UNIVERSITÀ DEGLI STUDI DI PADOVA
Department of Comparative Biomedicine and Food Science - BCA, Department of Agronomy, Food, Natural Resources, Animal and Environment- DAFNAE

Second Cycle Degree (MSc)
in Biotechnology for Food Science

Development Of Food Packaging Films Based On Natural Materials

Supervisor
Prof. Barbara Cardazzo
Co-supervisor
Prof. Federica Tonolo

Submitted by
Deniz Baldoğan
Student n.
2039464

THE ACADEMIC YEAR 2022-2023

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RIASSUNTO

Come suggeriscono gli obiettivi di sviluppo sostenibile 2030 del Programma delle Nazioni Unite per lo Sviluppo (UNDP), l'utilizzo dei rifiuti è uno dei temi di ricerca più importanti per il miglioramento degli alimenti e dei prodotti alimentari. L'imballaggio degli alimenti è il fattore primario che ne garantisce la qualità e la sicurezza per i consumatori. Protegge gli alimenti da contaminazioni esterne e permette ai consumatori di avere a disposizione alimenti di alta qualità per tutta la vita a scaffale. Tuttavia, principale costituente degli imballaggi è la plastica, la quale attualmente rappresenta una delle maggiori fonti di inquinamento. Pertanto, lo sviluppo di soluzioni di imballaggio sostenibili è fundalmentale per migliorare l'impatto ambientale, lo smaltimento dei rifiuti e conseguentemente salute pubblica.

Da questo punto di vista, la tesi fornisce indicazioni sullo sviluppo di pellicole di imballaggio sostenibili per i prodotti alimentari utilizzando i comuni materiali di scarto dell'industria dell'amido: il glutine. La tesi si propone di approfondire la capacità della proteina del glutine di formare fibrille amiloidi, la loro caratterizzazione ed infine la possibilità delle fibrille amiloidi a base di glutine di formare film di imballaggio per alimenti e prodotti alimentari. Nell'ambito della ricerca, il glutine viene idrolizzato e fabbricato tramite incubazione in condizioni controllate. Il glutine in queste condizioni produce fibrille amiloidi che possono essere utilizzate come elementi costitutivi per film a base biologica. La loro formazione è stata rilevata con il test della tioflavina T (ThT) e lo sviluppo deli film è stato effettuato utilizzando PVA e glicerolo. È stata quindi un'accurata caratterizzazione dei film formatisi. Le fibrille amiloidi sono state ampiamente studiate nella letteratura disponibile, ma quelle derivate da proteine vegetali richiedono ancora una comprensione più approfondita.

ABSTRACT

As the UNDP's 2030 sustainable development goals suggest, food waste utilization is one of the important research topics as well as the improvement of food and foodstuff. Food packaging is the primal factor that ensures food quality and safety for consumers. It protects food from contamination and allows consumers to reach high-quality foods as long as the shelf-life allows. However since plastic, a common petroleum product, is the main source of packaging today, the consumption of plastic is reaching frightening levels with the increase in food consumption. Therefore the development of sustainable packaging solutions is fundamental for the better utilization of waste and the improvement of the environment and public health.

From this perspective, the thesis provides insight into the development of sustainable packaging films for food products using common waste material from the starch industry: gluten. The thesis aims to create a deeper understanding of gluten protein's ability to form amyloid fibrils, their characterizations, and the ability of gluten-based amyloid fibrils to form packaging films for food and foodstuff.

For this purpose, gluten is hydrolyzed and its fabrication is achieved through incubation under controlled conditions. Incubated gluten yields amyloid fibrils which can be used as building blocks for bio-based films. These formed amyloid fibrils are detected using the Thioflavin T (ThT) assay. Development of amyloid fibril-based films was carried out using PVA and glycerol. Further characterization of those films was performed. Amyloid fibrils have been investigated widely in available literature however amyloid fibrils sourced from plant proteins still require deeper understanding.

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LIST OF SYMBOLS AND ABBREVIATIONS

AFM: Atomic force microscopy

FTIR: Fourier transform infrared spectroscopy

ThT: Thioflavin T

UNDP: United Nations Development Programme

CD: Circular Dichroism

PVA: Polyvinyl alcohol

TEM: Transmission Electron Microscopy

WG: Wheat gluten

1. Introduction

The research project aims to create sustainable food packaging films using wheat gluten as source material. Proteins have been the main ingredient in creating bio-based packaging films for a long time due to their advanced film-forming abilities, good mechanical properties, and high gas and liquid permeability. Plant proteins, however, have just gained importance within this research to support UNDP's sustainable development goals (UNDP, Sustainable Development Goals, 2023). Waste utilization is one of the goals of UNDP's Sustainable Development Goals, 12 - Responsible Consumption and Production, therefore the selection of wheat gluten as source material to develop sustainable films is a valuable asset of the research project. Plant proteins carry a high potential for forming food packaging films as they possess good mechanical properties, specifically good elasticity. Wheat gluten is selected because of its high abundance rate as a waste product in the industry (Chen H.Z. *et al*., 2020). Gluten itself has been studied as a source to form packaging films rather than amyloid fibrils formed by gluten. However, gluten that is processed in alkaline conditions is characterized by a yellow colour and an unpleasant smell, whereas films obtained through acidic dispersion contain large protein particles, which strictly limits its application as a packaging film for foodstuffs (Bastioli, 2020). The limitations and drawbacks that working with gluten itself brings, are the reasons why this research project fabricates the wheat gluten under controlled incubation conditions to form amyloid fibrils. Amyloid fibrils not only allow better mechanical properties but also gluten-sourced amyloid fibril-based films do not possess the same drawbacks as gluten-based packaging films.

Figure 1: Sustainable Development Goals by 2030, UNDP
(UNDP, Sustainable Development Goals, 2023)

However one of the major drawbacks of using gluten as a source for a material to be used in food packaging is its allergenicity. Celiac disease is a condition in which the immune system attacks the body's tissues in the gut (small intestine) lining (NHS UK, 2023). People who suffer from celiac disease not only cannot consume any food products that contain gluten but also cannot consume any food products that encounter gluten. The possible transfer from the food packaging film containing gluten to the food product inside the packaging possesses a great concern within this project. To overcome this problem, the transfer rate of gluten towards the food product will be evaluated as further research after this project as well as the ways to stop or minimize the transfer if applicable. Alternatively, chitosan is being investigated as an alternative to gluten as a side project. Chitosan is a large sugar molecule that possesses great film-forming properties that have been studied largely (Wang H. *et al*., 2018; Tardy B.L. *et al*., 2021). It is a biodegradable and cheap source of food packaging films that possesses great potential without allergenic drawbacks, unlike gluten.

1.1. Wheat Proteins

Wheat is a crop that is almost as old as humanity. It is cultivated for its seed, the most commonly consumed cereal grain around the world (Giraldo P. *et al*., 2019; Arshad M.J. *et al*., 2022). Wheat (*Triticum aestivum*) also known as common wheat is part of the wild grasses (*Gramineae*) family known to be native to Western Asia, however nowadays it is cultivated all over the world (Cornell, H. J., 2020). Being the main component of flour,

wheat is the key ingredient for many foods we consume even today, including foods that are valuable for human history such as bread and beer. The first cultivation of wheat is suggested to be around 10,000 years ago, accepted to be the first spark of the "Neolithic Revolution" which is known to be the transition from a hunting and gathering era to settled agriculture in human history (Giraldo P. *et al*., 2019). Currently, wheat is the second-most commonly produced cereal around the world after maize with the production of 761 million tonnes in 2020 covering over 220.4 million hectares of agricultural land (Pequeno D.N. *et al.*, 2021; FAO, 2022).

Wheat proteins, which are gliadins, glutenins, albumins, and globulin, are found in the endosperm which is about 80-85% of the total weight of the cereal's seed (Cornell, H. J., 2020). Wheat is an important crop for human nutrition, mainly because of its high protein content which is around 13%, known to be higher than most other cereals (CORDIS, 2016). The protein in wheat is responsible for the structure and texture of breadmaking and functionality in food science.

1.1.1. Wheat Gluten

Among the given wheat proteins, gluten, is the most abundant protein in the seed kernel, presenting up to 30% of the total protein content of the seed (Cornell, H. J.,2020). Gluten is a complex protein that consists of gliadin and glutenin. Within the entire protein content of wheat gluten is the major protein that is generally found to be 75-85% (Sharma N. *et al*., 2020). In food science gluten is particularly important for baking products because of its unique viscoelastic and adhesive properties, gives help the dough to become elastic, rise while baking, keep its shape, and give the known chewy texture (Sharma N. *et al*., 2020; Lau S. *et al*., 2021). In the food industry, it is used to improve the textural properties of food since it is a by-product of the starch industry and is highly available and cheap.

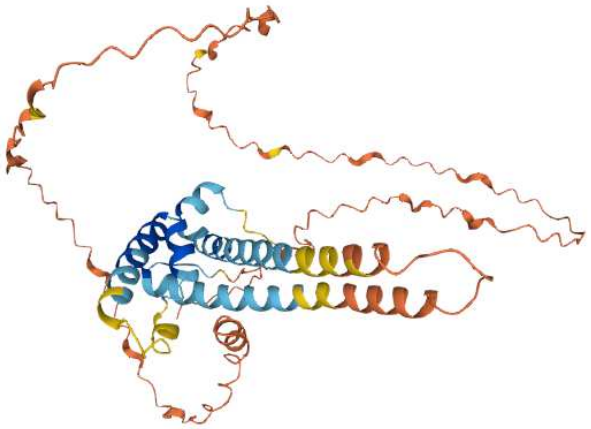


Figure 2: Glutenin Low Molecular Weigh Subunit Structure (P10385, GLTA, Uniprot, 2023)

Due to gluten being highly insoluble in water, its applications in the food industry are limited and its advanced mechanical and foaming properties are diminished. However, when gluten is hydrolyzed, its hydrolystes show similar properties and their efficiency in being used in the food industry in similar ways to gluten has been investigated largely (Akharume *et al*., 2021).

1.2. Gluten Hydrolysis

Hydrolyzation of gluten can be achieved via enzymatic hydrolyzation or non-enzymatic hydrolyzation (acid/base hydrolyzation) (Janssen F. *et al*., 2023). Enzymatic hydrolyzation of gluten can be achieved via multiple available enzymes such as trypsin, chymotrypsin, thermolysin, papain, and proteinase K. Among these (and many more) trypsin is the most common enzyme that is used in gluten hydrolyzation due to its unique ability to cut molecules in carboxyl sides of amino acids that allow numerous biotechnological processes (Lambrecht *et al*., 2021).

1.3. Trypsin

Trypsin is a proteolytic enzyme that is commonly found in the small intestine and is responsible for cutting longer protein chains into smaller pieces. The optimal pH for Trypsin to work is between 7 and 9 (Hu Q. *et al*., 2021). Trypsin was first discovered and isolated from the pancreas by rubbing glycerin on it, in 1876 by Wilhelm Kühne (Thapa S. *et al*., 2019). Using trypsin in the specificity of this research is important due to the enzyme's

ability to cut the proteins and peptides at the carboxyl group of lysine or arginine (Punekar, N. S., 2018). Trypsin as an enzyme has many commercial uses in the food industry. For instance, such applications of Trypsin can be listed as the following, improving the workability of dough, extracting seasonings and flavorings from vegetable or animal proteins, controlling aroma formation in cheese and milk products, improving the texture of fish products, and tenderizing meat, during cold stabilization of beer, to produce hypoallergenic food.

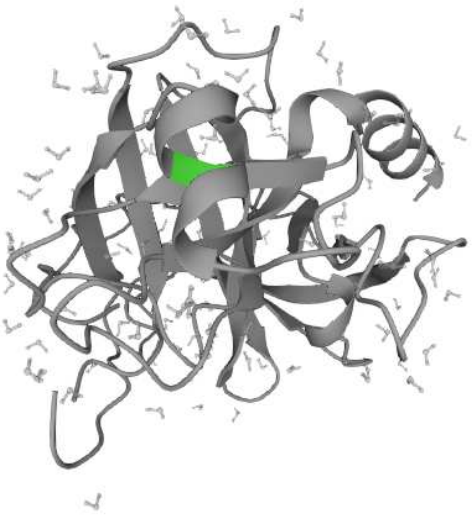


Figure 3: Porcine pancreas Trypsin enzyme 3D structure (P00761, Uniprot, 2023)

Within the scope of this project, trypsin allows the gluten to hydrolyze better in water. Since gluten is partially insoluble in water, for further processes amyloid fibrillation results better while gluten has smaller peptides. Trypsin is not the only enzyme that can be used for gluten hydrolysis since it has been investigated and mentioned in the study of Monge-Morera *et al*. from 2021(Monge-Morera *et al*., 2021). Other proteolytic enzymes such as chymotrypsin, thermolysin, papain, and proteinase K can be used as well but overall trypsin is proven to be the most effective.

1.4. Amyloid Fibrils

Amyloids or amyloid fibrils are highly specific aggregates of proteins that are usually characterized by a β-sheet secondary structure, also known as cross-β (Nguyen P.H. *et al*., 2021). They are known for having the ability to be stained by Congo Red and Thioflavin (Frenkel-Pinter, M. *et al*., 2020). They can be deposited by any source of protein however they are largely investigated in animal proteins since they are known to cause

various diseases in humans (Alberti, S., & Hyman, A. A., 2021). They were first identified by Rudolf Virchow since he thought they were deposits of starch, however, the availability of nitrogen in amyloids further allowed scientists to understand they are deposits on proteins (Oladoye, P. O., 2022). Today, it is known that all proteins can form amyloid structures under controlled conditions. However, plant-sourced amyloid fibrils such as gluten have not yet been studied deeply since animal-sourced amyloid fibrils are associated with several diseases in humans and are very important for medical science (Yiping *et al*., 2023). 37 different diseases are associated with amyloids in humans, such diseases include Alzheimer's disease, diabetes type 2, and spongiform encephalopathies (e.g., mad cow disease) (Alberti, S., & Hyman, A. A., 2021; Frenkel-Pinter, M. *et al*., 2020). Recently, amyloid fibrils have been deeply investigated for bionanotechnological applications, especially for forming nanotubular scaffolding since they exhibit strong structures and the ability to bind to metals (Buchanan, C. J., *et al*., 2022; Frenkel-Pinter, M. *et al*., 2020).

1.4.1. Structure of Amyloid Fibrils

Amyloid fibrils are insoluble and heterogeneous therefore the determination of their structure is difficult with common techniques. More complex and advanced techniques such as X-ray fibre diffraction, electron microscopy, and atomic force microscopy (Frenkel-Pinter, M. *et al*., 2020). Investigations that are performed with such techniques revealed that amyloid fibrils are long, straight, and unbranching, they are made out of individual subunits called “protofilaments” (Frenkel-Pinter, M. *et al*., 2020; Kent, S. A., *et al*., 2020). Later using cryo-electron microscopy it was discovered that each protofilament consists of a continuous β-sheet structure (Frenkel-Pinter, M. *et al*., 2020).

Figure 4: Structure of the Het-s prion amyloid from Podospora anserina
(Vlamakis *et al*., 2008)

Forming amyloid fibrils is a sensitive process however formed amyloid fibrils are incredibly stable and resistant to degradation. Amyloid fibrils are described to have a similar tensile strength to steel (Shimizu, *et al*., 2020). Therefore, this incredible strength makes them great building blocks for fabrication processes.

1.4.2 . Formation of Amyloid Fibrils

Amyloid fibrils are formed as a result of the polymerization of thousands of peptides or proteins forming a long fibre structure. Formation of amyloid fibrils involves a *lag* (*nucleation*), an *exponential* (*growth*), and a *plateau* (*saturation*) phase (Greenberg, S. M. *et al*., 2020; Almeida, Z. L., & Brito, R. M., 2020; Levin *et al*., 2020). Individual unfolded or partially unfolded polypeptide chains (monomers) transform into a nucleus (monomer or oligomer) via a thermodynamically unfavourable process that begins early in the *lag* phase in the simplest model of "nucleated polymerization" (Almeida, Z. L., & Brito, R. M., 2020). Further formed fibrils are grown in the exponential phase via the addition of more monomers to these formed nuclei. Another model called "nucleated conformational conversion" suggests that the often monomers form into highly disorganized oligomers that are distinct from the nuclei which then later reorganize in the nuclei and eventually form fibrils (Nguyen *et al*., 2021). More recently developed models showed that amyloid fibril formation also involves secondary events such as "fragmentation" where a fibril breaks into at least two or more smaller fibril fragments. Such secondary events increase the total

number of amyloid fibrils and put the overall mechanism into a positive feedback loop (Levin *et al*., 2020).

