Kutluay Kaan Tamer

MASTER OF SCIENCE THESIS

2024-ANTALYA

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AKDENIZ UNIVERSITY

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DEPARTMENT OF GENE AND CELL THERAPY

ADVANCEMENTS IN GLUTEN DETECTION: ANTIBODY DEVELOPMENT AND GOLD NANOPARTICLE CONJUGATION FOR A LATERAL FLOW IMMUNOASSAY

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ADVISOR

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To the Institute of Health Sciences;

This study has been approved by our jury as a Master of Science Thesis in the Department of Gene and Cell Therapy, Gene and Cell Therapy Master of Science program.

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DECLARATION

I hereby declare that this thesis is my own work; that it does not include any unethical act from the planning to the writing of the thesis; that I have acquired all the information included in this thesis in accordance with the ethical principles; that I have made due acknowledgements to all the information and comments that were not acquired by this thesis work; and that I have included all these references in the references list.

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You didn't get to see me finish it, but I'm dedicating this thesis to you, dad. Rest in peace.

ABSTRACT

Objective: Gluten-related disorders, like celiac disease (CD), affect 5% of the global population, with CD impacting 1.4% of individuals. Consuming gluten triggers inflammation in the intestines for those with CD. Following a gluten-free (GF) diet is crucial despite challenges like cross-contamination. Immunoassays like enzyme-linked immunosorbent assay (ELISA) and lateral flow immunoassay (LFIA) are preferred for detecting gluten due to their simplicity and cost-effectiveness. LFIA kits offer a portable option for analyzing gluten in food. The demand for reliable gluten detection methods highlights the need for accurate solutions for both food manufacturers and individuals with gluten-related disorders. Our research focuses on developing specific techniques using antibodies targeting gliadin, one of the main parts of gluten, to confirm the absence of gluten.

Method: The polyclonal antibodies obtained from rabbits were purified and tested for reactivity to gliadin using ELISA and Western blot techniques. They were then conjugated with gold nanoparticles (AuNPs) to develop an LFIA, with optimal conditions determined by pH and antibody concentration. The efficacy of the LFIA in detecting wheat gliadin, including the limit of detection, was investigated. Gliadin was extracted and analyzed from various food samples to establish a reliable method for detecting it in different products.

Results: Antibodies from three rabbits (R1, R2, and R3) were sensitive to gliadin in an ELISA. Western blot analysis confirmed common gliadin-specific epitopes in these antibodies. They did not react to GF food samples. The LFIA effectively detected wheat gliadin using a selected membrane and fibers, with a detection limit of 50 µg/mL. The LFIA consistently produced reliable results when testing samples from different food sources, making it a dependable tool for gliadin detection in various food samples.

Conclusion: Our study presents a technique for identifying gluten, including the development of antibodies, improving AuNP attachment, and establishing a reliable LFIA protocol. This methodology has the potential to ensure food safety and facilitate gluten analysis.

Keywords: Antibody development, Antibody conjugation, Lateral flow immunoassay

ÖZET

Amaç: Çölyak hastalığı gibi glütenle ilişkili rahatsızlıklar, küresel nüfusun %5'ini etkilemekte olup, çölyak hastalığı tek başına bireylerin %1,4'ünü etkilemektedir. Çölyak hastalığı olan kişilerde gluten tüketimi bağırsaklarda iltihaplanmayı tetikler. Çapraz bulaşma gibi zorluklara rağmen glütensiz bir diyet uygulamak çok önemlidir. ELISA ve LFIA gibi immünolojik testler, basitlikleri ve uygun maliyetleri nedeniyle gluten tespitinde tercih edilmektedir. LFIA kitleri, gıdalardaki gluteni analiz etmek için taşınabilir bir seçenek sunmaktadır. Güvenilir gluten tespit yöntemlerine olan talep, hem gıda üreticileri hem de glütenle ilişkili rahatsızlıkları olan bireyler için doğru çözümlere duyulan ihtiyacı vurgulamaktadır. Araştırmamız, gıdalarda gluten bulunmadığını doğrulamak için glutenin ana bileşenlerinden biri olan gliadini hedefleyen antikorlar kullanan özel teknikler geliştirmeye odaklanmaktadır.

Yöntem: Tavşanlardan elde edilen poliklonal antikorlar saflaştırıldı ve ELISA ve Western blot teknikleri kullanılarak gliadine karşı reaktiviteleri test edildi. Daha sonra, pH ve antikor konsantrasyonu ile optimal koşullar belirlenerek altın nanopartiküllerle konjuge edildi. LFIA'nın buğday gliadinini tespit etmedeki etkinliği, tespit limiti de dahil olmak üzere araştırıldı. Farklı gıda örneklerinden gliadin ekstrakte edilip analiz edildi ve farklı ürünlerde gliadini tespit etmek için güvenilir bir yöntem oluşturuldu.

Bulgular: Üç tavşandan (R1, R2 ve R3) elde edilen antikorlar, ELISA testinde gliadine duyarlıydı. Western blot analizi, bu antikorlarda ortak gliadin-spesifik epitopları doğruladı. Glütensiz gıda örneklerine tepki vermediler. LFIA, seçilen bir membran ve lifler kullanarak buğday gliadinini etkili bir şekilde tespit etti ve tespit limiti 50 µg/mL idi. LFIA, farklı gıda kaynaklarından alınan örnekleri test ederken tutarlı ve güvenilir sonuçlar verdi.

Sonuç: Çalışmamız, antikorların geliştirilmesi, altın nanopartikül bağlanmasının iyileştirilmesi ve güvenilir bir LFIA protokolü oluşturulması da dahil olmak üzere gluten tespiti için bir teknik sunmaktadır. Bu metodoloji, gıda güvenliğinin ve gluten analizinin kolaylaştırılması potansiyeline sahiptir.

Anahtar Kelimeler: Antikor geliştirme, Antikor konjugasyonu, Lateral akış immünoanalizi

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ICONS AND ABBREVIATIONS

AuNPs : Gold Nanoparticles

BSA : Bovine Serum Albumine

CD : Celiac Disease

DH : Dermatitis Herpetiformis

ELISA : Enzyme-linked Immunosorbent Assay

GF : Gluten-free

GRDs : Gluten-related Disorders

HLA : Human Leukocyte Antigen

HPLC : High-performance Liquid Chromatography

HRP : Horseradish Peroxidase

LFIA : Lateral Flow Immunoassay

MS : Mass Spectrometry

NCGS : Non-celiac Gluten Sensitivity

OD : Optical Density

PBS : Phosphate-buffered Saline

PBST : PBS with Tween-20

PCR : Polymerase Chain Reaction

TMB : 3,3',5,5'-Tetramethylbenzidine

1. INTRODUCTION

Gluten, a combination of proteins found in wheat, barley, and rye, plays a vital role in food texture but may pose challenges for specific individuals. Its distinct composition of gliadins and glutenins contributes to its functionality in cooking and its potential to cause health problems. When gluten is digested, it can provoke a range of immune responses based on the person's genetic susceptibility (Cukrowska et al., 2017). Fragments of gluten can stimulate an autoimmune reaction that leads to damage in the intestines. Individuals can experience a different, innate immune reaction that causes gastrointestinal discomfort. Also, gluten is part of a broader group of wheat proteins that incites an immediate allergic response in some people.

There are various conditions related to gluten, including celiac disease, non-celiac gluten sensitivity (NCGS), and wheat allergy (Dale et al., 2018). These conditions have adverse reactions to gluten but differ in their underlying mechanisms and clinical presentation. Individuals with celiac disease have an adverse immune response when they consume gluten. This response harms their small intestine, leading to problems like diarrhea, anemia, and fatigue. For individuals with celiac disease, avoiding gluten in their diet is crucial to manage the condition. While NCGS shares some symptoms with celiac disease, it is a distinct condition. Unlike celiac disease, NCGS does not trigger an autoimmune response or harm the intestines. People with NCGS may experience gastrointestinal distress and fatigue, which can be managed with a less restrictive GF diet than celiac disease. Individuals with a wheat allergy experience an immune response to wheat proteins, notably gluten. This reaction manifests in symptoms from mild dermatological irritations such as urticaria (hives) to severe, potentially fatal anaphylaxis. Complete avoidance of wheat products is necessary for managing wheat allergy.

The field of research dedicated to discovering new treatments for gluten-related disorders is advancing quickly. Researchers are currently investigating various strategies, including enzymatic degradation, where supplements containing enzymes like prolyl endopeptidases (PEPs) are being explored to help break down gluten in the system during unintended consumption (De Lourdes Moreno Amador et al., 2019). It is essential to highlight that these enzyme supplements are not meant to substitute for a GF diet. Research also focuses on immunomodulation; while the vaccine Nexvax2, designed to desensitize the immune system to gluten, has been discontinued (Tye-Din et al., 2023), exploration of alternative vaccines and

immunotherapies continues. Drug therapies such as larazotide acetate are being studied for their ability to decrease gut permeability and adjust the immune system's reaction to gluten (Hoilat et al., 2022). Exploration into manipulating the gut microbiome through probiotics, prebiotics, and fecal microbiota transplant (FMT) is another area showing promise for regulating the microbiome's composition and functionality (Rossi et al., 2023). Furthermore, the advancements in genomics and personalized medicine are seen as a way to customize treatments to individual genetic markers, with HLA typing and precision medicine strategies being particularly significant (Tye-Din et al., 2015). Also, newly explored strategies include using biotech methods such as CRISPR to develop grains utterly free of gluten or less likely to cause immune reactions (Verma et al., 2021). Despite the promise these new treatments bring to the table for managing gluten-related disorders more effectively, at present, adhering to a GF diet is the most reliable treatment method for these conditions.

The key to protecting individuals with celiac disease and gluten sensitivity is effectively identifying gluten in food. This can be achieved through various analytical methods, each with advantages and disadvantages (Osorio et al., 2019). One common approach is immunoassays, which use antibodies to detect gluten proteins. This includes methods such as the ELISA and the LFIA. ELISA can be used in both sandwich and competitive formats, offering various sensitivity levels, while LFIA allows quick and easy-to-understand results that are perfect for immediate testing. Molecular approaches such as the Polymerase Chain Reaction (PCR) are highly sensitive in detecting gluten DNA but require complex equipment. Mass Spectrometry (MS) and High-performance Liquid Chromatography (HPLC) are known for identifying and measuring gluten precisely through their distinct chemical characteristics. However, their use is restricted due to the need for advanced tools and expertise.

This study aimed to develop a precise and highly sensitive method for detecting gluten by creating and validating antibodies that target gliadin. The effectiveness of these antibodies was evaluated using techniques such as ELISA, Western blot, and LFIA that utilizes gold nanoparticles. This LFIA technique is quick, easy to use, and can be widely used to identify food contaminants, including allergens and harmful bacteria.

2. LITERATURE REVIEW

2.1. Gluten Structure and Characteristics

Wheat is a staple food for much of the world's population, with an average yearly consumption of 65.6 kilograms per person, showing its importance as a critical source of carbohydrates and protein (Shewry & Hey, 2015; Erenstein et al., 2022). Among these proteins, gluten is the predominant component (Biesiekierski, 2017). Wheat, barley, and rye all contain a protein called gluten, which is crucial in giving baked goods their texture and quality. This protein comprises two main components – gliadin and glutenin – giving the dough its unique elasticity and structure, which are essential for making good bread and pastries.

