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IMPACT OF MICROSTRUCTURE ON PROTEIN HYDROLYSIS AND ACID UPTAKE DURING IN VITRO GASTRIC DIGESTION OF EGG WHITE PROTEIN GELS

Author: *María García Guerrero*

UPV Tutor: *José Vicente García Pérez*

External Tutor: *Gail Michelle Bornhorst*

València, April 2019

Title:

Impact of microstructure on protein hydrolysis and acid uptake during in vitro gastric digestion of egg white protein gels

Abstract:

Scientific interest in evaluating the effects of food on human health has increased in the last few years, but the underlying mechanisms of digestion still need to be better understood. The aim of this study was to determine the influence of food microstructure on protein hydrolysis, acid and moisture uptake during in vitro gastric digestion of egg protein gels. Dispersions prepared with 11.26% egg white protein were adjusted to pH 3, 5 or 7.5 and heated at 90 ^{o}C for 1 hour to form different gel microstructures. Gels were cut into cubes (12 x 12 mm) and underwent in vitro oral digestion for 30 seconds (0.2 mL saliva/g gel, pH 7, 194.7 U/mL α-amylase) and in vitro gastric digestion for 15, 30, 60, 120, 180 or 240 minutes (6 mL gastric juice/g gel, pH 1.8, 2000 U/mL pepsin) in a shaking water bath (37^{o}C, 100 rpm). Free amino groups during digestion were quantified using the o-Phthalaldehyde (OPA) method. Acid penetration was measured by potentiometric titration to pH 8.2, and moisture uptake was measured gravimetrically. Changing the microstructure of the gel (due to different initial pH) impacted protein hydrolysis ($p < 0.05$). The amount of free amino groups present in the gel after 240 minutes digestion was 22.66 mg glycine eq/g dry mass in pH 3 gels, 1.03 in pH 5 gels, and 6.44 in pH 7.5 gels. The acid uptake was significantly ($p < 0.05$) influenced by gel microstructure. The moisture uptake during gastric digestion was significantly ($p < 0.05$) greater in gels at pH 3, but similar in gels at pH 5 and 7.5. Egg protein gel microstructure influenced protein breakdown and uptake of moisture and acid. The influence of initial food structure on acid uptake and subsequent protein hydrolysis may help predict nutrient release and inform design of new food products.

Keywords:

In vitro digestion, Egg white protein, Food microstructure, Protein hydrolysis, Acid uptake

Author: María García Guerrero

UPV Tutor: José Vicente García Pérez

External Tutor: Gail Michelle Bornhorst

València, April 2019

Título:

Efecto de la microestructura en la hidrólisis de proteínas y la absorción de ácido durante la digestión gástrica in vitro de geles de proteína de huevo.

Resumen:

El interés científico en el análisis de los efectos de los alimentos en la salud humana ha aumentado, pero los mecanismos subyacentes de la digestión todavía necesitan una mayor comprensión. El objetivo de este estudio consiste en determinar la influencia de la microestructura de los alimentos en la hidrólisis proteínica y en la absorción de ácido y humedad durante digestión gástrica in vitro de geles de proteína de huevo. Las dispersiones preparadas con un 11.26% de proteína de clara de huevo y ajustadas a pH 3, 5 o 7.5 se calentaron a 90 ^{o}C por una hora para formar geles con diferentes microestructuras. Estos geles fueron cortaron en cubos (12 x 12 mm) y sometidos a una digestión oral in vitro por 30 segundos (0.2 mL saliva/g gel, pH 7, 194.7 U/mL α-amilasa) y a una digestión gástrica in vitro por 15, 30, 60, 120, 180 o 240 minutos (6 mL jugo gástrico/g gel, pH 1.8, 2000 U/mL pepsina) en un baño de agua con agitación (37^{o}C, 100 rpm). Los grupos amino libres formados durante la digestión fueron cuantificados usando el método o-Phthalaldehyde (OPA). La penetración acida fue medida mediante valoraciones potenciométricas a pH 8.2, y la absorción de humedad fue medida gravimétricamente. Cambiar la microestructura del gel (dada por el pH inicial) ha afectado a la hidrólisis de proteínas ($p<0.05$). La cantidad de grupos amino libres presentes en el gel después de 240 minutos de digestión fue de 22.66 mg equivalente de glicina/g masa seca en geles a pH 3, 1.03 en geles a pH 5, y 6.44 en geles a pH 7.5. La absorción de ácido fue influida de manera significativa ($p<0.05$) por la microestructura del gel. La absorción de humedad durante la digestión fue considerablemente ($p<0.05$) mayor en geles a pH 3, pero similar en geles a pH 5 y pH 7.5. La microestructura del gel de proteína ha afectado la descomposición química de las proteínas y la absorción de ácido y humedad. La influencia de la estructura inicial de los alimentos en la absorción de ácido y la posterior hidrólisis proteínica puede ayudar a predecir la liberación de nutrientes y conformar el diseño de nuevos productos alimenticios.

Palabras clave:

Digestión in vitro, Proteína de clara de huevo, Microestructura de alimentos, Hidrólisis de proteínas, Absorción de ácido.

Autora: María García Guerrero

Tutor UPV: José Vicente García Pérez

Tutor externo: Gail Michelle Bornhorst

Valencia, Abril 2019

Títol:

Impacte de la microestructura sobre la hidròlisi de proteïnes i captació d'àcids durant la digestió gàstrica in vitro de gels proteics blancs d'ou.

Resum:

L'interès científic per avaluar els efectes dels aliments en la salut humana ha augmentat en els últims anys, però els mecanismes subjacents a la digestió encara han no són del tot compresos. L'objectiu d'aquest estudi va ser determinar la influència de la microestructura alimentària en la hidròlisi de proteïnes, l'absorció d'àcids i humitats durant la digestió gàstrica in vitro de gels proteics d'ous. Les dispersions preparades amb 11,26% de proteïna blanca d'ou es van ajustar a pH 3, 5 o 7,5 i es van escalfar a 90 ^{o}C durant 1 hora per formar diferents microestructures de gel. Els gels es van tallar en cubs (12 x 12 mm) i es va realitzar una digestió oral in vitro durant 30 segons (0.2 ml de saliva / g de gel, pH 7, 194.7 U / ml d'α-amilasa) i digestió gàstrica in vitro de 15, 30, 60 , 120, 180 o 240 minuts (6 ml de suc gàstric / g de gel, pH 1,8, 2000 U / ml de pepsina) en un bany amb agitació (37^{o}C, 100 rpm). Els grups amino lliures durant la digestió es van quantificar utilitzant el mètode o-Phthalaldehyde (OPA). La penetració àcida es va mesurar mitjançant una valoració potenciomètrica fins al pH 8.2, i es va mesurar la absorció d'humitat gravimètrica. Canviar la microestructura del gel (a causa del pH inicial diferent), va afectar a la hidròlisi de proteïnes ($p < 0,05$). La quantitat de grups amino lliures presents al gel després de 240 minuts de digestió era de 22.66 mg de glicina eq / g de massa seca en pH 3 gels, 1.03 en pH 5 gels i 6.44 en pH 7.5 gels. La captació d'àcids va ser significativament ($p < 0,05$) influenciada per la microestructura del gel. L'absorció d'humitat durant la digestió gàstrica va ser significativament ($p < 0,05$) major en gels a pH 3, però similar en gels a pH 5 i 7,5. La microestructura de gel de proteïnes d'ou va influir en la descomposició de proteïnes i l'absorció d'humitat i àcid. La influència de l'estructura alimentària inicial en la captació d'àcids i posterior hidròlisi de proteïnes pot ajudar a predir l'alliberament de nutrients i informar per al disseny dels nous productes alimentaris.

Paraules clau:

Digestió in vitro, proteïna blanca d'ou, microestructura alimentària, hidròlisi de proteïnes, captació d'àcids

Autora: María García Guerrero

Tutor UPV: José Vicente García Pérez

Tutor extern: Gail Michelle Bornhorst

València, Abril 2019

1. INTRODUCTION

1.1. PROTEIN GELS IN FOOD SCIENCE RESEARCH

1.1.1. Historical perspective

The word "jelly" appeared for the first time in the XIV century and it came from the Latin word *gelare* that means "to freeze". This word derived to the French word *gelée* that means "ice cream". The scientific term of the word "gel" was used for the first time by Thomas Graham, the father of colloids chemistry, in the middle of the XVII century (Alting, 2003). The word "gel" has had different meanings throughout the years but thirty years ago was described as "a system of solid characteristics in which the colloid particles form a consistent structure" (Oakenfull *et al.,* 1997).

1.1.2. Food gels: Characteristics and properties

Food gels are defined as viscoelastic substances in which the gelling agents are usually polysaccharides and proteins. In food gels, the polymer molecules are not cross-linked by covalent bonds with the exception of disulphide bonds in some protein gels (Banerjee & Bhattacharya, 2012). Instead, the molecules are held together by a combination of weak inter-molecular forces like hydrogen bonds, electrostatic forces, Van der Waals forces, and hydrophobic interactions that extends the volume of the liquid.

Studies using light scattering and microscopy has shown that the gelling process involves the aggregation of colloidal particles (Lattuada, Wu & Morbidelli, 2004). Moreover, it is known that the gels present a tridimensional branch structure more or less consistent that retain the liquid component and give elasticity and stiffness to the system (Stading & Hermansson, 1991). In food gels, this liquid is mostly water and the molecular network consists of proteins or polysaccharides, or a combination of both. These molecules are able to form cross-linking bonds in three dimensions, which is essential in order to form a gel. The properties of proteins are very important for the gelling process, as they give flexibility to the gel due to their ability to denaturalize and form extended chains by cross-linking (Spotti, 2013). In order to achieve this denaturalization process of proteins, heating of the solution is needed in order to expose the hydrophobic part of the molecule, as well as the sulfhydryl groups to the solvent. When the non-polar amino acids are exposed, the intermolecular interactions take place. This way, the regions of the protein that originally were involved on keeping the native structure are now available for creation of intermolecular bonds (Perez, Wargon & Pilosof, 2006) and the consequent formation of aggregates that join together to form gels. The way the protein gel is heated also modifies the structure of the gel and therefore, digestion process and the nutritional properties are affected (Opazo-Navarere *et al.,* 2018).

