Universidade de Aveiro
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Departamento de Química

ANA SOFIA BATISTA QUEIRÓS

GELIFICAÇÃO DE PROTEÍNAS DO SORO DO LEITE INDUZIDA POR ÁCIDOS FRACOS

ACID-INDUCED GELATION OF WHEY PROTEINS

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, ramo Bioquímica Alimentar, realizada sob a orientação científica do Doutor José António Teixeira Lopes da Silva, Professor Auxiliar do Departamento de Química da Universidade de Aveiro

Dedico este trabalho à minha família,

por todo o apoio e confiança.

O júri

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palavras-chave Proteínas do soro do leite, ácidos orgânicos, gelificação, desnaturação, reologia, calorimetria diferencial de varrimento.

resumo

Tradicionalmente, a gelificação das proteínas do soro do leite requer aplicação de calor, limitando a utilização destes agentes gelificantes em alimentos sensíveis a elevadas temperaturas. É possível a gelificação destas proteínas induzida por ácidos à temperatura ambiente, através do uso de acidulantes, requerendo, contudo, a aplicação de calor numa fase inicial do processo. De acordo com a literatura, foi possível gelificar proteínas miofibrilares de tubarão na presença de ácidos orgânicos fracos. No entanto, não existem registos que indiquem a utilização destas condições para gelificar proteínas do soro do leite.

Este trabalho teve como objetivo investigar a gelificação de proteínas do soro do leite, à temperatura ambiente, na presença de ácidos orgânicos fracos (fórmico, acético e propiónico). Os efeitos do tipo e concentração de ácido, concentração de proteína e pH sobre a transição de fase sol-gel foram estudados através da observação macroscópica de amostras em tubos de ensaio. Estabeleceram-se diagramas de fase para as proteínas do soro de leite em meio aquoso acidificado, em função das concentrações de proteína e ácido acético e do pH. Os tempos de gelificação e as propriedades viscoelásticas dos géis obtidos foram caracterizados através de ensaios reológicos dinâmicos a baixa deformação. A desnaturação destas proteínas, sob as diferentes condições em estudo, foi avaliada por calorimetria diferencial de varrimento.

Os resultados dos ensaios reológicos e da avaliação visual das amostras indicaram que todos os ácidos estudados induziram a gelificação do isolado de proteínas do soro do leite. Contudo, este processo demonstrou ser altamente dependente da concentração de proteína e de ácido, do pH e do tipo de ácido, fatores estes que também influenciam o aspeto final dos géis. Assim, o aumento da concentração de proteína e de ácido resultou em tempos de gelificação menores e na formação de géis cada vez mais turvos e opacos. A gelificação destas proteínas também aconteceu mais rapidamente à medida que o pH aumentava e se aproximava do ponto isoelétrico, originando géis inicialmente translúcidos que se tornaram mais turvos a pH mais elevado. A formação de géis aconteceu de forma mais rápida na presença de ácido propiónico, seguindo-se o ácido acético e o ácido fórmico. No primeiro caso, foram produzidos géis translúcidos e opacos, enquanto os outros ácidos formaram géis mais transparentes.

Os resultados de calorimetria mostraram a diminuição da temperatura de desnaturação do isolado de proteínas do soro de leite, de 78 para 58 ºC, para a concentração de ácido acético estudada mais elevada (2.8 mol L^{-1}, pH 3,2), indicando a influência da presença do ácido na estabilidade térmica das proteínas, provavelmente uma consequência de alterações nas interações intramoleculares e na conformação destas proteínas.

keywords whey proteins, organic acids, gelation, protein denaturation, rheology, differential scanning calorimetry.

abstract

Traditionally, whey protein (WP) gelation requires the application of heat, hence limiting the use of whey protein ingredients as gelling agents in foods sensitive to high temperatures. Acid-induced gelation has been shown to promote whey protein gel formation at room temperature, using acidulants. However, it requires the application of heat in the initial stages of the process to achieve partial denaturation of the protein and the formation of soluble aggregates. Gelation in the presence of weak organic acids at room temperature has been reported for shark myofibrils. Nevertheless, according to the literature, these conditions have not yet been tested in whey proteins.

Therefore, the aim of this study was to investigate whey protein gelation at ambient temperature upon addition of weak organic acids (formic, acetic and propionic). The effect of protein concentration, acid concentration, pH and acid type on the sol-gel protein phase behavior was investigated by macroscopic observation. Phase diagrams were established to define the physical state of the WP systems as a function of protein and acetic acid concentration, and pH. Small strain oscillatory rheological measurements were performed in order to characterize the gelation times and the viscoelastic properties of the obtained gels. Differential scanning calorimetry was applied to investigate the denaturation behavior of the WP, under the studied concentration and ionic conditions.

Rheological measurements and visual assessment of the prepared samples indicated that all formic, acetic and propionic acids have induced whey protein gelation. However, this process was shown to be highly dependent on protein concentration, acid concentration, pH and acid type, which also seemed to influence the appearance of the final gels. Therefore, increasing protein and acid concentrations resulted in decreased gelation times and led to the formation of increasingly turbid and opaque gels. WPI gelation was also shown to occur more rapidly as the pH increased towards the isoelectric point, promoting the formation of translucent gels which became more turbid at higher values of pH. Lastly, propionic acid was the fastest to induce gel formation, yielding opaquer gels, followed by acetic acid and formic acid which formed clearer gels.

DSC results showed a decrease in the denaturation temperature of WP in the presence of the highest acetic acid concentration studied (2.8 mol L^{-1}, pH 3.2) in relation to the protein with no added acid, from 78 to about 58 ºC, indicating the lower thermal stability of the proteins in the presence of high acetic acid concentrations, probably related to changes in the intramolecular interactions stabilizing the proteins and to consequent conformational changes in the proteins upon acid addition.

NOMENCLATURE - SYMBOLS AND ABBREVIATIONS

Δ - Relaxation exponent

ΔH – Enthalpy

aa - amino acid

BSA – Bovine Serum Albumin

Cp – heat capacity

FDA – Food and Drug Administration

G′ – Elastic or storage modulus

G″ – Viscous or loss modulus

G* – Complex modulus

GDL – Glucono-δ-lactone

GRAS – Generally Recognized As Safe

Mw – Molecular Weight

pI – Isoelectric Point

SAOS – Small Amplitude Oscillatory Shear

Td – Denaturation temperature

Tm – melting temperature

WP – Whey Protein

WPC – Whey Protein Concentrate

WPI – Whey Protein Isolate

α-LA – α-Lactalbumin

β-LG – β-Lactoglobulin

γ - Strain

δ – Phase angle

η* - Complex viscosity

σ - Stress

ω – Angular frequency

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BACKGROUND

The current food trends point to a growing preference for highly nutritious, protein-rich foods. Therefore, the food industry has been challenged to choose healthier, protein-based ingredients to control food texture which is one of the main factors determining consumer's acceptance [1]. Whey proteins (WP) have been widely used in food processing as gelling agents due to their nutritional and functional properties. One of the most important among the last is their ability to form gels [2]. The classical method for WP gelation requires application of heat. Therefore, over the last years, new techniques have been developed in order to promote a cold gelation, enabling the incorporation of these ingredients into heat-sensitive foods [3]. However, most of these techniques require an initial heat-denaturation step. That is the case of acid-induced gelation. Studies have shown that pre-denatured WP gel in the presence of acidulants such as hydrochloric acid (HCl), glucono-δ-lactone (GDL) and some organic acids, as a result of pH reduction [4]. Nevertheless, the influence of formic, acetic and propionic acids has never been reported for WP gelation at room temperature.

Formic, acetic and propionic acids are weak organic acids differing in the length of the main chain by one carbon. Moreover, acetic acid (E260) and propionic acid (E280) are authorized food additives. They are categorized as food preservatives, contributing to the shelf-life extension of food products due to their antimicrobial properties [5]. The development of a gelation technique in the presence of these acids and in the absence of a previous denaturation step would be of great advantage for texture control of acid heat-sensitive foods such as meat and dairy products, with lower production costs.

The present work aims to investigate whey protein isolate (WPI) gelation induced by formic, acetic and propionic acids, at ambient temperature. The effects of protein concentration, acid concentration, pH and acid type on protein denaturation, gel formation and final viscoelastic properties were studied by macroscopic observation, small strain oscillatory rheology and differential scanning calorimetry (DSC).

1. LITERATURE REVIEW

1.1. WHEY PROTEINS AS FUNCTIONAL INGREDIENTS

Whey proteins (WP) represent the soluble fraction of the milk protein system and are typically found in whey, amongst other components, such as sugar, minerals and fat [6,7]. Due to their high nutritional value and functional properties, such as gelation, thermal stability, foam formation and emulsification, WP have been widely used in the food and beverage industry as ingredients. They are mainly used as a fat replacers, stabilizers, thickeners, binders and texturizers in desserts, backed goods, dairy products, among others [2,8]. WP are commercially available in the form of concentrates (WPC) or isolates (WPI), depending on the purity of the product. WPC and WPI contain between 34 to 85 % and over 90 % protein content, respectively. These products are obtained from whey, which was previously processed by physical separation technologies to remove non-protein components [7]. Both WPC and WPI were notified by the Food and Drug Administration (FDA) as generally recognized as safe (GRAS) ingredients [9].

1.1.1. Physicochemical characteristics of whey proteins

WP comprise two main globular proteins, β-lactoglobulin (β-LG) and α-lactalbumin (α-LA), accounting for approximately between 70 to 80 % of the total protein content of bovine whey [10]. WP also include a minor fraction of protein/peptide components such as bovine serum albumin (BSA), immunoglobulins, glycomacropeptide, lactoferrin, lactoperoxidase, lysozyme and growth factors [7]. The main molecular features of the most abundant protein components of WP are shown in Table 1.

Table 1. Molecular properties of the major protein components in WP.

Protein	**pI**[a] [3]	**Td**[b] **(ºC)** [3]	**Mw**[c] **(kDa)** [3]	**aa**[d] **residues** [6]	**-SH** [11]	**S-S** [11]
β-LG	5.2	78	18200	162	1	2
α-LA	4.8-5.1	62	14200	123	-	4
BSA	4.8-5.1	64	66000	582	1	17

[a]pI – isoelectric point
[b]Td – denaturation temperature
[c]Mw – molecular weight
[d]aa – amino acid

β-Lactoglobulin

WP functionality has been mainly attributed to β-LG, as it is the dominant protein in bovine whey, accounting for about 50 % of its total protein content [10,12]. In terms of structure, β-LG contains a free cysteine residue (Table 1) which usually remains unexposed. However, in the presence of denaturing conditions (such as heat, pressure, etc.), it becomes available for intermolecular interactions [11]. For this reason, β-LG can have different quaternary structures in aqueous solutions, depending on the pH; it can exist as a dimer (pH between 3 and 7) or as a monomer (pH lower than 3) [10,13].

α-Lactalbumin

α-LA is the second most abundant protein in bovine whey, corresponding to 20% of its total protein content [10]. Structurally, α-LA does not have free thiol groups (Table 1). However, one of its four disulfide bonds is more sensitive to cleavage due to its instability. This protein also contains a binding site for cations, with higher affinity for Ca^{2+}, which contributes to its stability. The conformation of α-LA is also highly influenced by environmental conditions [11]. For instance, at low pH or near the pI, α-LA changes from the native to a partially folded state, also known as molten globule state [10,11].

1.2. WHEY PROTEIN GELATION

The ability to form gels is one of the most important functional traits of WP. Basically, gelation occurs upon intermolecular interactions established among WP resulting in the formation of a cross-linked three-dimensional network [14]. Generally, this process involves two steps. The first step consists in the partial or total unfolding of the protein (denaturation) which can be induced by heating, increase in hydrostatic pressure, addition of chemicals, changes in the net charge, or enzymatic hydrolysis. The second step encompasses aggregation of the denatured proteins through covalent and/or non-covalent interactions [14,15]. This section focuses on identifying the intrinsic and extrinsic factors that lead to structural changes in WP and, consequently, affect globular protein gelation. The existent mechanisms for WP gelation will also be reviewed, as well as the main WP gel structures and their characteristics.

1.2.1. Factors affecting protein gelation

Generally, functional properties are closely related to protein structural transitions. For instance, changes in the tertiary and secondary structures need to occur, usually through denaturation, to increase the amino acid surface exposure. This results in an increased interaction potential, enabling the formation of new molecular interactions [16]. This process is highly influenced by a combination of factors related to the native protein (intrinsic factors) and to the environmental conditions (extrinsic factors). Particularly for gelation, the factors involved are listed in Table 2. These factors determine not only gel formation, but also the type and properties of the final gels [17,18].

Table 2. Factors affecting protein gelation [18].

Intrinsic Factors	**Extrinsic Factors**
Hydrophobicity	Protein Concentration
Electrostatic Interactions	pH
Disulphide Bonds	Temperature
Molecular weight	Ionic Strength and ion type
Amino acid composition	Pressure

The importance of molecular interactions on gel formation

For the purpose of this work, molecular interactions, especially electrostatic and hydrophobic interactions (non-covalent), as well as disulphide bonds (covalent) are the most important intrinsic factors to consider [18]. Electrostatic interactions are established between charged species and may be either attractive or repulsive. When proteins are involved, these interactions are especially sensitive to medium pH and salt concentration. Protein charge changes depending on whether the pH is close or far from the pI. In turn, the presence of electrolytes might cause a screening effect, leading to the reduction of the magnitude and range of these interactions. Moreover, the presence of polyvalent ions may also cause the formation of bridges between molecules with an opposite charge to the ion through interactions of electrostatic nature. Ion bridging effects overlap screening effects [3].

Hydrophobic interactions are strong attractive forces between non-polar groups, in an aqueous media. These interactions are originated due to the repulsion between water molecules and the non-polar protein residues. Both temperature and pressure affect hydrophobic interactions [3,19].

Disulphide bonds are covalent interactions involving protein cysteine residues. Naturally, cysteine residues are present in proteins in the free sulfhydryl (-SH) or in the oxidized (S-S) forms [20]. However, as thiol and disulphide groups are usually located in the interior of the native protein, unfolding is necessary to expose them. Otherwise, they are unavailable to react [3]. High pressure, elevated temperatures, shear and solvents containing HCl or urea are known to increase disulphide bond formation rate. Disulphide crosslinking usually occurs by an oxidation reaction between two disulphide groups or by a thio-disulphide interchange reaction (Figure 1). Moreover, these interactions can be both inter or intramolecular [20].

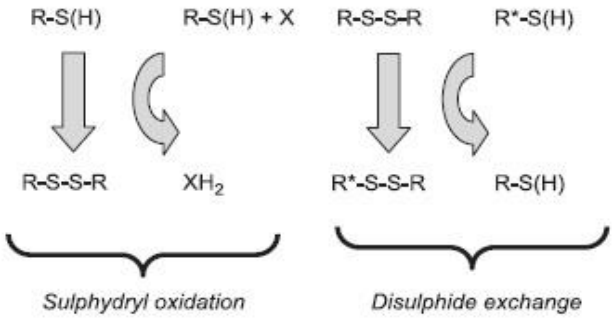

Figure 1. Pathways leading to the formation of disulphide bonds [20].

