FACULTY OF PHARMACEUTICAL SCIENCES

Impact of heat treatment on gliadin digestibility and immunogenic potential.

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A Master dissertation for the study programme Master in Drug Development / Pharmaceutical Care

Academic year: 2023 - 2024

GHENT UNIVERSITY

ABSTRACT

Introduction and objectives: Gliadins, a key component of gluten proteins, are integral to the structure and function of wheat flour. However, for those with celiac disease, they present a notable risk by eliciting an immune response. Understanding of gliadin complex nature is crucial for food processing and celiac disease management. Thermal processing of wheat can modify the allergenic and immunogenic properties of gluten proteins, potentially inducing adverse reactions. The impact of thermal treatment on wheat allergenicity has already been explored. But the influence of the heat treatment on gliadin, a gluten protein known to trigger an immune response in genetically predisposed individuals, has not been explored. The primary objective is to investigate the impact of thermal treatment on gliadin's digestibility and immunogenic potential.

Materials and methods: The experiment involved heating gliadin at various temperatures and assessing the digestibility through *in vitro* digestion. Gliadin underwent both a full (gastric and duodenal) and a partial (gastric) digestion cycle at each temperature. The degree of hydrolysis was determined by the OPA assay, the quantify of solubilized proteins was calculated in the supernatants, gel electrophoresis and size-exclusion chromatography were conducted to explore potential effects on the molecular weight of gliadin digested. Lastly, a peptide analysis was conducted to determine to presence of epitopes associated with celiac disease.

Results: The highest degree of hydrolysis was found at 180°C in the full digestion, then a decrease was observed for higher temperatures. The same trend was seen in the gel: the most intense band was visible at 180°C in the full digestion, for the following temperatures no bands were visible. This suggests that aggregates may be present, which was confirmed with the size-exclusion chromatography. The highest quantify of soluble proteins was observed at 60°C in the full digestion. For the peptide analysis, more epitopes were found in the gastric phase compared to the intestinal phase, only one epitope was present at all different temperatures of both digestion cycles.

Conclusion: According to our findings, the most optimal conditions for gliadin digestion involved heating at 180°C. Following this temperature, a decline in digestibility is observed, likely due to the formation of larger aggregates as the temperature increases. This is supported by the findings of the degree of hydrolysis, gel electrophoresis and size-exclusion chromatography. The peptide analysis indicated that while temperature does not clearly affect the number of epitopes present, it does influence which epitopes are detectable.

SAMENVATTING

Inleiding en doelstellingen: Gliadines, een belangrijk onderdeel van glutenproteïnen, zijn essentieel voor de structuur en functie van tarwemeel. Voor mensen met coeliakie vormen ze echter een aanzienlijk risico door het veroorzaken van een immuunrespons. Inzicht in de complexe aard van gliadine is cruciaal voor voedselverwerking en het medische beheer van coeliakie. Thermische verwerking van tarwe kan de allergene en immunogene eigenschappen van glutenproteïnen wijzigen, waardoor mogelijk allergische reacties ontstaan. De invloed van thermische behandeling op de allergeniciteit van tarwe is al onderzocht. Maar de invloed van de warmtebehandeling op gliadine, een glutenproteïne dat een immunologische reactie kan veroorzaken bij genetisch vatbare individuen, is nog niet onderzocht. Het primaire doel is om de invloed van thermische behandeling op de verteerbaarheid en immunogene potentie van gliadine te onderzoeken.

Materialen en methoden: Het experiment omvatte het verwarmen van gliadine bij verschillende temperaturen en het beoordelen van de verteerbaarheid door middel van *in vitro* spijsvertering. Gliadine onderging zowel een volledige (maag en twaalfvingerige darm) als een gedeeltelijke (maag) spijsverteringscyclus voor elke temperatuur. De mate van hydrolyse werd bepaald door de OPA-test, de hoeveelheid oplosbare eiwitten werd berekend in de supernatanten, gel-elektroforese en size-exclusiechromatografie werden uitgevoerd om mogelijke effecten op het moleculair gewicht van gliadine te onderzoeken. Tot slot werd een peptide-analyse uitgevoerd om de aanwezigheid van epitopen geassocieerd met coeliakie te bepalen.

Resultaten: De hoogste mate van hydrolyse werd gevonden bij 180°C van de volledige verteringscyclus, waarna een afname werd waargenomen. Dezelfde trend werd gezien in de gel: de meest intense band was zichtbaar bij 180°C van de volledige verteringscyclus, voor de daaropvolgende temperaturen waren geen banden zichtbaar. Dit suggereert dat er mogelijk aggregaten aanwezig zijn, wat werd bevestigd met de size-exclusion chromatography. Het hoogste aantal oplosbare eiwitten werd waargenomen bij 60°C in de volledige spijsvertering. Met de peptide-analyse werden meer epitopen gevonden in de maagfase dan in de darmfase; slechts één epitoop was bij elke verschillende temperatuur van beide verteringscycli aanwezig.

Conclusie: Volgens onze bevindingen zijn de meest optimale omstandigheden voor gliadinevertering het verwarmen ervan tot een temperatuur van 180°C. Na deze temperatuur wordt een afname in verteerbaarheid waargenomen, waarschijnlijk als gevolg van de vorming van aggregaten, die groter worden naarmate de temperatuur stijgt. Dit wordt ondersteund door de resultaten van de graad van hydrolyse, gel-elektroforese en size-exclusion chromatography. De peptide-analyse gaf aan dat hoewel de temperatuur het aantal aanwezige epitopen niet duidelijk beïnvloedt, het wel invloed heeft op welke epitopen detecteerbaar zijn.

ACKNOWLEDGEMENTS

Over the past several months, my journey of writing this thesis in Parma, Italy, has been truly remarkable. I am deeply grateful to the incredible individuals who supported me throughout this period.

First and foremost, my sincere thanks go to my promotors, prof. dr. Marthe De Boevre and prof. dr. Tullia Tedeschi, for entrusting me with the opportunity to explore such an interesting topic.

Furthermore, I express my sincere gratitude to my supervisor, dr. Barbara Prandi, whose guidance and expertise have been instrumental in shaping this research. Her insights have continually opened new insights which have contributed to the progress and quality of my work.

I also wish to express my gratitude to Alejandra Muñoz for her invaluable assistance in the laboratory, from day one. Her support not only within the lab but also in navigating life abroad has been indispensable to me.

A special thank you goes out to all the fellow students, PhD students, and post-doctoral researchers in Parma for helping me out when necessary and for creating an enjoyable environment.

Lastly, I want to express my deepest gratitude to my friends and family for their unwavering encouragement and for brightening my time abroad with their visits and support.

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List of used Abbreviations

A	Absorbance
ANOVA	Analysis of variance
BSA	Bovine serum albumine
CD	Celiac disease
DH	Degree of hydrolysis
HLA	Human leucocyte antigen
MHC	Major histocompatibility complex
m/z	Mass-to-charge ratio
OPA	*o*-phtaldialdehyde
PAGE	Polyacrylamide gel electrophoresis
qTOF	Quadrupole time of flight
RT	Retention time
SDS	Sodium dodecyl sulfate
SEC	Size exclusion chromatography
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
SSF	Simulated salivary fluid
T	Transmitance
TG2	Transglutaminase 2
TNBS	Trinitro-benzene-sulfonic acid
TOF	Time of flight

1. INTRODUCTION

1.1 GLUTEN PROTEINS

Gluten is a complex mixture of hundreds of related but distinct proteins. The main gluten proteins are divided into two groups: gliadin and glutenin. These proteins represent up to 80% of the wheat proteins. [1, 2]

1.1.1 Gliadin

Gliadins are one of the major gluten storage proteins of wheat. They account for 40-50% of the total storage proteins and they give wheat their viscous character. Past research has suggested that gliadin plays an important role in determining the functional property of wheat flour. However, toxicity studies have also revealed that gliadins trigger the occurrence of celiac disease in genetically susceptible individuals with human leukocyte antigen DQ2 (HLA-DQ2) of HLA-DQ8 haplotypes. [3]

1.1.2 Classification

Gliadins are a heterogenous mixture of single chained polypeptides soluble in 70% ethanol. Based on their electrophoretic mobility, they can be divided into four groups; α-, β-, γ- and ω-gliadins, where α -gliadins have the fastest mobility. [1, 3]

1.1.3 Structure

The overall structure of gliadins consists of a central domain with repetitive amino acid sequences that are rich in proline and glutamine and two terminal non-repetitive domains which are hydrophobic. This hydrophobic domain contains most of the ionizable amino acids such as histidine, arginine and lysine. The sequence of gliadin proteins is very important as they are the major determinants of the allergenicity and functionality in dough. [3]

Unlike glutenin, gliadins are monomeric. This is because either they lack cysteine residues or because the cysteine residues are entangled in intramolecular disulfide bonds. [1]

1.1.3.1 α- and β-gliadins

These two types of gliadins proteins have a similar primary structure consisting of 250 and 300 amino acids. The sequence consists of the N-terminal domain with five residues, a repetitive central domain with 113 to 134 amino acids rich in proline and glutamine and the C-terminal domain of 144 to 166 amino-acids. The protein structure is stabilized by the disulphide bonds formed between the cysteine residues. Because of their common features, α- and β- gliadins are considered a single gliadins type. [1, 3]

1.1.3.2 γ-gliadins

The γ-gliadin is also formed by three different regions. The sequence starts with a 20-residue signal peptide, followed by 12 residue N-terminal non-repetitive domain, a high variable repetitive domain of 72 to 161 residues, a non-repetitive domain containing most of the cysteine residues and the C-terminal non-repetitive domain. All the cysteine residues form intramolecular disulfide bonds. About 25% of the γ-gliadins contain an uneven number of cysteine residues. The free cysteine left after the formation of intramolecular disulfide bonds forms intermolecular disulfide bonds. [1,3]

1.1.3.3 ω-gliadins

About 80% of the total amino acids in ω-gliadins are proline, glutamine and phenylalanine. This percentage is higher compared to the other gliadins which contain about 50-60%. They may also contain a few or no methionine and cysteine. Because of this low presence, ω-gliadins are not able to produce a disulfide bond. A compact structure of ω-gliadins cannot be observed. They are classified as the S-poor prolamins as their structure lacks sulfur containing amino acids. [1, 3]

1.1.3.4 Function

Because of their structural features, gliadins take part in the development of the gluten network during dough making, through formation of hydrogen bonds and hydrophobic interactions between non-polar amino-acid side chains. These will also interact with the flour lipids. Gliadins are for these reasons important in determining the extensibility characteristic of dough [1]

The rheological and functional properties of gluten are dependent on the ratio of glutenin to gliadin and the interactions of these structures. Each component has slightly different functions crucial in determining the viscoelastic properties and the quality of the product. For example, purified hydrated gliadin contribute more to the viscosity and the extensibility of the dough, whereas hydrated glutenin contribute more to the elasticity and the strength of the dough. [2, 3]

1.1.4 Allergenicity

Gliadin contain peptide sequences, known as epitopes, that are highly resistant to the gastric, pancreatic and intestinal proteolytic digestion in the gastrointestinal tract by which they escape degradation in the human gut. [2]

Celiac disease is an autoimmune enteropathy that develops in genetically predisposed individuals after consumption of wheat gluten and related barley and rye prolamins. Common symptoms include diarrhea, weight loss, poor appetite and abdominal dissension. Currently, the most effective treatment is a gluten-free diet. [1, 4]

In Celiac Disease (CD) patients, peptides that originate from incomplete digestion of gliadins bind to HLA-DQ2 or HLA-DQ8 receptors or antigen presenting cells. Because of the binding, CD4+ T cells response gets activated and release pro-inflammatory cytokines. This leads to atrophy of the small intestinal villi and hyperplasia of crypts. [3]

The native gliadin acts as an antigen against which the genetically susceptible individuals produce an inappropriate T cell mediate immune response. [3]

1.2 CELIAC DISEASE

1.2.1 Epidemiology

Celiac disease is one of the most common autoimmune diseases worldwide, with a prevalence typically ranging from 0.5 to 2.0% in most countries among the general ppulation. However, regions characterized by low gluten consumption and those exhibiting a scarcity of CD-predisposing genes, such as parts of East Asia and sub-Saharan Africa, often fall outside this prevalence range. Celiac disease is more common in females than in males. Although this might be because men are more likely to remain undiagnosed. [5, 6]

Research has shown that the prevalence of celiac disease can change over time. Over the last 5 decades an increasing trend can be observed in some countries (e.g., Italy), probably due to changes in eating habits. The reason for this increase is unknown. Between 1980 and 1990 there were less than 2 per 100 000 people per year diagnosed with coeliac disease. This increases to more than 20 cases per 100 000 people in 2010. However, there are a lot of silent cases, in which the disease is asymptomatic but still damage is occurring to the intestine. Celiac disease is still largely under diagnosed especially in countries with healthcare limited resources. [5, 7]

1.2.2 Causes

1.2.2.1 Gluten

The major environmental factor responsible for the development of celiac disease is gluten. It represents a group of storage proteins present in wheat, rye and barley. These contain a high proportion of prolamins, which are proline and glutamine residues. Gluten proteins triggers the adaptive immune response in celiac disease. The pancreatic and gastric enzymes are unable to fully break down these proteins, due to their usual repetitive sequences. Also, a high proline content leads to poor digestibility, because proline has a cyclic side chain that hinders the activation of proteolytic digestive enzymes. [5]

1.2.2.2 Genetic factors

People with a family history of celiac disease have a 10-15% risk of developing the disease in their lifetime. The most important genetic factors are major histocompatibility complex

(MHC) class II genes. 90% of patients with celiac disease express HLA-DQ2.5. The remaining patients usually express HLA-DQ2.2 or HLA-DQ8. Patients who are homozygous for HLA-DQ2 have a higher risk of developing celiac disease than heterozygous patients. [5, 7, 8]

There are an additional 41 non-HLA-loci associated with celiac disease. These genetic factors contribute a small amount to the disease development, but they are involved in regulating various aspects of the immune system and barrier function. So, these genetic factors could modulate disease presentation and phenotype. [5]

Even tough 30 to 40% of the general population carries HLA-DQ2 or HLA-DQ8, only 3% develops celiac disease. The reason why only 3% and not more develops celiac disease remains an important question under active investigation by researchers. [9]

1.2.2.3 Additional environmental factors

Gluten is the environmental factor to trigger the disease, but other factors may also be involved such as the age for the introduction of gluten. The optimal time of introduction of gluten in the diet if a child has never been tested thoroughly but it is a long-standing practice to introduce gluten at the age of six months. Many pediatricians believe that the introduction of gluten should be delayed in children who have a family risk of celiac disease, this should allow the maturation of the intestinal barrier and the immune response. [10]

Intestinal infections such as rotavirus among infants might change the permeability of the gut. This can lead to the passage of immunogenic gluten peptides through the epithelial barrier. Research have associated this with an increased risk of developing celiac disease. In the last years, the rates of the intestinal infections have not been increased. Thus, these infections are not likely to drive the epidemic of celiac disease. [10, 11]

Studies have evaluated the role of the variables (breastfeeding, age at gluten introduction and infections) in influencing the risk of developing celiac disease. None of these variables had a significant effect in predicting the development of celiac disease. However, more research is necessary to determine if and how these factors can cause loss of gluten tolerance. [10, 11]

1.2.3 Pathophysiology

First, it is important to note that celiac disease in non IgE-mediated. The primary T-cell epitope involved in the immune response is derived from gliadin. This immunopathologic process takes place in five main stages (Figure 1.1). First, the ingested gluten is partially digested by a combination of host and microbial enzymes. Transglutaminase 2 (TG2) deaminated the glutamine residues of the ingested gliadin. Then the modified gliadin is taken up by antigen-presenting cells carrying HLA-DQ2 or HLA-DQ8. This will activate the gliadin specific CD4+ T cells. These cells produce pro-inflammatory mediators such as

interferon-γ and interleukin-12. Interleukin-12 induces a massive release of interleukin-15, this contributes to intestinal lesion. [2, 7, 8]