1.1.3. Egg white protein gels

Humans have used bird eggs since prehistoric times (Hirose, 2003). Eggs are capable of performing various useful functions in foods, including foaming, gelling or emulsifying (Woodward, 1990). Egg liquid components can form a gel through a heating treatment. This is an important property in the everyday

consumption of egg, as they could be scrambled, fried, boiled, or been used in the preparation of other meals as omelets, quiches or desserts among other things. The liquid portion of the eggs consists of about 67-70% egg white and 30-33% yolk (Powrie & Nakai, 1986). Egg white is more or less a colloidal suspension of different proteins in water. Ovalbumin (54%), ovotransferrin (12%), ovomucoid (11%), ovomucin (3.5%), and lysozyme (3.5%) are the most functionally important proteins of egg white (Abeyrathne & Lee 2013).

Due to their functional and nutritional properties, egg white protein gels have been widely used as a model food system. Egg white protein gels are three-dimensional continuous networks formed via connected biopolymers or proteins capable of retaining large amount of water (Croguennec *et al.* 2002). The structural properties of these gels depend on the aggregate morphology such as linear strands or spherical particles, which in turn depends on pH, ionic strength, protein concentration and heating time and temperature (Opazo-Navarrete *et al*., 2018). The isoelectric point of the egg white proteins is around 4.7, this means that as close the gel gets to that pH, the electrostatic interaction between the proteins forming the gel is stronger than the interaction between the proteins and the water particles. This leads to a lack of continuousness and homogeneity of the gel matrix. These properties of egg white make it an interesting model to investigate protein gelation because it can form a wide range of gel structures while keeping a constant chemical composition. Moreover, several studies have suggested that the microstructure of food is an important parameter that influences digestion within the gastrointestinal tract (Fardet *et al.*, 2013) and that, in the same way, the rate of digestion of gelled protein systems depends on the gel structure (Nyemb *et al.,* 2016). More specifically, the importance of food protein gelation on the nutritional properties of human foods has been investigated (Nyemb *et al.,* 2016). In addition, the potential of protein gelation for developing food structures that modulate digestion in now recognized (Norton *et al.*, 2015).

1.2. GASTRIC DIGESTION

1.2.1 Gastric digestion process

Gastric digestion consists in the absorption of energy and nutrients from foods, an essential step in human nutrition and health (Luo *et al.*, 2015). Food is disintegrated into small size in the mouth and stomach and the main nutrient absorption takes place in the small intestine (Kong & Singh, 2008).

The digestion process starts in the moment food is masticated and mixed with saliva. During chewing, saliva helps in preparing the food bolus by agglomerating the formed particles, and it initiates enzymatic food breakdown (Joubert *et al*., 2017). This cohesive food mass will undergo simultaneous physical and chemical transformations that will continue to release the nutrients that the body will absorb in order to perform the necessary life functions. (Johnson, 2014). The majority of the remaining food breakdown occurs in the gastric environment when bolus can be swallowed and transported through the esophagus to the stomach by the mechanism of peristalsis (F. Kong & Singh, 2008).

Functionally, the stomach can be separated into two regions: the fundus, or proximal stomach, comprising the cardia, fundus, and body; and the antrum, or distal stomach, comprising the antrum and the pylorus (Bornhorst & Singh, 2014). Ingested food will remain in the proximal stomach, acting as a food reservoir, before it moves into the distal stomach, where the physical breakdown of food happens due to the antral contraction waves (also called peristaltic contractions) that act to crush and grind food particles before they pass through the pyloric sphincter into the small intestine (Heuman *et al.* 1997), where the remaining chemical breakdown will occur, and the ingested nutrients will be absorbed for use in the body. The rate of food breakdown in the stomach plays a key role in determining other processes such as gastric emptying and nutrient absorption, but it is still not fully understood (Bornhorst & Singh, 2014).

During the gastric phase of digestion, the food is mixed with fluids that dramatically lower its pH to about 2 in one to two hours due to the production of HCl by parietal cells (Mat *et al.,* 2017). Enzymes as pepsin and gastric lipase are also secreted, initiating the hydrolysis of proteins and lipids, respectively. Compared to the intestinal phase, the extent of hydrolysis is relatively limited at the gastric stage, up to typically 10-15% (Norton *et al.,* 2014).

Current trends in the food industry are moving toward designing innovative foods with unique health benefits, such as increased satiety, larger nutrient availability, or decreased blood glucose response (Bornhorst & Singh, 2014). In order to achieve this, it is necessary to understand the food breakdown process, as well as determining the mixing kinetics in the stomach.

1.2.2 Models of gastric digestion in research

In the field of food sciences, in vitro digestion experiments present the great advantage of being a relatively fast and inexpensive way to study food digestion and allow to avoid, as much as possible, in vivo studies, which involve high cost, complexity and variability (Mat *et al.*, 2018). These in vitro digestion protocols mostly fall into two categories: dynamic and static approaches (Guerra *et al.,* 2012). In the dynamic approach, it is attempted to reproduce the main events encountered within the gastro-intestinal tract using dynamically-controlled transit fluxes, pH, mechanical constraints, using (or not) digestive fluid secretions, while static models consist in subsequently immersing the food in simulated gastric and intestinal fluids to perform gastro-intestinal digestions. This static approach offer simplicity, enough reason for this method to have more widespread use. These in vitro digestion methods constitute a powerful means to investigate the effects of food composition and structure on digestion being able to have greater repeatability with a larger number of samples, as well as the ability to generate results in shorter time without ethical restrictions, less use of human resources, less cost and being able to isolate specific parameters to study, among others reasons (Minekus *et al.,* 2014). Moreover, to overcome the lack of homogeneity in the protocols, a harmonized static protocol was recently proposed in 2014 by Mans Minekus, among other researchers, based on a consensus of international experts involved in the InfoGest COST action.

1.3. INFLUENCE OF pH IN FOOD BREAKDOWN

The gastric pH fluctuates in response to food consumption. When fasting, the human gastric pH is usually around 2 (Marieb & Hoehn, 2010). After food ingestion, the gastric pH rises depending on the volume and content of the food. The gastric pH gradually decreases, as gastric fluid is being secreted and the food is being digested and emptied from the stomach (Luo *et al.*, 2018).

Besides the physical breakdown of food that occurs during oral and gastric digestion due to the mastication and peristaltic contraction of the stomach, it also exists some chemical breakdown (Kong & Singh, 2010; Bornhorst & Singh, 2012) where the gastrointestinal fluids mix into the food matrix. This absorption process is possible thanks to acid and enzymatic hydrolysis, that break food matrices intro nutrient molecules (Bornhorst *et al.,* 2016).

It is commonly known that the optimal activity of pepsin occurs at around pH 2 (Kondjoyan *et al*., 2015) and that while for salivary α-amylase its optimal activity happens at physiological pH (Ramasubbu *et al*., 1996), its inactivation occurs at a low pH (Dona *et al.,* 2010). The pH of the gastric environment may vary depending on the amount, type and buffering capacity of the meal. This fact could impact pepsin activity and therefore, protein hydrolysis. Moreover, during gastric digestion, acid is secreted through the wall of the epithelium of the stomach and it is hypothesized that the only mechanism through which gastric acid will contact the food bolus is through diffusion (Mennah-Govela *et al.,* 2015). The rate of acid diffusion into the food bolus may have implications on the overall gastric breakdown of food.

1.1. DIGESTION OF PROTEINS

Protein is one of the most important macronutrients in food (Luo *et al.*, 2015). In spite of that scientific knowledge on protein gastric digestion is increasing very much over the last years, a big part of this studies is focused only on digestion of protein solutions, whereas most of the proteins in our food are present in solid foods (Lambers *et al.*, 2013). Knowledge about digestion of solid food is limited, especially on the underlying mechanism of the process (Bornhorst & Singh, 2014).

Pepsin is the major enzyme in gastric fluid. It is an aspartic protease and has a broad specificity with a preference for hydrophobic residues (Rawlings & Salvesen, 2012), especially the aromatic amino acid residues tyrosine and phenylalanine (Fruton & Bergmann, 1939) Pepsin, by definition, is a proteolytic enzyme maximally active at a highly acid pH and inactivated in neutral or alkaline solution (Magee, 1974).

The digestion of protein is mostly facilitated by the acid and pepsin in the stomach and subsequently by the pancreatic and intestinal enzymes in the small intestine (Whitney *et al.,* 1998). The enzymatic hydrolysis of protein was studied by Adler-Nissen in the 80s and his work still valid nowadays (Manrique, 2014). Regarding the kinetics of the enzymatic hydrolysis, some models are proposed to characterize the reaction during the proteolysis (Luo *et al.,* 2015). Linderstrøm-Lang introduced two types of reactions for native globular proteins: the “one-by-one” type and the “zipper” type (Linderstrøm-Lang, 1952). In the first case, the protein tends to be hydrolyzed as soon as a it is attacked by a protease. This process happens in one sequence to the final products, and intermediate products can hardly be detected. In the “zipper”

type, the initial stage of hydrolysis is fast, but the subsequent steps are much slower, which results in a wide range of intermediate products and peptides in solution (Ortiz & An, 2000). It is hypothesized that most proteases will act in between these two extreme models (Adler-Nissen, 1976). For example, some researchers have observed a 'one-by-one' mechanism for the peptic hydrolysis of native hemoglobin and a 'zipper' type reaction for the hydrolysis of denatured hemoglobin (Choisnard *et al.,* 2002).

1.5 UPTAKE OF ACID AND MOISTURE DURING DIGESTION

Previous studies have shown that the rate of both acid and moisture diffusion into the food bolus may have implications on the overall breakdown of the food (Mennah-govela & Bornhorst, 2016). As it has been previously said, the enzyme α-amylase is inactivated with low pH, but proteins in food strongly influence the gastric pH. This is due to the buffering capacity of proteins that comes from the ionizable groups on the side chains of Aspartic acid (Asp) and Glutamic acid (Glu) (Luo *et al.*, 2018), as well as from the amino and carboxyl ends of polypeptide chains and dissociable posttranslational modifications (PTM) (Poznanski *et al.*, 2013). However, the 3D structures or PTMs are not known for all proteins. Therefore, there are some knowledge available about of isoelectric points (pI), pK_a values or titration curves were approximately predicted from protein sequences alone (Bjellqvist, *et al.,* 1993). Also, in consequence of all postulated before, a solution of proteins can be considered as a buffer system composed of those ionizable groups (Luo *et al.,* 2018). Nevertheless, the exact nature of this buffering capacity also depends on the electrostatic interactions.

