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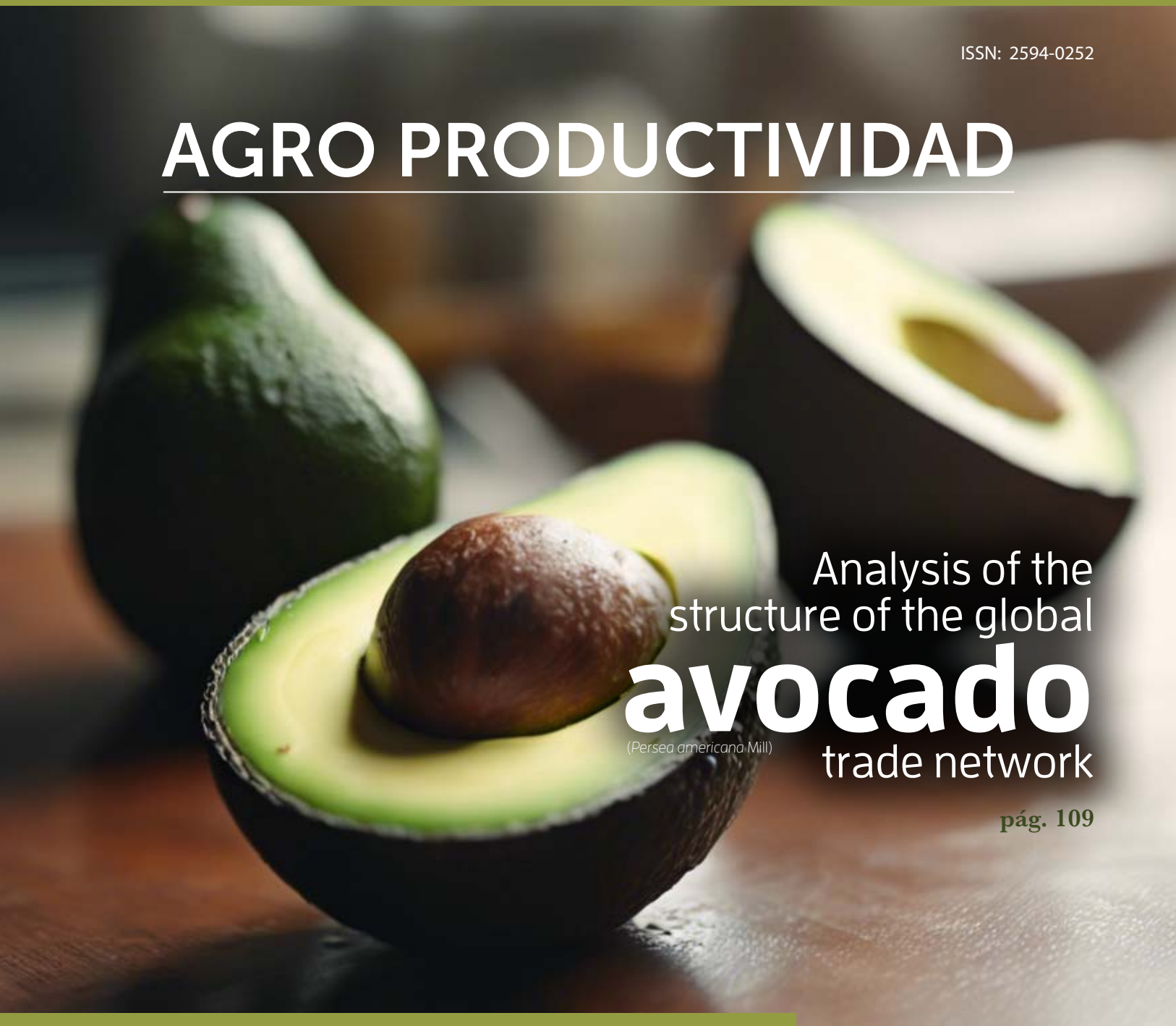

Analysis of the structure of the global avocado (*Persea americana* Mill) trade network

pág. 109

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Evaluation of the influence of ph modification on food proteins structure by FT-IR AND AFM

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ABSTRACT

Objective: The related research of proteins is important due to their wide applications in food science. The aim of this study was to evaluate the influence of pH variation (3.6, 4.6 and 5.6) on morphometric parameters and the secondary structure of proteins (ovalbumin and gliadin). The arithmetic mean roughness (Ra) and agglomerate size (AS) of the proteins were analyzed by atomic force microscopy (AFM), while their secondary structure was analyzed.

