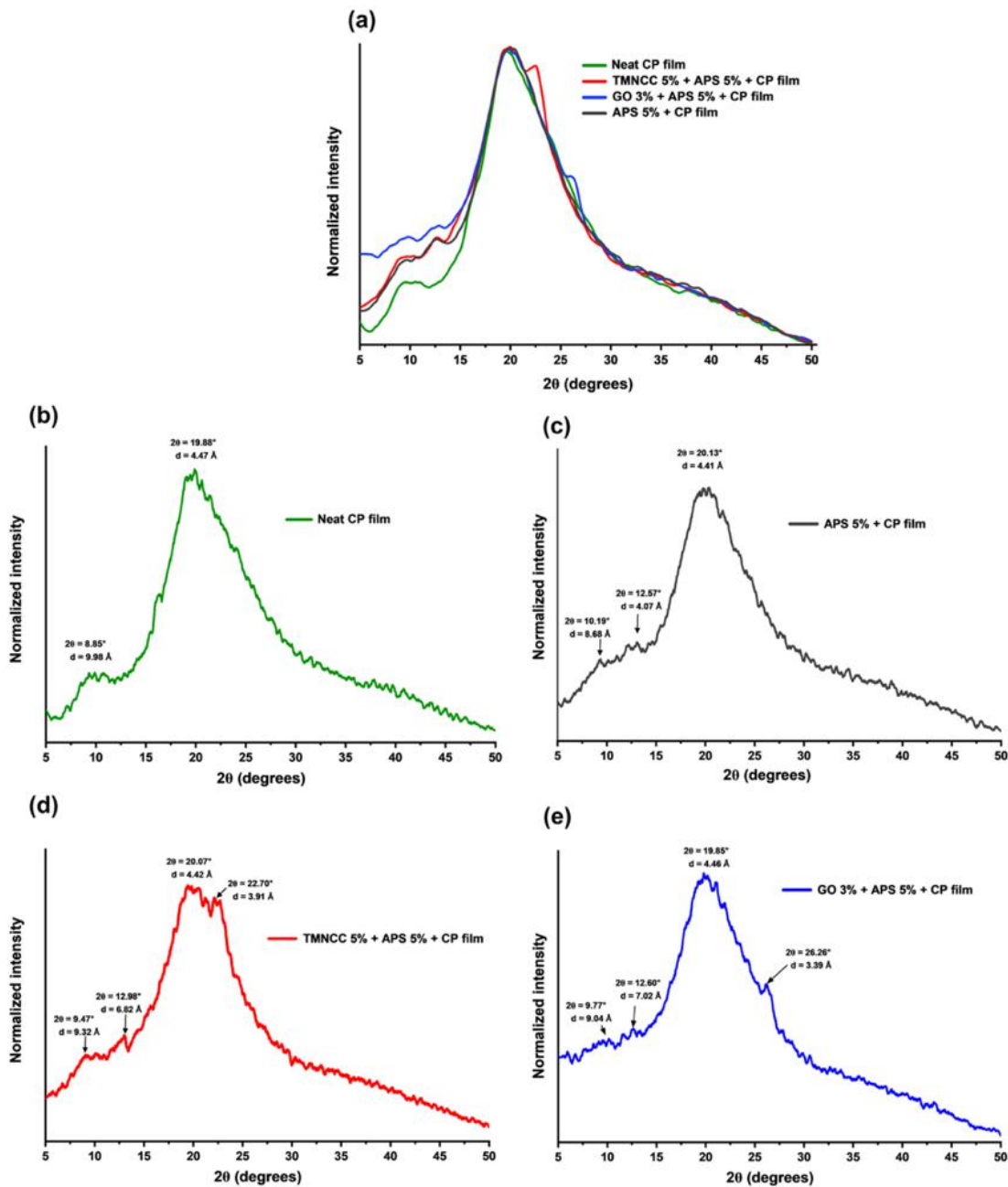


Figure 3.5. XRD patterns of the CP films (a) comparative illustration of all films (b) neat CP film (c) APS 5% + CP film (d) TMNCC 5% + APS 5% + CP film (e) GO 3% + APS 5% + CP film.

Figure 3.5 illustrates the XRD patterns of the canola protein films reinforced with different nanomaterials. The crystalline intensities of all treated films at 2θ value at 10° have increased, while no variations were observed at 2θ value at 20° compared to the neat canola film. Similarly, the interlayer d-spacing value of peak B has decreased from 9.98 Å in neat films to 8.68 Å in an APS 5%-added film. The protein modification increased the layer packing density of the packaging material. However, the d-spacing value has reduced to 9.04 Å in GO-added films, followed by 9.32 Å in the TMNCC-added film. This occurred due to the incorporation of negatively charged functional groups into the film matrix and the repulsive forces between them (Dissanayake et al., 2022). A new peak at 2θ of 12.57° appeared on the APS-modified films, and upon introducing nanomaterials, it shifted to 12.60° with TMNCC and 12.98° with GO, resulting in interlayer d-spacing values of 7.04, 7.02, and 6.82 Å, respectively. This might be a characteristic XRD peak for APS crosslinking. Harrington & Santiso (2021) explained that new weak reflections can arise from structural rearrangements induced by a chemical modification in a thin-film system. Minor peaks appeared at 22.70° in the TMNCC added film (Figure 3.5(d)) and 26.26° in the GO added film (Figure 3.5 (e)), confirming the validity of the modification. Similar peaks were reported for TMNCC-added films by Dissanayake et al. (2022) and for GO-added films by Chairez-Jimenez et al. (2024).

3.4.3.2. Surface morphology of the modified canola films

As shown in Figure 3.6, SEM micrographs were used to identify the surface and cross-sectional morphological characteristics of the modified canola films at magnifications of $\times 2000$ and $\times 3000$, respectively. As depicted in Figure 3.6(b), the film surface becomes smoother with APS modification, and this improvement is further enhanced with the addition of nanoparticles. Dissanayake et al. (2022) reported that some surface roughness remained on the film surface even after the addition of TMNCC, due to aggregation. However, in this study, the level of surface roughness was higher than in Dissanayake et al. (2022). This might be due to the collective effect of both APS 5% and TMNCC 5% on the CP film. As depicted in Figure 3.6 (c), the cross-section of the APS 5% added film showed an improved bottom layer with a less porous nature compared to the native CP film. The compact behaviour has been observed in the majority of nanomaterial-added films, with close packing and a more compacted structure. This might explain the improved WVP, tensile, and thermal properties of the reinforced films.

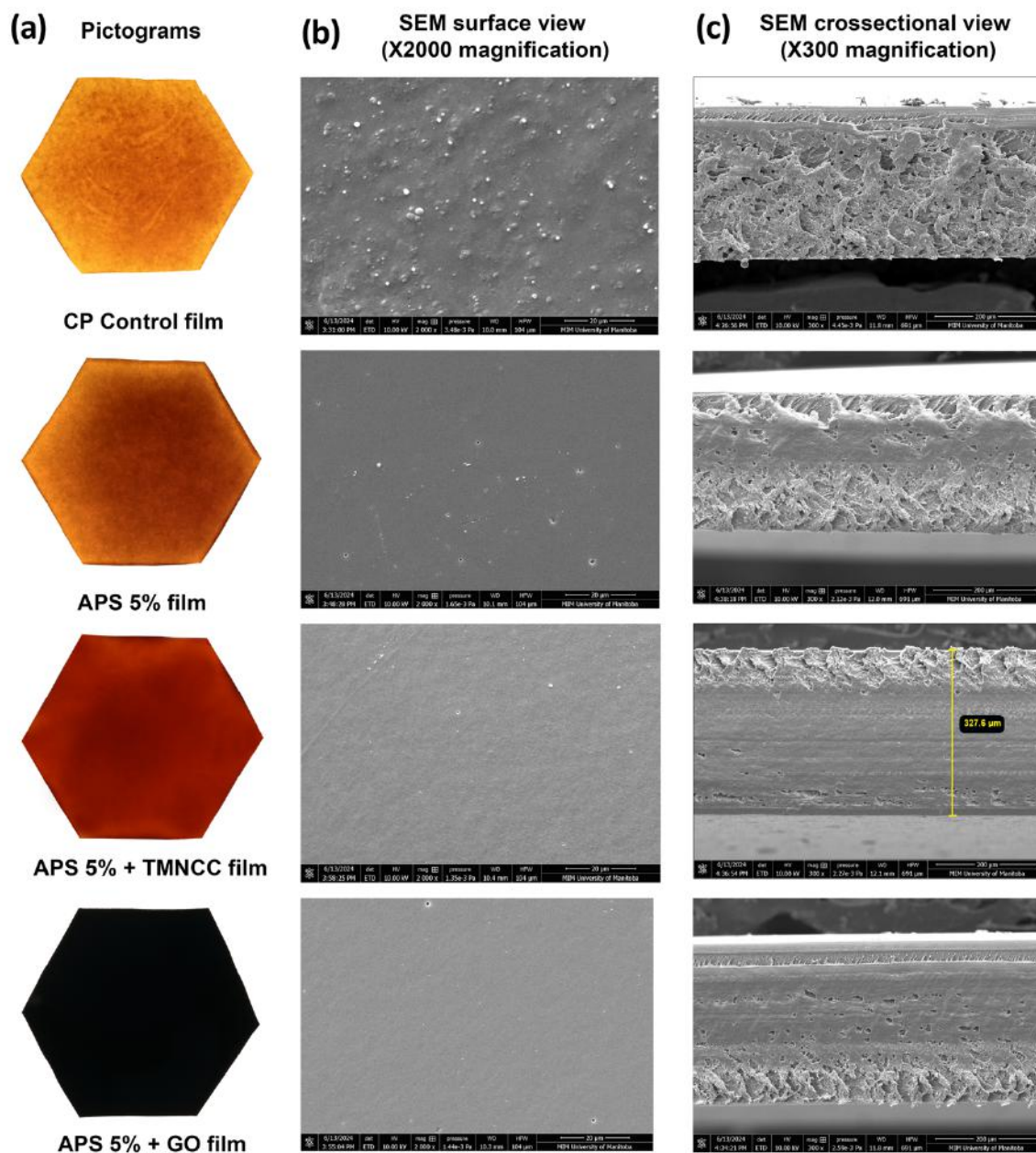


Figure 3.6. (a) pictograms of modified CP films (b) SEM images of the surface view of the modified CP films ($\times 2000$ magnification) (c) SEM images of the cross-sectional view of the modified CP films ($\times 300$ magnification).

3.4.3.3. Mechanical properties of the nano-reinforced canola protein-based films

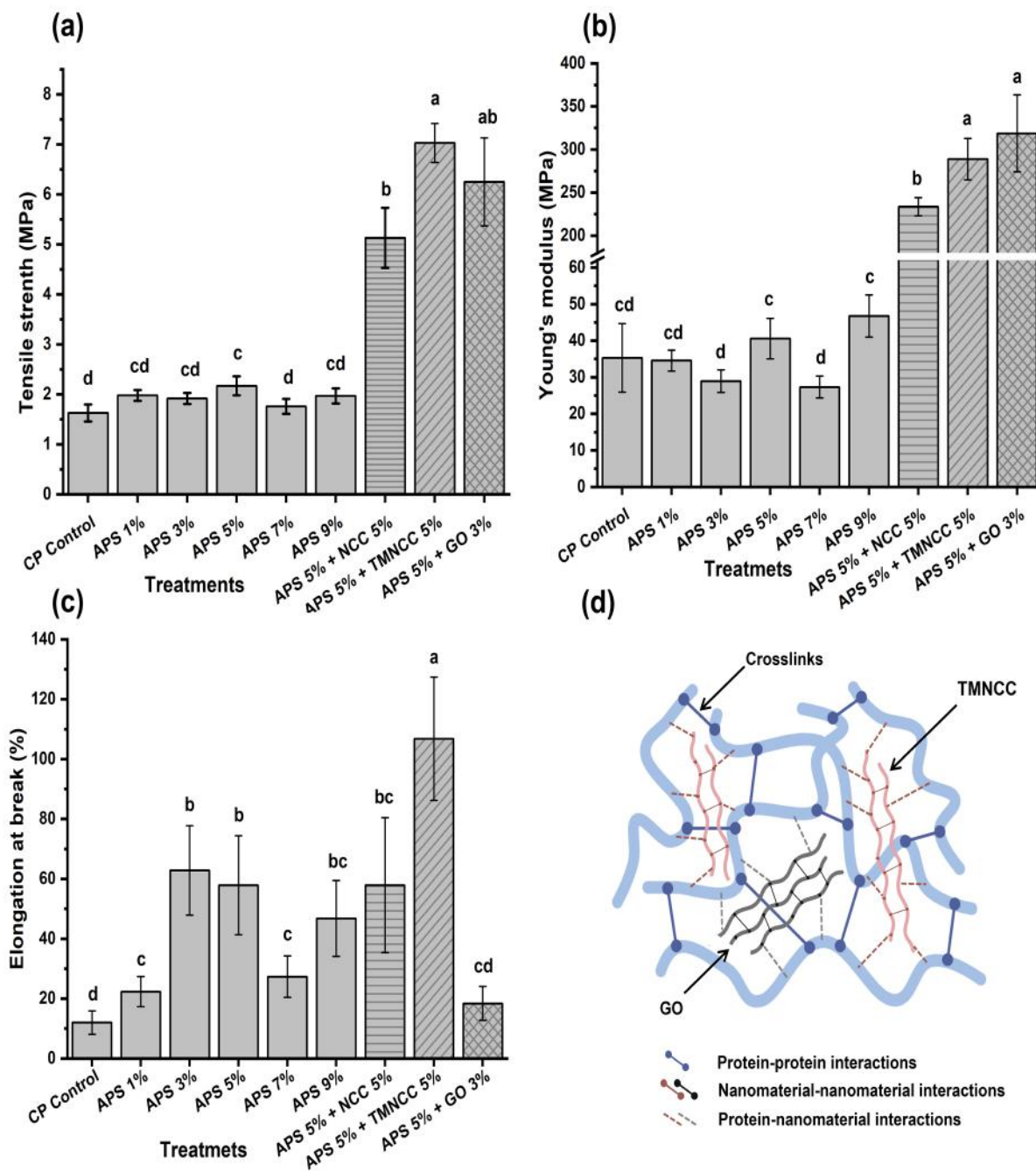


Figure 3.7. Mechanical and water barrier properties of the modified CP films (a) tensile strength (b) elongation at break (c) Young's modulus (d) Graphical illustration of different types of molecular interactions in the modified CP films. All values are shown as the average $\pm$ SD (n=5).

Mechanical properties are among the most important parameters for evaluating the strength and flexibility of a packaging film. Tensile strength (TS), Young's modulus (YM), and elongation at break (EB) were analyzed to identify the practical potential of the films. A tensile strength value of 1.63 ± 0.17 MPa was obtained for the neat films, which is in line with previously reported values for canola protein films (Chairez-Jimenez et al., 2024; Chang & Nickerson, 2014). According to the results shown in Figure 3.7 (a), there was no significant difference ($p < 0.05$) between them except for the APS 5% film compared to the neat control film. APS 5% treatment increased tensile strength by 1.33-fold compared with inorganic crosslinking. Li et al. (2017) studied the organic crosslinking effect by adding 1,4-Butanediol diglycidyl ether (BDDE) to the CP films and reported that the modification enhanced the TS values by approximately 1.8 times. On the other hand, Chang & Nickerson (2014) used Genipin as a natural crosslinking agent and reported a 1.25-fold increase in TS, following modification. The APS modification is at a higher position than the natural crosslinker (genipin) and below the organic crosslinker (BDDE). However, reinforcement using TMNCC (7.03 ± 0.39 MPa) and GO (6.25 ± 0.88 MPa) increased the TS by ~ 4 times compared to the neat film and ~ 3 times compared to the APS 5% films. A similar TS value was reported 5% TMNCC-added canola protein films (Dissanayake et al., 2022). A significant ($p < 0.05$) improvement was observed in the mechanical properties of NCC when TEMPO-modified NCC was compared with native NCC in CP films. Chairez-Jimenez et al. (2024) reported a lower TS value for 3% GO-added canola films, with a TS of 3.5 MPa. This might be due to the APS modification or to variations in the raw materials used for protein extraction (Zhang et al., 2021). Similar to the TS trend, EB also increased due to the addition of nanomaterials. However, the improvements are highly noticeable in the film's elasticity. As shown in Figure 3.7(c), the film elongations of APS 3% and APS 5% showed a significant ($p < 0.05$) improvement, whereas the APS 7% film showed weakening. This might be due to protein aggregation (Bandara & Wu, 2018). The YM represents the stiffness of the films, which was significantly ($p < 0.05$) increased by the addition of both nanomaterials, as depicted in Figure 3.7(b), while APS levels showed little variation. Different types of inter- and intra-molecular interactions form between proteins and nanomaterials, as illustrated in Figure 3.7(d). Protein-protein interactions / covalent bonds are formed due to the chemical crosslinking reactions of the canola protein chains. The conformational stability of the crosslinked protein matrix was governed by intermolecular hydrophobic

interactions in combination with a hydrogen-bonding network (Liu et al., 2025). Reduction of hydrophilic amino acid residues due to the APS modification might be a reason for the development of a strong molecular framework. Furthermore, modified nanomaterials form new inter- and intramolecular hydrogen bonds with crosslinked protein chains, thereby improving mechanical properties such as tensile strength and elongation at break (Abookleesh et al., 2024; Dissanayake et al., 2022).