Recent advances and the formation of the master equation also showed that the *lag phase* does not directly correspond to the formation of nuclei, similarly, the *exponential phase* also does not only correspond to fibril elongation. Such primary events and secondary events, as described above, occur spontaneously and contribute to fibril formation (Korendovych, I. V., & DeGrado, W. F., 2020).

Since amyloid fibrils become a popular topic for research, the formation of amyloid fibrils has been investigated deeply both to understand the mechanism and to replicate it. The mechanism of amyloid fibril formation is influenced by changes such as pH, temperature, and ionic strength. Food protein-sourced amyloid fibrils are investigated deeply in the study of Cao and Mezzenga from 2019 (Cao *et al*., 2019).

1.4.2.1. Heating Method

In the same study of Cao and Mezzenga, various food proteins are investigated to unravel the mechanism of amyloid fibril formation as well as the effectiveness of the mechanism. The most common experimental technique to form amyloid fibrils is defined as the heating method. The method is defined as heating the target protein above its denaturation temperature below its isoelectric point with constant stirring. The conditions of this method such as pH, temperature, ionic strength, and protein concentration are investigated. The most favourable heating conditions for most food proteins are low pH and low salt concentration (Cao *et al*., 2019; Jiang *et al*., 2022). Furthermore, an increase in either of these disrupts amyloid fibril morphology or causes different protein aggregates to form (Cao *et al*., 2019).

1.4.2.1.1. pH

Even though different pH values are defined for different protein sources, lower pH values are defined to be the most favourable conditions for amyloid fibril formation. The pH contributes to the cleavage of peptide bonds and the inflexion of electrostatic interactions (Cao *et al*., 2019). It is mentioned in the same study that in highly acidic conditions such as pH 2, peptides that contribute to amyloid formation cleaved 100 times greater than any other

peptide bond. Such cleaves allow more fibrillization-prone regions to become available therefore contributing to both the speed and formation efficiency of amyloid fibrils (Cao *et al*., 2019). It is also revealed that pH value not only contributes to the amyloid fibril formation but also the final structure and morphology of the formed fibrils. Found in the study that heating at pH values ranging between 1.6 to 3 formed semiflexible and long fibrils while fibrils formed at pH values greater than 3.5 were more flexible but shorter (Cao *et al*., 2019).

1.4.2.1.2. Temperature

To form amyloid fibrils, first proteins need to be unfolded which involves cleavage of bonds such as van der Waals interactions, hydrogen bonds, and hydrophobic interactions. The breakage of bonds is influenced greatly by increasing temperatures especially when the temperature is above the denaturation point (Jiang *et al*., 2022). Even though temperature favours amyloid fibril formation and results in higher formation kinetics, the highest fibril yield is not correlated with increasing temperatures (Cao *et al*., 2019). Multiple studies show that regardless of the type of protein the highest fibrillation yield happened to be between 75-120°C which shows maximum ThT fluorescence intensity. It is also mentioned (and most probably experienced) in many studies that prolonged incubation times in high temperatures might disrupt amyloid fibril formation and cause the destruction of already-formed fibrils (Jiang *et al*., 2022; Loveday *et al*., 2017).

1.4.2.1.3. Protein Concentration

Protein concentration is another important factor for amyloid fibrillation which in this study, is taken as a constant and not evaluated further. In a study conducted by vandenAkker *et al.*, β-lactoglobulin is heated at 80°C and pH 2 for 16 hours with different protein concentrations of 3% and 7.5%. The incubation resulted in long semiflexible fibrils at a 3% protein concentration while short worm-like fibrils were formed at a higher protein concentration of 7.5% (Hosseinpour *et al*., 2020).

1.4.2.1.4. Physical Treatments

Especially when working with food proteins physical treatments are more desirable than chemical treatments because they do not include an introduction of chemicals to the overall structure. The most common physical treatments are stirring, sonication, and high pressure which are also commonly used in food processing (Cao *et al*., 2019).

It is reported in many studies that shear flow and stirring can cause protein unfolding and further facilitate amyloid fibril formation to an extent (Meng *et al*., 2022; Wei *et al*., 2017). Shorter and more rigid fibrils are reported to be found at higher shear rates, therefore it can be said that higher shear rates cause disruption of long semiflexible fibrils into shorter ones (Jansens *et al*., 2019).

The mechanism behind sonification forming amyloid fibrils is complex but mainly caused by liquid-gas interfaces, reactive oxygen species, high local temperatures, and shear stress (Pawlowski *et al*., 2020; Cao *et al*., 2019).

1.4.2.1.5. Chemical Treatments

Proteins in general are very sensitive to the changes in the environment around them which include physical (temperature, pH, etc.) and chemical changes such as enzymatic activity and the presence of chaotropic agents (Cao *et al*., 2019).

Enzymatic cleavage of proteins is used as a pre-treatment before incubation to make protein regions that are prone to fibrillization more available. Gao *et al*. used several proteases like trypsin, pepsin, and proteases M and A in their study to modify whey proteins before heat-triggered fibrillation (Gao *et al*., 2013; Meng *et al*., 2022). A similar technique is applied to wheat gluten in this study with trypsin.

The most chaotropic agent used for this application is ethanol which is a food-grade chaotropic agent. Ethanol can affect protein structure by lowering the dielectric constant of the solution therefore leading to the weakening of the hydrophobic interactions (Cao *et al*., 2019)

1.4.3. Thioflavin T

Thioflavin T is a dye that can bind to the amyloid fibrils (Alberti, S., & Hyman, A. A., 2021). When Thioflavin T (ThT) binds to amyloid fibrils, ThT fluorescence displays

dramatic shifts in the excitation wavelength (from 385 to 450 nm) and the emission wavelength (from 445 to 482 nm) (Alberti, S., & Hyman, A. A., 2021; Liu *et al*., 2020). ThT was first described as a fluorescent indicator for amyloid fibrils in 1959 and since then it has become the gold standard method for the identification of amyloid fibrils and is used worldwide (Cao *et al*., 2019). Before Thioflavin T became the gold standard, Congo red was used to identify amyloid fibrils. However, Congo Red requires the use of polarized light microscopy and the interpretation of the results is often difficult (Moloney *et al*., 2021). Moreover, staining with Congo red needed to be followed by washing therefore causing a lot of background staining. Research showed that, Thioflavin T staining results in large deposits in specifically amyloid regions resulting in much easily identifiable and reproducible results. Its high solubility in water and moderate affinity towards fibrils make ThT applicable to many experimental systems containing both synthetic and biological sources (Liu *et al*., 2020).

1.5. Food Packaging and Plastic Films

Packaging is crucial in food manufacturing in order to preserve the quality of food until it reaches the final consumer. Common packaging materials for foodstuffs are plastics, cartons, metal, and glass. The packaging can be formed into a box, jar, pouch, or bag depending on the type of food and the needs of the consumption of the food product. Even though the main purpose of packaging is to protect the overall quality of the food, packaging technologies must balance food protection with environmental consciousness, energy and material costs, waste management, and pollutant usage (Priyadarshi, R., & Rhim, J. W., 2020).

Nowadays food packaging consists of a mixture of many materials in order to exploit all the barrier properties of different materials to protect the food. Plastic is by far the most used food packaging material due to its high formability to various shapes and sizes and good barrier properties against liquids and gasses. According to US Packaging and Wrapping LCC, food-grade plastic wrap, also known as saran wrap or cling wrap, is commonly made out of Polyvinylidene Chloride (PVC) but in recent years alternatives to PVC have been developed (US Packaging and Wrapping LCC, 2023). The most popular alternative to PVC is Low-Density Polyethylene (LDPE) due to its environmental concerns with the chloride associated with PVC plastic wrap.

1.5.1. Packaging Waste

In the ever-growing and consuming world, as food consumption increases, the production of packaging waste also increases in concerning numbers. According to the data presented by the European Parliament, 53 million metric tons of plastic waste were generated in the European Union only, and around 40% of this plastic waste was sourced from packaging materials (Ian Tiseo, 2022). The situation is not different outside of the EU either, US Environmental Protection Agency (EPA) declared that approximately 31% of all municipal solid waste (all items commonly thrown away from homes) consists of food packaging. These numbers are not surprising as food is the only item that is consumed at least 3 times a day, every day by almost all individuals.

The increasing concern due to the environmental impacts of plastic usage has increased the global interest in the development of more sustainable plastic solutions. The first example of biodegradable plastics made from cellulose was presented in 1862 (Priyadarshi, R., & Rhim, J. W., 2020). Since then many other ingredients have been investigated as a solution for sustainable plastic alternatives. Such materials include starches, proteins, and extraskeletal structures such as chitin.

1.6. Polyvinyl Alcohol (PVA)

Polyvinyl Alcohol (PVA) is a hydrophilic synthetic polymer that is synthesized from vinyl acetate and vinyl alcohol. The formula of PVA is $[CH_2CH(OH)]_n$. It is a colourless (white) and odourless substance that is usually found as beads or as a clear solution in water (Polman *et al*., 2021). Functionalities of PVA include elasticity, film-forming ability, biocompatibility, and high-water retention capacity making the PVA a potential polymer to be used as an ingredient for applications for sustainable plastic solutions (Mehta *et al*., 2023).

1.7. Glycerol

Glycerol is a simple triol compound that is found as a liquid that is colourless and odourless. Since it is non-toxic, the compound has wide applications in the food industry and medicine (Périard *et al*., 2021). In the food industry, glycerol is used as a humectant, solvent, thickening agent, and sweetener that can help food to be preserved better. In the

medical industry, glycerol can be used in several treatments as it possesses mildly antimicrobial and antiviral properties (Gerardy *et al*., 2020).

1.8. Discussion of Previous Literature

1.8.1. Amyloid Fibril Formation from Various Protein Sources

Amyloid fibrils are special protein deposits with characteristic molecular structures. Such structure allows amyloid fibrils to have unique properties of strength and flexibility. Over the years animal-sourced amyloid fibrils were investigated widely due to their negative effects on human health, which today we know that they cause various diseases. However, their structural benefits on film-forming abilities have not been deeply investigated especially for applications of materials.

Amyloid fibrils can be deposited from all kinds of protein sources. Food sources proteins have become popular within food science research to improve the textural properties of food and recently on food packaging. The main reason for food-sourced proteins to be popular in amyloid fibril research is the decreased risks to human health and lowered toxicity. As mentioned in the study by Cao and Mezzenga in 2019, amyloid fibrils that are derived from food protein sources have the potential to become advanced materials in biomedicine, environmental science, nanotechnology, material science as well as in food science (Cao *et al*., 2019).

In the same study from 2019, many food-sourced proteins are investigated and the types of proteins that can yield amyloid fibrils are identified. The mentioned potential proteins are given in the table below.

Table 1: Source of food proteins and potential amyloid fibrils (Cao *et al*., 2019)

Source of Proteins			Types of Proteins
Animal		Milk	β-lactoglobulin, α-lactalbumin, α_{S2}-casein, β-casein, κ-casein
		Egg	Ovalbumin, lysozyme, ovotransferrin
		Blood	Bovine serum albumin, hemoglobin, ferritin
Plant	Cereals	Wheat	Glutelin, prolamin/gliadin
		Corn	α-zein
		Rice	Albumin, globulin
	Legumes, and Pulses	Soybeans	Soy protein isolates: 7S / β-conglycinin, 11S / glycinin
		Peas	Pea protein isolates
		Kidney beans	7S / vicilin / phaseolin
	Oil seeds	Rapeseed, peanut, sunflower, hempseed, cottonseed	7S / congossypin

Furthermore, there have been many studies that investigated the given proteins and found the most favourable conditions for amyloid fibrillation. Even though food-grade protein fibrillation has been investigated deeply, the ability of these fibrils to form functional materials is still a novel technique that requires further research. The most favourable experimental conditions to form amyloid fibrils from given proteins are shared below in the table.

Table 2: Potential food-sourced amyloid fibrils fibrillization conditions (Cao *et al*., 2019)

Protein	Fibrillization conditions	References
β-lactoglobulin (Whey)	75~120 °C, pH 2~3.5, low salt content	(Aymard *et al.*, 1999),(Sagis *et al.*, 2004), (Lasse M. *et al.*, 2016)
α-lactalbumin (Whey)	37~55 °C, pH 2, 50 mM NaCl; 37~55 °C, pH 2~4.5, 100mM NaCl, Ca2+-depleted α-lactalbumin	(Goers J. *et al.*, 2002),(Wang S.S.S. *et al*. 2011),(Yang Jr.F. *et al.,* 2006)
κ-casein (Whey)	37 °C, pH 7 physiological conditions 20 mM DTT, 37 °C, pH 7 reducing disulfide bonds 90 °C, pH 2	(Pan, K., & Zhong, Q., 2015),(Chou W.L. *et al.*, 2014),(Koudelka T. *et al.,* 2012),(Farrell H.M. *et al*., 2003)
Ovalbumin (Egg)	80~90 °C, pH 2, 0~35 mM NaCl 70~90 °C, pH 7.5~8, 0~200 mM NaCl	(Veerman *et al.*, 2003),(Sagis *et al.*, 2004)
Wheat Gluten	Step 1: hydrolyzed by trypsin; Step 2: 37 °C, pH 8	(Ridgley, D. M., & Barone, J. R., 2013),(Ridgley D.M. *et al.*, 2012)
Wheat Gliadin	Step 1: hydrolyzed by trypsin; Step 2: 37 °C, pH 8,	(Ridgley, D. M., & Barone, J. R., 2013),(Ridgley D.M. *et al*., 2012)
Soy Protein Isolate	85 °C, pH 2, shear flow; 80 °C, pH 2 Step 1: hydrolyzed by papain; Step 2: 90 °C, pH 2	(Akkermans *et al*., 2007),(Lasse M. et al., 2016),
Kidney bean Vicilin	85 °C, pH 2, 20 mM NaCl; 80 °C, pH 1.6	(Ridgley, D. M., & Barone, J. R., 2013),(Tang C.H. *et al.*, 2010)
Cotton seed Congossypin	90 °C, pH 2	(Zhou J. Z., 2009)

Optimum experimental conditions for amyloid fibril formation differ depending on the protein type and unfortunately, such outcomes are still so far from such techniques to apply to the industry. Therefore, more research is needed to discover the benefits of amyloid fibrils both in food science and in further advanced material applications.

The most common food-sourced proteins have been whey proteins and egg albumin for amyloid fibril research however recently plant-based food source proteins such as gluten and pea protein became very popular. In the studies of Sagis *et al*. and Goers *et al*., whey proteins β-lactoglobulin and α-lactalbumin were investigated for their amyloid

fibril-forming abilities. The confirmation of amyloid fibrils was done via ThT fluorescence while further analysis such as length distributions and morphology were determined via more advanced techniques such as TEM and CD Spectra (Sagis *et al*., 2004; Goers J. *et al*., 2002). Similarly in the studies of Ridgley *et al.*, wheat gluten is investigated for its amyloid fibril forming abilities and similar techniques are used such as AFM and SEM to visualize the fibrils and determine the morphology (Ridgley, D. M., & Barone, J. R., 2013; Ridgley D.M. *et al*., 2012). Such techniques are commonly used in amyloid fibril research and followed in this study.

1.8.2. Formation of Amyloid Fibril-based Films

The formation of amyloid fibril-based films is a considerably new technique and not many scientific articles are available on the topic. Especially considering that plant protein-based amyloid fibril research has only been around for the past few decades, film-forming properties of plant-sourced amyloid fibrils have not been widely investigated. However other sources of protein deposits have been investigated for material-forming abilities.

The process of amyloid fibrillation for advanced material forming allows for eliminating the negative traits of the raw material that was previously used in the material making. Gluten as an example, when processed in alkaline conditions results in a yellow colour and unpleasant smell therefore undesirable as a food packaging material due to its unattractive appearance. Whereas films obtained in acidic conditions contain large protein particles, which strictly limits its application as a high-performance polymer for food packaging (Bastioli, 2020). It is mentioned in the study of Tanada-Palmu and Grosso from 2005 that strawberries that are covered with a biolayer edible packaging made from gluten are not found attractive by consumers due to their waxy, dull, and unnatural appearance. It was noted in the same study that the unnatural colour and appearance of the gluten film made it harder for consumers to see the real colour of the strawberries (Tanada-Palmu, P. S., and Grosso C. R., 2005). There are other ways to improve the functionality of such food-grade protein-based packaging films however biofabrication both eliminates the extensive reagent usage and also improves energy consumption. Therefore forming amyloid fibrils from food-grade proteins has become popular for food packaging research.

The most common food-grade proteins for amyloid fibril-based food packaging research recently are gluten, egg albumin, whey protein, and other seed proteins. In the study of Bagnani *et al*. from 2023, rapeseed, a common oil seed from the family Brassicaceae, has been investigated for its abilities to form functional amyloid fibrils and production of bioplastics (Bagnani M. *et al*., 2023; Georgiev R. *et al*., 2022). In this study amyloid fibrillation from rapeseed proteins was sustained via incubation at low pH (pH 2) and 95°C for 24 hours. Further AFM results confirmed the formation of amyloid fibrils and that the formation of amyloid fibrils was affected by different treatments such as protein extraction in high salt concentration and hexane depletion of rapeseed cake (Bagnani M. *et al*., 2023).