Design/methodology/approach: by Fourier transform infrared spectroscopy (FT-IR), both at different pH. Subsequently, a correlation analysis of the morphometric changes of the proteins with their secondary structure was performed.

Results: Highlighting that it was found that, protein agglomerate size is influenced by changes in β-sheets and turn conformations.

Findings/conclusions: The novelty of this contribution consists in demonstrating that there is a close structure-functionality relationship between the morphometric parameters of proteins and their secondary structure, combining microscopy and spectroscopy techniques. This allows a clear and deep understanding of protein behavior to select the appropriate pH conditions to improve the properties of many foods.

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INTRODUCTION

The development of research related to proteins in general plays a fundamental role not only because they are essential for human growth, but also because proteins provide the structural basis for several functional properties of foods that have a profound impact on food quality. They commonly serve as gelling, binding, emulsifying and foaming agents, which are functions that modify processing and behavior in food systems (Yada, 2007).

Although it is important to consider that some protein properties are affected by various factors, such as pH, protein concentration and ionic strength (Chang *et al.*, 2016; Mine *et al.*, 1991). An important protein to study is ovalbumin, the most abundant protein in egg white is useful for its good functionalities, including gelling properties and foaming activities. It is a glycoprotein consisting of 385 amino acids (mostly of which are hydrophobic). Due to its surface hydrophilic nature, it is not a good emulsifier in its raw state (Mine, 1995; Mleko *et al.*, 2007). But the emulsion stability and emulsifying activity of this protein are considerably lower than those of β-lactoglobulin, bovine serum albumin, κ-casein, soybean β-conglycinin and blood globin (Kato *et al.*, 1983; Nakamura *et al.*, 1984). However, the pH-dependent structural changes of ovalbumin lack characterization. Although, it is known that the structural state of ovalbumin is highly sensitive to pH variation because of surface charge alteration (Chen *et al.*, 2018).

Likewise, another protein of importance for its study is gliadin, which is the major storage protein in the byproducts of wheat starch. Wheat gliadins are monomeric, disulfide-bonded proteins, which are abundant in α-helices and β-turns (Tatham & Shewry, 2012). Besides, it should be noted that the peculiar sequence (abundant in prolines and glutamines) and structure of gliadin has already been proven to be responsible for severe anaphylaxis such as celiac disease and food allergy (Kim *et al.*, 2004). Moreover, the gliadin protein has the property of generating foam such as ovalbumin but also has viscoelastic and mechanical properties that are implemented in bread that provide the extensibility to stretch the bread dough without tearing (Quester *et al.*, 2014; Thewissen *et al.*, 2011). But, compared to other proteins, some properties of gliadin are still poorly explored (Wan *et al.*, 2015).

In addition, non-destructive techniques, such as molecular spectroscopy, are available to determine the behavior of proteins at the molecular scale. Different spectroscopy techniques (X-ray diffraction, nuclear magnetic resonance, Fourier transform infrared (FT-IR) and Raman spectroscopy) have been widely used for decades to elucidate the molecular structure of different types of proteins (Horne, 2002; Wang *et al.*, 2017). As a result of the development of high analytical capacity and the ease of acquisition of spectroscopy equipment, its applications have increased dramatically within the food industry. To evaluate the conformational changes of proteins produced by their processing and pH variations, which has been useful to understand their structure-functionality relationship. It should also be noted that FT-IR spectroscopy has been suitable for assessing conformational modifications of secondary and tertiary structures of food-derived proteins (Carbonaro & Nucara, 2010). On the other hand, atomic force microscopy (AFM) has been used to determine protein topography, as reported by McMaster *et al.* (1999), who have shown that the dimensions of A-gliadin fibers can change at different pH values and concluded that fiber shape and size depend on pH. Furthermore, Vié *et al.* (2002) demonstrated by AFM that the diameter of casein micelles is reduced due to their hydrophilic and lipophilic properties. Likewise, Grácia-Juliá *et al.* (2008) studied the dispersion at high pressures of whey protein and reported that the size distributions at 200 MPa ranged between 5 and 8 nm, while at >250 MPa between 60-170 nm and concluded that at pressures >250 MPa the protein agglomerates. Therefore, to have a better understanding of the influence of pH on protein structure and the development of proteins as multifunctional components

for the food industry, the conformational and morphometric changes produced in ovalbumin and gliadin were evaluated in the present study. With the main objective of unveiling the importance of pH-dependent structural features for their properties using AFM microscopy and FT-IR spectroscopy as tools to deepen the analysis of this type of biomolecules, because there is a certain complexity of such systems in terms of composition and spatial organization.