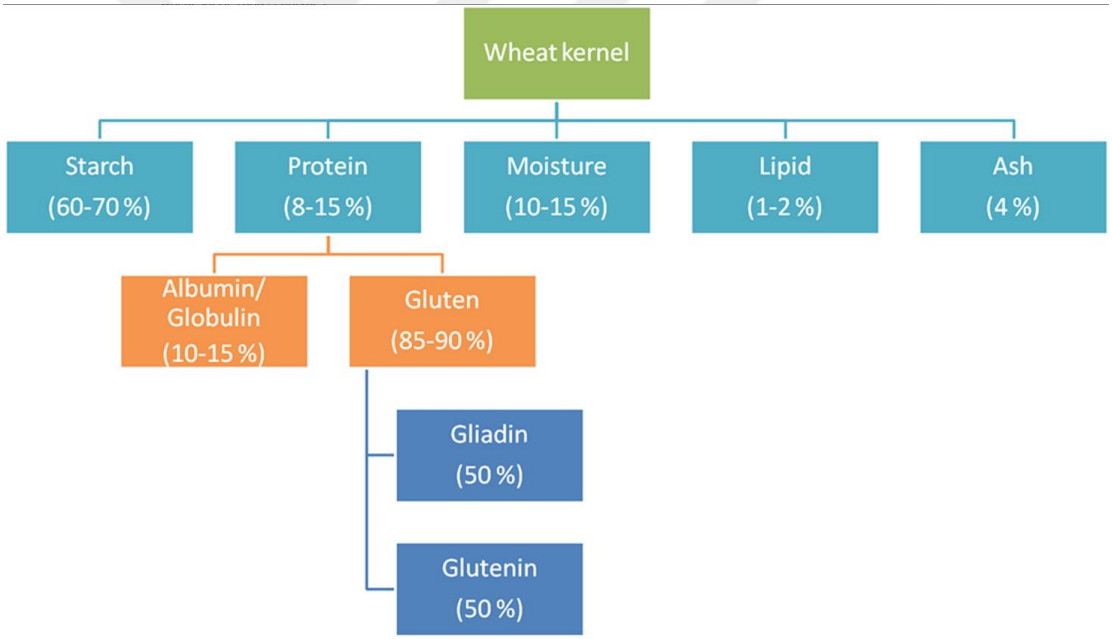

Figure 2.1. Approximate wheat components (Biesiekierski, 2017)

Gliadin, one of the main proteins in gluten, belongs to the prolamin family of storage proteins, which are also found in barley (as hordein) and rye (as secalin). These proteins, which dissolve in alcohol, have differences in their structure due to complex gene expression patterns that affect the formation of disulfide bonds and their solubility (Wang et al., 2020; Yang et al., 2021). Despite these differences, prolamins share common characteristics, including a high proline and glutamine content and repetitive sequences in their structure (Shewry, 2019). Gliadins are single-chain proteins that are crucial in determining the extensibility and ability of dough to resist deformation. Gliadins are individual molecules, unlike glutenins, which consist of several protein

chains. Their high proline and glutamine content gives them distinct properties that affect the texture of the dough and makes them hard to digest fully. These amino acids are essential for interacting with water and forming hydrogen bonds, crucial for developing the dough. Due to this unique structure, digestive enzymes cannot completely break down gliadins, leading to increased intestinal permeability and an immune response (Silano et al., 2009). A hallmark of these proteins is their ability to dissolve in 70% ethanol solutions. They have a notably compact structure due to internal disulfide bonds within the molecule.

Gliadins are classified into four main categories: α-gliadins, β-gliadins, γ-gliadins, and ω-gliadins. This classification is based on their behavior during electrophoresis and their distinct amino acid profiles (Barak et al., 2014). α-Gliadins have a moderate pace during electrophoresis and are crucial for dough extensibility. β-Gliadins are similar to α-gliadins but with less distinct characteristics. γ-Gliadins move more slowly in electrophoresis and contribute to gluten's structural integrity and functionality. On the other hand, ω-gliadins, with the highest molecular weight and the slowest movement in electrophoresis, are mainly associated with immune reactions in people with celiac disease.

Gliadins, especially the α variants, are known to pose health risks as they carry amino acid sequences that can cause adverse reactions in people prone to such conditions (Herrera & Dodero, 2021). In individuals with celiac disease, these sequences trigger an autoimmune reaction that leads to inflammation and harm to the small intestine. Specific peptides from gliadins, such as the 33-mer peptide in α-gliadin, are particularly problematic due to their strong immune response and resistance to being broken down by digestive enzymes. Additionally, although it does not involve the same autoimmune response, gliadins are also linked to non-celiac gluten sensitivity, where individuals exhibit symptoms similar to those of celiac disease. Moreover, gliadins can act as allergens, eliciting immune reactions in those with wheat allergies and causing various allergic symptoms. The focus is usually on gliadins, and this is because recent studies suggest that gliadin is the primary protein responsible for triggering the inflammatory immune response in individuals (Chen et al., 2024).

Glutenins are large, complex proteins formed by assembling numerous protein subunits linked together through intermolecular disulfide bonds. Unlike gliadins, they are insoluble in alcohol-water solutions and have relatively higher molecular weights due to their polymeric nature.

Glutenins are categorized by their size as either high-molecular-weight (HMW) or low-molecular-weight (LMW) subunits. They form extensive, interconnected networks within the dough, providing elastic and cohesive qualities crucial for its strength and the resulting bread's volume.

2.2. Overview of Gluten-related Disorders (GRDs)

In recent times, there has been a significant increase in interest in gluten-related disorders (GRD) within both the scientific community and the food industry. This growing interest can be attributed to the increasing global prevalence of GRD, which is estimated to affect around 5% of the population (Taraghikhah et al., 2020). This number is expected to rise as diagnostic methods improve and awareness increases. The development of GRD involves a complex set of mechanisms, which can generally be divided into three main categories: autoimmune, allergic, and neither autoimmune nor allergic (Sabença et al., 2021). Consequently, gluten sensitivity can manifest through various conditions, including celiac disease, wheat allergy, non-celiac gluten sensitivity, dermatitis herpetiformis (DH), and gluten ataxia, which pose significant challenges for those affected. Celiac disease is a condition in which the consumption of gluten triggers an autoimmune response, leading to damage in the small intestine. Its diagnosis is confirmed through antibody tests and an intestinal biopsy. The condition is managed by strictly avoiding gluten. DH, characterized by itchy skin rashes, is commonly associated with celiac disease and is treated with a GF diet and sometimes medication. Gluten ataxia, which impairs coordination, can be diagnosed with antibody tests, and symptom improvement has been noted with a GF diet. Non-celiac gluten sensitivity mimics the symptoms of celiac disease without the autoimmune component. It is diagnosed by ruling out other conditions, and a GF diet is recommended for management. Wheat allergy is an immune system reaction precisely to wheat proteins, identified through allergy testing, with avoidance of wheat as the primary treatment. For all these conditions related to gluten, following a GF diet is crucial for management.

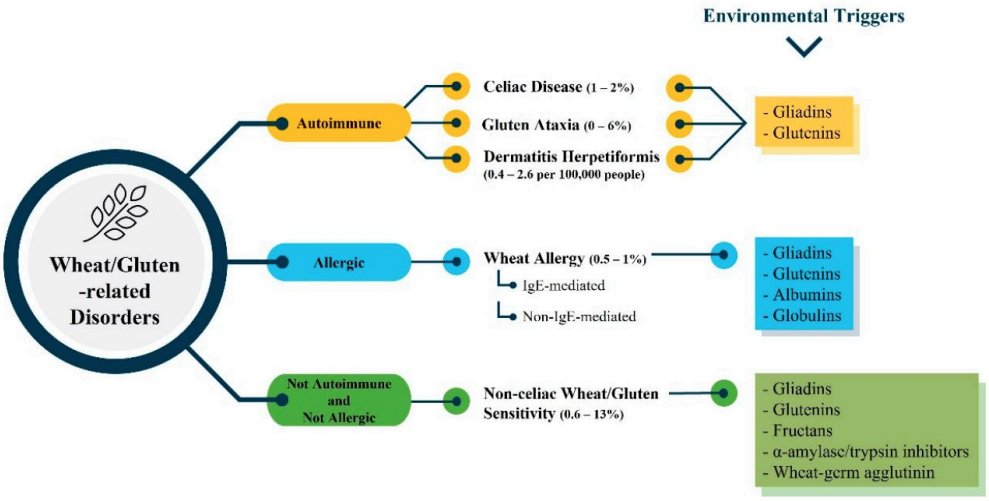

Figure 2.2.1. Gluten-related disorders, their known prevalence and triggers (Sabença et al., 2021)

Celiac disease is a chronic autoimmune condition that affects more than 1.4% of the global population (Singh et al., 2018). It mainly affects the small intestine and develops in genetically susceptible individuals who ingest gluten, triggering an abnormal immune response (Al–Toma et al., 2019). This immune reaction damages the small intestine's lining and can lead to various symptoms. Although new therapies are emerging, a GF diet remains the cornerstone of effective management (Ghazanfar et al., 2023).

The development of celiac disease is a complex process influenced by genetic, environmental, and immune factors. Essential genes in the human leukocyte antigen (HLA) system, such as HLA-DQ2 and HLA-DQ8, are critical to increasing susceptibility to the disease by enabling the immune system to recognize gluten proteins, which triggers an autoimmune response. However, having these genes does not automatically mean a person will develop celiac disease, as they are also found in a significant portion of the general population. Because of this, additional non-HLA genes that affect the likelihood of developing the condition have been identified, such as B cell receptor (BCR) and T cell receptor (TCR) genes (Iversen & Sollid, 2023).

Gluten in wheat, barley, and rye is the primary external trigger for celiac disease. The partial breakdown of gluten proteins, particularly prolamins like gliadin, leads to protein fragments remaining in the gut. These fragments trigger an immune response, causing inflammation of the intestinal lining. People with a genetic predisposition to celiac disease may develop because of viral infections like enterovirus and rotavirus, which can damage the intestinal lining and alter the immune system's response. Additionally, introducing gluten early and in large quantities

during infancy can increase the risk of developing celiac disease.

Recent studies also suggest that disruptions in gut flora (known as dysbiosis) may contribute to the development of celiac disease by impacting the gut's permeability and the immune system's response to gluten. This increased gut permeability, often called "leaky gut," allows gluten peptides to pass through the intestinal barrier more easily, leading to a heightened immune reaction (Aboulaghras et al., 2022). An important factor contributing to increased permeability is the disruption of tight junctions, which normally act as a barrier between epithelial cells (Jauregi-Miguel, 2021).

Celiac disease stems from a complex immune response triggered by the consumption of gluten. Gluten peptides are modified by an enzyme found in the intestinal mucosa, tissue transglutaminase (tTG), through a process called deamidation, converting glutamine residues to negatively charged glutamic acid, which enhances their ability to bind to specific immune receptors (HLA-DQ2 and HLA-DQ8) located on antigen-presenting cells (APCs), such as dendritic cells. Once altered, these gluten peptides are exposed to CD4+ T cells in the lamina propria of the small intestine. This exposure activates the CD4+ T cells, releasing inflammatory substances, including interferon-gamma (IFN-γ) and interleukin-21 (IL-21).

Once activated, T cells stimulate B cells, which then produce specific autoantibodies against tissue transglutaminase (anti-tTG), endomysium (EMA), and deamidated gliadin peptides (Caio et al., 2019). These autoantibodies are essential for diagnosing celiac disease. At the same time, the pro-inflammatory cytokines released by activated T cells drive inflammation and damage within the small intestine. This leads to characteristic tissue changes, such as villous atrophy (shortening of the villi), crypt hyperplasia (increased crypt cell growth), and increased intraepithelial lymphocytosis (higher numbers of lymphocytes within the intestinal lining). Consequently, the damaged intestinal lining cannot properly absorb nutrients, resulting in various clinical symptoms such as diarrhea, weight loss, anemia, osteoporosis, and reproductive issues (Sharma et al., 2020; Aboulaghras et al., 2022). Clearly, celiac disease affects more than just the digestive system. The immune response extends throughout the body, with autoantibodies in the bloodstream and widespread inflammation causing diverse symptoms in various organs.