During protein hydrolysis, the results products of the peptide bond when this is broken (the carboxyl and amino groups) are released and they will ionize. This ionization will depend at the same time on the current pH of the reaction medium and the pK values of the carboxyl and amino groups. A change in the environmental pH may also be caused by this ionization (Luo *et al.,* 2018).

Some interesting research has been done in this area. For example, regarding the importance of the microstructure of food in acid uptake, Mennah-Govela *et al.* found that the structure of food may difficult the acid penetration into the food matrix (Mennah-Govela *et al.,* 2015). Along with Qui Luo *et al*., who postulated in 2018 that the buffer reaction reduces acid diffusivity in gels, finding that the quantification of the buffering capacity can be used to predict the acid uptake of the proteins and the pH change during gastric digestion, even though the buffer capacity and the hydrolysis kinetic parameters of certain proteins need to be experimentally determined (Luo *et al.*, 2018). By means of the mechanistically quantification of the interaction of acid and proteins under the gastric condition, the interrelated processes in gastric digestion could be better understood.

2. MAIN OBJECTIVE

The overall aim of this study was to investigate the impact of the microstructure of egg white protein gels during in vitro gastric digestion in the protein hydrolysis and in the uptake of acid and moisture of the gel. The differences in microstructure were obtain by adjusting the protein dispersion at different pH prior to the heat treatment.

2.1 SPECIFIC GOALS:

With the purpose of achieving the general goal, some specific goals have been determined:

- Study the amount of free amino groups formed during digestion (fifth teen minutes to four hours) in egg gel samples.
- Measure the acid and moisture uptake of the samples after digestion.
- Analyze the differences in all exposed above between samples which pH was controlled during digestion and samples which pH was not.

3. Material and methods

3.1. Egg white protein dispersions and gels preparation

Egg white protein dispersions with 13.75 g of dehydrated egg white protein powder (81.89% protein content, Michael Foods, MN, USA) per 100 g of gel were made by dissolving the protein powder in Milli Q water and stirred at room temperature for around 2 hours. The pH of the solution was adjusted at either 3, 5 or 7.5 and then heated in the water bath for 1 hour at 90°C. After that time, the beaker was removed from the water bath and placed in ice for a thermal shock. The gels were let cool down in the fridge overnight for use the following day. The gels were cut into ½ inch cubes (12 mm^2) using the French fry cuter and a kitchen knife as it is shown in *Figure 1*.

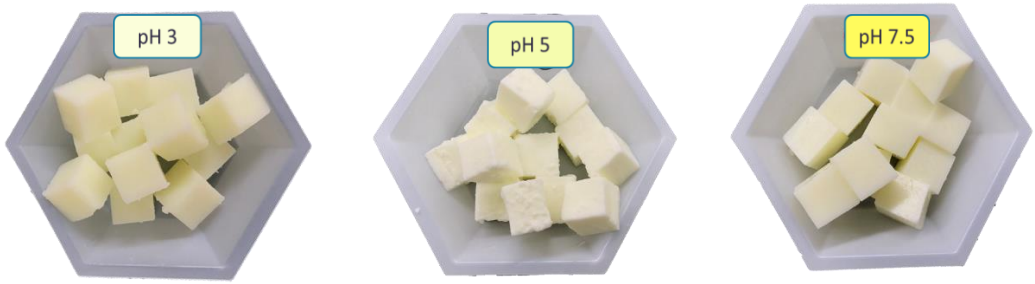

Figure 1. Samples of cubes of each type of egg white protein gel before digestion was performed.

3.2 In vitro oral and gastric digestion

All salts and pepsin for simulated oral and gastric fluids were purchased from Fisher Scientific (Waltham, MA, USA), unless otherwise specified.

3.2.1 Simulated saliva and gastric juice composition

Simulated saliva was prepared by dissolving 1 g of mucin (from porcine stomach, Type III, Sigma Aldrich; St. Louis, Missouri, USA), 0.117 g of NaCl (Avantor Performance Materials, PA, USA), 2.10 g of NaHCO3, 0.149 g of HCl, and 1.18 g of α-amylase (from *Bacillus subtilis*, activity 194.7 U/mL α-amylase; MP Biomedicals, Santa Ana, CA, USA) per liter of Milli Q water used (18.2 MΩ*cm at 25°C). The pH was adjusted to 7 using 1 M HCl (Bornhorst & Drechsler, 2018).
Simulated gastric juice was prepared by dissolving 0.4 g of pepsin (from porcine gastric mucosa, activity of 2000 U/mL), 1.50 g of mucin and 8.78 g of NaCl in 1 L of Milli Q water at pH 1.8, adjusted with 3M HCl. Regarding the low-fat content present on the egg protein gels no lipase was used (Bornhorst & Drechsler, 2018).

3.2.2 In vitro digestion procedure

Egg gel cube samples were digested at several time points: 0 (control), 15, 30, 60, 120, 180, 240 minutes (Mennah-Govela & Bornhorst, 2016). Twelve cubes of egg protein gel were used for each digestion timepoint and correspond to one sample. Each sample was weighted and mixed with 0.2 mL of saliva/g of sample for 30 seconds, to simulate oral digestion (Gaviao *et al*., 2004), and immediately after, it was mixed with 6 mL of gastric juice/g of sample (both digestion juices were preheated at 37°C in the static water bath). After this procedure, the beaker was placed in the shaking water bath at physiological temperature (37°C) and 100 rpm to simulate the peristaltic movement of the stomach *(Figure 2)*. Each digestion was performed in triplicate.

After digestion, cubes were drained from the excess gastric fluid using a kitchen sieve. The cubes were immediately weighed, and aliquots of both cubes and gastric juice were taken for further analysis.

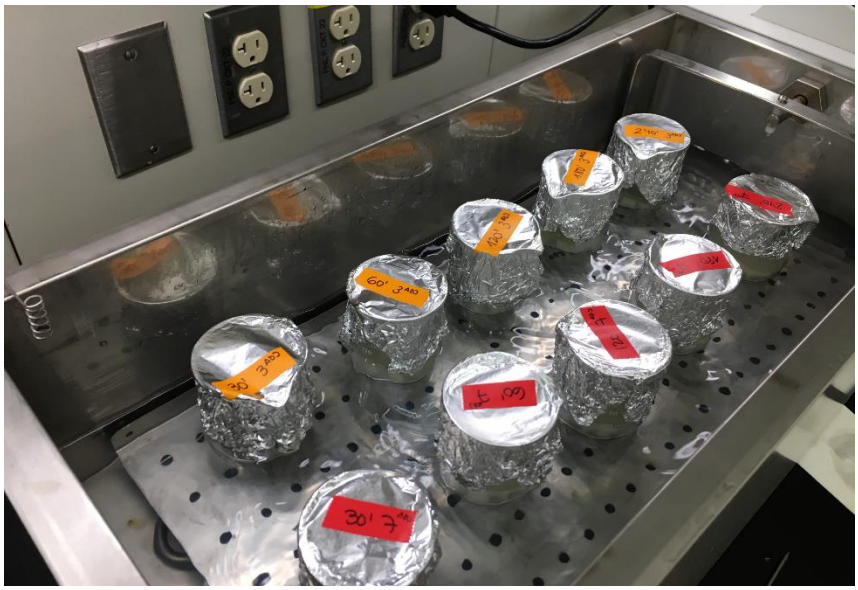

Figure 2. Beakers with sample during digestion in shaking water bath.

3.2.3. Digestion varying factors: pH adjusted and unadjusted samples

The gels were adjusted at pH 3, 5 and 7.5 and two full digestions were done to each gel (with three replicates each). In the pH-adjusted samples, the pH was kept between 1.8 and 2.2 by checking each beaker at 5, 15, 30, 60, 120 and 180 minutes after its first placement in the shaking water bath. 1M HCl was used to adjust the pH of the sample and the mL used were recorded. In the pH-unadjusted samples the pH was recorded before and after digestion, but it was not adjusted. This exhaustive control of the pH of the sample during digestion tries to simulate the gastric environment and to study how the activity of pepsin – that works better in acidic environment – affected the properties of egg white gels studied after digestion.

3.3 Digestion sample analyses

3.3.1 pH and Brix

The pH of the liquid gastric fluid was measured after digestion using the pH meter (Thermo Fisher Scientific, Waltham, MA, US). The degrees Brix (°Bx) were also measured using the refractometer (Model HI 96801, Hanna Instruments, RI, USA).

3.3.2 Moisture content

Moisture content was determined gravimetrically after drying to constant weight at 110°C in a Fisher Scientific Vacuum Oven (Model 285, Fisher Scientific Waltham, MA, USA) (Bornhorst *et al.,* 2014) *(Figure 3)*. Moisture content measurements were completed in duplicate for each digestion.

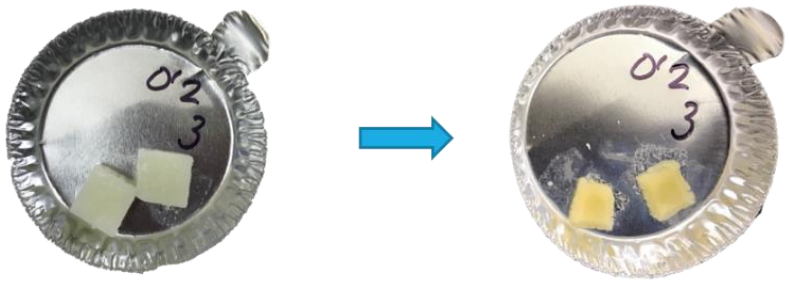

Figure 3. Control moisture content pans before (left) and after (right) 24h at 110°C in the vacuum oven.

3.3.3 Acid uptake

Three cubes were weighted and homogenized (Ultra Turrax T18 digital with S18N-19G disperser, IKA Works, Wilmington, NC) with 10 ml of Milli Q water in a 50 mL graduated polypropylene centrifuge tube (Catalog No. 14375150, Fisherbrand™, ThermoFisher Scientific, PA, USA). Extra 5 mL of MilliQ water were used to rinse the homogenizer in order to avoid solid loss. The tubes were placed in the fridge for its analysis the following day. Each analysis was performed in duplicate.

The content of the tube was transferred to a beaker and the tube was rinsed with other extra 5ml of MilliQ water and the initial pH of the mixture was recorded. A 50 mL burette was filled up with 0.05M NaOH and it was slowly added to the mixture until the pH rises to around 8.2 as it is shown in *Figure 4*. The initial and the final mL of NaOH were recorded.