MATERIALS AND METHODS

Samples

Ovalbumin was acquired by the supplier Hycel (Cat. 568, México) and Gliadin was acquired by Sigma-Aldrich as wheat gliadin (G3375-25G, USA). Gliadin and ovalbumin were used 1% w/v dissolved in MiliQ water; pH adjusted to corresponding using 0.1 M HCl at room temperature (25 °C). The pH values for proteins studied were 3.6, 4.6 and 5.6. The solutions were mixed by a sonicator (VCX130, SONICS Vibra cell™, 90 Newton CT, USA) with 50% of amplitude at 20 kHz and 130 W with a 120 V generator (CV13) and was used a standard probe of the sonicator (length of 113 mm and diameter of 6 mm).

Atomic force microscopy

Proteins were observed by AFM (Bruker, Bioscope Catalyst ScanAsyst, USA). AFM images of gliadin and ovalbumin were acquired at different pH values (3.6, 4.6 and 5.6 pH). Samples were placed on a glass slide, dried at room temperature for 10 min and mounted on to equipment. ScanAsyst mode was used, $5 \times 5\ \mu m^2$ scans were performed and 3 images of each sample were selected from the different pH values. The study was carried out at ambient conditions and the cantilevers used in this study were silicon cantilevers (DNP-10A) with a spring constant of 0.540 Nm^{-1} and a resonance frequency of 1 kHz. For image processing the software NanoScope Analysis v2.0 (Bruker Nano, Santa Barbara, CA, USA) was used.

Fourier transformed infrared spectroscopy

The analysis of the secondary structure of the proteins was carried out by FT-IR spectroscopy (Agilent Cary model 630, USA). A small amount of each sample was placed on the attenuated total reflectance (ATR) diamond crystal of the analyzer. The samples were pressed against the diamond crystal using the attached pressure clamp with a slip clutch press on the clamp that prevents over-tightening. It was operated in the 1000 to 1800 cm^{-1} wavenumber ranges and 64 scans at 4 cm^{-1} of resolution at room temperature (25 °C). For evaluation, a necessary baseline and smoothing correction was performed on the spectrum using OriginPro 8 software (v8.0724, USA). Finally, the deconvolution method was used to evaluate the areas of the regions of interest.

Statistical analysis

Measurements were expressed as mean values with a precision of standard deviation. The data obtained by the previous analyses were compared using the ANOVA-Tukey test, and significant differences were considered significant when $p<0.05$. Both statistical

analyses were performed using SigmaPlot v.12 software (Systat Software Inc. USA). In addition, using XLSTAT software (2020.1.3, Addinsoft, USA) a Pearson analysis was performed on all acquired variables and a visualized correlation matrix was produced.

RESULTS AND DISCUSSION

Analysis of morphometric parameters by AFM

Figure 1 shows a selection of AFM height images for protein solution at different pH values: 3.6, 4.6 (close to its isolelectric point) and 5.6. These pH values considered are within the range used in food systems (Mleko *et al.*, 2007; Thewissen *et al.*, 2011). Figures 1(a-c) correspond to AFM height images of ovalbumin, where it is noted that larger agglomerates are formed at pH 4.6 compared to pH 3.6 and 5.6. This is considered to be because the protein is quite close to its isoelectric point and therefore most likely has minimal solubility and possesses a neutral charge. While at pH 3.6 and 5.6, although they also present less agglomeration, at these pH values they are mostly soluble, and their surface is more uniform. The agglomeration size (AS) in a certain way presents relationship, as shown also in Figures 1(a-c), with the average roughness values, where the Ra values are higher at the same pH of 4.6 (4.05±2.60 nm), due to the increase of agglomeration. Since, as observed the Ra at pH 3.6 was 3.40±0.54 nm and that at pH 5.6 was 2.59±0.84 nm. Which suggests that the protein is immobilized with a slight net positive charge (Lahiri *et al.*, 1999).