3.4.3.4. Barrier properties of the modified canola protein-based films

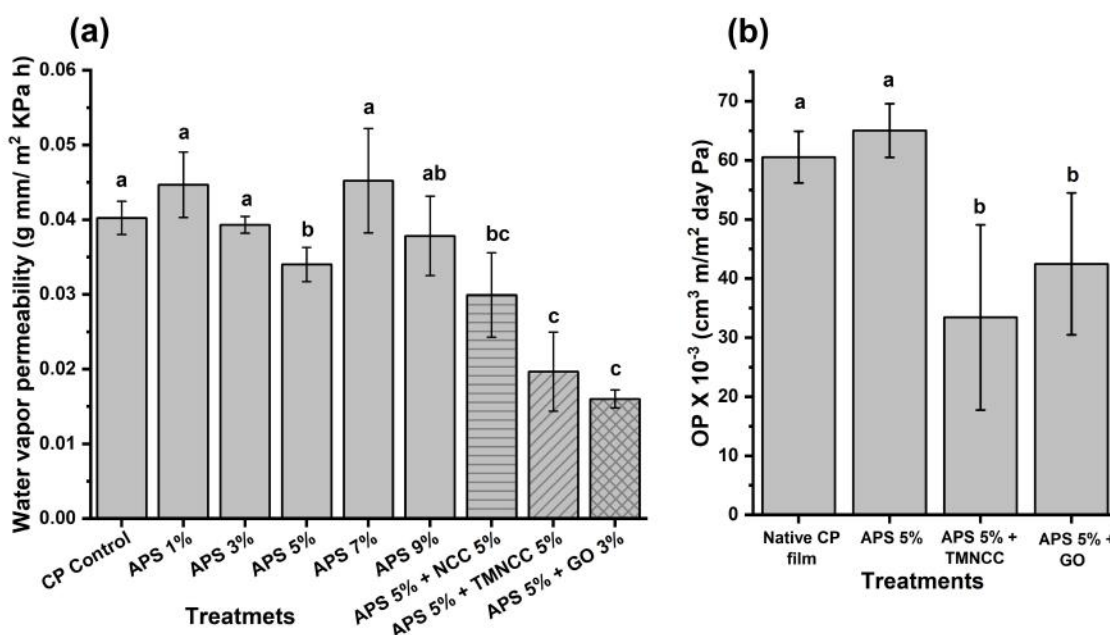


Figure 3.8. Water and oxygen barrier properties of the modified CP films (a) water vapour permeability (b) oxygen permeability of the modified CP films. All values are shown as the average $\pm$ SD (n=3).

The barrier properties of a packaging material are crucial parameters that affect the overall quality of the food item. Water vapour permeability (WVP), Water solubility, Contact angle, and Oxygen permeability (OP) were evaluated to determine the film barrier properties. WVP is the diffusion rate of water molecules through a unit area of a film in a unit period of time. A higher WVP rate induces microbial spoilage and food deterioration, reducing shelf life and overall quality (Santhosh & Sarkar, 2022). According to Figure 3.8(a), the WVP for the neat CP film is 0.040 ± 0.002 g mm/m² kPa h. APS-modified films showed fluctuating values around the control CP, but these differences were not significant ($p > 0.05$) except for APS 5%. That treatment reduced the WVP

value up to $0.034 \pm 0.002 \text{ g mm/m}^2 \text{ kPa h}$. The protein modification reduced the number of hydrophilic amino acid residues and improved hydrophobic interactions within the protein matrix, thereby increasing the protein's water-resistance (Li et al., 2017). The addition of NCC, TMNCC, and GO ($0.0160 \pm 0.0005 \text{ g mm/m}^2 \text{ kPa h}$) improved the water vapour resistance by 133%, 205%, and 252%, respectively, compared to the neat CP film. Well-dispersed nanomaterials can increase the tortuosity of the protein matrix for water molecules, which ultimately increases the water resistance of the films (Hubbe et al., 2017). Comparably higher literature values of WVP were reported as 0.500, 0.060, 0.054, and 0.068 $\text{mm/m}^2 \text{ kPa h}$ for genipin-modified, TMNCC, oleic acid-modified NCC, and GO-incorporated CP films, respectively (Chairez-Jimenez et al., 2024; Chang & Nickerson, 2014; Dissanayake et al., 2022, 2023).

Solubility represents the ability to remain stable in prolonged contact with moisture. There was no significant difference observed between the modified CP films in this study, and all the values fluctuated within a 33-36 % range. The solubility of the films is expected to be reduced due to the incorporation of nanomaterials and the new interactions formed (González et al., 2020). However, the hydrophilic nature of the TMNCC and GO might be the reason for this behaviour. Similar observations were reported by Dissanayake et al. (2023) and Chairez-Jimenez et al. (2024) for CP-passed films.

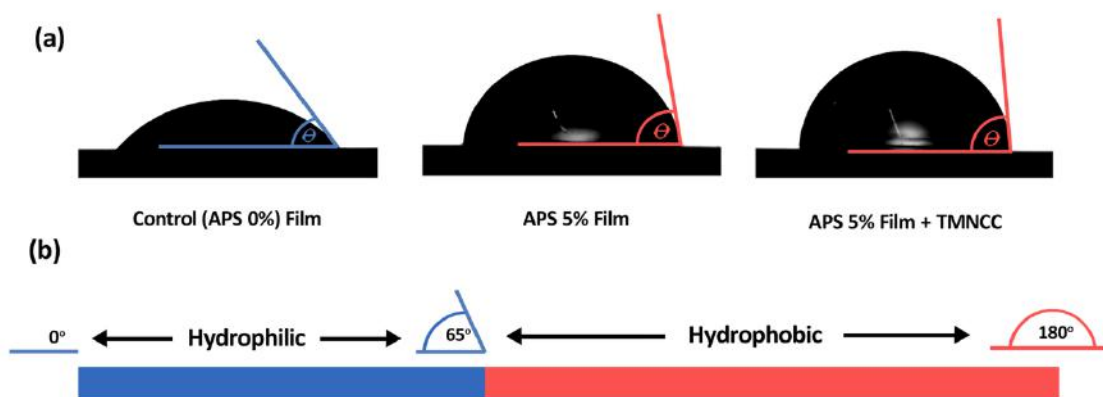


Figure 3.9. (a) Microscopic images of the water droplets with the contact angle of CP films (b) Graphical illustration of the film contact angle and the surface hydrophobicity of the films adopted based on the information from (Rocca-Smith et al., 2016).

Contact angle measurements represent the surface hydrophobicity of the packaging films. Higher values indicate a reduction in film wettability, causing water droplets to bounce off the surface due to stronger adhesive forces between water molecules; lower values result in water droplets sticking to the surface because cohesive forces exceed adhesive forces (Good, 2012). As illustrated in Figure 3.9 (b), the higher contact angles over 90° would be perfectly hydrophobic film surfaces, and the reference value for hydrophobic film surfaces is over 65° (Rocca-Smith et al., 2016). According to Table S1.2, in this study, the APS 3% film showed the highest contact angle value ($80.90 \pm 5.91^\circ$) among the APS treatments, while higher APS levels reduced the contact angle values. Tadele et al. (2023) reported that protein modification can reduce the interfacial tension of the modified protein, as evidenced by the contact angle. The authors further discussed that the reduction in interfacial tension of the modified protein might positively impact the tensile properties and impact resistance of the material. Neat CP film and APS 1% samples showed a more hydrophilic nature, while others showed hydrophobic film surfaces, which is a well-needed property of packaging films. However, with the addition of TMNCC, the contact angle of the APS 5% treatment further increased to $85.22 \pm 1.26^\circ$, indicating the highest value. Surface views of the SEM images support these results. Similar variations in contact angle results were reported when amaranth protein films were reinforced with NCC extracted from waxy maize (Condés et al., 2015).

Oxygen permeability (OP) is another important barrier property that indicates the film's susceptibility to oxygen migration. A packaging film's ability to limit contact with atmospheric oxygen will prolong the shelf life of food items by preventing lipid oxidation. In this study, OP was reduced in both the nanoparticle-added films compared to the APS 5% film. However, no significant difference was observed in the neat CP film (Figure 3.8(b)). No significant difference in the OP was identified due to the APS modification. The available free polar hydroxyl groups in the molecular structure of the CP films have been reduced with the APS modification. This improves the oxygen resistance by limiting the permeability to non-polar O_2 molecules (Alves et al., 2024; W. Liu et al., 2021). The behaviour OP correlated with WVP variation, highlighting the importance of adding nanomaterials to improve barrier properties. This might be due to improved interactions between the new protein and the nanomaterial. $OP < 39 \text{ cm}^3 \mu\text{m}/\text{m}^2/\text{day kPa}$ was indicated as an excellent oxygen barrier material for food packaging films (Xu et al., 2019). Similar results were reported Chairez-Jimenez et al. (2024) in GO-added CP films.

3.4.3.5. Thermal properties of the modified canola protein-based films

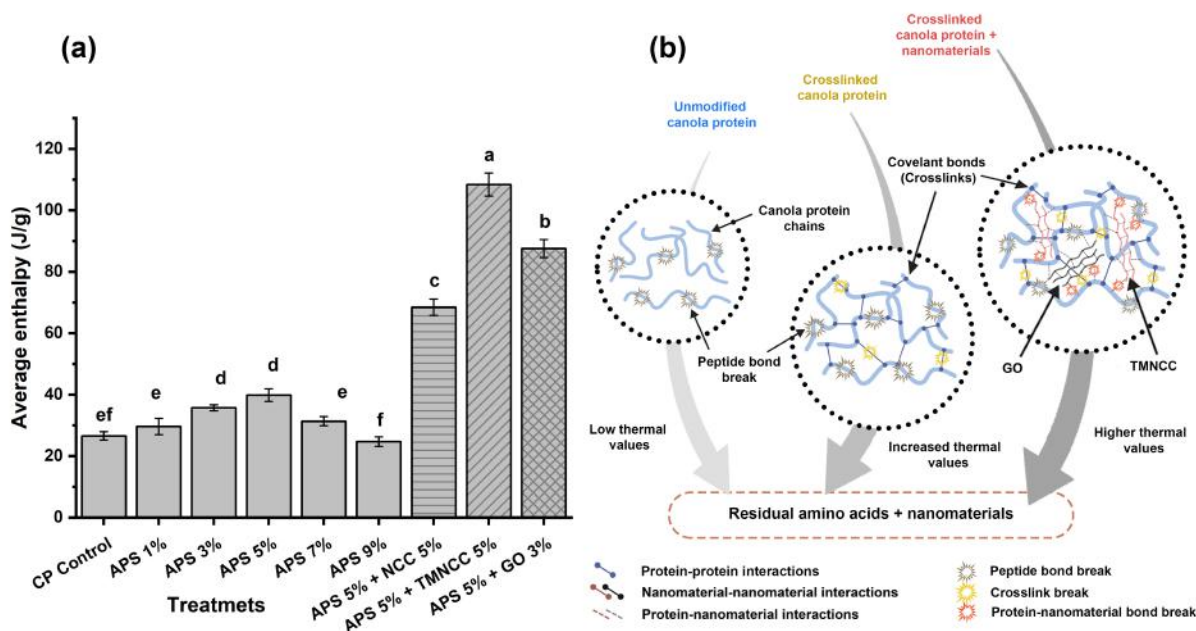


Figure 3.10. Thermal and oxygen barrier properties of the modified CP films (a) Average enthalpy values of the modified CP films (b) Graphical illustration of different types of interactions responsible for thermal properties. All the values are illustrated as the average of three specimens $\pm$ SD.

The effects of the APS modification and the nano-reinforcement on CP films could be identified by analyzing their thermal properties. Average enthalpy and peak temperature were analyzed to identify changes within the films. Enthalpy is the measure of heat energy absorbed by a film material during a thermal transition. Furthermore, it represents the energy required to break protein-protein interactions, such as hydrogen bonds or crosslinks. As depicted in Figure 3.10 (a), the higher ΔH indicates a more ordered or strongly bonded structure, often due to APS-induced oxidation or crosslinking. The addition of higher concentrations of APS led to increased random coils in protein secondary structures, weakening the protein matrix and inducing protein aggregation (Senthilkumaran et al., 2022). This might be the reason for the reduction in thermal values at higher concentrations. Figure 3.10 (b) depicts the graphical illustration of different types of bonds formed due to crosslinked proteins and due to the intermolecular interactions between proteins and nanomaterials. A greater number of bonds requires more thermal energy to break,

thereby elevating the average enthalpy of the films. In nanomaterial-incorporated films, additional nanomaterial-nanomaterial interactions can break, which might be the reason for the drastic increase in the films' thermal stability.

Peak temperature is the temperature marked by an endothermic peak at which the protein's native structure unfolds its organized conformation (Xu et al., 2019). The peak temperature of the APS-modified protein films ranged from 95 to 96 °C, with no significant difference (Table S1.3). This unfolding of the native protein structure depends mainly on intramolecular hydrogen bonds and hydrophobic interactions within the protein molecule, rather than on intermolecular crosslinks between different protein chains. This might be why the peak temperature remains unchanged due to the APS modification.

TMNCC-added films showed the highest enthalpy (Figure 3.10(a)), followed by the GO-added film, with values of 108.40 J/g and 87.55 J/g, respectively. TEMPO modification formed new intermolecular hydrogen bonds between nanocellulose and protein chains, thereby improving the thermal properties (Abookleesh et al., 2024; Dissanayake et al., 2022). However, the narrow endothermic peak of TMNCC indicated uniform exfoliation across the film, with a lower peak temperature (113.74 °C). GO films exhibit a higher peak temperature (150.48 °C) and a broader enthalpy peak, indicating gradual denaturation and structural diversity of the reinforced protein over a wide temperature range. XRD results for the GO-added films confirmed increased crystallinity in some peaks relative to the TMNCC-added films, corroborating the thermal results.

3.5. Conclusion

The present study successfully demonstrated the chemical modification of canola protein using APS as an inorganic crosslinker, followed by nanomaterial reinforcement of the modified protein matrix with TEMPO-oxidized NCC and GO. APS initiated selective oxidation and crosslinking reactions among redox-active amino acid residues, resulting in a more compact, structured protein matrix. FTIR, XRD, and particle size analyses confirmed the chemical and structural rearrangements and increased crystallinity, with APS 5% supporting enhanced molecular ordering. However, excessive APS content ($\geq 7\%$) promoted protein aggregation, reducing crystallinity. Tensile strength values of APS 5% significantly improved by $\times 1.33$ times compared to the neat protein film, while nano-reinforcements with TMNCC and GO further enhanced tensile strength by $\sim 4X$ times. The optimized films showed improved water vapour and oxygen barrier properties

and exhibited hydrophobic surface characteristics. Thermal analysis supported the structural observations, with increases in enthalpy for APS 5% films, confirming enhanced crosslink density and bonding energy. The addition of nanomaterials further improved both barrier and thermal properties. Overall, APS modification at moderate concentrations, coupled with nanoscale reinforcement, produced canola protein-based films with superior mechanical, barrier, and thermal performance, positioning them as viable biodegradable alternatives for food packaging and sustainable material applications.

