Physical, structural and antioxidant properties of brewer's spent grain protein films

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***Running title*:** Film forming properties of brewer's spent grain proteins

Abstract

BACKGROUND: The development of brewer's spent grain protein films with potential active packaging properties was investigated. Films were prepared by casting protein dispersions at different pHs (2, 8, 11), plasticizers (polyethylene glycol –PEG– or glycerol), and levels (0 – 0.25 g g^{-1}) of PEG. Mechanical, water-barrier and solubility, optical, antioxidant (reducing power, $ABTS^{\bullet+}$ and lipidic radical scavenging), and antimicrobial properties of films were determined. Also structural characteristic of films were evaluated by ATR-FTIR.

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RESULTS: Only films prepared at pH 2 and plasticized by PEG were homogeneous in appearance and could be manipulated, thus different levels of PEG were studied at this pH. Higher PEG concentrations increased water solubility, water vapor permeability and elongation at break and decreased tensile strength and elastic modulus. PEG increased α-helix structure only when 0.10 g PEG g^{-1} BSG-PC was used. This could be related with the better mechanical properties of $F_{0.10}$ films (higher tensile strength, and elastic modulus) respect to the others films. Antioxidant activity depended on PEG concentration, whereas no antimicrobial properties against *Bacillus cereus*, *Salmonella newport* and *Penicillium corylophylum* were detected.

CONCLUSION: The formulations with 0.10 and 0.15 g PEG g^{-1} BSG-PC appear to be the most promising, balancing mechanical, water-barrier properties and antioxidant capacity of these films. Moreover, BSG proteins could be a cheap alternative for the preparation of biodegradable films, capable to be used as active food packaging.

Keywords: brewer's spent grain, protein films, active film, polyethylene glycol, antioxidant properties.

1. Introduction

Global beer production amounted to about 1.94 billion hectoliters in 2018.[1] Beer manufacturing produces several by-products, brewers' spent grain (BSG) being the most abundant. The main application of BSG has been basically limited to animal feeding because of its high content of protein and fiber.[2] It contains about 150 - 250 g kg^{-1} protein, 500 - 700 g kg^{-1} fiber as hemicellulose, cellulose, and lignin, 50 - 100 g kg^{-1} fat, and 20 - 50 g kg^{-1} ash.[3] Due to the significant amount produced annually, the difficulty of its disposal and its low current market value, BSG can represent an interesting by-product to process and give greater added value. Its use in the preparation of protein concentrates with good functional properties is particularly promising. In this regards, it has been reported that rich protein fractions can be obtained from BSG.[4,5]

The ability of BSG proteins to interact strongly among their polypeptide chains could be conducive for the development of bio-based and biodegradable materials.[6] As it is known, protein films stabilized by disulfide bonds are commonly more resistant and elongable, less soluble in water and have better barrier properties.[7] Moreover, modifying protein structure and the interactions between protein molecules by adjusting the pH of the film-forming dispersion it is possible to improve film formation and its properties.[8]

In general, protein-based film formulations require the addition of a plasticizing agent to reduce the film's brittleness and confer certain plastic properties.[9] Among them, the most utilized are sorbitol, polyethylene glycol, and glycerol. Plasticizer molecules have the ability to position themselves within the three-dimensional protein network and

disrupt protein-chain hydrogen bonding, thus increasing the free volume and intermolecular spacing,[10] which improve film flexibility and extensibility.[11] However, plasticizers also decrease the mechanical strength and barrier properties of the films.[12] Thus, both the type of plasticizer and its concentration could modify films properties.

As far as we know, the film forming properties of protein products derived from brewers' spent grain has not been practically studied. Only, Lee *et al.*[13] prepared and characterized BSG-protein-chitosan composite films. However, there is no information about films prepared only using these proteins, neither on the effects of pH or plasticizer type and concentration on film forming properties of BSG-proteins. The aims of this work were to examine film forming capacity of BSG-proteins under different pHs and type of plasticizers, and to study structural characteristics, physicochemical and bioactive properties of films made with different concentrations of PEG.

2. Materials and Methods

This section is in the Online Resource (ESM 1).