The influence of extrinsic factors on gel formation

Protein concentration is critical to gel formation, particularly during the aggregation step. Below a minimum concentration, a viscous solution is typically obtained instead of a gel; above a critical concentration, protein aggregation can originate a precipitate or a self-supporting gel [21]. Protein concentration also influences gel strength and deformability [18].

The net charge of protein molecules depends on the pH. For instance, when the environment pH is below or above the pI, proteins are positively or negatively charged, respectively. When the pH equals the pI, the protein net charge is equal to zero. The further the pH is away from the pI of the protein, the greater the electrostatic repulsions between

protein molecules. As a result, the interactions required for gel formation are impaired [3,18]. Not only protein-protein interactions are affected, but also protein-solvent interactions [22].

Temperature is one of the most important factors in protein gelation, influencing gelation rate and the final properties of the formed gels, particularly gel strength. Regarding gelation rate, higher temperatures lead to increased denaturation and aggregation rates [3,18].

The effects of ionic strength on gelation are very similar to those of pH. Increasing ionic strength, by adding salts, results in reduction or neutralization of repulsive forces between protein molecules, promoting gelation [22]. Therefore, increased salt concentrations lead to increased gelation rates. Unlike monovalent salts, divalent salts, originating divalent cations, act like bridges between the negatively charged carboxylic groups in the proteins, promoting gelation. Moreover, they have a higher charge screening effect than monovalent cations. For this reason, lower concentrations of divalent ions are needed to promote gelation. The effects of ionic strength are also extended to gel structure. Adding salts increases gel turbidity and may decrease gel water holding capacity, above a critical concentration. Gel strength and brittleness are also affected [3].

Pressure effects on proteins are based on the principle of *Le Chatelier*, which states that an increase in pressure results in a decrease in volume and vice-versa. Therefore, when proteins are submitted to high pressures, they are compressed and their volume decreases [19]. This implicates the rupture of non-covalent interactions within protein molecules, resulting in protein denaturation; covalent bonds are unaffected. Depending on the applied pressure, denaturation might be reversible or irreversible. Moreover, new intra and intermolecular interactions can be established, particularly hydrophobic interactions and disulphide bonds, leading to aggregation and gelation [23].

1.2.2. Gelation mechanisms

There are several methods reported in the literature to induce WP gelation. These can be classified as chemical (gelation promoted by salts, enzymes and acids) and physical methods (such as heat-set and pressure-induced gelation). Only the most relevant in terms of food processing will be reviewed.

Heat-set gelation

Heat-set gelation is the classical method to obtain gels from WP and consists in the application of heat, under controlled conditions. At a molecular level, the increased temperatures cause the proteins to unfold, exposing some of its hydrophobic and free –SH groups, which remain buried in the protein core at ambient temperature. Then, physical and chemical aggregation of the unfolded proteins takes place. Both polar and hydrophobic groups can associate physically through non-covalent interactions such as hydrogen bonds, electrostatic and hydrophobic interactions. Free –SH groups associate chemically through covalent cross-linking [14].

Cold-set gelation

The need to extend the use of WP as a gelling agent in foods sensitive to heat led to the development of a cold-set gelation technology [2]. The production process of cold-set gels comprises two steps: (1) heat-polymerization and (2) induction of gelation at low temperatures [2,3]. The first step consists in the production of a heat denatured WP dispersion containing filamentous protein aggregates. As the process involved is the same one responsible for heat-set gelation, careful control of the heat (temperature and holding time) and solution conditions (pH, mineralization, and protein concentration) is needed. Otherwise, the solution might gel. During the second step, the solution is cooled and aggregation takes place as salt (usually NaCl or $CaCl_2$) is added. Consequently, the electrostatic repulsion decreases, allowing the filaments to associate and form strands. During this phase, the manipulation of environmental conditions yields solutions or gels with different characteristics. Cold-set gels are usually stronger and more transparent than those prepared by heat-set gelation. The main advantage of this gelation technique is the possibility of thickening solutions and creating gels at ambient and refrigeration temperatures, enabling the production of foods with improved textures, appearances and properties [3]. This gelation mechanism is also known as salt-induced gelation.

Pressure-induced gelation

High pressure treatment is a relatively new processing technology in the food industry. This technology has a wide range of applications, including modification of

functional properties of foods such as protein gelation [23]. Generally, pressure-induced changes in proteins are related with variations in the volume of the system, which shifts in favor of the state with the lowest overall volume. For this reason, quaternary, tertiary and secondary structures of the protein are highly affected by breakdown and reformation of hydrogens bonds, rupture of hydrophobic interactions and separation of ion pairs, resulting in protein denaturation. Consequently, proteins unfold and the buried hydrophobic groups are exposed, leading to protein aggregation [24]. During this phase, disulfide bond interactions occur, contributing to the stabilization of the resultant gel [25]. Similarly to the previous gelation techniques, gel formation and the characteristics of the final gel depend on protein environment and processing parameters (exposure time, temperature and pressure) [24]. Oppositely to heat treatments, high pressure processing affects only the structure of macromolecules, allowing the preservation of important qualities related with food quality, such as the nutritional value, taste and color [23].

Over the last years, other physical technologies have been developed in order to promote protein modification, as these methods are considered safer than chemical ones. Ultrasonication and pulsed electric fields are non-thermal technologies which have been recently shown to affect WP gelation, especially when combined with heat treatments [26,27].

Enzyme-induced gelation

Enzyme treatments are another method used in the food industry to enhance milk proteins functional properties through structure modification. This method has been used both in the presence and absence of a thermal pre-treatment. There are two main mechanism for whey protein gelation induced by enzymes: (1) cross-linking using transglutaminase and (2) hydrolysis using a proteolytic enzyme [28]. The enzyme transglutaminase catalyzes the formation of covalent crosslinking, by donating acyl groups from a γ-carboxyamide group of peptide-bound glutamine residues to primary amines. Gels obtained with the use of this enzyme are different from heat-set gels. They can be produced at a lower protein content and both elastic moduli and breaking strength are increased [29]. In turn, proteolysis works by promoting protein hydrolysis. The proteolytic enzymes used to promote gel formation in WPI were *Bacillus licheniformis* Protease and Alcalase 2.4L. However, there is still lack of

agreement concerning the mechanisms underlying peptide-induced gelation of WPI in the presence of these enzymes [30].

Acid-induced gelation

Acid-induced gelation of WP is usually referred in the literature as a heat or cold-set gelation carried out under acidic conditions. As previously mentioned, lowering the pH influences the establishment of electrostatic interactions by changing the net charge of proteins, reducing or increasing the repulsive forces as the pH is near or below the pI, respectively. Consequently, the aggregation process is affected, leading to the formation of gels with different microstructures and physical characteristics (see section 1.2.3. Structural characteristics of whey protein gels) [31,32]. From a practical point of view, understanding acid-induced WP gelation can be an asset for food processing and ingredient manufacturers, as it enables altering properties, improving WP ingredients performance and selecting the most suited acids according their needs and restrictions [33].

The main techniques described in the literature for acid-induced WP gelation include adding acidulants, such as glucono-δ-lactone (GDL), which in contact with water slowly hydrolyses to gluconic acid and promotes a slow pH lowering, HCl and organic acids [33–35]. Depending on the procedure, these substances are added prior or after heating. In case of heat-set gelation, acidulants are added prior to heating [33], whereas in cold-set gelation acidulants are added in the second step of the process, instead of salts [36]. The acidulants used affect not only protein denaturation and aggregation, but also the overall kinetics of the process [33]. A study [33] performed in acid-induced cold-set β-LG gels analyzed the effect of different types of acidulants on the properties of these gels. It was hypothesized that certain acids might stabilize proteins due to the effects of their anions, which react with the solvent, water, disrupting its structure and consequently promoting conformational changes in the dispersed protein. The effectiveness of the anions in promoting protein stability follow the Hofmeister series: SO_4^{2-} > HPO_4^{3-} > acetate > citrate > Cl^- > NO_3^- > I^- > SCN^-, where the anions on the left are known to increase protein stability and those on the right promote protein instability.

WP gelation in the presence of weak organic acids, at room temperature, has never been reported. However, Venugopal and colleagues [37] studied the gelation of shark myofibrillar proteins in the presence of organic acids, at ambient temperature. Interestingly,

these proteins were able to form gels in the presence of acetic acid and lactic acid, but no gels were obtained in the presence of citric and tartaric acids, as well as HCl. According to the authors, organic acids would favor protein unfolding by slowly lowering the pH, inducing conformational changes in the protein.

1.2.3. Structural characteristics of whey protein gels

Food gels can have different structure networks depending mostly on electrostatic conditions (pH and salt concentration) and protein concentration [16]. The two main protein gel structure networks identified in food systems are designated as "particulate" and "fine stranded" (Figure 2) [38]. Particulate gels are formed near or at the isoelectric point, where the protein is minimally charged; or at high ionic strength, due to charge screening [16]. The resultant network is composed of relatively large particles, capable of scattering light, which are coarsely bound, creating a porous structure. For these reasons, particulate gels are opaque and have poor water-holding capacity [3]. On the other hand, fine-stranded gels are formed when electrostatic repulsion prevails among molecules, what leads to the association of strands or small diameter particles, unable to scatter light [3,16,39]. Thus, these gels are usually translucent, with smaller and more homogenous pores and higher water holding capacity due to capillary forces [3], than particulate gels. These types of structures and the influence of parameters such as pH, salt and protein concentrations have been reported for heat induced whey protein gels [40,41]. The kinetics of the gelation process was also shown to play an important role in determining gel structure. The factors that lead to the development of a particulate and fine-stranded gels vary depending on the primary aggregation process. Hence, heat-set and cold-set gels may display different structures even when produced in similar conditions [21]. Interestingly, in terms of mechanical properties, for heat-set β-LG particulate and fine-stranded gels no correlation was observed between the quite different network structures and the elastic modulus of the gels [42].

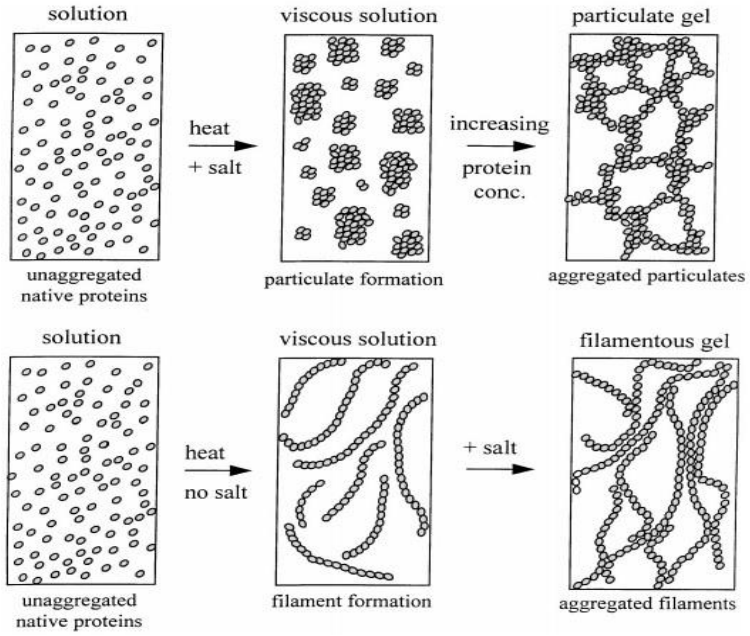

Figure 2. The effect of ionic strength on the development of particulate and fine-stranded gel structures induced by heat and cold-set gelation, respectively [3].

For acid-induced gels obtained by thermal gelation of WP, translucent fine stranded gels with a dense and regular microstructure were observed, similarly to gels formed at a pH > pI. However, acid gels showed to be less firm in comparison to the latter [43]. The acidulant type used to lower the pH during gelation was also shown to influence the final structural network of these gels. In a study performed by Resch and colleagues [33], the effect of different acids on cod-set β-LG gels was investigated. Different structures were observed, depending on the acidulants used. For instance, citric acid led to the formation of particulate gels whereas HCl, lactic and phosphoric acids originated fine-stranded gels concomitant with a slower sol-gel transition.

1.3. CHARACTERIZATION OF FOOD GELS

Characterization of gels obtained from food macromolecules can be achieved using different techniques probing at molecular (< 10 nm), macromolecular (10-10^4 nm) and supramolecular (10^4-10^7 nm) distance scales [44]. The main techniques which have been applied to characterize food gels are identified in Table 3.

Table 3. Techniques employed in gel characterization and respective applications [22,44,45].

Methods	Techniques	Applications
Spectroscopy	NMR (Nuclear Magnetic Resonance) FTIR (Fourier-Transfer Infrared) Raman	Molecular characterization (conformation changes, molecular structure…) [22].
Scattering and diffraction	Light scattering X-Ray diffraction	Structural characterization (particle size, area of the granules,…) [22].
Microscopy	SEM (Scanning electron microscopy) TEM (Transmission Electron microscopy) CLSM (Confocal Laser scanning microcopy) AFM (Atomic Force Microscopy)	Characterization of the microstructure of gels (structural arrangements and distribution of gel particles); characterization of gel nanoparticles [22].
Rheology	SAOS (Small amplitude Oscillatory Shear) Stress relaxation Creep tests Large deformation (TPA, …)	Characterization of viscoelastic behavior of gels [46]; characterization of textural properties [45].
Thermal analysis	DSC (Differential Scanning Calorimetry) TGA (Thermogravimetric analysis) DMA (dynamic mechanical analysis)	Information on protein thermal transitions (denaturation and aggregation) [47].

In this work, oscillatory rheology at low strain, as well as DSC were the preferred techniques. The first technique was chosen because it provides continuous rheological data regarding the gelation process, including gelation time, viscoelastic behavior of the sample, etc [48]. The second technique was chosen because it provides useful information regarding thermal transitions of molecules, including denaturation an even aggregation, enabling a better understanding of the gelation process [47]. These techniques will be briefly discussed below.

1.3.1. Gel rheology

Dynamic rheological measurements are often used to characterize food gels and to provide information regarding the processes of gel formation and melting (structure loss). More specifically, small-amplitude oscillatory shear (SAOS) tests are the best suited to obtain data regarding gel structure [45,48] due to their non-destructive nature, thus not interfering with gelation and melting processes. Moreover, they are relatively fast to perform and their results can be related to the molecular changes occurring during the formation of

the gel network and viscoelastic properties, as molecular structure controls the rheological response of the materials [49].

SAOS tests are characterized by operating at very low strains (or stresses), typically between 1 and 5 %, to assure that the material is in the linear viscoelastic range. Hence, it is essential to determine the limit of linear viscoelasticity before performing these tests [48]. During a SAOS test, a small sinusoidal strain (γ) or stress (σ) is applied, with a certain oscillatory frequency (ω). Then, the resultant time-dependent stress (σ(t)) (or strain) is measured (Equation 1) and the stress response (σ_0) of the sample is, in turn, expressed in terms of an elastic or storage modulus (G′) and a viscous or loss modulus (G″) (Equation 2) [48,49].

$$\sigma\,(t) = \sigma_0 \sin(\omega t + \delta) \quad (1)$$

$$\sigma_0(t) = \gamma_0 G'(\omega) \sin(\omega t) + \gamma_0 G''(\omega) \cos(\omega t)\ , \quad (2)$$

where t is the time, γ_0 the maximum preset strain amplitude and δ is the phase angle.