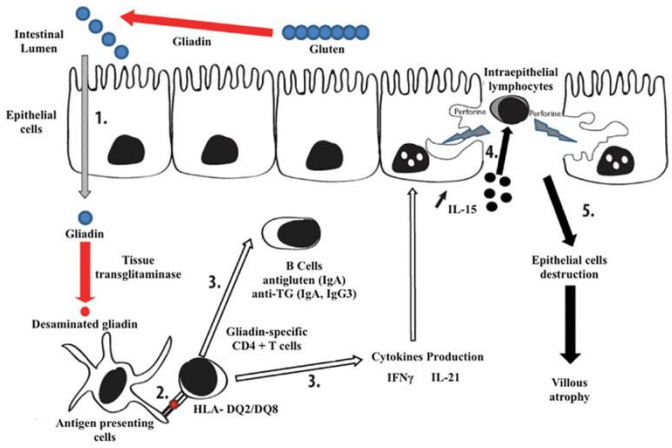

Figure 1.1: Immunopathological process of celiac disease. Gliadin is transformed to deaminated gliadin, then it is taken up by antigen-presenting cell that carries HLA-DQ2 or HLA-DQ8. This will activate specific T cells: these will produce pro-inflammatory mediators. [7]

1.2.4 Symptoms

Celiac disease can present itself at any age with various manifestations. The biggest difference between adults and pediatric patients is that in adults there is a higher prevalence of developing extra-intestinal manifestations and long-term complications. [1]

Depending on the clinical features at the time of diagnosis, celiac disease can be divided into three forms: classical, non- classical and subclinical. [4, 5]

1.2.4.1 Classical

The classic form is more common in children younger than five years. Celiac disease presents itself with chronic diarrhea, poor appetite, weight loss, abdominal dissension, muscle wasting and mood changes. [4, 5]

1.2.4.2 Non-classical

The non-classical presentation of celiac disease is the most common. It is characterized by non-specific intestinal complaints (for example bloating, recurrent abdominal pain, diarrhea or constipation), extra intestinal or other systemic manifestations. [4, 5, 7]

1.2.4.3 Subclinical

The subclinical is a clinically silent form of celiac disease. It can be identified through either screening programmed in the general population or case-finding in risk groups, such as relatives of individuals with celiac disease. [4, 5]

1.2.5 Diagnose

1.2.5.1 Serological markers

The detection of serum autoantibodies has a central role in the diagnostic procedure. Anti-transglutaminase 2 (TG2) antibodies represent the primary auto antigen associated with celiac disease. In individuals on a gluten-containing diet, the presence of IgA class anti-TG2 is highly sensitive and specific for active celiac disease. Screening for these antibodies combined with the measurement of the total IgA serum, to exclude selective IgA deficiency, is the first level screening test for celiac disease. [4, 5, 12]

1.2.5.2 Intestinal biopsy

Small intestinal biopsies are conducted by upper gastrointestinal endoscopy. Multiple biopsies are taken to increase the diagnostic yield of celiac disease: at least two samples from the duodenal bulb and at least four samples from the descending portion of the duodenum. [4, 5]

The typical histological picture of celiac disease shows a severe villous atrophy with crypt hypertrophy and an increase in intraepithelial lymphocytes. [5]

The intestinal damage is classified using the Marsh-Oberhuber classification system, where grade zero is a normal small intestinal mucosa and grade one to three classify the variable degree of damage found in celiac disease. [5]

It is important to be careful when interpreting the results from an intestinal biopsy to avoid over diagnosis or under diagnosis. These are concerns especially for patients with borderline histology abnormalities. [5, 6]

1.2.5.3 Diagnostic criteria

If children meet the following criteria, it might be possible to avoid collecting an intestinal biopsy: they have characteristic symptoms of celiac disease, the level of IgA TG2 is greater than ten times the upper limit and they present homozygosity or heterozygosity for HLA-DQ2 or HLA-DQ8. According to a study this approach can identify children with celiac disease with a positive predictive value of 99.75%. There have been some studies to test this strategy in adults, but because of the use of different serologic tests, it is still too early to recommend a biopsy-free approach in adults. [5, 6]

1.2.6 Treatment

1.2.6.1 Gluten-free diet

Treatment of celiac disease is based on a gluten-free diet. According to guidelines from international regulatory agencies, available gluten-free items must contain less than 20 mg/kg of gluten to remain within the safety threshold. [5, 8]

A gluten-free diet is an intervention that makes remission of the disease possible, including mucosal healing, without any major risks. However, there are studies that have shown that a gluten-free diet can lead to an insufficient intake of some nutrients over the long term. We are talking about fibre, calcium, iron, folate and other vitamins. Gluten-free items can contain more sugars and fat than gluten-containing products. Yet, most studies haven't found any increased risk of obesity or dyslipidemia related to a gluten-free diet. [5]

The complete elimination of gluten from a diet is more complex than it might seem. Gluten-free items, like oats and lentils, can be cross-contaminated with gluten at different steps of the food-processing chain. Gluten is also an ingredient that is often added for its viscoelastic and stabilizing properties, it can be found in many unexpected items such as ice cream and hamburgers. [5, 7]

Gluten is a staple food in many countries, so eliminating this from one's diet can have a negative impact on the quality of life. Especially in a social environment such as eating out at a restaurant can be difficult. [5]

1.2.6.2 Additional therapies

A 2020 meta-analysis investigated the efficacy of probiotics in celiac disease. They concluded that although probiotics can improve the persistent gastrointestinal symptoms in a subset of patients with celiac disease on a gluten-free diet. The overall quality of the evidence was very low. [5, 13]

Many patients with celiac disease regularly consume over-the-counter probiotics because of their persistent symptoms. However, some of these medicines could be contaminated with gluten and thus make their symptoms worse. The pharmacist plays an important role here, they must verify the gluten-free status of the probiotics and every other medicine before giving it to a celiac patient. [5, 7, 13]

1.2.6.3 Follow-up

After a patient is diagnosed with celiac disease, a periodic follow-up is recommended. The patient can be monitored to follow their progress but also to check their wellbeing. Most patients on a strict gluten-free diet show improvement during the first six months of the treatment. Yet it is common to see patients with persistent symptoms, this can be the result

of continuing ingestion of gluten or the emergence of another condition such as lactose intolerance or irritable bowel syndrome. [4, 5, 7]

During a follow-up a dietician should be involved to overview the patient's current nutritional status. They can help analyze eating habits and identity potential factors that affect the gluten-free diet. When a patient has deficiencies and excesses, a dietician can help come up with a solution. [5, 7]

1.3 DIGESTION OF GLUTEN

A normal Western diet includes 15 to 29g of gluten per day. During digestion, the proteins are digested by the pepsin in the stomach, passed to the small intestine and then they are attacked by the pancreatic enzymes. Gluten proteins have a high amount of proline and glutamine; thus they can't be completely hydrolyzed by the human proteases. [2, 8, 14]

Studies have showed that the feces from new-born babies contain proteins that are capable of gluten degradation. These proteins can hydrolyze potential toxic and immunologic peptides derived from gliadin. This leads to the conclusion that the human digestive tract system has the necessary tools to hydrolyse gluten proteins from birth, before starting gluten intake. This suggests that diet and the surrounding environment may affect the early development of the infant intestinal microbiome and therefore the efficiency of the gluten digestion. When sample from babies older than 12 months were analyzed, it was noticed that there is a decrease in the fecal gliadin degradative capacity. This decrease can be related with the changes in the human diet. During the first few months, infants are fed with breast or formula milk. Around the age of 6-9 months foods that contain gluten are included in their diet to replace the milk. The fact that there is a decrease in in the fecal gliadin degradative capacity from 6 months old, supports the hypothesis that a gluten-containing diet may negatively impact the human gluten digestive capacity. [14, 15, 44]

1.4 THERMAL TREATMENT

Wheat is a typical example of a food that can't be consumed without some type of thermal processing, including cooking, baking, etc. The digestibility of proteins can be strongly affected by thermal processing, which induces modifications of the physicochemical and immunological characteristics of the potential allergens of a given food. [15]

Many of the food allergens are stable proteins that are very resistant to digestion by gastrointestinal enzymes or can be digested into high molecular weight peptide fragments which retain IgE binding and T-cell stimulation. Thermal treatments are usually applied to food to improve the microbiological quality or to process the food. Heat treatment of food proteins can induce modifications such as denaturation, hydrolysis of peptide bonds, aggregation by non-covalent and disulphide bonds and reactions with other foods components. Because of this whole range of different reactions, thermal processing can

have a strong influence on the protein allergenicity by either reducing it, by loss of epitopes, or enhancing it, by exposure of hidden epitopes or the creation of new ones. [16]

1.4.1 Temperature

Different processing conditions of the same food may lead to different effects. Different temperatures can lead to variable changes in the protein structure. In general, a temperature of 70-80°C leads to the loss of the secondary structure. Whereas formation of new bonds and rearrangements of disulphide bonds take place at a temperature of 80-90°C. Further at a much higher temperature, 90-100°C, aggregate formation can occur. [16, 17]

1.4.2 Impact

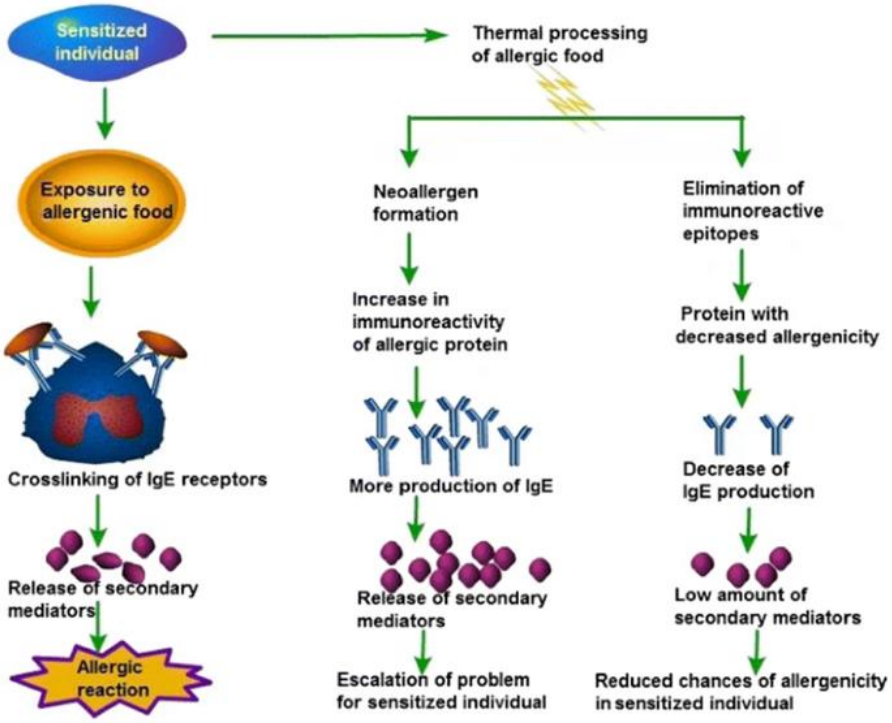

Figure 1.2: The effect of heat treatment. Two things can happen because of thermal processing: neo-allergen is formed which increases the allergenic reaction or immunoreactive epitopes are eliminated which decreases the allergenic reaction. [17]

Food processing affects the structural and allergenic properties of allergens by changing the stability and other physicochemical properties. In most of the cases, the protein digestibility increases, and the heat treatment may also increase the absorption in the gastrointestinal tract. Because of this, the possibility of getting an allergenic response

decrease. However, in some cases, heat processing may reduce the digestibility of an allergen (Figure 1.2). [17]

Thermal treatment can also lead to the formation of neoantigens that are not originally present (Figure 1.2). The creation of these neoantigens may enhance the allergenic reaction in genetically susceptible individuals. [17]

1.4.3 IgE-binding

In addition to celiac disease (described in paragraph 1.2), gluten proteins can also be involved in IgE-mediated allergic reactions.

The antibodies recognize and interact with IgE-binding epitopes, that are present on allergenic proteins. When an allergen cross-links the IgE-antibodies present on mast cells, it degranulates the cell. This results in release of allergenic mediators like histamine. [17]

There are two types of IgE-binding epitopes based on the structural properties: linear and conformational. In linear epitopes, the amino acids are arranged in a linear order in polypeptide chains. These epitopes are generally resistant to heat. While in the conformational epitopes the amino acids are far apart in the primary sequence, they may come together during folding of the polypeptide chains. But these bonds may easily break after thermal processing. This means that after thermal processing different allergens have a different fate which is evident by decreased, enhanced or no change on antigenic behavior. [17]

Research has shown that heat treatment on gluten epitopes doesn't decrease the allergenicity of a protein allergen. However, it is almost certain that it lessens the native structure of a protein. Heat treatments can lead to different allergenicity, even in the same food. It depends on the protein component. [18]

1.4.4 Effect of thermal processing on wheat

Wheat is one of the world's major food sources consumed, cultivated and traded worldwide. It is a product that is usually consumed as an ingredient in bread or pasta, that have already undergone a heat treatment. Heat treatment on wheat has only been studied for wheat allergy. There are no papers that have studied the effect of heat treatment on pure gliadin. [2, 3]

Research has shown that baking does not affect the IgE-binding of wheat allergens as a part of bread compared to the dough. It seems that baking protects the wheat allergens from digestive enzymes by strong molecular linkages different than disulphide bonds or hydrophobic interactions. On the contrary was the IgE-binding potential of heated wheat flour reduced after heating. [16]

Based on clinical studies, it is established that cooked pasta is dangerous for allergic patients, they develop gastrointestinal symptoms. Loss of solubility of wheat proteins is a drastic effect of heat treatment. It has been found that wheat proteins of boiled pasta became insoluble up to 23 and 39% when dried at 110 and 180°C. Because of this extensive heating, protein aggregates were formed. This led to an enhanced resistance to the digestion, which might favor the presence of allergenic structures in the gut and the risk of eliciting an immune response. [16]

1.5 ANALYSES

1.5.1 O-phthaldialdehyde

O-phthaldialdehyde (OPA) is a method used for determining the degree of hydrolysis (DH), it is based on the reaction of primary amino groups with OPA in the presence of *N*-acetylcysteine, forming a colored compound detectable at 340nm in a spectrophotometer (Figure 1.3). [19, 20]

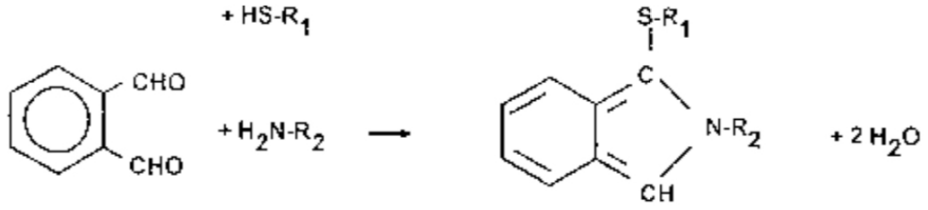

Figure 1.3: OPA reacts with primary amino groups and an SH-compound (*N*-acetylcysteine) to form a compound that will absorb light at 340nm. [19]

The degree of hydrolysis is defined as the percentage of cleaved peptide bonds:

$$DH = \frac{h}{htot} * 100\%$$

Where htot the total number is of peptide bonds per protein and h is the number of hydrolyzed bonds. htot is dependent on the amino-acid composition of the raw material. [19]

Another method for determining the degree of hydrolysis is the trinitro-benzene-sulfonic acid (TNBS) method. However, this method cannot follow a hydrolysis reaction continuously and it includes hazardous and unstable chemicals. Because of this, the OPA method is more accurate, has a broader application range, is easier and faster to carry out and it is safer for the environment than the TNBS method. [19]

1.5.1.1 Spectrophotometer

Spectrophotometry is a quantitative analysis. Each chemical compound absorbs or transmits light over a certain range of wavelengths. Spectrophotometry measures how much the compound absorbs or transmits within this wavelength range. [21, 22]

The spectrophotometer measures the intensity of the light that passes through the sample solution. By analyzing this intensity, you can identify certain dissolved substances and determine their concentration. The more the light is that a substance absorbs, the higher its concentration is. [21, 22]

There are two types of spectrophotometers: the UV-visible and the IR spectrophotometer. The UV-visible spectrophotometer uses light over the ultraviolet (185 – 400nm) and the visible range (400 – 700nm) of the electromagnetic radiation spectrum. The IR spectrophotometer uses light over the infrared range (700 – 15 000nm). [21]

The amount of light goes trough the cuvette and into the sector depends on the length of the cuvette and the concentration of the sample. Once the intensity of the light after it passes thought the cuvette is know, the transmittance (T) can be calculated. The transmittance is the fraction of the light that passes through the sample. [21, 22]

$$T = \frac{It}{Io}$$

Where It is the intensity of the light after the light passes through the sample and Io is the light intensity before it passes through the cuvette (figure 1.4). Based on the transmittance, the absorbance (A) can be calculated:

$$A = -log(T) = -\log\left(\frac{It}{Io}\right)$$

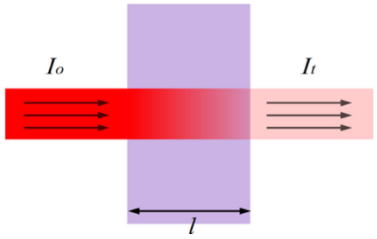

Figure 1.4: Transmittance. Io is the intensity of the light that passes through the length of the cuvette (l), It is the intensity of the light after the light passes through the cuvette. [21]

When the absorbance is determined, you can determine the unknown concentration of the sample by using Beer-Lambert Law.

$$A = \epsilon l c$$

Where A stands for the absorbance, ϵ is the molar extinction coefficient (L x mol-1 x cm-1), l is the path length (cm) and c is the concentration (mol/L). The molar extinction coefficient is given as a constant, it varies for each molecule. The absorbance has no unit, thus the units of the molar extinction coefficient must cancel out the units of the path length and the concentration. [21, 22]

1.5.2 Gel electrophoresis

SDS-PAGE stands for sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). This is a method that involves denaturation of proteins with the detergent sodium dodecyl sulfate and an electric current to pull them through a polyacrylamide gel. SDS binds strongly to proteins, approximately one detergent molecule binds to two amino acids. When the proteins are heated with SDS, they gain a negative charge that is in proportion to their molecular size. Then the proteins will travel in the polyacrylamide gel according to their molecular sizes. The smaller the size of a protein, the faster it travels through the pores of the gel to the positive charge. [23, 24]

1.5.3 LC-MS

1.5.3.1 Liquid chromatography

Liquid chromatography involves the separation of analytes based on their different interaction with a stationary phase and a mobile phase. The sample mixture is dissolved in a solvent, which is called the mobile phase. This mobile phase passes through a chromatographic column packed with a stationary phase. As the components of the sample interact differently with the stationary phase, they are eluted at different rates. These lead to their separation. [25]

1.5.3.2 Mass spectrometer

A mass spectrometer operates by converting the molecules of the analyte to a charged state. After this, the ions and their fragments created during this ionization process are analyzed based on their mass-to-charge ratio (m/z). There are different technologies available for both the ionization process and the analysis. This results in many different types of mass spectrometers. [26].