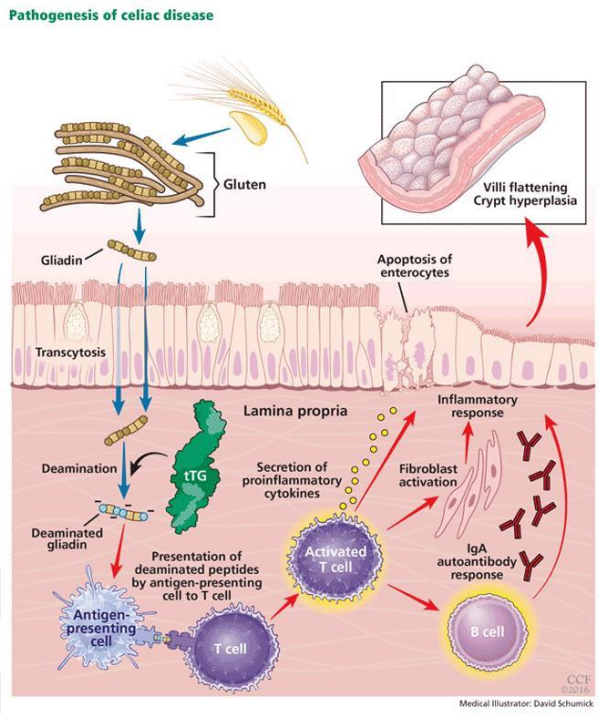

Figure 2.2.2. Simple presentation of celiac disease pathogenesis (Kochhar et al., 2016)

Dermatitis herpetiformis (DH) is a long-lasting skin condition characterized by itching and often associated with celiac disease. It appears as small blisters and bumps, primarily on the elbows, knees, buttocks, and scalp. This condition occurs due to the immune system's response to gluten, where IgA antibodies attack the skin's epidermal transglutaminase, accumulating immune complexes and inflammation (Reunala et al., 2021). Diagnosis of DH requires a thorough clinical evaluation, a skin biopsy demonstrating IgA deposits through direct immunofluorescence, and positive tests for celiac disease antibodies. The mainstays of treatment involve strictly avoiding gluten in the diet for life, which can help clear the skin lesions and relieve symptoms, although complete improvement may take up to two years. Additionally, dapsone can be used for rapid symptom relief but requires careful monitoring for adverse effects.

Gluten ataxia is a condition where the immune system's response to gluten damages the cerebellum, a part of the brain that controls coordination and balance, due to an autoimmune reaction. This disorder can be linked to celiac disease or sensitivity to gluten without celiac disease. It occurs when antibodies produced in response to gluten mistakenly attack the cerebellum tissue, specifically targeting a protein called transglutaminase 6 (tTG6) (Gala et al., 2022). This attack destroys Purkinje cells within the cerebellum, essential for controlling movement. People affected by gluten ataxia may experience symptoms such as difficulty

walking, loss of coordination in arms and legs, slurred speech, and involuntary eye movements. Continued consumption of gluten can worsen these symptoms over time. To diagnose this condition, doctors typically evaluate ataxia symptoms, perform blood tests for specific antibodies, including anti-tTG and anti-tTG6, and use brain MRI scans to identify any shrinkage in the cerebellum. The most effective treatment is strictly and permanently avoiding gluten, which can help relieve symptoms and prevent further damage. In addition to a GF diet, therapies focused on physical, speech, and occupational rehabilitation can also be beneficial in managing the symptoms.

An allergic reaction to the proteins found in wheat, known as IgE-mediated wheat allergy, can cause various symptoms. These can include skin issues such as eczema and hives, digestive problems like vomiting and nausea, respiratory troubles similar to asthma, and, in severe cases, potentially fatal anaphylaxis. This allergy is characterized by the production of IgE antibodies, which lead to the release of histamine and other substances that cause inflammation after exposure to wheat (Cianferoni, 2016). To diagnose this condition, reviewing the patient's medical history, conducting skin prick tests, and measuring blood levels of specific IgE antibodies against wheat proteins is essential. A definitive diagnosis may require an oral food test under medical observation. The primary strategy for managing IgE-mediated wheat allergy is to avoid wheat and its derivatives altogether. It is essential for affected individuals to always have an epinephrine auto-injector on hand to manage potential anaphylactic episodes. Medications such as antihistamines, bronchodilators, and corticosteroids may be recommended for symptom relief and management. On the other hand, non-IgE-mediated wheat allergy is characterized by a delayed immune response to wheat proteins through the action of T cells instead of IgE antibodies. Symptoms may not show up for hours or days after consuming wheat and usually impact the digestive system (including chronic diarrhea, and abdominal pain) as well as the skin (such as eczema). To diagnose this condition, healthcare professionals review the patient's medical history, suggest a wheat-free diet, and perform food challenge tests. The primary treatment involves strictly avoiding wheat and providing nutritional support for more severe cases.

NCGS occurs when individuals experience gluten-related symptoms without having celiac disease or a wheat allergy. The exact cause of NCGS is unclear, but it may involve factors such as an innate immune response, sensitivity to gluten or other wheat components, such as amylase-

trypsin inhibitors (ATIs), FODMAPs (fermentable sugars), and fructans (Catassi et al., 2023). Symptoms of NCGS range from gastrointestinal issues such as abdominal pain, bloating, diarrhea, and constipation to non-digestive symptoms like headaches, fatigue, joint pain, and mental health effects like depression, anxiety, and brain fog. These symptoms usually appear shortly after consuming gluten and improve when following a GF diet. Diagnosing NCGS is complex and mainly involves ruling out other conditions. It requires a thorough clinical history, blood tests to exclude celiac disease and wheat allergy, and observing how symptoms respond to a GF diet. Occasionally, a gluten challenge may be performed to confirm the diagnosis.

2.3. Gluten Usage Areas

Due to gluten's unique properties, it plays a critical role in various food industry applications. In baking, the elasticity and strength provided by gluten are essential for achieving the desired rise and texture in bread, pastries, and cakes. It also contributes to the chewiness and firmness characteristic of pasta products. When brewing beer, gluten aids in fermentation and enriches the flavor profile. Moreover, gluten is used as a meat substitute known as seitan, prized for its high protein content and similarity to meat's texture. Its thickening properties make it a popular choice for enhancing the consistency of sauces and soups. As a food additive, gluten plays multiple roles; it acts as a binder to keep ingredients together, stabilizes mixtures to prevent separation, and helps extend product volume and shelf life, thus boosting production efficiency (Biesiekierski, 2017). Gluten also enhances the chewiness of candies, the crunchiness of snack foods, and the distinct shape and texture of pretzels. Beyond its culinary applications, gluten is utilized in other sectors as well. It is found in cosmetics and personal care items as a conditioning or texturizing agent, serves as a pharmaceutical binder, and is included in animal feed as a protein-rich supplement.

Gluten is found in many food products, and the potential for contamination and errors in labeling poses challenges for people who follow a GF diet (Comino et al., 2012). The extensive use of gluten in various food items and production methods presents a significant risk of cross-contamination. It is especially essential for individuals with celiac disease or gluten sensitivity, as even small amounts of gluten can cause adverse health effects. Leading health and food safety organizations, including the Codex Alimentarius Commission of the WHO, the European Commission (EC41/2009), and the U.S. Food and Drug Administration (FDA), have established

that the maximum allowable gluten content in foods claiming to be GF must not exceed 20 ppm (FDA, 2013; Al-Toma et al., 2019). It is crucial to ensure the accuracy of food labels and implement strict measures to prevent cross-contamination to safeguard the health of those sensitive to gluten. The extensive usage of gluten in various industries underlines its importance but also brings to light the difficulties experienced by those who must avoid it. This has led to an increased demand for GF alternatives and ongoing research into the health implications of gluten consumption.

2.4. Treatment Strategies for Gluten-related Disorders (GRDs)

Research into gluten-related disorders is an evolving field with continuous efforts to deepen understanding and generate novel treatment approaches. The focus is particularly sharp on celiac disease, given its autoimmune characteristics and the necessity for individuals to adhere to stringent dietary guidelines for life.

One exciting area of study involves enzyme supplements designed to decompose gluten proteins in the gut before they can initiate an immune reaction. Early-phase clinical studies have revealed optimistic results for a dual protease formula known as ALV003, which appears to effectively break down gluten and lessen intestinal damage in celiac patients following gluten exposure (De Lourdes Moreno Amador et al., 2019). Additionally, AN-PEP, an enzyme originating from a specific fungus, has been shown to disintegrate gluten within the stomach successfully and small intestine, offering relief for those with gluten sensitivity.

Investigations into immune modulation therapies are also underway to alter the immune system's reaction to gluten. This could reduce the inflammation and tissue damage prominent in celiac disease. Latiglutense is under review for its dual potential in diminishing inflammation and mucosal harm and acting as an enzymatic agent (Murray et al., 2022). Nexvax2, a peptide-based vaccine, is another innovative approach aiming at condition-specific immune cells that play a role in the autoimmune aspect of celiac disease, striving for a state of immune tolerance to gluten (Tye-Din et al., 2023). Nexvax2 is a treatment to reduce the immune system's response to gluten peptides. It is designed for individuals with specific HLA-DQ2 genes commonly found in patients with celiac disease. Although there have been encouraging outcomes from clinical trials, establishing a durable tolerance has proven to be a significant hurdle.

Research on microbiome-based treatments is primarily focused on using gut bacteria to manage celiac disease. Ongoing research aims to identify probiotic strains that help break down gluten and improve intestinal health. Another method being investigated is fecal microbiota transplantation (FMT), where gut bacteria from healthy individuals are transferred to those with celiac disease to create a healthier microbiome and potentially reduce symptoms (Rossi et al., 2023). Another experimental therapy is oral tolerance induction, which involves gradually giving small amounts of gluten to the patient. The goal is to retrain the immune system to tolerate gluten through controlled escalation of gluten consumption.

Research into chemical modifiers is also advancing. These agents have the potential to alter gluten in a way that reduces the likelihood of causing an immune reaction or interfering with the mechanisms that trigger immune responses. Antagonists of zonulin, a protein that increases intestinal permeability, are being studied; reducing permeability might decrease the gluten-induced immune reaction (Hoilat et al., 2022). One example of these efforts is larazotide acetate, a drug targeting the zonulin receptor, which has shown promising results in clinical tests by alleviating symptoms and decreasing intestinal permeability in celiac patients after gluten exposure.

Genetic and molecular biology advancements are creating new possibilities for addressing gluten-related conditions. Techniques such as CRISPR/Cas9 promise to modify genes associated with celiac disease and gluten intolerance, although this research is still in its early stages (Verma et al., 2021). Similarly, RNA interference (RNAi) technology aims to deactivate specific genes in the immune response to gluten.

Although progress is being made, the treatment of gluten-related disorders continues to be difficult due to the differences in sensitivity and immune reactions to gluten among individuals. New treatments show potential and bring hope, yet they require extensive research on their long-term safety and effectiveness and must be cost-effective for general use. The novel approaches could diminish the need for a stringent GF diet, enhance the quality of life, and even lead to a cure for conditions such as celiac disease. Ongoing research is crucial to making these treatments available to patients. Recent advancements in treatments provide hope for better control of gluten-related disorders, but adhering to a GF diet remains the most reliable approach for treatment.

2.5. Gluten Detection Techniques

The detection of gluten is crucial for ensuring the safety of GF products, particularly for individuals with celiac disease or gluten sensitivity. Various methods are available to identify gluten in food and the environment, including traditional laboratory techniques and modern rapid tests (Osorio et al., 2019).