3. Results and discussion

3.1. Brewer's spent grain proteins

Protein profiles from BSG and BSG-PC were determined using SDS-PAGE with a reducing agent (β-mercaptoethanol). The BSG proteins (Fig. 1B) presented components higher than 90 kDa molecular weight (no clear protein bands), bands between 89-61 kDa and between 43-29 kDa corresponding to D, C, and B hordeins, respectively of barley (Fig. 1B). Celus *et al.*[14] reported that BSG had D hordeins (greater than 94 kDa), C hordeins (between 80 and 55 kDa), B hordeins (between 35 and 50 kDa), and

albumins and globulins (smaller than 20 kDa). Yalcin and Celik[15] reported different hordeins fractions for barley flour: D hordeins (greater than 66 kDa), C hordeins (66 - 45 kDa), B hordeins (45 - 29 kDa), and albumins and globulins (smaller than 29 kDa). During malting, barley proteins are in part degraded to amino acids and small peptides by a range of proteolytic enzymes and also are extracted in sweet wort.[16] Thus, proteins remained in BSG are insoluble and non-water extractable. Only polypeptides of 47.5 kDa and 27.7 kDa were present in BSG-PC (Fig. 1C). Moreover, FPLC (Fig 1D) showed fractions of 3000 Da and 108 Da corresponding to oligopeptides and free amino acids obtained during alkaline extraction. In this regard, Niemi *et al.*[17] reported that proteins solubilized from BSG by alkaline pH (9.5) presented bands lower than 10 kDa in a SDS–PAGE.

Regarding protein solubility, BSG-PC had lower solubility at acidic (pH 2: 390.1 ± 9.3 g kg^{-1}; pH 4: 381.4 ± 7.3 g kg^{-1}) than at neutral (pH 6: 427.7 ± 17.0 g kg^{-1}) or alkaline pH (pH 8: 515.5 ± 18.9 g kg^{-1}; pH 10: 517.5 ± 13.6 g kg^{-1}).

On the other hand, isoelectric point was at 2.92. This value was lower than 3.3 and 3.8 reported by Arauzo *et al.*[18] and Connolly *et al.*[19] for BSG proteins, respectively.

Amino acid profile is showed in Table 1. It is showed that large hydrophobic and small neutral side chain amino acids account 59.6% of amino acids. Moreover, BSG-PC is rich in S-containing amino acids, which could be involved in protein-protein interactions.

3.2. Film forming conditions

Taking into account that there are no studies reported in the scientific literature of films produced with BSG protein concentrates, a rapid screening was initially carried out to evaluate the conditions in which these proteins can form films. Only Lee *et al.*[13] prepared films with BSG proteins, but they filtered the film forming dispersions to prepare composite films with chitosan using glycerol as plasticizer. Thus, these films were very different than those studied in this work.

It has been reported that some peptides of low molecular weight or compounds such as polyphenols could plasticize protein matrices.[20,21] Thus, considering that some components of low molecular weight in the concentrate could exercise as plasticizers, an attempt was made to obtain, in the first instance, films without plasticizers. Films prepared with BSG-PC at pH 8.0 and 11.0 were not homogeneous in appearance and could not be demolded. However, films prepared at pH 2 were homogeneous in appearance, without visible pores or cracks brittle areas or bubbles. However, they were not easy to detach from the surface on which they were formed. Mauri and Añón[22] studied the effect of pH on soy protein film forming capacity. They reported that the pH affected the charge and the degree of denaturation of the proteins, which influenced the way in which the peptide chains interact during the formation of the films and finally the hydrophilic-hydrophobic nature of protein films. Although BSG-PC showed to have lower solubility at acidic than at alkaline pH suggesting a higher protein-protein interaction at this pH, proteins could be well dispersed in the filmogenic dispersion, allowing obtaining homogeneous films. Moreover, protein-protein interactions are favored at pH near the pI, which could favor film formation.

Plasticizers, generally of low molecular weight, reduce the extensive interactions among protein molecules and thus decrease the material brittleness with a consequent increase in its flexibility and handling.[23] The use of two plasticizers commonly used for this purpose, with different hydrophobicity was analyzed: glycerol and PEG at three different pHs: 2, 8 and 11. Fig. 2 shows the appearance of these films. Again, only those formulated at pH 2 were homogeneous and could be easily demolded from the support, while the rest of the studied formulations resulted in very hygroscopic films that could not be removed from the mold. Those formulated with PEG at pH 2 seemed to be the best and the most interesting to continue being studied.