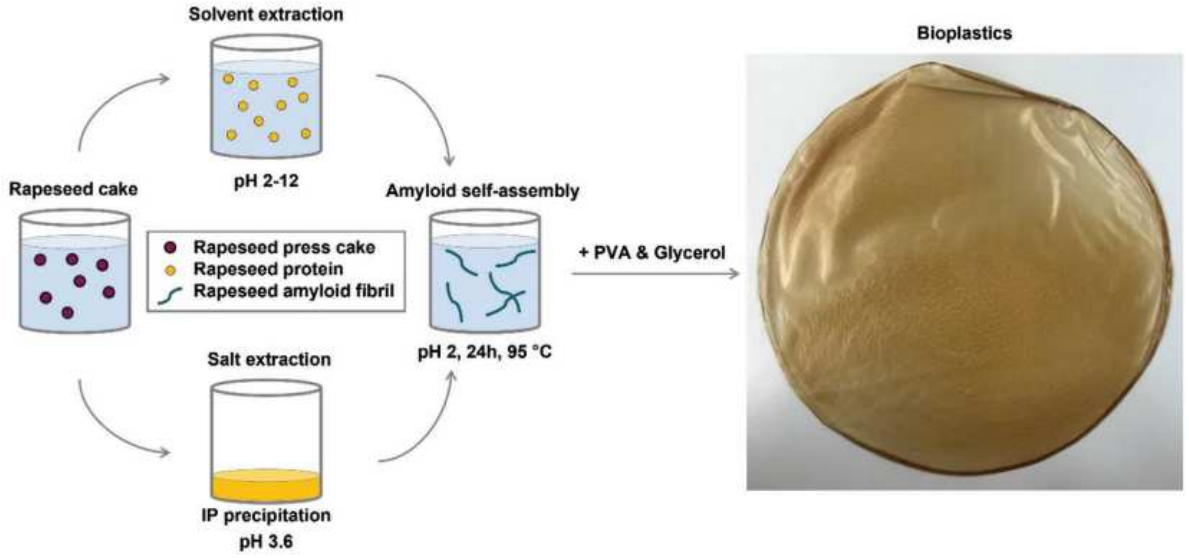


Figure 5: Formation of bioplastics from rapeseed cake protein (Bagnani M. *et al*., 2023)

For the formation of bioplastic films, amyloid fibrils were blended with Polyvinyl alcohol (PVA) and glycerol with a ratio of 55% rapeseed extracts, 41.5% PVA, and 3.5% glycerol. The final bioplastic film was characterized by decreased water absorption rate, higher hydrophobicity, and elongation at break >600% which is more than double compared to the films obtained from native monomers (Bagnani M. *et al*., 2023).

In another study by Li T. *et al.* from 2023, 11 different legumes were investigated for their abilities to form amyloid fibrils and formation of amyloid fibril-based materials, these legumes include kidney bean, black bean, cowpea, mung bean, chickpea, lentil, and pumpkin seed which showed excellent fibril forming abilities (Li T. *et al*., 2023). Amyloid fibrillation was sustained by incubating the protein isolates at pH 2 and 90°C with constant

stirring. Confirmation and characterization of amyloid fibrils were done with Thioflavin T fluorescence assay (ThT) and CD Spectra and the morphological properties of fibrils were identified with Atomic Force Microscope (AFM) and Transmission Electron Microscope (TEM) (Li T. *et al*., 2023). Further, the formed amyloid fibrils were used to form functional biodegradable materials such as hydrogel fabrication, bioplastic film production, and membranes for water purification. To form bioplastic films PVA and glycerol are used as a common procedure and the ratio of formulation between fibrils, PVA, and glycerol is set to be 3:4:4, as followed in this study (Li T. *et al*., 2023).

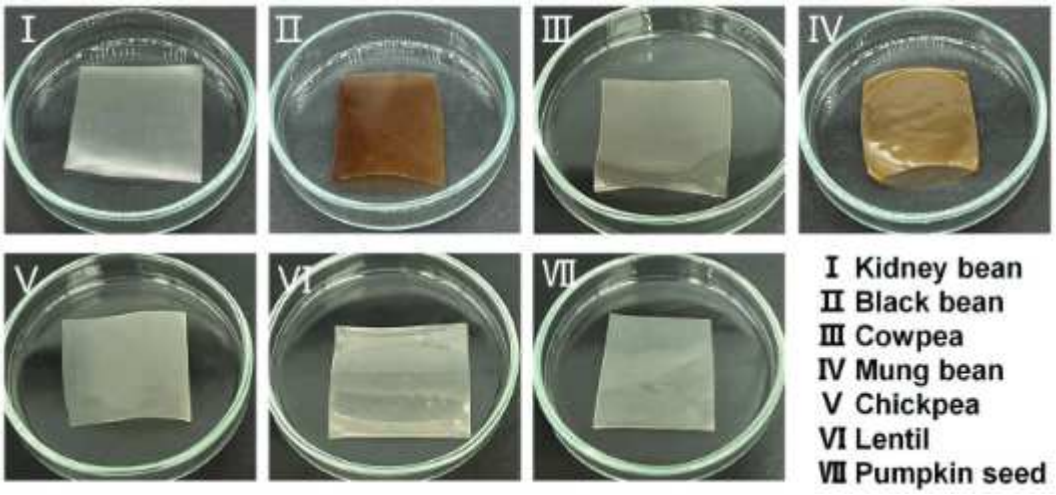


Figure 6: Formed bio-packaging films from various types of legumes and seeds (Li T. *et al*., 2023)

In the study by Paydayesh M. *et al*., from 2021, whey protein was investigated for its ability to form amyloid fibrils and applications of functional materials (Peydayesh, M. *et al*., 2021). For the fibrillization process whey protein isolate was mixed with PVA and cellulose, and the mixture was incubated at 90°C and pH 2 for 5 hours. The microstructure and the morphology of the formed amyloid fibrils were determined using atomic force microscopy (AFM). The solvent of the mixture was evaporated and the remaining mixture was cast into a mould and let to be dried fully (Peydayesh, M. *et al*., 2021). The procedure can be seen in the figure below.

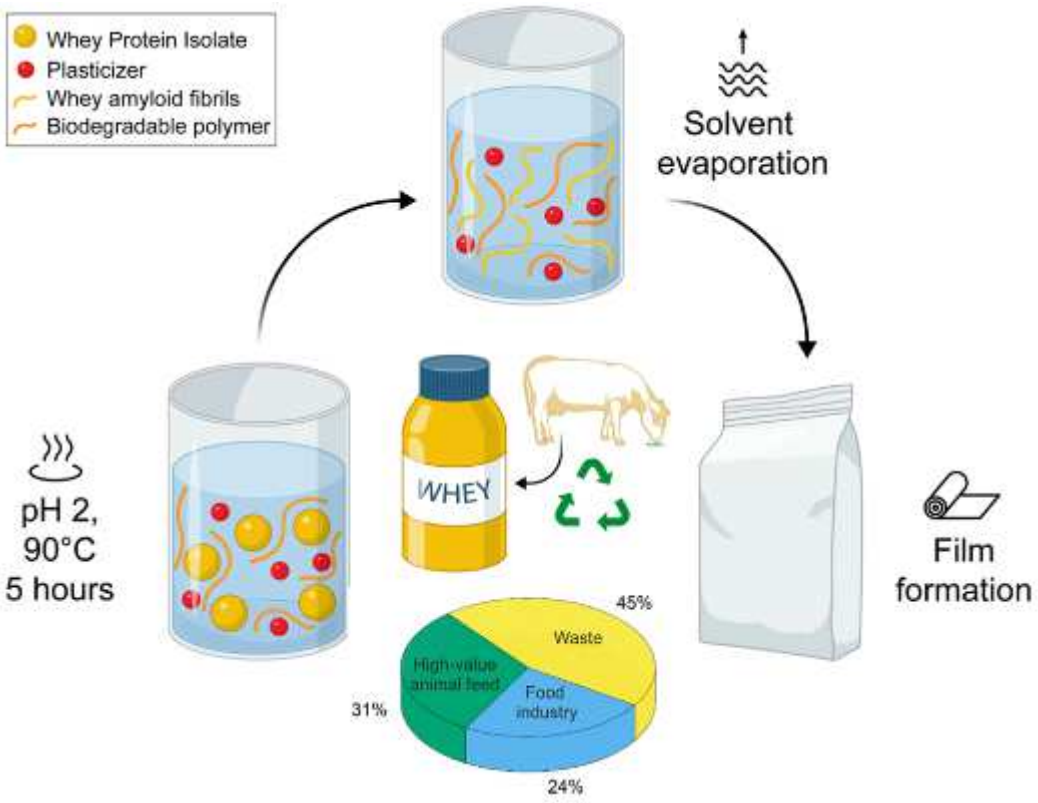


Figure 7: Procedure of forming films from whey protein (Peydayesh, M. *et al*., 2021)

The same study also evaluated the formed films for hydrophobicity, water stability, water vapour permeability, mechanical properties such as maximum stress at break, ultimate elongation, and toughness as well as optical properties such as transparency (Peydayesh, M. *et al*., 2021).

2. Aim of the Thesis

In the past few decades, in the light of rapidly developing technological advancements, modern manufacturing, and other related industrial activities have gained momentum, and due to the unpreventable population growth, world consumption is more than ever before. Despite all the benefits of modern technology, the unconscious use of fossil fuels has led to a decrease in oil reserves and irreversible damage to the world's ecosystems. The increasing observable effects of global warming and increasing environmental pollution have pushed industries to change their production methods under the principles of sustainability. In this direction, the use of environmentally friendly and recyclable techniques and materials has become increasingly widespread.

This thesis project aims to prepare food packaging using wheat gluten amyloid fibril-based films. The thesis topic is divided into two main stages. The first stage includes the evaluation of wheat gluten protein to form amyloid fibrils under specific incubation conditions. The evaluated incubation conditions include the change in temperature, pH, and overall incubation time. The ability of proteins to form amyloid fibrils has been investigated widely in the literature however the investigation specifically on the plant proteins, especially wheat gluten is still very limited. First, the wheat gluten is hydrolyzed to its monomers using the trypsin enzyme. The effect of the hydrolyzation degree is observed via changing the enzyme content. Moreover, the ability of gluten to form amyloid fibrils is investigated. This natural phenomenon is observed under changing conditions of temperature, pH, and incubation time, and the results of the fibrillation degree are measured via ThT fluorescence.

In the second part of the thesis, the ability of the formed amyloid fibrils to form film structures is evaluated. PVA and glycerol are used as plasticizers while forming films. The proper formulation ratio between the ingredients of the mixture is evaluated as well as the proper drying time. For the evaluation and characterization of the films several measurements are performed such as thickness, mechanical properties (tensile strength and elongation), optical properties (transparency, and gloss), and static water contact angle analysis.

In conclusion, with this project and the thesis we aimed to investigate the possibility of using wheat gluten as an alternative, natural, and sustainable source to form food packaging films.

3. Materials and Methods

3.1. Utilization of Wheat Gluten Fibrillation

To achieve amyloid fibril fibrillation, the wheat gluten protein is first hydrolyzed to its monomers. After hydrolyzation to achieve the continuity of the further processes, hydrolyzed gluten samples are freeze-dried. Freeze drying is carried out in a desiccator -55°C (the drying process took about 4-6 days at depending on the sample size). Then the hydrolyzed protein samples are suspended in water and incubated to achieve protein fibrillation. The optimization of the fibrillation process is accomplished by changing the incubation temperature and the initial pH of the sample. For further analyses, the enzyme ratio in the hydrolyzation process is changed and its effects on protein fibrillation are evaluated.

3.1.1. Hydrolyzation of Wheat Gluten Protein

Wheat gluten is obtained from Tereos Starch and Sweeteners Company in Aalast, Belgium NV. 2wt% WG protein solution is prepared using purified water (2 g WG Protein/100 mL water). Prepared WG Protein solution is hydrolyzed using the trypsin enzyme with a ratio of 1:22.5 and 1:45. Trypsin is obtained from Sigma derived from porcine pancreas (T0303).

Figure 8: The experimental setup for hydrolyzation of wheat gluten

The entire hydrolyzation process was run in a 40°C water bath with constant stirring. Stirring is achieved using magnetic stirrers and the stirring speed was 300 rpm. 50 mL of the solvent (water) is separated to dissolve trypsin. Wheat gluten is added to the remaining water gradually to prevent forming clots. The pH of the solution is around pH 5, and, to ensure trypsin works properly, is set to be pH 8 before adding the enzyme and during the whole procedure. The process is finished when the entire amount of 0.05M NaOH solution is added (83 mL). To deactivate the enzyme and stop the reaction the pH is decreased reaching pH 7. To observe the effects of the pH over fibrillation the final pH of the hydrolyzed wheat gluten solution is set to pH 7. The same procedure is followed for both samples containing respectfully 1:45 and 1:22.5 enzyme ratios.

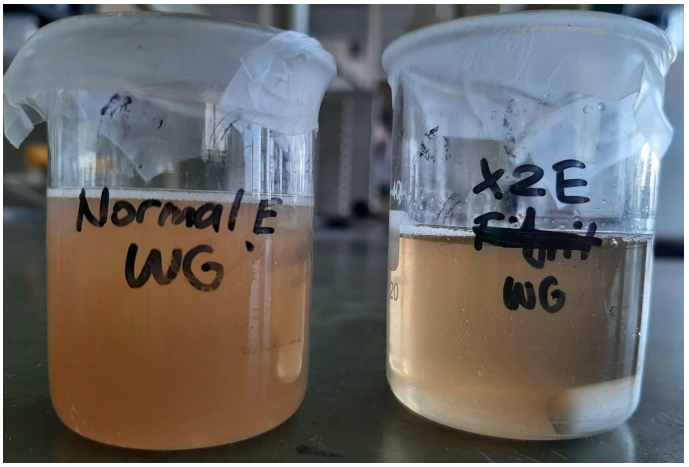


Figure 9: The solutions obtained from wheat gluten that were hydrolyzed with 1:22.5 and 1:45 enzyme ratios respectively

After the hydrolyzation has been finalized the solution is centrifuged at 18600g for 15 mins and the sediment is removed. The liquid after the centrifuge is collected and freeze-dried to collect hydrolyzed WG Protein monomers.

3.1.2. Incubation, Forming Amyloid Fibrils

Freeze-dried WG protein samples are re-sustained in purified water with a concentration of 2% w/w (400 mg sample/20 mL). Two sets of samples are prepared, one pH 7 and one pH 2. The pH is adjusted using 1M HCl solution.

Figure 10: Wheat gluten samples at pH 2 and pH 7

For the incubation of the samples, an oil bath is prepared, making sure the sample is not touching the bottom of the oil bath and is stirred continuously using a magnetic stirrer set to 300 rpm. The first incubation is performed following the literature written by Monge-Morera from 2021 at 85°C for 40 hours (Monge-Morera *et al*., 2021). To decrease the length of the incubation, samples at pH 2, and 95°C are evaluated since available literature on amyloid fibril formation has suggested that lower pH values promote amyloid fibril formation. Samples are taken at the given hours at respectfully, 1h, 2h, 3h, 4h, 5h, 6h, 7h, 8h, 9h, 10h, 15h 20h, 25h, 30h. Then initial 40-hour run is preserved and the change in pH is evaluated with several runs with both pH values. Finally, to observe the change in ThT fluorescence, samples are analyzed every hour for 40 hours at 95°C and pH 7, specifically using a 1:22.5 enzyme ratio. Incubation is finalized after the desired time of 40 hours is reached and the samples are put into a cold water bath to stop the heating immediately. After the samples are cooled down to room temperature further analyses are performed to measure the amyloid fibril formation.

Figure 11: Experimental setup for the incubation of the wheat gluten protein samples

3.1.2.1. Thioflavin T (ThT) Assay

After the incubation period, the formation of the fibrils is checked using ThT. 2 mM ThT solution is prepared using purified water. 300 µL of samples are collected from each sample diluted to 1:20 (to 6 mL). 2 mM ThT solution is added in the ratio of 1:100 (0.06 mL). The fibril formation is measured using ThT fluorescence with fluorimetry. The excitation and the emission wavelength are set to be 440 nm and 480 nm respectively. After 4 and 6 days the samples are measured again with ThT fluorescence to make sure that the amyloid fibrils are stable.