Viscoelastic Properties

The storage modulus (G′) expresses the magnitude of energy stored in the sample that is recoverable per cycle of deformation. Therefore, it represents the elastic response of the material [48,49]. In turn, the loss modulus (G″) expresses the magnitude of energy dissipated as heat per cycle of deformation, representing the viscous response of the material [49]. ′The values of G′ and G″ are influenced by temperature, frequency and strain. However, for strain values within the linear viscoelasticity range G′ and G″ are independent of strain. The loss tangent (tan δ) is also an important viscoelastic property, where δ represents the phase angle. This angle corresponds to the mismatch between the sinusoidal curves of the applied strain and the resultant stress response. It ranges between 0 and π/2 rad, as the viscous component increases (Fig. 3). Additionally, tan δ represents the ratio of dissipated energy to that stored per cycle of deformation (Equation 3). Hence, higher values of tan δ point to a more viscous behavior. There are also other dynamic rheological properties used to characterize the viscoelastic behaviour of materials, such as the complex modulus (G*) (Equation 4), which represents the overall resistance to deformation; and the complex viscosity (η*) (Equation 5) [49].

$$tan\delta = G''/G' \quad (3)$$

$$G^* = \sqrt{(G')^2 + (G'')^2} \quad (4)$$

$$\eta^* = G^*/\omega \quad (5)$$

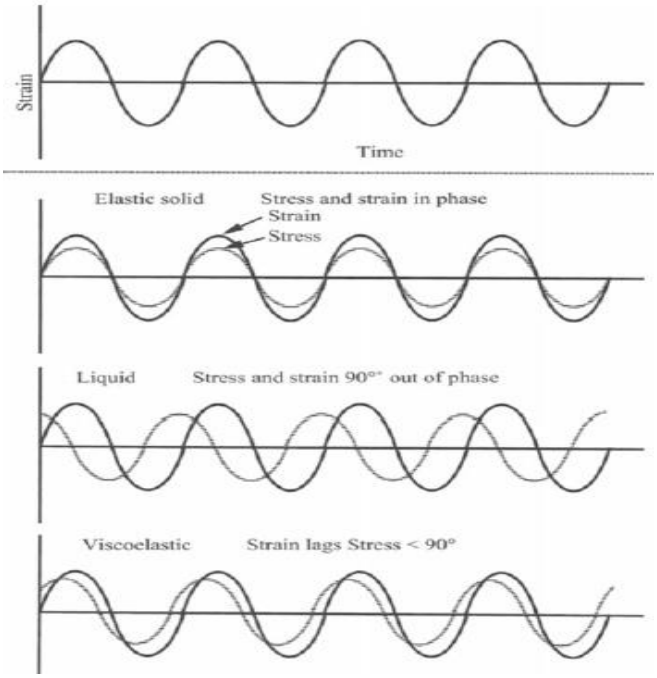

Figure 3. Comparison between the stress vs. strain response of an ideal liquid, a perfect solid and viscoelastic liquid in dynamic tests [49].

Types of SAOS tests and applications

There are three main types of dynamic tests possible to be performed in the linear viscoelastic region: (1) frequency sweep (G′ and G″ vs. ω at a fixed temperature) to determine the viscoelastic properties of the gel; (2) time sweep (G′ and G″ vs. time, at fixed ω and temperature) to determine the structure development in gels (curing and kinetic information); and (3) temperature sweep (G′ and G″ vs. temperature at fixed ω) to provide data on the gelation during cooling or heating [49].

However, as it was previously mentioned, before performing these tests it is essential to submit the sample to a strain or stress sweep test (G′ and G″ vs. γ or σ) performed at a low frequency (e.g. 1 Hz), in order to determine the linear viscoelastic region. The limit of

linearity is identified when rheological properties (G′ and G″) shift from their constant value (Figure 4) [48,49].

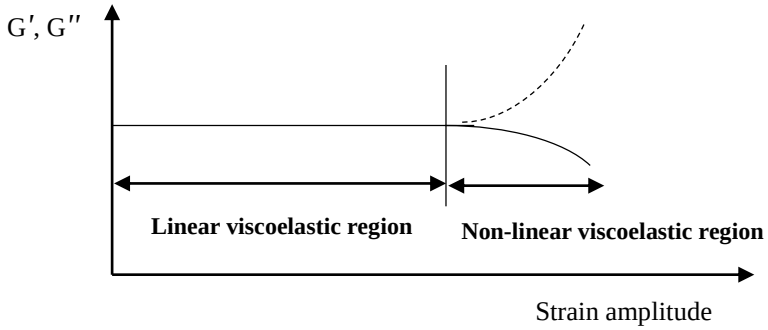

Figure 4. Schematic illustration of the strain sweep test to determine the linear viscoelastic region. Adapted [48].

Determining the gel point

During gelation, a phase transition between a liquid to a solid state occurs. The moment in time or the temperature at which the system undergoes this phase transition can be considered the "gel point" [50]. In terms of rheological measurements, the SAOS technique has been widely used to detect this critical point. It provides continuous rheological data, making it possible to follow the evolution of the viscoelastic properties throughout the gelation process [48,50]. Moreover, due to the small applied strains, the molecular structure modifications caused by shear are minimized [50].

There is not a universal technique to determine the gel point. Instead, there are several rheological measurements possible to be performed, each one having different limitations. Therefore, the choice of one technique over the other should be based on the main purpose of the study, on the equipment limitations and on the characteristics of the sample [48,51]. The cross-over method, the Winter-Chambon method, and the threshold G′ value are some of the techniques used to detect sol-gel transitions. The cross-over method is based on the assumption that the gel point occurs when G′ and G″ cross each other, i.e. when $\tan \delta = 1$, at a given ω. The main concern of this method is the fact that the dynamic moduli cross-over depends on the ω [50]. However, if the chosen ω is sufficiently low, this method might lead to a cross-over time very close to the sol-gel transition time [48]. In turn, according to the Winter-Chambon method, the gel point occurs at the moment when $\tan \delta$ becomes independent of ω. Therefore, a range of frequencies need to be considered [48]. At the gel

point, both dynamic moduli and ω are related through a relaxation exponent (Δ) (Equation 6), which depends on several factors (such as concentration, molecular weight, gel molecular structure…). This method is considered the most objective and well-founded [51]. Lastly, the threshold G′ method consists in detecting the gel point when G′ increased to a value higher than the background noise [48,50]. This criterion is not very rigorous. Nevertheless, it is useful in systems whose lowest detected G′ is already greater than G″[50] .

$$G'(\omega) \sim G''(\omega) \sim \omega^{\Delta} \quad (6)$$

Dynamic rheology and gel structure

In general, aqueous biopolymer gels have a very similar and characteristic behavior during structure development, which is possible to assess with a time sweep test. Typically, the gelation process is characterized by the dominance of the viscous behavior (G″ > G′) during the initial phase of network formation. However, in the final stages of gel formation, the elastic behavior prevails (G′ >> G″), as a result of a gradual increase of G′ throughout the process. In the beginning, due to the rapid formation of junction zones within the gel network, it is possible to observe an increase in both moduli, especially G′ which intersects and exceeds G″. After this point, G′ keeps rising steadily until reaching a plateau region, as the formation and rearrangement of the junction zones occur more slowly [50].

The analysis of the viscoelastic behavior of fully developed gels is achieved by studying the effect of frequency on G′ and G″ with a frequency sweep test. By looking at the resultant mechanical spectra, it is possible to infer whether the gel networks tends to be more or less developed and, consequently, more or less elastic [51]. For instance, the mechanical spectra of gels possessing a perfect tridimensional network typically show a G′ higher than G″, with both moduli almost independent of ω. These gels are also known as “strong” or “true gels”. On the other hand, there are gels whose network is easily broken down, due to the existence of less junction zones or more labile ones. The typical mechanical spectrum of these gels usually indicates a small difference between the moduli values and shows a higher dependence of both moduli on ω. These gels are also called “weak” gels [50].

1.3.2. Differential Scanning Calorimetry

DSC is a calorimetric technique that measures the absorbed or produced heat as function of time or temperature. It has several applications, including studying temperature-induced conformational changes (transitions) in proteins and other biomolecules, as well as to predict their thermal stability [52]. Numerous studies have used DSC [15,47,53,54] to investigate WP gelation due to its ability to detect denaturation and aggregation, as they are both temperature-inducible processes. DSC is performed using a calorimeter, which comprises two matched compartments: a reference cell, which usually contains the solvent, and the sample cell, both located in an adiabatic chamber. During the experiment, both cells are heated simultaneously and at constant rate, under a small pressure (2-3 atm) to avoid bubble formation and evaporation. [55]. The instrument works by measuring the electrical energy provided by the heaters to maintain the two cells at the same temperature (power compensation), or by measuring the heat flow as a function of the sample temperature (differential temperature). As a result, DSC outputs a curve of power or heat flow vs. temperature or time. The heat flow can be converted to heat capacity (Cp), by being divided by the heating rate of the experiment (Equation 7) [55].

$$C_p = \frac{q}{\Delta T} \quad , \tag{7}$$

where q *i*s the heat flow over a certain time and ΔT is the change in temperature over the same time.

In order to analyze and interpret DSC data, certain thermodynamic parameters, such as excess heat capacity (Cp), melting temperature (Tm) and enthalpy variation (ΔH) need to be calculated, using the information available in the respective thermogram (Figure 5). As previously mentioned, Cp consists in the difference between the heat capacity of the cells and represents the capacity of the system to store energy [55]. In turn, Tm corresponds to the mid-point of the transition (peak), where the concentrations of both folded and unfolded proteins are equal. Finally, ΔH is calculated by integration of the DSC curve (Equation 8) [52,55]. These parameters must be normalized to the protein concentration, so that results can be compared [56].

$$\Delta H = \int_{T0}^{T1} Cp.dT \quad , \tag{8}$$

where T1 and T0 represent the temperature that corresponds to the limits of the peak

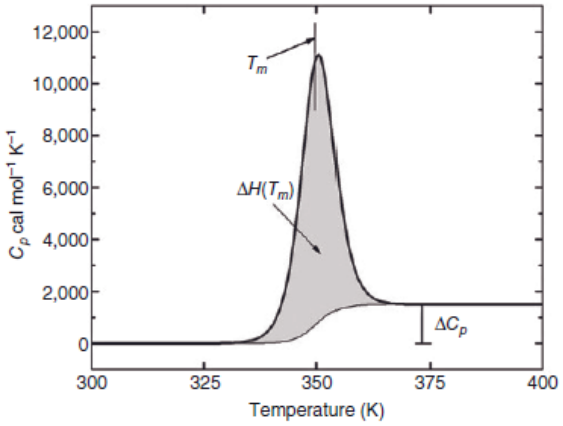

Figure 5. Schematic representation of important thermodynamic parameters in a simulated DSC curve of a globular protein in a dilute solution. ΔCp represents the difference betwee the Cp of both native and denatured states of the protein [55].

Analysis of protein denaturation and aggregation

Protein DSC thermograms usually show the heat capacity of the native and of the unfolded state, separated by an endothermic peak which signals the heat absorption associated with protein denaturation. Basically, at Td, protein molecules store the energy required to unfold in the denatured molecules. When every molecule is unfolded, this mechanism no longer operates, hence heat capacity decreases to a relatively low value (post-transition baseline). It is important to note that for "complex" proteins, several or even overlapping peaks might be detected [52]. For instance, DCS scans of WP usually show two peaks, which correspond to α-LA and β-LG respectively, as these are the most abundant proteins of WP (Figure 6) [47].

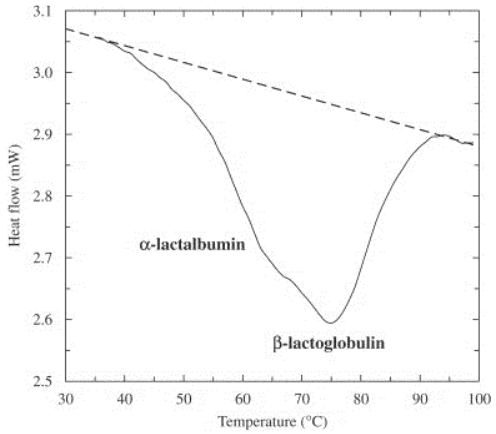

Figure 6. DSC heating scan (1.0 ºC/ min) of 3.0 wt. % WPI (pH 7.0). Endo down [47].

In turn, protein aggregation is an exothermic process that involves the formation of new bonds between protein molecules, hence producing an exothermic peak in the thermogram. However, according to Fitzsimons and colleagues [47], this transition isn't usually visible in most studies on WP gelation, as conventional calorimeters (typical sample load ~ 15-50 mg) are used, instead of micro-calorimeters (sample mass ~ 850 mg). This lack of resolution is due to a smaller sample mass load in conventional calorimeters, which promotes a faster heat transfer, causing a rapid denaturation process, leading to the overlap of the exothermic heat flow by the endothermic heat flow.

2. MATERIALS AND METHODS

2.1. MATERIALS

Whey protein isolate powder (BiPRO, 97.7 % protein, 0.5 % fat, 1.9 % ash), α-lactalbumin (BioPURE) and β-lactoglobulin (BioPURE) were obtained from Davisco Foods International (USA). Acetic acid glacial 100 % and propionic acid for synthesis (99 %) were purchased from Merck (Germany). Hydrochloric acid (37 %) was bought from Riedel-de-Haën (Germany) and formic acid (98 – 100% puriss.pa) was obtained from Sigma-Aldrich (Germany).

2.2. METHODS

2.2.1. Preparation of whey protein solutions

WPI, α-LA and β-LG solutions 20 % and 25 % (w/w) were prepared by gradual dispersion of the respective powder in deionized water, under mild agitation to avoid foam formation. The solutions were stirred for three hours at room temperature until completely dispersed and kept in the refrigerator (5 ºC) until usage in the following 24 to 48 hours. The pH remained unadjusted (6.7-6.9 for WPI solutions and 6.9-7.0 for α-LA and β-LG solutions).

2.2.2. Gelation of whey protein isolate in the presence of weak organic acids

Influence of acid type and concentration

In order to assess the influence of acid type and concentration on WPI gel formation, three acids differing in the length of their main chain were tested (formic, acetic and propionic acid). Samples were prepared by mixing WPI stock solution with an appropriate volume of 6 mol L^{-1} acid and deionized water. Three samples were prepared for each acid. These contained a final protein concentration of 12 % (w/V) and a final acid concentration of 0.5, 1.0 and 1.5 mol L^{-1}. The pH of each sample was measured after their preparation. The

samples were kept at room temperature and were assessed visually for gel formation after 24, 48 and 216 hours.