In this thesis we will couple two types of mass spectrometer to a liquid chromatography. We will do an analysis with a VIon-quadrupole Time of flight (QToF) mass spectrometer and an ORBITrap.

1.5.3.3 Electrospray ionization

With electrospray ionization, liquid samples are pumped through a metal capillary maintained at 3 to 5kV. The samples are sprayed at the end of the capillary to form a fine spray of charged droplets. The droplets are rapidly evaporated by the application of heat

and dry nitrogen. The residual electrical charge on the droplets is transferred to the analytes. These analytes are then transferred into the mass spectrometer. The ions are then separated based on their m/z ratio and detected by a mass analyzer. [26]

1.5.3.4 Orbitrap

The Orbitrap analyzer consists of three electrodes, where the outer two electrodes face each other and are separated by a dielectric central ring. The central electrode is spindle shaped and holds the trap. The trap is aligned towards the outer two electrodes. The electric field between the outer electrodes, the central electrode and the centrifugal force pushes the ion to take up harmonic axial oscillations. The outer electrodes detect these axial oscillations, and this signal is transformed into a mass spectrum. [27]

1.5.3.5 VIon-qToF

The quadrupole analyzer consists of a set of four parallel metal rods. A combination of constant and varying voltages allows the transmission of a narrow band of m/z values along the axis of the metal rods. By changing the voltages over time, it is possible to scan a range of m/z values. This will result in a mass spectrum. [26]

The time-of-flight (TOF) analyzer accelerates ions through a high voltage. The speed of the ions and the time it takes them to travel through a flight tube and reach the detector, depends on their m/z values. [26]

The quadrupole time-of-flight analyzer is a hybrid analyzer. It uses the combination of different mass analyzers: the quadrupole analyzer and the time-of-flight analyzer. [26]

1.5.4 Size Exclusion Chromatography

The basic principle of size exclusion chromatography (SEC) is that molecules are separated according to their molecular size. Solubilized molecules are passed through a column with porous beads. Smaller molecules penetrate through the pores of these porous beads while larger molecules such as aggregates do not. As a result, the larger molecules elute first while smaller molecules elute later. [28, 29]

2. OBJECTIVES

Extensive research has explored the impact of thermal treatment on wheat allergenicity, digestibility, and solubility. However, the specific influence of thermal treatment on gliadin, a gluten protein known to trigger allergic reactions in genetically predisposed individuals, remains unexplored. Consequently, the primary objective of this thesis is to investigate the impact of thermal treatment on gliadin digestibility and allergenic potential.

To achieve this goal, gliadin will undergo thermal treatments at various temperatures. Following these heat treatments, gliadin will be subjected to *in vitro* digestion. For each temperature, the heat-treated gliadin will undergo both a full digestion cycle and a half digestion cycle. In the half digestion cycle, the digestion process will be halted after the gastric phase. From here on this procedure will be referred to as “half digestion”.

Various analyses will be conducted on the digested gliadin. The objective is to assess the impact of different temperatures on gliadin and to identify any differences between full and half digestion cycles.

The degree of hydrolysis will be determined using an OPA assay, measuring the absorbance at 340 nm. The quantity of soluble proteins will be quantified using a Qubit fluorometer. Gel electrophoresis and size-exclusion chromatography will be conducted to investigate potential effects of the different temperatures and the digestion cycles on the molecular weight profile of digested gliadin. Finally, peptide analysis will be performed using LC-MS to explore the presence of epitopes associated with celiac disease.

3. MATERIALS AND METHODS

Table 3.1: all the products used with their Lot-number and their supplier.

Product	Lot-number	Supplier
Gliadin	118K7004	Sigma-Aldrich, USA
Ethanol	23F014008	VWR Internationals, France
Methanol	22B034009	VWR Internationals, France
Glacial acetic acid	21L064005	VWR Internationals, France
Coomassie brilliant blue	BCB41219	Sigma-Aldrich, USA
KH_2PO_4 (≥ 99%)	SZE90420	Sigma-Aldrich, USA
$NaHCO_3$ (≥ 99.5%)	SLBK2545V	Sigma-Aldrich, USA
NaCl (≥ 99%)	SZBC3530V	Sigma-Aldrich, USA
$MgCl_2$ (≥ 98%)	SLBK9099V	Sigma-Aldrich, USA
$(NH_4)_2CO_3$	SZBE0630V	Sigma-Aldrich, USA
$CaCl_2$	BCBN5264V	Sigma-Aldrich, USA
NaOH	V3L037154A	Carlo ERBA reagents, France
HCl	STBK1740	Sigma-Aldrich, USA
KCl (≥ 99%)	BR303007A	Farmaitalia, Carlo ERBA, Milano
Bile	SLCDO888	Sigma-Aldrich, USA
Pepsin	0000249346	Sigma-Aldrich, USA
Pancreatin	SLCD7175	Sigma-Aldrich, USA
Sodium dodecyl sulfate (SDS)	8B015082	Pancreas Applichem, Germany
Di-sodium-tetra-borate-decahydrate	BCBN2987V	Sigma-Aldrich, USA
Tergitol™ 15-S-9	1003439443	Sigma-Aldrich, USA
o-phtaldialdehyde	VOBO62040C	Carlo ERBA reagents, France
Dithiothreitol (DTT)	6T015360	PancReac, Applichem, Germany
Sodium dodecyl sulfate (SDS) buffer	64509368	Biorad, USA
Reducing agent (20x)	64509368	Biorad, USA
Running buffer	64236556	Biorad, USA
Gel marker	1610363	Biorad, USA
Pullulan	-	Sigma-Aldrich, USA
Lys-tyr-lys	061K1311	Sigma-Aldrich, USA
Isoleucine (≥ 99%)	MKBQ2672V	Sigma-Aldrich, USA
Bovine serum albumine	AO428182	Acros Organics, Belgium

Table 3.1 (continued)

Product	Lot-number	Supplier
Lactose	L3625-5KG	Sigma-Aldrich, USA
α-lactalbumin	SLCD1189	Sigma-Aldrich, USA
Qubit protein buffer	2549287	Thermo Fisher, USA
Qubit protein reagent	2549296	Thermo Fisher, USA

3.1 SDS-PAGE GEL-ELECTROPHORESIS

To determine the state of a gliadin standard an SDS-PAGE assay was performed following the procedure described by Gasparini et al. (2020) with modifications. 5 mg of gliadin was dissolved in 70% ethanol. Three 200 µL disposable plastic tubes with a volume of gliadin solution equivalent to approximately 30 µg, 40 µg and 50 µg of protein were prepared. Then 6.25 µL SDS buffer and 1.25 µL reducing agent 20x were added to each and vortexed. [30]

The samples were placed in the thermocycler (Thermo Hybaid, Euroclone S.p.A., Italy) at 95°C for 5 minutes and then at 20°C for 1 minute.

The separation of proteins was performed with a precast 10% Bis/Tris Criterion XT gel (Bio-Rad, Hercules, CA, USA). First, the running buffer was prepared by mixing 95% distilled water and 5% buffer (1x).

Each sample was added in a specific well by using a Hamilton syringe. The selected voltage was 150V. This process lasted for 45 minutes.

After 45 minutes the voltage was switched off and the gel was removed. The gel was washed with distilled water and a coloring step was performed with the staining solution (50% distilled water, 40% methanol, 10% glacial acid and 1 g/L Coomassie Brilliant Blue). The gel was covered with aluminum foil and put under agitation overnight on a tilting platform (20 rpm, room temperature) (SSL3, Bibby Sterilin LTD, UK).

The next day, the staining solution was removed, and the gel was washed with distilled water. Then the destaining solution (50% distilled water, 40% methanol and 10% glacial acetic acid) was added, the gel was covered with aluminum foil and put on a tilting platform at 20 rpm for 20 minutes. The destaining solution was removed. This process was repeated four times. After the fourth time, the residues were removed with distilled water. Finally, the gel was scanned with a GS-800 TM scanner with the Quantity-One BIO-RAD software (Biorad, USA).

3.2 SAMPLE PREPARATION

Different ratios of gliadin to water were prepared, to see which ratio had the same structure as dough. Three different ratios were tried: 50:50, 40:60 and 30:70. Gliadin and water were weighed in a test tube, then mixed with a spatula until an even mixture was obtained. The ratio 40:60 resembled the structure of a dough to most.

3.3 HEAT TREATMENT

For the heat treatment, eight different temperatures were studied: 25°C, 60°C, 90°C, 120°C, 150°C, 180°C, 210°C and 245°C. 25°C was used as the control. For every temperature, triplicates were made. The oil bath was used for every temperature, except for the control temperature. When the oil bath reached desired temperature, the tree test tubes were put in the oil bath for 30 minutes. After, they were put in an ice bath for a couple minutes. This process was repeated for every temperature.

For the control temperature, 25°C, the three test tubes were placed in the incubator at 25°C for 30 minutes (ES-20, Biosan, Latvia). After, the test tubes were put in an ice bath for a couple minutes.

3.4 DIGESTION

To evaluate the digestibility of gliadin, the INFOGEST *in vitro* static gastro-intestinal digestion procedure was chosen. This procedure consists of three different phases: the oral phase, the gastric phase and the intestinal phase. This model mimics closely the human physiology. [31]

Normally for this procedure 1 g of sample is used but here, since a pure protein was used the amount of gliadin in wheat was calculated in order to establish the necessary amount needed for digestion. The protein content in wheat is around 12%. Since the two major proteins that count for approximately 80-85% is gliadin (30%) and glutenin (50%), the amount of gliadin needed for the digestion trials was calculated as follows: [32, 33]

(3.4)

$$0.12 \text{ x } 0.85 = 0.102$$

$$\frac{30}{80} \text{ x } 100 = 37.5\%$$

$$37.5\% \text{ x } 0.102 = \ 0.038\text{g}$$

First, the salt's aqueous solutions were prepared at a known concentration and brought up to volume with ultrapure water in a volumetric flask (see table 3.2):

Table 3.2: amount of each substance used to prepare the salt's aqueous solutions.

Substance	Amount	Volumetric flask (mL)
KCl	1.87g	50mL
KH_2PO_4	0.68g	10mL
$NaHCO_3$	8.4g	100mL
NaCl	5.85g	50mL
$MgCl_2$	0.0715g	5mL
$(NH_4)_2CO_3$	0.096g	2mL
$CaCl_2$	3.3226g	100mL
NaOH (4M)	25mL	100mL
HCl (1M)	100mL	100mL
HCl (6M)	100mL	100mL

Then the simulated salivary fluid (SSF), simulated gastric fluid (SGF) and the stimulated intestinal fluid (SIF) were prepared as described in table 3.3, by mixing the different salt stock solutions. The solutions were brought to volume in a 500 mL volumetric flask with ultrapure water (ddH_2O).

Table 3.3: amount of the salt stock solutions used to prepare SSF, SGF and SIF.

Compound	SSF (mL)	SGF (mL)	SIF (mL)
KCl	18.875	8.625	8.5
KH_2PO_4	4.625	1.125	1
$NaHCO_3$	8.5	15.625	53.125
NaCl	-	14.75	12
$MgCl_2$	0.625	0.5	1.375
$(NH_4)_2CO_3$	0.075	0.625	-
HCl (6M)	0.1125	1.625	0.875

3.4.1 pH Trial

In order to determine the stability of the pH during the digestion a pH trial was performed. In this process, the digestion was done without enzymes. The pH was checked for every phase: 6 for the salivary phase, 3 for the gastric phase and 7 for the intestinal phase.

For the pH trial 0.038 g gliadin was weighed three times and 0.962 g distilled water was added. Three solutions were made as described in 3.4, three other solutions were

prepared: solution 1 for the oral phase, solution 2 for the gastric phase and solution 3 for the intestinal phase (see table 3.5). For these solutions, enzymes were prepared as described in table 3.4.

Table 3.4: enzyme solutions used for digestion.

Enzyme	Concentration	Solvent
Pepsin	2000 U/mL	ddH_2O
Pancreatin	8 USP	SIF
Bile	160 mM	SIF

Table 3.5: amount of each substance and solution to prepare the oral, gastric and intestinal phase for the pH trial.

Solution 1 - Oral		Solution 2 - Gastric		Solution 3 - Intestinal	
SSF	4.8 mL	SGF	8 mL	SIF	10 mL
$CaCl_2$	0.030 mL	$CaCl_2$	0.005 mL	$CaCl_2$	0.030 mL
ddH_2O	1.170 mL	ddH_2O	1.785 mL	ddH_2O	2.858 mL
		HCl (1M)	0.210 mL	NaOH (4M)	0.113 mL
				Bile solution	1.875 mL
Total volume	6 mL	Total volume	10 mL	Total volume	15 mL

For the oral phase, the sample was added with solution 1 in a 1:1 ratio. Then it was incubated at 180 rpm at 37°C for 2 minutes. To reconstruct the gastric phase, solution 2 was added in a 1:1 ratio. The samples were incubated at 180 rpm at 37°C for 2 hours. The pH was checked every 30 minutes. For the intestinal phase, solution 3 was added in a 1:1 ratio. Then the samples were again incubated at 180 rpm at 37°C for 2 hours. The pH was checked every 30 minutes.

If needed, the pH was corrected with the previously made HCl (1M) or NaOH (4M). The added volumes were noted (see attachment 1). These volumes were needed to correct the pH during the process with the enzymes present.

The samples were heated at 90°C for 15 minutes in a water bath (Buchi, Switzerland), and then the samples were centrifuged (3220g, 4°C) for 45 minutes (5810R Eppendorf, Hamburg, Germany).

The supernatants were transferred to a disposable plastic tube and stored at -18°C.

3.4.2 Digestion cycles

The solutions for the three phases were made as described in table 3.6.

Table 3.6: amount of each substance and solution used to prepare the oral, gastric and intestinal phase for the digestion.