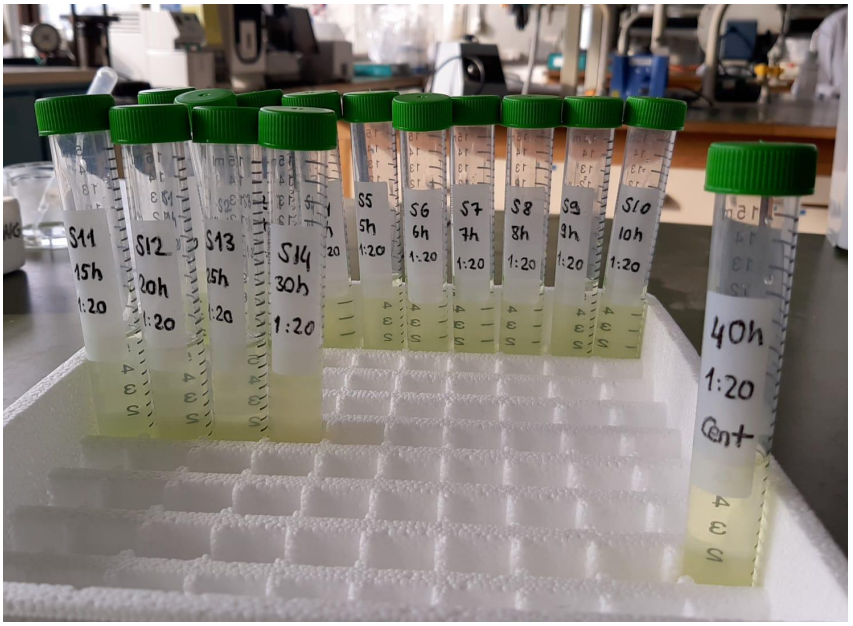


Figure 12: Prepared ThT fluorescence assay samples

3.2. Utilization of Amyloid Fibril-based Packaging Films

The 2% w/w gluten protein samples are set to 2% w/w. The samples are incubated under 95°C, pH 7 for 40 hours with constant stirring under 300 rpm as explained in the procedure above. After the incubation ThT measurements are taken to ensure the formation of amyloid fibrils and mixed with PVA and glycerol.

PVA and glycerol are used for the formation of packaging films. PVA is obtained from Sigma-Aldrich (St. Louis, Missouri, USA) and glycerol is obtained from thermo-scientific (Waltham, Massachusetts, USA). 10wt% PVA solution is prepared using pure water and PVA. To allow PVA to completely dissolve before use the solution is heated to 90°C with constant stirring until the entire amount of PVA is dissolved. Then the solution is cooled before adding the fibril solution. Lastly, glycerol is added directly on top, and the mixture is stirred until homogeneity is reached. Then 10 mL of this mix is poured into plastic cups in a thin layer. The formed films are left to be dried under the hood and regularly checked every 24 hours up to 72 hours.

The formulations for each sample and the drying times can be observed in Table 3, reported below. The formulation of Sample 2 which is written in red, is taken from the literature by Li *et. al.*, from 2023 “Plant Protein Amyloid Fibrils for Multifunctional Sustainable Materials” (Li T. *et al*., 2023).

Table 3: The formulations of developed gluten amyloid fibril-based films and drying times

Sample number	Enzyme ratio used in WG hydrolyzation	Set amyloid fibril concentration	Glycerol/Fibril	The ratio on wet basis	Drying time
1	1:45	5%	1.2	3.12% Fibrils 3.75% PVA 3.75% Glycerol	-
2	1:45	2%	0.75	1.7% Fibrils 1.3% PVA 1.3% Glycerol	48
	1:22.5	2%	0.75		
3	1:45	2%	7.5	0.8% Fibrils 6% PVA 6% Glycerol	48
	1:22.5	2%	7.5		
4	1:45	2%	4.5	1.05% Fibrils 4.73% PVA 4.73% Glycerol	48
	1:22.5	2%	4.5		
5	1:22.5	2%	2.5	1.3% Fibrils 3.3% PVA 3.3% Glycerol	48
6	1:22.5	2%	1.5	1.66% Fibrils 1.66% PVA 2.5% Glycerol	72
7	1:22.5	2%	0.5	1.66% Fibrils 1.66% PVA 0.8% Glycerol	48
8	1:22.5	2%	1.5	1.53% fibrils 2.3% PVA 2.3% Glycerol	72
9	1:22.5	2%	0.5	1.81% fibrils 0.9% PVA 0.9% Glycerol	48

According to the observed results of these formulations, Sample 3 (7.5 ratio) with the enzyme ratio 1:22.5 has been selected to be the most successful one therefore more trials for film utilization have been performed based on the formulation of Sample 3.

For the second trial of the film utilization, the same formulations from Sample 2 (replica from the literature), Sample 3, Sample 4, and Sample 9 are replicated because, in the first trial, the samples became unusable before adequate characterization can be done. Moreover, new formulations are formulated by keeping the PVA and Glycerol amounts the same and only changing the concentration of the fibril solution, ranging from 0%

concentration to up to 4% of fibril concentration, (therefore changing the total amount of fibrils that is added in the formulation).

The replicated samples are prepared in the same way that was explained before (95°C incubation for 40 hours in pH 7 with a set fibril concentration of 2%), meanwhile, the new formulations' incubation was carried out under the conditions of 95°C, pH 7 for 10 hours with constant stirring under 300 rpm. After the incubation the solution is cooled and ThT measurements are taken to ensure the formation of amyloid fibrils. In this batch, the drying process was aimed to be monitored. To monitor the drying process each plate is weighed individually and kept in a desiccator under 25°C (in an incubator). The process is followed for 48 hours to create a drying curve. For drying first saturated NaCl solution is prepared to mimic 75% humidity in the desiccator. After 15 hours of drying, it was observed that the films had started gaining weight rather than losing. It is assumed that the RH inside the desicator has reached an equilibrium therefore the drying was interrupted. The NaCl solution was changed with silica beads to overcome this struggle and the drying continued. However, the desired drying has never been achieved therefore the entire batch is eliminated.

The same batch was reproduced again using the same formulations. This time to eliminate the mold that can grow in the samples, 0.02% NaN_3 (0.02% of the end solution) is added for each sample during the sample preparation. The total amounts for each formulation are doubled and half of the number of plates are dried under the hood with no controlled atmosphere and temperature while the other half is again dried in the desiccator with silica beads in the 25°C incubator to control the atmosphere. For both samples, they are weighted for 48 hours to create a drying curve.

According to this system, the new formulations can be found in Table 4 below.

Table 4: The formulations of newly developed gluten amyloid fibril-based films

Sample number	Enzyme ratio used in WG hydrolyzation	Set amyloid fibril concentration	Glycerol/Fibril	The ratio on the wet basis	The ratio on the dry basis
2	1:22.5	2%	0.75	1.7% Fibrils 1.3% PVA 1.3% Glycerol	40% Fibrils 30% PVA 30% Glycerol
3	1:22.5	2%	7.5	0.8% Fibrils 6% PVA 6% Glycerol	6.25% Fibrils 46.87% PVA 46.87% Glycerol
4	1:22.5	2%	4.5	1.05% Fibrils 4.73% PVA 4.73% Glycerol	10% Fibrils 45% PVA 45% Glycerol
9	1:22.5	2%	0.5	1.81% fibrils 0.9% PVA 0.9% Glycerol	50% fibrils 25% PVA 25% Glycerol
1	-	0%	-	50% PVA 50% Glycerol	-
2	1:22.5	1%	15	0.4% fibrils 6% PVA 6% Glycerol	3.22% fibrils 48.38% PVA 48.38% Glycerol
3	1:22.5	2%	7.5	0.8% Fibrils 6% PVA 6% Glycerol	6.25% Fibrils 46.87% PVA 46.87% Glycerol
4	1:22.5	3%	5	1.2% Fibrils 6% PVA 6% Glycerol	9.37% Fibrils 45% PVA 45% Glycerol
5	1:22.5	4%	3.75	1.6% Fibrils 6% PVA 6% Glycerol	11.76% Fibrils 44.11% PVA 44.11% Glycerol

3.2.1. Characterization of Amyloid Fibril-based Packaging Films

3.2.1.1. Mechanical Properties - Tensile Strength (Puncture Test)

The puncture test is performed with Stable Micro Systems, TA.XT.Plus Texture analyzer. Using the probe SMS P/0.5S with a ball tip. The pre-test speed is set to 0.5mm/sec, the test speed is set to 0.1mm/sec, and the total distance that the probe will go is set to 15mm to make sure that the probe will be able to puncture the film. Trigger force was set to 0.1g, which is when the machine realized the obstacle surface and started to obtain data.

Figure 13: SMS Tensile Strength (Puncture test) Machinery and the platform that is designed to hold the films

To make the films fit into the texture analyzer sample platform, a custom-made platform is designed. The platform consists of 2 thick plastic plates that the film is being squished in between in order to stabilize the film. The 2 plates are secured with 4 bolts from 4 sides of the plates. The platform has a hole in the middle slightly bigger than the ball tip of the probe so that the probe can puncture through the film. The films are centred on the platform and the most suitable place for the puncture to occur is selected (not having any prior damage). Fortunately, during the experiment, it was observed that the films broke from where the ball tip pushing instead of the sides of the hole which provided us with better results that correlated with the force applied to the films.

As the probe's tip gets lowered onto the films, the machine starts to generate data as the tip touches the film and at least 0.1g of force is applied. Later the machine puts more force into the push until the film breaks. The generated data can be observed as a graph that gives a peak where the film broke. From the graph, the total distance that the probe moved until the breaking point can also be observed which corresponds to the elasticity of the films.

Figure 14: Probe pushing through the film

3.2.1.2. Thickness

The thickness of the films is measured using a micrometre with the ability to measure 0-25mm in total. The micrometre has a sensitivity of 0.001 scale. Since some of the samples could not be removed from the petri dish after drying, the measurement was performed while the samples were still in the petri dishes. First, the thickness of the petri dish was measured and that value was subtracted from the total value. The total dry content of the films is correlated with the thickness of the films after drying.

Figure 15: Measuring the thickness of the films with a micrometre

3.2.1.3. Gloss Measurements

Gloss measurements are performed with a BYK Single angle gloss meter 75°, and reported in gloss units. Before starting the measurement, the calibration is performed by measuring the gloss of a black marble surface which is provided as a setup of the machinery. The machine works by shooting a light beam at a 75° angle and measuring the light that was reflected from the surface again at a 75° angle. If the surface is not uniform the machine cannot measure any scattering reflecting light from the surface.

Measuring with the gloss of the films was not a usual measurement procedure that is often performed with this machinery due to the films being transparent. The films being transparent, it is estimated that some amount of light is going through the films and therefore cannot be measured. Moreover, the petri dish that the films are moulded in is extremely reflective therefore it was estimated that the petri dish would heavily affect the measurements. The films are first removed from the petri dishes. Since not all the films were able to be removed from the petri dishes, not all the different formulations of the films were able to be subjected to gloss measurement. To standardize the measurements, all films are put on a sheet of paper, first, the glossiness of the paper is measured and that is considered as a blank. Then the glossiness of each film is measured and the gloss units are subtracted. Therefore, it is considered that the amount of light that only reflects from the surface of the

films is measured rather than the light beams that go through the film and reflect from the surface of the paper.

Since the surface of the films is not uniform, the results are affected by the different variations of texture and thickness of the films. Moreover, since the drying process was performed in the hood which films' surfaces were subjected to open air circulation it is estimated that some amount of physical contamination was possible that also affected the results of the gloss measurement.

3.2.1.4. Static Water Contact Angle Analyses (Hydrophobicity)

Static water contact angle analysis is performed to measure the hydrophobicity of the films. The principle suggests that when a water droplet is put on the surface of the film, the angle between the surface of the film and the surface of the droplet changes as time passes. The initial angle and the change of the angle over time give information about how hydrophobic a surface is.

The droplet is measured with a microsyringe and dropped on the film surface. In total 8 µl of water is measured for each droplet. When a droplet was dropped on the surface of the films it was observed that the droplet was not able to hold a proper shape. The droplet has flattened out to a degree that it was not possible to be observed on the camera. Additionally, using oil was proposed instead of water. In theory, since water and oil show exactly opposite hydrophobicity characteristics, it was assumed that oil would behave opposite to water on the films. Therefore, the measurement can be correlated as the more hydrophobic the oil would act, the more hydrophilic the water is expected to be. However, it was further explained by Prof Paul van Der Meeren, the promoter of the project and the head of the research group PainT, that the wettability is mainly governed by the surface tension of the liquid: liquids of high surface tension (especially mercury, but also water) do not spread on hydrophobic surfaces. On the other hand, liquids of low surface tension (i.e. apolar liquids, such as oil) spread on all surfaces (both hydrophobic and hydrophilic). Hence, if water spreads on a surface, oil will surely do. Therefore, no contact angle measurements could be performed.

4. Results and Discussion

4.1. Analysis of Fibril Formation

After the incubation period, the samples were collected as explained in the Materials and Methods section. Furthermore, additional samples were collected at the given times respectively 1h, 2h, 3h, 4h, 5h, 6h, 7h, 8h, 9h, 10h, 15h, 20h, 25h, 30h, 35h, and 40h. Those samples were sent to ETH Zurich for further analysis.

4.1.1. ThT Measurements

ThT measurements were carried out with an excitation wavelength of 440 nm and an emission wavelength of 480 nm. The results of each measurement can be seen in Table 5. Additionally, the graphs below demonstrate the relationship between the change in the enzyme ratio and pH on the ThT measurements.

Table 5: ThT results of the incubation samples

WG Powder Sample	Sample No	Incubation Temperature	pH	Incubation Time	ThT Fluorescence results
Freeze-dried product from Sample1 Protein hydrolysis Tyripsin enzyme ratio 1:45	1	85	7	40	3.902
	4	95	7	40	36.656
	5	95	2	40	11.82
	6	95	7	40	82.4
	7	95	2	40	7.392
	8	95	7	40	20.47
	11	95	7	40	54.759
	12	95	7	40	21.373
	14	95	7	40	29.576
Freeze-dried product from Sample2 Protein hydrolysis Tyripsin enzyme ratio 1:22.5	10	95	7	40	53.185
	13	95	7	40	47.705
	15	95	7	40	36.283
	16	95	2	40	15.155
	18	95	7	40	44.39

Table 6 demonstrates the average values of ThT results with two different given enzyme ratios and the standard deviation of the ThT measurements.

Table 6: ThT Results with standard deviation with determined experimental conditions (95°C, pH 7, 40 hours)

WG Powder Sample	Incubation Temperature	pH	Incubation Time	Average ThT Fluorescence result	Standard deviation
Freeze-dried product from Sample1 Protein hydrolysis Tyripsin enzyme ratio 1:45	95	7	40	40.872	21.82
Freeze-dried product from Sample2 Protein hydrolysis Tyripsin enzyme ratio 1:22.5	95	7	40	45.39	6.12

It can be observed from the results given in Table 6 that the ThT results are not very stable. This instability can be explained by several errors caused by the experiment setup. First, there was some loss in volume that was caused by the improper selection of incubation bottles. This volume loss caused the overall concentration of the amyloid fibril solution to increase and affect the mixing ratios of ThT. Later this volume loss was eliminated by changing the bottles for incubation followed by film formation.

Another error that should be mentioned can be due to differences in heating units. For some of the incubations, more than one heating unit is used for the oil bath. Even though the thermometers are used to ensure the temperature is stable throughout the whole process, it can still be possible that there are calibration errors between different thermometers.

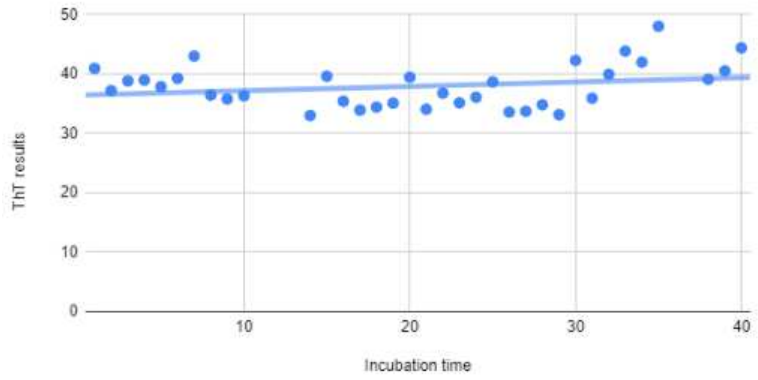


Figure 16: Change of ThT values with the increasing incubation time on 95°C, pH 7, 1:22.5 enzyme ratio sample

Figure 16 represents the ThT values that are taken from incubation at 95°C with pH 7 using the hydrolysed WG sample with a 1:22.5 enzyme ratio. The samples for the ThT measurements are taken every hour. Additionally, at the given hours 1h, 2h, 3h, 4h, 5h, 6h, 7h, 8h, 9h, 10h, 15h, 20h, 25h, 30h, 35h and 40h, 5ml samples are taken and sent to ETH Zurich for further analysis. It can be observed that there is a trend of increasing the ThT values by the length of the incubation. However other studies showed a much apparent increase in ThT values which was also the case for the incubation performed at 95°C for 30 hours at pH 2 with a 1:45 enzyme ratio. Accordingly, the results obtained in the same conditions reported in Figure 17 showed increasing ThT values.