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Chapter 4 : Functionality of Canola Protein-Based Nanocomposite Film Prepared Using the Extrusion Moulding

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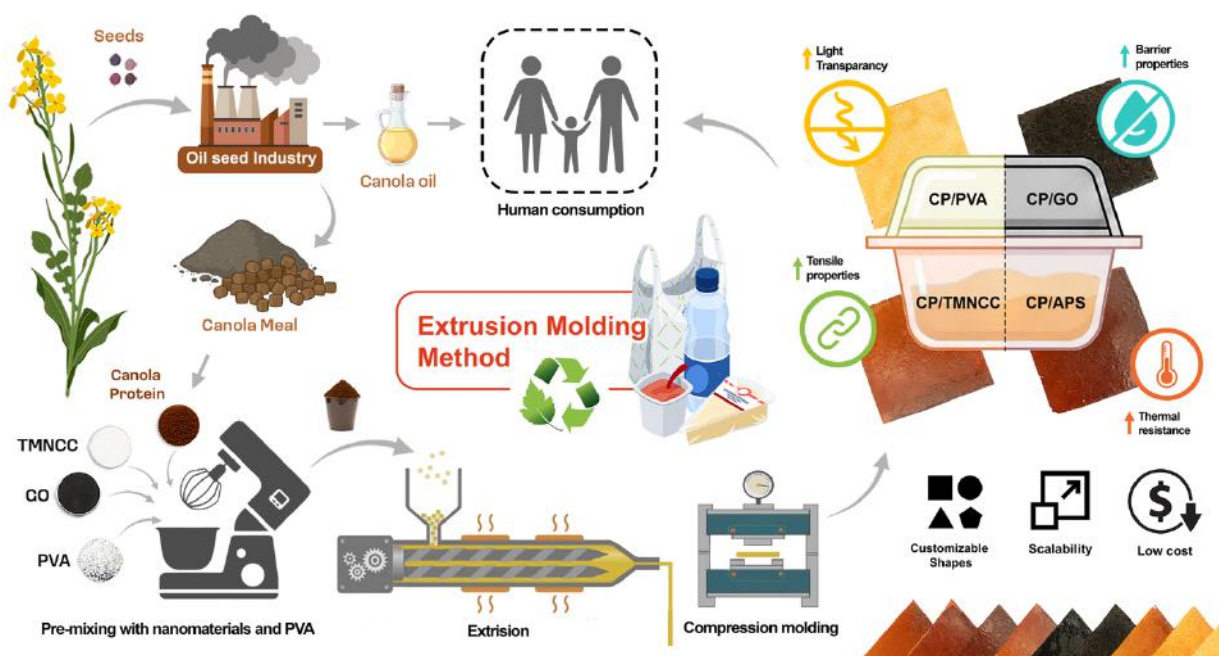
4.1. Abstract

The development of sustainable bioplastics has gained significant attention as an alternative to petroleum-based plastics. Canola meal, a major byproduct of the oil milling industry, is rich in canola protein (CP), biodegradable, and renewable, and has excellent film-forming capability. However, solvent-cast canola films have been observed to lack sufficient barrier and mechanical properties. Extrusion moulding is the preferred industrial method for preparing bioplastics due to its low cost, continuous production, and customization. However, canola protein has not yet been extensively studied for this application. In this study, alkaline-extracted canola protein from industrial canola meal was plasticized with glycerol at a 65:35 ratio, selected from five formulations. Various polymers, crosslinkers, and fillers were added to the initial blend, including polyvinyl acetate (PVA), ammonium persulfate (APS), nanocrystalline cellulose (NCC), and graphite oxide (GO). Furthermore, crosslinking of CP using APS was performed in the extruder (Reactive extrusion -RE) to assess the effect of the extrusion method on CP crosslinking. Polymer blends were extruded using a twin-screw extruder, and the films were hot-pressed using compression moulding. The mechanical, barrier, thermal, and light-transmittance properties of the extruded films were characterized. The highest tensile strength was observed in the CP/TMNCC blend (4.53 ± 0.23 MPa), with a 150% improvement over the control. The highest elongation properties were identified in the CP/APS and CP/TMNCC blends. Water vapour permeability was reduced by up to 500% in GO-added films ($0.016 \pm 0.002 \times 10^{-3}$ g mm/m² Kpa h). The highest light

transparency (62%) was observed in the blend with PVA, while GO-added films were opaque, making them ideal for light-sensitive food packaging applications. Both the storage modulus and thermogravimetric analysis showed improved thermal stability and elasticity in the improved CP blends compared to neat films. APS modification prior to extrusion resulted in higher film properties than those of the reactively extruded blends. Overall, this study showcases the feasibility of scaling up the production of biodegradable plastic food packaging while promoting environmental sustainability and reducing reliance on synthetic polymers.

Keywords: Canola Protein, Extrusion Molding, Nanocomposite Film, Environmental Sustainability

Graphical abstract



Author Contribution

Funds acquisition: Bandara N.; Conceptualization: Bandara N. and Abeysinghe V.; Methods: Abeysinghe V., Tadele D., and Bandara N.; Analysis: Abeysinghe V.; Writing the first draft: Abeysinghe V.; Editing and reviewing: Bandara N., Mekonnen T. and Abeysinghe V.; Research Supervision: Mekonnen T. and Bandara N.; Student academic supervision: Bandara N.

4.2. Introduction

The growing environmental concerns associated with petroleum-based plastics have intensified global interest in sustainable, biodegradable alternatives for food packaging. Conventional plastics persist for decades, contribute significantly to microplastic pollution, and rely heavily on non-renewable resources (Aquilina et al., 2024; Rajendran et al., 2025). In response, researchers and industry are increasingly exploring biopolymer-based materials that offer comparable functional performance while reducing ecological impact. Plant proteins have emerged as promising film-forming agents due to their renewability, biodegradability, and ability to create cohesive polymeric networks with desirable mechanical and barrier properties (Chang et al., 2023; Jafarzadeh et al., 2023). Canola protein (CP), an underutilized byproduct of the oilseed processing industry, has gained attention for its strong film-forming properties and its availability as a raw material for large-scale industrial extraction. Canola meal, the main byproduct of the canola oil milling industry, is reported to have 37-40 % w/w of protein (Wang et al., 2014) of CP, with a high level of glucosinolate content, which reduces the consumer perception of human consumption as a protein source rather it is currently used for animal feed formulation (Višnjevec et al., 2021). Canola protein was identified as a sustainable source for the development of plant protein-based food packaging materials (Dissanayake et al., 2025). Previously prepared canola protein-based films indicated key limitations due to the phase separation of the canola protein and the plasticizer, and low water barrier properties (Chang & Nickerson, 2014), very high water absorption, and comparatively weak mechanical performance (Shi & Dumont, 2014), and the limitation of the film fabrication methods. Therefore, nanomaterial incorporation and nanomaterial modification to further improve the film properties were investigated for canola protein-based films prepared by the solvent casting method (Abookleesh et al., 2024; Chairez-Jimenez et al., 2024; Dissanayake et al., 2023). Chemical crosslinking agents have been identified as a promising technique to modify canola protein films and avoid the aforementioned limitations.

Ammonium persulfate (APS) is a chemical crosslinking and oxidizing agent commonly used in polymer chemistry and a GRAS-approved food preservative widely applied in food processing (H. Shi et al., 2017). Beyond its oxidizing capability, APS thermally decomposes into sulphate radicals ($\text{SO}_4^{\bullet-}$), which are highly reactive species capable of initiating radical-chain reactions between acid side chains such as cysteine, tyrosine, and tryptophan (Bandara & Wu, 2018; Mascheroni et al., 2016). APS has been previously studied for initiating graft copolymerization on cellulose

nanocrystals and for canola protein modification (Bandara & Wu, 2018; Wang et al., 2014). Based on the conclusions of Chapter 3 on optimization of the suitable chemical crosslinking (APS) level for canola protein modification, the addition of 5% (w/w APS/protein) APS was identified as the optimum level for APS modification of CP in the solvent casting method.

TEMPO-mediated oxidation, using 2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPO), converts the primary hydroxyl groups of cellulose nanocrystals (NCC) into carboxyl groups, increasing surface polarity and improving interfacial compatibility with protein matrices. Dissanayake et al. (2022) reported that TEMPO-modified NCC significantly enhanced the mechanical performance of solvent-casted canola protein films, with the highest tensile strength (8.36 ± 0.85 MPa) observed in films containing 5% (w/w of protein) TM-NCC. Graphite oxide (GO) has also been identified as an effective nanofiller, offering a cost-efficient alternative to graphene while providing excellent barrier characteristics. According to Chairez-Jimenez et al. (2024), incorporation of 3% (w/w of protein) GO reduced the oxygen permeability of solvent-casted canola protein films to 5.65×10^{-3} cm³·m/m²·day·Pa, corresponding to a 665% decrease relative to neat films. Polyvinyl acetate (PVA) is an amorphous, random copolymer of ethylene and vinyl acetate groups (Emblem, 2012). Compared with other synthetic polymers, such as polyethylene (PE) and polypropylene (PP), which are highly hydrophobic and incompatible with biopolymers, PVA offers better interfacial interactions with protein matrices due to its acetate functional groups. Unlike polyvinyl alcohol (PVOH), which is produced by hydrolysis of PVA, it exhibits greater water sensitivity due to its hydrophilic OH groups and a higher material cost (Emblem, 2012; Kaboorani & Riedl, 2015). PVA provides a balance of flexibility, adhesion, and oxygen barrier properties, making it a suitable auxiliary polymer for enhancing the mechanical performance of protein-based packaging film.

However, most of the previously reported CP-based films were produced by solvent casting, a laboratory-scale method unsuitable for industrial production due to long drying times, solvent requirements, and limited scalability (Hernandez-Izquierdo et al., 2008). Therefore, the extrusion-based compression moulding method is recognized as a continuous, solvent-free, and industry-compatible approach for manufacturing biopolymer films (Ochoa-Yepes et al., 2019). Extrusion moulding method melts and shapes polymers by applying heat and shear, then compression moulding shapes the material into uniform films (Pommet et al., 2003). Extrusion exposes proteins to controlled combinations of heat, shear, pressure, and moisture, enabling structural modification, denaturation, and crosslinking. Extrusion has been successfully applied for zein (Tadele et al.,

2023), soy (Jiménez-Rosado et al., 2022b; Ogale et al., 2000), wheat gluten (Jiménez-Rosado et al., 2019), lentil (Ochoa-Yepes et al., 2019), and rice (Jerez et al., 2007) protein-based bioplastic preparation. Extrusion and compression moulding methods have been applied to canola industry byproducts such as canola meal. Liu et al. (2025) used Glycidyl methacrylate as a chemical crosslinking agent to modify canola meal via compression moulding. Li et al. (2018) used poly(styrene-co-maleic anhydride-co-glycidyl methacrylate) (PSMG) as a crosslinking agent to modify canola meal-based extrusion-moulded films. However, its compatibility in film production using canola protein alone as the raw material remains limited (Zhang et al., 2016).

These findings demonstrate the strong potential of APS, TMNCC, GO, and PVA as functional crosslinking agents, reinforcement nanomaterials, and compatible polymers, respectively. To address these gaps, this study focuses on the development of canola protein-based food packaging films using an extrusion-moulding approach, combined with protein modification using APS modification (reactive extrusion) and nanomaterial (TMNCC, GO) reinforcement to enhance film properties, and to identify the effects of CP/PVA polymer blend in the extrusion moulding method.

4.3. Materials and methods

4.3.1. Materials

Defatted Canola meal pellets and native NCC were donated for research purposes by Richardson International (Lethbridge, AB, Canada) and InnoTech Alberta (Edmonton, AB, Canada), respectively. TEMPO (98.0% purity) was purchased from TCI America (PA, USA). Graphite ($\geq 99.5\%$ purity), glycerol ($\geq 99.5\%$ purity), NaOH ($\geq 97.0\%$ purity), HCl (12N), H₂SO₄ ($\geq 95\%$ purity), Ammonium persulfate ($\geq 98\%$ purity), and 3.5 kDa dialysis tubes (snakeskin) were obtained from Fisher Scientific in Ottawa, ON, Canada. Additionally, NaNO₃ ($\geq 97\%$ purity), NaBr (99.5% purity), KMnO₄ ($\geq 99\%$ purity), NaClO (14.5% available chlorine), along with anhydrous ethanol, glycerol ($\geq 99.5\%$ purity), and PVA [Molecular weight (M_w) = 113,000 (GPC/Gel Permeation Chromatography)], were purchased from Sigma-Aldrich (Sigma Chemical Co, St. Louis, MO, USA). All chemicals employed were of analytical grade.

4.3.2. Canola protein extraction

Protein extraction from canola meal was conducted according to the method of Manamperi et al. (2010) with minor modifications. Initially, defatted canola meal pellets were milled twice using a Hosokawa milling system (Hosokawa Micron Powder Systems, Summit, NJ, USA) and passed

through 0.75 mm and 0.25 mm sieves to enhance extraction efficiency. The milled material was mixed with distilled water at a 1:10 (w/v) ratio, and the slurry pH was adjusted to 12.0 with 3 M NaOH. The mixture was stirred at 300 rpm for 1 h at room temperature to solubilize the proteins. Insoluble material was removed by centrifugation (Beckman Coulter Avanti JXN-30, CA, USA; 10,000 g, 15 min, 4 °C), and the protein-rich supernatant was collected. Protein precipitation was induced by lowering the supernatant pH to 4.0, and the precipitate was recovered via centrifugation under the same conditions. The resulting protein was freeze-dried in a semi-pilot plant freeze dryer (SP VirTis, Oakville, ON) and stored at −20 °C until further use. Proximate analysis of the extracted CPI revealed protein, fat, ash, and moisture content according to the AOAC methods (AOAC 990.03), (AOAC 920.39), (AOAC 942.05), and (AOAC 925.10), respectively.

4.3.3. APS modification of canola protein

Chemical modification of Canola protein was performed following the method Wang et al. (2014) with minor adjustments. Briefly, 3 g of freeze-dried CPI was dissolved in 90 mL of deionized water, and the pH was adjusted to 7.0 using 1 M NaOH. The protein dispersions were placed in a circulating water bath, and all samples were purged with N₂ gas for 5 minutes before APS addition. The water bath temperature was maintained at 30 ± 2 °C throughout the procedure. The optimal APS concentration (5% w/w APS relative to protein) was added to the dispersions, followed by an additional 5-minute N₂ purging. The flasks were then sealed and stirred at 300 rpm for 4 hours at 30 ± 2 °C. Afterward, the mixtures were centrifuged at 10,000 g for 15 minutes at 4 °C to collect the precipitate. The APS-modified CPI was subsequently freeze-dried (SP VirTis, Oakville, ON) and stored at −20 °C until further use. The average ash (AOAC 942.05) and moisture (AOAC 925.10) contents of the modified CPI samples were analyzed according to the AOAC methods.

4.3.4. Modification of Nanomaterials

Previously prepared TEMPO-oxidized nanocrystalline cellulose (TMNCC) was prepared according to the method described by Dissanayake et al. (2022), while graphite oxide (GO) was synthesized following the procedure reported by Chairez-Jimenez et al. (2024). Both materials were used as nanofillers in this study.

4.3.5. Canola protein blend preparation

Biopolymer blend preparation was performed according to the procedure described in Tadele et al. (2023) with slight modifications. Glycerol was used as the plasticizer, and the optimal canola protein:glycerol (65:35) ratio was determined in preliminary trials. Both TMNCC and Go were sonicated in 10 g of water for 5 min at 60% amplitude, 20 kHz frequency, and 500 W power (Fisher Brand Model 505, Fisher Scientific, Connecticut, USA) to facilitate the dispersion in the blend matrix. PVA pellets were ground using liquid N₂ until the particle size was reduced to less than 0.5 mm in diameter. Premixing was performed using a KitchenAid® mixer (KitchenAid Classic Series, K45SSWH, USA) for 45 min until the mixture formed a homogeneous powder, as indicated in Table 4.1. The premixed blend powders were kept overnight in Ziplock bags before being put into the extruder at 4°C.