The ELISA is the most used method due to its accuracy and ability to differentiate gluten. There are different types of ELISA, including Sandwich and Competitive ELISA. Sandwich ELISA uses two antibodies to capture and identify gluten proteins, making it highly precise and capable of detecting gluten at very low levels (parts per million, ppm). It is considered the industry standard. Competitive ELISA is used when gluten proteins are somewhat broken down, and although it is less sensitive than Sandwich ELISA, it can help detect gluten in hydrolyzed products.

LFIAs are quick test strips that provide results within minutes. They are convenient for on-site testing but generally have lower sensitivity than ELISA. LFIAs capture gluten proteins on a membrane and display a visible signal like a colored line.

The PCR technique detects gluten by amplifying DNA sequences associated with gluten-containing grains such as wheat, barley, and rye. While PCR is highly accurate, it does not measure the actual amounts of gluten protein but identifies the DNA from these grains, which helps pinpoint sources of contamination.

Mass Spectrometry (MS) is a sophisticated method that detects gluten peptides by analyzing their mass-to-charge ratios. MS offers precise information about gluten proteins, including confirming gluten presence and identifying specific peptides. This technique demands specialized equipment and expertise.

High-performance Liquid Chromatography (HPLC) sorts gluten proteins according to their physical and chemical properties. When used alongside MS, it delivers extensive data about the composition of gluten. HPLC is predominantly employed in research settings to validate findings from alternative testing methods.

Biosensors are emerging as a rapid and sensitive method for detecting gluten. These devices utilize biological molecules, such as antibodies or enzymes, to recognize gluten proteins. They can be combined with electronic systems for immediate monitoring, highlighting their potential for future applications.

Factors such as sensitivity, specificity, speed, cost, and intended use influence the choice of detection method. ELISA and MS are notable for their sensitivity and specificity. LFIAs provide immediate results but may differ in sensitivity compared to ELISA or MS. Regarding cost, LFIAs, and some ELISA kits are generally more affordable than MS. For routine testing within the food industry, ELISA is commonly preferred, while research settings might opt for MS or HPLC. Food industry professionals prefer immunological tests because they offer enough sensitivity and specificity, quick results, user-friendliness, and affordability (Jing-Qing et al., 2019; Wieser et al., 2021; Momeni et al., 2022). The R5 Competitive ELISA, the industry standard since 2013, is a prime example of such assays (FDA, 2013; FDA, 2020). On the other hand, LFIA is known for its simplicity and the lack of additional equipment required (Momeni et al., 2022), making it ideal for end-users. Several compact LFIA kits have been developed, as highlighted in research by Jing-Qing et al. (2019) and Hnasko et al. (2021).

It can be challenging to identify gluten due to matrix effects, where components in food can interfere with its detection. Developing validation techniques for various food categories is crucial. Detecting gluten in processed products, such as hydrolyzed or fermented ones, is difficult because gluten proteins break down into smaller peptides. Standardizing detection methods and laboratories is essential for consistent results, requiring uniform reference materials and procedures.

Looking to the future, advancements in gluten identification include the development of rapid tests that are more sensitive and specific for immediate use, the integration of biosensors and digital technology for continuous monitoring, and the improvement of global standards and reference materials to ensure consistent gluten detection efforts.

These challenges underscore the increasing need for reliable and easy-to-use gluten detection methods that people with gluten-related disorders can employ in their everyday lives.

3. MATERIALS and METHODS

3.1 Production of Anti-gliadin Polyclonal Antibodies

Three antibodies (R1, R2, and R3) that specifically target wheat gliadin were developed in rabbits by LAMPIRE Biological Laboratories, Inc. (Pipersville, US-PA). The antibodies were obtained from three female New Zealand White rabbits aged between 10 and 14 weeks. Pre-immune blood samples were collected from these rabbits to assess prior immune response against gliadin. Then, these rabbits were vaccinated using a combination of wheat gliadin (Sigma-Aldrich, Inc., US-MO; G3375) and Freund's incomplete adjuvant over a 57-day schedule. Subsequent booster shots were administered on day 21 and day 42 after the first immunization. On day 30, 10 ml of blood was drawn for screening of immune response, and on day 50, 20 ml production blood was drawn. ELISA tests were performed on these dates to monitor the concentrations of the antibodies. After the final blood collection (ranging from 60 to 100 mL of serum per rabbit), the antibodies underwent purification using Protein G affinity chromatography.

3.2 Characterization of Anti-gliadin Polyclonal Antibodies

3.2.1 ELISAs

All ELISAs were carried out on 96-well plates (Greiner Bio-One GmbH, AT-4; 655180) under room conditions unless stated otherwise. For indirect ELISAs, a solution of wheat gliadin (Sigma-Aldrich, Inc., US-MO; G3375) was prepared at 200 µg/mL concentration in 70% ethanol. In contrast, sandwich ELISAs utilized a mouse monoclonal anti-gliadin antibody (Sigma-Aldrich, Inc., US-MO; SAB4200864) at a dilution of 1:1000. Both these preparations were diluted and affixed onto the plate wells overnight at 4 ℃ in a 0.1 M carbonate-bicarbonate buffer solution (pH 9.6). Phosphate-buffered saline (PBS, pH 7.4) was used as diluting agent (unless otherwise stated), and PBS with 0.05% Tween-20 (PBST) was used for washings. Blocking was achieved using 1% bovine serum albumin (BSA) (Sigma-Aldrich, Inc., US-MO; A2153) for one hour at room temperature (indirect ELISA) or overnight at 4 ℃ (sandwich ELISA). As the common basic method for detection, antibodies conjugated with horseradish peroxidase (HRP) were used, and the chromogenic substrate employed was 3,3',5,5'-Tetramethylbenzidine (TMB) (Sigma-Aldrich, Inc., US-MO; T4444). Absorbance readings (OD) were taken using a

SpectraMax iD3 Multi-Mode Microplate Reader (Molecular Devices, LLC, US-CA). In indirect ELISAs, the primary antibodies (R1, R2, R3) were incubated for an hour in triplicate wells at various concentrations (1:1000 to 1:64000 in 1:2 ratio decrement), followed by the washing step with 250 µL PBST of each well three times. Next, the procedure continued with the addition and an hour incubation of HRP-conjugated goat anti-rabbit secondary antibody (Sigma-Aldrich, Inc., US-MO; AP307P) diluted 1:3000. In sandwich ELISAs, a reaction buffer made of half PBST and half SuperBlock™ blocking buffer (Thermo Fisher Scientific Inc., US-MA, 37515) was used to dilute grain/food alcohol extracts and wheat gliadin at different concentrations, followed by a one-hour attachment period. Detection was performed using a rabbit anti-gliadin (wheat)-peroxidase-conjugated antibody (1:1000, Sigma-Aldrich, Inc., US-MO; A1052) mixed in reaction buffer and incubated for an hour. Then, plates were washed thrice with 250 µL PBST of each well. Next, all the ELISA plates were incubated in TMB at room temperature for approximately 15 minutes, after which the reactions were stopped using 50 µL of a 1:10 sulfuric acid solution for each well. Finally, the optical density (OD) values at 426 nm were obtained using the SpectraMax iD3 to determine the gliadin content in the sample tests.

3.2.2 Western Blot

The Western blotting procedure involved isolating 20 µg of wheat gliadin (Sigma-Aldrich, Inc., US-MO; G3375) through SDS-gel electrophoresis using a Bolt™ Bis-Tris Plus protein gel (Thermo Fisher Scientific Inc., US-MA, NW04120BOX). The proteins were then transferred to a nitrocellulose membrane using the iBlot™ 2 Dry Blotting System (Thermo Fisher Scientific Inc., US-MA; IB21001). The SeeBlue™ Plus2 prestained protein standard (Thermo Fisher Scientific Inc., US-MA; LC5925) was used as a molecular weight reference. Subsequently, the membrane was incubated for an hour at room temperature with mild shaking in a 3% BSA solution in PBS. After blocking, the membrane was incubated at 4 ℃ overnight with anti-gliadin primary antibodies (R1, R2, and R3) at a 1:1000 dilution in 2.5% BSA diluted with 0.05% PBST. The membrane was then washed three times with 0.05% PBST for ten minutes with gentle agitation. This was followed by a one-hour incubation at room temperature with mild agitation using an HRP-conjugated goat anti-rabbit secondary antibody (Sigma-Aldrich, Inc., US-MO; AP307P) also diluted to 1:1000 in 2.5% BSA with 0.05% PBST. Visualization was achieved by a three-minute incubation with TMB substrate (Sigma-Aldrich, Inc., US-MO; T0565), and the reaction was stopped using distilled water.

3.3 Conjugation of Antibodies with Gold Nanoparticles (AuNPs)

3.3.1 Optimization of Colloidal Gold Conjugation Conditions

All reagents were brought to room temperature. To prepare the custom antibodies (R1, R2, and R3) for attachment, they underwent dialysis using a Slide-A-Lyzer™ MINI device (Thermo Fisher Scientific Inc., US-MA; 88405). A 50 ml falcon tube was labeled for stocking unconjugated colloidal gold. Additionally, five 15 ml falcon tubes were labeled with desired pH levels (7, 7.5, 8, 8.5, 9) for the colloidal gold. Thirty 2 ml microcentrifuge tubes were labeled according to planned antibody concentrations (0.012, 0.0010, 0.008, 0.006, 0.004, 0.002 mg/ml) alongside their corresponding pH levels. Approximately 16 mL of the unconjugated colloidal gold (DCN Dx, US-CA; CG-020) was transferred to the 50 mL falcon tube. This stock was then divided, dispensing 3.12 mL into the five 15 mL falcon tubes labeled with their specific pH values. The pH of these colloidal gold solutions was adjusted using 0.1 M potassium carbonate to reach the desired levels, starting from the initial pH of the unconjugated colloidal gold stock (pH 4.69). The procedure continued with the 0.5 mL of the pH-adjusted colloidal gold distribution from the 15 ml falcon tubes into the corresponding 2 ml microcentrifuge tubes. Various volumes of R1 anti-gliadin antibody (6, 5, 4, 3, 2, 1 µL) were added to achieve the targeted concentrations. The setup involved placing all the tubes in a water bath and shaking them at 150 rpm for 5 minutes, then adding 100 µL of 10% NaCl to each tube and continuing shaking for another 5 minutes to stabilize the conjugation. The samples from these tubes were then transferred into the 96-well plate with 200 µL per well. A 400-700 nm spectrophotometric scan was performed to analyze the samples using the SpectraMax iD3. The optimal conditions for conjugation, showing peak wavelength and absorbance values similar to those of unconjugated colloidal gold, were found at a pH of 9.08 and an antibody concentration of 0.008 mg/mL. These conditions were selected for future applications based on their favorable optical properties.

Next, the process involved conjugation with the gliadin antibody. 10 mL colloidal gold with pH value 9.06 was prepared in a 15 mL falcon tube. 0.08 mL R1 antibody was pipetted into the falcon tube to get 0.008 mg/mL antibody concentration. The tube was let to shake at 150 rpm for 15 minutes in the waterbath at room temperature. Following this, 1 mL of gold conjugate block buffer (DCN Dx, US-CA; 10006) was added to the tube and shaken for 30 minutes in the water

bath. The solution was centrifuged at 14,000 x *g* at 4°C for 30 minutes. After centrifugation, the supernatant was aspirated and discarded, leaving the pellet undisturbed. To this, 0.9 mL of conjugate diluent buffer (DCN Dx, US-CA; 10007) was added to achieve a total volume of 1 mL of the conjugate, and the mixture was gently shaken. The resulting solution was homogeneous and dark red. For evaluation, the prepared solution and unconjugated colloidal gold were scanned at 540 nm using the SpectraMax iD3 to compare their OD values. The unconjugated gold had an OD of 0.246, while the sample had an OD of 2.639, 10.73 times that of the unconjugated gold. According to the manual from DCN Dx kit (US-CA; CKCG-010), a successful conjugate should be homogeneous and dark red and have an OD ten times greater than unconjugated colloidal gold at least.