Influence of acid and protein concentration

Phase diagrams were constructed as a function of protein and acid concentration, for both acetic and propionic acids. Samples were prepared as mentioned above to obtain protein and acid concentrations ranging between 10 % - 14.5 % (w/V) and 0.1 – 2.4 mol L^{-1}, respectively. The pH of each sample was adjusted to 3.20 using 1.0, 0.5 or 0.1 mol L^{-1} HCl as required. The samples were kept at room temperature and were macroscopically observed after 24, 48 and 240 hours for gel formation, turbidity and color.

Influence of pH

Samples were prepared as previously described to obtain a final acid concentration of 0.9 mol L^{-1} and a protein content varying between 10 and 14.5 % (w/V). The pH was adjusted to 3.0, 3.2, 3.4 and 3.6 using 1.0, 0.5 and 0.1 mol L^{-1} HCl. The samples were also kept at room temperature and assessed visually at 24, 48 and 240 hours for any changes in their appearance and gel formation. Both acetic and propionic acids were tested. A phase diagram was established for acetic acid, as a function of pH and protein content.

2.2.3. Rheological characterization of WPI gels

Small amplitude oscillatory measurements were performed using a controlled stress rheometer (AR 1000 TA Instruments) equipped with a cone geometry (4 cm diameter, 3.59 º angle). In order to determine the linear viscoelastic region, strain sweep measurements of G′ and G″ were performed. Time and frequency sweep measurements were carried out at 25 ºC and within the linear viscoelastic limit, at a strain amplitude of 1 %. Time sweep tests were performed at a constant oscillatory frequency (2 rad s^{-1}), whereas frequency sweep tests were performed within the interval 0.05 - 100 rad s^{-1}. Solvent evaporation was prevented by covering the exposed surface of the samples with a thin layer of mineral oil. The gelation time was determined as the G′- G″ crossover point.

2.2.4. Differential Scanning Calorimetry (DSC)

DSC scans were carried out using a power compensated differential scanning calorimeter (PYRIS Diamond DSC PerkinElmer) to assess the effects of HCl and acetic acid on WPI, α-La and β-LG denaturation. Samples were sealed in stainless steel pans (typical loading ~ 40 mg); an empty pan was used as a reference. The samples and the reference were heated from 25 to 100 ºC, at a heating rate of 10 ºC min^{-1}. For each sample, the enthalpy of the process (ΔH) was calculated as the area under the transition peak, using a straight extension of the baseline, whereas the denaturation temperature (Td) was determined as the temperature at maximum deflection of the baseline. These parameters were determined using PerkinElmer PYRIS Software, version 7.0.0.0110. The obtained values were then adjusted to the WP content present in the sample (Equation 7). As no information regarding the purity of α-LA and β-LG was available, the protein content of these reagents was considered to be the same as that of WPI (97 %) for enthalpy calculations purposes. The results were expressed in J g^{-1}.

$$\Delta Hr = \Delta H \times \frac{m\,(WPI\ in\ the\ sample)}{m\,(WP\ in\ the\ sample)} \times 0.97 \quad (7)$$

3. RESULTS AND DISCUSSION

3.1. SOL-GEL TRANSITIONS

3.1.1. Influence of acid type and concentration

Weak organic acid-induced gelation of WPI was first studied by visually assessing gel formation in the presence of formic, acetic and propionic acid, using three different acid concentrations. Observations were made at 24, 48 and 216 hours (Table 4). Figure 7 shows the appearance of the samples at 24 and 216 hours (9 days). The results indicated that WPI gelation occurred in the presence of all three acids, at room temperature. Nevertheless, this process was highly influenced by acid type and concentration. All samples containing propionic acid gelled at 24 hours and the obtained gels were opaque and showed syneresis after 48 hours. However, the sample containing 0.5 mol L^{-1} propionic acid, unlike the remaining, was brittle and presented syneresis at 24 hours. Acetic acid also promoted WPI gelation, but at a slower rate when compared with propionic acid. Samples containing 1.0 and 1.5 mol L^{-1} acetic acid gelled after 24 hours, whereas the sample containing 0.5 mol L^{-1} acetic acid gelled between 48 and 216 hours. Moreover, these gels did not show syneresis during the considered time interval. Lastly, formic acid caused the slowest gelation process. Although the samples containing 0.5 and 1.0 mol L^{-1} formic acid did not apparently gel, a transitioning sol-gel state was observed for the sample containing 1.5 mol L^{-1} formic acid.

The variations on the pH of the samples might have influenced these results, suggesting that below the pI, increasing values of pH lead to faster gelation times. This hypothesis is also supported by the differences in the appearance of the obtained gels, as gels formed in the presence of higher values of pH were more opaque, brittle and showed a lower water holding capacity, concomitant with a rapid sol-gel transition [33]. However, more data is needed in order to confirm these effects. Nevertheless, the influence of acid type and its concentration seemed to prevail over the influence of pH. For instance, it was observed that samples containing 0.5 mol/L acetic and propionic acid, which had approximately the same pH, had different gelation times and yielded gels with different visual characteristics. It was also observed that samples containing increasing concentrations of formic acid had the lowest pH values. However, a gel was obtained only for the highest formic acid

concentration, suggesting that effects of acid concentration and acid type override the possible effects of pH.

Table 4. Macroscopic observations of 12 % (w/V) WPI dispersions added with weak organic acids. The pH remained unadjusted.

Acid concentration (mol L^{-1})	Acid Type	pH	Visual Observations		
			24 hours	48 hours	216 hours
0.5	Formic	3.1	sol	sol	sol
	Acetic	4.0	sol	sol	translucent gel
	Propionic	4.0	opaque brittle gel	white brittle gel	white brittle gel
1.0	Formic	2.7	sol	sol	sol
	Acetic	3.7	translucent gel	translucent gel	translucent gel
	Propionic	3.8	opaque gel, syneresis	opaque gel, syneresis	opaque gel, syneresis
1.5	Formic	2.5	sol	sol	viscous sol
	Acetic	3.5	translucent gel	translucent gel	translucent gel
	Propionic	3.7	opaque gel, syneresis	opaque gel, syneresis	opaque gel, syneresis

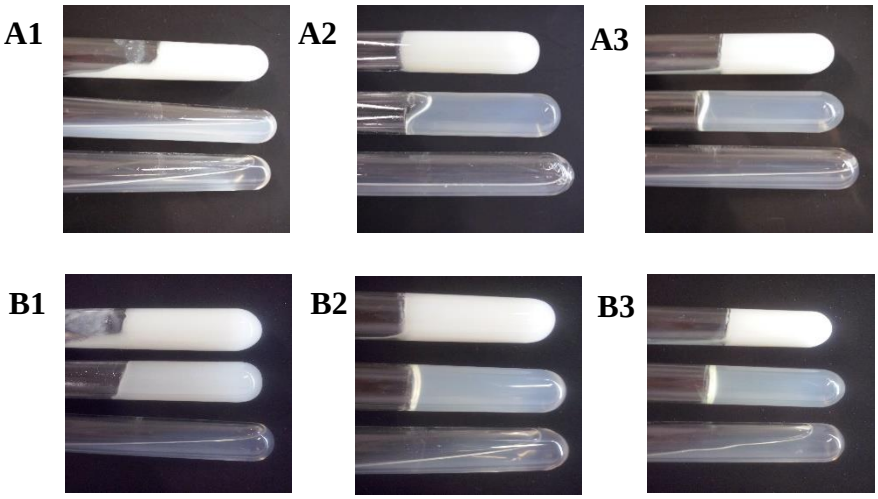

Figure 7. The effects of acid type and acid concentration on 12 % (w/V) WPI, at room temperature. Pictures were taken after 24 hours (**A**) and 216 hours (**B**); Concentrations of 0.5 mol L^{-1}; 1.0 mol L^{-1} and 1.5 mol L^{-1} were tested for propionic (top), acetic (middle) and formic acid (bottom). The pH remained unadjusted.

3.1.2. Influence of acid and protein concentrations

After demonstrating that WPI gelation was promoted by formic, acetic and propionic acids, the effects of acid concentration and protein concentration on WPI sol-gel transition

were investigated, at constant pH (3.20). For this purpose, phase diagrams were built based on macroscopic observations of the prepared samples in test tubes. Figure 8 illustrates the criteria used to determine whether a sample was in the sol, gel or transition state. It is important to note that the pH of the samples was adjusted to 3.20 with HCl. Previous observations of test tubes containing 12 % (w/V) WPI and HCl at a pH 3.20 confirmed that this acid did not promote WPI gelation.

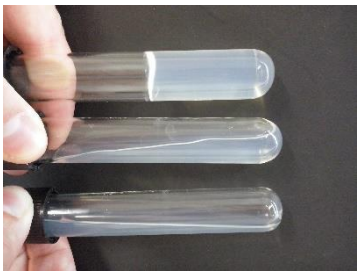

Figure 8. Classification of samples according to their appearance: gel (top), transition (middle) or sol (bottom).

Phase diagrams showing how the occurrence of sol and gel phases depend on protein and acid concentrations are shown in Figures 9 and 10 for acetic and propionic acids, respectively. In both cases, it was observed that gel formation was highly dependent on acid concentration and WPI concentration. For instance, as protein concentration increased, lower acid concentrations were needed to promote gel formation. Moreover, the acid used to induce WPI gelation was also shown to influence this process. In the presence of acetic acid, higher concentrations of protein and/or acid were required to produce gels. Oppositely, lower concentrations of protein and acid were needed for propionic acid-induced gelation. Additionally, for both acids, observations made at 48 and 240 hours (10 days) showed that some sol samples transitioned to a gel state, whereas gelled samples increased in rigidity which confirms the influence of time in this process.

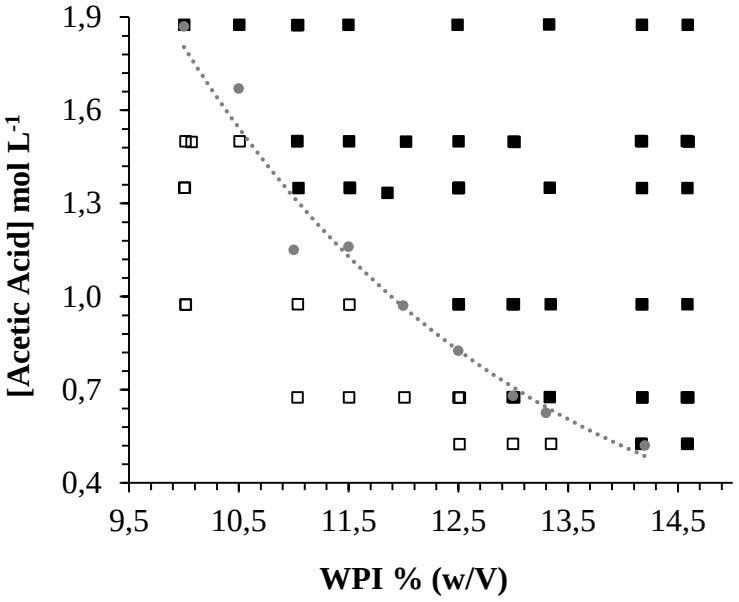

Figure 9. Gelation phase diagram of WPI in the presence of acetic acid at pH 3.20, determined by macroscopic observation after 24 hours. (□) sol; (■) gel (●) sol-gel transition.

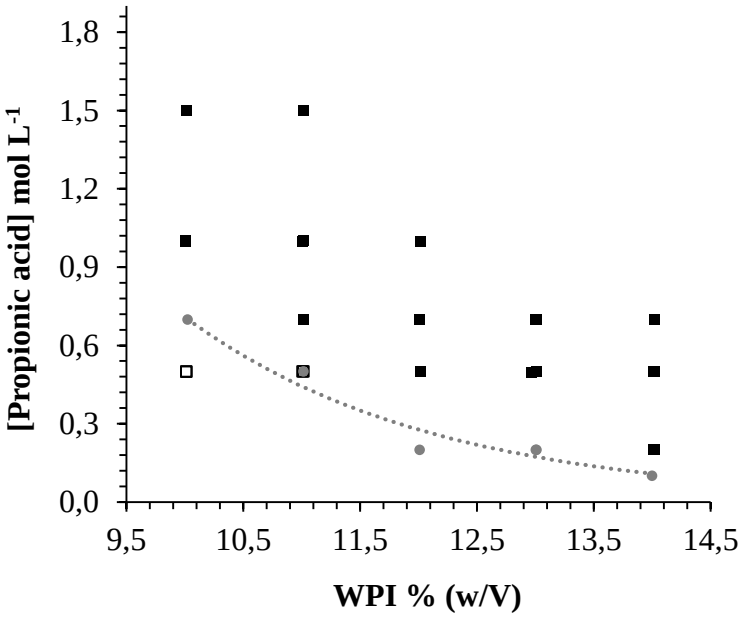

Figure 10. Gelation phase diagram of WPI in the presence of propionic acid at pH 3.20, determined by macroscopic observation after 24 hours. (□) sol; (■) gel (●) sol-gel transition.

Varied combinations of protein and acid concentrations yielded gels with different characteristics. At higher acid and protein concentrations, stronger and more opaque gels were formed and the propensity to develop syneresis increased, reflecting a more particulate

and aggregated structure with lower water holding capacity. Some variations in gel appearance were found depending on acid type. For instance, acetic acid-induced gels were more transparent and showed signs of syneresis later than propionic acid-induced gels. Curiously, the color of every prepared sample acquired a yellow tone that darkened over time, independently on the acid used. Tubes containing higher concentrations of acid were darker than those containing lower acid concentrations. Samples containing higher protein concentrations did not darkened as much as samples containing lower protein concentrations. Figure 11 illustrates the color changes of two samples differing in storage time.

Figure 11. The effect of storage time on sample color. 12 % (w/V) WPI gels containing 0.9 mol L^{-1} acetic acid, at pH 3.20 after being stored for one month (top) and one week (bottom).

3.1.3. Influence of pH

The influence of pH was also tested for both acetic and propionic acid-induced gelation of WPI by observation of gel formation in test tubes. The sol-gel phase diagram as a function of pH and protein concentration, keeping the acetic acid concentration at 0.9 mol L^{-1} is shown in Figure 12. It was observed that higher pH and higher protein concentrations favor gel formation. In terms of gel appearance, no substantial differences were found. However, it was possible to observe that samples containing higher protein concentrations were slightly more turbid and rigid than those containing lower protein concentrations. In turn, in the presence of 0.9 mol L^{-1} propionic acid, under the same range of pH and WPI concentration, all samples have gelled after 24 hours. Therefore, no phase diagram is shown. However, unlike acetic acid-induced gelation, there were many discrepancies in terms of gel appearance. For the same acid concentration, samples containing higher values of pH resulted in totally or partially opaque gels (Figure 13). These characteristics were also found

in propionic acid-induced gels containing increasing protein concentrations, at high pH (Figure 14).