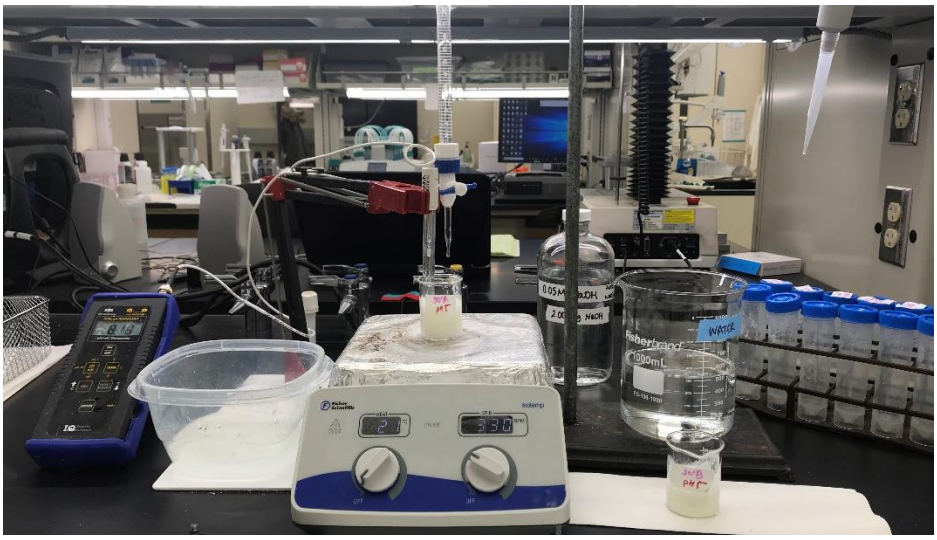

Figure 4. Equipment used to perform potentiometric titrations.

3.3.4 Hydrolysis of proteins

The degree of hydrolysis of the proteins in the sample was determined with the *o*-phthalaldehyde (OPA) method (Nielsen, 2001). For the liquid part of the sample, the gastric fluid, an aliquot of 2 ml was taken from each digested sample to a 15 mL polypropylene centrifuge tube (Corning 352196 Falcon™, Fisher Scientific, PA, USA) and 10 ml of 0.5M Na_2CO_3 was added for enzyme inactivation. In order to make sure the reaction was completely stopped, the centrifuge tube used was placed in ice.

Regarding the solid part of the sample, two cubes were weighted and homogenized (Ultra Turrax T18 digital with S18N-19G disperser, IKA Works, Wilmington, NC) with 20 ml of 0.5M Na_2CO_3 in a 50mL graduated polypropylene centrifuge tube (Catalog No. 14375150, Fisherbrand™, ThermoFisher Scientific, PA, USA), in order to inactivate enzyme activity. Extra 10 ml of 0.5M Na_2CO_3 were used to rinse the homogenizer, in order to avoid solid loss. After this procedure, the centrifuge tube used was placed in ice. Two replicates of each analysis were performed. Before the OPA analysis, an aliquot of 100 µL was taken and subsequently mixed with 1.5 mL of Tetraborate Extraction Buffer (0.0125M, 2% SDS, pH 9) in an Eppendorf tube (Eppendorf™ Snap-Cap Microcentrifuge Flex-Tube™, ThermoFisher Scientific, PA, USA) for one hour at room temperature. After this time, the tubes were centrifuged (Sorvall™ Legend™ Micro 21 Microcentrifuge, ThermoFisher Scientific, PA, USA) at 4000 g for 10 minutes.

Standard solutions with a known number of amino groups were prepared by mixing tetraborate extraction buffer and glycine solution (1% w/v) at different volumes. The absorbance of these standard solutions is going to be used to calculate the amount of free amino groups present in the samples.

The absorbance of two empty microplates per sample (one for the OPA analysis and one for the blank) was measured with microplate reader (BioTek Instruments Inc., Winooski, VT, US) at 340 nm *(Figure 6c)*.

In each microplate, 20 µL of each sample were added in triplicate, as it is shown in *Figure 5*. Following the arrangement shown in *Figure 5*, three aliquots of 20 µL of each standard for the standard solution were pipetted to the microplate (Catalog No 9205, 96 wells Microtiter™ microplate, ThermoFisher Scientific, PA, USA), 20 µL of the gastric fluid at each digestion timepoint and 20 µL of each solid sample at each digestion timepoint. On top of that, 200 µL of OPA solution (2.5% SDS, 0.2% 2-mercaptoethanol and 2% of OPA dissolved in methanol). For the blank or no-OPA solution, only methanol was added to the solvent *(Figure 6a).* OPA reaction is time sensitive, the microplate must be read 4 minutes after the addition of the solution *(Figure 6b)*. The reaction with no-OPA solution is not time sensitive.

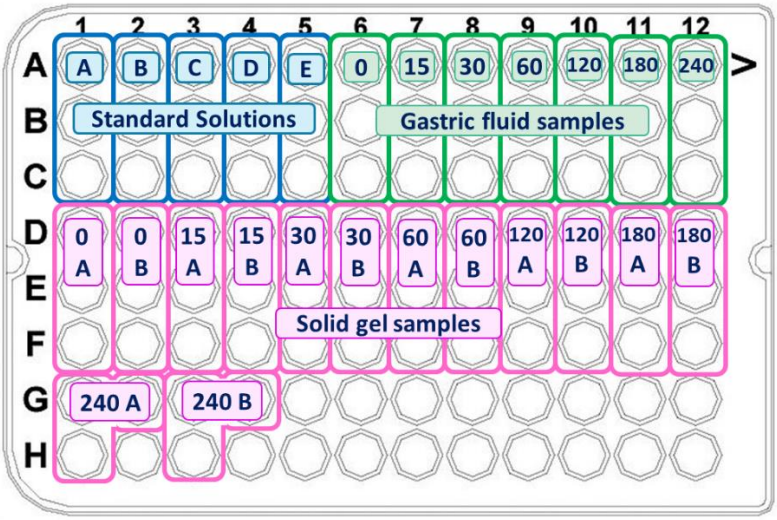

Figure 5. Layout of samples in the microplate. The number represents the digestion timepoint of the sample and the letter (A. B) correspond to each duplicate.

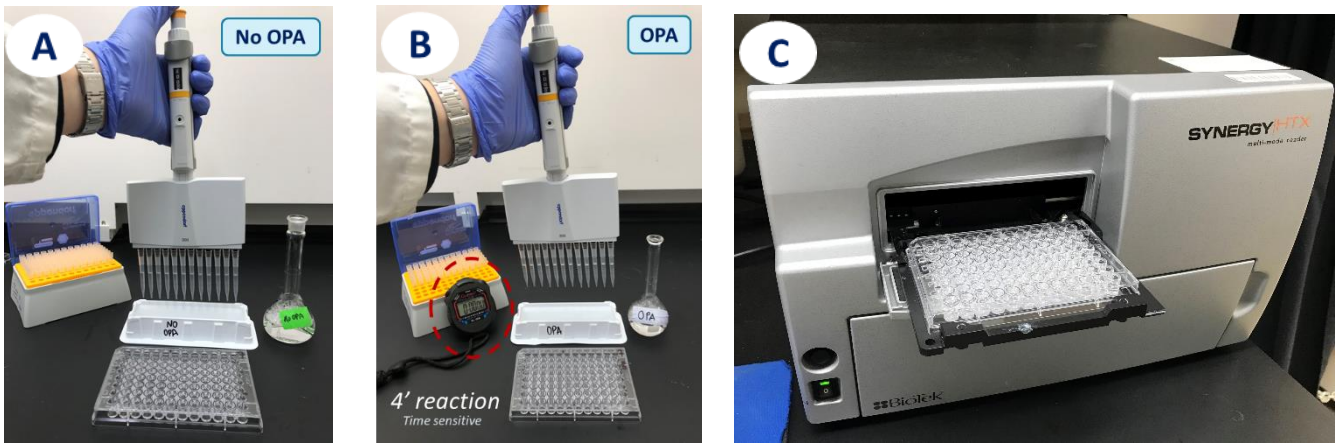

Figure 6. Pipetting no-OPA (A) and OPA solution (B). Microplate reader with microplate (C).

4. RESULTS AND DISCUSSION

4.1 Macroscopic analysis of egg white protein gels

The different gel structures in of egg white protein gels were performed by adjusting the protein dispersion at three different pH (3, 5 and 7.5) prior to heat-induced gelation.

The macrostructure of the three different gels was analyzed by visual examination. The day after the preparation of the gels, each type had a different macroscopic appearance. Gels at pH 7.5 were the firmest. This is due to a dominance of attractive forces (mainly hydrophobic) between the protein molecules over the repulsive forces (mainly electrostatic, hydration and entropic forces) (Weijers *et al.,* 2006). These electrostatic interactions are holding the proteins to the water forming a solid network. On the contrary, gels at pH 3 where the softest and most fracturable. Dupont *et al.* studied the microstructure of these type of gels using SEM methods and obtained that gels at low pH are comprised of both spherical aggregates (of a minimal size) and linear aggregates organized forming a tight mesh (Dupont *et al.*, 2015). In the case of gels at pH 5, they look spongy and lumpy and not completely homogeneous. This is because of the pH of the gel is very close to the isoelectric point (pI) of most egg white proteins (pI=4.7), thus the protein net charge and the electrostatic repulsions between proteins are minimized, thereby enhancing protein-protein interactions (Chou & Morr, 1979). Under these conditions, protein aggregation is dominant over protein unfolding, which explains the lack of continuousness in the gel matrix (Dupont *et al.*, 2015).

4.2 pH profile

The pH of the gastric fluid increases during digestion in samples which pH was not controlled, as it can be observed in *Figure 7*. This increase in the pH during digestion is due to the buffering capacity of the proteins of the gels. The buffering capacity is defined as the quantitative measure of the resistance of a buffer solution to pH change on addition of hydrogen or hydroxide ions (Slyke, 1922).

This difference between the initial and final value of the pH of the unadjusted samples is higher in the gels with higher pH. There is only an increase of 0.36 units between the first and the final pH values in unadjusted pH gels at pH 3, while this difference rises up to 1.32 units in pH 7.5 gels. Since the gastric environment pH is always around 2 (Malagelada, *et al.*, 1976), the samples with controlled pH during digestion have a pH profile between 1.8 and 2.2 during the whole digestion time, as it can be seen in *Figure 7*. In controlled pH 3 gels, it was not necessary to adjust the pH of the sample until three hours of digestion. On the contrary, pH 5 and 7.5 samples had to be adjusted to a lower pH from 5 to 15 minutes of digestion, because the pH of the gel increased the pH of the gastric fluid very quickly.