In fact, PEG is a relatively small hydrophilic molecule which can be easily compatible to the BSG-PC network. Due to its low molecular weight, PEG-400 has high polarity and solubility, which favor hydrogen bonding ability and the interaction with protein chains,[6] without the higher disruption of protein–protein interactions induced by glycerol. Theoretically, plasticizers containing more polar groups (-OH) should behave as better plasticizers for hydrophilic polymers due to the development of more protein-plasticizer interactions within the film, mainly through hydrogen bond.[24] However, molecule size, solubility, and polarity of plasticizers also affect the ability of hydrogen bonding and the effectiveness of the plasticizer.[25,26]

3.3. Effect of PEG concentration on physicochemical properties of films

The effects of different ratios of PEG to BSG-PC (0.05 to 0.25 g g^{-1}) over different properties of films prepared at pH 2 were evaluated. Also, films without PEG were prepared as a control, but the characteristic brittleness of these control films caused

them to break when were removed from the mold, allowing only properties requiring small pieces of film were analyzed.

All films were homogeneous and thin. Properties of BSG-PC films prepared at pH 2 and different concentrations of PEG are shown in Table 2.

Moisture values were within the range of 95.4 – 148.1 g kg^{-1}. It increased with PEG content and this could be related with the water-holding capacity of the plasticizer.[27]

The solubility in water of BSG-PC films increased from 704 to 898 g kg^{-1} when increasing PEG content. Generally, the addition of a plasticizer raises the solubility due to the increase of the hydrophilic groups, which increase the interaction with water, and also decrease of cross-linking between polymer chains.[28]

The WVP of edible films ranged from 8.49×10^{-11} to 1.00×10^{-10} (g H_2O $Pa^{-1}.s^{-1}.m^{-1}$). The $F_{0.05}$ and $F_{0.25}$ films did not resist permeation cell (with silica inside, HRc = 0). These results indicate that 0.10 and 0.20 g PEG g BSG-PC were the lower and the higher levels of plasticizer that allow obtaining handled films with good water-barrier properties, $F_{0.10}$ being the film with the lower WVP value. The WVP values were significantly lower than those of 100:0 and 30:70 BSG proteins: chitosan films ($2.93 \pm 0.20 \times 10^{-9}$ and $2.72 \pm 0.15 \times 10^{-9}$ g H_2O $Pa^{-1}.s^{-1}.m^{-1}$, respectively) reported by Lee *et al.*[13] using glycerol as plasticizer. Also, WVP for $F_{0.10}$ was lower than those obtained for films made with other proteins and glycerol 50% (w/w, based on protein content), like gelatin (10.2×10^{-10}g Pa^{-1} s^{-1} m^{-1}), soy protein isolate (7.54×10^{-10} g Pa^{-1} s^{-1} m^{-1}), and whey protein concentrate (1.54×10^{-10}g Pa^{-1} s^{-1} m^{-1}).[29] The amino acid profile of BSG-

PC (Table 1) indicated that 38% of amino acids have large hydrophobic side chain, which can be involved in the good water-barrier properties of these films.

Water susceptibility of films, analyzed by moisture content, solubility in water and WVP, is an important characteristic of films as determined their possible future application. Solubility of edible films, for example, is essential when selecting a film to pack water-rich foods and also is a significant factor that determines biodegradability and the release of bioactive compounds.[30]

On the other hand, films presented thickness values within the range of 83.10 – 106.84 µm, a maximum value being observed for $F_{0.10}$, decreasing over this ratio of PEG (Table 2).

Also, films $F_{0.10}$ and $F_{0.15}$ presented the higher difference of color (ΔE^* values). Thus, the addition of PEG induced a change on color depending on its concentration. Color is greatly affected by several factors including plasticizer addition, thermal treatment, fabrication process, and storage conditions.[31] However, for protein-based films, color is most affected by protein concentration than by film treatments.[32] In this case, the amount of proteins did not change among film formulas and the ratio protein/total solids decrease from 0.57 to 0.45 g protein g^{-1} total solids when increasing PEG addition. Considering films added with PEG, there was a lineal direct relationship between thickness and ΔE^* values (r: 0.6800).