Following reagent combinations were tested for the optimization of conjugation:
- 0.012 mg/mL R2 anti-gliadin polyclonal antibody (OD (540 nm): 2.882)
- 0.020 mg/mL R3 anti-gliadin polyclonal antibody (OD (540 nm): 2.201)
- Colloidal gold with pH of 9.72, 0.020 mg/mL R3 anti-gliadin polyclonal antibody, and extra 15 minutes centrifugation at 14, 000 x g and at room temperature (OD (540 nm): 4.000)
- Colloidal gold with pH of 9.64, 0.020 mg/mL R3 anti-gliadin polyclonal antibody, and extra 15 minutes centrifugation at 14, 000 x g and at room temperature (OD (540 nm): 4.000)
- Colloidal gold with pH of 9.69 and 0.020 mg/mL R1 anti-gliadin polyclonal antibody
- Colloidal gold with pH of 9.8 and 0.020 mg/mL R3 anti-gliadin polyclonal antibody

Ultimately, the custom anti-gliadin antibody, R3, was attached to colloidal gold nanoparticles using a DCN Dx kit (US-CA; CKCG-010), resulting in a dark red conjugate solution with an OD (540 nm) of 4. The process involved adjusting the pH level of the unconjugated colloidal gold solution and the amount of the R3 antibody. The most effective attachment was achieved by combining the colloidal gold with the R3 antibody at 20 µg per mL of colloidal gold at a pH level between 9.5 and 10.0 for 15 minutes at room temperature. After that, the mixture was blocked using the block buffer for 30 minutes at room temperature. Following centrifugation at 14,000 x *g* at 4°C, the supernatant was discarded, and the resulting conjugate was suspended in the diluent buffer to achieve an OD (540 nm) of 4 for the final product.

3.3.2 Performance evaluation of the conjugate

Colloidal gold conjugates were evaluated through a lateral flow half-strip assay (without a conjugate pad). Constructing the lateral flow half-strip involved utilizing DCNovations' base card, a nitrocellulose membrane (FF170HP; Cytiva), and cotton fibers (Ahlstrom; 222) serving as the sample and absorption pads. To test the strips, one μL of homogenous gliadin solution (1.1 mg/ml; obtained from DCN Dx) and PBS mixtures at varying concentrations (1.1 mg/ml, 0.55 mg/ml, and 0.275 mg/ml) alongside PBS as a control, were added to the bottom portion of each membrane, adjacent to the lower pad. These strips were then allowed to air dry for 20-30 minutes. A mixture of 32 μL of conjugated antibody and 160 μL of PBS as a diluting agent was prepared. From this 192 μL mixture, 20 μL was deposited onto each strip's upper pad. Following this, an initial attempt was made to facilitate the conjugate flow using a surplus of PBS, but it failed. Eventually, adding 40+40 μL of PBST (0.1%) as a flow medium to each strip brought outcomes.

Following combinations had been tried before the last decision was made, respectively:
- Each lateral flow strip had one μL of the 0.55 mg/mL solution of gliadin in PBS applied to its central section to form the control line. Following this, the strips were incubated at 37°C and left to dry for an hour. Subsequently, 30 μL of a conjugate antibody that had been diluted 1:3 was applied to the top pad of each strip. This was followed by adding 100 μL of PBST (0.1%) as the running buffer. The procedure was conducted at room temperature for one hour, after which the outcomes were visually recorded.
- 30 minutes drying at 37℃, 25 μL undiluted conjugate and 50 μL 0.1% PBST run, and 30 minutes drying at room temperature
- 1 mg/mL gliadin dissolved in 70% and 96% EtOH
- 0.5% PBST use as the running buffer
- Glass fiber (Ahlstrom; 8951) as the sample pad
- 1 μL (1 mg/mL) gliadin dissolved in 70% EtOH, one hour drying at 37℃, 10 μL undiluted conjugate and 50 μL 0.5% PBST run, and 30 minutes drying at room temperature

3.4 The Development of LFIA

The lateral flow strips were constructed using a DCN Dx Lateral Flow Construction Kit (DCN Dx, US-CA; MATL-005). All membrane types included in the kit were tested (FF120HP, CN95, CN140, FF170HP). As such, 1 μL of 0.55 mg/mL of gliadin in PBS was applied to the center of

each lateral flow strip to develop the control lines. These strips were then incubated at 37°C and dried for an hour. Subsequently, 30 µL of a 1:3 dilution of conjugate antibody was dispensed onto each strip's upper pad, followed by 100 µL of PBST (0.1%) as the flow medium. This setup was incubated at room temperature for one hour. After testing different nitrocellulose membranes (Table 3.4.), we chose a 0.2 mm thick membrane called FF170HP (Cytiva) for the final LFIA construction. Glass fiber (Ahlstrom; 8951) was selected as the sample pad. The nitrocellulose membrane (25 mm) was attached to a 60 mm DCN Dx backing card, and both the sample pad and a cotton absorbent pad (Ahlstrom; 222) were placed on the membrane with a slight overlap of 2-3 mm (Fig. 3.4.). To assess the effectiveness of the immunoassay, we tested various dilutions of wheat gliadin on the strips. To establish a hook zone, we deposited duplicate aliquots of wheat gliadin concentrations (1000, 500, 400, 300, 200, 100, and 50 µg/mL) at 1 µL each on the central part of the membrane. We allowed them to incubate overnight at room temperature. Subsequently, we applied 10 µL of conjugate solution to each sample pad and added 50 µL of PBST. Following 30 minutes incubation at room temperature, the strips were screened for formation of a signal.

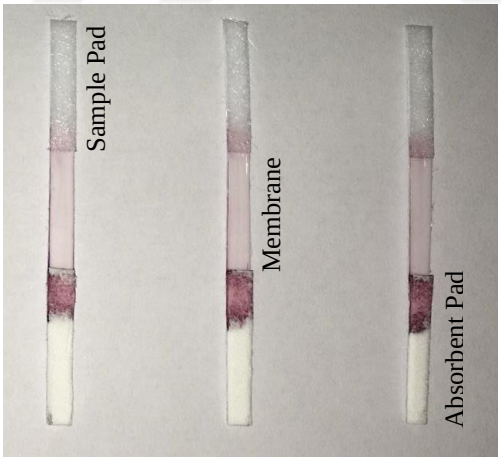

Figure 3.4. Three samples of LFIA strip assembled (negative controls) showing each part – Sample pad, Membrane (Nitrocellulose), Absorbent pad

Table 3.4. Types of cellulose membranes offered in the DCN Dx kit (US-CA; MATL-005)

Item	Vendor	Thickness (mm)	Wicking Rate s/4 cm
FF120HP	Cytiva	0.200	90-150
CN95	Sartorius	0.240-0.270	90-130
CN140	Sartorius	0.225-0.250	95-155
FF170HP	Cytiva	0.200	140-200

3.5 LFIA evaluation

A decision was made to prepare a test line comprised of capture antibodies. A 0.55 mg/mL gliadin-PBS solution was prepared, and 1 µL was placed on each lateral flow strip (lower part) for control line formation. Four types of antibodies were prepared: three custom anti-gliadin polyclonal antibodies (R1, R2, and R3) and one commercial anti-gliadin monoclonal antibody (Sigma-Aldrich, Inc., US-MO; SAB4200864) for test line formation. Each type of antibody was serially diluted in 1:1000 and 1:10,000 ratios with PBS in duplicates, resulting in a total of 16 samples. Following this, 1 µL from these samples was placed on the lateral flow strips (upper part) for test line formation. The strips were then placed in a 37°C incubator for an hour to dry. Subsequently, the stock gliadin (1.1 mg/mL) was diluted with PBS in a 1:200 ratio for running on the strips. This solution placed 20 µL and 50 µL PBST (0.1%) as a running buffer on each upper pad. The solution was allowed to run at room temperature for 30 minutes. The conjugated antibody was then diluted in a 1:6 ratio with PBS. To each upper pad, 20 µL from this solution and 50 µL PBST (0.1%) as a running buffer were placed. The solution was allowed to run at room temperature for 30 minutes.

Following conditions were tested for optimization of the assay conditions:

- The stock gliadin (1.1 mg/mL) was diluted with PBS in 1:200, 1:600, 1:1800, 1:5400, 1:16200 ratio to run on strips.
- Each type of antibody was serially diluted in 1:100, 1:500, and 1:1000 ratios with PBS in duplicates.
- The conjugated antibody was diluted in a 1:3 ratio with PBS.
- Each type of antibody was used undiluted.
- The stock gliadin (1.1 mg/mL) was used undiluted to run on strips.
- The conjugated antibody was used undiluted.

- Pre-mix (undiluted gliadin + undiluted conjugate + PBST) run
- 1 mg/mL gliadin dissolved in 70% and 96% EtOH
- Antibody dilutions in 1:25, 1:50, 1:100, and 1:1000 ratio in ultra-pure water (ddH2O)
- 0.5% PBST use as the running buffer
- 0.5 µL 1:25 R2 antibody in ddH2O and 0.5 µL 1:50 R2 antibody in ddH2O inoculation, overnight incubation, 25 µL undiluted gliadin (in 70% EtOH) and 25 µL 0.5% PBST run, three hours incubation, 10 µL undiluted conjugate and 25 µL 0.5% PBST run, and 30 minutes incubation

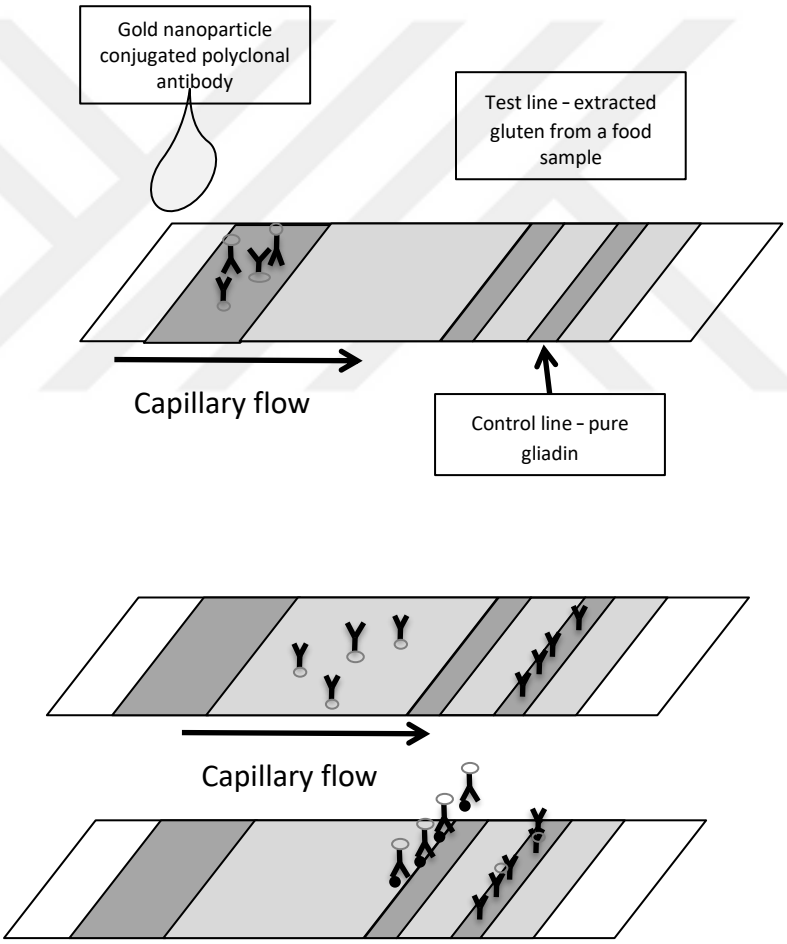

Figure 3.5. The revised lateral flow format for detection of gluten from food samples.