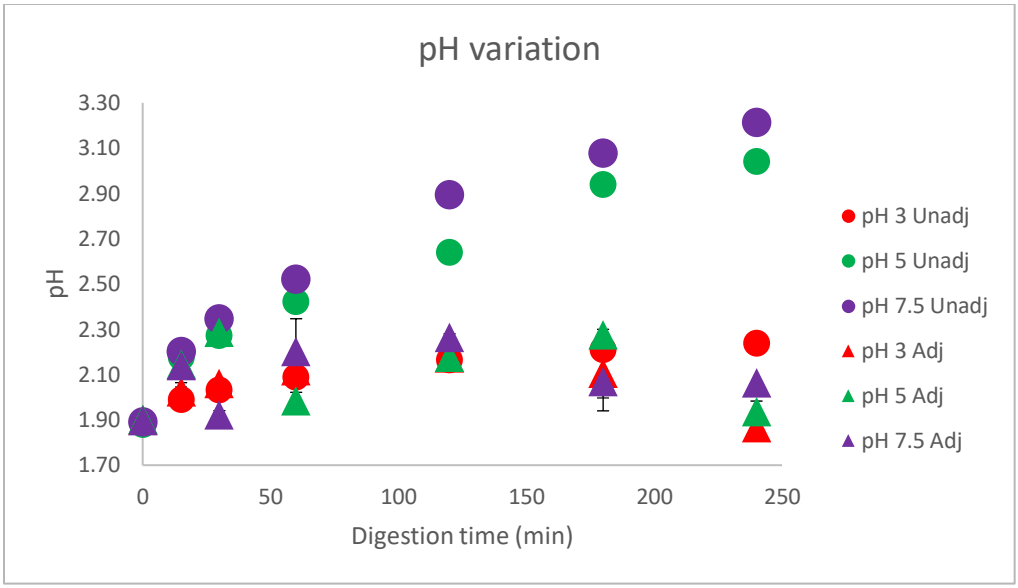

Figure 7. pH profile of samples during digestion. Gels at pH 3 are shown in red, pH 5 are shown in green and pH 7.5 are shown in purple. Circles represent unadjusted pH samples and triangles pH-adjusted samples. Values represent the average (n=3) ± standard error.

4.3. Protein hydrolysis in gels

4.3.1. Protein hydrolysis in the liquid phase

The amount of free amino groups present in the liquid phase of the sample (gastric juice) during digestion is shown in *Figure 8.* The microstructure of the gel significantly ($p<0.05$) influenced the amount of free amino groups present in the gastric juice as well as the interaction Gel x pH treatment ($p<0.05$).

The initial increase in free amino groups was compared to the initial values of a preliminary trial with no enzymes added and the results were very similar. The amount of free amino groups present after 15 minutes of digestion for unadjusted pH 7.5 gels with no enzyme added was 546 µg glycine equivalent/mL gastric fluid, while for a gel with the same characteristics besides that pepsin and α-amylase were added this time this value was 544 ± 10 µg glycine equivalent/mL gastric fluid. All values shown in this report are represented as averages (n=3) ± standard error of the mean. This results concur with previous studies where there was no increase in the amount of free amino groups in digestions performed without pepsin even after 24 h compared to the equivalent experiments using enzymes (Luo *et al.*, 2015).

The burst release observed in the beginning of the graph *(Figure 8)* could suggest that there are peptides and proteins that are coming out of the gel, besides the proteins that pepsin is hydrolyzing. Also, an increase in the amount of free amino groups in the liquid phase during digestion is observed. This increase could be a result of diffusion of more peptides out of the matrix or hydrolysis by pepsin. It is known that hydrolysis of proteins in heterogeneous gels structures takes place in by the diffusion of the enzymes into the network, accessing of the cleavage sites, adsorption and final hydrolysis (Dupont *et al.,* 2015).

In the present study, because proteolysis was performed on gelled systems, enzyme diffusion was assumed based on previous studies on egg white protein hydrolysis in gels and pepsin molecular structure (Sielecki, *et al.,* 1990; Luo, *et al.,* 2015; Dupont *et al.,* 2015), where the Linderstrøm-Lang's theory is proposed as a model for enzymatic hydrolysis of protein gels (Linderstrøm-Lang, 1952).

The products of this hydrolysis may be coming out to the liquid phase and observed in this graph *(Figure 8)*. The amount of free amino groups present in the liquid phase was not significantly ($p>0.05$) influenced by pH treatment, this means that controlling the pH during digestion at pH 2 did not change the amount of free amino groups produced. That implies that the amount of free amino groups formed during digestion are not from hydrolysis that takes place in the liquid phase, given that the environment is better for pepsin to work.

In summary, *Figure 8* shows that there is an initial diffusion of peptides to the liquid phase and that hydrolysis happens inside the gel matrix.

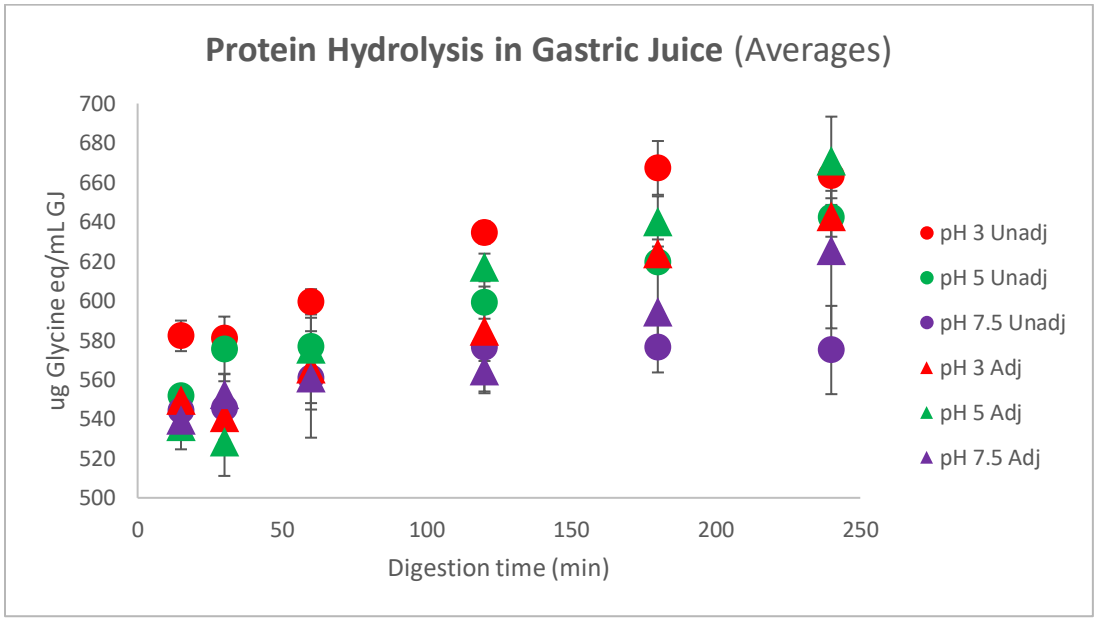

Figure 8. Amount of free amino groups present in the liquid phase during in-vitro gastric digestion of gels at pH 3 (red), pH 5 (green) and pH 7.5 (purple). Circles correspond to unadjusted pH samples and triangles represent pH adjusted samples. Values represent the average (n=3) ± standard error.

4.3.2. Protein hydrolysis in the solid phase

The amount of free amino groups present in the solid phase of the sample (gel) during digestion was significantly ($p<0.05$) influenced by its microstructure and the overall pH treatment, but not by their interaction ($p>0.05$).

There is a slightly increase in protein hydrolysis during digestion in every gel, as it is shown in *Table 1*. The initial values correspond to the amount of free amino groups present in the control sample (egg white gel undigested) and the final values correspond to the amount of free amino groups in the digested sample after four hours of in vitro gastric digestion. This increase between the initial and final value could be a result of protein hydrolysis products that remain in the gel matrix after the action of pepsin as previous researchers have suggested (Luo *et al.*, 2015). Luo *et al.* postulated in 2015 that pepsin needs to penetrate into the gel network, where the proteins are immobilized, to perform the hydrolysis of proteins. After pepsin cleaves a peptide bond, the two resulting fragments generally are still bound to network. Moreover, any protein fragment that is not bound to the network anymore will have to diffuse out, but the bigger the fragment the slower the diffusion and these fragments can be further hydrolyzed by the pepsin in the way out within the gel pores.

Table 1. Initial and final amount of free amino groups present in the solid phase during in vitro gastric digestion of gels at pH 3, pH 5 and pH 7.5 in each pH treatment. Values represent averages (n=3) ± standard error of the mean.

Microstructure	pH treatment	Initial Value	Final Value
pH 3	Unadjusted pH	1.33E+05 ± 4.44E+03	1.55E+05 ± 3.37E+03
	Adjusted pH	1.31E+05 ± 3.13E+03	1.48E+05 ± 2.91E+03
pH 5	Unadjusted pH	1.12E+05 ± 1.06E+03	1.13E+05 ± 4.59E+03
	Adjusted pH	1.09E+05 ± 6.51E+03	1.19E+05 ± 7.63E+03
pH 7.5	Unadjusted pH	1.14E+05 ± 2.01E+03	1.20E+05 ± 2.42E+03
	Adjusted pH	1.08E+05 ± 1.26E+03	1.23E+05 ± 1.17E+03

Regarding the kinetics of the hydrolysis of protein in the solid phase of the sample, the egg white protein gel cubes, it can be observed in *Figure 9* that the amount of free amino groups present in pH 3 gels is higher throughout the whole digestion process, and values are very similar between pH adjusted and unadjusted samples (the interaction Microstructure × Gel treatment ($p>0.05$) is not significant). This event of finding a high amount of free amino group without any enzyme added will be discussed in *section 4.3.3.1*. On the other hand, pH 5 and pH 7.5 results are very similar and follow the same trend (no significant differences ($p>0.05$) between them).