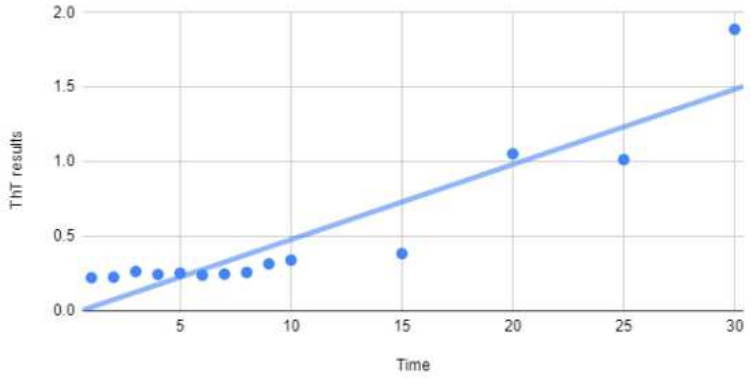


Figure 17: Change of ThT values with the increasing incubation time at 95°C and pH 2 with a 1:45 enzyme ratio sample

The difference between the two graphs can be explained by the differences in pH. Even though it is further explained that pH 2 overall resulted in much lower ThT intensities for gluten, it might still result in a more even distribution in the increase of ThT. Another important point to mention can be that prolonged incubation time (such as 40 hours) might cause the disruption of already formed amyloid fibrils which can explain the stabilization and even the decrease in ThT intensity in Figure 16. Such an effect of prolonged incubation is mentioned in multiple studies (Cao *et al.,* 2019: Jiang *et al.,*2022; Loveday *et al.,*2017).

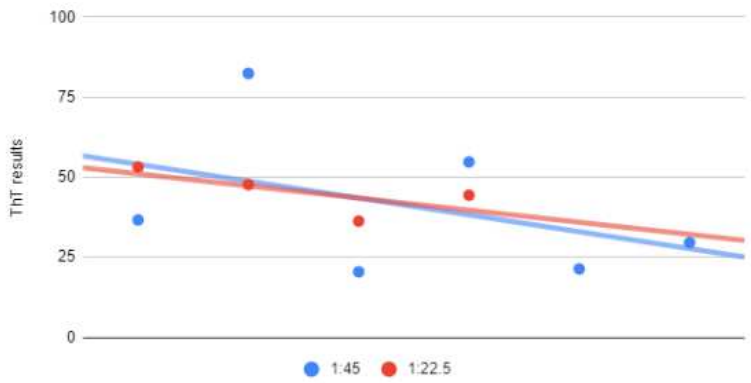


Figure 18: Change of ThT values with different enzyme ratios used in hydrolyzation of gluten

Figure 18 represents the change in ThT value when different enzyme ratios are used for the hydrolyzation of wheat gluten. In these incubations, temperature, pH, and the total incubation time were set to be the same (95°C, pH7, 40 hours), only the enzyme ratio was changed between 1:22.5 and 1:44.5. It can be seen that the general trend in ThT observations did not yield a significant difference between the samples with different enzyme ratios. Therefore, we can conclude that the amount of enzyme used for wheat gluten hydrolyzation is not adequate for amyloid fibril formation since it does not affect the hydrolyzation degree.

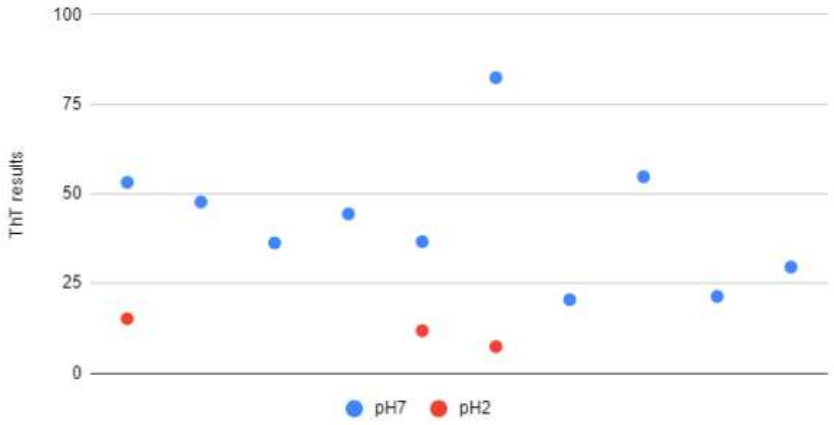


Figure 19: Change of ThT values with different pH values of the final solution regardless of the enzyme ratio

Figure 19 represents the change in ThT values with the change in pH regardless of the enzyme ratio. The enzyme ratio was not considered to have a significant effect since in Figure 18, it can be seen that the change in the enzyme ratio did not cause any significant change in the ThT values. It can be seen in the graph that lower pH values did not yield

higher amyloid fibril formation. This can be due to the usage of gluten. Many animal and plant proteins have the potential to yield amyloid fibrils with incubation under controlled conditions. However, the conditions of the incubation and the performance of fibrillation depend on the type of protein. It can be seen in other literature of Cao *et al.* from 2019, Monge-Morera *et al*. from 2021 and Lambrecht *et al.* from 2021 that for gluten the optimal conditions are defined at higher pH values for amyloid fibrillation (Cao *et al.*, 2019; Monge-Morera *et al*., 2021; Lambrecht *et al.*, 2021).

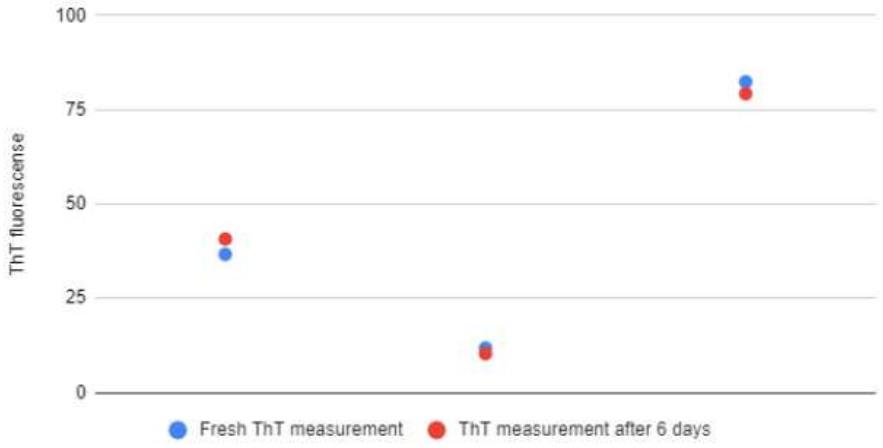


Figure 20: Stability of amyloid fibrils

Finally, Figure 20 represents the ThT measurements from the same samples first fresh and after 6 days. All the samples are stored in a fridge under 4°C for the whole time. It can be observed from the graph that the amyloid fibrils are quite stable and did not show a decrease in quantity even after 6 days.

4.2. Physical Observations of Amyloid Fibril-based Films

After the incubation, the solution contains amyloid fibrils formed by the wheat gluten protein. The presence of amyloid fibrils is proven with ThT measurements. The final solution with a protein concentration of 2wt% is used to prepare films. The solution was mixed with PVA and glycerol with different ratios to find the optimal formulation for the film formation. In Table 7, different formulations that were experimented with for film formation can be seen.

Table 7: Formulations of film samples #1

Trial Number	Enzyme ratio used in WG hydrolyzation	Set Protein Concentration	Drying time	Key ratio (Glycerol/fibril)	Ratio Wet Basis
2	1:45 1:22.5	2wt%	48	0.75	1.7% Fibrils 1.3% PVA 1.3% Glycerol
3	1:45 1:22.5	2wt%	48	7.5	0.8% Fibrils 6% PVA 6% Glycerol
4	1:45 1:22.5	2wt%	48	4.5	1.05% Fibrils 4.73% PVA 4.73% Glycerol
5	1:22.5	2wt%	48	2.5	1.3% Fibrils 3.3% PVA 3.3% Glycerol
8	1:22.5	2wt%	72	1.5	1.53% fibrils 2.3% PVA 2.3% Glycerol
9	1:22.5	2wr%	48	0.5	1.81% fibrils 0.9% PVA 0.9% Glycerol

The pictures reported in Figure 21 are taken after the drying hours indicated in the table when the film was able to get out from its support. Some of the films were not able to get removed from their support due to several different reasons such as; being too attached to the support and their very delicate structure. Overall pulling the film out of the support was quite hard due to its stickiness and delicate structure. However, the samples with lower ratios of glycerol were a lot less sticky both to their support and to themselves compared to the ones with a higher ratio of glycerol.

Figure 21: A) Amyloid Film Sample 2, Enzyme ratio 1:45, B) Amyloid Film Sample 2, Enzyme ratio 1:22.5, C)Amyloid Film Sample 3, Enzyme ratio 1:45, D) Amyloid Film Sample 3, Enzyme ratio 1:22.5 E) Amyloid Film Sample 4, Enzyme ratio 1:45, F) Amyloid Film Sample 4, Enzyme ratio 1:22.5, G) Amyloid Film Sample 5, Enzyme ratio 1:22.5, H) Amyloid Film Sample 8, Enzyme ratio 1:22.5 I) Amyloid Film Sample 9, Enzyme ratio 1:22.5

After the forming of the films, it was observed that the films formulated with the amyloid fibril solution prepared from the WG hydrolyzed with the enzyme ratio 1:45 (the double amount that was suggested in the literature (Monge-Morera *et al*., 2021) did not form a proper film. Instead, the texture was very sticky and waxy therefore these samples were not able to be taken out from their support. It was evaluated that even though the amyloid fibril formation was observed to be very similar with ThT measurements for both samples, a

solution with the enzyme ratio 1:45 is not able to form proper films under the same conditions. Therefore samples 5-9 (Figure 21.G and 21.I) were continued with only the enzyme ratio 1:22.5.

For samples 3 and 4 (Figure 21.D and 21.F) where the amount of glycerol and PVA added is way higher than the amount of fibril in the solution, respectively 7.5 and 4.5 times, the resulting film was very bouncy and elastic. The film was sticky both to its support and itself but due to its elastic nature it was easy to handle. However, after around 7 days, the surface of the film became very greasy and the film got very brittle.

For samples 5 and 8 (Figure 21.G and 21.H) it can be said that as the ratio of glycerol and fibrils get closer together the elasticity and the bounciness of the formed films decrease, instead the films get very moist and brittle. Therefore, these samples were hard to handle, however, this moistness allowed them to be less sticky to the surfaces and themselves.

Lastly, for samples 2 and 9 (Figure 21.B and 21.I) which the glycerol ratio is lower than the fibrils, the formed films had the texture of a paper. The surface of the films was not shiny, there was no bounce and the texture was rough. However, they were still elastic. The films were not sticky to the surface or themselves at all and came off from the support easily.

After around 7-10 days all the films were stuck to their support and brittleness was increased. Samples 3 and 4 became greasy at the surface and very brittle. Therefore, it can be concluded that even though some formulations seem to work, there is more work to be done for their stability if the desired use is planned to be for food packaging applications.

For the second trial of film forming, as indicated above, the 1:45 Trypsin enzyme ratio was not applied at all due to its inability to form films. The new formulations applied for the trial are given in Table 8 and the procedure is explained in Materials and Methods (Paragraph 3.2).

Table 8: Formulations of film samples trial #2

Sample number	Enzyme ratio used in WG hydrolyzation	Set amyloid fibril concentration	Glycerol/Fibril	The ratio on the wet basis	The ratio on the dry basis
2	1:22.5	2%	0.75	1.7% Fibrils 1.3% PVA 1.3% Glycerol	40% Fibrils 30% PVA 30% Glycerol
3	1:22.5	2%	7.5	0.8% Fibrils 6% PVA 6% Glycerol	6.25% Fibrils 46.87% PVA 46.87% Glycerol
4	1:22.5	2%	4.5	1.05% Fibrils 4.73% PVA 4.73% Glycerol	10% Fibrils 45% PVA 45% Glycerol
9	1:22.5	2%	0.5	1.81% fibrils 0.9% PVA 0.9% Glycerol	50% fibrils 25% PVA 25% Glycerol
1	-	0%	-	50% PVA 50% Glycerol	-
2	1:22.5	1%	15	0.4% fibrils 6% PVA 6% Glycerol	3.22% fibrils 48.38% PVA 48.38% Glycerol
3	1:22.5	2%	7.5	0.8% Fibrils 6% PVA 6% Glycerol	6.25% Fibrils 46.87% PVA 46.87% Glycerol
4	1:22.5	3%	5	1.2% Fibrils 6% PVA 6% Glycerol	9.37% Fibrils 45% PVA 45% Glycerol
5	1:22.5	4%	3.75	1.6% Fibrils 6% PVA 6% Glycerol	11.76% Fibrils 44.11% PVA 44.11% Glycerol

In this trial, to understand the drying mechanics, the weight of each film sample is recorded from the very beginning until the drying curve reaches a balance. When the drying curve has reached a plateau, it is assumed that the drying stopped therefore a film has been formed. At this stage, the developed film is removed from the support and prepared for further characterization processes. Unfortunately, not all films were able to be removed from the support. This is due to the differences between formulations. In general, it is observed that the formulations that contain higher amounts of PVA and glycerol content are much

more elastic, durable, and easy to remove from the support, while others tend to be more fragile and cannot be removed from the support.

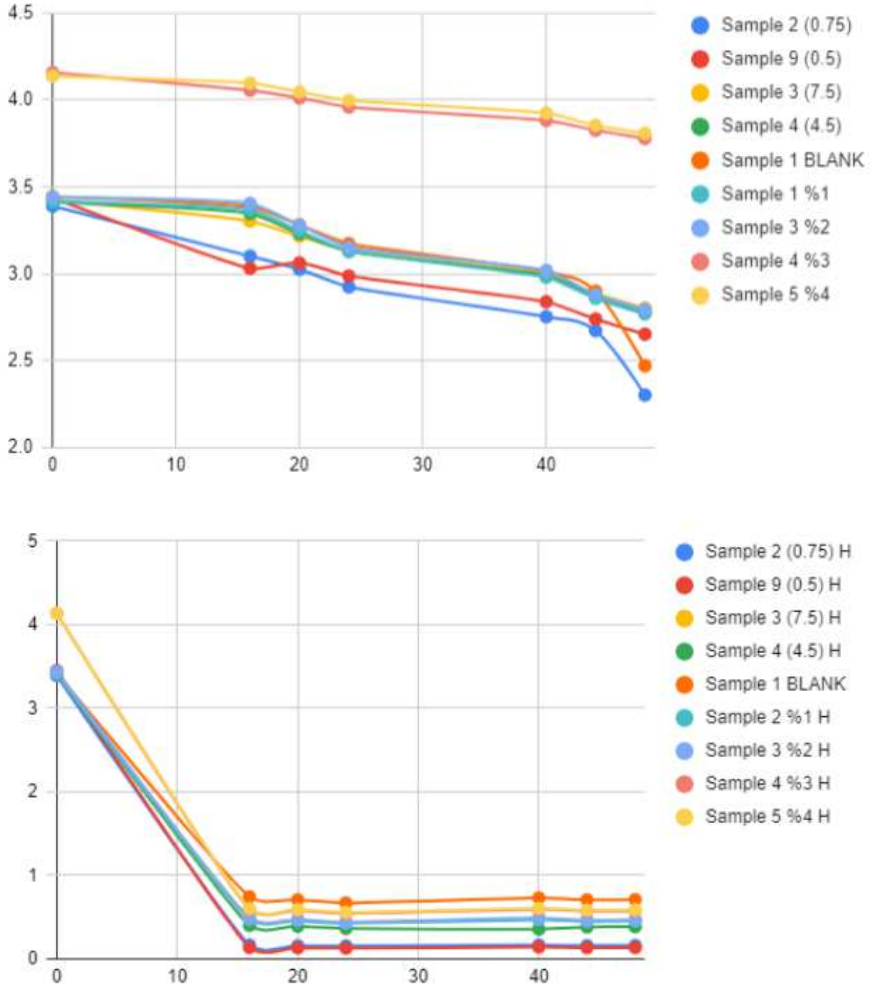


Figure 22: A: Drying curve of the samples that were dried in the desiccator, B: Drying curve of the samples that dried under the hood

The drying curves of the samples that dried under two different conditions are given in Figure 22. It can be seen in the graphs that the samples that dried in the desiccator (containing silica beads, at 25°C) have never reached the equilibrium in their drying. These samples have lost so little weight over time that they did not go from a liquid to a solid state at all until it was concluded that this drying method was ineffective and the experiment had failed. On the contrary, the samples that dried under the hood reached the equilibrium state within the first 15 hours. Therefore, only the samples that were dried under the hood have been used for further characterization measurements.