Solution 1 - Oral		**Solution 2 - Gastric**		**Solution 3 - Intestinal**	
SSF	12.8 mL	SGF	24 mL	SIF	10 mL
$CaCl_2$	0.080 mL	$CaCl_2$	0.015 mL	$CaCl_2$	0.116 mL
ddH_2O	3.120 mL	ddH20	3.855 mL	ddH_2O	11.05 mL
		HCl (1M)	0.630 mL	NaOH (4M)	0.44 mL
		Pepsin solution	1.5 mL	Bile solution	7.25 mL
				Pancreatin solution	14.50 mL
Total volume	16 mL	Total volume	30 mL	Total volume	58 mL

Based on the calculations in section 3.4, 0.095 g of heated gliadin and 0.905 g distilled water were mixed. For every digestion cycle, three blanks were prepared using 1 g of distilled water.

The same process was repeated as in section 3.4.1. For the half digestion, the digestion cycle was stopped after the gastric phase. The samples were inactivated under the same conditions (90°C, 15 minutes). The pH was checked every 30 minutes, if necessary NaOH (4M) or HCl (1M) were added to correct the pH.

3.4.3 Controls

For the controls of the digestion, no enzymes were used. Only the bile solution was added since it can affect the pH. The solutions for the three phases were made as described in table 3.7.

Table 3.7: amount of each substance and solution to prepare the oral, gastric and intestinal phase for the controls.

Solution 1 - Oral		**Solution 2 - Gastric**		**Solution 3 - Intestinal**	
SSF	12.8 mL	SGF	24 mL	SIF	39.15 mL
$CaCl_2$	0.080 mL	$CaCl_2$	0.015 mL	$CaCl_2$	0.116 mL
ddH_2O	3.120 mL	ddH_2O	5.355 mL	ddH_2O	11.05 mL
		HCl (1M)	0.630 mL	NaOH (4M)	0.44 mL
				Bile solution	7.25 mL
Total volume	16 mL	Total volume	30 mL	Total volume	58 mL

The samples were prepared the same as in section 3.4.2. The procedure for the controls of the full digestion and half digestion was the same as described in section 3.4.2.

3.4.4 Soluble proteins

The amount of soluble proteins present in the supernatants from digestion was obtained by the protein content quantified by Qubit fluorometer (Thermo Fisher Scientific, Waltham, USA) multiplied by the volume of the supernatants. The protein content of the standard was determined by applying the conversion factor wheat, 5.7. [34]

(3.4.4)

$$Soluble\ proteins\ (\%) = \frac{soluble\ proteins}{protein\ content\ of\ standard} * 100$$

3.5 PEPTIDE ANALYSIS

To prepare the samples for the analysis, the supernatants obtained from the *in vitro* digestion were centrifuged for 15 minutes (3220g, 4°C). The supernatants were filtered with a 0.45 µm filter. Then, 1000 µL was pipetted into a vial. The triplicates from every temperature and the blanks of the complete and half digestion were analysed.

3.5.1 UPLC-HRMS analysis

The samples were analyzed with liquid chromatography couple to high-resolution mass spectrometry, and the detection was obtained by Vion IMS Q-ToF Mass Spectrometer (Waters, Milford, MA, USA). Chromatographic separation was achieved using a reverse phase column (ACQUITY UPLC Peptide BEH C18 Column, 300Å, 1.7 µm,

2.1 mm x 150 mm, Waters Milford, MA, USA) in a UPLC system (UPLC Acquity I-class, Water, Milford, MA, USA). Eluent A was Milli-Q H_2O + 0.1% HCOOH (MS grade). Eluent B is CH_3CN (UHPLC-MS grade, Scharlab, Barcelona, Spain) + 0.1%HCOOH (Scharlab, Barcelona, Spain). [35]

The analysis parameters were as follows. The flow was maintained at 0.25 mL/min and the gradient applied was: 0−3.5 min, 100% A; 3.5−25 min, from 100% A to 50% A; 25—26.3 min, 50% A; 26.3−26.5 min, from 50% A to 0% A; 26.5−29.1 min, 0% A; 29.1−29.5 min, from 0% A to 100% A; 29.5−36 min, 100% A. The run time was 36 min; the column temperature was 35°C; the sample temperature was 10°C; the injection volume was 1 µL. The detection parameters were as follows: experiment type: peptide map (IMS); experiment type: MS^E; source type: ESI; polarity: positive; analyzer mode: sensitivity; mode: standard transmission; capillary: 3.00 kV; sample con voltage 40 V; source offset voltage: 80 V; source temperature: 120°C; desolvation temperature: 450°C; cone gas: 50 L/h; desolation gas: 800 L/h. MS^E mode: high definition MS^E; acquisition time: 3.5-29.1 min; scan range: 100-2000 m/z; scan time: 0.4 s; low collision energy: 6 V; high collision energy ramp: 20 V to 45 V; automatic lock correction (leucine enkephaline). [35]

The data processing was performed using UNIFI software (Waters, Milford, MA, USA). The parameters were as follows: high energy threshold: 150 counts, low energy threshold: 500 counts; maximum number of peaks to keep per channel 20000. Non-specific digestion reagent, minimum sequence length: 2; amino acid modifications: none. [35]

The components, target proteins, used are described in table 3.8.

Table 3.8: Description for the target proteins used for the peptide analysis.

Component	**Descripition**
U5U6N3	ω-gliadin
B2ZRD3	Prolamin
A7XDG3	Prolamin
A0A385YFZ7	Prolamin
A0A3B5XTS9	ω-gliadin
A0A290XZ51	γ-gliadin
A0A290XZ34	γ-gliadin
A0A290XZ04	γ-gliadin

Table 3.8 (continued)

Component	Description
A0A0E3Z5U5	Prolamin
A0A060N056	ω-gliadin

3.5.2 Orbitrap specifications

The samples were also analysed with UHPLC (Dionex Ultimate 3000, Sunnyvale, CA) coupled to an Orbitrap LTQ XL mass spectrometer (Thermo Scientific, Waltham, MA). The software used for detection was Xcalibur 2.0.7 (Thermo 330 Scientific, Waltham, MA). Eluent A was H_20 +0.2% HCOOH, eluent B was CH_3CN with 0.2% HCOOH. The sample loading conditions were as follows: µ-Precolumn Cartridge, Acclaim PepMap100 C18 5 µm, 100 Å, 300 µm × 5 mm, loading flow: 30 µL/min, 50% eluent A and 50% eluent B. The sample elution condition were as follows: Column: Phenomenex Jupiter 4 µm Proteo 90 Å 150 mm × 0.3 mm, column temperature: 35°C, flow: 5 µL/min, gradient: 0-4 min 10% B, 4−60 min linear from 10% B to 50% B, 60−62 min from 50 to 95% B, 62−72 min 95% B (column washing), 72−73 min from 95% B to 10% B, 73−82 min 10% B (column equilibration).

The high-resolution mass spectrometer acquisition was performed through five subsequent events: event 1, full scan acquisition from 250 to 2000 m/z in high resolution mode (resolution at 400 m/z = 30,000); events from 2 to 5, data dependent scan, at each cycle the four most intense ions (with charge z > 1 and with a minimum signal of 500 counts) identified in event 1 were fragmented. The same ion (tolerance 10 ppm and isolation window 2 m/z) can be observed for a maximum of two cycles, and then it is automatically inserted in the exclusion list for a maximum time of 20 seconds. FTMS max ion time was 250 ms and micro scans 1; ion trap max ion time was 200 ms and micro scans 1. Fragmentation is performed in the linear trap of the instrument in CID mode with collision energy of 35 (arbitrary units). [36]

The proteins were identified with Proteome Discoverer (Thermo Scientific, Waltham, MA). The parameters were as follow: precursor ion tolerance 5 ppm, fragment ion tolerance 0.8 Da, decoy database search: strict 0.01, relaxed 0.05, fixed modifications: none. [36]

3.6 OPA

First, the following reagents were prepared: SDS 10% (w/w) in ethanol, borate buffer solution (0.1M) in distilled water, Tergitol^{TM} 15-S-9 solution (100 g/L) in distilled water, OPA solution (40 g/L) in ethanol and stock solution of isoleucine (2 mg/mL) in distilled water.

After all the solutions were made, the calibration curve was prepared in duplicates. For our calibration curve seven points were made by doing sequential dilutions of the isoleucine stock solution (see table 3.9).

Table 3.9: calibration curve used for OPA.

N Standard	Concentration (mg/mL)	Standard N°	Volume (µL)	dH_2O (µL)
1	2	Stock	1000	0
2	1	1	500	500
3	0.5	2	500	500
4	0.25	3	500	500
5	0.125	4	500	500
6	0.0625	5	500	500
7	0.0312	6	500	500

The OPA reagent was prepared by mixing: 200 mg DTT, 25.0 mL SDS 10% (w/w) solution, 125.0 mL borate buffer solution (0.1M), 12.5 mL Tergitol™ 15-S-9 solution (100 g/L) and 5.0 mL OPA solution (40 g/L). The solution was brought to volume with dH_2O in a 250 mL volumetric flask that was covered from light.

The samples were diluted with the borate buffer (0.1M) as follows. For the samples of the full digestion a dilution factor of 1:36 was used. For the half digestion a dilution factor of 5:36 was used. For the controls, there was no dilution factor used.

For the analysis of the calibration curve 32 µL of the standards were mixed with the 928 µL OPA reagent and incubated at 30°C for 10 minutes in the dark. After, the absorbance was read at 340 nm with the spectrophotometer (B530 JASCO, Oklahoma City, USA). The same procedure was followed for reading the absorbance for all the samples and their controls, as well as as water blanks.

The degree of hydrolysis was obtained by calculating the concentration of free aminogroups determined by the OPA assay, divided by the total concentration of aminogroups determined by the protein content.

(3.6)

$$DH(\%) = \frac{Conc.Free\ AG(M)}{Conc.Total\ AG(M)} * 100$$

3.7 STATISTICAL ANALYSIS

The data retrieved from the OPA assay and the soluble proteins were analysed by using SPSS version 26.0 software (SPSS Inc., Chicago, IL., USA). First, the data was subjected to the test of homogeneity of variances. Since the variances were equal, the one-way analysis of variance (ANOVA) was conducted. The Duncan test was used to determine differences between temperatures ($p < 0.05$).

3.8 GEL-ELECTROPHORESYS AFTER HEAT TREATMENT

For the gel, one replicate from each temperature from the full digestion, the half digestion and their controls were used. For each of the digestion type a volume of supernatants from each sample or control equivalent to 50 ug was mixed with the buffer and the reducing agent 20x. The same procedure was followed as in section 3.1.

3.9 SIZE-EXCLUSION CHROMATOGRAPHY

To prepare the samples for the analysis, the supernatants from the digestion were centrifuged for 15 minutes (3220g, 4°C). Then they were filtered by using a 0.45 µm filter in to an HPLC vial. The following standard solutions were prepared with a concentration of 1 mg/mL: pullulan (22 kDa, 49.4 kDa), tripeptide (lys-tyr-lys) (424 Da), isoleucine (132 Da), lactose (342 Da), α-lactalbumin (14 kDa), gliadin (28 – 50 kDa) and bovine serum albumin (BSA) (66 kDa).

The column used for the analysis was a PL aquagel-OH mixed-M (7.5 × 300 mm, 8 µm particle size, Agilent Technologies, Santa Clara, CA, USA) with a molecular mass range between 1 kDa and 500 kDa. The separation was performed under isocratic conditions at a flow rate of 0.7 mL/min with milli-Q water as the mobile phase, keeping the column temperature at 45°C.

4. RESULTS

4.1 GEL-ELECTROPHORESIS

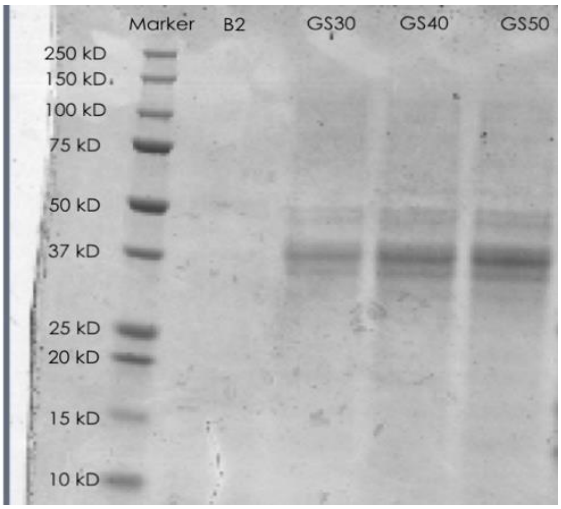

Figure 4.1: Electrophoretic pattern of gliadin (1 mg/mL) in three different concentrations: 30 µL (GS30) , 40 µL (GS40) and 50 µL (GS50).

The initial observation reveals a correlation between increased volume and band intensity on the gel. Gliadin typically ranges in molecular weight from 28-55 kDa, and the bands on the gel appear around 37 kDa, consistent with the gliadin standard, therefore it can be used for further analysis. [45]

4.2 HEAT TREATMENT

After the heat treatment, differences in texture of the gliadin mixture were evident. The lower temperatures (25°C, 60°C and 90°C) had a higher viscosity, while the higher temperatures (120°C, 150°C, 180°C, 210°C and 240°C) resulted in gliadin hardening.

4.3 DIGESTION

After the digestion process, samples subjected to full digestion formed firm pellets upon centrifugation, whereas samples stopped at the gastric phase did not. Notably, the higher temperatures correlated with looser pellet formation.

Attached there is a comprehensive overview of the pH checks during each digestion cycle (see attachment 1).

4.4 DEGREE OF HYDROLYSIS

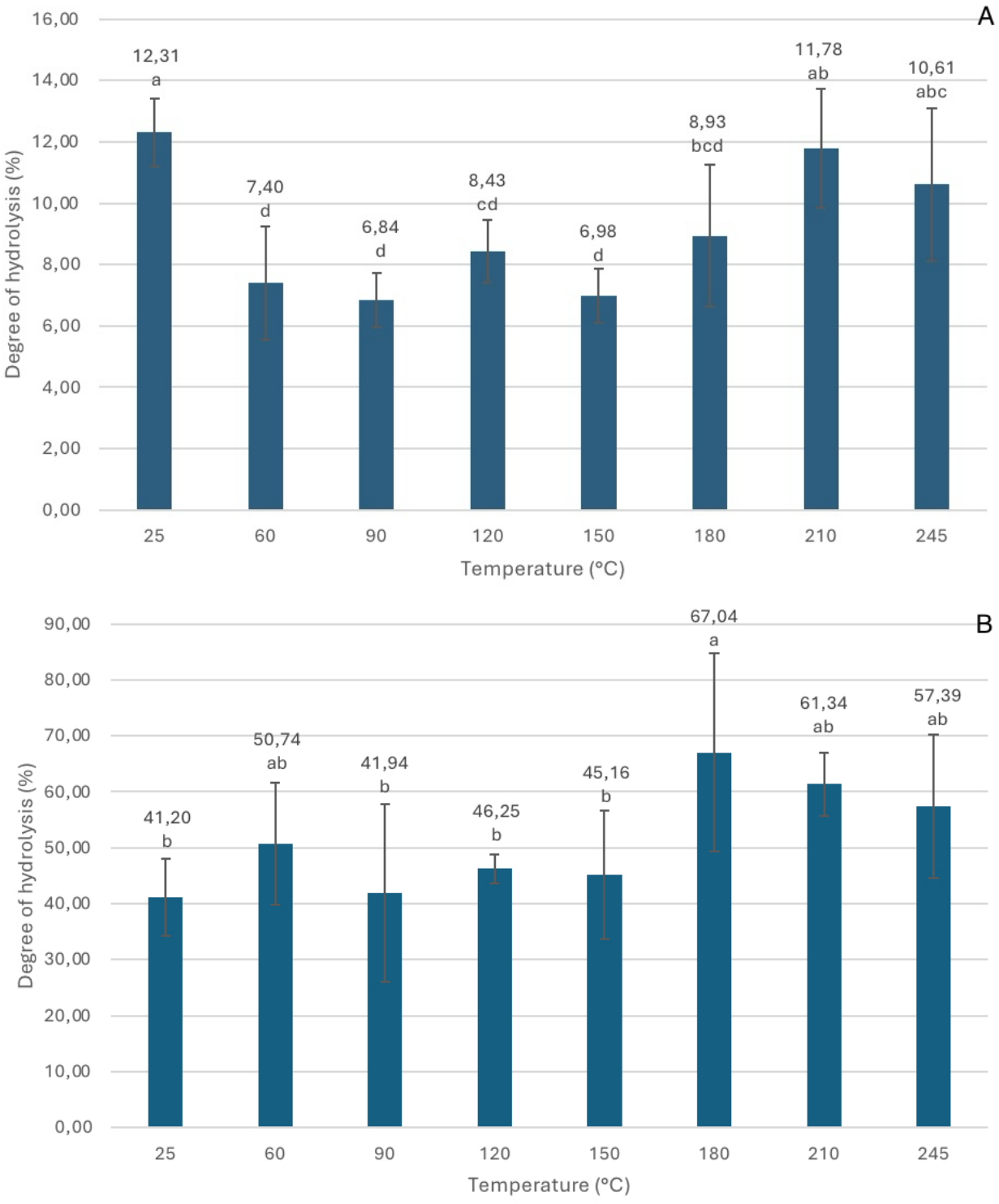

Figure 4.2: Degree of hydrolysis obtained by the OPA assay. A: DH for the half digestion, B: DH for the full digestion. Different letters mean statistically different samples ($p < 0.05$) (one-way ANOVA, Duncan test).