We encountered challenges with the sandwich immunoassay format in our experiment, so we decided to modify the design of our rapid gluten detection test. Our approach involved using the sample as a test line on the membrane and conducting the lateral flow assay using the conjugated polyclonal antibody, as illustrated in Fig. 3.5.

3.5.1 Extraction and Testing of Gliadin Using the LFIA

The method for extracting gluten from food samples was based on a previously described technique involving PBS and ethanol (Güven & Azizoglu, 2023). To extract gliadin from flour and various ground food samples, as outlined in Table 4.3, 0.25 g samples were combined with 2.5 mL of PBS and 7.5 mL of 80% ethanol. This mixture was then vigorously shaken for one minute and incubated for one hour at room temperature. Next, centrifugation was performed on 600 µL of the mixture at 2500 x g for 5 minutes, and 400 µL of the clear supernatant was gathered as the extract. This extract was then analyzed using lateral flow strips to evaluate the efficiency of the extraction process. The tested flour included GF, all-purpose, and pastry wheat flour. Additionally, ground food items were acquired from a local supermarket for analysis, including GF pretzels, GF bread, GF coconut cookies, potato chips, whole wheat cookies, biscuits, pretzels, and roasted peanuts. For each sample, two strips were tested. A 1 µL droplet of the extracted solution was applied to the center of the strip to form what is known as a hook zone. The strips were then left to dry overnight at room temperature. The following day, 10 µL of a conjugate and 50 µL of PBST were added to the sample pads. The results were recorded after incubation for 30 minutes at room temperature. In addition, flour and ground sample extracts were cross-checked with sandwich ELISA protocol used in the characterization of the antibodies.

3.6 Statistical Analysis

The data analysis was conducted using SPSS Statistics 23 software. To ensure the reliability of the findings, each treatment condition was repeated at least two times. The differences among treatments were analyzed using a one-way ANOVA test, and a p-value of less than 0.05 was considered statistically significant.

4. RESULTS

4.1 Assessment of the Antibodies through ELISA and Western Blot

Three anti-gliadin polyclonal antibodies (R1, R2, R3) were produced, purified, dialysed and tested using an indirect ELISA approach. Each antibody was tested in three trials. Although there were minor variations in sensitivities, all antibodies effectively detected purified gliadin, as demonstrated in Figure 4.1.1. The results of the indirect ELISA showed a direct correlation between antibody concentration and OD response. Specifically, the R1 antibody displayed significantly higher reactivity toward purified gliadin than the R2 and R3 antibodies (p-value < 0.05). However, there was no substantial difference in gliadin binding affinity between the R2 and R3 antibodies (p-value > 0.05). For example, at a 1:8,000 dilution, the OD (426 nm) reading for R1 was around 2.6, while for R2 and R3, the readings were approximately 1.5 under the same conditions in the indirect ELISA, as illustrated in Figure 4.1.1. Further analysis of these antibodies through Western blotting revealed that despite being produced in separate rabbits, all three recognized similar epitopes specific to gliadin, as depicted in Figure 4.1.2. Gliadin fragments were mostly aggregated on 35-55 kDa interval. Furthermore, no cross-reactivity of antibodies was detected when tested against proteins relevant to GF food samples.

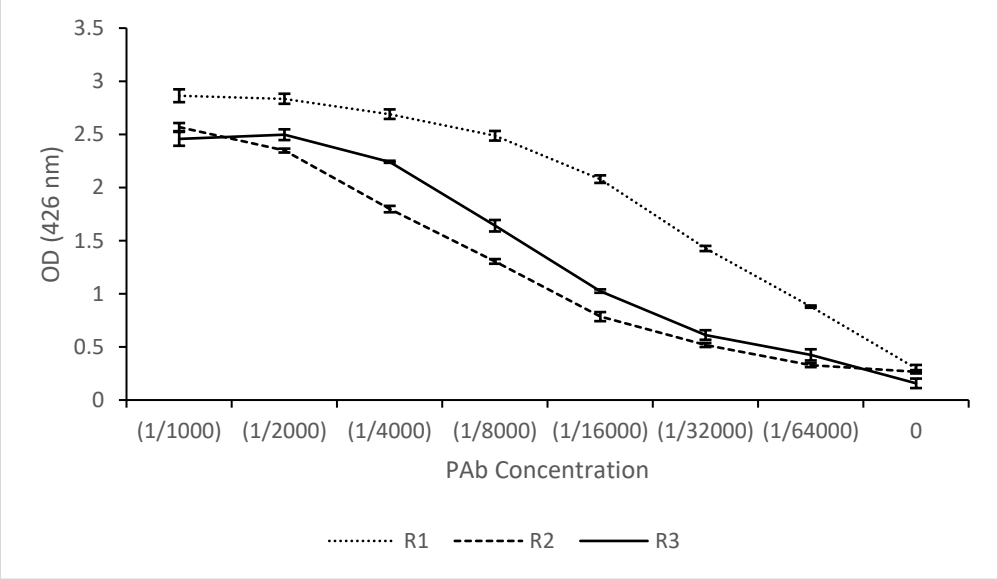

Figure 4.1.1. Optical density (OD) readings at 426 nm for a series of diluted concentrations of the custom anti-gliadin polyclonal antibodies (PAb: R1, R2, and R3). In order to ensure accuracy and reliability, three technical replicates were conducted for each measurement. The average OD values were calculated and represented graphically using error bars to indicate the standard deviations.

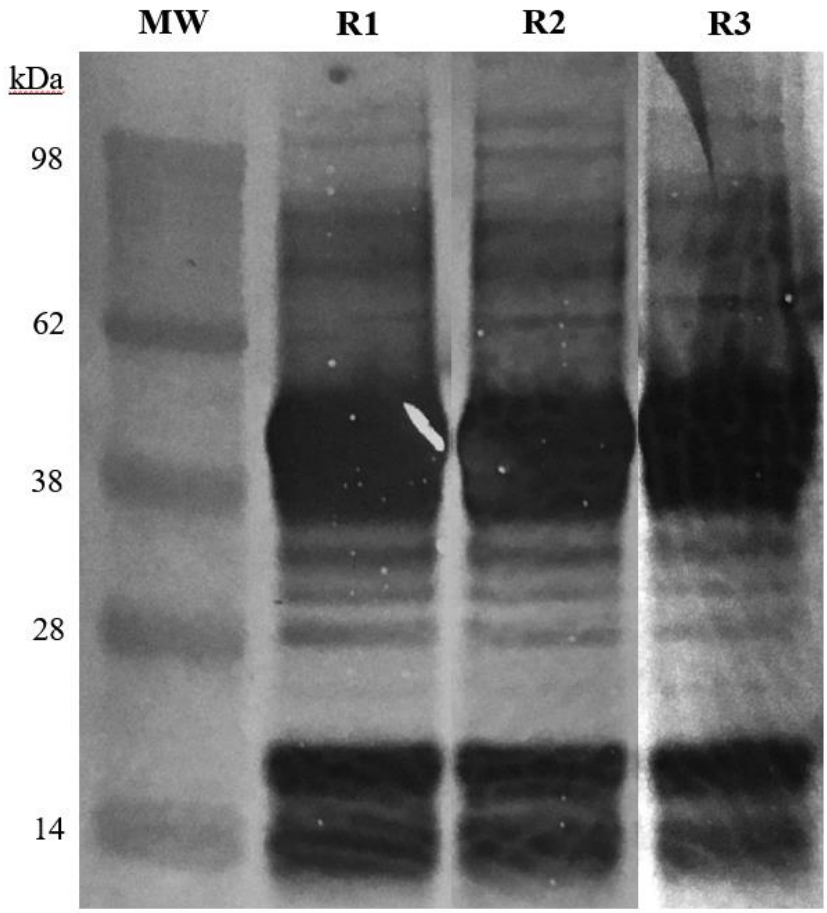

Figure 4.1.2 Western blot analysis of wheat gliadin fractions with custom anti-gliadin antibodies (R1, R2, and R3). Each image indicating different antibodies was taken from triple replicate images (data not shown). MW: molecular weight ladder in kilodaltons (kDa).

4.2 Optimization of the Attachment of Antibodies to Gold Nanoparticles (AuNPs)

The research involved attaching gold nanoparticles to the custom rabbit polyclonal antibodies to create detection antibodies for developing LFIA. The optimal conditions for conjugation were determined through experiments with various pH levels and antibody concentrations. The exploration of pH levels was supported by optimization strategies and antibody concentration data from studies by Jing-Qing et al. (2019) and Hnasko et al. (2021). The evaluation focused on a colloidal gold pH range from 7 to 10, in increments of 0.5, with a pH range between 9.5 and 10 proving to be the most suitable for conjugation. Antibody concentrations ranging from 0.002 mg/mL to 0.020 mg/mL were tested, with the highest concentration (0.020 mg/mL) being the most effective for conjugation.

Following the guidelines of the DCN Dx kit (US-CA; CKCG-010), the conditions that resulted

in the peak wavelength and absorbance values closest to those of the unconjugated gold were chosen for the conjugation process. The stock colloidal gold had a peak absorbance value at a wavelength of 500 nm, while all the conjugated samples had their highest OD value at 550 nm. Since there is no significant difference between the OD value of stock gold at 500 (OD: 0.3824) and 550 nm (0.3735), the OD values at 550 nm were compared. Among various conditions combining these pH levels and antibody concentrations, three conditions stood out due to their unique OD patterns across different wavelengths, as shown in Fig. 4.2. Among these testing conditions, the one demonstrating a trend most closely matching the original colloidal gold (referred to as condition 1) was highlighted as the most successful in the optimization process with the OD of 0.3716 at 550 nm, as recommended by the DCN Dx's kit manual, also noted in Fig. 4.2.

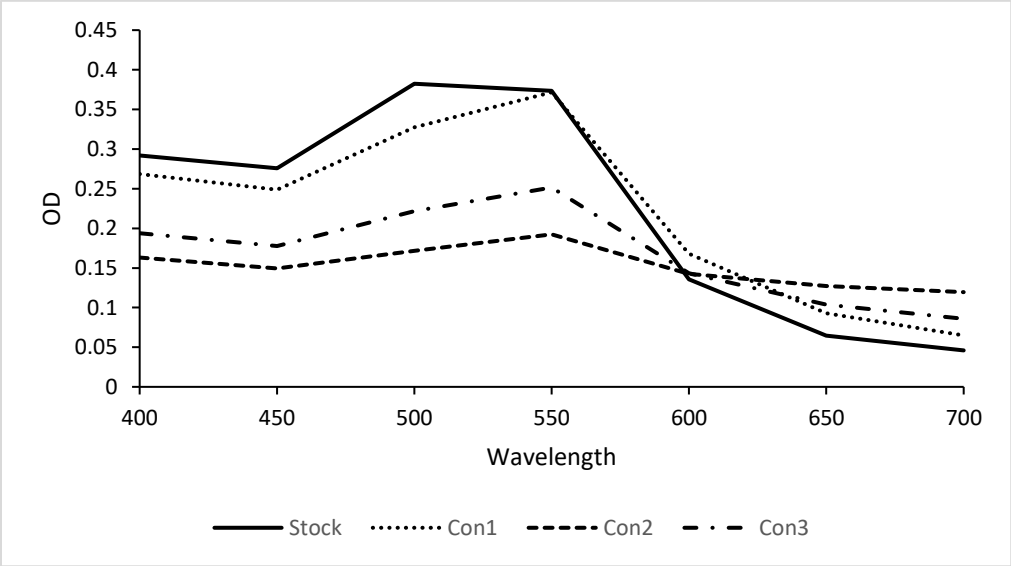

Figure 4.2. Optical density (OD) measurements of the unconjugated colloidal gold stock and various selected conditions for several wavelengths. These three conditions were selected from a multitude of options (data not shown) due to their distinctiveness. Stock: unconjugated colloidal gold, pH = 4.69; Condition 1 (Con1): pH = 9.08, R1 antibody concentration = 8 µg/mL; Condition 2 (Con2): pH = 7.55, R1 antibody concentration = 12 µg/mL; Condition 3 (Con3): pH = 8.03, R1 antibody concentration = 12 µg/mL.