A twin-screw extruder (Process 11 Parallel Twin-Screw Extruder, Thermo Fisher Scientific) with a length of 440 mm, L/D of 40:1, and an 11 mm diameter was used to extrude the blends. From die to feed, an extrusion temperature profile of 130/135/140/150/150/140/135/130 °C was employed, along with a screw speed of 100 rpm and a feed rate of roughly 2 g/min. Extrusion parameters were determined through preliminary tests based on morphology, tensile testing, and compression film casting. The extrudates had a moisture content below 5% (DWB) and were stored in airtight containers at 4 °C until further use.

Table 4.1. Composition of the CP blend formulations

Treatment identifier	Treatment description	Canola Protein (%)	Plasticizer/ Glycerol (%)	Water (%)	APS (%)	5% TMNCC (%)	3% GO (%)	PVA (%)
Neat CP	Control	65	35	10	-	-	-	-
CP/APS EXT	APS modification using extrusion	65	35	10	5% (3.25 g)	-	-	-
CP/APS	Modified CP with 5% APS	65	35	10	-	-	-	-

CP/TMNCC	TEMPO NCC	65	35	10	-	5%	-	-
	with CP					(3.25 g)		
CP/GO	GO with CP	65	35	10	-	-	3%	-
							(1.95 g)	
CP/TMNCC	GO &	65	35	10	-	5%	3%	-
+ GO	TEMPO NCC					(3.25 g)	(1.95 g)	
	with CP							
CP/PVA	PVA with CP:	52	35	10	-	-	-	13%
(80/20)	blend1							(13 g)
CP/PVA	PVA with CP:	39	35	10	-	-	-	26%
(60/40)	blend 2							(26 g)
CP/PVA	PVA with CP:	26	35	10	-	-	-	39%
(40/60)	blend 3							(39 g)

4.3.6. Compression moulding of films

The bioplastic films were fabricated using a hydraulic hot-press (Carver press, USA). Approximately 3 g of extrudates from each extruded CP blend were compression-moulded between two stainless steel plates with 0.20 mm spacer blocks (length x width = 7.5 x 7.5 cm). The hot-presses were preheated for 5 min each, and the films were compressed for an additional 5 min at 155 °C and 5000 psi. The resulting films measured varied in thickness levels (0.20 - 0.50 mm) due to the different blend materials. Before testing, the films were conditioned at 23 °C temperature and 50% RH for at least 48 hr.

4.3.7. Characterization of CP-based extruded films

4.3.7.1. Fourier transform infrared spectroscopy (FTIR)

The structural modifications of the extruded CP films were investigated using Fourier transform infrared spectroscopy (ATR-FTIR; Invenio S, Bruker, Leipzig, Germany). A background scan was

performed prior to each measurement to minimize noise. IR spectra of the CP blend films were recorded over a range of 400–4000 cm^{-1} , with 120 scans at a resolution of 4 cm^{-1} . The spectra were baseline-corrected in the amide I region (1600–1700 cm^{-1}) and normalized using Origin 2025 software (OriginLab Corporation, MA, USA). The second derivative of the amide I region was calculated using the Savitzky–Golay method with a nine-point smoothing window, following the approach described by Yang et al. (2015). Peak fitting to determine protein secondary structure components was performed using Gaussian functions. FTIR measurements were conducted in triplicate for each extruded film replicate, averaged, and then subjected to second-derivative analysis.

4.3.7.2. X-ray diffraction (XRD)

The crystallinity of the extruded CP film samples was examined using X-ray diffraction (XRD) on a Siemens/Bruker D5000 diffractometer (BrukerNano Inc., Madison, WI, USA) equipped with a Cu X-ray source and operated in Bragg–Brentano reflection mode. Diffraction data were collected over a 2θ range of 5° to 50° with a step size of 0.02° and a dwell time of 1 s per step. Background correction and preliminary data processing were performed using Jade software (Materials Data and the International Centre for Diffraction Data, Newton, USA). Interlayer d-spacing values were calculated using XpovderXTM software (XpovderXTM, Granada, Spain), and final analysis and graphical representation of the results were completed with OriginPro 2025 (OriginLab Corporation, Northampton, MA, USA).

4.3.7.3. Scanning electron microscopy (SEM)

Surface morphology of the extruded CP film samples was examined using a field emission scanning electron microscope (FE-SEM; Quanta 650 FEG, FEI Company, OR, USA). Modified canola protein films were sectioned into 1×1 cm squares prior to analysis, and both surface and cross-sectional views were examined. To enhance conductivity, samples were sputter-coated with a metal layer composed of 60% gold (Au) and 40% palladium (Pd) under a nitrogen atmosphere (50 mTorr pressure, 45 mA current, for 60 s) using a Denton Vacuum Desk II coater (Denton Vacuum Inc., NJ, USA). Surface views and the cross-sectional views of the SEM images were captured under $\times 2000$ and $\times 300$ magnifications, respectively.

4.3.7.4. Light and UV transmittance of blends

The extruded CP film samples were cut into 1 cm wide strips, which were fit into the cuvette of the UV-vis spectrophotometer (Genesys 150, Thermo Fisher Scientific, USA), and the transmittance values were measured between 200 nm and 800 nm wavelength range.

4.3.7.5. Mechanical properties

Mechanical properties of the extruded CP film samples were evaluated according to ASTM D882 (ASTM D882–18, 2018). Before evaluation of mechanical properties, the canola protein films were equilibrated at 23 °C and 50% relative humidity for 48 h. The effects of modifying CP blends on tensile strength (TS), elongation at break (EAB), and elastic modulus (EM) were determined using an Instron 68TM-50 universal testing machine (Illinois Tool Works Inc., MA, USA) equipped with a 5000 N load cell. Film specimens were prepared with dimensions of 50 mm (length) × 10 mm (width) × t mm (varying thickness) and tested at a crosshead speed of 5 mm/min. Each treatment was tested in 5 replicates, and the results were expressed as mean ± standard deviation (SD).

4.3.7.6. Water vapour permeability (WVP)

The WVP of the extruded CP film samples was evaluated using a gravimetric method according to the ASTM E96/E96M-16 standard (ASTM E96/E96M-16, 2016). Before testing, the canola protein films were equilibrated at 23 °C and 50% relative humidity for 48 h in accordance with standard conditioning requirements. Water vapour permeability experiments were conducted in a controlled climate chamber (Mettmert HPP 260 Constant Climate Chamber, Germany) using disposable plastic cups with a mouth diameter of 4.24 cm and a height of 4.02 cm. The cups were filled with deionized water to approximately 75% of their total volume. Film specimens were cut to match the cup opening and securely affixed using a water-impermeable adhesive. The initial mass of the assembled system, consisting of the cup, water, and film, was recorded prior to placement in the chamber, which was maintained at 23 °C and 50% RH for 48 h. Following the test period, the mass loss attributable to water vapour transmission was determined, and the water vapour permeability (WVP) was calculated using Equation (4.1).

$$WVP = \frac{\text{Weight change (g)} \times \text{Film thickness (mm)}}{\text{Area of the cup mouth (m}^2\text{)} \times \text{vapour pressure difference (kPa)} \times \text{time (h)}} \quad (4.1)$$

4.3.7.7. Water solubility

The aqueous solubility of the extruded CP film samples was quantified using a refined protocol adapted from (González et al., 2019). Briefly, the films were sectioned into 2×2 cm pieces and oven-dried at 105 °C for 24 h to determine the initial dry mass (W_i). The dried specimens were then individually immersed in 50 mL of deionized water and maintained in a temperature-controlled water bath at 25 °C with continuous orbital agitation at 100 rpm for a 24 h period to promote dissolution. Following incubation, the residual, non-solubilized film fractions were carefully retrieved and oven-dried again at 105 °C for 24 h to obtain the final dry mass (W_f). The percentage water solubility of each film was subsequently calculated using Equation (4.2).

$$\text{Water solubility} = \frac{w_f - w_i}{w_i} \times 100\% \quad (4.2)$$

4.3.7.8. Oxygen permeability (OP)

The oxygen permeability (OP) of the extruded CP film samples was determined using a custom-made bubble flow rate system. The films were securely positioned within a dual-chamber cartridge, forming an airtight barrier between the two compartments. One chamber was supplied with ultra-high-purity oxygen, while the opposing chamber was connected to a bubble flow meter. A constant pressure differential of 30 psi was applied across the system to drive oxygen permeation through the film and the sealing interface. Gas transmission was quantified by measuring the time required for oxygen to displace a 100 mL volume in the flow meter. The OP values were subsequently calculated using Equation (4.3) and reported in units of $\times 10^{-3}$ cm³·mm/m²·day·Pa.

$$\text{OP} = \frac{V}{t \times A} \times \frac{L}{P_2 - P_1} \quad (4.3)$$

where: $\frac{V}{t \times A}$ = Oxygen flux

L = Film thickness

($P_2 - P_1$) = Pressure difference across the film

4.3.7.9. Dynamic Mechanical analysis (DMA)

Tan delta (δ) analysis of the extruded CP film samples were performed using a dynamic mechanical analyser (Q800 DMA, TA Instruments, USA). The temperature range of -60 °C to 180 °C was used

to measure the damping factor ($\tan \delta$). A continuous heating rate, frequency, and strain of 3 °C/min, 1 Hz, and 0.2%, respectively, were applied to a film tension clamp.

4.3.7.10. Thermogravimetric analysis (TGA)

A thermogravimetric analyzer (TGA-2, Mettler Toledo, Mississauga, ON, Canada) was used to examine the thermal stability of extruded CP film samples. Approximately 5–10 mg of the samples were heated to 500 °C at a rate of 10 °C/min under a continuous nitrogen flow (30 mL/min) to obtain the thermogravimetric curves. OriginPro 2025 was used for data visualization and analysis of thermogravimetric curves and their first-order derivatives (OriginLab Corporation, Northampton, MA, USA).

4.3.8. Statistical analysis

Each experimental condition was evaluated with 3-5 independent replicates, and the results are presented as the mean $\pm$ standard deviation. The effects of treatments were statistically analyzed using one-way analysis of variance (ANOVA), with $p < 0.05$ considered statistically significant. Differences among group means were further examined using Tukey's multiple comparison test. All statistical computations were performed using OriginPro 2025 software (OriginLab Corporation, Northampton, MA, USA).

4.4. Results and Discussion

Proximate analysis of the extracted CPI revealed protein, fat, ash, and moisture content of $81.07 \pm 0.85\%$, $7.85 \pm 0.68\%$, and $2.47 \pm 0.02\%$ (DWB), and $4.14 \pm 0.16\%$ (WWB), respectively. The average ash and moisture content of the APS-modified CPI were $1.84 \pm 0.05\%$ (DWB) and $1.05 \pm 0.02\%$ (DWB), respectively.

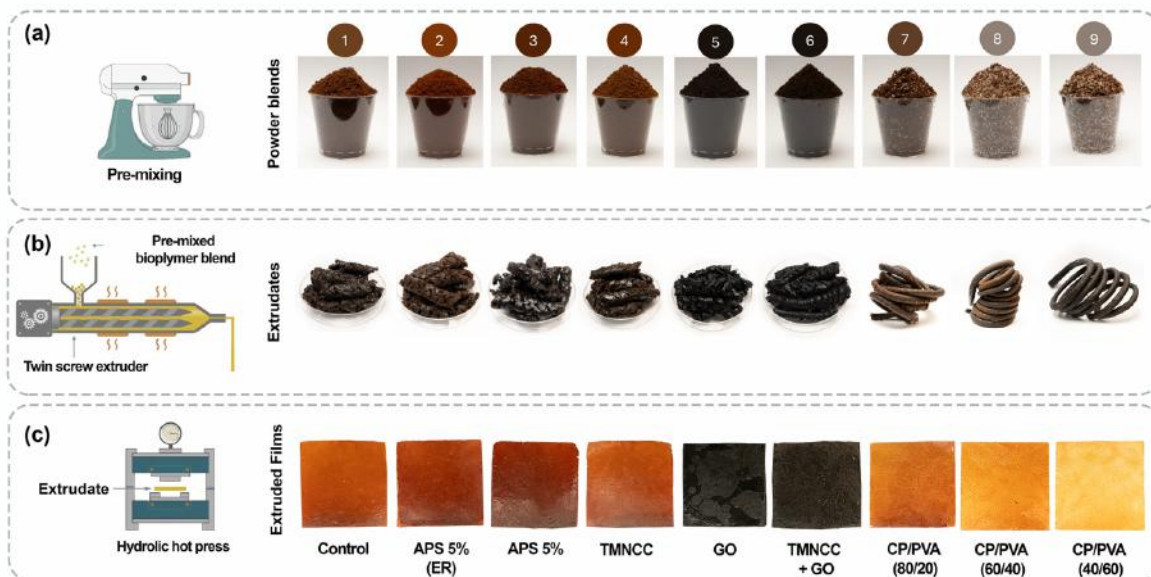


Figure 4.1. Graphical and pictorial illustration of CP blends used for extrusion moulded film preparation (a) Premixing CP blends (b) extrudates after extrusion (c) extruded films after compression moulding of the extrudates.

Figure 4.1 illustrates the pictorial representation of the CP powder blends used as the ingredient for the extrusion process, the extrudates as the result after the extrusion and the final compression-moulded extruded films obtained using the respective CP extrudates.

4.4.1. Fourier transform infrared analysis (FTIR)

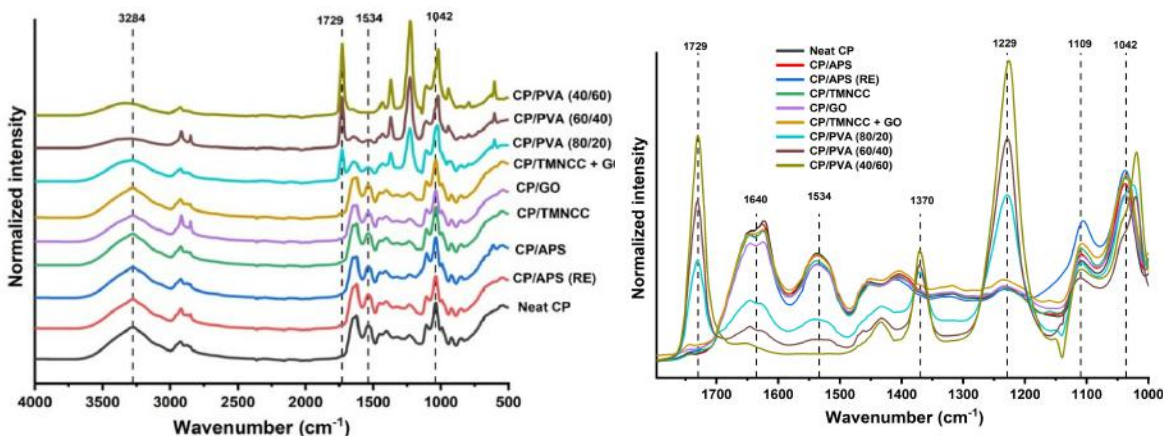


Figure 4.2. FTIR spectra of the CP polymer blends (a) full spectral view (b) enlarged FTIR spectral view

The molecular interactions and chemical changes in the neat and CP polymer blends were identified using Fourier transform infrared spectroscopy. Figure 4.2 illustrates the normalized intensities of the respective blends. The intensities of the characteristic amide II peak at 1534 cm^{-1} and the broad OH peak at 3284 cm^{-1} indicated a slight reduction due to the APS modifications compared to the neat CP films. APS is a radical initiator that forms interactions between amino acid residues, leading to protein crosslinking. Bandara & Wu (2018) reported that the reduction of the tyrosine -OH residues peak at 1534 cm^{-1} might be an indication of the initiation of the crosslinking reactions between Try-Try amino acid residues. In this study, the APS-modified protein showed lower peak intensity than the APS modification that occurred during reactive extrusion. Furthermore, the peak intensity corresponding to the C-O-C asymmetric vibration at 1109 cm^{-1} has increased and shifted to 1105 cm^{-1} in CP/APS. This might be due to interactions between protein and glycerol molecules in the matrix, or to new interactions induced by the APS modification (Kachel-Jakubowska et al., 2017; Rabelo et al., 2015). According to the APS modification of the CP procedure described by Wang et al. (2014), this reaction takes approximately 4h to complete, and the reaction chamber was purged with N_2 to provide a deoxygenated environment, facilitating the reaction. Reactive extrusion was faster and required a higher extrusion temperature, which were not ideal conditions for the crosslinking of the CP matrix. Although the modification was not efficient, it still showed higher film properties than neat

CP film, highlighting the importance and potential of reactive extrusion for APS-modified CP films using the extrusion moulding method. Similar trends were observed with the nanomaterial- and PVA-incorporated films. This could be due to the incorporation of CP amino acid residues with the additive materials. Similar observations were reported by Tadele et al. (2023) in a zein/ PBAT polymer blend.