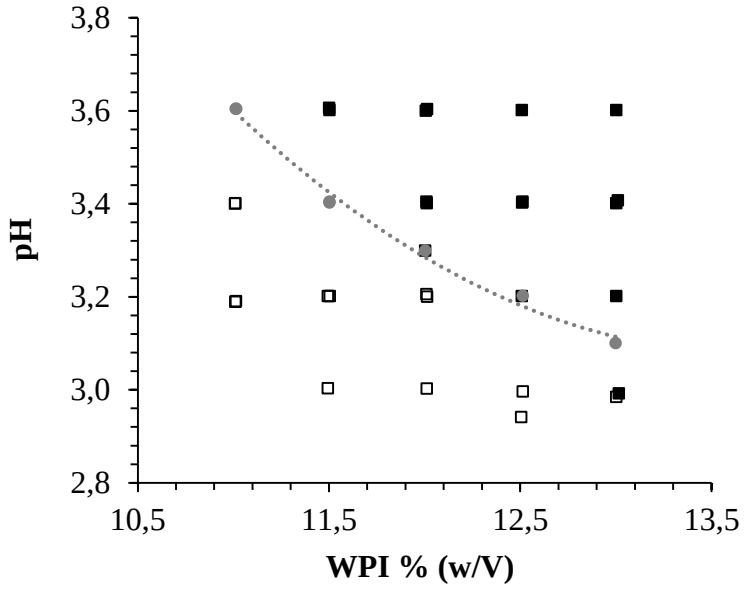

Figure 12. Gelation phase diagram of WPI in the presence of 0.9 mol L^{-1} acetic acid determined by macroscopic observation of gel formation after 24 hours. (□) sol; (■) gel (●) sol-gel transition.

A **B**

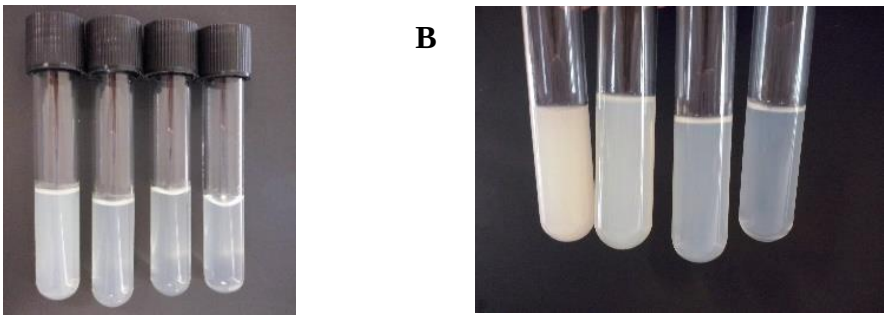

Figure 13. The effect of pH on acetic and propionic acid-induced gels. (**A**) Gels prepared with 13 % (w/V) WPI and 0.9 mol L^{-1} acetic acid and (**B**) gels containing 12 % (w/V) WPI 0.9 mol L^{-1} propionic acid. The pH was ranging from 3.00 (right) to 3.60 (left). Pictures taken after 48 hours.

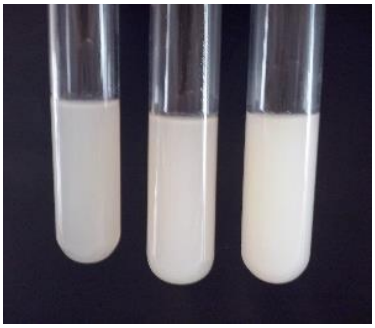

Figure 14. Gels containing 0.9 mol L^{-1} propionic acid and 11, 12 and 13 % (w/V) WPI, respectively (from left to right), at pH 3.6. Picture taken after 48 hours.

3.2. GEL RHEOLOGY

3.2.1. Influence of acid type

During most of the performed experiments, the pH of the samples was adjusted with HCl, so that results could be directly compared at the same pH. Although test tube experiments have shown that HCl did not promote WPI gelation by its own, it was important to confirm whether this acid was or not influencing this phenomenon. Figure 15 illustrates the gelation kinetics of two systems induced by acetic acid, in the presence and in the absence of HCl. Both samples showed a similar viscoelastic behavior and a small difference of 10 minutes was found between the assessed gelation times. The sample containing HCl was the last to gel. In terms of mechanical properties (Figure 16), both gels had an identical behavior. These results confirmed that, for the purpose of this work, the influence of HCl on the viscoelastic behavior of the obtained gels could be despised.

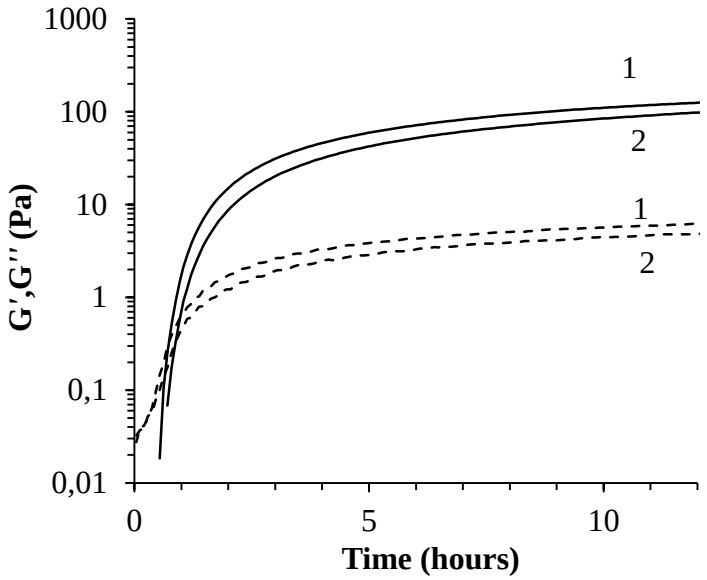

Figure 15. Evolution of the viscoelastic moduli as a function of time, measured at 25 ºC, at an angular frequency of 2 rad/s and 1% strain, for 12 % (w/V) WPI gels containing 2.4 mol L^{-1} acetic acid under two different pH conditions: (1) at pH 3.3 unadjusted and (2) at pH 3.2 adjusted with HCl. Full lines represent G′; dashed lines represent G″.

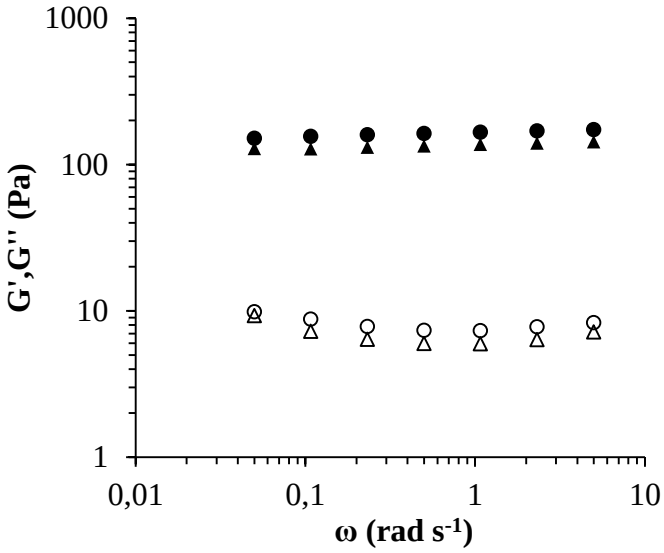

Figure 16. Viscoelastic moduli (G', G") as a function of angular frequency (ω), measured at 25 ºC, 1% strain, for 12 % (w/V) WPI gels containing 2.4 mol L^{-1} acetic acid at pH pH 3.3 unadjusted (●) and at pH 3.2 adjusted with 1.0 and 0.5 mol L^{-1} HCl (▲). Full symbols represent G′; empty symbols represent G″.

Effects of formic, acetic and propionic acids on WPI gelation time and on mechanical properties of the final gels were then investigated. Figure 17 represents the evolution of viscoelastic properties as a function of time for four gels differing in acid type. As gelation

times differ greatly among the three acids, propionic and formic acid gels were separately compared with gels induced by acetic acid. Propionic acid was shown to promote gelation faster than acetic acid. In turn, acetic acid promoted gelation faster than formic acid.

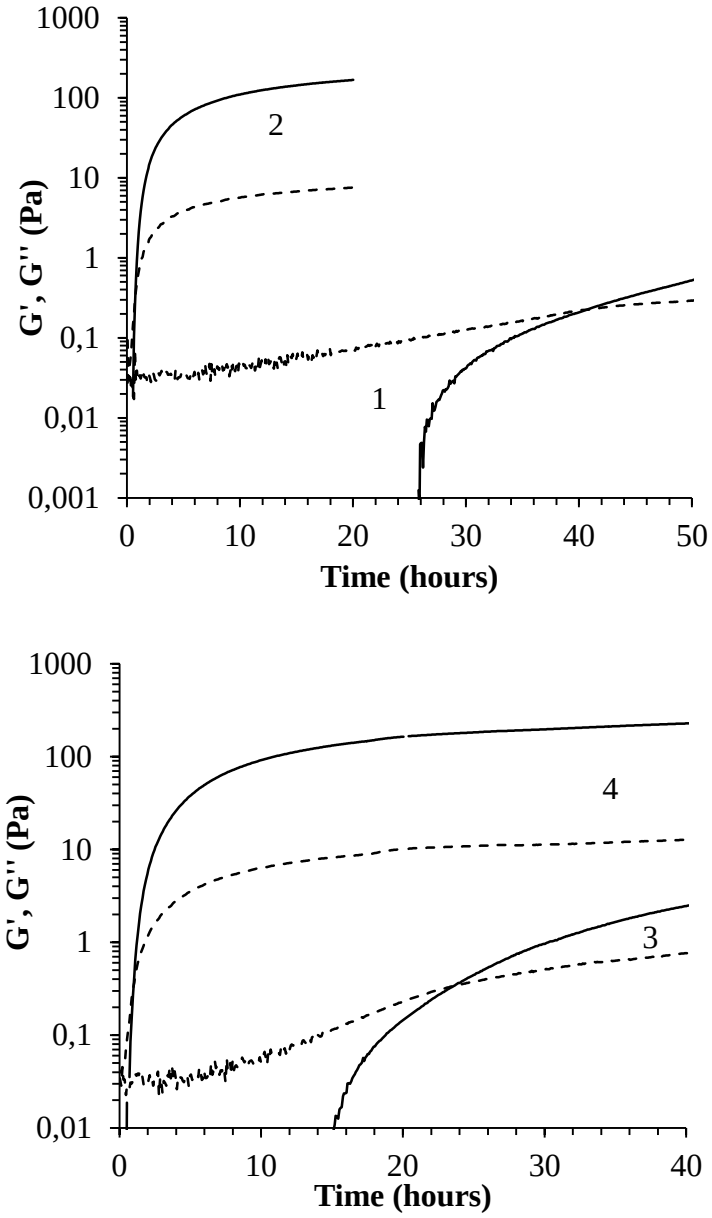

Figure 17. Influence of acid type on the gelation kinetics of 12% (w/V) WPI, measured at 25 ºC and at an angular frequency of 2 rad s^{-1}, 1% strain. The samples contained (1) 2.4 mol L^{-1} formic acid at pH 2.2 unadjusted; (2) 2.4 mol L^{-1} acetic acid at pH 3.3 unadjusted; (3) 0.9 mol L^{-1} acetic acid at pH 3.2 adjusted with HCl; (4) 0.9 mol L^{-1} propionic acid at pH 3.2 adjusted with HCl. Full lines represent G′; dashed lines represent G″.

Figure 18 represents the mechanical spectra of the previous samples. It was observed that every gel had different mechanical behaviors, depending on the acid type. For the same acid concentration, formic acid yielded a weaker gel than acetic acid, as G′ and G″ had lower and closer values and G′ had a higher dependency on ω. In turn, propionic acid yielded a stronger gel than acetic acid, as the difference between G′ and G″ was higher and G′ was shown to be practically independent on ω, oppositely to that of acetic acid. Therefore, although further studies are needed to better characterize the mechanism involved, the gelation process seems to be dependent on the size of the acid's hydrocarbon chain, which suggests that hydrophobic interactions and destructuring of water (solvent) play a decisive role.

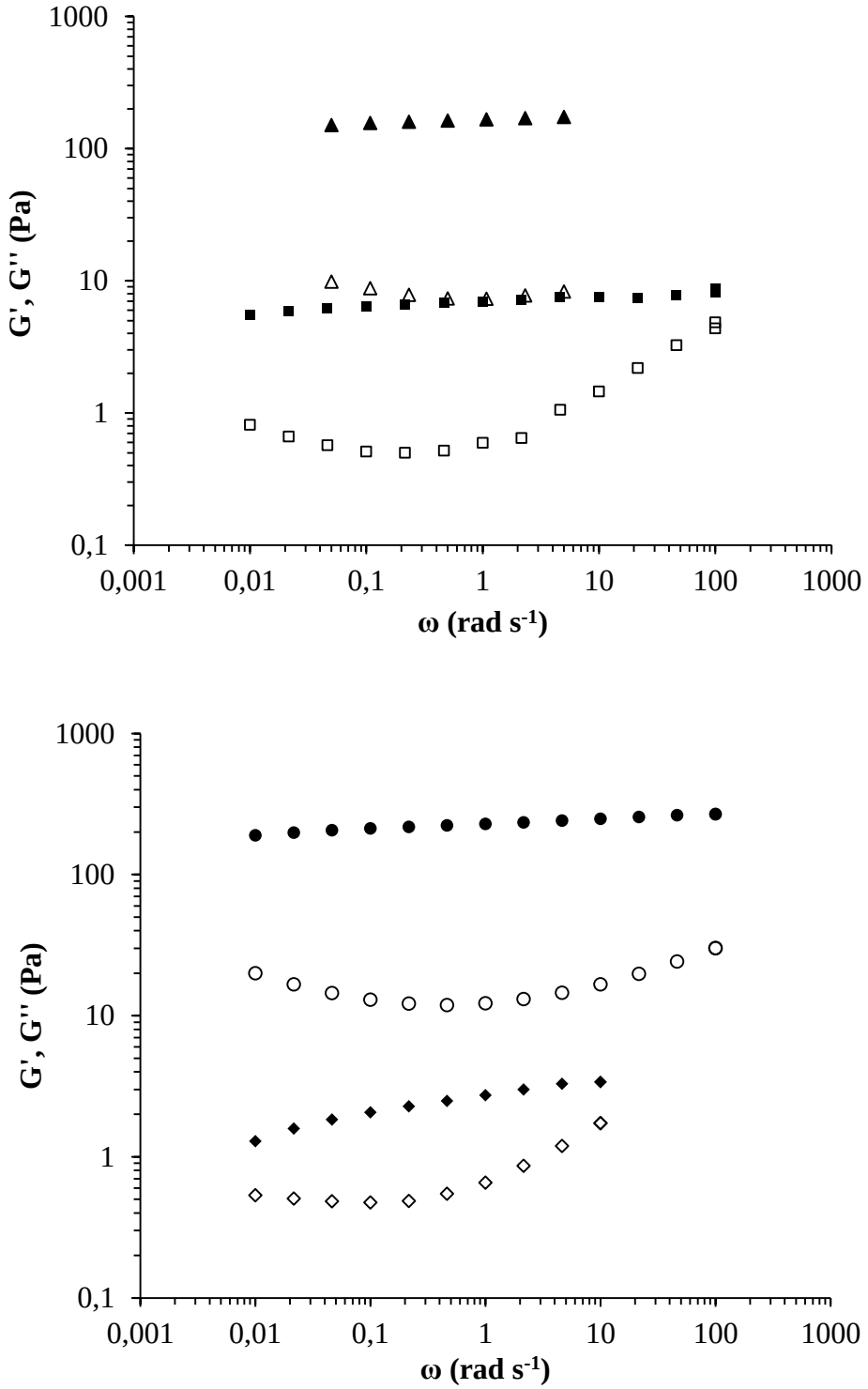

Figure 18. Influence of acid type on the mechanical properties of 12 % (w/V) WPI gels, measured at 25 ºC, 1% strain. The samples contained (■) 2.4 mol L^{-1} formic acid at pH 2.2 unadjusted; (▲) 2.4 mol L^{-1} acetic acid at pH 3.3 unadjusted; (◆) 0.9 mol L^{-1} acetic acid at pH 3.2 adjusted; (●) 0.9 mol L^{-1} propionic acid at pH 3.2 adjusted. Full symbols represent G′; empty symbols represent G″.