4.3 LFIA Assessment

After evaluating nitrocellulose membranes from two suppliers, Cytiva (FF120HP, FF170HP) and Sartorius (CN95, CN140), we found that the membrane from Cytiva (FF170HP) provided the highest LFIA signal (Fig. 4.3.1). Additionally, we decided to use glass fibers (Ahlstrom; 8951) instead of cotton fibers (Ahlstrom; 222) for the sample pad due to their superior performance in the assay.

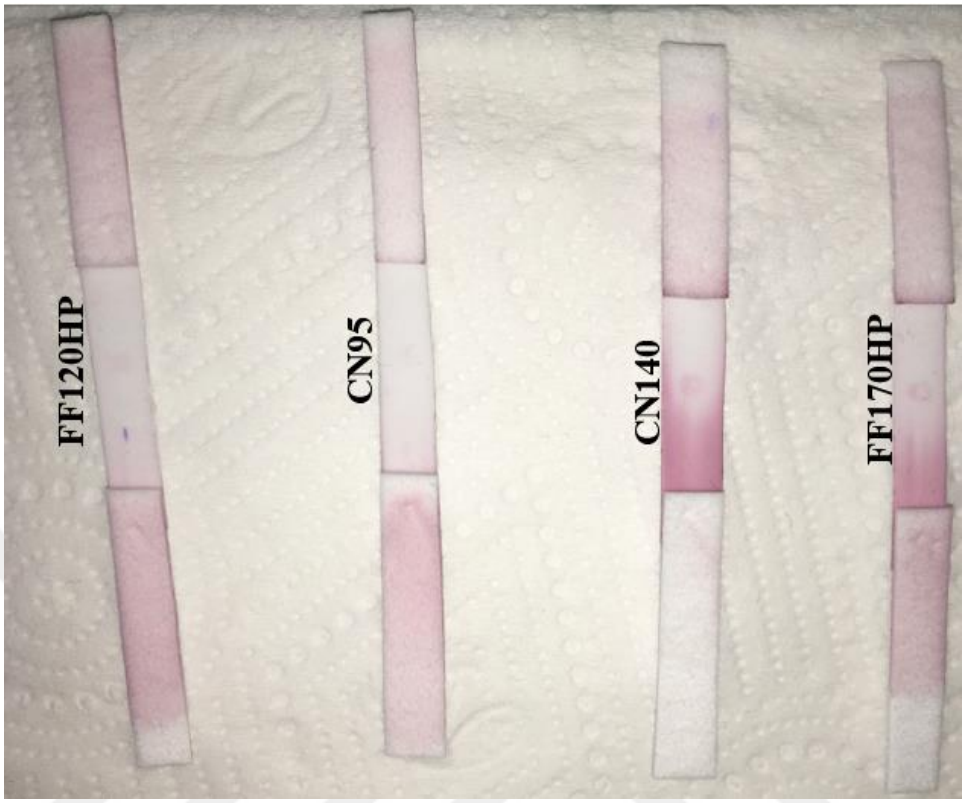

Figure 4.3.1. LFIA evaluation of nitrocellulose membranes from two suppliers, Cytiva (FF120HP, FF170HP) and Sartorius (CN95, CN140).

The effectiveness of our LFIA was evaluated using a dilution series of wheat gliadin (Fig. 4.3.2A) and various sample extractions (Fig. 4.3.2B). The results, depicted in Fig. 4.3.2A, showed a consistent reduction in wheat gliadin concentrations, aligning with the expectations. We detected a discernible signal from a sample containing wheat gliadin at a concentration as low as 50 µg/mL, suggesting that this could be the detection limit of our assay. Furthermore, a correlation was found between the LFIA results (Fig. 4.3.2B) and those obtained from sandwich ELISA for the tested samples (Fig. 4.3.3), consistently indicating the presence of gluten. Although their signal intensities do not match the LFIA results, the results from the sandwich ELISA confirmed the presence and absence of gliadin, which correlated with the LFIA results. Samples with high OD values (>1.5 at 426 nm) contained gliadin, such as all-purpose wheat flour, whole wheat cookies, biscuits, pretzels, and pastry wheat flour. Conversely, samples with low OD values (<1 at 426 nm), such as GF pretzels, GF bread, GF coconut cookies, GF flour, potato chips, and roasted peanuts, did not contain significant amount of gliadin. Consistent results were also found regarding the presence of gluten, as reported on the packaging of flour and food samples, as well as the findings from LFIA and sandwich ELISA tests (Table 4.3). Specifically, being on the upper

side of Fig. 4.3.2B, signals from pastry wheat flour (high), all-purpose wheat flour (high), the whole wheat cookie (medium), biscuit (medium), and pretzel (low) were reported. On the other hand, no signals were recorded from samples on the lower side of Fig. 4.3.2B, which are GF pretzels, GF bread, GF coconut cookies, GF flour, potato chips, and roasted peanuts.

No test line could be obtained on LFIA membranes despite the efforts outlined in section 3.5. A successful test line should include capture antibodies, captured analyte, and detector antibodies. Our analyte (gliadin) and detector antibodies (separately or together) were always stuck at the beginning of the capture antibodies zone; hence, no signals were observed.

Table 4.3. Comparison of gluten presence indicated on packages of flour and food samples and detection test results by LFIA and sandwich ELISA. N: no; Y: yes; NEG: negative; POS: positive

Sample	Gluten Indication	Test Result
GF Pretzel	N	NEG
GF Bread	N	NEG
GF Coconut Cookie	N	NEG
GF Flour	N	NEG
All-purpose Wheat Flour	Y	POS
Potato Chips	N	NEG
Whole Wheat Cookie	Y	POS
Biscuit	Y	POS
Pretzel	Y	POS
Roasted Peanuts	N	NEG
Pastry Wheat Flour	Y	POS

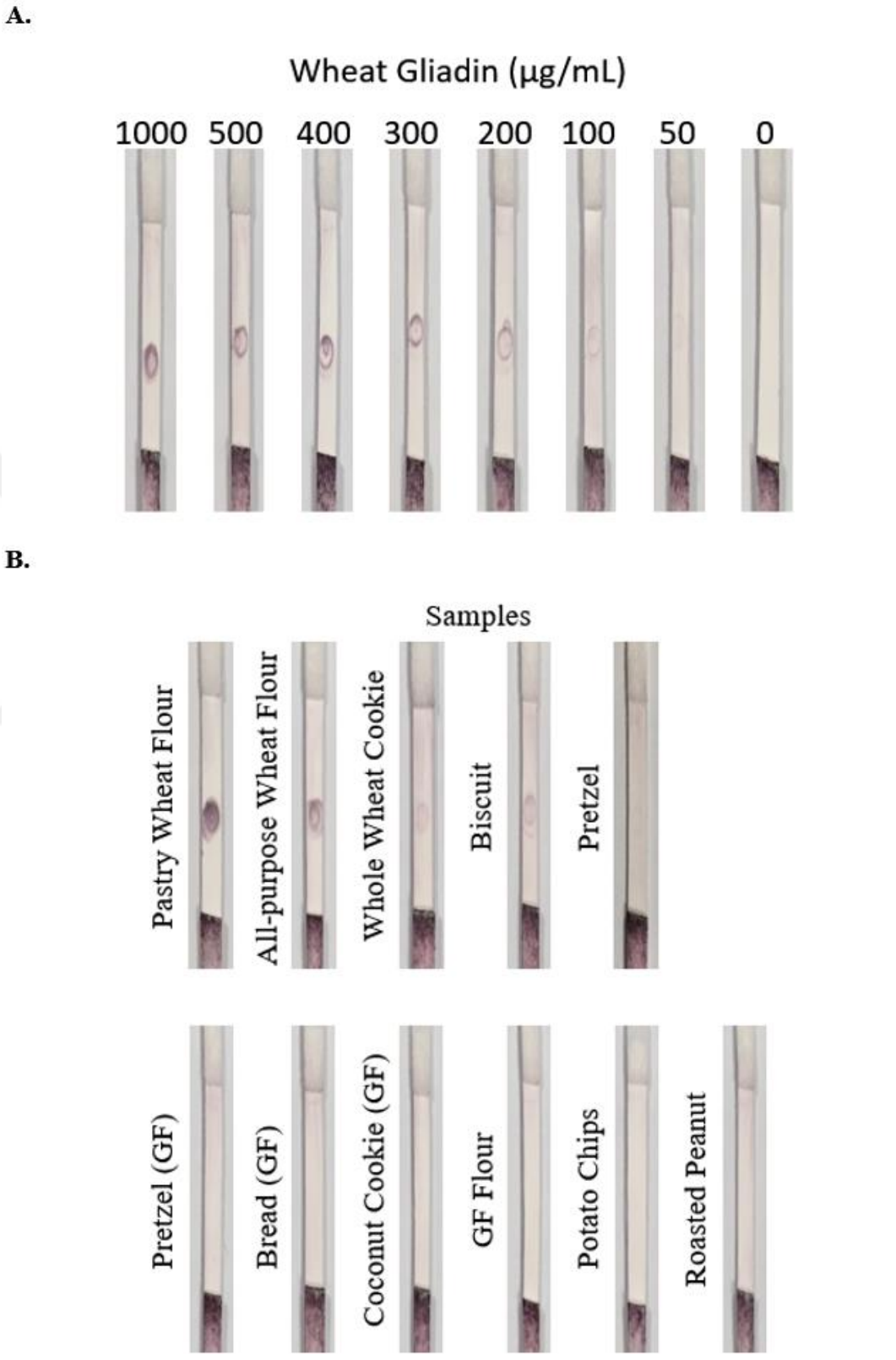

Figure 4.3.2. Detection of wheat gliadin by LFIA. Hook zones containing wheat gliadin were reported to be visible on strips. The observation of sample pads (up), membranes (middle), and wick pads (bottom) is possible. Decreasing detection signal on wheat gliadin dilution series was reported for 1000, 500, 400, 300, 200, 100, 50, and 0 µg/mL concentrations (A). Extracted wheat gliadin detection from flour and food samples (B). GF: Gluten-free.