4.5 SOLUBLE PROTEINS

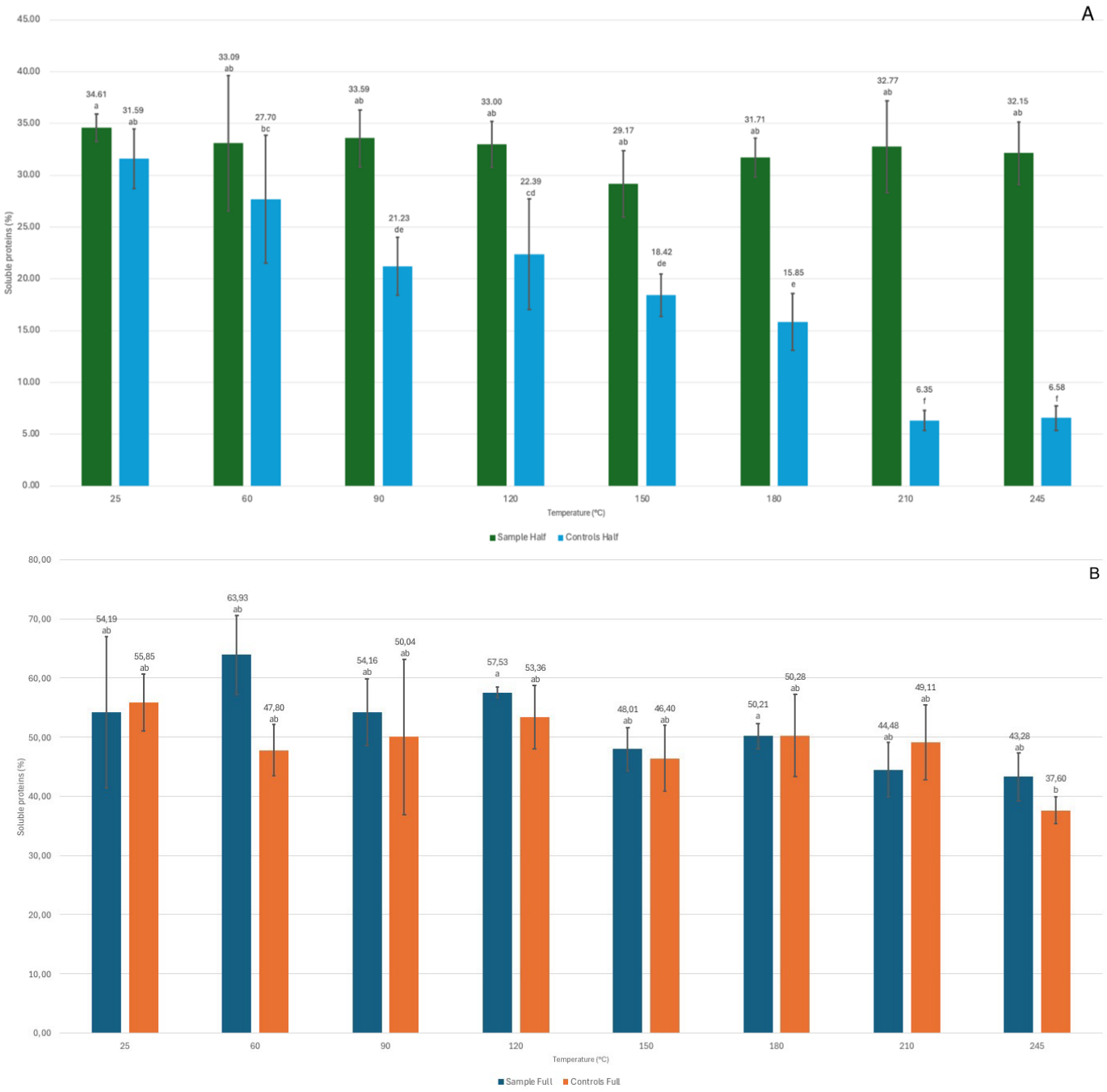

Figure 4.3: Quantified soluble proteins present in the supernatants measured by Qubit Fluorometer. A: comparing between the sample its controls for the half digestion, B: comparing between samples and its controls for the full digestion. Different letters mean statistically different samples ($p < 0.05$) (one-way ANOVA, Duncan test).

4.6 GEL-ELECTROPHORESIS AFTER HEAT TREATMENT

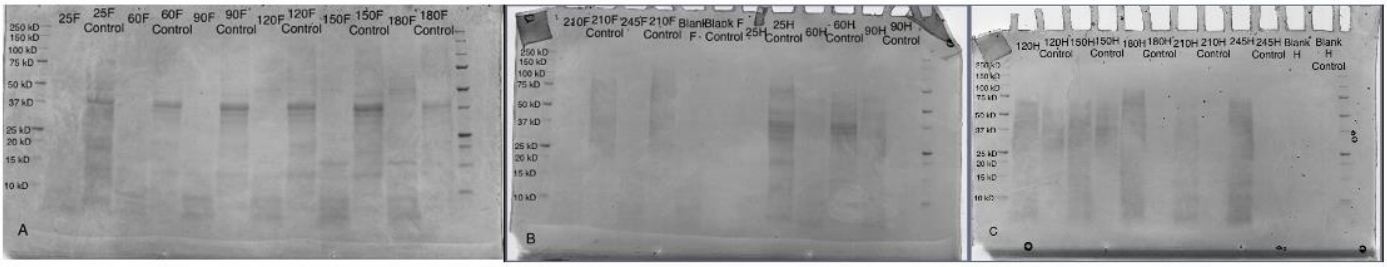

Figure 4.4: Electrophoretic pattern of gliadin after heat treatment. A: from 25°C full digestion to 180°C full digestion control. B: from 210°C full digestion to 90°C half digestion control. C: from 120°C half digestion to blank half digestion control.

4.7 SIZE-EXCLUSION CHROMATOGRAPHY

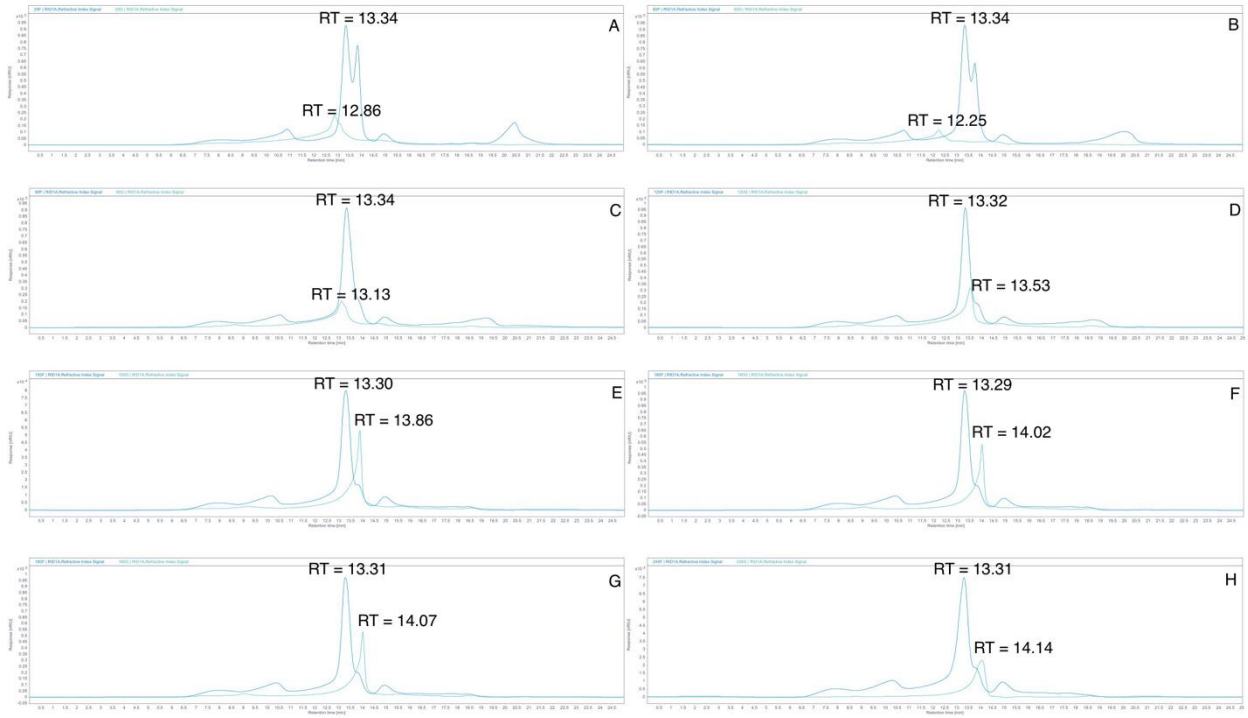

Figure 4.5: Chromatograms from the size-exclusion chromatography. The y-axis represents the response and the x-axis presents the retention time (minutes). The comparison between the gliadin heated in the complete digestion and the half digestion. A: 25°C, B: 60°C, C: 90°C, D: 120°C, E: 150°C, F: 180°C, G 210°C and H: 245°C. The blue curve represents the pattern of the complete digestion, the light green represents the half digestion.

4.8 PEPTIDE ANALYSIS

Table 4.1: identified peptides in the heat treated gliadin mixture in the half and full. The peptides found have epitopes that are known to cause celiac disease. [35, 36, 46, 47]

Sequence	Protein code	Protein name	Response (Mean±SD)	Retention time (minutes)	m/z
Half digestion at 25°C					
LQPQQPFPQ	A0A3B5XTS9	ω-gliadin	$1.51^{E}06\pm5.26^{E}04$	9.11	427.48
PQPQQQFPQ	A0A290XZ34	γ-gliadin	$2.60^{E}05\pm5^{E}04$	9.38	1005.98
LQPQQPFPQ	A0A3B5XTS9	ω-gliadin	$1.51^{E}06\pm5^{E}04$	9.11	427.48
QGSFQPSQQ	A0A0E3Z5U5	Prolamin	$8.73^{E}04\pm3^{E}04$	9.69	624.85
LQPQQPFPQ	U5U6N3	ω-gliadin	$3.79^{E}05\pm1.67^{E}04$	9.74	1003.73
QYSQPQQPI	B2ZRD3	Prolamin	$5.69^{E}05\pm1.05^{E}05$	9.793	636.82
QQPQQPFPQ	A0A290XZ51	γ-gliadin	$4.25^{E}05\pm6.47^{E}04$	10.07	793.39
QQPFPQQPQ	A0A290XZ51	γ-gliadin	$4.25^{E}05\pm6.47^{E}04$	10.07	793.39
PQQSFPQQQ	A0A290XZ34	γ-gliadin	$2.90^{E}05\pm1.05^{E}05$	10.59	511.86
QGSVQPQQL	A0A0E3Z5U5	Prolamin	$1.17^{E}05\pm2.15^{E}04$	10.62	947.78
FRPQQPYPQ	A0A290XZ34	γ-gliadin	$1.77^{E}06\pm1.29^{E}06$	10.82	1004.44
PFPQPQLPY	A0A0E3Z5U5	Prolamin	$4.81^{E}05\pm2.29^{E}05$	10.92	1148.24
PYPQPQLPY	A0A0E3Z5U5	Prolamin	$4.81^{E}05\pm2.29^{E}05$	10.96	843.11
QGSVQPQQL	B2ZRD3	Prolamin	$1.91^{E}05\pm4.06^{E}04$	11.23	996.01
Half digestion at 60°C					
LQPQQPFPQ	A0A3B5XTS9	ω-gliadin	$1.13^{E}06\pm8.83^{E}05$	8.91	611.26
QGSFQPSQQ	A0A0E3Z5U5	Prolamin	$7.01^{E}04\pm9.47^{E}03$	8.93	479.73
FRPQQPYPQ	B2ZRD3	Prolamin	$1.12^{E}06\pm1.29^{E}03$	9.34	527.27
FRPQQPYPQ	B2ZRD3	Prolamin	$1.12^{E}06\pm1.29^{E}03$	9.34	527.27
QQPQQPFPQ	A0A290XZ51	γ-gliadin	$6.45^{E}04\pm2.55^{E}03$	9.72	864.93
LQPQQPFPQ	U5U6N3	ω-gliadin	$3.33^{E}03\pm4.94^{E}04$	9.74	1003.73

Table 4.1 (continued)

QQPFPQQPQ	A0A290XZ51	γ-gliadin	$1.56^{E}05\pm1.58^{E}04$	9.84	841.08
QYSQPQQPI	B2ZRD3	Prolamin	$3.69^{E}05\pm2.89^{E}05$	10.03	712.26
QGSVQPQQL	A0A0E3Z5U5	Prolamin	$1.09^{E}05\pm6.00^{E}03$	10.62	947.79
PQQSFPQQQ	A0A290XZ51	γ-gliadin	$3.27^{E}05\pm6.78^{E}04$	10.65	511.87
PQPQLPYPQ	A0A0E3Z5U5	Prolamin	$6.37^{E}048\pm1.67^{E}04$	10.67	673.69
PYPQPQLPY	A0A0E3Z5U5	Prolamin	$6.37^{E}048\pm1.67^{E}04$	10.67	673.69
PFPQPQLPY	A0A0E3Z5U5	Prolamin	$3.02^{E}05\pm7.15^{E}04$	10.81	1291.13
FRPQQPYPQ	A0A0E3Z5U5	Prolamin	$9.18^{E}05\pm1.07^{E}06$	10.82	1147.79
Half digestion at 90°C					
PQPQQQFPQ	A0A290XZ34	γ-gliadin	$5.30^{E}05\pm2.03^{E}04$	9.38	1005.9
QQPFPQQPQ	A0A290XZ51	γ-gliadin	$6.46^{E}0\pm1.18^{E}04$	9.73	864.92
QQPQQPFPQ	A0A290XZ51	Prolamin	$7.15^{E}05\pm1.18^{E}04$	9.73	864.92
LQPQQPFPQ	U5U6N3	Prolamin	$6.40^{E}05\pm1.25^{E}05$	9.74	1003.73
QYSQPQQPI	B2ZRD3	Prolamin	$6.23^{E}05\pm6.64^{E}04$	9.79	636.81
QGSVQPQQL	A0A0E3Z5U5	Prolamin	$1.45^{E}05\pm4.93^{E}04$	10.25	890.42
LQPQQPFPQ	A0A3B5XTS9	ω-gliadin	$7.86^{E}05\pm9.16^{E}05$	10.31	703.15
QGSFQPSQQ	A0A0E3Z5U5	Prolamin	$6.25^{E}04\pm7.52^{E}03$	10.37	720.68
PQPQLPYPQ	A0A0E3Z5U5	Prolamin	$4.31^{E}05\pm4.42^{E}05$	10.43	796.75
PYPQPQLPY	A0A0E3Z5U5	Prolamin	$3.15^{E}05\pm1.40^{E}04$	10.61	833.44
FRPQQPYPQ	A0A0E3Z5U5	Prolamin	$2.60^{E}05\pm9.72^{E}05$	10.82	1291.13
PFPQPQLPY	A0A0E3Z5U5	Prolamin	$1.18^{E}05\pm3.62^{E}05$	11.12	862.44
QQPFPQQPQ	A0A290XZ04	γ-gliadin	$6.46^{E}05\pm4.33^{E}03$	11.30	802.36
QQPQQPFPQ	A0A290XZ04	γ-gliadin	$6.46^{E}05\pm4.33^{E}03$	11.30	802.36
Half digestion at 120°C					

Table 4.1 (continued)