In particular, a drastic weight loss occurred in the first hours of drying and the curve reached an equilibrium within 6-8 hours (Figure 23).

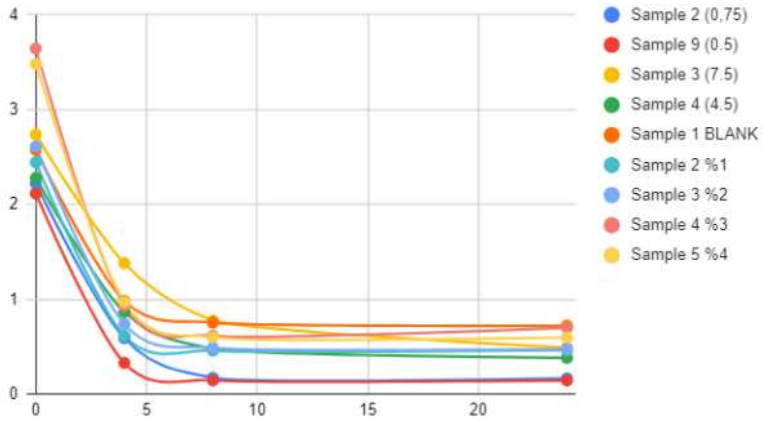
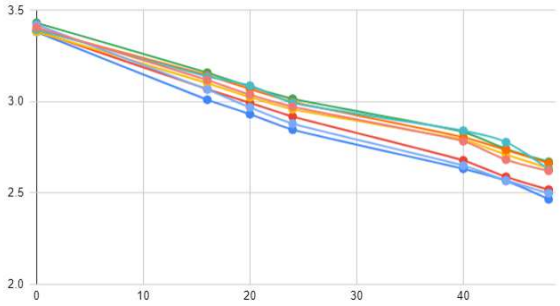


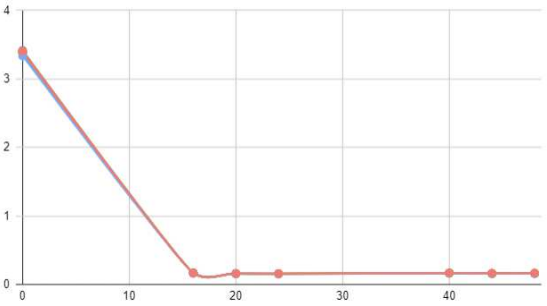
Figure 23: Drying curve for samples under the hood, the first 24 hours

As reported in Figure 24 the samples that contain higher amounts of PVA and glycerol have dried to be slightly heavier than the other samples as their final weight (Figure 24.C, 24.D, 24.E, 24.F, 24.G, 24.H, 24.I). This is due to the total amount of dry matter present in these samples due to their content of PVA. Overall, it can be concluded that the samples under the hood have shown much more successful drying characteristics while the samples in the desicator (with silica beads under 25°C) did not dry at all. All samples that were inside the desiccator were eliminated after 92 hours. This replication of drying proves that the drying occurs more efficiently under the hood where the samples can be in direct contact with the airflow. In the future, to understand the drying characteristics of amyloid-fibril-based films better, drying the films in a chamber would provide controlled temperature, relative humidity, and airflow would be beneficial for the research.

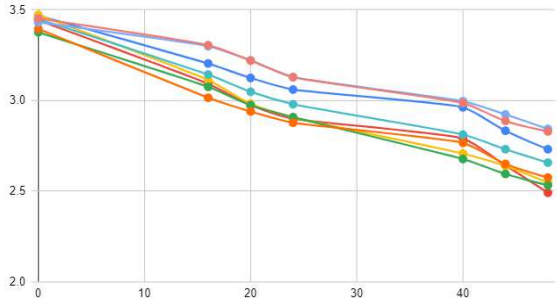
Sample 2 (0.75) In desicator (25C, Silica beads)



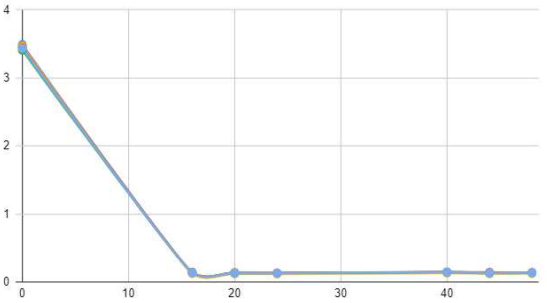
Sample 2 (0.75) under the hood



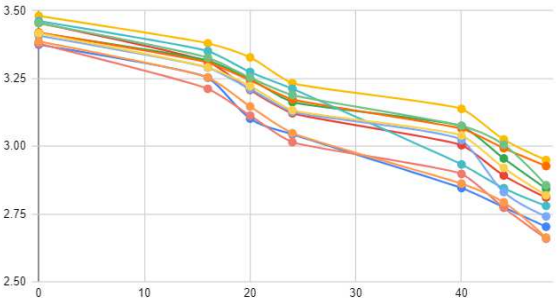
Sample 9 (0.5) In desicator (25C, Silica beads)



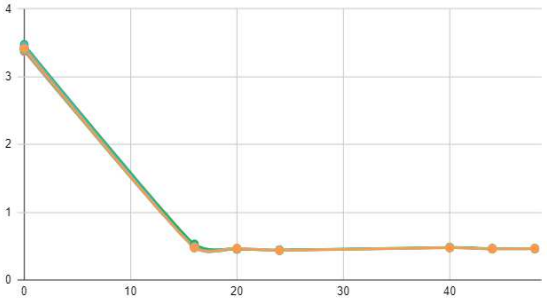
Sample 9 (0.5) under the hood



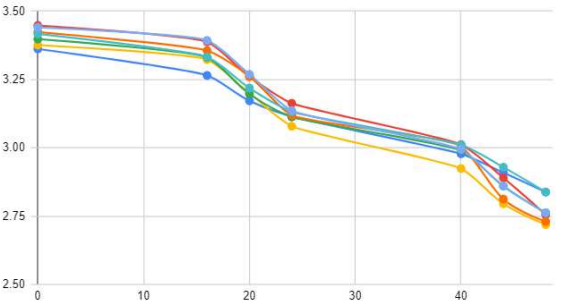
Sample 3 (7.5) In desicator (25C, Silica beads)



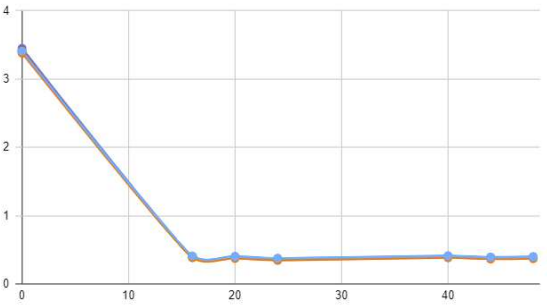
Sample 3 (7.5) under the hood



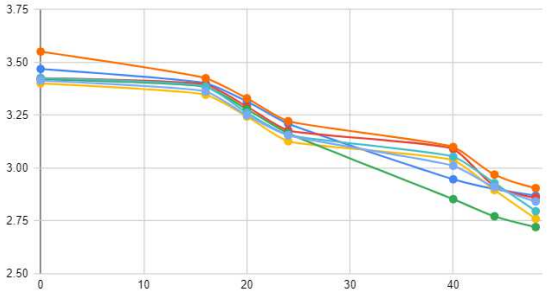
Sample 4 (4.5) In desicator (25C, Silica beads



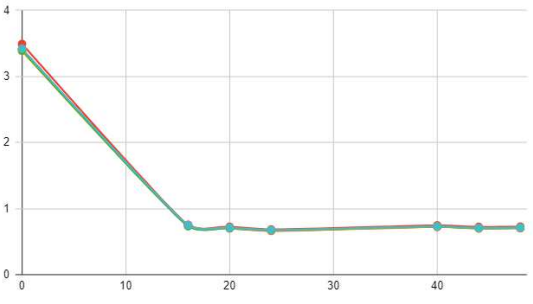
Sample 4 (4.5) under the hood



Sample 1 BLANK, In desicator (25C, Silica beads)



Sample 1 BLANK, under the hood



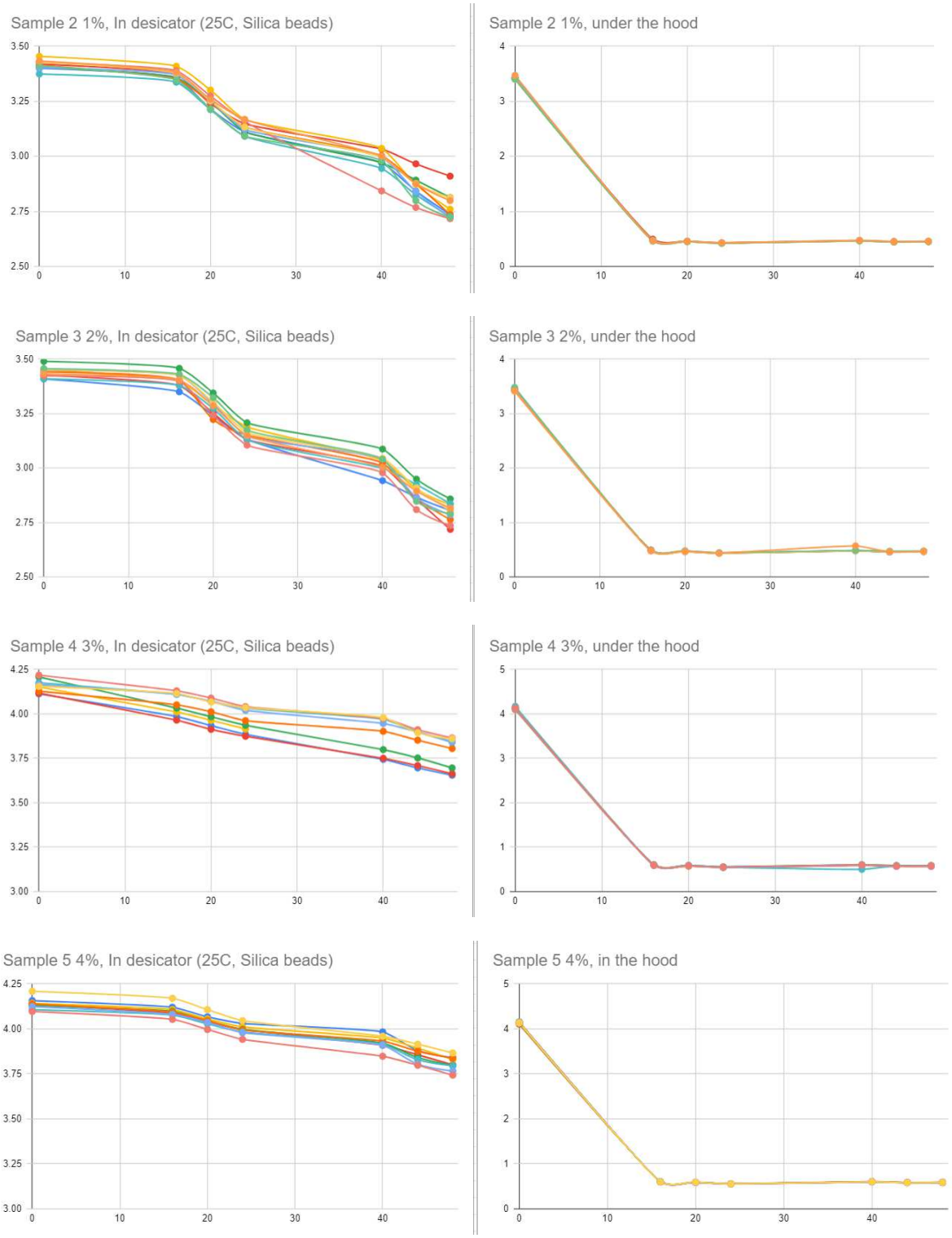


Figure 24: Drying curves for each sample both for the ones that are left in the desiccator and under the hood

After the drying is concluded the films are removed from their supports and prepared for further measurements. Most of the films were used for puncture tests since multiple replicas need to be done for proper results. In Figure 25 can be seen that some of the films

are placed on paper, and prepared for the gloss test. It can be observed from the blurriness of the lines on the paper that the films are quite transparent with a slight tint of yellow colour.

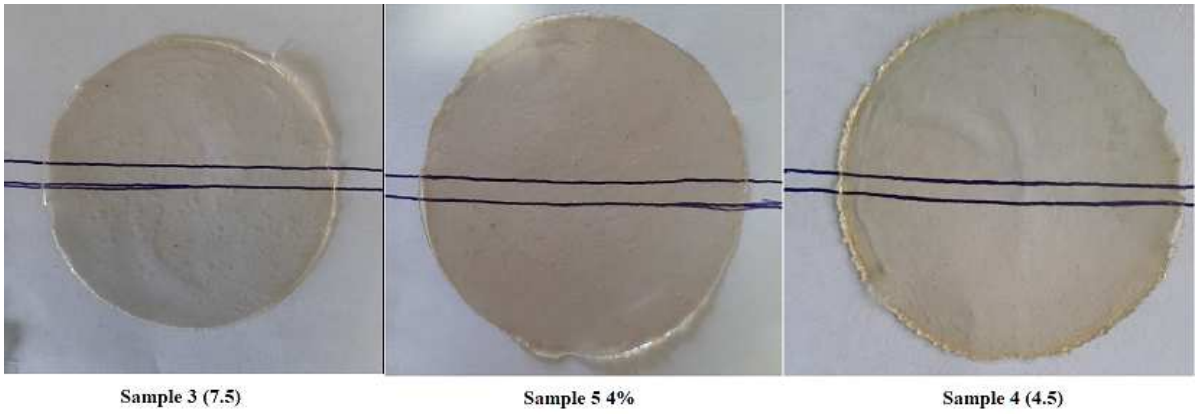


Figure 25: Characterization of films obtained from gluten

4.3. Characterization of Amyloid Fibril-based Films

4.3.1. Mechanical Properties - Tensile Strength (Puncture Test)

Tensile strength measurements are performed with Stable Micro Systems, TA.XT.Plus Texture analyzer. Using the probe SMS P/0.5S with a ball tip. The experimental conditions and the setup are explained in the Materials and Method section (paragraph 3.2.1.1). For each sample that was able to be removed from the support, multiple films were tested in order the create replicable results. Since some of the films have raptures, it is expected that some results do not reflect the actual strength of the films well due to the puncture occurring where the rapture is rather than the absolute centre of the film. Nevertheless, most films have raptured right in the middle, as expected from the puncture test. Below can be found the photos of each sample after the puncture test where the puncture hole can be observed as being centred.