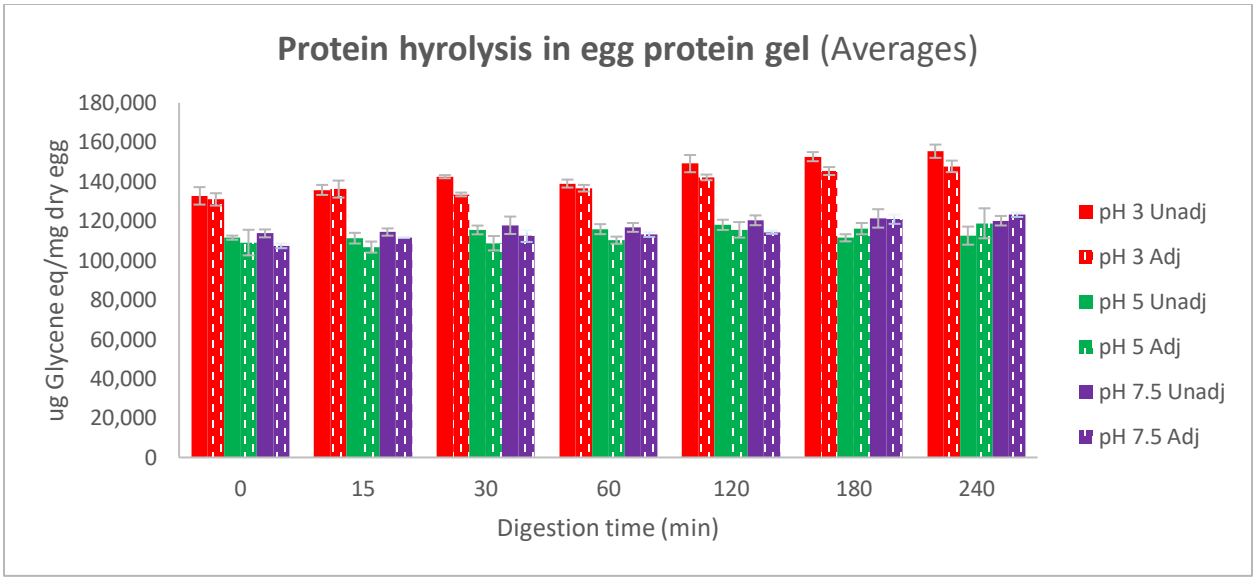

Figure 9. Amount of free amino groups present in the solid phase during in-vitro gastric digestion of gels at pH 3 (red), pH 5 (green) and pH 7.5 (purple). Solid bars correspond to unadjusted pH samples and dotted bars represent pH adjusted samples. Values represent the average (n=3) ± standard error.

4.3.3. Protein hydrolysis in dispersions

The amount of free amino groups present in the protein dispersions during in vitro gastric digestion is shown in *Figure 10*. The dispersion underwent the same digestion procedure as the gels in order to isolate the pH effect from the microstructure effect. The pH profile of the unadjusted pH dispersions is shown in *Table 2* and the pH profile of the pH adjusted dispersions is shown in *Table 3.* The pH adjusted dispersions were controlled at around pH 1.8 – 2.2 using 1M HCl, yet the pH of unadjusted pH dispersions was not corrected throughout the digestion process.

The graph represented in *Figure 10* shows that dispersions at different pH have different trends, even though all samples have very similar initial values. Moreover, when these dispersions are adjusted to the same pH (around pH 2), in order to contribute to a better environment for pepsin to work (Mat *et al*., 2018), their behavior is the same, which does not happen in the gel matrix digestions. Although dispersions and gels are at the same pH, their microstructure is different.

These results show that the pH significantly ($p<0.05$) influences the microstructure of the gel and that differences in protein hydrolysis among the gels (regardless if they are pH-adjusted or not) are due to the differences in their structure.

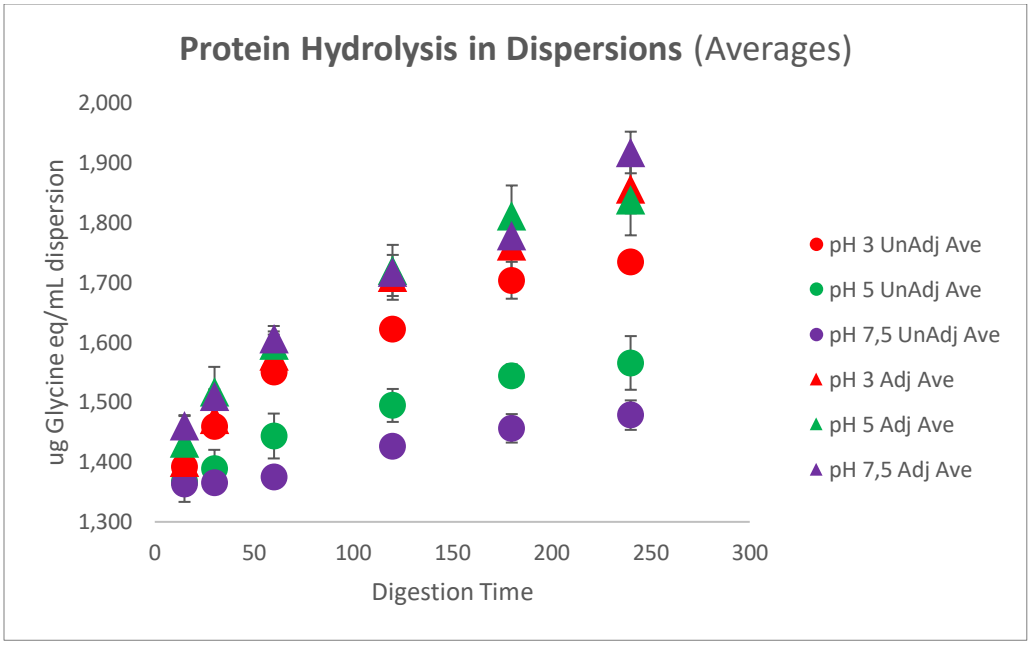

Figure 10. Amount of free amino groups present in the dispersions during in-vitro gastric digestion. The dispersions were adjusted at pH 3 (red), pH 5 (green) and pH 7.5 (purple). Circles correspond to unadjusted pH samples and triangles represent pH adjusted samples. Values represent the average (n=3) ± standard error.

Table 2. pH profile of unadjusted pH dispersions during in vitro gastric digestion. Dispersions were adjusted at either pH 3, 5 or 7.5 prior to digestion. Values represent the average (n=3) ± standard error.

	Unadjusted pH dispersions		
	pH 3	pH 5	pH 7.5
Initial pH	2.23 ± 0.003	3.52 ± 0.025	4.15 ± 0.023
Final pH	2.27 ± 0.003	3.68 ± 0.018	4.25 ± 0.015

Table 3. pH profile of pH adjusted dispersions during in vitro gastric digestion. Dispersions were adjusted at either pH 3, 5 or 7.5 prior to digestion and pH was controlled at pH 2 ± 0.2 using 1M HCl. Values represent the average (n=3) ± standard error of the mean.

	Adjusted pH dispersions		
	pH 3	pH 5	pH 7.5
Initial pH	2.22 ± 0.009	3.50 ± 0.024	4.13 ± 0.015
Final pH	2.06 ± 0.020	1.95 ± 0.015	2.07 ± 0.015
mL of HCl added	0.45	1.1	1.2

4.3.3.1 Protein hydrolysis in dispersions with lower concentration of protein

In order to study the event that it is observed in *Figure 9,* where the gels at pH 3 seem to have a higher rate of protein hydrolysis at the initial point compared to the gels at pH 5 and pH 7.5, and also to test the hypothesis of a acid catalyzed hydrolysis, a set of digestions of heated and non-gelled dispersions were performed.

The hypothesis of a possible acid catalyzed hydrolysis in pH 3 gels falls on the low pH of the gel and the high temperature used in the gelling procedure. To test this hypothesis, dispersions with a percentage of protein that it is already known that will not gel were adjusted at three different pH (3, 5 and 7.5) and, as the limiting factor, one replicate was heated at 90 °C for 1 hour, as it have been done for the previous protein gels, and the other replicate was digested without going through any heating process. This way the impact of the heat treatment in the hydrolysis of the sample is studied and afterwards compared to the results for protein hydrolysis in the gels.

Regarding previous studies in the gelation of egg white proteins (Ahmed *et al.,* 2007; Arzeni *et al.,* 2011; Cold, 2003; Spotti, 2013; Weijers *et al.*, 2006) and based on empirical experiments, it was decided use a percentage of egg white protein of 2.5% for these dispersions. This percentage of protein has seemed to be the highest amount possible in dispersion without gelification happening in the case of our egg white protein. All the protein dispersions in these experiments were pH controlled during the digestion process at pH 2 ± 0.2 using 1M HCl and the experiments were performed in triplicate.

The hydrolysis of protein during digestion of 2.5% egg white protein dispersion are shown in *Figure 11.* It can be observed in this graph that pH 3 samples have the lowest amount of free amino groups during digestion, which does not match with the results from the gels. On the contrary, the results for pH 5 and pH 7.5 dispersions are very similar between them and between both heat treatments (heated and not heated). This does not happen in pH 3 samples, where the values for not heated samples are far from the heated results. The fact that the amount of free amino groups present in pH 3 heated samples is the lowest one (even lower than pH 3 unheated samples) does not support the idea of a possible acid catalyzed hydrolysis. Therefore, the interaction between pH × Heat treatment did not cause any large differences in the hydrolysis of proteins in the dispersion samples, so that cannot be the explanation for the differences in the gels and hence further experiments may be performed in this aspect.

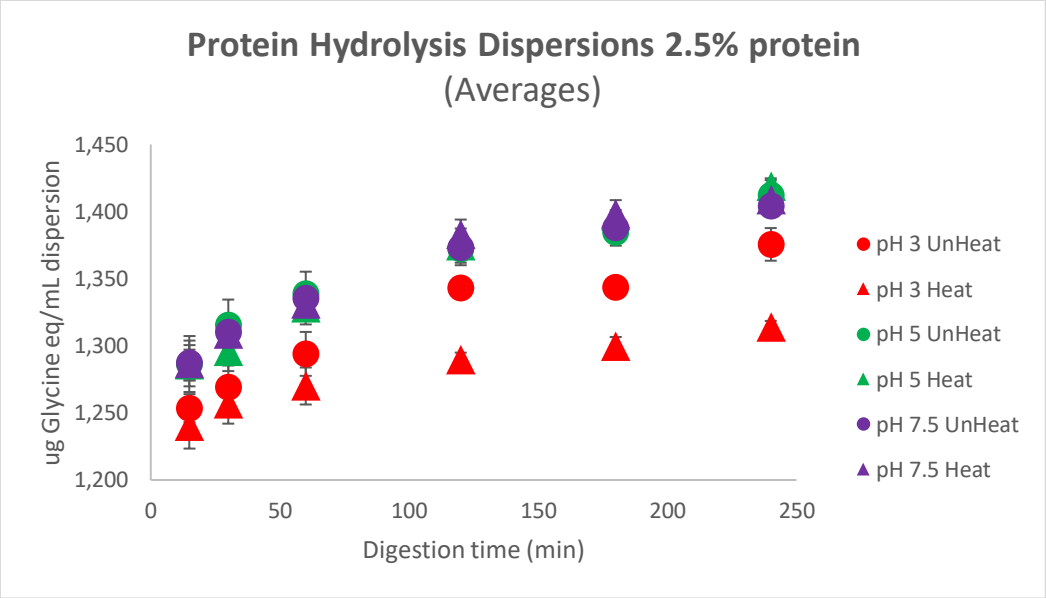

Figure 11. Amount of free amino groups present in 2.5% egg white protein dispersion during in-vitro gastric digestion. The dispersions were adjusted at pH 3 (red), pH 5 (green) and pH 7.5 (purple). Circles correspond to not heated samples and triangles represent heated samples. All the dispersions were adjusted at pH 2 ± 0.2 during the digestion process. Values represent the average (n=3) ± standard error of the mean.