Sequence	Protein code	Protein name	Response (Mean±SD)	Retention time (minutes)	m/z
PQPQQQFPQ	A0A290XZ34	γ-gliadin	$3.89^{E}05\pm1.97^{E}05$	9.38	1005.9
QQPFPQQPQ	A0A290XZ51	γ-gliadin	$7.46^{E}04\pm3.09^{E}04$	9.73	864.92
LQPQQPFPQ	U5U6N3	ω-gliadin	$5.36^{E}05\pm1.47^{E}05$	9.75	1003.7
QYSQPQQPI	B2ZRD3	Prolamin	$4.21^{E}05\pm8.82^{E}04$	9.79	636.81
QQPQQPFPQ	A0A290XZ51	γ-gliadin	$1.38^{E}05\pm1.23^{E}05$	10.06	889.80
QGSVQPQQL	A0A0E3Z5U5	Prolamin	$1.52^{E}05\pm3.13^{E}04$	10.28	935.24
PQQSFPQQQ	A0A290XZ51	γ-gliadin	$1.67^{E}05\pm1.05^{E}05$	10.34	840.40
QGSFQPSQQ	A0A0E3Z5U5	Prolamin	$6.02^{E}04\pm8.37^{E}03$	10.38	720.68
PYPQPQLPY	A0A0E3Z5U5	Prolamin	$2.29^{E}05\pm8.69^{E}03$	10.88	833.44
PFPQPQLPY	A0A0E3Z5U5	Prolamin	$2.29^{E}05\pm8.69^{E}03$	10.88	833.44
LQPQQPFPQ	A0A3B5XTS9	ω-gliadin	$9.75^{E}04\pm3.13^{E}04$	11.55	935.24
FRPQQPYPQ	B2ZRD3	Prolamin	$5.54^{E}04\pm9.82^{E}03$	11.57	696.01
Half digestion at 150°C					
LQPQQPFPQ	A0A3B5XTS9	ω-gliadin	$1.20^{E}06\pm1.69^{E}04$	9.33	427.49
QQPFPQQPQ	A0A290XZ51	γ-gliadin	$5.22^{E}04\pm6.43^{E}03$	9.74	864.93
QQPQQPFPQ	A0A290XZ51	γ-gliadin	$5.22^{E}04\pm6.43^{E}03$	9.74	864.93
QQPQQPFPQ	A0A290XZ34	γ-gliadin	$5.57^{E}04\pm7.94^{E}03$	10.13	576.30
QGSVQPQQL	A0A0E3Z5U5	Prolamin	$9.98^{E}04\pm1.56^{E}04$	10.41	962.12
PQQSFPQQQ	A0A290XZ51	γ-gliadin	$1.04^{E}05\pm8.23^{E}04$	10.86	682.02
QYSQPQQPI	B2ZRD3	Prolamin	$1.00^{E}05\pm2.08^{E}04$	11.11	840.91
PYPQPQLPY	A0A0E3Z5U5	Prolamin	$9.44^{E}05\pm1.93^{E}05$	11.13	862.44
PFPQPQLPY	A0A0E3Z5U5	Prolamin	$9.44^{E}05\pm1.93^{E}05$	11.13	862.44
Half digestion at 180°C					

Table 4.1 (continued)

Sequence	Protein code	Protein name	Response (Mean±SD)	Retention time (minutes)	m/z
LQPQQPFPQ	U5U6N3	ω-gliadin	$6.87^{E}05\pm3.91^{E}05$	9.62	811.65
QQPQQPFPQ	A0A290XZ51	γ-gliadin	$7.39^{E}04\pm2.88^{E}04$	9.75	864.92
QQPFPQQPQ	A0A290XZ51	γ-gliadin	$7.39^{E}04\pm2.88^{E}04$	9.75	864.92
QYSQPQQPI	B2ZRD3	Prolamin	$3.21^{E}05\pm5.07^{E}04$	9.81	636.81
QGSVQPQQL	A0A0E3Z5U5	Prolamin	$1.09^{E}05\pm2.00^{E}04$	10.42	962.12
QQPQQPFPQ	A0A290XZ34	γ-gliadin	$1.62^{E}05\pm6.89^{E}04$	10.55	1077.1
PQPQQQFPQ	A0A290XZ34	γ-gliadin	$1.62^{E}05\pm6.89^{E}04$	10.55	1077.1
PQQSFPQQQ	A0A290XZ51	γ-gliadin	$9.44^{E}04\pm9612$	10.67	1012.3
PYPQPQLPY	A0A0E3Z5U5	Prolamin	$1.21^{E}06\pm1.3.83^{E}05$	11.136	862.45
PFPQPQLPY	A0A0E3Z5U5	Prolamin	$1.21^{E}06\pm1.3.83^{E}05$	11.136	862.45
Half digestion at 210°C					
QQPQQPFPQ	A0A290XZ34	γ-gliadin	$2.24^{E}05\pm5.96^{E}04$	9.39	1005.9
PQPQQQFPQ	A0A290XZ34	γ-gliadin	$2.24^{E}05\pm5.96^{E}04$	9.39	1005.9
LQPQQPFPQ	U5U6N3	ω-gliadin	$3.16^{E}05\pm8.70^{E}04$	9.75	1003.7
QQPQQPFPQ	A0A290XZ51	γ-gliadin	$1.90^{E}05\pm1.56^{E}04$	9.85	841.08
QQPFPQQPQ	A0A290XZ51	γ-gliadin	$3.90^{E}05\pm3.33^{E}04$	10.07	793.39
QGSVQPQQL	A0A0E3Z5U5	Prolamin	$9.18^{E}04\pm5.93^{E}03$	10.63	947.78
QYSQPQQPI	B2ZRD3	Prolamin	$2.68^{E}05\pm2.26^{E}05$	10.67	772.88
PYPQPQLPY	A0A0E3Z5U5	Prolamin	$1.31^{E}06\pm5.13^{E}04$	11.13	862.44
PFPQPQLPY	A0A0E3Z5U5	Prolamin	$1.31^{E}06\pm5.13^{E}04$	11.13	862.44
Half digestion at 245°C					
QQPQQPFPQ	A0A290XZ34	γ-gliadin	$1.44^{E}05\pm1.27^{E}05$	9.39	1006.0
LQPQQPFPQ	U5U6N3	ω-gliadin	$2.72^{E}05\pm5.88^{E}04$	9.75	1003.7

Table 4.1 (continued)

Sequence	Protein code	Protein name	Response (Mean±SD)	Retention time (minutes)	m/z
QYSQPQQPI	B2ZRD3	Prolamin	$4.54^{E}05\pm5.01^{E}04$	9.80	636.82
QQPFPQQPQ	A0A290XZ51	γ-gliadin	$4.19^{E}05\pm1.23^{E}04$	10.08	793.39
QQPQQPFPQ	A0A290XZ51	γ-gliadin	$4.19^{E}05\pm1.23^{E}04$	10.08	793.39
QGSVQPQQL	A0A0E3Z5U5	Prolamin	$9.73^{E}04\pm3.39^{E}03$	10.41	962.12
PYPQPQLPY	A0A0E3Z5U5	Prolamin	$8.58^{E}05\pm6.54^{E}05$	11.05	852.78
PFPQPQLPY	A0A0E3Z5U5	Prolamin	$1.22^{E}06\pm5.56^{E}04$	11.14	848.22
Full digestion at 25°C					
QQPQQPFPQ	A0A060N0S6	ω-gliadin	$1.70^{E}05\pm1.75^{E}05$	9.89	686.33
QYSQPQQPI	B2ZRD3	Prolamin	$2.\ 57^{E}05\pm1.75^{E}05$	10.50	513.59
QQPFPQQPQ	U5U6N3	ω-gliadin	$1.14^{E}05\pm4.58^{E}04$	10.53	518.92
QGSFQPSQQ	A0A0E3Z5U5	Prolamin	$1.69^{E}05\pm4.40^{E}04$	10.55	726.34
Full digestion at 60°C					
QYSQPQQPI	B2ZRD	Prolamin	$7.36^{E}04\pm1.83^{E}04$	10.39	531.59
QGSFQPSQQ	A0A0E3Z5U5	Prolamin	$8.13^{E}04\pm1.29^{E}04$	10.52	884.30
QGSVQPQQL	A0A0E3Z5U	Prolamin	$1.35^{E}05\pm3.52^{E}04$	10.98	551.616
Full digestion at 90°C					
QGSFQPSQQ	A0A0E3Z5U5	Prolamin	$5.64^{E}04\pm6.63^{E}03$	10.40	782.2
QYSQPQQPI	B2ZRD3	Prolamin	$9.02^{E}04\pm4.55^{E}04$	10.51	513.5
Full digestion at 120°C					
QYSQPQQPI	B2ZRD3	Prolamin	$4.31^{E}04\pm1.32^{E}04$	10.43	513.59
QGSFQPSQQ	A0A0E3Z5U5	Prolamin	$6.38^{E}02\pm2.19^{E}04$	10.59	818.26
Full digestion at 150°C					
QQPQQPFPQ	A0A060N0S6	ω-gliadin	$2.06^{E}05\pm1.33^{E}05$	9.28	686.33

Table 4.1 (continued)

Sequence	Protein code	Protein name	Response (Mean±SD)	Retention time (minutes)	m/z
QYSQPQQPI	B2ZRD3	Prolamin	$1.77^{E}05\pm9.28^{E}04$	9.66	513.59
QQPFPQQPQ	A0A290XZ51	γ-gliadin	$9.01^{E}04\pm5.60^{E}04$	9.85	613.13
QGSFQPSQQ	A0A0E3Z5U5	Prolamin	$2.98^{E}05\pm1.02^{E}05$	9.94	832.71
QGSVQPQQL	A0A0E3Z5U5	Prolamin	$9.87^{E}04\pm2.86^{E}04$	9.98	551.62
Full digestion at 180°C					
QGSVQPQQL	A0A0E3Z5U5	Prolamin	$1.88^{E}05\pm2.83^{E}04$	9.13	551.62
QQPQQPFPQ	A0A060N0S6	ω-gliadin	$1.12^{E}05\pm7.64^{E}04$	9.26	686.33
QQPFPQQPQ	A0A060N0S6	ω-gliadin	$1.12^{E}05\pm7.64^{E}04$	9.26	686.33
QYSQPQQPI	B2ZRD3	Prolamin	$1.16^{E}05\pm9.96^{E}04$	9.62	513.59
QGSFQPSQQ	A0A0E3Z5U5	Prolamin	$1.83^{E}05\pm2.75^{E}04$	9.91	832.71
Full digestion at 210°C					
QQPQQPFPQ	A0A060N0S6	ω-gliadin	$1.70^{E}05\pm7.76^{E}04$	9.27	686.33
QYSQPQQPI	B2ZRD3	Prolamin	$1.79^{E}05\pm4.74^{E}04$	9.64	513.59
QGSFQPSQQ	A0A0E3Z5U5	Prolamin	$2.51^{E}05\pm1.08^{E}05$	9.94	832.71
Full digestion at 245°C					
QGSFQPSQQ	A0A0E3Z5U5	Prolamin	$1.97^{E}05\pm2.56^{E}04$	9.93	832.71
QGSVQPQQL	A0A0E3Z5U5	Prolamin	$6.28^{E}04\pm2.54^{E}04$	9.58	551.62
QYSQPQQPI	B2ZRD3	Prolamin	$1.06^{E}05\pm1.18^{E}04$	9.64	513.59

Table 4.2: overview of the epitope sequences found in the digestion cycles at the different temperatures. In the left column the temperatures is given and the type of digestion cycle. H: half digestion and F: full digestion. [35, 36, 46, 47]

	PFPQPQLPY (DQ2.5-glia-α1a)	FRPQQPYPQ (DQ2.5-glia-α3)	PQQSFPQQQ (DQ2.5-glia-γ1)	PQPQQQFPQ (DQ2.5-glia-γ4b)	QQPQQPFPQ (DQ8-glia-γ1a)	QQPFPQQPQ DQ8-glia-γ1b)	QGSFQPSQQ (DQ8-glia-α1)	LQPQQPFPQ (DQ2.5-glia-γ4e)	QGSVQPQQL (DQ2.2-glia-α1)	QYSQPQQPI (DQ2.2-glia-α2)	PYPQPQLPY (DQ2.5-glia-α1b)	PQPQLPYPQ (DQ2.5-glia-α2)
25H	X	X	X	X	X	X	X	X	X	X	X	
60H	X	X	X		X	X	X	X	X	X		X
90H	X	X		X	X	X	X	X	X	X	X	X
120H	X	X	X	X	X	X	X	X	X	X	X	
150H	X		X		X	X		X	X	X	X	
180H	X		X	X	X	X		X	X	X	X	
210H	X			X	X	X		X	X	X	X	
245H	X				X	X		X	X	X	X	
25F					X	X	X			X		
60F							X		X	X		
90F							X			X		
120F							X			X		
150F					X	X	X		X	X		
180F					X	X	X		X	X		
210F					X		X			X		
245F							X		X	X		

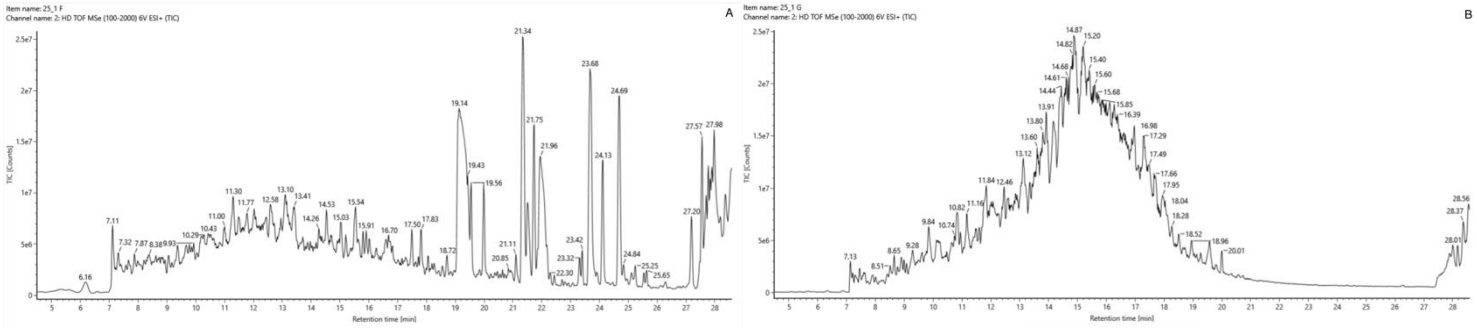

Figure 4.6: Chromatograms obtained from the peptide analysis by Vlon. A: the peptides identified at 25°C in the full digestion, B: the peptides identified at 25°C in the half digestion.

5. DISCUSSION

5.1 DEGREE OF HYDROLYSIS

The degree of hydrolysis indicates the proportion of peptide bonds in a protein that have been cleaved during hydrolysis. As expected, a higher DH is observed in samples subjected to complete digestion compared to those undergoing half digestion. During half digestion, the samples do not complete the full digestion cycle and interact with fewer enzymes, resulting in less digestion and thus less cleavage. This finding is supported by the literature. [35]

Samples subjected to complete digestion exhibited similar DH at the initial temperatures (25°C, 60°C, 90°C, 120°C, and 150°C). From 150°C to 180°C, the degree of hydrolysis increases, followed by a subsequent decrease. The highest DH is observed at 180°C, indicating that gliadin is most effectively digested at this temperature. Up to 180°C, it can be concluded that higher temperatures lead to better digestion.

The heat treatments may induce the formation of aggregates. The process of heating proteins can induce unfolding, resulting in the loss of their secondary and tertiary structure. This can lead to an increase in surface hydrophobicity, which may be significant enough to drive aggregation. OPA is used to measure the free amino groups (refer to figure 1.3), but when aggregation occurs, the primary amino group is no longer free and cannot participate in the reaction. As stated above, the highest DH is observed at 180°C, suggesting that gliadin may be fully denatured at this temperature. This denaturation allows enzymes to cleave bonds effectively, resulting in the highest DH. A possible explanation for the decrease in DH values at 210°C and 245°C could be that at these high temperatures, gliadin begins to crosslink and forms aggregates. Consequently, enzymes are unable to cleave all bonds effectively, leading to reduced digestion and lower DH values. [35, 37, 38, 39]

Initially, the degree of hydrolysis appears quite similar across all temperatures for the half digestion. However, upon closer examination through statistical analysis, significant differences between temperatures become evident. Surprisingly, the DH at 25°C is equal to the DH at the highest temperatures: 210°C and 245°C according to the statistical analysis. Interestingly, there are more pronounced differences between temperatures in half digestion compared to complete digestion. This indicates that temperature clearly influences digestion during the acidic phase.