Mine, (1996) reported the particle size of ovalbumin with a mean of 317.3 nm to 752.6 nm due to using pH 7.5. And in a different study of the particle size of the mixture of proteins including ovalbumin, finding Ra values of 70-295 nm because the treatment was at pH 3 and with thermosonication. Previous research reported the globular structure of ovalbumin and the size of about 24-25 nm because the protein was subjected to heat

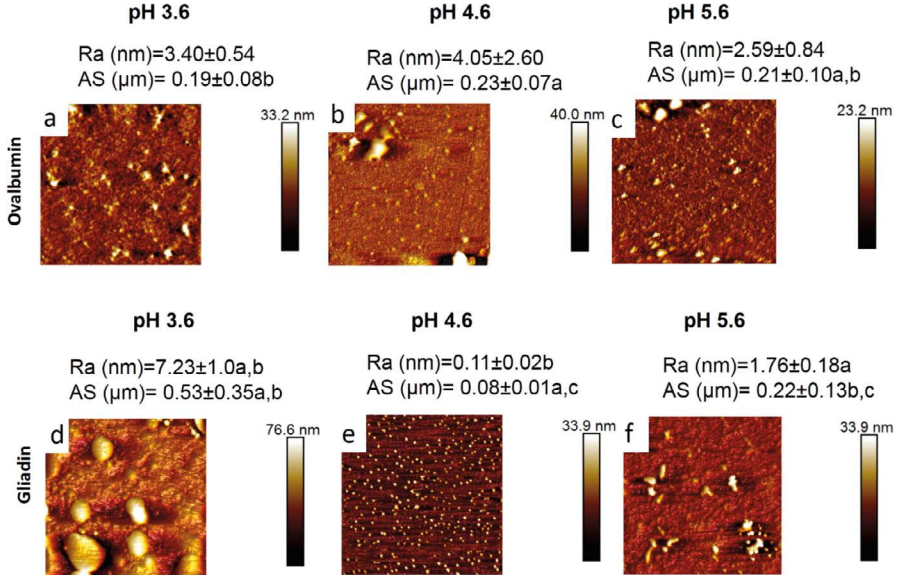

Figure 1.

treatment (Najbar *et al.*, 2003). Another study likewise confirmed the shape of the globular structure of ovalbumin and the size of 78-143 nm (Taheri *et al.*, 2012).

For gliadin, the same pH values were used and in the AFM height images shown in Figures 1(d-f) it is observed that the largest Ra values were at pH 3.6, since this is where larger and heterogeneous agglomerates are present compared to pH 4.6 and 5.6. When the protein is close to the isoelectric point (pH 4.6) it has homogeneous agglomerates of 0.08 ± 0.01 μm and larger agglomerates than at pH 3.6 and 5.6 due to protein precipitation. The Ra values vary as a function of the number of gliadin agglomerates that are not dissolved in solution. This is consistent with the characteristic size found for each pH condition. Therefore, these results show, that the size of the protein as well as the topographical characteristics depend on the specific pH value used for its precipitation and affect the different properties for food use (Moitzi *et al.*, 2011).

Research has reported average 100 nm sphenoidal structures of gliadin mixed at 70% aqueous ethanol by AFM analysis (Terence J. McMaster *et al.*, 1999). In addition, work found gliadin aggregates in egg cake substitution around 100-200 nm (Lin *et al.*, 2017). While McMaster *et al.* (2000) found α-gliadin fibrils at pH 3 with a width of 15 to 80 mm and a length of 100 nm to 2 μm. Since the agglomerate size observed in the present work is similar to that reported by previous literature, it is likely that the change in morphology of ovalbumin and gliadin as size and shape at different pH values is associated with reassembly at the molecular level.

Analysis of secondary structure by FT-IR

Proteins dissolved at different pH values exhibit modifications in morphology and solubility properties through protein rearrangement at the molecular level, but their correlation remains unknown. Consequently, the secondary structure of proteins was studied to associate these behaviors with their morphometry of the protein evaluated at different pH values. Figure 2 shows the FT-IR spectra with the characteristic bands of the protein. There is information concerning amide vibrational bands and stretching, bending and other values. The first band observed in the spectrum of ovalbumin is due to stretching of the C-C and C-O bonds (1100 cm^{-1}).