3.2.2. Influence of acid and protein concentrations

The influence of acid concentration on gelation time and mechanical properties of the final gels was investigated for acetic and propionic acids. These effects were not tested for formic acid due to its very long gelation times. For both propionic and acetic acids, increasing acid concentrations led to decreasing gelation times (Figure 19). Once more, propionic acid was shown to promote gelation faster than acetic acid, as lower acid concentrations were needed to promote WPI gelation.

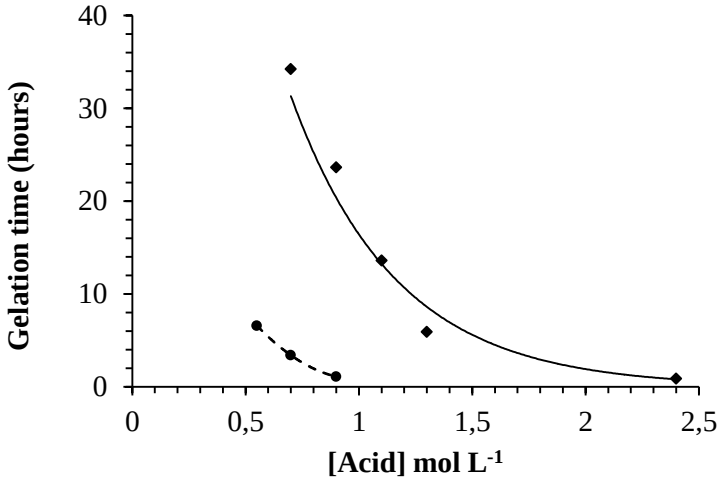

Figure 19. The effect of acetic (◆) and propionic acid (●) concentration on WPI gelation kinetics, at 25 ºC and at an angular frequency of 2 rad s^{-1} and 1% strain. Samples contained 12 % (w/V) WPI and different concentrations of acetic and propionic acids, at pH 3.20 (adjusted). The gelation time was defined as the critical time corresponding to the G'-G" crossover.

The effect of acid concentration on the mechanical properties of the gels was also investigated. Figure 20 shows the evolution of viscoelastic moduli as a function of ω for different acid concentrations. The results show that increasing acid concentration induces the formation of stronger gels, for both tested acids. These results reinforced the ability of propionic acid to produce stronger gels than acetic acid, especially at low concentrations.

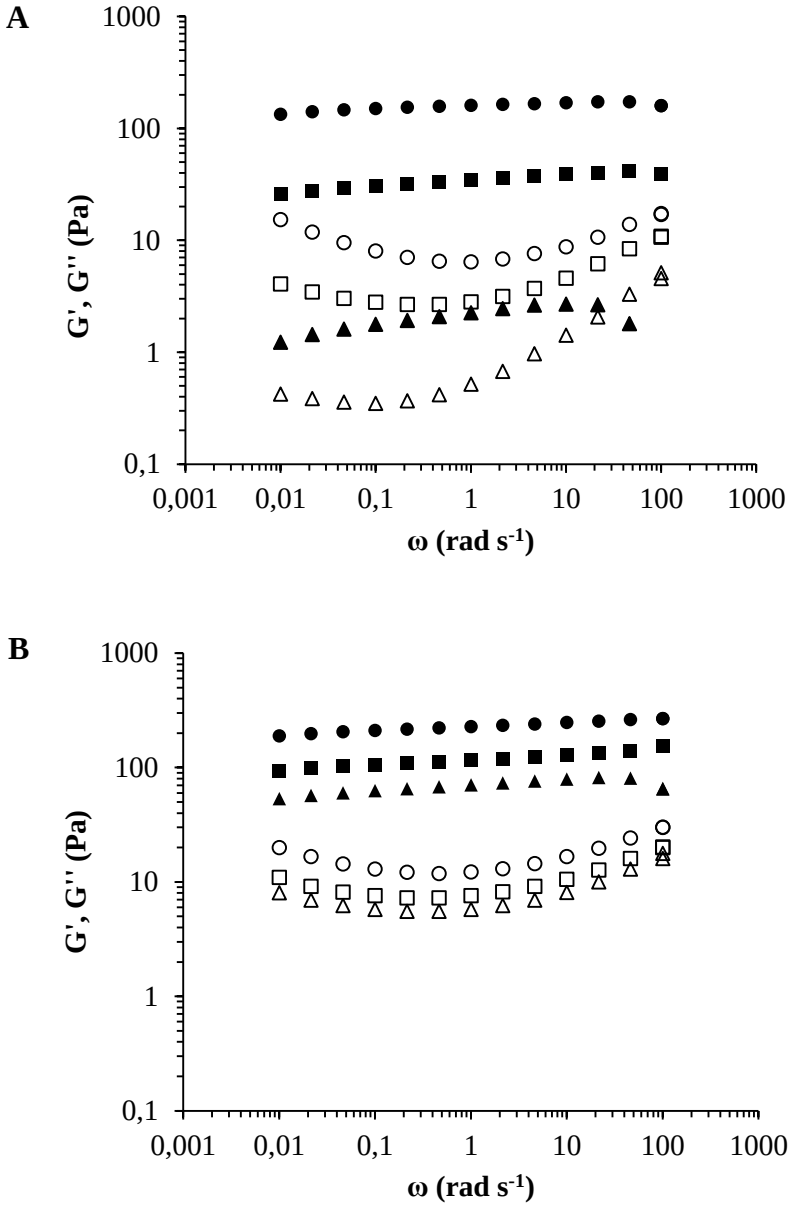

Figure 20. The effect of acid concentration on the mechanical properties of 12% WPI (w/V) gels induced by (●) 2.4; (■) 1.3 and (▲) 0.7 mol L^{-1} acetic acid (**A**) and induced by (●) 0.9, (■) 0.7 and (▲) 0.55 mol L^{-1} propionic acid (**B**). The pH was adjusted to 3.20 with HCl. Measurements were performed at 25 ºC and 1% strain. Full symbols represent G′; empty symbols represent G″.

The influence of WPI concentration on gelation time and mechanical properties was tested for acetic acid-induced gels. Samples varying in protein content containing the same acid concentration at pH 3.20 were assessed. Results are illustrated in Figure 21. As for the mechanical properties of the obtained gels, mechanical spectra are illustraded in Figure 22. Gelation time was found to decrease in the presence of higher concentrations of protein whereas gel strength was observed to increase.

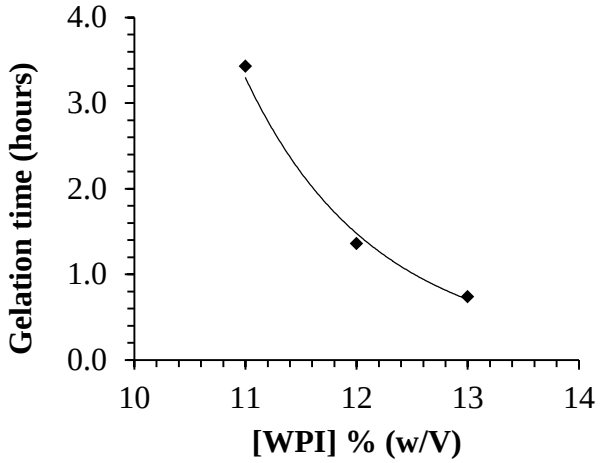

Figure 21. The effect of protein concentration on WP gelation kinetics, measured at 25 ºC at an angular frequency of 2 rad s^{-1} and 1 % strain. Samples contained 11-13 % (w/V) WPI added with 1.1 mol L^{-1} acetic acid at pH 3.20 adjusted with HCl. The gelation time was defined as the critical time corresponding to the G'-G'' crossover.

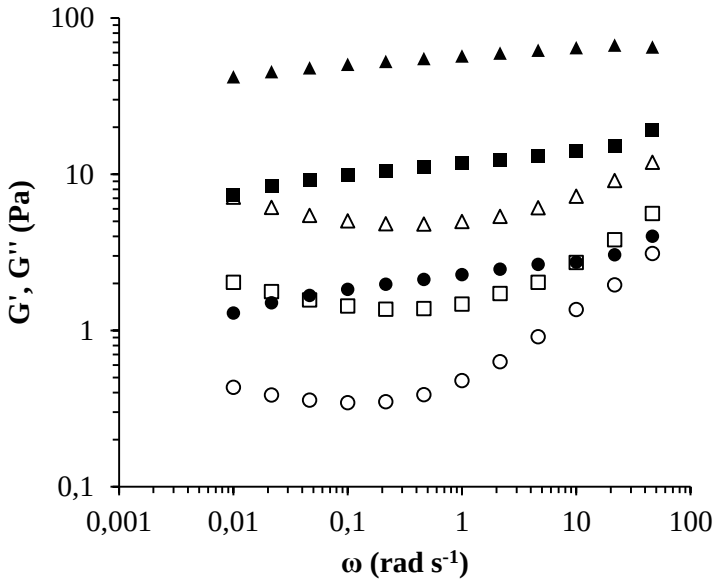

Figure 22. The effect of protein concentration on the mechanical characteristics of WPI gels, at 25 ºC and 1 % strain. Samples contained (▲)13, (■) 12 and (●) 11 % (w/V) WPI added with 1.1 mol L^{-1} acetic acid at pH 3.20 adjusted with HCl.

3.2.3. Influence of pH

The influence of pH on acetic and propionic acid-induced gelation of WPI was investigated in terms of gelation time and mechanical properties. Figure 23 shows the relation between gelation time and pH of samples prepared with acetic acid and propionic acid. The pH had a significant influence on the gelation times, for both acids, with gelation occurring faster as the pH increases within the analyzed range.

In terms of mechanical properties, Figure 24 shows the evolution of viscoelastic moduli as a function of ω for acetic and propionic acid-induced gels. Although these gels could be considered weak due to the proximity of the viscoelastic moduli values and their dependency on ω, increasing pH leads to an increasing gel strength.

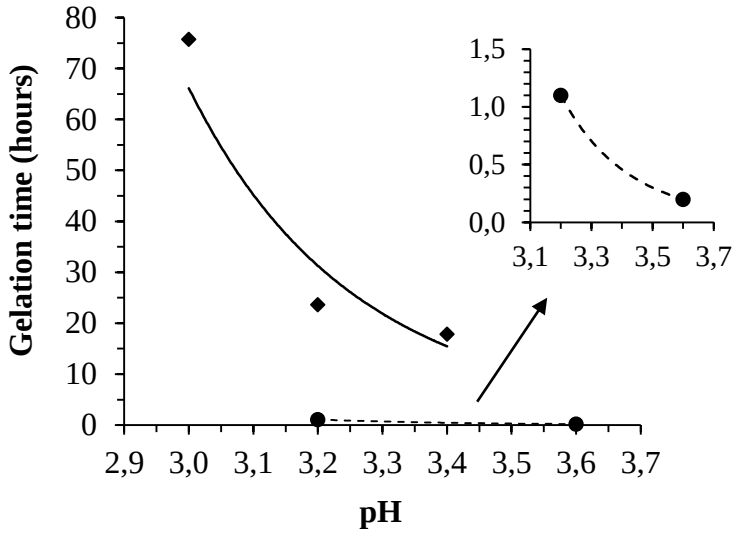

Figure 23. The effect of pH on gelation kinetics of WPI, induced by (◆) acetic and (●) propionic acid (enlarged). Measurements were performed at 25 ºC and at an angular frequency of 2 rad s^{-1} and 1% strain. Samples were prepared with 12 % (w/V) WPI added with 0.9 mol L^{-1} acetic acid at pH ranging between 3.0 and 3.4, adjusted with HCl. The gelation time was defined as the critical time corresponding to the G'-G" crossover.

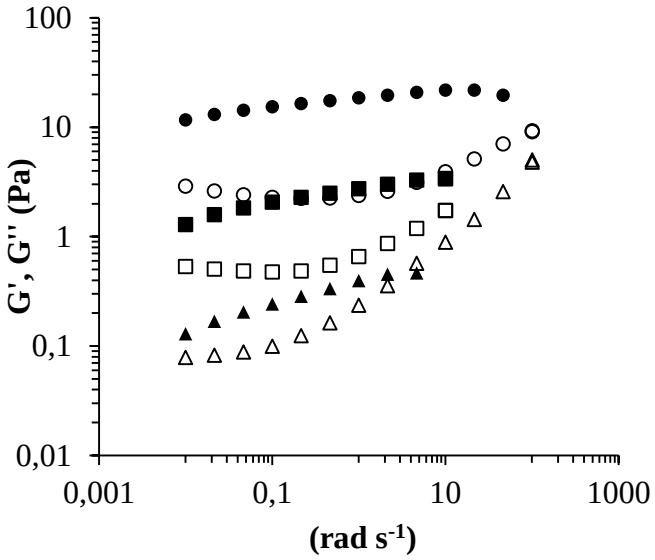

Figure 24. The effect of protein concentration on the mechanical characteristics of WPI gels, at 25 ºC, at 1% strain. Samples were prepared with 12 % (w/V) WPI added with 0.9 mol L^{-1} acetic acid at pH (●) 3.4; (■) 3.2 and (▲) 3.0, adjusted with HCl.

The acid-induced gelation mechanism is very dependent on the type of acid, but for each acid, increasing the pH towards the isoelectric point, i.e. the decrease of the charge density in the peptide chains and the decrease of electrostatic repulsions, also seems to favor the gelation process.

3.3. CALORIMETRIC ASSESSMENT

3.3.1. Influence of acid type

Thermal analysis was performed by differential scanning calorimetry (DSC) in order to understand the effect of acetic acid on WPI denaturation during acid-induced gelation. Figure 25 shows the effect of two different acids, HCl and acetic acid (AA), at pH 3.2, on the DSC thermograms obtained for 12% (w/V) WPI, comparing to the observed behavior in water (pH 6.9). Results showed distinct endothermic peaks and different enthalpy variations (ΔH). The WPI sample in water presented two peaks which corresponded to the denaturation endotherms of the main whey globular proteins, α-lactalbumin (α -LA) and β-lactoglobulin (β -LG) [47]. Therefore, the first peak, with a smaller amplitude, represents α-LA

denaturation, whereas the dominant endotherm corresponds to the denaturation of the most abundant WP, β-LG [53]. The temperature of the main peak was assessed (here defined as the denaturation temperature, T_d), as well as the value of denaturation enthalpy of both proteins (ΔH).