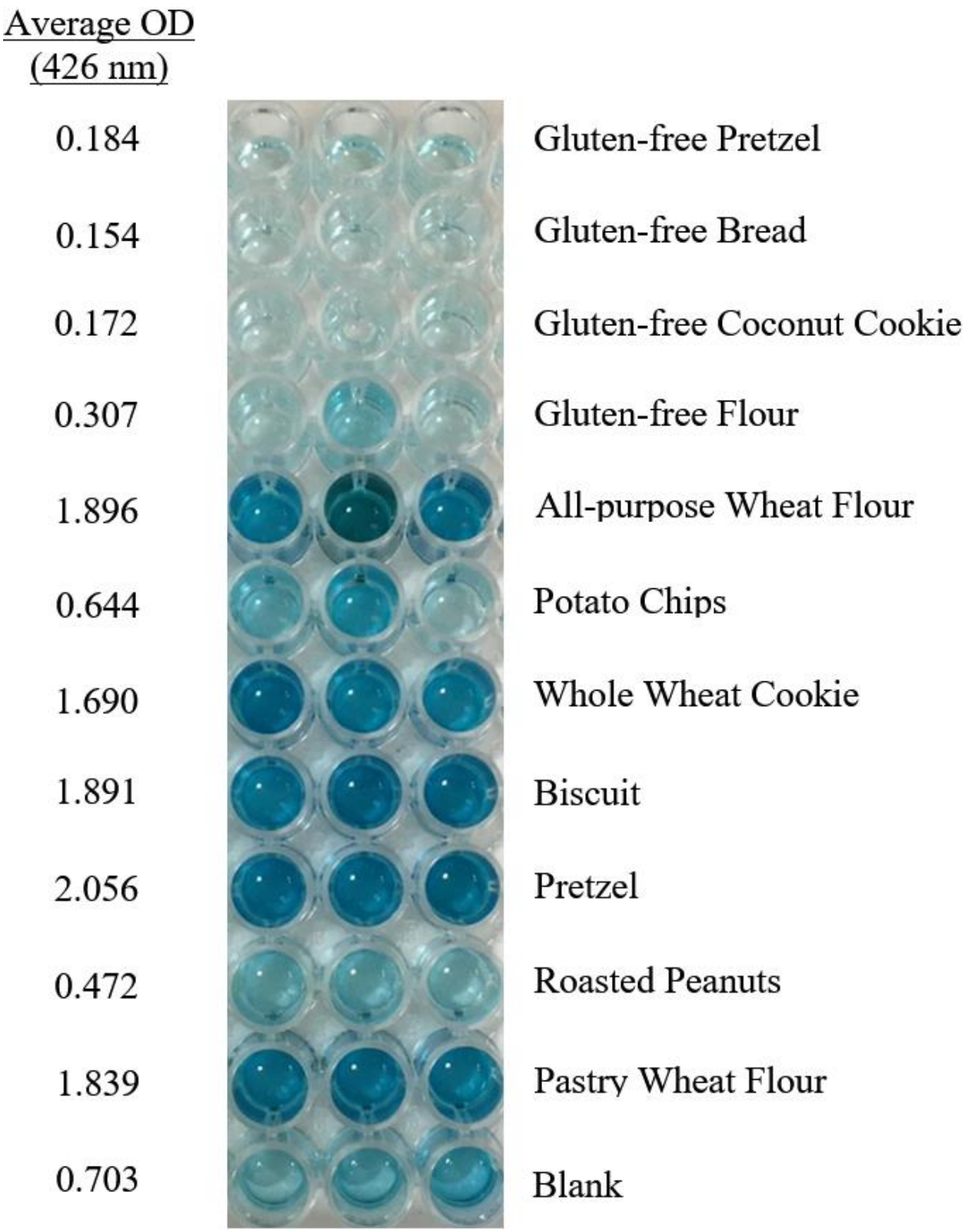

Figure 4.3.3. Triplicate sandwich ELISA results from flours and grounded samples regarding gliadin presence. High OD values (>1.5) represent gliadin presence.

5. DISCUSSION

Testing for gluten has crucial implications for individuals with gluten intolerances, as well as for verifying the authenticity of GF products and upholding regulatory standards. These tests are essential for people with celiac disease or gluten sensitivities to avoid unintended consumption of gluten and related health issues. They also enable consumers to make well-informed decisions about their dietary choices and trust the safety of their food. In the food industry, these tests are critical for quality control, ensuring products live up to their GF claims and minimizing the risk of cross-contamination to maintain consumer confidence. Regulatory agencies rely on these methods to enforce food labeling laws and to ensure product safety. Moreover, these tests are valuable for scientific research and diagnosing gluten-related disorders. Their compact, user-friendly design and quick results suit various settings, including home kitchens, dining establishments, and travel. Rapid gluten detection tests are instrumental in promoting better health outcomes, guaranteeing product reliability, and assisting with regulatory compliance, benefiting consumers and the food industry.

When deciding between monoclonal and polyclonal antibodies for immunoassay applications, it is vital to consider their advantages and disadvantages. Monoclonal antibodies offer high specificity and consistency by targeting a single epitope, but they can be more expensive and take longer to develop. On the other hand, polyclonal antibodies can detect multiple epitopes, providing greater sensitivity and a quicker, more cost-effective production process. Nevertheless, there is a higher chance of cross-reactivity for the same reason. This cross-reactivity happens due to structural similarities and amino acid sequence alignments among proteins containing linear and non-linear epitopes, allowing antibodies to bind (Sharma et al., 2016). Moreover, given that they are obtained from animals, batch-to-batch differences can occur. In immunoassays, sensitivity refers to detecting small amounts of the antigen, reducing false negatives. It is generally more significant when using polyclonal antibodies. On the other hand, specificity accurately recognizes the intended antigen, reducing false positives. This tends to be superior in monoclonal antibodies. Polyclonal antibodies are beneficial for detecting low levels or different forms of antigens, while monoclonal antibodies are preferred for their precise targeting and minimal cross-reaction. Monoclonal antibodies are often preferred for diagnostic purposes due to their superior specificity, ensuring accurate identification of targets with minimal cross-

reactivity and false positives. They are valued for delivering consistent and reproducible outcomes, essential for test standardization. Additionally, they offer precise quantification in assays, low background interference, the ability for large-scale production, and long-term stability, making them particularly suitable for reliable and precise diagnostic applications. We started developing monoclonal antibodies. However, we observed no immune response in mice injected with gliadin, as they displayed no signs of an immune reaction.

The standard method for generating polyclonal antibodies in rabbits begins with preparing and purifying the antigen. The antigen is then injected into rabbits with an adjuvant to enhance the immune response. Blood is collected from the rabbits through immunizations and booster shots over several weeks. The antibody serum is isolated and purified using protein A/G affinity chromatography. Finally, the antibodies are assessed for their specificity, affinity, and effectiveness against the target antigen. The ELISA and Western blot tests have confirmed that the method effectively produces polyclonal antibodies that target gliadin. Evaluation of these antibodies, including their sensitivity, specificity, and likelihood of cross-reaction, indicated that each one holds fair promise for inclusion in the LFIA designed in this research.

Several crucial steps must be followed to effectively attach antibodies to gold nanoparticles (AuNPs) to ensure successful binding and functionality. There are various methods for conjugation, with passive adsorption and covalent bonding being among the most commonly utilized. The passive adsorption technique involves attaching antibodies to the surface of AuNPs through non-covalent means such as electrostatic forces and hydrophobic interactions (Jazayeri et al., 2016). One key aspect of this method is adjusting the pH of the solution containing AuNPs to promote electrostatic attraction between the charged nanoparticles and the antibodies' charged regions. Optimizing the pH when conjugating antibodies with AuNPs is critically important because of its significant effects on charge, stability, and binding efficiency (Ruiz et al., 2019).
The charge of AuNPs is determined by the stabilizer used in their production. Colloidal gold nanoparticles are typically stabilized with citrate, giving them a negative charge due to the ionization of the carboxyl groups. Thus, colloidal gold nanoparticles usually display a negative surface charge. However, the nanoparticles may exhibit a positive surface charge if different stabilizers are used, such as positively charged polymers or ligands.

Antibodies' isoelectric point (pI) is another crucial factor in pH optimization for conjugation with

AuNPs (Trilling et al., 2013). The pI represents the pH level at which the antibody has no net electrical charge, impacting its solubility and interactions with AuNPs. The pI for polyclonal antibodies is generally within the pH 6-8 range (Lea & Gross, 1992). Therefore, when adjusting the pH for conjugation, it is essential to select pH levels that will charge the antibodies positively or negatively to enable electrostatic solid interactions with AuNPs. For example, the antibodies will have a positive charge at a pH below the pI (e.g., pH 5-6), facilitating their attachment to negatively charged AuNPs. On the other hand, a pH above the pI (e.g., pH 8-9) will result in negatively charged antibodies, possibly necessitating adjustments to the AuNPs' surface charge for practical attachment. By selecting an optimal pH range of 9.5-10 for our conjugation process, we ensured the antibodies were negatively charged, aligning with the positively charged AuNPs.

Incorporating Tween-20 into the buffers used in lateral flow assays significantly improves the assays' performance and reliability. This is achieved by reducing non-specific binding and enhancing fluid movement. As a surfactant, Tween-20 decreases the surface tension, which promotes the uniform distribution of samples and reagents across the assay (Lee et al., 2001). It also aids in stabilizing proteins, preventing them from aggregating and helping maintain their functionality, leading to increased signal clarity and sensitivity of the assay (Heller et al., 2019). Additionally, Tween-20 ensures the membrane remains hydrated, avoiding drying and blockage and facilitating consistent capillary action.

The strip layout in lateral flow tests ensures accurate results by controlling the sample flow and reagent interaction (Omidfar et al., 2023). The sample pad plays a critical role in controlling the release of the sample, conditioning it, and managing the flow rate, significantly improving the assay's performance and reliability. Glass fiber pads offer consistent and controlled flow rates, are chemically inert, and handle higher throughput, making them perfect for quick assays. Choosing nitrocellulose membranes with different wicking rates in lateral flow assays can impact their performance. Membranes with low wicking rates enhance sensitivity due to extended interaction times, making them more effective for detecting analytes at low concentrations.

Several factors could lead to the absence of a signal at the test line in our lateral flow assay. These include inadequate capillary flow, unsuccessful capture antibody immobilization, ineffective release of the labeled antibody, improper membrane drying, suboptimal buffer conditions, and incorrect storage practices. Likewise, several reasons may be why the targeted detection

threshold (20 ppm) could not be reached in our lateral flow test. These include inadequate antibody affinity or specificity, poor reagent and membrane quality, suboptimal sample processing, and buffer conditions.

The distinct signal differences between flours and thermally processed foods result from how heating impacts the solubility of gliadin. This is due to the aggregation and structural modifications that arise from disulfide bond formation during gluten's heat treatment (Marston et al., 2016; Güven & Azizoglu, 2023). Specifically, α/β- and γ-gliadins are prone to denaturation and aggregation when heated, while ω-gliadins are unaffected owing to their lack of disulfide bonds (Wieser, 1998; García et al., 2005). The ethanol extraction method primarily recovers ω-gliadins and only small amounts of denatured α/β- and γ-gliadins (Mothes & Stern, 2003; García et al., 2005), leading to the recommendation of using reducing and disaggregating agents for extracting components from heat-processed foods (García et al., 2005; Svigelj et al., 2017).

6. CONCLUSION and SUGGESTIONS

To summarize, the development and evaluation of three anti-gliadin polyclonal antibodies have shown promising results in terms of sensitivity and specificity. This was demonstrated through both ELISA and Western blot assays. These antibodies do not cross-react with GF food items, which makes them potentially useful. The next step involved fine-tuning the conjugation process of these antibodies with gold nanoparticles for use in LFIAs. This required careful pH levels and antibody concentration adjustment to determine the most effective settings. The LFIA performed reliably, achieving a detection threshold of 50 µg/mL for wheat gliadin, which was consistent with the findings from sandwich ELISA tests. Variations in detection signals across different food samples were observed and linked to how thermal processing influences gliadin's solubility, emphasizing the importance of choosing suitable extraction techniques. Furthermore, carefully selecting membrane types and materials was vital in successfully deploying the LFIA methodology. In conclusion, these results highlight the effectiveness of the custom antibodies and the refined LFIA method in the sensitive and precise identification of gliadin in various food items, providing a valuable resource for gluten content analysis.

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RESUME

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Work Experience

Position	Organisation	Year
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Intern	İstanbul Medipol University Regenerative and Restorative Medicine Research Center (REMER)	2019

Foreign Languages	Name of the Exam	Score
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English	YDS	93,75
English	YÖKDİL	98,75

Project Experience

Project Name	Supporting Institution	Duration
Development of Rapid Detection Tests through Novel Immunoassay Applications and Biological Sample Processing	TÜBİTAK	2021-2023 (Actively involved as a researcher)