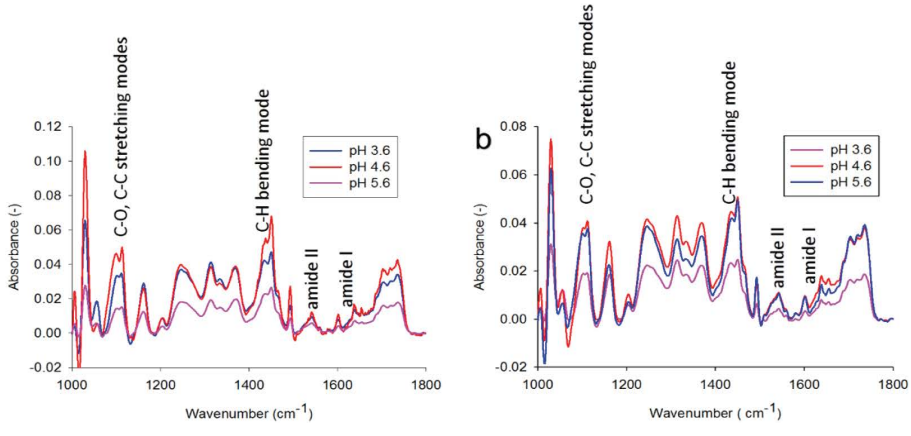

Figure 2.

The corresponding intensity is higher at pH 4.6 compared to those at pH 3.6 and 5.6. (Carbonaro & Nucara, 2010). A similar behavior had to C-H bending (1420 cm^{-1}) the lowest intensity found at pH 5.6 following that at pH 3.6. The band of amide I of ovalbumin shows a reduction of the area under the curve when pH decreases (Figure 3).

Amide II has observed a high valley on the far right generated by all peaks. Table 1 shows that at pH 4.6, ovalbumin decreases the percentage of α-helix conformation and increases the β-antiparallel conformation compared to pH 3.6 and 5.6. It is concluded that at pH 4.6 ovalbumin unfolds due to decreased solubility and hydrophobic, covalently bound interactions (Wang *et al.*, 2017).

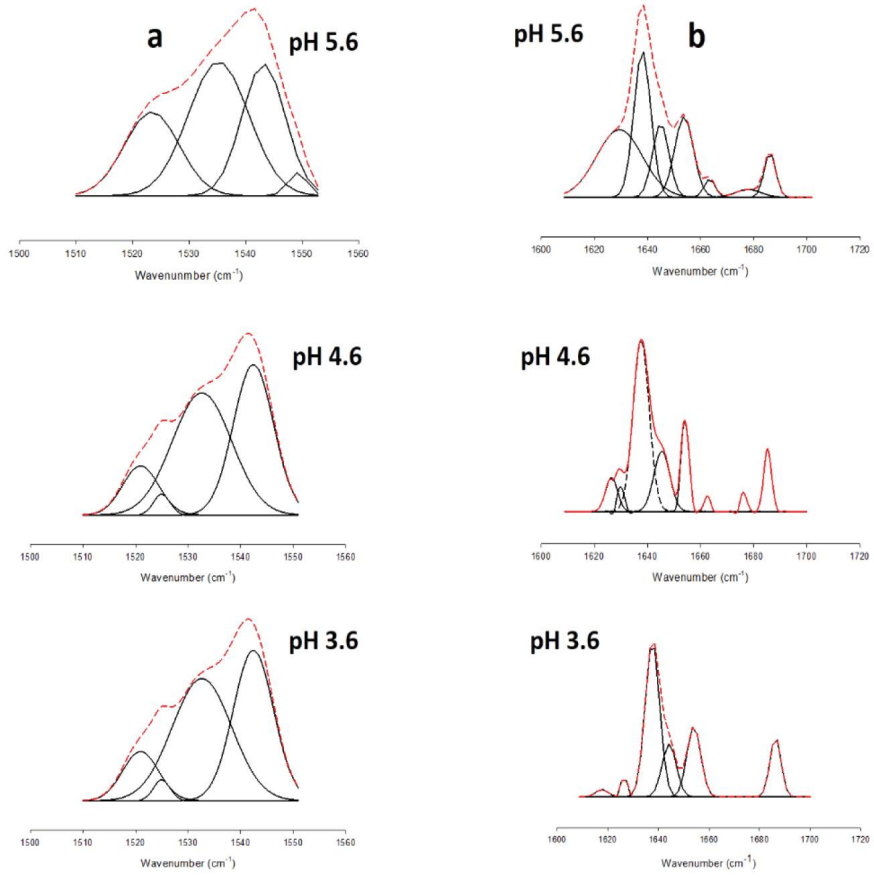

Figure 3.