The degree of hydrolysis for the controls of the digestion was zero, indicating that the presence of enzymes essential is for cleaving gliadin into smaller peptides during digestion.

One study calculated the degree of hydrolysis on heat-treated, digested whey proteins. Notably, they used three temperatures for their heat treatments: 30°C, 60°C, and 90°C.

Comparing our results to theirs, we find similarities when examining samples subjected to the complete digestion, as both show an increase in the degree of hydrolysis from 30°C to 90°C. Differences arise when comparing results from half digestion. While they also observe an increase in DH from 30°C to 90°C, we do not observe such an increase at these temperatures. However, an increase is observed in our samples at the higher temperatures. Several factors may contribute to these differences. Firstly, they conducted experiments on whey protein while our experiments were focused on gliadin. Additionally, their heat treatment duration was 3 hours, whereas ours was only 30 minutes. Lastly, differences in the OPA method used to determine the degree of hydrolysis could also contribute to the observed differences. [35, 40]

When comparing the degree of hydrolysis (DH) results between full digestion and half digestion, two significant factors are involved: time and pH. In the half digestion, the pH of samples is acidic (pH = 2-3), whereas samples undergoing complete digestion have a neutral pH (pH = 7-8) and undergo a longer digestion cycle. As mentioned earlier, the DH is higher in samples subjected to the complete digestion cycle. Comparing our findings to a study that explored the individual effects of pH and time on DH, similarities are observed, with an increase in DH for both factors. [41]

Based on the results obtained, it can be concluded that the optimal conditions for the digestion of gliadin involve heating it to 180°C.

5.2 SOLUBLE PROTEINS

The quantified soluble proteins present in the supernatants obtained from the digestion cycles should correlate with the degree of hydrolysis. On first sight, when comparing these results with the DH on the complete digestion, a similar trend is observed among the different temperatures. However, the most significant difference between the two set of results is that the highest amount of soluble proteins is found at 60°C, rather than at the expected temperature of 180°C. Firstly, it’s important to consider that the degree of hydrolysis reflects the proportion of peptide bonds cleaved during digestion, whereas the amount of soluble proteins indicate the quantity of proteins that have dissolved in the solution. A possible explanation could be that at 180°C, although there was efficient cleavage of peptide bonds leading to a higher degree of hydrolysis, the high temperature might also have caused the formation of aggregates or insoluble complexes. This reduces the solubility of proteins despite their increased DH. On the other hand, at 60°C while the DH might have been lower compared to 180°C, the proteins remained more soluble due to less extensive aggregation or degradation at this temperature.

Following the temperature of 180°C, a decrease is observed for 210°C and 245°C possibly due to aggregation as mentioned earlier. Based on the obtained results, it can be

concluded that insolubility increases with higher temperatures, this is consistent with findings in the literature. [35]

The quantify soluble proteins for the half digestion is consistent across the different temperatures. A literature search revealed that gliadin is soluble in acid and basic pH solutions, with over 90% solubility. Gliadin is poor soluble near neutral pH values, which correspond to its isoelectric point (Figure 5.1). Interestingly, the results from the half digestion cycle are significantly lower than those from the full digestion, despite the expectation that gliadin should be more soluble in acidic pH than in neutral pH. One potential explanation could be the amount of solvent is not enough or the presence of salts and enzymes during digestion, in addition to gliadin. [42]

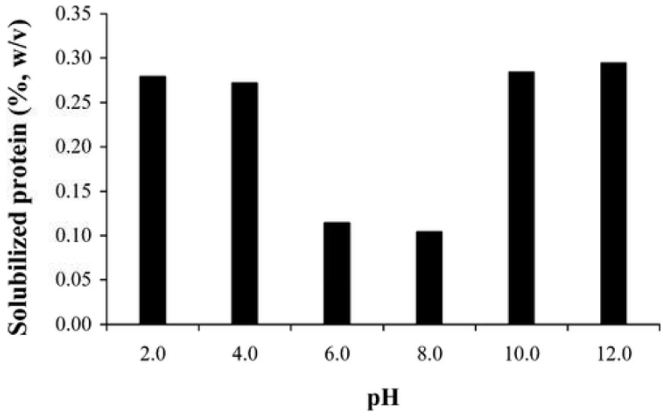

Figure 5.1: Protein solubility (%, w/v) of wheat gliadin dispersions (0.3%, w/v) as a function of pH. [42]

There is a notable contrast observed between the controls and the samples from both the full and the half digestion. The amount of soluble proteins remains mostly consistent between the controls and the samples of the full digestion. This similarity in the full digestion suggests that enzymatic activity may not influence the solubility. However, the exact state of the solubilized proteins remains uncertain: they could exist as cleaved peptides, which would result in a higher degree of hydrolysis or as aggregates. Interestingly, the DH value for the controls was zero, indicating that the proteins are solubilized as a whole or as small aggregates rather than as cleaved peptides. This aligns with existing literature, as heat treatments are known to induce aggregate formation. [35,39]

Furthermore, in the half digestion, a significant difference is observed between the samples and the controls. As previously noted, higher temperatures are associated with lower solubility. This trend is evident in the control samples. It is suspected that enzymes influence solubility in this phase. To fully understand the gastric phase dynamics, repeating

the half digestion with identical conditions, as described in the materials and methods section, and with an increased solvent volume is recommended to asses potential differences in the soluble protein levels.

5.3 GEL-ELECTROPHORESIS

Upon comparing the gel of the gliadin standard with the gel containing gliadin after various heat treatments and digestion, the initial observation was that the bands in the latter gels appeared less visible and defined. This is consistent with findings in the literature. [43]

For the heated samples that underwent complete digestion, similar trends were observed as in the degree of hydrolysis and the soluble proteins. Initially, the bands at the starting temperatures (25°C, 60°C, 90°C, 120°C, and 150°C) were faint, becoming more visible with increasing temperature. The most intense band appeared at 180°C, the bands at 210°C and 245°C were no longer visible. As previously mentioned, a potential hypothesis is that strong aggregation occurs due to these high temperatures, resulting in the formation of larger molecules with a high molecular weight, possibly exceeding 250 kDa, and thereby not displaying any visible bands in the gel. Another possibility is that these high temperatures induce degradation, in this case, gliadin is cleaved into into very small peptides with a low molecular weight, also lacking visible bands in the gel. However, this possibility is ruled out by the measurements of the degree of hydrolysis. At lower temperatures, the structure may not be fully cleaved by the enzymes, as indicated by the DH results. This could explain the faint bands. [35, 37]

In the controls of the complete digestion, a visible band at 37 kDa is evident for the temperatures ranging from 25°C to 180°C. These bands correspond to the one observed in the gel for the gliadin standard prior to heat treatment and digestion. In these controls, the full digestion cycle was conducted without enzymes, resulting in undigested gliadin. These temperatures did not influence the molecular weight of gliadin. However, for the controls at temperatures 210°C and 245°C, no visible bands at 37 kDa is observed. Suggesting that these temperatures possibly influence the structure such as aggregation or degradation. [38]

As for the half digestion, no visible bands are observed at any temperature or their respective controls. Initially, it was speculated that gliadin might not have solubilized in the acidic environment. As seen in our results from the solubilized proteins. It is also possible that no visible bands were observed because the volume injected into the gel had a concentration that was too low. The calculation of the injection volume was based on the results from the gel of the gliadin standard. In that gel, the band for GS50 was the most intense, as expected. Therefore, we used 50 µg of gliadin to calculate the injection volume needed, considering the dilution factor. Additionally, there is a possibility of a strong degradation or aggregation effect induced by the extreme acidic pH. [35, 37]

In theory, the digested gliadin sample with the highest DH, i.e., 180°C, could show a greater number of bands on the gel. This is because the DH indicates the total number of peptide bonds in a protein that have been cleaved during hydrolysis. The higher this value, the more bands should be visible on the gel, resulting in more intense bands at lower molecule weight. This observation is reflected in our gel, where the gliadin is heated to 180°C exhibits the most intense band at around 15 kDa. Additionally, several more bands are visible around 50 kD.

These findings led to the decision to perform an additional analysis using size-exclusion chromatography to assess the presence of aggregation or degradation.

5.4 SIZE-EXCLUSION CHROMATOGRAPHY

In size-exclusion chromatography, molecules with higher molecular weights elute first, while those with smaller molecular weights have longer retention times.

Several observations can be made when analyzing the results obtained from size-exclusion chromatography. Firstly, for the temperatures of 25°C and 60°C, the peak of the half digestion appears before the peak of the full digestion. This suggests that the molecules are larger in the half digestion compared to those in the full digestion. Secondly, at temperatures of 90°C and 120°C, both peaks overlap with each other. However, from the temperature of 150°C, the peak of the half digestion elutes later than the peak of the full digestion, indicating that the molecules in the half digestion are smaller compared to those in the full digestion. It can be concluded that for the half digestion, the molecules become smaller with the increasing temperature. In contrast, the peaks of the full digestion always elute at the same moment, with a retention time (RT) around 13.25 minutes (Figure 4.5). Additionally, it is evident that aggregates are present in the full digestion, see figure 5.2. These aggregates increase with the increasing temperature, see figure 4.5. [35]

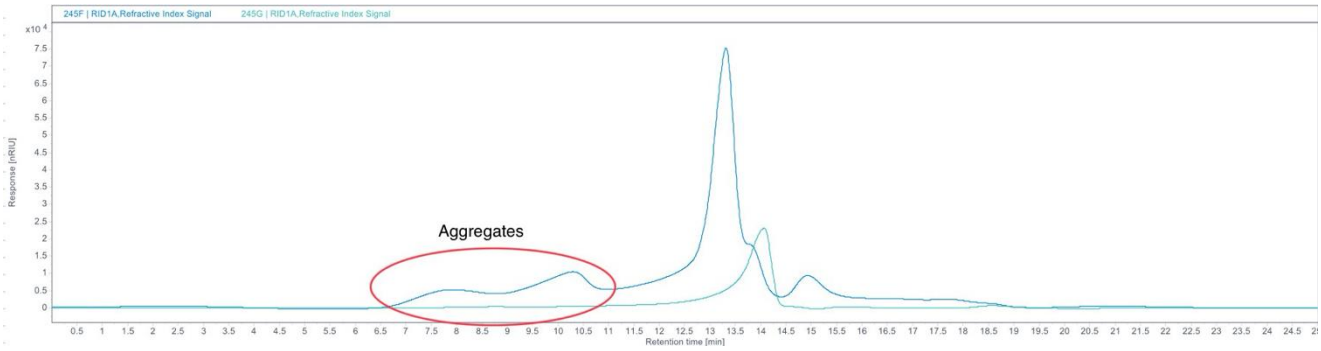

Figure 5.2: The comparison between the gliadin heated at 245°C in the complete digestion and the half digestion. Aggregates are present in the full digestion.

As a result of these findings, our hypothesis, derived from the DH, soluble proteins and the gel analysis, has been strengthened. At the temperatures of 210°C and 245°C, aggregates

are formed. These aggregates likely contribute to the decrease in the DH and the quantity of soluble proteins observed after the temperature of 180°C. It also explains the absence of any visible bands for these two temperatures in the gel. Additionally, based on these results, it may be concluded that there is no aggregation or degradation present in the gastric phase. It is possible that the injection volume used had too low of a concentration, as mentioned earlier. Therefore, it would be advisable to repeat the gel analysis using the same injection volume as used for the gel of the full digestion.

Further research is required to fully understand the processes occurring during both the gastric and the intestinal phase of digestion to draw definitive conclusions.

5.5 PEPTIDE ANALYSIS

ORBITrap analysis run on a series of digested grain samples provided a list of peptides detected in the samples and the list of proteins from which these peptides are are derived. This list (Table 3.8) served as input for processing of the data from the Vion analysis, which ultimately provided the peptide sequences present in the digested gliadin samples. These peptide sequences were then compared to known epitope sequences responsible for celiac disease, which are T cell epitopes recognized by $CD4^+$ T cells. [46, 47]

As a result, numerous epitopes were identified, as described in Tables 4.1 and 4.2. Several observations can be made. First, more epitopes were found during half digestion (the gastric phase) than during the intestinal phase, suggesting that most epitopes are still present in the gastric phase. During half digestion, most epitopes remain present throughout all temperatures of the heat treatment, although some, such as DQ2.5-glia-α3, DQ2.5-glia-γ1, DQ2.5-glia-γ4b, DQ8-glia-α1 and DQ2.5-glia-α2 disappear.

After the full digestion, far fewer epitopes are present. Notably, only one epitope, DQ2.2-glia-α2, is consistently present at every temperature in both digestion cycles. Interestingly, some epitopes that are detected at lower temperatures, disappear, and then reappear at higher temperatures. This suggests that the temperature may affect the denaturation of gliadin's structure, causing epitopes to be present but undetectable due to denaturation. Another interesting observation is that the epitope DQ2.5-glia-α2 appears for the first time at 60°C during half digestion, not at the control temperature. One possible hypothesis is that this epitope may be exposed (therefore available for digestive enzymes capable of liberating it from the protein sequence) after heating the gliadin due to structural modifications.

This is a semi-quantitative analysis, where the response can be compared for the same peptide across different temperatures. The response is directly proportional to the amount of epitope, with a higher response indicating a greater presence of peptide. Observing the response, there are no obvious trends between the temperatures of each cycle. Generally, it can be concluded that the number of peptides containing a CD epitope is higher during

half digestion than during full digestion, suggesting that heat treatment does not have a clear trend on the amount of epitope presence and that the epitopes are more exposed in the gastric phase.

Upon examining the chromatograms (Figure 4.6) from the peptide analysis, several comparisons can be made. Comparing the full digestion with the half digestion at 25°C reveals differences. The full digestion chromatogram exhibits peaks over the entire range with retention times from 6 to 28 minutes. Considering that after 19 minutes the peaks are due to the elution of bile salts, the elution range of the peptides is between approximately 6 and 20 minutes. Similarly, half digestion displays peaks with retention times ranging from 7 to 20 minutes. Generally, higher retention times correspond to larger molecular weights of peptides. In the half digestion, higher peaks at higher retention times (thus probably peptides with higher molecular weights) are observed, potentially indicating the presence of larger polypeptides than in the full digestion. In general it can be concluded that peptides from half digestion have a higher molecular weight than peptides from full digestion. This is alignment with the results of the SEC.

6. CONCLUSION

The study investigated the impact of thermal treatment on gliadin digestibility and immunogenic potential in a full (gastric + duodenal) and a gastric digestion cycle.

The findings for the complete digestion cycle revealed that the most optimal conditions for gliadin digestion involved heating it to a temperature of 180°C. However, the temperatures exceeding 180°C led to aggregation, as evidenced by the degree of hydrolysis, the gel electrophoresis and the SEC findings. Additionally, the soluble protein analysis revealed unexpected results, with the highest amount of observed at 60°C rather than 180°C, possibly due to the formation of larger aggregates at 180°C.

The results from the half digestion cycle indicated a lower degree of hydrolysis compared to the full digestion, which is expected since they were not exposed to all the necessary enzymes for a complete digestion. Additionally, the quantity of soluble proteins was lower compared to the full digestion, which is surprising considering that gliadin is known to be soluble in acidic pH but not in neutral pH. It is possible that the amount of solvent used was insufficient for proper solubilization or the presence of salts and enzymes affected the solubility. The gel analysis showed no visible bands, likely due to an injection volume with a concentration that was too low. Size-exclusion chromatography revealed no aggregates for the half digestion, but gliadin appeared to decrease in size with the increasing temperatures.

For both digestion cycles, the controls showed a degree of hydrolysis of zero, indicating the necessity of enzymes for cleaving gliadin into smaller peptides. For the soluble protein analysis, there was no difference between the controls and the samples of the full digestion, suggesting that the enzymes do not have influence the solubility of gliadin. However, a significant difference was observed between the controls and the samples of the half digestion for this analysis. The controls have a significant lower quantity of soluble proteins compared to the samples, with the amount decreasing with the increasing temperatures. Suggesting that pepsin does have an affect on the solubility. In the gel, the bands for the controls of the full digestion are present till the temperature of 180°C, indicating the presence of aggregates at higher temperatures. For the half digestion, no bands were visible for the controls, possibly due to the low concentration of the injection volume.