When HCl was added, both Td and ΔH increased from 78.4 ± 0.9 to 86.3 ± 0.4 ºC and from 10.4 ± 1.2 to 11.6 ± 0.2 J g^{-1}, respectively. This suggests that WPI thermal stabilization was promoted when the pH decreased (pH 3.2) below the pI, using HCl. Increased thermodynamic stability of WP in acidic conditions has been demonstrated [33,53,57]. For β-LG, this was explained by the establishment of additional hydrogen bonds or the loss of unfavorable electrostatic interactions [57]. At the same pH (3.2) but upon addition of acetic acid, the peak temperature (T_d) abruptly dropped to 58.5 ± 0.8 ºC, as well as ΔH, which decreased to 6.48 ± 0.03 J g^{-1}. These results clearly indicated that acetic acid was influencing and promoting changes in WP conformation, as less energy was needed to promote denaturation. As the only difference between the analyzed samples was the presence of a different acid, the observed effects might be related with acid structure. According to previous works [33,58], the observed differences between the effect of HCl and acetic acid on β-LG heat-set gels was explained by the different effects of their anions, chloride and acetate, respectively, in protein stabilization, as well as their concentration. For instance, due to the weak nature of acetic acid, acetate would be produced in much lower amounts when compared with chloride, which results from a total dissociation of HCl.

Basically, upon addition of an acid, as the pH lowers below the pI, the protein positive net charge increases, leading to increased intramolecular repulsion between charged groups and weakened van der Waals interactions, causing the protein to unfold [57,58]. Studies on acid-induced folding of other proteins, including apomyoglobin, [58,59] hypothesized that as anions (from the acids) are being introduced, electrostatic interactions are favored between these species and the positively charged groups of the proteins. This would cause a decrease in the internal repulsive forces, leading to the establishment of hydrophobic linkages and protein folding, to a certain extent. This conformational transition would be mainly dependent on the kosmotropic or chaotropic nature of the anion, as the previous was found to be more effective in promoting protein folding than the latter. Acetate is known to be a kosmotropic species, hence it is easily hydrated and have a tendency to react more strongly with water. Moreover, because it enhances hydrophobic bonding and

decreases the solubility of non-polar molecules, it promotes protein hydration and folding to a greater extent than chloride. By reacting less strongly with water, chloride, which is known to have a neutral or chaotropic effect, would promote interactions between chaotropes and the protein, causing it to unfold so as to maximize their surface area [33].

Simultaneously, macroscopic observations of these samples at 24 hours indicated that only the sample containing acetic acid gelled. According to some studies on thermal gelation of WP, the presence of HCl at pH 3 did not impair gelation of these proteins [15,33]. However, the same study that reported acetic-acid induced gelation of shark myofibrils at room temperature, also observed no gel formation in the presence of HCl [37]. A possible explanation for the fact that the sample containing HCl did not form a gel might be related to the increased thermal stability of WP at low pH upon HCl addition. In this case, higher amounts of energy in the form of heat would be needed in order to promote conformational changes in the protein, leading to gelation.

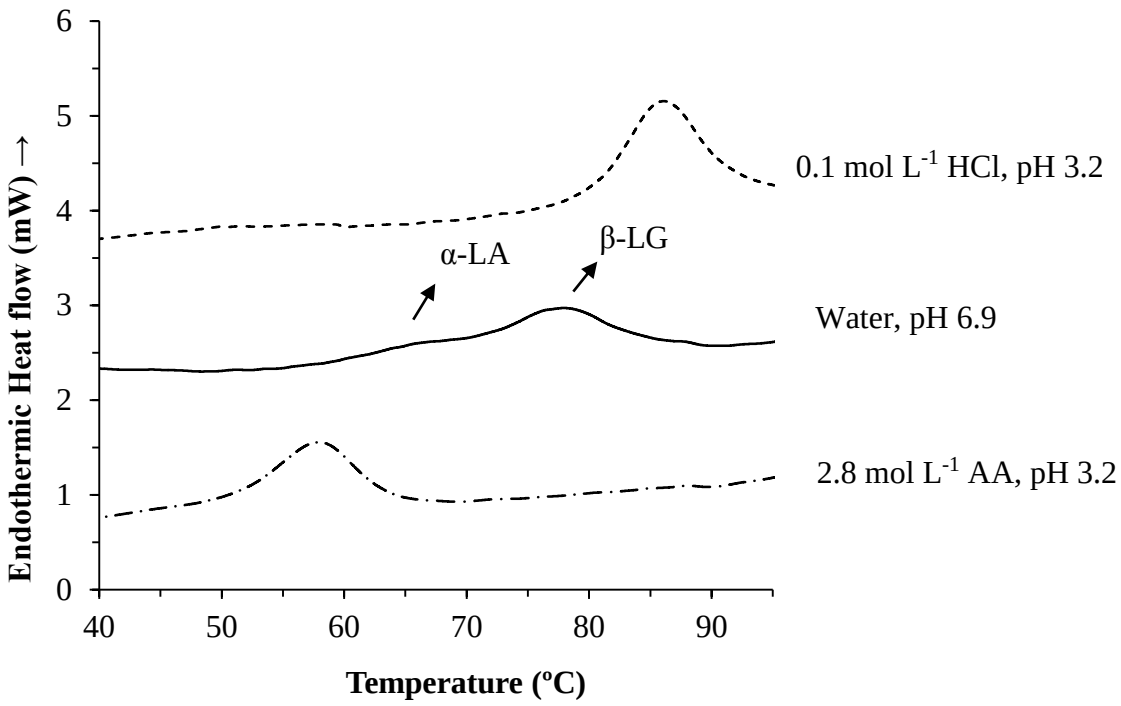

Figure 25. Representative DSC thermograms obtained at a heating rate of 10 ºC min^{-1}. (——) 12 % (w/V) WPI in water, at pH 6.9; (------)12 % (w/V) WPI added with 0.1 mol L^{-1} HCl, at pH 3.2 and (– – –) 12 % (w/V) WPI added with 2.8 mol L^{-1} acetic acid (AA), at pH 3.2.

In order to obtain more information regarding the denaturation process of WPI in the presence of acetic acid, a second scan of the pans containing the reference sample and the sample containing acetic acid was performed. No curve was detected for the reference sample, conversely to the sample containing acetic acid whose representative thermogram showed a Td of 60.2 ºC with an enthalpy of 0.82 J g^{-1}. The obtained temperature peak was close to that of the first scan of the sample (58.5 ± 0.8 ºC). These results indicated that during the first scan WPI in water suffered a complete denaturation, whereas WPI added with acetic acid suffered only a partial denaturation. This suggests that acetic acid might be promoting the establishment of stronger bonds between molecules and/or inducing conformational changes that require more energy to be disrupted. As the first denaturation was not complete, maybe during cooling new intramolecular linkages were established, which could also explain these results.

3.3.2. Influence of acid concentration

In order to assess the influence of acid concentration and pH on the initial step of WPI gelation, samples containing 12 % (w/V) WPI and acetic acid or HCl in different concentrations were analyzed. Table 5 shows the transition enthalpy and peak temperature (denaturation temperature, T_d) for each sample containing acetic acid and a reference sample in water with no added acid, immediately after preparation and after 24 hours. DSC curves of the samples are also depicted in Figure 26 to facilitate the comparison of the results. First, it was possible to observe that increasing concentrations of acid which inevitably resulted in the lowering of pH led to a progressive decrease in the T_d of WPI. Interestingly, these results are in accordance to those obtained for α-LA [53], whose T_d decreased with decreasing pH; but are the complete opposite of what was demonstrated for β-LG and for WPC [43,57]. Surprisingly, the sample which contained the least amount of acetic acid (0.5 mol L^{-1}) was the most thermally stable, even when compared with the reference, and did not form a gel. In this case, the increased thermal stability of the sample could be related to the fact that its pH (4.0) is very close to the pI (4.5). A study on β-LG explained the increase in the thermal stability of the protein near the pI to be due to a reduction in the electrostatic repulsions which would facilitate a closer packing of groups in the interior of the protein, leading to increased hydrophobic interactions and van der Waals attractions [57]. As a consequence of a more stable protein, no gel was formed at ambient temperature after 24 hours. In terms of

ΔH, it was difficult to establish a tendency due to the high standard deviation values. For the same reason, it was not possible to infer whether the values after 24 hours differed meaningfully from those obtained after sample preparation. In order to obtain more precise information, a higher number of replicates would be needed.

Table 5. Comparison between the denaturation temperatures (Td) and enthalpy values of 12 % (w/V) WPI samples containing different concentrations of acetic acid, immediately after preparation ($Td_0/\Delta H_0$) and after 24 hours ($Td_{24}/\Delta H_{24}$).

Acetic Acid concentration (mol L^{-1})	**pH**	**Td_0 (ºC)**	**ΔH_0 (J g^{-1})**	**Td_{24} (ºC)**	**ΔH_{24} (J g^{-1})**	**Observations (24h)**
0	**6.9**	78.4 ± 0.9	10.4 ± 1.2	ND	ND	transparent sol
0.5	**4.0**	85.9 ± 0.9	8.0 ± 1.1	86.7 ± 1.0	11.0 ± 1.0	opaque sol
1.2	**3.6**	79.8 ± 0.4	9.15 ± 0.07	80.4 ± 1.5	9.5 ± 0.2	translucent gel
2.8	**3.2**	58.5 ± 0.8	6.48 ± 0.03	59.4 ± 2.4	5.0 ± 0.4	translucent gel

ND – Not determined

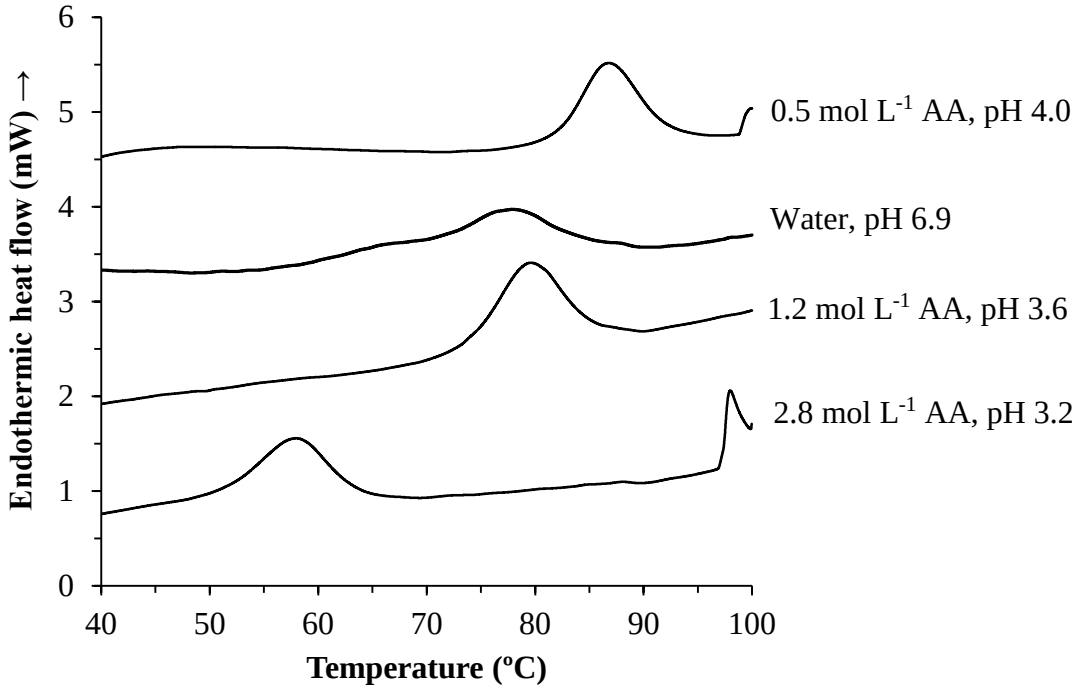

Figure 26. Representative DSC thermograms obtained at a heating rate of 10 ºC min^{-1} for samples containing 12 % (w/V) WPI in water (reference sample) and added with acetic acid (AA) in different concentrations shown in the figure (0.5; 1.2; 2.8 mol L^{-1}). The samples were analyzed immediately after sample preparation.

The influence of HCl concentration and pH on the thermal behavior of WP dispersions was also assessed. Figure 27 shows the DSC scans of the analyzed samples. Two samples were added with 0.13 and 0.07 mol L^{-1} HCl, to obtain samples with a final pH of 3.2 and 4.0, respectively. Increasing concentrations of HCl and consequent pH drop led to a decrease in the denaturation temperatures relatively to the reference sample with no added acid (Table 6). As both samples containing HCl presented a T_d higher than that of the reference and no gel formation was observed, it was deduced that below the pI, increasing values of pH lead to an increase in the thermodynamic stability of WP and consequently no gel was formed. A similar effect on thermal stability upon pH lowering was observed for β-LG [57]. The fact that in the presence of HCl the thermal behavior of WP was similar to that of β-LG and in the presence of acetic acid, WP tended to behave more like α-LA suggests that the relative composition of the WP samples, β-LG/ α-LA ratio, might play an important role in acetic acid-induced gelation of WPI. After 24 hours, new DSC scans were performed and the results were inconclusive, as there was no consistency between the obtained data for each sample pair.

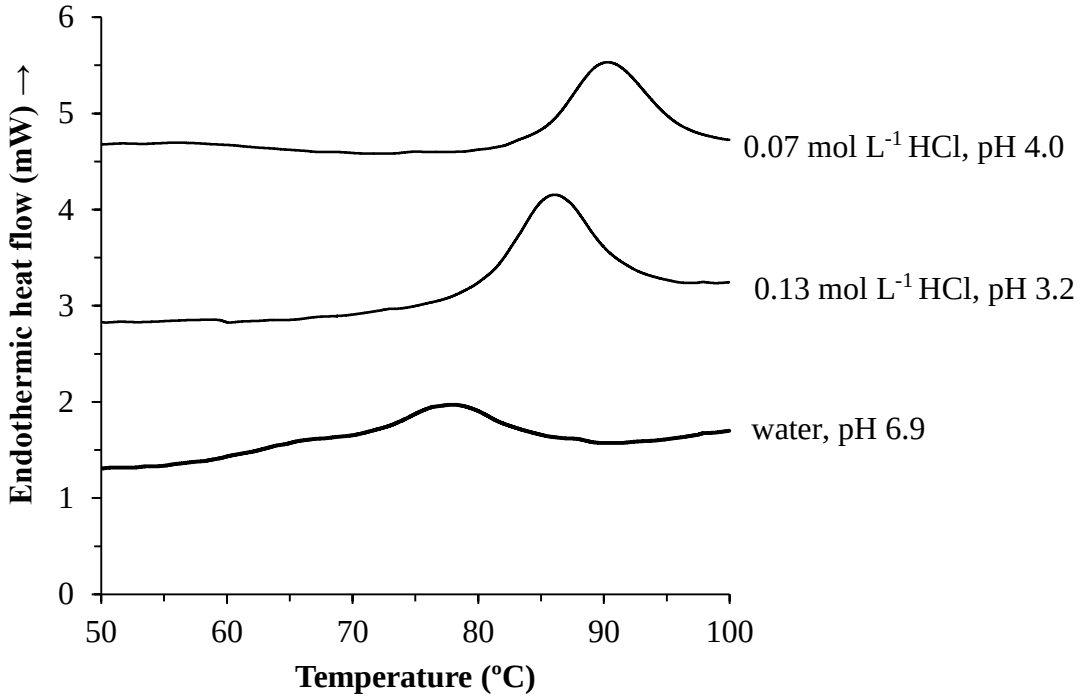

Figure 27. Representative DSC thermograms obtained at a heating rate of 10 °C min^{-1} for samples containing 12 % (w/V) WPI in water (reference sample) and added with HCl in different concentrations shown in the figure (0.07 and 0.13 mol L^{-1}). The samples were analyzed immediately after sample preparation.