TEMPO modification converts the primary -OH groups on the NCC surface into -COOH groups via catalytic oxidation (Dissanayake et al., 2022b). Elevated peak intensity at 3284 cm^{-1} of both in the CP/TMNCC and CP/TMNCC+GO blends indicates the existence of supplementary OH groups resulting from hydrogen bonding among the nanofillers, glycerol, and protein chains (Wang et al., 2015). Furthermore, Abookleesh et al. (2024) reported a peak associated with the -C-O vibration in glycerol, which explained the hydrogen bonding interactions between nanofillers, CP, and the plasticizers within the matrix. In this study, a similar peak was observed, and a shoulder peak shifted from 1042 to 1038 cm^{-1} with the addition of nanomaterials, indicating improved interactions between the protein and nanomaterials.

Due to the presence of acetate monomers of the PVA polymer of the CP/PVA blends, characteristic peaks at 1732 , 1370 , 1229 , and 1018 cm^{-1} were identified representing the -C=O , -CH_3 symmetric bending, C-O-C , and -C-O (secondary ester linkage) stretching vibrations, respectively (Dibbern-Brunelli et al., 1998). Moreover, Dibbern-Brunelli et al. (1998) reported that, with increased strong absorption of the carbonyl (-C=O) peak, a shoulder shift toward lower wavenumbers indicated hydrogen-bonded carbonyl groups. Similar shoulder shifts were observed in this study at 1732 to 1729 cm^{-1} in the -C=O peak and at 1229 to 1226 cm^{-1} in the C-O-C peak, indicating stronger interactions in the CP/PVA blend matrix with increasing PVA content.

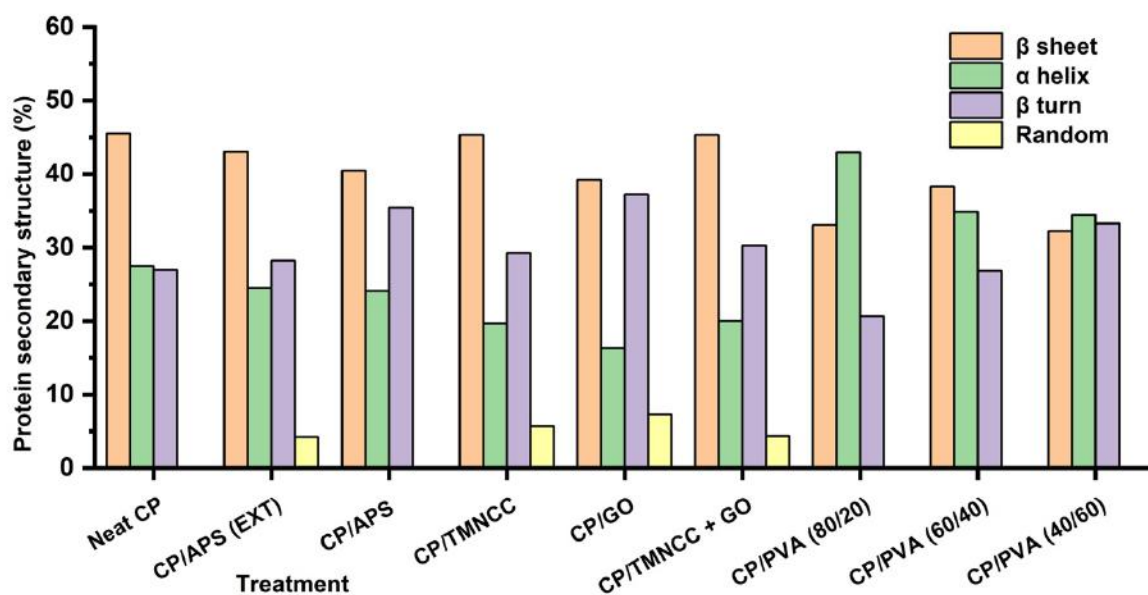


Figure 4.3. FTIR analysis, relative proportions of the protein secondary structures in the amide I region of CP blends

Shifts in the characteristic absorption bands corresponding to protein secondary structures, including β -sheets (1620 , 1627 and 1634 cm^{-1}), random coils (1443 cm^{-1}), α -helices (1650 cm^{-1}), and β -turns (1660 , 1668 , 1681 and 1694 cm^{-1}), indicate alterations in the conformational arrangement of the proteins (Kong & Yu, 2007). The secondary structural changes in different CP polymer blends due to modifications are shown in Figure 4.3. The decrease in α -helix content and the corresponding increase in β -turns in the modified canola protein films can be attributed to the disruption of intramolecular hydrogen bonds, oxidative side-chain modifications, and thermomechanical unfolding during processing. These factors destabilize the helical conformation and promote the formation of compact β -turn structures, which are thermodynamically favoured in partially denatured or crosslinked protein matrices (Dissanayake et al., 2022b). Furthermore, increased protein–protein and protein-nanomaterial interactions, and this increased tortuosity of the protein matrices, explain and support the mechanical and barrier properties of the films, as discussed in the next sections (Bandara & Wu, 2018). As depicted in Figure S2.1, Random coil structures at 1643 cm^{-1} indicate protein aggregation during the extrusion process featured in the CP/APS (EXT) blend. This might be due to the higher heat treatment during the extrusion process for the APS modification. A similar peak was observed in nanocellulose-added films.

4.4.2. X-ray Diffraction (XRD) Analysis.

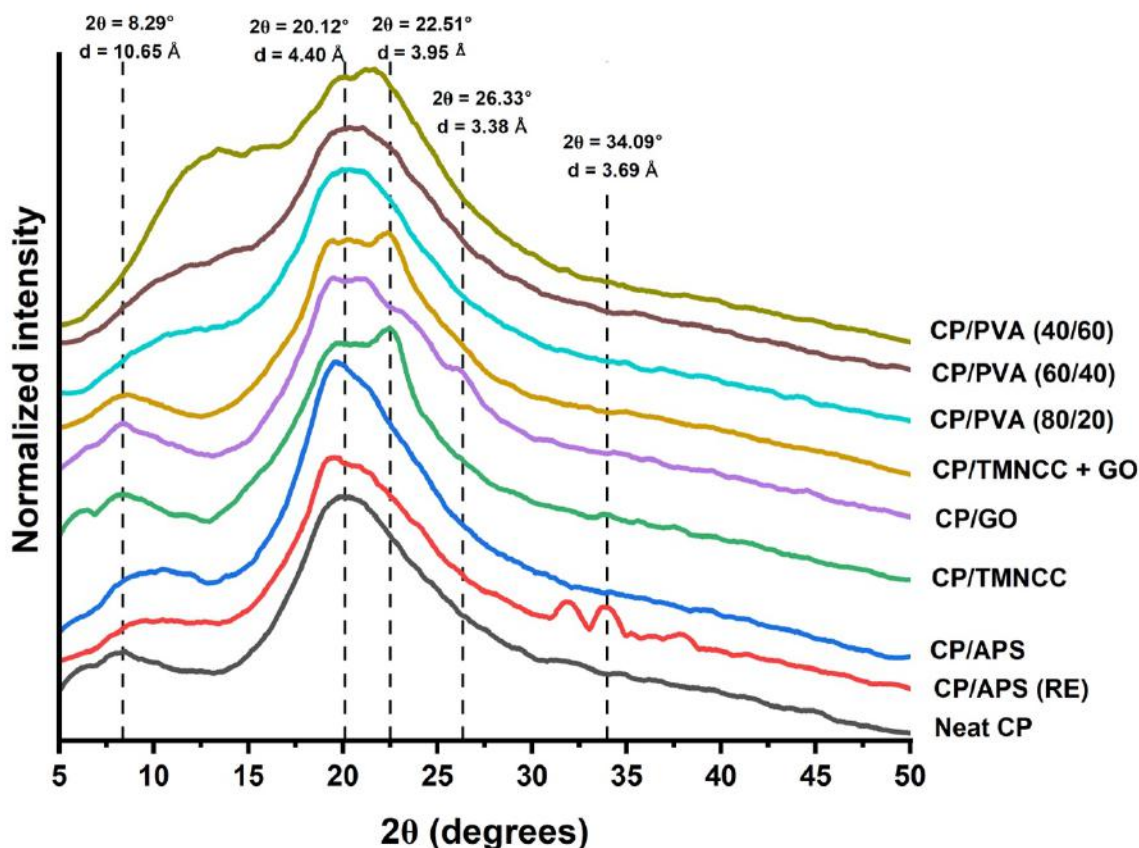


Figure 4.4. XRD patterns of the modified CP blend films

Figure 4.4 shows the X-ray diffraction (XRD) profiles of CP blends, indicating the crystalline characteristics of the material blend. The diffraction angle (2θ) and corresponding interplanar distance (d -spacing) can be determined using Bragg's law ($n\lambda = 2d \sin \theta$). Canola protein typically exhibits prominent diffraction peaks near 20° and 10° , consistent with previously reported findings (Dissanayake et al., 2022b). With the APS modification, the intensity of the characteristic XRD peak at 20° was increased in both CP/APS blends compared to the neat CP blend. In the meantime, the peak of the neat CP blend at 8.29° was shifted to higher glancing angles, resulting in a lower interlayer d -spacing. This might be due to the increased crystallinity of the CP matrix resulting from the APS modification, which leads to closer packing. However, new peaks appeared in the

higher glancing-angle region of the CP/APS (EXT) blend, which were absent in the CP/APS blend. This might be due to protein aggregation due to adverse heat conditions. Furthermore, this result coincides with the appearance of the aggregated random coil peak in the protein secondary structure analysis.

The addition of TMNCC and GO showed characteristic peaks at glancing angles of 22.21° and 26.33°, respectively. Similar peaks were reported in previous studies on NCC, TMNCC, modified montmorillonite, and GO-added CP films (Abookleesh et al., 2024; Chairez-Jimenez et al., 2024; Dissanayake et al., 2022). Dissanayake et al. (2022) explained that this might be due to TMNCC aggregation at higher concentrations ($\geq 5\%$ w/w, NCC/Protein). Furthermore, the addition of nanofillers reduced the glancing angle from 20.12° to 19.38° while increasing the interlayer d-spacing from 4.40 Å to 4.58 Å. Increased interlayer spacing might result from repulsive forces arising from the addition of new -COO⁻ and oxidized graphite groups during the nanomaterial modification process (Dissanayake et al., 2022b). Increased peak intensities at both $2\theta=8.29$ and 10.43° indicate the semi-crystalline behaviour of the CP/nanomaterial blends. These structural changes are responsible for the increased tensile properties, which underscore the potential of the nanomaterials as reinforcement materials in extruded CP films. PVA is an amorphous polymer. The peak intensity at 13.47° increases with increasing PVA content, indicating greater stiffness in the CP/PVA blend.

4.4.3. Light and UV transmittance

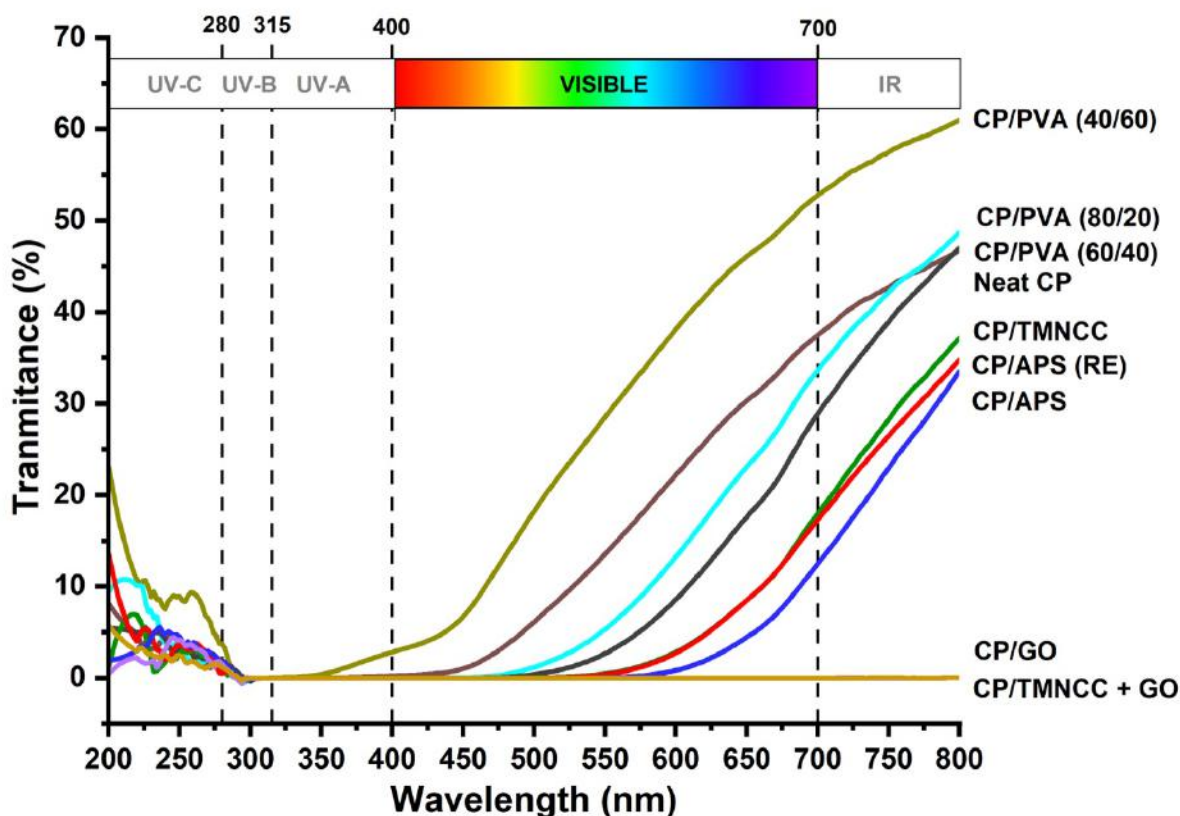


Figure 4.5. Light, UV transmittance and opacity of the CP blends due to modifications

Optical attributes such as transparency and opacity, combined with UV-shielding capacity, play a pivotal role in defining the functional performance of films and sheets. These properties directly affect product protection, consumer visibility, and overall suitability for applications such as food packaging and material containment. Film transparency was measured in the UV and visible regions (200-800 nm), as illustrated in Figure 4.5. The alkaline extraction of canola meal extract brown colour CP due to the oxidation of phenolic compounds (sinapic acid derivatives) and the protein-phenolic interactions (Sharma et al., 2024). Phenolic-rich extruded neat CP film transmits 30% visible light at 700 nm wavelength, while the transparent PVA polymer incorporated CP/PVA (40/60) blend reached the maximum of 54% of light transmittance, making the films translucent. Similar results were reported in Tadele et al. (2023) for modified zein PBAT blend films. However, the addition of GO completely reduced light transmittance and gave the film an opaque

appearance, which might be important for light-sensitive food packaging applications. Except for the CP/PVA (40/60) blend, all other blends showed good UV barrier properties in the UV-A and UV-B regions but were susceptible to UV-C. Exposure to UV-A and UV-B wavelengths can initiate photochemical reactions that deteriorate sensitive food components, including vitamins, proteins, lipids, and pigments, ultimately resulting in undesirable off-flavours, discoloration, and diminished nutritional quality and shelf life of the product (Tripathi et al., 2024). In this study, GO has served as an active food packaging agent, reducing the UV transmittance of extruded CP films.