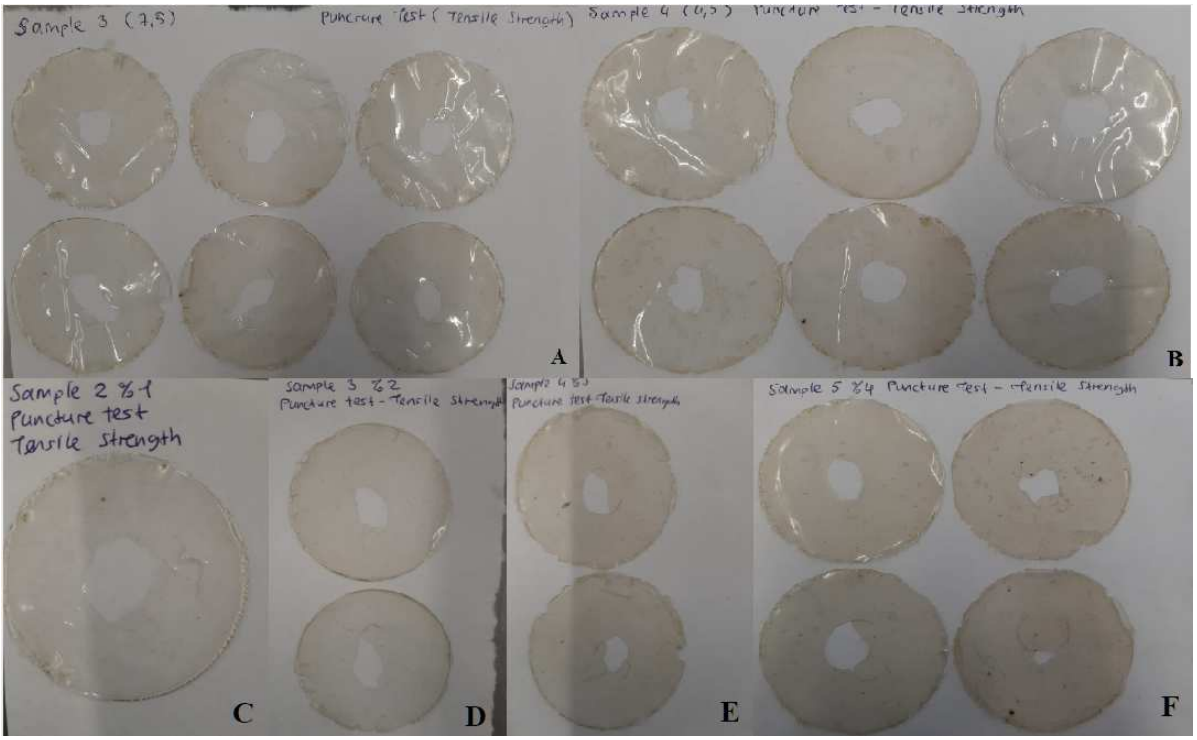


Figure 26: A) Punctured films from the sample 3 formulation 7.5x, B) Punctured films from the sample 4 formulation 4.5x, C) Punctured films from the sample 2 formulation 1% fibril concentration, D) Punctured films from the sample 3 formulation 2% fibril concentration, E) Punctured films from the sample 4 formulation 3% fibril concentration, F) Punctured films from the sample 5 formulation 4% fibril concentration

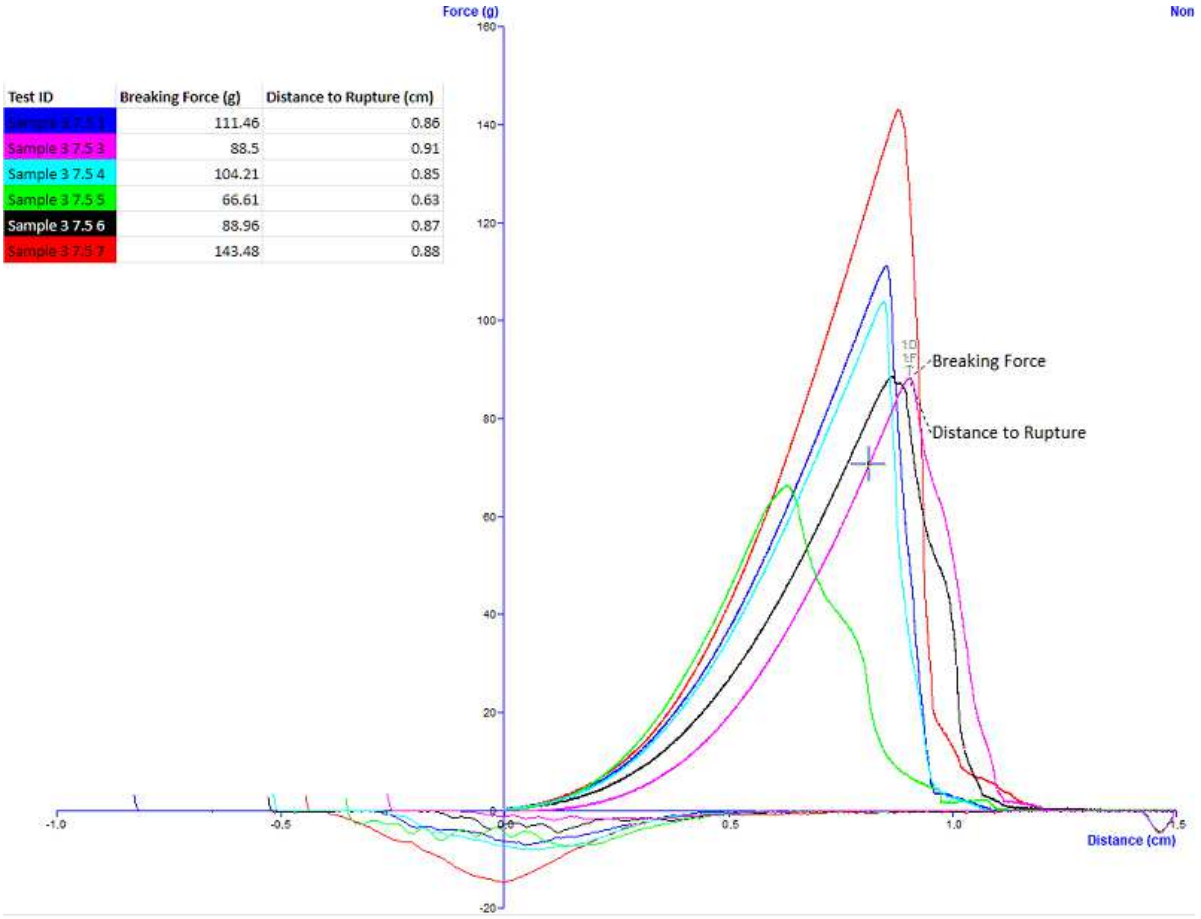
The tensile strength of the films is measured in terms of the amount of force the films can withstand (in grams) and the amount of distance they stretch from the beginning positions (in cm). The averages of the results are given in Table 9 followed by graphs for individual films together (Figure 27).

Table 9: The withstand force (g) and stretch distance (cm) of the films

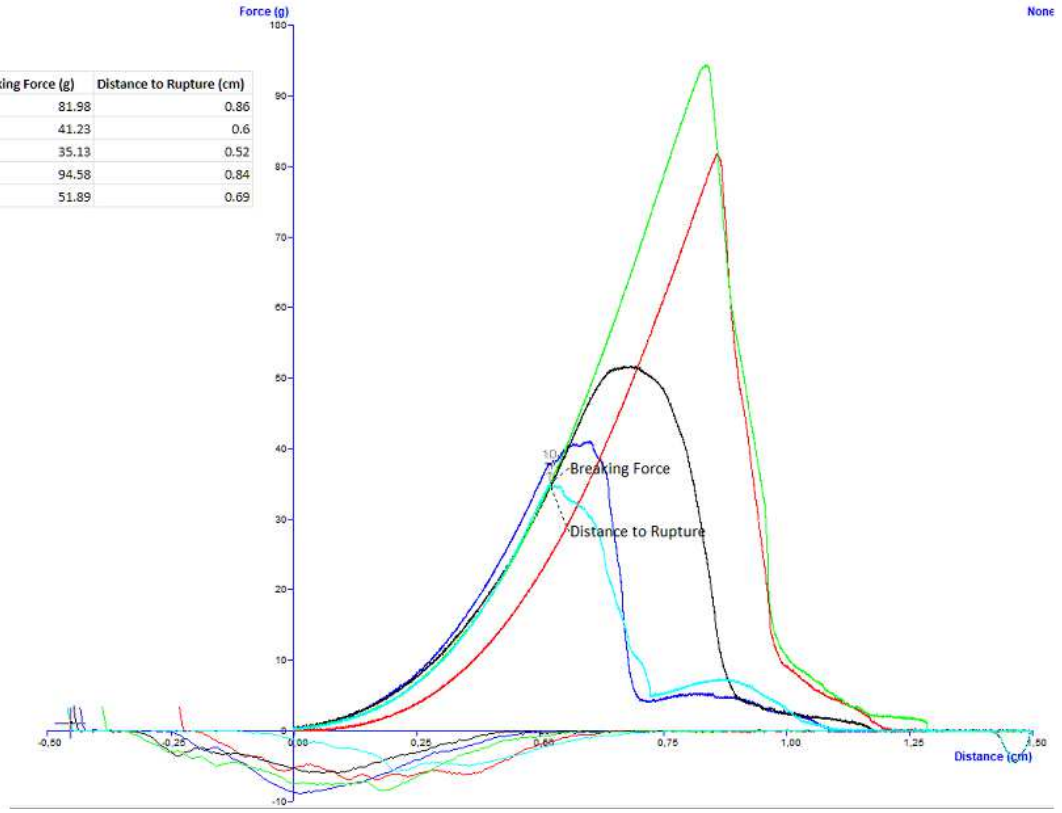
Sample Title	**Average Breaking Force (g)**	**Average Distance to Rapture (cm)**
Sample 3 7.5	100.54	0.83
Sample 4 4.5	59.05	0.68
Sample 2 %1	72.04	0.82
Sample 3 %2	68.15	0.8
Sample 4 %3	78.73	0.72
Sample 5 %4	101.27	0.81

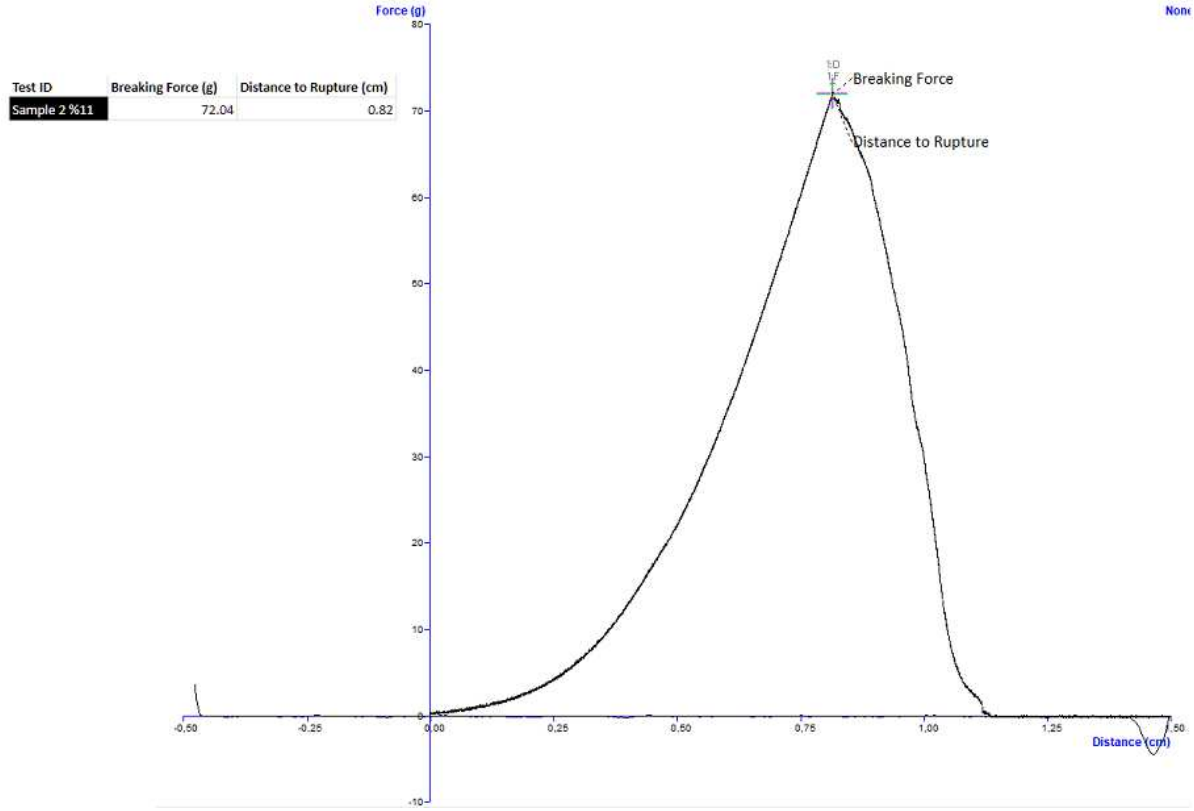
The graphs representing both the breaking force and the distance to rapture for each sample are given in Figure 27.

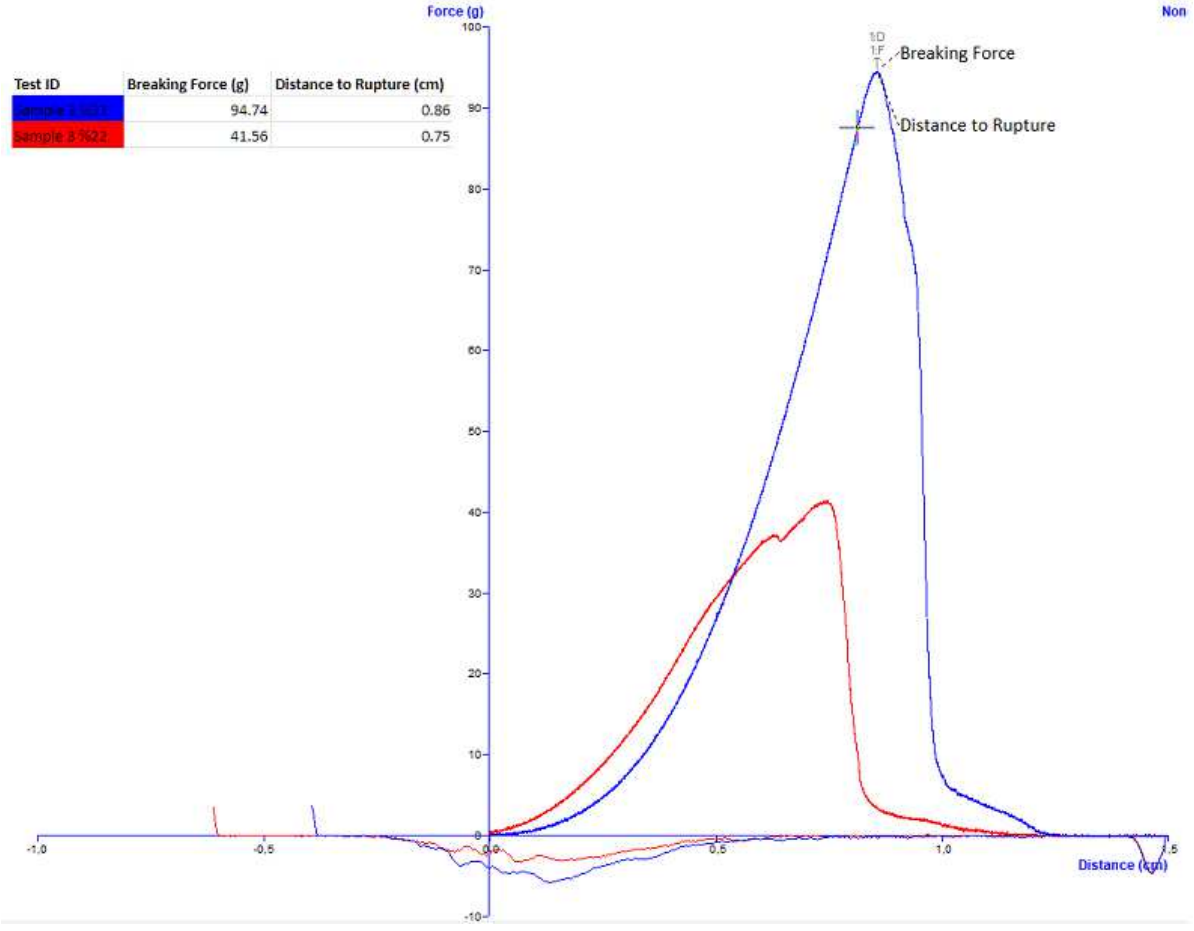
Test ID	Breaking Force (g)	Distance to Rupture (cm)
Sample 3 7.5 3	111.46	0.86
Sample 3 7.5 3	88.5	0.91
Sample 3 7.5 4	104.21	0.85
Sample 3 7.5 5	66.61	0.63
Sample 3 7.5 6	88.96	0.87
Sample 3 7.5 7	143.48	0.88



Test ID	Breaking Force (g)	Distance to Rupture (cm)
Sample 4 4.5 1	81.98	0.86
Sample 4 4.5 2	41.23	0.6
Sample 4 4.5 4	35.13	0.52
Sample 4 4.5 5	94.58	0.84
Sample 4 4.5 6	51.89	0.69




Force (g)
Test ID
Breaking Force (g)
Distance to Rupture (cm)
Sample 2 %11
72.04
0.82
Breaking Force
Distance to Rupture
Distance (cm)


Force (g)
Test ID
Breaking Force (g)
Distance to Rupture (cm)
94.74
0.86
41.56
0.75
Breaking Force
Distance to Rupture
Distance (cm)