Table 6. Comparison between the denaturation temperatures (Td) and enthalpy values and of 12 % (w/V) WPI samples containing different concentrations of HCl, immediately after preparation ($Td_0/\Delta H_0$) and after 24 hours ($Td_{24}/\Delta H_{24}$).

HCl concentration (mol L^{-1})	pH	Td_0 (ºC)	ΔH_0 (J g^{-1})	Td_{24} (ºC)	ΔH_{24} (J g^{-1})	Observations (24h)
0	**6.9**	78.4 ± 0.9	10.4 ± 1.2	ND	ND	transparent sol
0.07	**4.0**	90.3	9.4	90.0	9.47	opaque sol
0.13	**3.2**	86.3 ± 0.4	11.6 ± 0.2	86.5	10.4	transparent sol

ND – Not determined

In order to observe the effect of acetic acid concentration at pH 3.2, samples containing 12 % (w/V) WPI and two distinct concentrations of acetic acid, 1.1 and 2.8 mol L^{-1}, were prepared and analyzed by DSC immediately after preparation and 24 hours later. The first sample was added with HCl to have the pH adjusted, whereas the same was not necessary in the latter. The obtained results are illustrated in Figure 28 and were compared with the behavior of 12% (w/V) WPI in water. Both Td and ΔH of the analyzed samples are shown in Table 7.

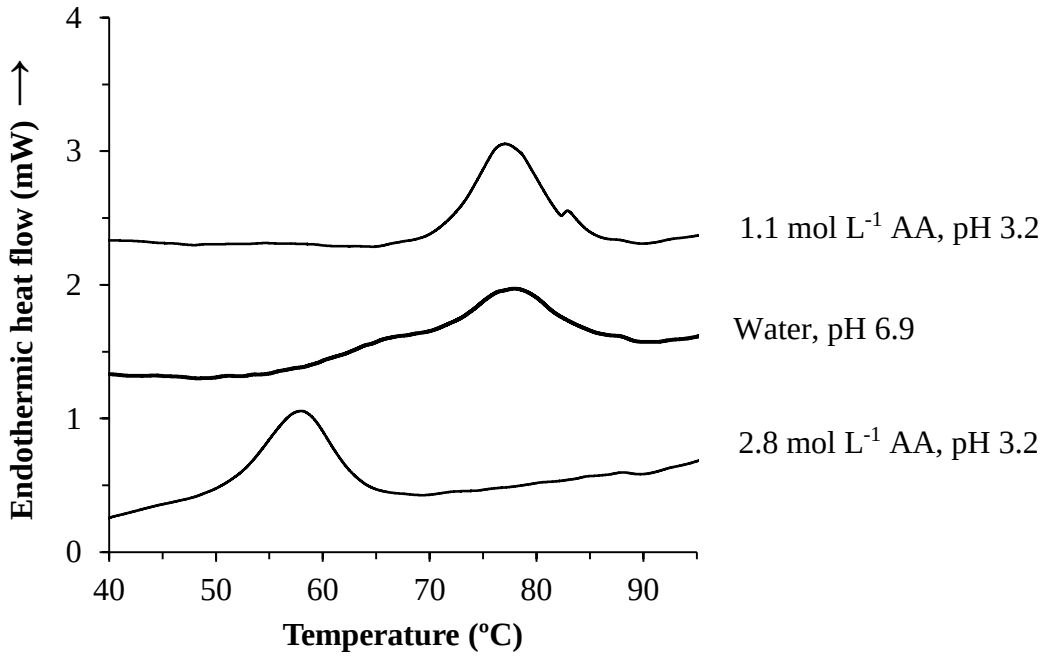

Figure 28. Representative DSC thermograms obtained at a heating rate of 10 ºC min^{-1} of samples containing 12 % (w/V) WPI in water at pH 6.9 and 12 % (w/V) WPI added with 1.1 or 2.8 mol L^{-1} acetic acid (AA) at pH 3.2, adjusted with 1.0 and 0.1 M HCl, when required. The samples were analyzed immediately after preparation.

Table 7. Comparison between the denaturation temperatures (Td) and enthalpy values and of 12 % (w/V) WPI samples containing different concentrations of acetic acid at pH 3.2, immediately after preparation ($Td_0/\Delta H_0$) and after 24 hours ($Td_{24}/\Delta H_{24}$).

Acetic acid concentration (mol L^{-1})	**pH**	**Td_0 (ºC)**	**ΔH_0 (J g^{-1})**	**Td_{24} (ºC)**	**ΔH_{24} (J g^{-1})**	**Observations (24h)**
0	6.9	78.4 ± 0.9	10.4 ± 1.2	ND	ND	transparent sol
1.1	3.2	77.1	9.7	77.7	9.6	Transition sol-gel
2.8	3.2	58.5 ± 0.8	6.48 ± 0.03	59.4 ± 2.4	5.0 ± 0.4	translucent gel

ND – Not determined

It was demonstrated that increasing concentrations of acetic acid cause a decrease in the protein thermal stability, possibly due to the presence of a higher concentration of acetate. The results obtained for the sample containing the lowest acetic acid concentration might seem surprising, as they were very close to those of the sample in water. However, it is important to note that the concentration of acetate is quite low, so the effects of this anion on protein thermal stability might not be as noticeable as in the sample containing the highest concentration of acetic acid. Despite the similarities in Td and ΔH between the reference sample and the sample containing 1.1 mol L^{-1} acetic acid, gel formation only occurred in the sample containing the acid, as expected. The sample containing the highest acetic acid concentration (2.8 mol L^{-1}) also gelled after 24 hours and the obtained gel was more rigid than that of the previous sample. These observations are in agreement to the results obtained previously, as increasing acid concentrations lead to the formation of stronger gels.

3.3.3. The involvement of α-lactalbumin and β-lactoglobulin

Samples of α-LA and β-LG were also analyzed by DSC so as to obtain a deeper insight regarding acid-induced gelation of WP. First, samples containing 12% (w/V) α-LA and β-LG were compared with a dispersion of WPI at an identical concentration (Figure 29). The results show that the Td of α-LA (66.7 ºC) and β-LG (78.4 ºC) coincided with the curves which corresponded to these proteins in WPI, 66.4 ± 1.1 and 78.4 ± 0.9 ºC, respectively. Then, β-LG and α-LA samples were added with 2.8 mol L^{-1} acetic acid, in order to obtain a final pH of 3.20 .The obtained DSC curves were then compared (Figure 30). Unexpectedly, no curve was observed for α-LA, whereas the peak observed regarding β-LG (60.0 ºC) was

very close the one of WPI sample (58.5 ± 0.8 ºC). In terms of ΔH, denaturation of WPI was shown to require less energy (6.48 ± 0.03 J g^{-1}), comparing to β-LG (7.02 J g^{-1}). These results indicated that α-LA suffered a complete denaturation upon addition of acetic acid at room temperature, conversely to β-LG. In order to form gels, proteins need to suffer denaturation so that aggregation can take place [14]. Therefore, α-LA denaturation previous to thermal treatment confirms this protein fraction as the main responsible for WPI acetic acid-induced gelation. These findings were quite surprising as, according to the literature, α-LA has a poorer gelling capacity when compared with β-LG [60].

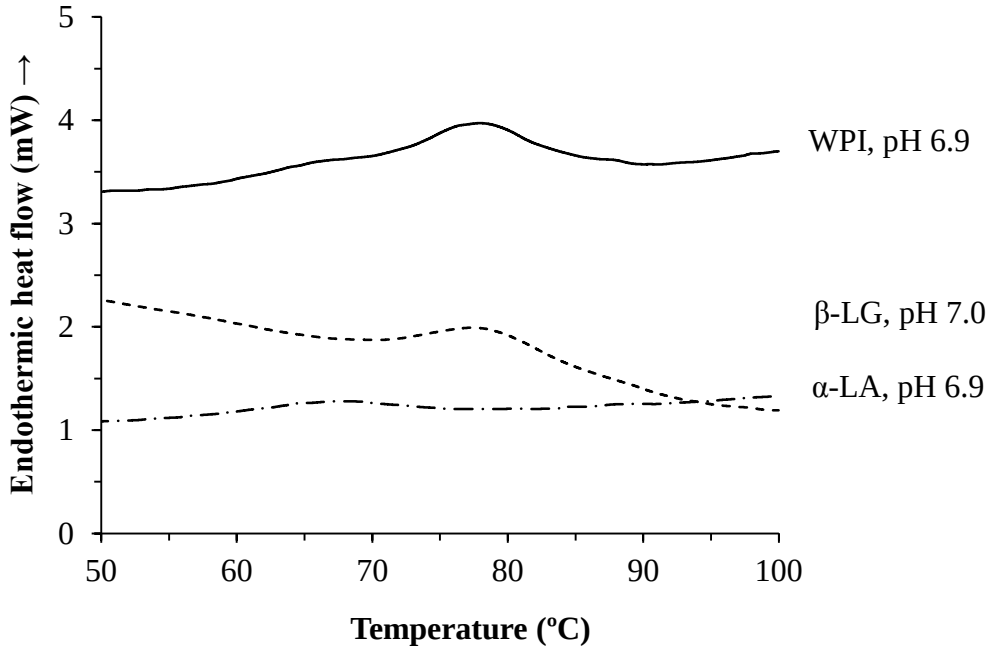

Figure 29. Representative DSC thermograms obtained at a heating rate of 10 ºC min^{-1} of samples containing 12 % (w/V) WPI (___), α-LA (_ _ _) and β-LG (- - - -) in water. The samples were analyzed immediately after sample preparation.

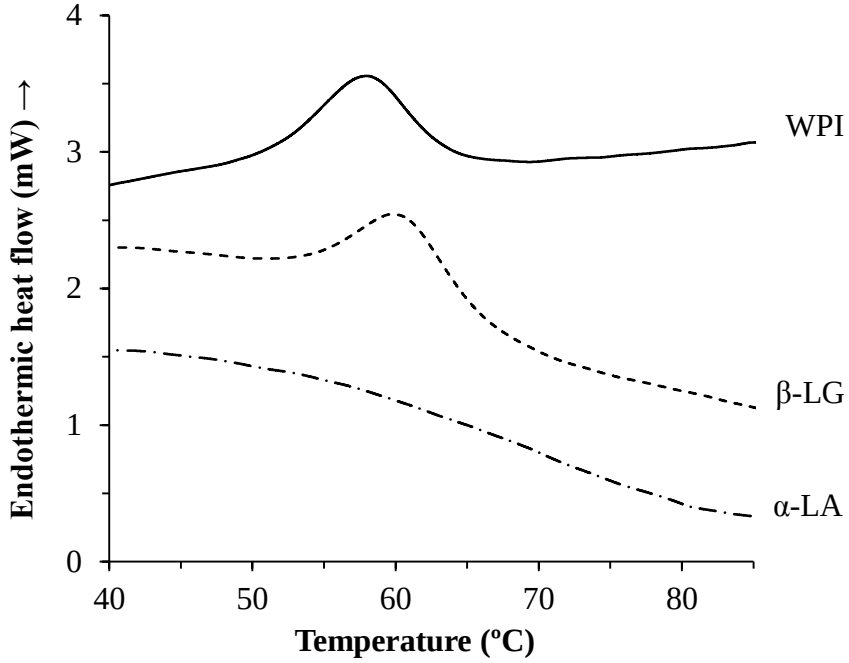

Figure 30. Representative DSC thermograms obtained at a heating rate of 10 ºC min^{-1} for samples containing 12 % (w/V) WPI (——), α-LA (– . – .) and β-LG (- - - - -) added with 2.8 mol L^{-1} acetic acid, at pH 3.2. The samples were analyzed immediately after sample preparation.

4. CONCLUDING REMARKS AND PERSPECTIVES FOR FUTURE WORK

All three acids used in this work - formic, acetic and propionic - promoted WP gelation, at room temperature, possibly due to the presence of the corresponding anion. This anion was shown to decrease WP thermal stability and because of its kosmotropic nature, it might had reacted with the solvent, water, inducing conformational changes in WP, which might have included their partial unfolding and aggregation. Less specific interactions cannot be discarded, including those of hydrophobic nature which probably also play an important role. Propionic acid, however, produced gels with the lowest gelation times, followed by acetic and formic acids. It was hypothesized this would occur due to the increase in the length of the hydrocarbon chain of the acid, what supports the hypothesis of the important role of hydrophobic interactions,. Independently of the acid, increasing acid and protein concentrations, as well as higher pH values (within the range below pI) were observed to be correlated with diminished gelation times. Gel appearance and viscoelastic behavior were also found to vary depending on the previous variables. Therefore, in the presence of conditions that favored faster sol-gel transitions of WP, gel appearance shifted from translucent to opaque with signs of syneresis, suggesting these gels might have a particulate network structure. The viscoelastic behavior of these gels also showed higher values of G′, which were demonstrated to be independent of frequency, as well as progressively decreasing values of G″. Lastly, α-LA and β-LG were both shown to gel in the presence of acetic acid, yielding clear and opaque gels, respectively. Surprisingly, α-LA was shown to suffer a total denaturation in the presence of acetic acid, unlike β-LG. Although the influence of both formic and propionic acids was not tested for the isolated fractions of the main globular WP, it was inferred the same would happen in the presence of these acids, as all of them were shown to lead to WP gelation. Therefore it could be concluded that the α-LA fraction was very likely the main responsible for WP acid-induced gelation at ambient temperature.

In terms of future work, as this is a novel topic, there are many features which would be interesting to explore. In order to have a better understanding of the process, more DSC experiments could be performed using a wider number of replicates, and the effect of each acid on WP and on α-LA and β-LG, separately could also be assessed; other organic acids could also be tested in order to evaluate the effect of the respective anions or of their own

hydrocarbon chain on WP gelation. Spectroscopic methods could also be used to determine the conformational changes that occur in the different structures of WP upon the addition of weak organic acids. The use of some microscopic techniques could also be useful in the determination of the microstructure of these gels.

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