4.4.4. Scanning electron microscopy (SEM)

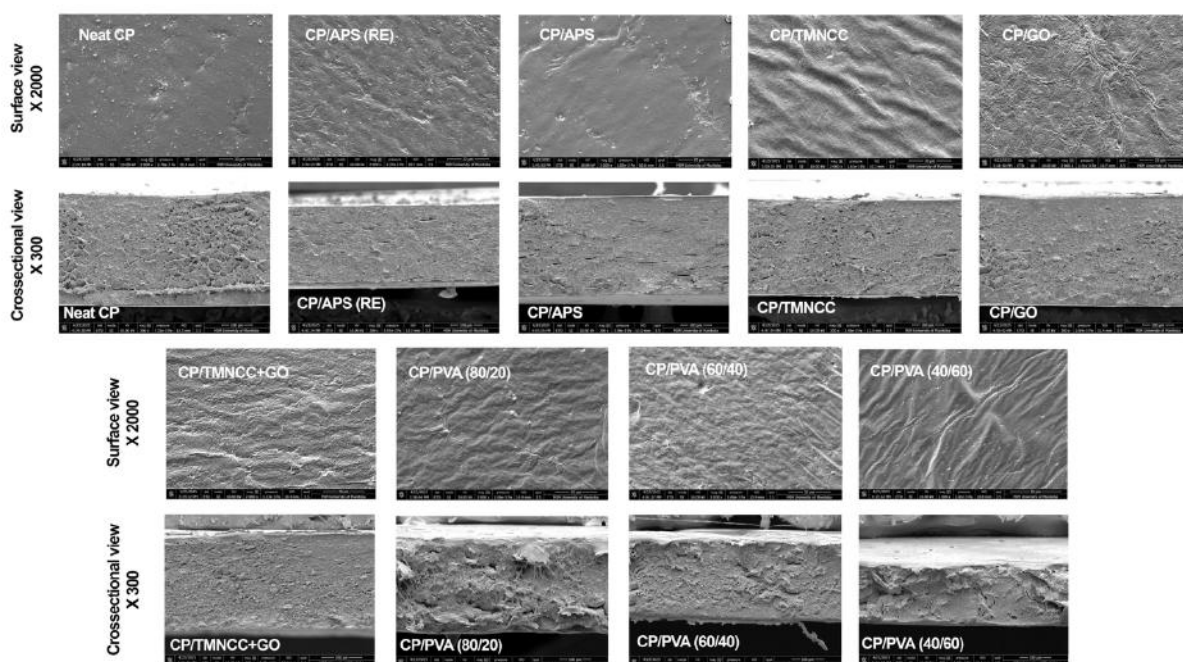


Figure 4.6. SEM microstructural morphology of the CP blends

Morphological understanding of polymer blends is important, as it helps to describe the film's strength, barrier properties, and compatibility (Gong et al., 2022). The microstructural morphology of the CP blends was characterized by scanning electron microscopy (SEM) in both surface and cross-sectional views, as shown in Figure 4.6. The APS modification has increased the surface smoothness of the CP films. Protein modification reduces interfacial tension at the film surface by smoothing the film (Tadele et al., 2023). Moreover, the CP/APS blend showed a more uniform, smoother surface and a more compact cross-sectional packing view than CP/APS (RE). Chang & Nickerson (2014) reported visual changes in the cross-sectional views of genipin-crosslinked CP

films, with crosslinking increasing the films' compactness. They further explained that films with larger pores had higher WVP values. According to the SEM results of this study, reactive extrusion of APS has produced less crosslinking effect than the other modification. This might be the reason the CP/APS blend performs better in barrier properties (WVP, solubility, and OP) (Zhang et al., 2016). However, both APS-modified films showed improved crosslinking nature in the cross-sectional view. Incorporation of TMNCC showed a wavy surface, while GO incorporation indicated a clustered surface in CP/TMNCC and CP/GO blends, respectively. A simultaneous effect was observed in the CP/TMNCC + GO blend. This might be due to the aggregation of nanomaterials/protein on the surface. As depicted in Figure 4.3, this might be due to protein aggregation with nanomaterials, leading to random coil structures in the protein secondary structure. Dissanayake et al. (2022) observed similar results in SEM analysis at higher TMNCC addition levels (5%) in CP films. The authors suggested that the nanomaterials started to aggregate at that level of addition. However, the cross-sectional views of the respective blends were quite uniform, and both nanomaterial-incorporated blends showed reduced porosity compared to the CP/TMNCC blend, highlighting the importance of mixing the two nanomaterials to obtain better film properties. This effect is significantly observed in WVP and oxygen permeability results. CP/PVA blends show relatively better mixing at lower PVA addition levels up to 40% (w/w PVA/protein). More than 50% of PVA addition showed phase separation between the protein and polymer materials. This might be the reason for the rough surface of the CP/PVA (40/60) blend. The thickness of the film reduced with the PVA addition, as the films were made under constant compression pressure and temperature. This might be due to the thermo-sensitivity of the PVA polymer (Kaboarani & Riedl, 2015). Sharp $\tan \delta$ peak indicates high thermo-viscoelasticity of PVA, and this tends to high chain mobility with the heat treatment. Jiménez-Rosado et al., (2022) reported a higher number of voids and increased porosity in the extruded soy-protein-based bioplastics. Extruded CP films were less porous and more compact due to proper plasticization, demonstrating the potential of CP-based bioplastics in this study.

4.4.5. Mechanical properties

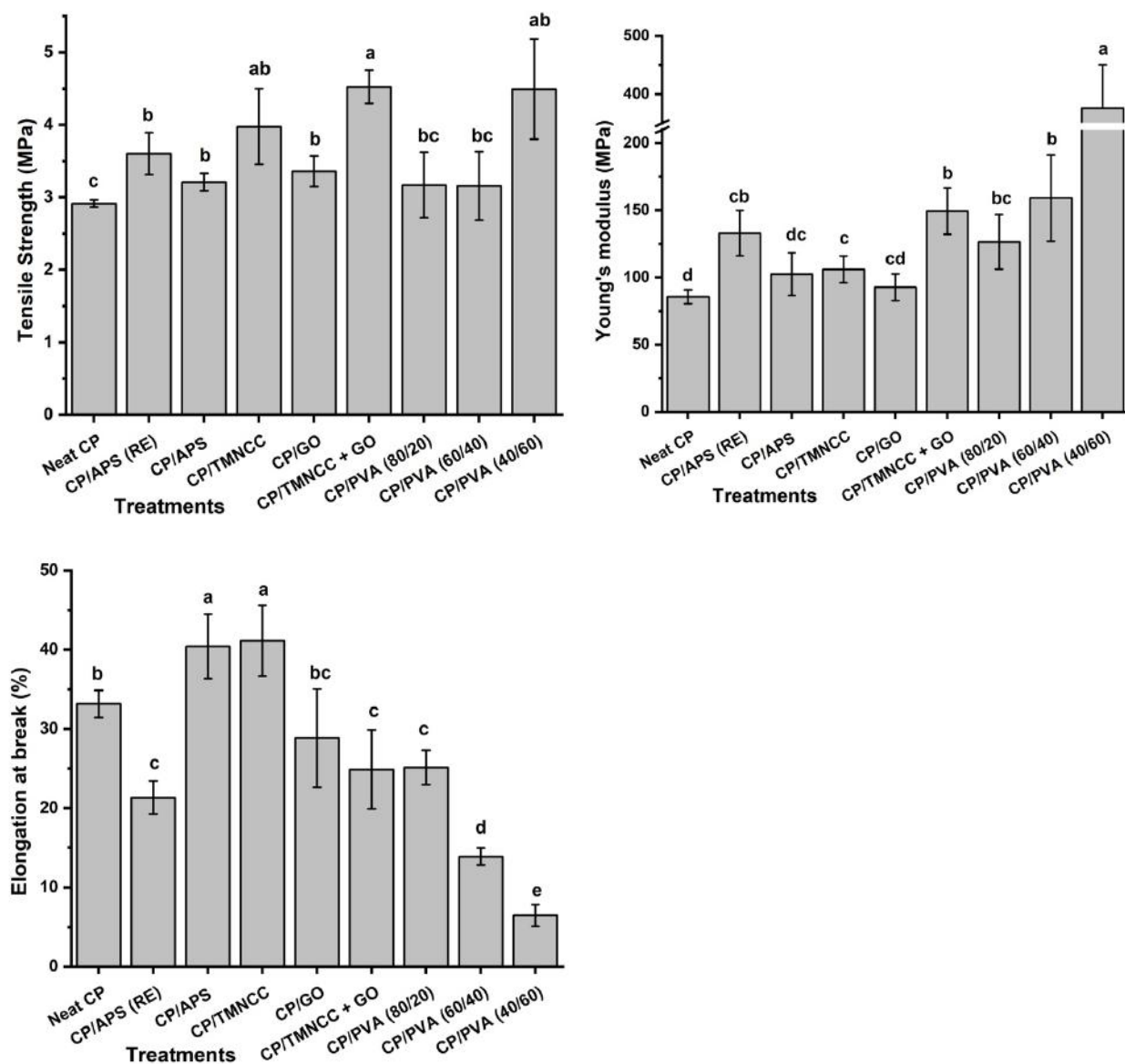


Figure 4.7. Mechanical and water barrier properties of the modified CP films (a) tensile strength (b) Young's modulus (c) elongation at break. All the values are illustrated as the average $\pm$ SD (n=5).

Mechanical characteristics play a key role in determining the strength and flexibility of packaging films. To assess functional suitability, extruded films, tensile strength (TS), Young's modulus (YM), and elongation at break (EB) were examined as depicted in Figure 4.7. As anticipated, the lowest TS was obtained from the neat CP blend (2.90 ± 0.05 MPa). The CP/APS blend resulted

YM than that of CP/APS (EXT), while TS was not significantly affected. This might be due to the higher protein-protein interactions of the radical-driven protein crosslinking mechanism of the APS modification (Bandara & Wu, 2018). The FTIR and XRD results support and clarify this phenomenon, showing that the structural improvements introduced by APS modification of the canola protein prior to processing enabled the specific reactions that occurred during reactive extrusion. However, both APS modifications have increased the mechanical properties of the neat CP blend, highlighting the potential of APS modification of CP in the biopolymer industry. TMNCC showed greater reinforcement of mechanical properties than GO nanomaterials. These results are consistent with previous studies (Chairez-Jimenez et al., 2024; Dissanayake et al., 2022). The CP/TMNCC+GO blend exhibited the highest tensile properties, with a TS of 4.53 ± 0.23 MPa. These improvements may result from the synergistic effects of TMNCC and GO, along with the uniform exfoliation and dispersion of these nanomaterials within the protein matrix, as confirmed by the XRD and SEM results (Bandara & Wu, 2018). There were no significant ($p < 0.05$) differences identified in the lower-level PVA-added CP blends. However, when the PVA content exceeds the CP in the blend, the TS increased up to 4.49 ± 0.69 MPa.

The EB is responsible for the elasticity of the films, which showed higher values in CP/APS and CP/TMNCC blends. The amount of α -helix was decreased with the increasing amounts of β -turns, as depicted in Figure 4.3 Yoshioka et al. (2016) proposed that this could be due to the conversion of α -helices into β -turns via a solid-solid structural transformation during hot stretching of the protein matrix. Moreover, the storage modulus (Figure 4.9) of the CP blends confirmed this observation, showing that the materials' elasticity increased in the mentioned blends compared to the neat CP blend. The increased hydrogen bonding between the plasticizer and protein molecules improves the flexibility of the blends. However, the elongation of the reactive extruded blend was lower than that of the neat film, which might be due to uneven APS modification under unfavourable high-heat conditions. The addition of GO reduced the films' stretchability. Incorporation of PVA polymer restricts the film's elasticity due to the increased crystalline structural behaviour. Furthermore, YM is a measure of a material's stiffness and quantifies the resistance to elastic deformation under tensile stress. The YM shows a reciprocal behaviour compared to EB. Higher YM values in CP/APS (RE) and CP/TMNCC + GO blends confirm the less elastic nature illustrated in Figure 7(c). Therefore, PVA blends showed higher YM values, suggesting greater potential for injection-moulded rigid and semi-rigid bioplastic production.

4.4.6. Barrier properties

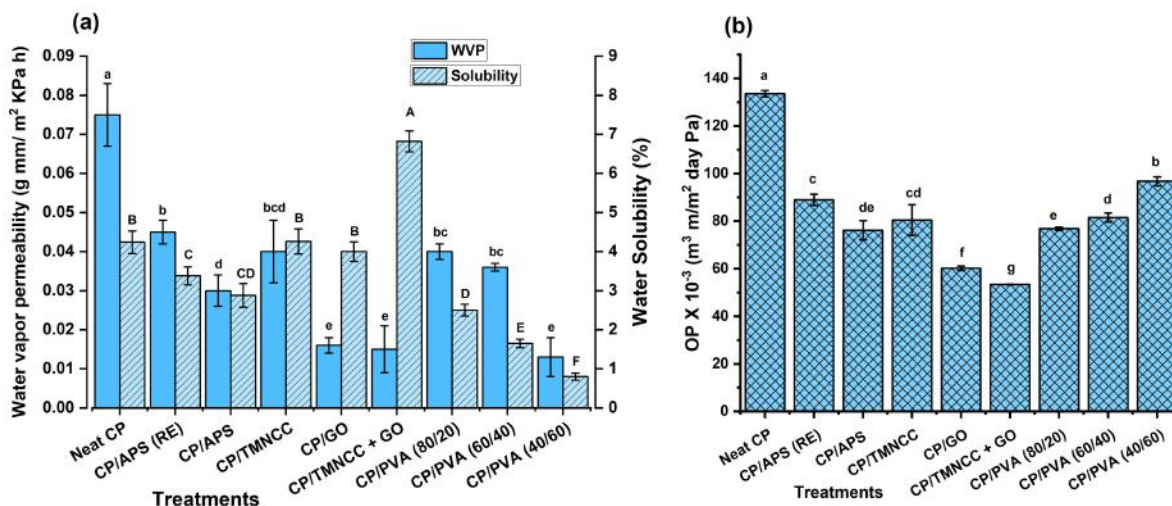


Figure 4.8. Barrier properties of the CP blends (a) water vapour permeability (WVP) and water solubility (b) oxygen permeability

The water vapour permeability (WVP), water solubility, and oxygen permeability (OP) were examined to evaluate the overall barrier properties of the extruded CP blends, as shown in Figure 4.8. Neat CP blends showed the highest WVP value of 0.075 ± 0.008 g mm/m² KPa h, while the CP/TMNCC+GO blend showed the highest water solubility of 6.82 ± 0.27 % (DWB). The APS modification has reduced both WVP and solubility relative to the neat CP blend. The protein modification decreased the availability of hydrophilic amino acid residues and enhanced hydrophobic interactions within the protein matrix, thereby improving the water-resistant properties of the films (S. Li et al., 2017). The addition of GO as a barrier-enhancing agent for water molecules increased the water vapour barrier property by 500% compared with the neat CP blend. The lowest WVP values were observed in the CP/TMNCC + GO and CP/GO blends, at 0.015 ± 0.006 and 0.016 ± 0.002 g mm/m² KPa h, respectively. GO nanosheets form a barrier layer by closely packing both on the surface and inside the protein matrix, as illustrated in SEM images (Figure 4.6). This packing behaviour is again evident in the XRD patterns (Figure 4.4) due to the increased crystallinity. Even though TMNCC had lower water barrier properties than GO, it still improved WVP by 187.5% compared to the neat CP blend. The higher solubility of the CP/TMNCC + GO blend may be due to the presence of charged functional groups and the hydrophilic amino acid residues remaining in the protein blend (Dissanayake et al., 2022b).