4.4. Acid uptake in gels

The acidity of the gels during digestion are shown in *Figure 12*. Acid uptake in the gels has been significantly ($p<0.05$) influenced by the type of gel, as well as by the pH treatment and their interaction.

It can be observed that pH 3 gels have the highest acidity values, as they are the most acidic gels, but they have the lowest acid uptake (the initial value is 0.999 ± 0.03 mmol H^+/ g of dry mass and the final value is 1.048 ± 0.02). The greatest acid uptake happens in pH 7.5 gels when its pH is controlled during digestion (the initial value is 0.016 ± 0.006 mmol H^+/g of dry mass, compared to the final value of 0.781 ± 0.02 mmol H^+/ g of dry mass). The values for acidity in pH 7.5 gels with no pH adjustments during digestion range from 0.012 ± 0.003 mmol H^+/g of dry mass to 0.327 ± 0.01. This differences in values between pH 7.5 gels pH-adjusted and unadjusted might represent that controlling the pH during digestion is what influences acid uptake.

Comparing the acidity behavior of the gel with the hydrolysis of proteins it could be seen that each gel follows a different trend due to the differences in microstructure. As previous researches have observed, heat denaturation eliminates the resistance of the native conformation of proteins to pepsin hydrolysis (Ki *et al*., 2007). Moreover, hydrolysis disintegrates the gel particles, which may increase the accessibility of some ionizable groups in the protein aggregates and is reflected in the acid uptake (Luo *et al.,* 2018).

However, no differences can be seen between pH-adjusted and unadjusted samples in protein hydrolysis. Controlling the pH of the samples to a low pH influences the acidity of the gel, but not the protein breakdown.

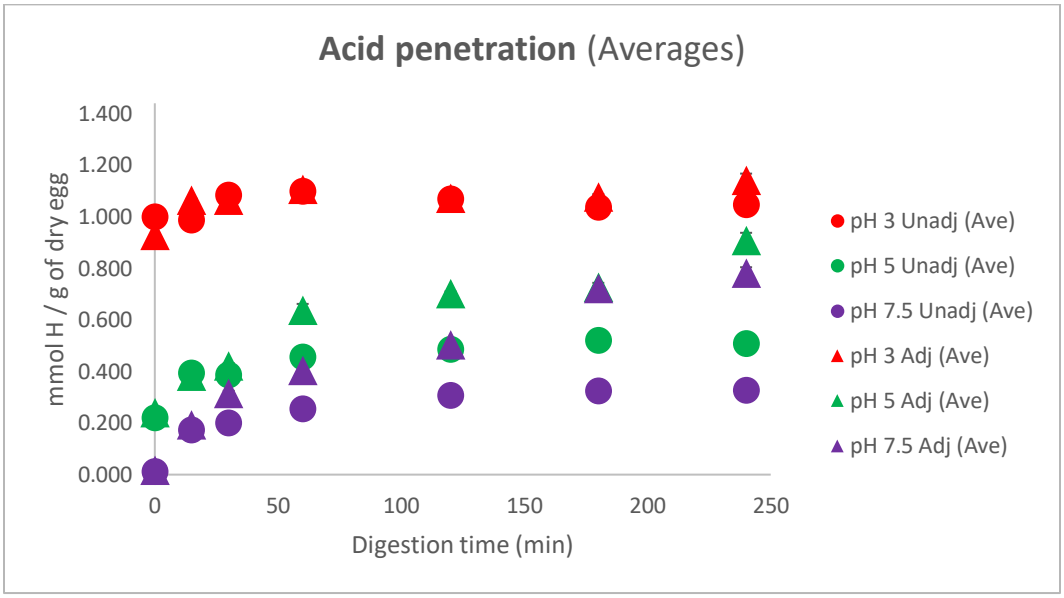

Figure 12. Acid uptake during in-vitro gastric digestion of gels at pH 3 (red), pH 5 (green) and pH 7.5 (purple). Circles correspond to unadjusted pH samples and triangles represent pH adjusted samples. Values represent the average (n=3) ± standard error.

4.5. Moisture uptake in gels

The moisture content dry basis of the gels during digestion are shown in *Figure 13*. Moisture uptake has been significantly ($p<0.05$) influenced by the type of gel and the pH treatment but not by their interaction. It could be seen that even though the initial values for all the gels are very similar (6.86 ± 0.06 g/g dry matter for both unadjusted pH 3 and 5 samples and 6.84 ± 0.05 for unadjusted pH 7.5 samples), they have different trends. The highest moisture uptake happens in the most acidic gel, at pH 3, with no differences between pH-adjusted and unadjusted samples (8.03 ± 0.02 g/g dry matter in both treatments in pH 3 samples after four hours of digestion). It could also be observed that controlling the pH during digestion has affected the moisture uptake of the gels with a higher initial pH. Values of moisture content at final timepoint of digestion for pH 7.5 gels are 7.02 ± 0.02 g/g dry mass for unadjusted pH samples and 7.49 ± 0.09 g/g dry mass for adjusted pH samples.

Comparing the acid penetration behavior of the gels to the moisture uptake, it can be seen that pH 3 has the highest moisture content change, but the lowest acid uptake. On the contrary, pH 7.5 has the least moisture change but the highest acid uptake. These results show that gel does not seem to take up moisture and acid at the same time, as previous studies have suggested for other model foods as sweet potatoes (Mennah-govela & Bornhorst, 2016) or rice (Mennah-Govela *et al.,* 2015), where it is postulated that, in addition to structural changes, the biochemical conditions present during digestion may modify the mass transport properties, such as moisture and acid diffusion into food matrices. Studying the rate in which acid and water diffuses through the food matrix may be important to estimate the rate of breakdown and nutrient release in the later stages of digestion.

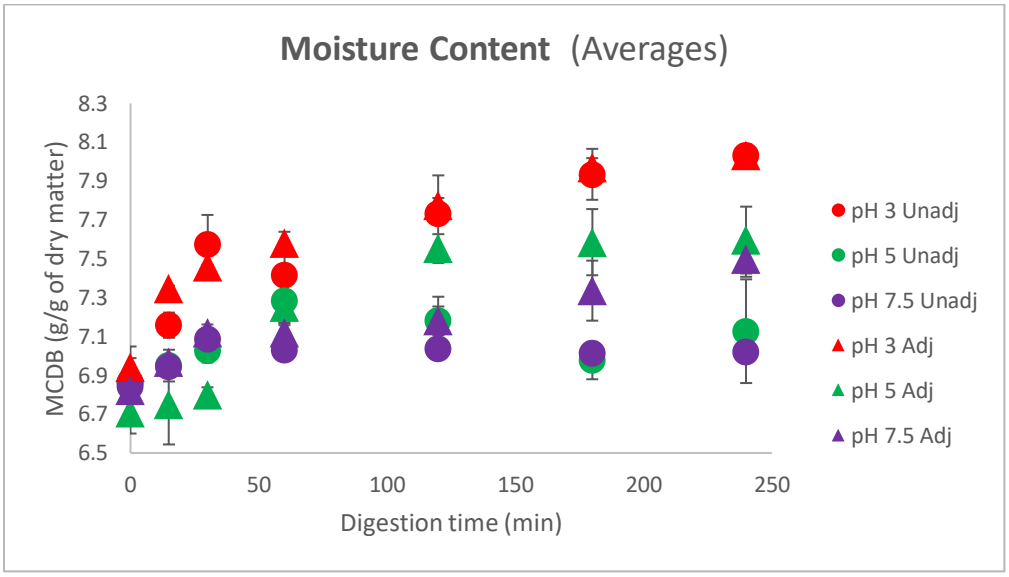

Figure 13. Moisture uptake during digestion in gels at pH 3 (red), pH 5 (green) and pH 7.5 (purple). Circles correspond to unadjusted pH samples and triangles to pH-adjusted samples. Values represent the average (n=3) ± standard error.

5. CONCLUSION

This study shows how changing the microstructure of the gel by adjusting the pH of the dispersion prior to gelation influences the protein hydrolysis and acid and moisture uptake of the gel. It also exposes how controlling the pH of the gastric fluid during digestion affects to the properties previously mentioned.

In summary, last findings indicated that microstructure of the gel affects protein hydrolysis, as well as the uptake of moisture and acid. For this reason, the behavior of the dispersions and gels adjusted to the same pH during in vitro gastric digestion was not the same. Moreover, controlling the pH during this process has shown to influence both acid and moisture uptake but not the hydrolysis of protein. It would be necessary to perform a deeper analysis of acid and enzyme diffusion, as well as analyze gels with different characteristics to gain a better understanding of the behavior of egg white protein gels during digestion. Some examples of these different attributes could be study dispersions with variable percentage of protein that could cause non-gelled dispersions after the heating treatment, which could be very interesting to investigate.

The information presented here creates a relationship between the properties and microstructure of egg white protein gels and their behavior during digestion. The influence of initial food structure on acid uptake and subsequent protein hydrolysis may help predict nutrient release and inform design of new food products.