Table 1. Determination of the secondary structure percentages by FT-IR of the ovalbumin solution (0.01%) at different pH values.

Protein structures	pH 3.6 (%)	pH 4.6 (%)	pH 5.6 (%)
β-Antiparallel	2.75	21.53	16.09
β-Parallel	17.26	45.46	50.14
Turn	0	3.65	4.30
Random coil	15.47	16.76	12.97
α-helix	20.21	12.57	16.48

The previously mentioned characteristics allow water retention and increase gelling properties (Li *et al.*, 2014). Due to the exposure hydrophobic groups and sulfhydryl groups (Campbell *et al.*, 2003). On the other hand, at pH 3.6, the results are different, they do not have a turning percentage but develop a higher content of the α-helix structure. This most probably means that the protein is folded. This most likely means that the protein is folded and disordered. At pH 5.6, the results are different; it develops a higher content of the β-parallel and twist structures. There is also a reduction of the α-helix conformational structure, so the protein was found to be unfolded. While in gliadin the bands show the lowest intensity for 3.6 pH compared to 4.6, and 5.6 pH these behaviors affect the bands C-H bending mode, C-O and C-C stretching mode. Gliadin had similar behavior in the amide I and amide II regions. Figure 4 shows that the amide I region at 4.6 pH and 3.6 pH, have similar behavior compared to pH 5.6 which has a heterogeneous distribution. In amide II a high valley was observed at the right end generated by all the peaks. At pH 4.6, ovalbumin increases the α-helix but decreases the β-parallel, since the protein was partially disordered and folded and when the protein is folded, it acquires energy transfer properties due to the closeness of protein-protein interaction (Chakraborty & Basak, 2007).

While the pH of 5.6 and 3.6 of gliadin (Table 2) decreases the α-helix and decreases the β-parallel conformations. The protein was partially disordered and unfolded.

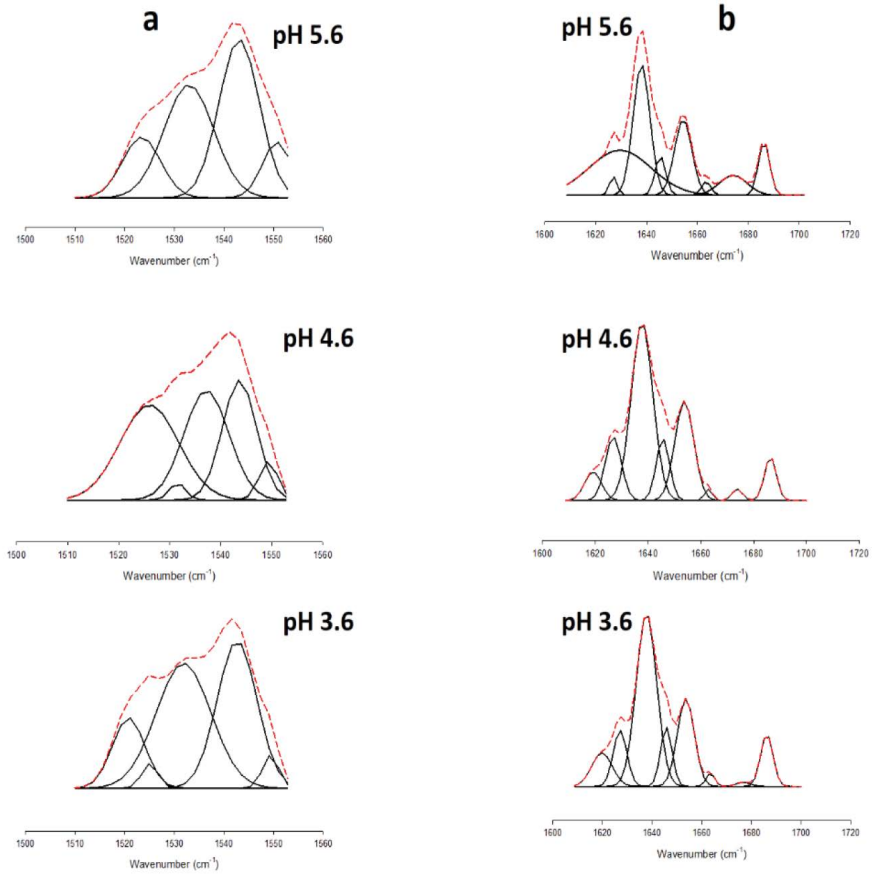

Figure 4.