However, both solubility and WVP values obtained in this study were much lower than those reported (Abookleesh et al., 2024; Alves et al., 2024; Jiménez-Rosado et al., 2022b). PVA is a water-insoluble amorphous polymer with promising water barrier properties (Emblem, 2012). Therefore, the PVA content shows an indirect relationship with WVP and water solubility. When the PVA content is higher than 50% in the CP blend, the WVP properties act similarly to those of GO-added films, as illustrated in Figure 4.8 (a).

Oxygen permeability (OP) is a measure of how easily oxygen gas can diffuse through the polymer blend under a concentration gradient. It reflects the material's ability to act as a barrier against oxygen transmission. OP of the CP blends, shown in Figure 4.8(b), followed a similar trend to WVP, except for the CP/PVA blends. APS modification of CP and the incorporation of nanomaterials increased the number of polar functional groups within the molecular structure, enhancing the film's oxygen barrier performance, as these polar groups hinder the diffusion of non-polar oxygen molecules (W. Liu et al., 2021b). The lowest OP was again identified in GO incorporated films, highlighting the lowest values as 53.35 ± 0.15 and $60.18 \pm 0.95 \times 10^{-3} \text{ m}^3 \text{ m}^{-2} \text{ day Pa}$, in CP/TMNCC + GO and CP/GO CP blends, respectively. The abundance of polar functional groups, such as secondary amines and carboxyl groups in the protein matrix and oxygen-containing groups in GO, impedes the diffusion of non-polar oxygen molecules, making their diffusion through the film structure less favourable.(Chairez-Jimenez et al., 2024; Chang et al., 2023). The OP should be reduced, along with the addition of PVA, due to the increased polarity of the acetate (CH_3COO^-) groups on the PVA polymers. However, the OP has increased due to the addition of PVA. This might be due to limited miscibility between the CP and PVA polymer chains, leading to partial incompatibility within the blended film matrix. This incompatibility promotes microphase separation, leading to the formation of heterogeneous domains and interfacial discontinuities. Furthermore, due to intermolecular interactions (protein-protein and protein-nanomaterials), free volume within the polymer matrix is reduced, thereby creating a less favourable pathway for the diffusion of non-polar oxygen molecules (Alves et al., 2024; Chairez-Jimenez et al., 2024; Dissanayake et al., 2022). SEM images in Figure 4.6 support the identification of morphological structural changes underlying the barrier properties of the CP blends.

4.4.7. Thermal properties

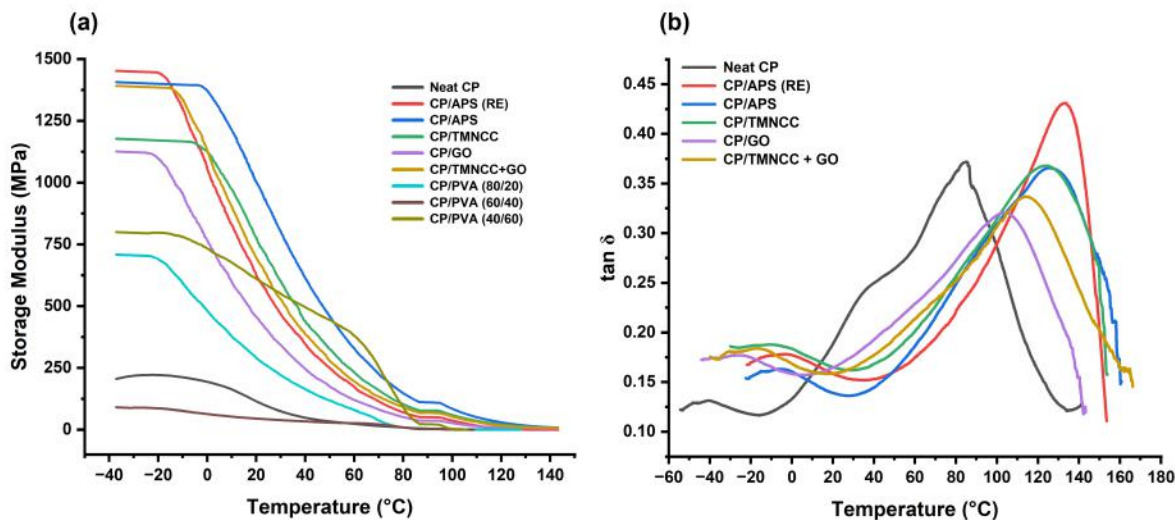


Figure 4.9. DMA analysis of CP blends (a) Storage modulus (b) $\tan \delta$ values

The thermomechanical properties of the extruded CP blends were evaluated using dynamic mechanical analysis, and the storage modulus (G') and the $\tan \delta$ values were illustrated in Figure 4.9. As depicted in Figure 4.9 (a), all the CP blends showed the three characteristic regions of the storage curve with the temperature ranges of -40 to 10 °C, 10 to 90 °C, and 90 to 140 °C, explaining the glassy, glass transition, and rubbery regions, respectively (Zhang et al., 2019). The storage modulus of the G' curves of the APS and nanomaterial modified CP blends was higher than that of the neat CP blend. A similar type of behaviour was reported in modified zein/PBAT polymer blend due to the internal resistance of the protein materials to deformation of external forces (Tadele et al., 2023). Furthermore, the glass transition temperature ranges were broader than those of the neat CP blend. The incorporation of nanomaterials might have restricted the molecular mobility of the protein chains, leading to strong interfacial interactions. That enhances the rigidity and thermal stability of the CP matrix, requiring higher thermal energy for the polymer segments to undergo the glass–rubber transition.

DMA analysis of extrusion-moulded CP films has not yet been studied. Therefore, the reported values for other plant protein sources were compared. Jerez et al. (2007) reported two characteristic $\tan \delta$ peaks in wheat gluten-based compression-moulded films with α and β transitions from 80–90 °C and around -50 °C, respectively. Similar peaks were reported by Zhang et al. (2001) in soy

protein isolate (SPI) sheets. As shown in Figure 4.9(b), the δ values in this study coincide with the previously reported two characteristic transition peaks, α and β . The α transitions are responsible for the glass transition of the plasticized polymer blend, while β transitions could be due to the molecular motion of abundant glycerol, hydrated side chains, and/or crankshaft motions of the main canola protein chains (Lim et al., 1999). According to Table S2.1, α and β transitions were observed at 85 °C and -40 °C, respectively, for neat CP films. A shift of the β transition peak to higher temperature indicates a less hygroscopic nature in the modified films compared to the neat film (Lim et al., 1999; Zhang et al., 2001). The highest α transition peak was observed in the CP/APS (RE) blend, attributed to incomplete protein denaturation during prior thermomechanical processing (Jerez et al., 2007). Furthermore, upon the addition of nanomaterials, the peak intensity of the α transitions decreased compared to the neat CP blend. This might be due to restricted molecular motion and intermolecular interactions between the nanomaterials and the protein matrix (Bandara & Wu, 2018; Dissanayake et al., 2022). Similar variations were reported by Tadele et al. (2023) in modified zein protein blends. Zhang et al. (2006) reported split $\tan \delta$ peak domains at their α transitions in SPI/PLA blends due to the lower compatibility of the two polymers. In this study, all the CP/PVA blends showed only a single domain in their α -transition peaks. This represents the compatibility of PVA as a potential candidate for extruded polymer blending in film preparation.

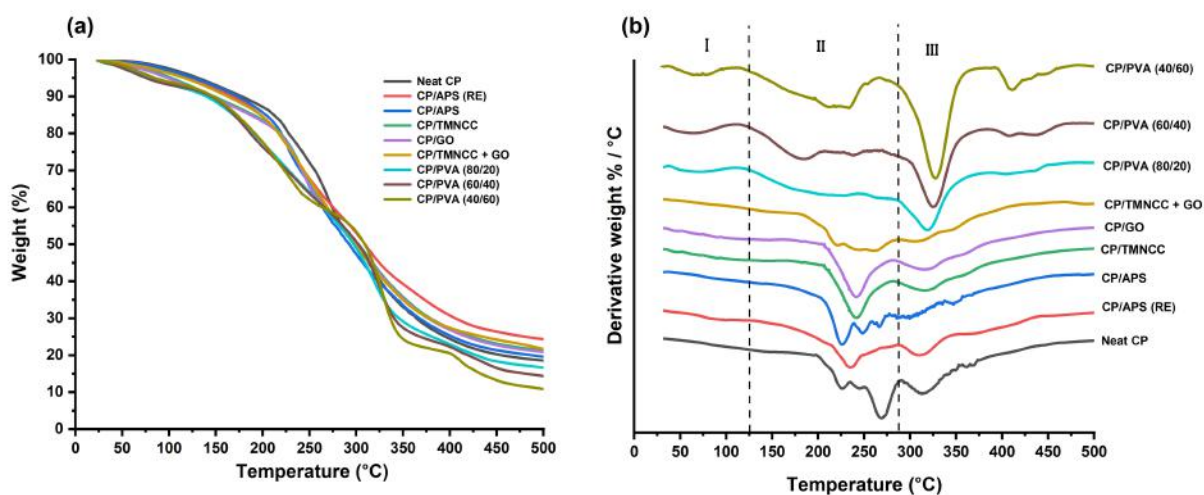


Figure 4.10. TGA analysis of the CP blends (a) TGA curve (temperature vs change of the weight percentage) (b) DTGA curve (1st derivative of weight loss with temperature)

The thermal stability of the extruded CP blends was evaluated using thermogravimetric analysis and differential scanning calorimetry. Figure 4.10 (a) illustrates the percentage of weight loss as a function of temperature. The highest weight loss was observed in CP/PVA blends. After that, the highest weight loss was observed in the neat CP blend, and all other modified films showed thermal resistance against thermal degradation. The improved thermal properties could be due to strong interactions between nanomaterials and the protein matrix (Chairez-Jimenez et al., 2024; Dissanayake et al., 2022). Figure 4.10 (b) shows the percentage values of the 1st derivative of weight loss with temperature. Three characteristic plateaus were reported in DTGA curves, corresponding to moisture evaporation, glycerol evaporation, and the degradation of proteins, polymers, and nanomaterials at 30-125 °C, 125-280 °C, and 280-500 °C, respectively (Osorio-Ruiz et al., 2019; Rojas-Lema et al., 2021). The remaining weight percentages of the CP blends at 500 °C indicated that the reactive-extruded CP/APS (RE) blend showed improved thermal resistance compared with the CP/APS blend (Table S2.2). This trend was consistent with the observed tensile strength values, suggesting a correlation between enhanced thermal stability and mechanical performance. Furthermore, according to Liu et al. (2025), the narrowing of the protein degradation temperature range in the CP/APS blend indicates improved thermal behaviour, which may be attributed to hydrogen-bonding interactions between the plasticizer molecules and protein chains. The blend's corresponding FTIR spectra support this interpretation. Overall, APS incorporation enhanced the thermal properties of the extruded CP films, with both APS-induced CP blends exhibiting improved thermal stability. As the moisture content prior to compression moulding was maintained below 5% (DWB), the moisture peaks were less prominent in the DTGA curves, except for PVA-added CP blends, due to PVA's immiscibility with water (Emblem, 2012). Glycerol evaporation was reduced in the APS-modified CP blends and the CP/TMNCC+GO blend at the second DTGA plateau compared with the neat film. In contrast, the peak intensities of these blends were reduced, indicating a slower decomposition rate. A lower peak often suggests the material requires more energy to break bonds. This might be due to increased crosslinking within the polymer blend or Stronger intermolecular interactions resulting from the nanomaterial addition (Omosola et al., 2022). Furthermore, the narrowing of the protein degradation range upon the addition of nanomaterials and crosslinking indicated enhanced molecular interactions in the CP films (Liu et al., 2025). A similar DTGA pattern was reported by Dissanayake et al. (2023) in TMNCC-reinforced CP films.

4.5. Conclusions

This study demonstrates that targeted chemical modification using APS, incorporation of nanomaterials (TMNCC, GO), and blending with PVA of canola protein have the potential to develop food packaging materials using the extrusion-moulding method. Tensile properties of the APS 5% were increased by 1.33 times, while nanomaterial incorporation increased ~4 times compared to neat films. APS treatment improved intermolecular interactions, crystallinity, and crosslinking, leading to superior mechanical strength, reduced water permeability, and greater thermal stability. Nanomaterial incorporation, particularly the combined TMNCC+GO system, further reinforced the matrix by increasing tortuosity, restricting molecular mobility, and enhancing both tensile and barrier properties. FTIR and XRD analyses confirmed these structural improvements, while SEM revealed reduced porosity and more compact morphologies. The addition of GO increased the WVP by 500% compared to neat films, indicating improved barrier properties. The highest light transparency (60%) was observed in the CP/PVA (40/60) blend, while GO-added films completely blocked light transmission, making them ideal for opaque, light-sensitive food packaging materials. Modified films showed good UV-blocking properties, suitable for food protection. PVA-containing blends performed well at moderate inclusion levels, though miscibility limitations influenced oxygen permeability at higher ratios. DMA results verified increased rigidity and expanded glass-transition ranges in modified films, supporting the observed mechanical and barrier enhancements. Overall, the synergistic effects of APS modification and nanomaterial reinforcement highlight the strong potential of CP-based blends as sustainable, high-performance bioplastic films for packaging applications. According to the existing literature, no other studies have examined canola protein-based extruded films. As the extrusion moulding method is the industrial method for biopolymer processing, this study would help improve byproduct utilization and the circular economy of the canola oil industry.

4.6. References

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Chapter 5 : Conclusions and future trends

Utilization of the main by-product of the canola oil milling industry was limited to animal feeds. The high potential of the extractable canola protein (CP) content in canola meal suggests promising applications in the development of plant protein-based food packaging materials. Ammonium persulfate (APS) showed promising results as a potential crosslinking agent towards strengthening the properties of the neat canola protein films. Modification of nanocrystalline cellulose (NCC) using TEMPO and graphite oxide (GO) resulted in substantial improvements during film formation, increasing the application potential of the food packaging films. Packaging films developed by the extrusion moulding method exhibited better film properties than those developed by the solvent casting method. This is very important for industrial scalability. This thesis practicum contains two packaging film studies conducted using both solvent casting (study 1) and extrusion moulding (study 2).

5.1. Major findings of the study 1: APS modification of CP films and reinforcement using nanomaterials

- The degree of oxidation of the TEMPO-mediated oxidation of NCC was 28.37 ± 2.76 %.
- FTIR and XRD patterns confirmed the conversion of primary OH groups on the surface of the NCC to COOH groups via TEMPO modification.
- The C/O ratio was reduced from 46.76 ± 0.45 to 2.63 ± 0.05 due to the conversion of raw graphite to GO.
- XPS and XRD analysis confirmed the modification by quantifying the increased oxygen peaks.
- APS modification reduced the FTIR peaks of the amino acid residues such as Tyrosine -OH, Tryptophane-CN, and Histidine -NH.
- The particle sizes of the modified protein were increased with the addition of APS content, while the zeta potential was reduced, confirming the APS modification. XRD patterns also supported these results.
- Higher addition levels ($\geq 7\%$) negatively impact the film properties. Therefore, APS at 5% (w/w protein) was identified as the optimal level of APS modification for CP-based packaging films. Nanomaterial reinforcement was performed only at the optimized level.
- SEM images showed reduced porosity in the APS-modified CP, indicating crosslinking.

- Tensile properties of the APS 5% were increased by 1.33 times, while nanomaterial incorporation increased ~4 times compared to neat films.
- APS (5%) modification reduced the WVP from 0.040 ± 0.002 to $0.034 (0.002 \text{ g mm/m}^2 \text{ kPa h})$ compared to neat films.
- Lowest WVP values were observed in GO incorporated films, indicating a value as $0.0160 \pm 0.0005 \text{ g mm/m}^2 \text{ kPa h}$. This is nearly a 250% improvement compared to neat films.
- No significant difference in water solubility was observed among the films. All the values fluctuated within a 33-36 % range.
- The contact angle values improved from $53.43 \pm 8.01^\circ$ to $85.22 \pm 1.26^\circ$ in the TMNCC-incorporated film, highlighting the surface hydrophobicity of the modified films.
- The oxygen permeability of the films decreased with the addition of both nanomaterials. However, no significant difference due to the APS modification.
- The thermal properties were improved with the APS modification and further enhanced with the incorporation of nanomaterials.