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7. ANNEXES

7.1. Gel preparation protocol

1) Turn on the Static Water Bath at 90°C (Reserve it for at least 2 hours)

2) Weight egg protein and water
   - 1L Beaker → 68.75 g egg / 431.25 g water
   - 2L Beaker → 137.5 g egg / 862.5 g water

   [13.75% egg in water]

3) Add egg protein slowly into water in a stirring plate at ~350 rpm to avoid too much foam formation
   - If coagulation occurs, stir cross-current with a spatula

   → Wait for 2h or until it is well dissolved ←

4) ADJUST pH [3 / 5 / 7.5]

5) Strain mixture into plastic container (to get rid of foam)
   - 1 container for 500ml gel
   - 2 containers for 1L gel

6) Label and cover container with foil

7) Place container into water bath

   → Wait for 1h ←

8) Go for ice!

9) Place container in ice (~10 min)

10) Place container in the fridge (to digest it the following day)

11) Cut the gel into perfect cubes (½ x ½ inch)
    - Use the sweet potato cutter blade
    - Use Alex's cutting board to cut borders (½ inch mark)

Note: Prepare as much gel sample as you will be putting into digestion at once on the following day

7.2. Simulated saliva and gastric juice preparation

7.2.1 Simulated saliva preparation procedure

For 500 mL of simulated saliva:

1) Add 200 mL of Milli Q water to a 600 mL beaker. Add a stir bar and place it on a stir plate.

2) Weigh 0.5 g of mucin and add it very slowly to stirring water.

3) Weigh out 1.05 g $NaHCO_3$, 0.058 g NaCl, and 0.0745 g of KCl. Add to the solution.

4) Wait for about 20 minutes for the mucin to dissolve. Cover and label the beaker during this time.

5) Pour the solution into a 500 mL volumetric flask.

6) Rinse out any remaining solution from the beaker into the flask. Add water up to the line.

7) Cover the top of the flask securely with parafilm and mix carefully.

8) Pour the solution back into the 600 mL beaker and adjust the pH to 7 using 0.1 M HCl or NaOH.

7.2.2 Simulated gastric juice preparation procedure

For 1 L of simulated gastric juice (GJ):

1) Add approximately 600mL of MilliQ water into a 1L beaker. Add a stir bar and place it on a stir plate.

2) Add 5.3 mL of 3 M HCl using the 10 mL Eppendorf pipet.

3) Weigh out 1.5 g of mucin and add it very slowly to the stirred water.

4) Weigh out 8.78 g of NaCl and SLOWLY add the salt to the stirred water.

5) Wait for around 20 minutes for the mucin to dissolve. Cover and label the beaker during this time.

6) Add the contents of the beaker to a 1000 mL volumetric flask

7) Rinse out any remaining solution from the beaker into the 1000 mL volumetric flask. Continue to add water to the line on the flask.

8) Cover the top of the flask securely with parafilm and mix carefully.

9) Add the contents of the 1000 mL volumetric flask to a labelled flask with a cap. Store in the fridge.

7.3. In vitro digestion protocol

1) Measure pH of Gastric Juice (1.8) and Saliva (7) and correct it if necessary using 1 M HCl or NaOH.

2) Heat up Gastric Juice and Saliva in water bath (37°C - 20 min)

3) Weight beakers with samples
    - Label beakers with digestion time points (30, 60, 90, 120, 180, 240 min)
    - Use 12 egg cubes in each beaker

4) Calculate how much Saliva and Gastric Juice I need
    - 0.2 ml Salvia / g sample
    - 6 ml GJ / g sample

5) Calculate how much enzymes I need to add to saliva and Gastric Juice
    - Saliva: 1.18 g a-amylase / L solution
    - GJ: 2000 units pepsin / L solution

6) Prepare the volume of saliva and the GJ needed for digestion

7) Add enzymes → At this point time starts!!

8) Add Saliva to sample

    → Wait 30 seconds ←

9) Add Gastric Juice to sample

10) Place beaker in the Shaking Water Bath (37 C - 100rpm)

11) Record the time you placed the beaker in the Shaking Water Bath.

    → After digestion ←

12) Strain solids with a kitchen sieve for subsequent analysis
    - Free amino groups quantification
    - Acidity titrations
    - Moisture content

13) Measure liquids
    - pH
    - Brix (in duplicate)

14) Clean up: Pour the Gastric Juice into the Gastric Juice container under the sink.

Note: pH-controlled samples are adjusted at 5, 15, 30, 60, 120 and 180 minutes after their first placement in the shaking water bath with 1M HCl. The pH before and after the adjustment and the volume of acid used have to be recorded.

7.4. Analyses procedure

7.4.1. OPA method protocol

1) Get ready in the fume hood:
   - Sodium Tetraborate → Solid Chemical Shelf
   - SDS → Solid Chemical Shelf
   - Glycine → Solid Chemical Shelf
   - Tetraborate Extraction Buffer → Buffer cabinet
   - OPA → Fridge
   - Methanol → Flammables cabinet
   - 2-mercaptoethanol → Flammables cabinet

2) Prepare the buffers and standard solutions:

   - **1* Sodium Tetraborate Solution (0.1M)**
     - 19.05 g Sodium Tetraborate (Na2B4O7)
     - 400ml Milli Q water

     ↳ Heat the solution a little bit to solubilize it and let it cool down afterwards

     ↳ Fill up to 500 mL with Milli Q water

     ↳ Adjust pH at 9.3

   - **2* SDS 20%**
     - 20 g SDS
     - 50 mL of Milli Q water

     ↳ Heat the solution a little bit to solubilize it and let it cool down afterwards

     ↳ Fill up to 100 mL with Milli Q water

   - **3* Tetraborate Extraction Buffer (0.0125M) (SDS 2%)**
     - 4.77 g of Sodium Tetraborate Solution (1*)
     - 10 mL SDS 20% (2*)
     - 800 mL of Milli Q water

     ↳ Fill up to 1 L with Milli Q water

     ↳ Adjust pH at 9

   - **4* Standard Solution**
     - 100 mg Glycine
     - 100 ml Tetraborate Extraction Buffer (3*)

     ↳ Mix it in a stirring plate in a 150ml beaker

4) Prepare the OPA and no-OPA solutions:

- Solution without OPA:
    - 1.25 mL of SDS 20% (2*)
    - 100 µL de 2-Mercaptoethanol
    - 1 mL Methanol

  ↳ Use a volumetric flask of 50 mL → Keep this flask labelled as no-OPA for future experiments

  ↳ Make up to 50 mL with Na-tetraborate solution (1*)

- Solution with OPA:
    - 1.25 mL of SDS 20% (2*)
    - 100 µL de 2-Mercaptoethanol
    - 1 mL Methanol-OPA *(see below)*

  ↳ Use a volumetric flask of 50 mL → Keep this flask labelled as OPA for future experiments

  ↳ Make up to 50 mL with Na-tetraborate solution (1*)

  → It's very important to use the same OPA flask every time to avoid contamination ←

- Methanol-OPA:
    - 40 mg de OPA (*o*-phthaldialdehyde)
    - 1 mL Methanol

  ↳ Weight OPA directly in the Eppendorf tube (to avoid loss)

  ↳ Vortex the tube until the solution is completely homogenized

Note: These solutions have to be prepared right before the experiment.

5) Prepare the standards for the standard curve as it is given in the *Figure 14*:

- Glycine stands for standard solution (4*)
- Extraction buffer stands for Tetraborate Extraction Buffer (3*)

Note: These solutions have to be prepared every time the experiment is performed.

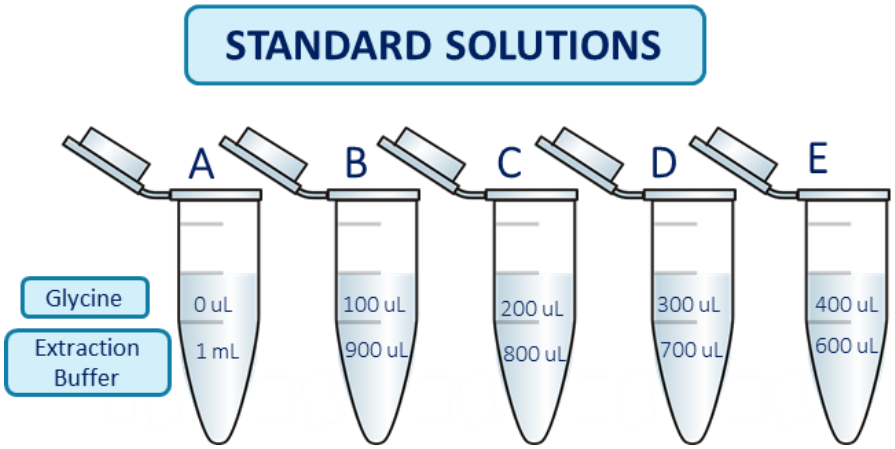

Figure 14. Graphic representation of standard solutions. Each Eppendorf tube has a different concentration of glycine with a known amount of free amino groups.

6) Read the absorbance at 340 nm of two empty microplates (OPA and no-OPA). Copy the absorbance chart in an excel spreadsheet.

7) Inject three replicates of 20 µL of all the samples in both microplates.

8) Inject 200 µL of no-OPA solution in the wells of one microplate. Watch out for bubbles as they interfere in the reading. This reaction in not time-sensitive.

9) Read the absorbance at 340 nm of no-OPA microplate. Copy the absorbance chart next to the no-OPA blank reading in the excel spreadsheet.

→ Prepare the chronometer ←

10) Inject 200 µL of OPA solution in the wells of the other microplate. Watch out for bubbles they interfere in the reading. This reaction is time-sensitive and the microplate must be read 4 minutes after the addition of the OPA solution.

11) Read the absorbance at 340 nm of OPA microplate. Copy the absorbance chart next to the OPA blank reading in the excel spreadsheet.

12) Plot the amount of free NH_2 (µg/mL) vs. absorbance (OPA – no OPA) to determine the standard curve slope and intercept to use for further calculations.

7.4.2. Acidity titrations protocols

1) Calibrate pH meter.
2) Prepare stirring plate and burette and 0.05M NaOH.
3) Add a stir bar in the sample and place it on a stir plate.
4) Measure and register initial pH.
5) Start pouring NaOH until the pH 8.2
6) Register volume spent of NaOH to reach that pH.

7.4.3. Moisture content protocols

1) Label and dry moisture content pans in the vacuum oven. There will be two pans for each time point.
2) Weight and record the mass of the empty moisture content pan.
3) Place sample on the pan and record the mass of the pan and sample combined.
4) Place the pans in the oven at a temperature of 110°C for 24 hours.
5) After drying, record the mass of each pan with the dried sample.
6) Calculate moisture content by mass difference relative to initial mass.