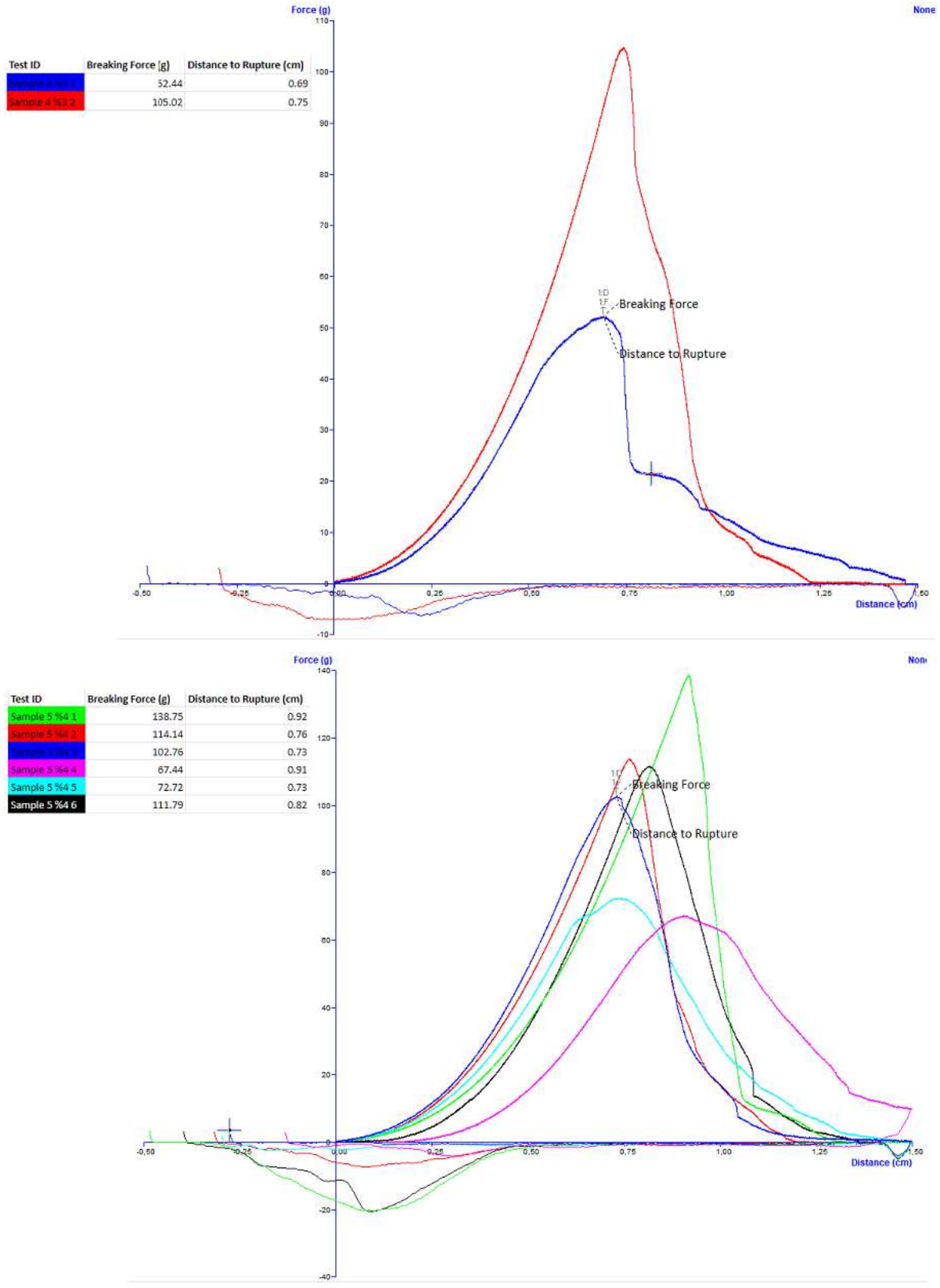


Figure 27: Graphs representing the breaking force and the distance to rupture for each film

It was suggested that the films that contain more plasticizers (PVA and glycerol) would be stronger (able to withstand more force) and stretchier but also expected that the

amount of fibrils in the formulation to make the films stronger. To express this correlation the total percentage of plasticizer content of each formulation is compared with the average of the breaking force (g) and the distance to rupture (cm) in Table 10 and Figure 28.

Table 10: The withstand force (g), stretch distance (cm), and plasticizer content (%) of the films

Sample Title	Average Breaking Force (g)	Average Distance to Rapture (cm)	% Total Plasticizers (PVA+Glycerol)
Sample 3 7.5	100.54	0.83	93.74
Sample 4 4.5	59.05	0.68	90
Sample 2 %1	72.04	0.82	96.76
Sample 3 %2	68.15	0.8	93.74
Sample 4 %3	78.73	0.72	90.9
Sample 5 %4	101.27	0.81	88.2

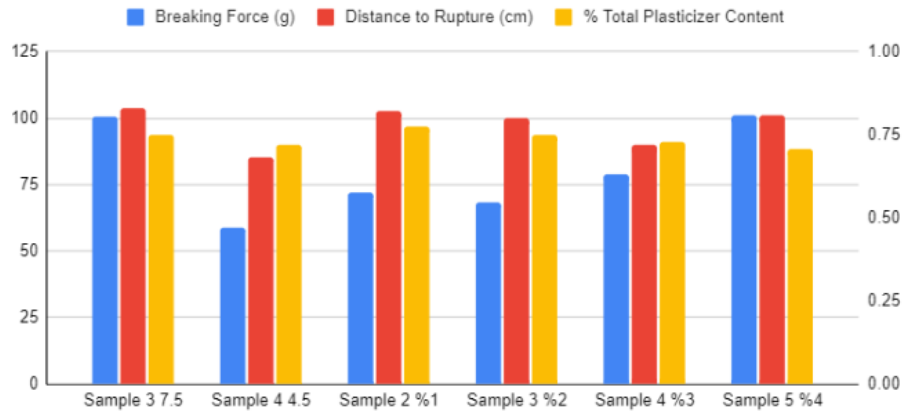


Figure 28: Comparison between the breaking force (g), distance to rupture (cm) and the contained total amount of plasticizers (%) for each film sample

It can be observed that the breaking force seems to be related to the change in the content of the total plasticizer apart from the sample 2 1% (Figure 28). As the total plasticizer content decreases and the fibril ratio increases, the breaking force increases accordingly. Therefore, it can be said that the increasing fibril ratios contribute to the tensile strength of the films. However, the distances that each sample stretched until rupture are not associated with the total amount of plasticizers they contain. Additionally, results are unstable and not replicable since Sample 3 (7.5) and Sample 3 (2%) have the same formulations but yield significantly different results. These results suggest that there is more

to consider than just the amount of plasticizers used in the formulations to understand the strength of the final material.

4.3.2. Thickness

Thickness measurements are performed for all the samples whether they are removed from the support or not. The special micrometre allowed the measurement to be done while samples were still attached.

Figure 29: Measurement of the thickness of the film with a micrometre

The results of each thickness measurement on samples are given in Table 11. The thickness of the films is correlated with the total amount of dry matter they contain in their formulation, which is demonstrated in the graph provided below. It can be observed that the total amount of dry matter is correlated with the thickness of the films after drying.

Table 11: Thickness and the total dry matter content of the films

Sample Name	Thickness (mm)	Dry matter content (g)
Sample 2 (0.75x)	0.043	1.75
Sample 9 (0.5x)	0.032	1.5
Sample 3 (7.5x)	0.131	5.1
Sample 4 (4.5x)	0.109	3.3
Sample 2 %1	0.086	4.8
Sample 3 %2	0.134	5.1
Sample 4 %3	1.161	5.4
Sample 5 %4	0.163	5.7
Sample 1 Blank	0.155	4.5

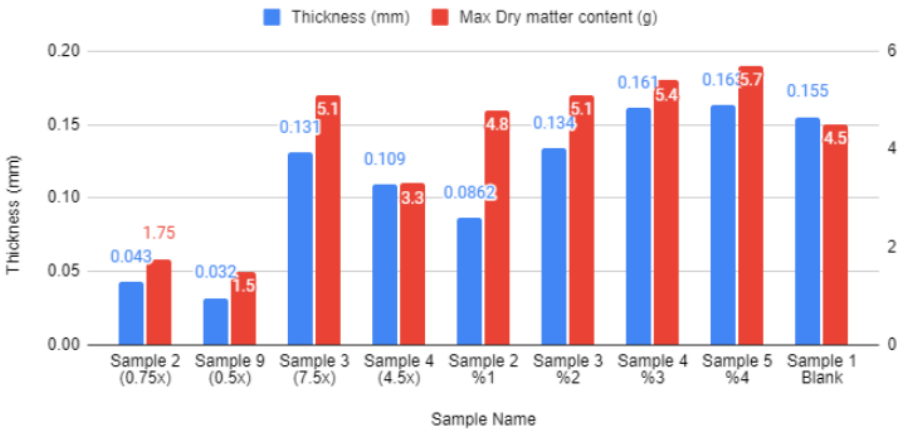


Figure 30: Thickness (mm) and max dry matter content of the films

The average thickness of the films is correlated with the total dry matter content in the formulation (Figure 30). The formulations are correlatable within themselves since there are 2 sets of trials (the ones that fix the ratio and the ones that fix the fibril content). Once the films were dried their total weight was lower than the dry matter previously added into the formulation. This was an unexpected result but since all of the dry ingredients are fully soluble in water (fibrils and PVA) and the glycerol is added in the liquid form, the total dry

matter did not represent the minimum weight of the films at the end of drying. Therefore, this affected the thickness of the films as a slight inconsistency between the formulations.

4.3.3. Static Water Contact Angle Analysis (Hydrophobicity)

Due to the reasons explained in the materials and methods section, no static water contact angle analysis was performed. The films are described to be “extremely hydrophilic”.

5. Discussion

The increasing usage of petrol-based plastics and other unsustainable packaging materials in the food industry possesses a great threat to the environment. Recently there has been a shift to use more sustainable materials in all aspects of life which affected the way packaging is produced and used. Such a shift has also been effective in science and yielded more research on sustainable packaging solutions.

Amyloid fibrils have been a great deal of research in medicine for several decades due to their negative effects on human health. However, nowadays, amyloid fibrils are under great investigation for their benefits in food science. Amyloid fibrils are specific deposits of proteins that can affect food texture positively and can be used as building blocks for functional materials. In recent years both animal and plant protein-based amyloid fibril formation and their functional properties have been investigated.

Within this study gluten protein-based amyloid fibril-based packaging films are investigated. The study is divided into two parts, the first part of the study covers the formation of amyloid fibrils from wheat gluten and investigates the amyloid fibril formation conditions and characteristics. The second part of the study covers the formation and characterization of amyloid fibril-based films and investigates the characteristics of different formulations for film formation. For amyloid fibril-based film formation plasticizers such as PVA and glycerol are used. For the characterization of films for a potential alternative to food packaging, thickness, hydrophobicity, elasticity, and gloss measurements are performed.

In this study, gluten is selected because of the potential reflection of its known mechanical properties in food into packaging materials as a plant-based protein source. However, selecting gluten as the main source of protein comes with several disadvantages such as the allergenicity of gluten and the partial hydrolysis in water. Gluten is only partially soluble in water which decreases the efficiency of the amyloid fibril forming process. Therefore, in this study, gluten is first hydrolyzed with trypsin enzyme in order to free the protein bonds that will eventually yield amyloid fibrils following the technique presented in the article from Monge-Morera *et al.,* in 2021 (Monge-Morera *et al.,* 2021). The allergenicity of the gluten has been considered however not yet been investigated within this study. To understand the possibility of gluten particles' migration to food from packaging,

the first development of packaging needs to be excelled. In case of gluten migration from the developed packaging material, alternative plant protein chitin will be replaced with gluten and further investigation will be performed.

After the hydrolyzation, gluten protein is incubated to form protein fibrils. The formation of amyloid fibrils eliminates the negative traits of gluten in the end product, such as its unappealing and dull yellow colour and unpleasant smell (Bastioli, 2020). The optimum incubation condition is investigated and decided to be at 95°C and pH 7 for 40 hours with constant stirring. This evaluation has been done according to the research from Lambrecht *et al.,* in 2021 which states the best conditions for incubation to be at 85°C and pH 7 for 38 hours with constant stirring (Lambrecht *et al.,* 2021). Lower pH values (pH 2) were evaluated during this research according to the common knowledge of amyloid fibrils being formed more frequently in lower pH values. However, the ThT results have shown that amyloid fibril formation has not been affected positively in lower pH. Temperature increase from 85°C to 95°C was estimated to increase the overall formation of amyloid fibrils. As a result of the temperature increase the ThT values of the samples have increased compared to the study that was followed (an average of 45.39 in this research compared to the 6.9 in the study of Lambrecht *et al*.). Even though the efficiency of amyloid fibril formation seems to increase with the temperature increase, the stability of the process has been affected negatively as can be seen by the increased value of the standard deviation (6.12 in this research compared to the 0.3 in the study of Lambrecht *et. al*.). Additionally, the increase of ThT fluorescence value did not follow the typical curve when measured every hour of the incubation. Instead, the increase was rapid and stayed stable during the entire period of incubation. This observation suggests that the prolonged hours of incubation under high temperatures promoted amyloid fibril formation until a degree, then formed fibrils are rapidly degraded and formed again during the incubation. Such mechanism might have affected the structure of the amyloid fibrils therefore affecting the quality but the characterization of the amyloid fibrils is not investigated in this study and further investigation is needed using advanced techniques such as FTIR, AFM and CD spectra.

Several formulations were investigated for the forming of films from gluten-sourced amyloid fibril-based solutions using PVA and glycerol. For the formulations of the films, similar ratios that are used in the study of Li T. *et al*., in 2023 are followed (Li T. *et al*., 2023). However, the formulation that followed did not yield good films. The formulation of Sample 7 (7.5) showed the highest tensile strength with the given formulation 0.8% Fibrils/% PVA/6% Glycerol on a wet basis and 6.25% Fibrils/46.87% PVA/46.87% Glycerol

on a dry basis, while the Sample 2 (the same ratios of the study of Li T. *et al.*, 2023) could not be removed from the support. The article that followed worked with different types of seeds and legumes as the main protein source to form functional materials. This can be given as the main reason why the same formulation did not work for this study due to different protein sources needing different ratios of plasticizers to form films.

After the film solutions drying process was conducted primarily under the hood and in the dessicator to follow the weight and create a curve for the drying process. The samples that were placed in desiccators did not dry as expected since there was no airflow inside the desiccator and the silica beads were not enough to collect the moisture. The samples that were placed under the hood dried with better characteristics in 24 hours. However conducting the drying process under the hood comes with multiple problems, such as not being able to control the environmental conditions (temperature, airflow, moisture content) and the contamination of the samples. Due to the inability to control outside temperature, humidity content and consistent airflow, the created drying curves do not represent true scientific data. To be able to understand the drying characteristics of gluten-sourced amyloid fibril-based films, a drying chamber that will allow us to control such measures should be created in further research. Moreover, having airflow through the hood (from outside of the hood with no control/filtering) caused contamination of samples. After 24 hours of drying, particles of dust and other physical contaminants were observed on the surface of the films. Such contaminants are critical because most characterization measurements such as tensile strength, static water contact angle, thickness and gloss, are affected by the impurities on the surface of the films. Therefore, possible contamination of the films should be added as an error value to all the characteristic measurements conducted in this study and should be avoided in further studies to achieve better results.

Finally, even though gluten, as a plant-based protein source has formed functional packaging films the final samples are far from applicable to the industry. For instance, as such food packaging film application is thought to be an alternative to Saran Wrap, the physical and mechanical properties of Saran Wrap are quite advanced compared to biodegradable alternatives. The average thickness of Saran Wrap is 12.7 microns (0.0127mm), meanwhile, in this research, the average thickness of gluten-sourced amyloid fibril-based films is 223 microns (0.223mm) while the sample with the highest tensile strength performance's (sample 3, 7.5x) thickness is 131 microns (0.131mm). Both such results are still much thicker than commercially available products (MatWeb, 2023). Within

this study, the thickness of the films was found to be directly correlated with the total dry matter content of the films. The tensile strength at the break for Saran Wrap is found to be 103MPa (10.50 kg/sq.mm) meanwhile in this study sample 3 7.5x, the one with the highest tensile strength performance is measured to be 0.10054 kg/sq.mm (100.54 g/sq.mm) which is 10000 times lower than how much Saran Wrap can carry (MatWeb, 2023). Moreover, within this study, thickness is also found to be directly correlated with tensile strength. However, as commercial petroleum products are much thinner with higher tensile strength, the ability to develop thinner gluten-based films with stronger mechanical properties still needs further research.

Lastly, within this study, static water contact angle analysis for hydrophobicity has shown that such film formed with gluten, PVA and glycerol is extremely hydrophilic and not at all suitable for food packaging. This is an expected result considering all the ingredients used in the formulation are water soluble, however in order to make such product applicable for industry, it is necessary to increase its hydrophobicity.

6. Conclusion

In conclusion, the application of gluten protein to form sustainable food packaging films is a novel advancement that needs further research for improvement. The known mechanical properties of gluten possess a potential for mechanical applications like packaging films however successfully replicating the plastic alternatives already widely available in the market is a great challenge. In the future, a deeper understanding of plant-based amyloid fibrils and the improvement of experimental procedures might allow such applications to be a potential replica of petroleum-based plastic food packaging films.

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