The peptide analysis revealed numerous epitopes, with more epitopes present in the gastric phase than in the intestinal phase, suggesting that epitopes are more exposed and resistant during this phase. One epitope, DQ2.2-glia-α2, was consistently present at every temperature during both digestion cycles. Generally, the number of epitopes was higher during the half digestion compared to complete digestion.

According to our finding, the most effective conditions for gliadin digestion involves heating gliadin to 180°C. Beyond this temperature, a decrease in effectiveness is observed, likely due to formation of larger aggregates as the temperature increases. This conclusion is supported by the finding of the degree of hydrolysis, gel electrophoresis and size-exclusion

chromatography. The peptide analysis indicated that while temperature does not clearly affect the number of epitopes present, it does influence which epitopes are detectable.

Further research is required for a deeper understanding of the processes occurring during both the gastric and the intestinal phase of digestion to draw definitive conclusions.

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8. ATTACHMENT

8.1 ATTACHMENT 1

Table 1.1: pH during the different phases of the pH trial

ORAL PHASE				
	Replicate	**Initial pH**	**V_{NaOH}/V_{HCl}**	**pH after adjustment**
	1	pH = 6		
	2	pH = 6		
	3	pH = 6		
GASTRIC PHASE				
	Replicate	**Initial pH**	**V_{NaOH}/V_{HCl}**	**pH after adjustment**
Minute 0	1	pH = 3-4	10 µL HCl (1M)	pH = 2-3
	2	pH = 3-4	10 µL HCl (1M)	pH = 2-3
	3	pH = 3-4	10 µL HCl (1M)	pH = 2-3
Minute 30	1	pH = 4	20 µL HCl (1M)	pH = 2-3
	2	pH = 4	20 µL HCl (1M)	pH = 2-3
	3	pH = 4	20 µL HCl (1M)	pH = 2-3
Minute 60	1	pH = 2-3		
	2	pH = 2-3		
	3	pH = 2-3		
Minute 90	1	pH = 2-3		
	2	pH = 2-3		
	3	pH = 2-3		
Minute 120	1	pH = 2-3		
	2	pH = 2-3		
	3	pH = 2-3		
INTESTINAL PHASE				
	Replicate	**Initial pH**	**V_{NaOH}/V_{HCl}**	**pH after adjustment**
Minute 0	1	pH = 7		
	2	pH = 7		
	3	pH = 7		
Minute 30	1	pH = 7		
	2	pH = 7		
	3	pH = 7		
Minute 60	1	pH = 7		

Table 1.1 (continued)

	Replicate	Initial pH	V_{NaOH}/V_{HCl}	pH after adjustment
	2	pH = 7		
	3	pH = 7		
Minute 90	1	pH = 7		
	2	pH = 7		
	3	pH = 7		
Minute 120	1	pH = 7		
	2	pH = 7		
	3	pH = 7		

Table 1.2: Volume supernatans after pH trial

Replicate	Volume supernatans (mL)
1	7.6
2	7.4
3	7.6

Table 1.3: pH during the different phases of the complete digestion

ORAL PHASE				
		Initial pH	V_{NaOH}/V_{HCl}	pH after adjustment
	25 °C	pH = 6		
	60 °C	pH = 6		
	90°C	pH = 6		
	120 °C	pH = 6		
	150°C	pH = 6		
	180°C	pH = 6		
	210°C	pH = 6		
	245°C	pH = 6		
GASTRIC PHASE				
		Initial pH	V_{NaOH}/V_{HCl}	pH after adjustment
Minute 0	25 °C	pH = 3-4	10 µL HCl (1M)	pH = 2-3
	60 °C	pH = 3-4	10 µL HCl (1M)	pH = 2-3
	90°C	pH = 3-4	10 µL HCl (1M)	pH = 2-3
	120 °C	pH = 3-4	10 µL HCl (1M)	pH = 2-3

Table 1.3 (continued)

		Initial pH	V_{NaOH}/V_{HCl}	pH after adjustment
	150°C	pH = 4	20 µL HCl (1M)	pH = 2-3
	180°C	pH = 4	20 µL HCl (1M)	pH = 2-3
	210°C	pH = 3-4	10 µL HCl (1M)	pH = 2-3
	245°C	pH = 3-4	10 µL HCl (1M)	pH = 2-3
	Blank	pH = 2-3		
Minute 30	25 °C	pH = 2-3		
	60 °C	pH = 2-3		
	90°C	pH = 2-3		
	120 °C	pH = 2-3		
	150°C	pH = 3- 4	10 µL HCl (1M)	pH = 2-3
	180°C	pH = 3-4	10 µL HCl (1M)	pH = 2-3
	210°C	pH = 3-4	10 µL HCl (1M)	pH = 2-3
	245°C	pH = 3-4	10 µL HCl (1M)	pH = 2-3
	Blank	pH = 2-3		
Minute 60	25 °C	pH = 3-4	10 µL HCl (1M)	pH = 2-3
	60 °C	pH = 2-3		
	90°C	pH = 2-3		
	120 °C	pH = 2-3		
	150°C	pH = 2-3		
	180°C	pH = 2-3		
	210°C	pH = 2-3		
	245°C	pH = 2-3		
	Blank	pH = 2-3		
Minute 90	25 °C	pH = 2-3		
	60 °C	pH = 2-3		
	90°C	pH = 2-3		
	120 °C	pH = 2-3		
	150°C	pH = 2-3		
	180°C	pH = 2-3		
	210°C	pH = 2-3		
	245°C	pH = 2-3		
	Blank	pH = 2-3		

Table 1.3 (continued)

		Initial pH	**V_{NaOH}/V_{HCl}**	**pH after adjustment**
Minute 120	25 °C	pH = 2-3		
	60 °C	pH = 2-3		
	90°C	pH = 2-3		
	120 °C	pH = 2-3		
	150°C	pH = 2-3		
	180°C	pH = 2-3		
	210°C	pH = 2-3		
	245°C	pH = 2-3		
	Blank	pH = 2-3		
INTESTINAL PHASE				
		Initial pH	**V_{NaOH}/V_{HCl}**	**pH after adjustment**
Minute 0	25 °C	pH = 7		
	60 °C	pH = 7		
	90°C	pH = 7		
	120 °C	pH = 7		
	150°C	pH = 7		
	180°C	pH = 7		
	210°C	pH = 7		
	245°C	pH = 7		
	Blank	pH = 7		
Minute 30	25 °C	pH = 7		
	60 °C	pH = 7		
	90°C	pH = 7		
	120 °C	pH = 7		
	150°C	pH = 7		
	180°C	pH = 7		
	210°C	pH = 7		
	245°C	pH = 7		
	Blank	pH = 7		
Minute 60	25 °C	pH = 7		
	60 °C	pH = 7		
	90°C	pH = 7		
	120 °C	pH = 7		
	150°C	pH = 7		
	180°C	pH = 7		
	210°C	pH = 7		
	245°C	pH = 7		
	Blank	pH = 7		

Table 1.3 (continued)

		Initial pH	**V_{NaOH}/V_{HCl}**	**pH after adjustment**
Minute 120	25 °C	pH = 7		
	60 °C	pH = 7		
	90°C	pH = 7		
	120 °C	pH = 7		
	150°C	pH = 7		
	180°C	pH = 7		
	210°C	pH = 7		
	245°C	pH = 7		
	Blank	pH = 7		

Table 1.4: pH during the different phases of the half digestion

ORAL PHASE				
		Initial pH	**V_{NaOH}/V_{HCl}**	**pH after adjustment**
	25 °C	pH = 6		
	60 °C	pH = 6		
	90°C	pH = 6		
	120 °C	pH = 6		
	150°C	pH = 6		
	180°C	pH = 6		
	210°C	pH = 6		
	245°C	pH = 6		
GASTRIC PHASE				
		Initial pH	**V_{NaOH}/V_{HCl}**	**pH after adjustment**
Minute 0	25 °C	pH = 3-4	10 µL HCl (1M)	pH = 2-3
	60 °C	pH = 3-4	10 µL HCl (1M)	pH = 2-3
	90°C	pH = 3-4	10 µL HCl (1M)	pH = 2-3
	120 °C	pH = 2-3		
	150°C	pH = 2-3		
	180°C	pH = 2-3		
	210°C	pH = 2-3		
	245°C	pH = 2-3		
	Blank	pH = 2-3		
Minute 30	25 °C	pH = 3-4	10 µL HCl (1M)	pH = 2-3

Table 1.4 (continued)

		Initial pH	**V_{NaOH}/V_{HCl}**	**pH after adjustment**
	60 °C	pH = 3-4	10 µL HCl (1M)	pH = 2-3
	90°C	pH = 2-3		
	120 °C	pH = 2-3		
	150°C	pH = 2-3		
	180°C	pH = 2-3		
	210°C	pH = 2-3		
	245°C	pH = 2-3		
	Blank	pH = 2-3		
Minute 60	25 °C	pH = 2-3		
	60 °C	pH = 2-3		
	90°C	pH = 2-3		
	120 °C	pH = 3-4	10 µL HCl (1M)	pH = 2-3
	150°C	pH = 2-3		
	180°C	pH = 2-3		
	210°C	pH = 2-3		
	245°C	pH = 2-3		
	Blank	pH = 2-3		
Minute 90	25 °C	pH = 2-3		
	60 °C	pH = 2-3		
	90°C	pH = 2-3		
	120 °C	pH = 2-3		
	150°C	pH = 2-3		
	180°C	pH = 2-3		
	210°C	pH = 2-3		
	245°C	pH = 2-3		
	Blank	pH = 2-3		
Minute 120	25 °C	pH = 2-3		
	60 °C	pH = 2-3		
	90°C	pH = 2-3		
	120 °C	pH = 2-3		
	150°C	pH = 2-3		
	180°C	pH = 2-3		
	210°C	pH = 2-3		
	245°C	pH = 2-3		
	Blank	pH = 2-3		

Table 1.5: pH during the different phases of the control complete digestion

ORAL PHASE				
		Initial pH	**V_{NaOH}/V_{HCl}**	**pH after adjustment**
	25 °C	pH = 6		
	60 °C	pH = 6		
	90°C	pH = 6		
	120 °C	pH = 6		
	150°C	pH = 6		
	180°C	pH = 6		
	210°C	pH = 6		
	245°C	pH = 6		
GASTRIC PHASE				
		Initial pH	**V_{NaOH}/V_{HCl}**	**pH after adjustment**
Minute 0	25 °C	pH = 3-4	10 µL HCl (1M)	pH = 2-3
	60 °C	pH = 2-3		
	90°C	pH = 2-3		
	120 °C	pH = 2-3		
	150°C	pH = 2-3		
	180°C	pH = 2-3		
	210°C	pH = 2-3		
	245°C	pH = 2-3		
	Blank	pH = 2-3		
Minute 30	25 °C	pH = 2-3		
	60 °C	pH = 2-3		
	90°C	pH = 2-3		
	120 °C	pH = 2-3		
	150°C	pH = 2-3		
	180°C	pH = 2-3		
	210°C	pH = 2-3		
	245°C	pH = 3-4	10 µL HCl (1M)	pH = 2-3
	Blank	pH = 2-3		
Minute 60	25 °C	pH = 2-3		
	60 °C	pH = 2-3		
	90°C	pH = 2-3		
	120 °C	pH = 2-3		
	150°C	pH = 3-4	10 µL HCl (1M)	pH = 2-3

Table 1.5 (continued)

		Initial pH	**V_{NaOH}/V_{HCl}**	**pH after adjustment**
	180°C	pH = 3-4	10 µL HCl (1M)	pH = 2-3
	210°C	pH = 3-4	10 µL HCl (1M)	pH = 2-3
	245°C	pH = 2-3		
	Blank	pH = 2-3		
Minute 90	25 °C	pH = 2-3		
	60 °C	pH = 2-3		
	90°C	pH = 2-3		
	120 °C	pH = 2-3		
	150°C	pH = 2-3		
	180°C	pH = 2-3		
	210°C	pH = 2-3		
	245°C	pH = 2-3		
	Blank	pH = 2-3		
Minute 120	25 °C	pH = 2-3		
	60 °C	pH = 2-3		
	90°C	pH = 2-3		
	120 °C	pH = 2-3		
	150°C	pH = 2-3		
	180°C	pH = 2-3		
	210°C	pH = 2-3		
	245°C	pH = 2-3		
	Blank	pH = 2-3		
INTESTINAL PHASE				
		Initial pH	**V_{NaOH}/V_{HCl}**	**pH after adjustment**
Minute 0	25 °C	pH = 7		
	60 °C	pH = 7		
	90°C	pH = 7		
	120 °C	pH = 7		
	150°C	pH = 7		
	180°C	pH = 7		
	210°C	pH = 7		
	245°C	pH = 7		
	Blank	pH = 7		
Minute 30	25 °C	pH = 7		
	60 °C	pH = 7		
	90°C	pH = 7		
	120 °C	pH = 7		
	150°C	pH = 7		

Table 1.5 (continued)

	180°C	pH = 7		
	210°C	pH = 7		
	245°C	pH = 7		
	Blank	pH = 7		
Minute 60	25 °C	pH = 7		
	60 °C	pH = 7		
	90°C	pH = 7		
	120 °C	pH = 7		
	150°C	pH = 7		
	180°C	pH = 7		
	210°C	pH = 7		
	245°C	pH = 7		
	Blank	pH = 7		
Minute 120	25 °C	pH = 7		
	60 °C	pH = 7		
	90°C	pH = 7		
	120 °C	pH = 7		
	150°C	pH = 7		
	180°C	pH = 7		
	210°C	pH = 7		
	245°C	pH = 7		
	Blank	pH = 7		

Table 1.6: pH during the different phases of the control half digestion

ORAL PHASE				
		Initial pH	**V_{NaOH}/V_{HCl}**	**pH after adjustment**
	25 °C	pH = 6		
	60 °C	pH = 6		
	90°C	pH = 6		
	120 °C	pH = 6		
	150°C	pH = 6		
	180°C	pH = 6		
	210°C	pH = 6		
	245°C	pH = 6		
GASTRIC PHASE				
		Initial pH	**V_{NaOH}/V_{HCl}**	**pH after adjustment**
Minute 0	25 °C	pH = 3-4	10 µL HCl (1M)	pH = 2-3
	60 °C	pH = 3-4	10 µL HCl (1M)	pH = 2-3

Table 1.6 (continued)

		Initial pH	**V_{NaOH}/V_{HCl}**	**pH after adjustment**
	90°C	pH = 3-4	10 µL HCl (1M)	pH = 2-3
	120 °C	pH = 2-3		
	150°C	pH = 2-3		
	180°C	pH = 2-3		
	210°C	pH = 2-3		
	245°C	pH = 2-3		
	Blank	pH = 2-3		
Minute 30	25 °C	pH = 3-4	10 µL HCl (1M)	pH = 2-3
	60 °C	pH = 3-4	10 µL HCl (1M)	pH = 2-3
	90°C	pH = 2-3		
	120 °C	pH = 2-3		
	150°C	pH = 2-3		
	180°C	pH = 2-3		
	210°C	pH = 2-3		
	245°C	pH = 2-3		
	Blank	pH = 2-3		
Minute 60	25 °C	pH = 2-3		
	60 °C	pH = 2-3		
	90°C	pH = 2-3		
	120 °C	pH = 2-3		
	150°C	pH = 2-3		
	180°C	pH = 3-4	10 µL HCl (1M)	pH = 2-3
	210°C	pH = 3-4	10 µL HCl (1M)	pH = 2-3
	245°C	pH = 3-4	10 µL HCl (1M)	pH = 2-3
	Blank	pH = 2-3		
Minute 90	25 °C	pH = 3-4	10 µL HCl (1M)	pH = 2-3
	60 °C	pH = 2-3		
	90°C	pH = 2-3		
	120 °C	pH = 2-3		
	150°C	pH = 2-3		
	180°C	pH = 2-3		
	210°C	pH = 2-3		
	245°C	pH = 2-3		
	Blank	pH = 2-3		

Table 1.6 (continued)

		Initial pH	**V_{NaOH}/V_{HCl}**	**pH after adjustment**
Minute 120	25 °C	pH = 2-3		
	60 °C	pH = 2-3		
	90°C	pH = 2-3		
	120 °C	pH = 2-3		
	150°C	pH = 2-3		
	180°C	pH = 2-3		
	210°C	pH = 2-3		
	245°C	pH = 2-3		
	Blank	pH = 2-3		

Master dissertation submitted to the faculty of Pharmaceutical Sciences, performed in collaboration with the Laboratory Food Science, University of Parma.

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