Table 2. Determination of the secondary structure percentages by FT-IR of the gliadin solution (0.01%) at different pH values.

Protein structures	pH 3.6 (%)	pH 4.6 (%)	pH 5.6 (%)
β-Antiparallel	11.88	23.90	10.20
β-Parallel	58.65	42.87	60.31
Turn	2.20	2.43	8.34
Random coil	8.47	9.34	5.07
α-helix	18.78	21.43	16.05

Correlation analysis

Pearson's analysis provided a representation of the correlation of morphometric parameters with protein secondary structure. Figure 5 shows an image of a Pearson correlation matrix, where the range of its correlation coefficients is indicated. The intensity of the green and blue colorings shows which variables have the highest positive or negative correlations (green(α)=1 to 0.81, red (α)=−1 to −0.81) with F (dotted red line). Pearson matrix of ovalbumin observed positive correlation random coil conformation with roughness protein and agglomerate size with β-Antiparallel, β-Parallel and turn conformations. The negative correlation agglomerated protein with random coil and α-helix conformations. Roughness and agglomerate size also have negative correlation with α-helix.

The secondary structures of ovalbumin are correlated with each other, the positive correlation was β-Parallel with β-Antiparallel and twist conformations. In addition, the negative correlation was α-helix with β-Antiparallel. In contrast, the Pearson matrix of gliadin has positive correlation agglomerate size with β-Parallel conformation, but agglomerate size has negative correlation with β-Antiparallel. Secondary structures there are correlations with each other, negative correlation was α-helix conformation with β-Parallel and twist conformations, another negative correlation was β-Parallel with β-Antiparallel. Positive correlations were α-helix with β-Antiparallel, random coil and α-helix, another positive correlation was random coil with β-Antiparallel.

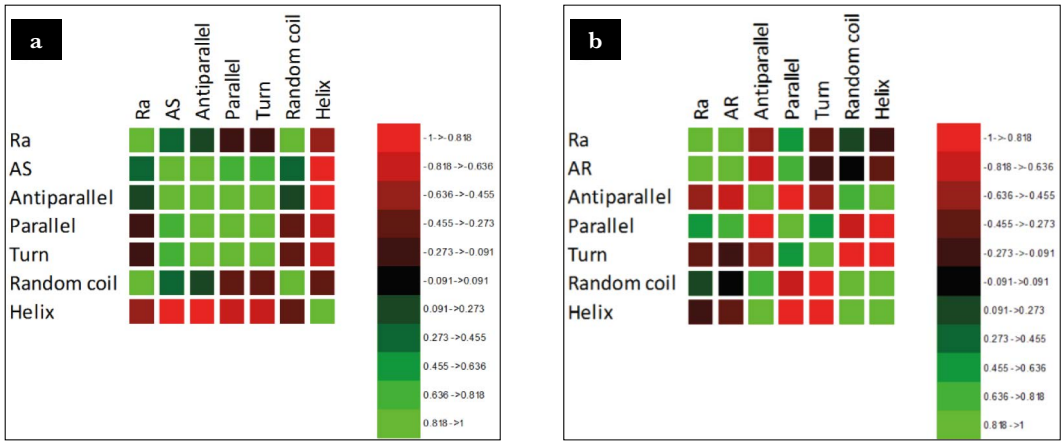

Figure 5.

CONCLUSION

Changes in pH provided ovalbumin and gliadin with useful information to correlate molecular parameters and topographical parameters. The information collected indicates that the regions of secondary structure with the greatest influence on agglomerate size are the β-Parallel conformation for proteins. Additionally, for ovalbumin the agglomerate size was β-Antiparallel and twist conformations. Overall, Ra is shown to have a higher correlation with random coil conformation for ovalbumin. Structural changes at the microstructural and molecular level in proteins and secondary structure at different pH values. This information can be valuable for understanding protein behavior and selecting appropriate pH conditions to improve some food properties.

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