Overall, the APS modification improved the properties of the canola protein film, and the addition of modified nanomaterials further enhanced the value of the food packaging material.

5.2. Major findings of the study 2: Canola Protein-Based Nanocomposite Film Prepared Using the Extrusion Moulding

- The canola protein: glycerol (65:35) ratio was identified as the optimal blend in preliminary trials and served as the control blend.
- Extrusion temperature profile of 130/135/140/150/150/140/135/130 °C, a screw speed of 100 rpm, and a feed rate of roughly 2 g/min were identified as the optimized extruder conditions through preliminary trials.
- Compression moulding of 5 min of both preheating and compression heating cycles was performed at 5000 psi and 155 °C for film preparation. These parameters were optimized through preliminary trials.
- Moisture content of the extrudates was varied from 2-4 % (DWB) and maintained below 5% prior to extrusion.
- FTIR, XRD, and SEM analysis helped to identify the structural properties of the films.

- The highest light transparency (60%) was observed in the CP/PVA (40/60) blend, while GO-added films completely cut off light transmission, making them ideal for light-sensitive opaque-type food packaging materials. Except for the CP/PVA (40/60) blend, all other blends showed good UV barrier properties in both the UV-A and UV-B regions.
- Significantly higher tensile properties were identified in most of the modified blends compared to the neat film. The highest tensile strength was identified in CP/TMNCC blend (4.53 ± 0.23 MPa) with 150% improvement.
- The highest elongation properties were identified in the CP/APS and CP/TMNCC blends.
- The addition of GO (0.016 ± 0.002 g mm/m² Kpa h) as a barrier agent for water molecules increased the water vapour barrier property by 500% with respect to the neat CP blend.
- The lowest Oxygen permeability was again identified in GO incorporated films, highlighting the lowest values as $53.35 \pm 0.15 \times 10^{-3}$ m³ m/m² day Pa.
- The modified and reinforced CP blends showed higher storage moduli than the neat CP blend.
- The tan δ values represent the increased Glass transition values due to the additives, showing improved thermal properties in DMA analysis.
- TGA analysis of the modified CP, using APS modification and nanomaterial incorporation, has increased its thermal stability by reducing its susceptibility to thermal degradation.

5.3. Future research trends

The development of canola protein-based packaging materials aligns closely with the global shift toward sustainable and biodegradable alternatives. As demonstrated in this thesis, CP possesses strong potential as a functional biopolymer for film development, and several promising research directions can further advance its application.

Before blending polymers for extrusion moulding, several key material properties must be carefully considered to ensure stable processing and desirable final performance. The polymers should possess compatible thermal properties, particularly overlapping melting or glass transition temperatures and sufficient thermal stability at the processing conditions. Comparable melt viscosities and similar rheological behavior are essential to achieve uniform dispersion and prevent phase separation during extrusion. Chemical compatibility, including polymer polarity and functional groups, governs interfacial adhesion and may necessitate the use of compatibilizers in immiscible systems. In addition, molecular weight characteristics, moisture sensitivity, and

resistance to shear-induced degradation must be controlled, as these factors strongly influence processability, blend morphology, and the mechanical and functional properties of the extruded product. If the materials are not properly blended together, a “compatibilizer” can be introduced apart from a plasticizer to improve the adhesion and the blending of the two materials by reducing the interfacial tension.

Further research on the structural engineering of CP through controlled protein unfolding, enzymatic modification, or physical restructuring offers a pathway to enhance intermolecular interactions and improve film-forming capacity without relying heavily on chemical modifiers.

Reinforcement with advanced nanomaterials, such as TEMPO-NCC and GO, has already shown substantial improvements in mechanical, thermal, and barrier performance. Future studies may explore hybrid nanofiller systems, bio-inspired crosslinkers, or tailored nanocellulose functionalization to expand material performance and application versatility further. The potential of adding graphene or graphene oxide can be further studied to improve barrier properties; this research can be extended. The incorporation of active agents, such as antimicrobial or antioxidant compounds, also offers opportunities to develop multifunctional CP-based active packaging systems.

A major future direction lies in scaling up CP-based film production using extrusion technologies. The findings of this thesis clearly demonstrate that extrusion processing provides superior film homogeneity, reproducibility, and mechanical robustness compared to solvent casting. Given that extrusion is already widely used in the plastics industry, CP-based extrusion blends hold high potential for large-scale industrial trials. Future work should therefore focus on optimizing melt rheology, establishing continuous feed systems, integrating co-extrusion or multilayer configurations, and evaluating process stability during long production runs. Developing CP-based masterbatch formulations that are compatible with commercial twin-screw extruders could significantly accelerate industrial adoption. Combining CP with complementary biodegradable polymers, such as PLA, PHA, PBAT, or thermoplastic starch, may yield synergistic improvements in flexibility, durability, and environmental resistance. Furthermore, due to the observed thermoplastic properties of the modified CP blends, the development of single-use semi-rigid and rigid plastics, such as straws, cutlery, coffee cup lids, and packaging crates, can be further studied with polymer blending to identify the true potential of CP-based bioplastics. Without limiting to the studied solvent casting and extrusion-moulding methods, other bioplastic preparation methods,

such as injection moulding, electrospinning, and 3D printing, can be tested using the optimized CP blends to assess their potential.

Environmental and economic assessments, including biodegradability studies, life cycle analysis (LCA), and techno-economic evaluations, will be essential for validating the market readiness of CP-based films. Additionally, regulatory evaluations, such as migration testing, storage stability testing, and safety assessments, will be required to support their use as food-contact materials.

Overall, future research should emphasize CP structural engineering, advanced nanocomposite design, and, especially, the transition from laboratory-scale extrusion to industrial-scale processing, positioning CP-based films as a viable next-generation sustainable packaging solution within the circular bioeconomic framework.

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Appendix

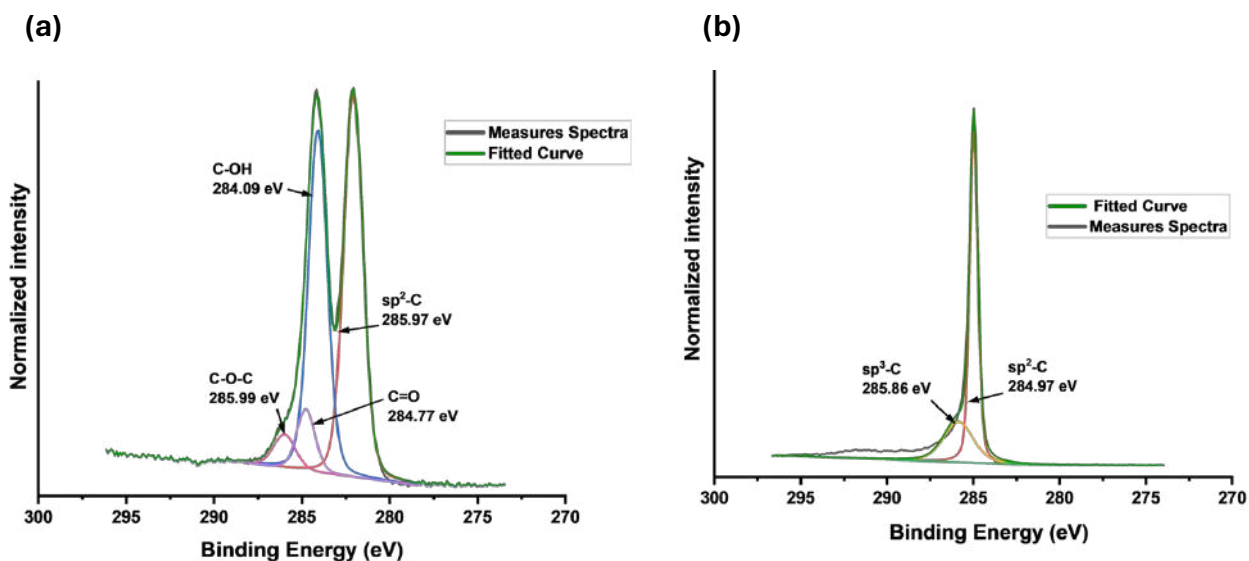


Figure S1.1. Deconvoluted peaks of the high-resolution C1 spectra of the modified graphite oxide and raw graphite (a) modified GO (b) raw graphite

Table S1.1. Particle size distribution of APS-modified canola protein isolates

Treatments	D _v (0.1)	D _v (0.5)	D _v (0.9)
APS 0%	6.34± 0.10	40.43±1.11	125.33±1.53
APS 1%	8.16± 0.23	70.90±2.50	187.33±5.51
APS 3%	8.40±0.24	79.53±2.60	202.67±4.51
APS 5%	9.40±0.20	80.43±1.76	200.00±2.65
APS 7%	14.83±0.15	130.67±0.58	309.33±2.31
APS 9%	17.20±0.30	109.00±1.00	292.33±4.73

D_v(0.1): 10th volume percentile, D_v(0.5): 50th volume percentile, D_v(0.9): 90th volume percentile. All data were analyzed by analysis of variance followed by Tukey's range test (n = 3) and expressed on DWB. Different superscripts within a column indicate a significant difference (p <0.05).

Table S1.2. Barrier properties of the modified CP films.

Treatments	Solubility (%, DWB)	Contact Angle (degrees)
CP Control	34.31 ± 1.84 ^a	53.43 ± 8.01 ^d
APS 1%	35.18 ± 2.62 ^a	55.26 ± 2.53 ^d
APS 3%	33.78 ± 1.16 ^a	80.90 ± 5.91 ^{ab}
APS 5%	34.69 ± 2.79 ^a	78.33 ± 1.40 ^b
APS 7%	35.41 ± 0.94 ^a	73.47 ± 0.54 ^c
APS 9%	35.98 ± 1.30 ^a	70.52 ± 2.82 ^c
APS 5% + NCC 5%	34.12 ± 2.05 ^a	80.74 ± 3.41 ^{ab}
APS 5% + TMNCC 5%	35.33 ± 3.74 ^a	85.22 ± 1.26 ^a
APS 5% + GO 3%	33.27 ± 1.30 ^a	77.42 ± 2.97 ^b

All the values are reported as the average of three specimens ± SD. All data were analyzed by analysis of variance followed by Tukey's range test (n = 3). Different superscripts within a column indicate a significant difference (p < 0.05).

Table S1.3. Peak temperature values of the modified CP films.

Treatments	Peak temperature (°C)
CP Control	96.05 ± 0.16 ^c
APS 1%	95.99 ± 0.59 ^c
APS 3%	96.01 ± 0.38 ^c
APS 5%	95.84 ± 0.30 ^c
APS 7%	95.93 ± 0.64 ^c
APS 9%	95.34 ± 0.68 ^c
APS 5% + NCC 5%	108.36 ± 2.14 ^b
APS 5% + TMNCC 5%	113.74 ± 2.61 ^b
APS 5% + GO 3%	150.48 ± 3.10 ^a

All the values are reported as the average of three specimens $\pm$ SD. All data were analyzed by analysis of variance followed by Tukey's range test ($n = 3$). Different superscripts within a column indicate a significant difference ($p < 0.05$).

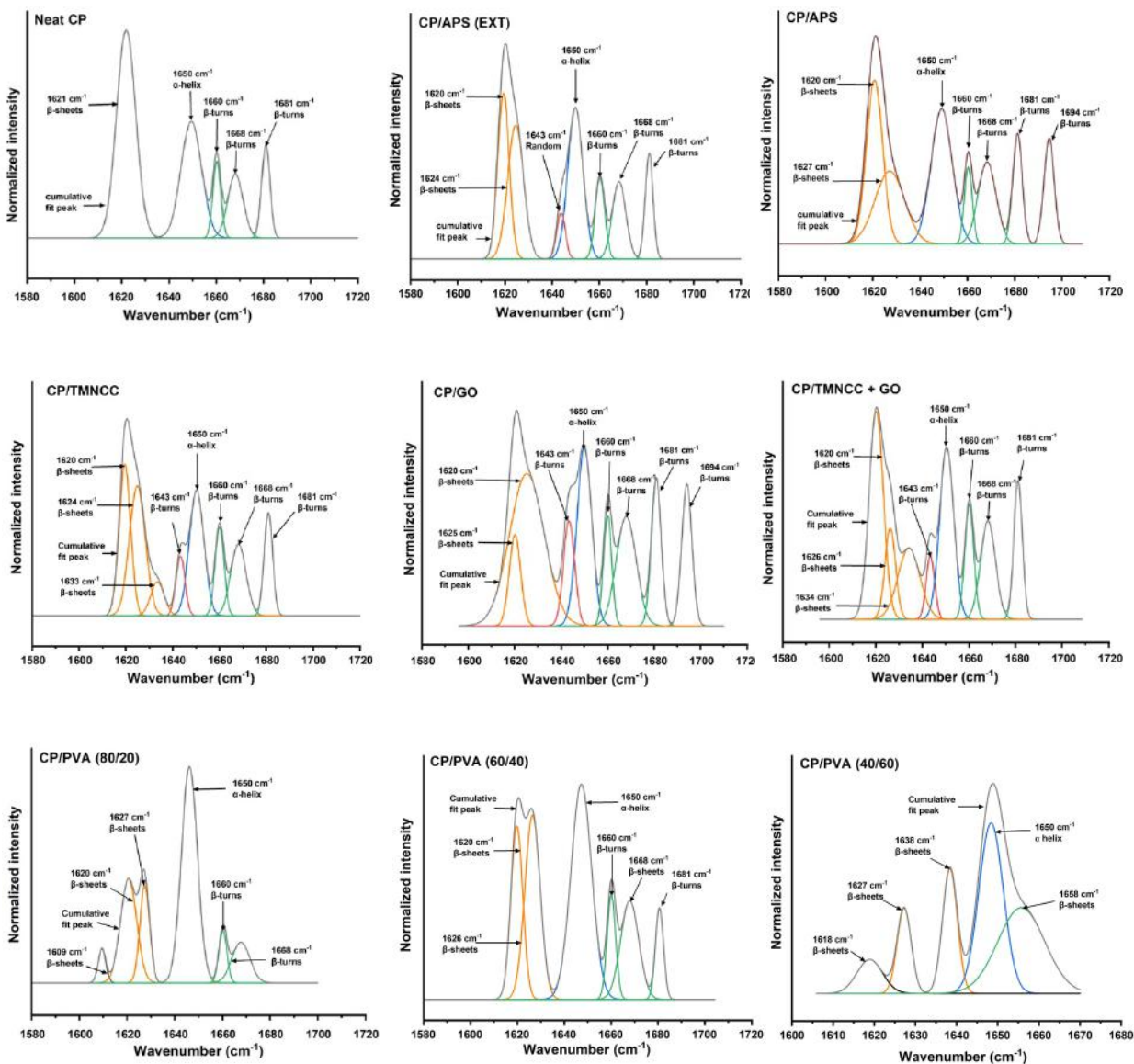


Figure S2.1. Secondary structural FTIR peaks of the CP blends using the 2nd derivative peak fitting method in the amide-I peak

Table S2.1. α and β transition peaks of the CP films in DMA analysis

Treatments	α transition ($^{\circ}\text{C}$)	β transition ($^{\circ}\text{C}$)
Neat CP	85	-40
CP/APS (RE)	132	-3
CP/APS	126	-4.5
CP/TMNCC	123	-8.3
CP/GO	104	-25
CP/TMNCC + GO	114	-16
CP/PVA (80/20)	78	-25
CP/PVA (60/40)	81	-23
CP/PVA (40/60)	85	-16

Table S2.2. Remaining weight percentage of the CP films at 500 $^{\circ}\text{C}$ in TGA analysis

Treatments	Weight % at 500 $^{\circ}\text{C}$
Neat CP	18.58
CP/APS (RE)	24.40
CP/APS	19.63
CP/TMNCC	21.28
CP/GO	20.88
CP/TMNCC + GO	21.69
CP/PVA (80/20)	16.68
CP/PVA (60/40)	14.36
CP/PVA (40/60)	10.80