Regarding opacity, there was a significant difference in film opacity according to PEG concentration. There was a trend to increase opacity as PEG content increased. However, $F_{0.10}$ presented the lower value according at least in part to the fact this film

had the higher thickness. It is important to mention that films did not show transmittance from 200 to 400 nm, thus they could be an important barrier to UV radiation. Opacity is important mainly when films are used to package fatty foods, in order to slow the oxidative degradation catalyzed by light, extending the shelf life of the products.[33]

Results of mechanical properties of BSG-PC films are showed in Fig.3. The $F_{0.25}$ films were very hygroscopic and could not be analyzed regarding mechanical properties. It was observed that the addition of PEG at low concentrations (0.05 - 0.10 g g^{-1} solid) resulted in higher tensile strength (Fig. 3A) and elastic modulus values (Fig. 3B), and lower elongation at break (Fig. 3C) than films formed at higher concentrations of PEG (0.15 - 0.20 g g^{-1}). Guerrero and de la Caba[34] reported that increasing the amount of glycerol as plasticizing in soy protein films caused a decrease of tensile strength and an increase of elongation at break due to the fact that plasticizing reduces the interactions between protein chains, thus increasing their mobility.

Lee *et al*.[13] reported values of 4.32 MPa for tensile strength and 36.38% elongation for films made with BSG proteins and 40% glycerol (w/w, BSG-PC basis), but they used only soluble proteins heat treated to prepare their films.

These mechanical properties were related with the presence of secondary protein structures present in films. Fig. 3D shows deconvoluted amide I band from ATR-FTIR spectra of F_0 and $F_{0.10}$ BSG-PC films. Table 3 shows the percentages of α-helix, β-sheet, γ-turn and random coil calculated from the amide I band of ATR-FTIR spectra. The concentration of plasticizer affected the content of secondary structures, values ranging

17.9 to 24.9% for α-helix; 8.0-14.6% for β-turns, 5.4-7.0% for β-sheets, 6.9-14.85% for random coils, and 9.9-16.0% for antiparallel β-sheets. Yun *et al.*[35] reported that α-helix and β-turns structures are formed through intramolecular hydrogen bonding and β-sheet structures are formed through intra/intermolecular hydrogen bonding. However, if the plasticizer successfully disrupts the hydrogen network of the protein, the secondary structure will be altered significantly. It was observed that PEG increased α-helix rate only when 0.10 g g^{-1} BSG-PC ($F_{0.10}$) was used. Generally, the increase in the elongation of protein films by addition of plasticizers will be the result of increased α-helix, β-turn or random coil structures, which are more stretchable than the extended β-sheet structure.[36] This could be related with the better mechanical properties of $F_{0.10}$ films (higher tensile strength, and elastic modulus) respect to the others.

3.4. Effect of PEG concentration on bioactive properties of films

Fig. 4 shows antioxidant capacity evaluated through the scavenging of $ABTS^{\bullet+}$ radical (TEAC), reducing power (RP) and β-carotene bleaching inhibition (BBI). These values were expressed taking into account the proteins solubilized from films.

It was observed that $ABTS^{\bullet+}$ scavenging capacity depended on PEG concentration, it being higher at low levels of PEG (0.05 and 0.10 g g^{-1} BSG-PC) (Fig. 4A). This result can be attributed to the solubilization of antioxidant low molecular weight protein components. As mentioned before, BSG-PC had oligopeptides (3000 Da) and free amino acids (108 Da). Several studies have shown that low molecular weight protein components generally possess higher radical scavenging capacity than high molecular weight proteins or peptides.[37,38] In this regard, it has been suggested that low molecular

weight protein components would access more easily to the oxidant system and lead to high values of TEAC.[39]

Regarding RP, the higher capacity was observed for films with PEG concentration higher than 0.10 g g^{-1} (Fig. 4B). Thus, RP of films depended on PEG concentration. This can be due to solubilization of antioxidant high molecular weight protein components. Note that high PEG concentrations in films formulation increased water solubility (Table 2). Therefore, the solubilization of high molecular weight protein components was favored. In this regard, Sonklin *et al.*[40] reported that protein components with molecular weight higher than 10 kDa from mungbean meal protein hydrolysates showed the greatest RP at all concentrations evaluated. Thus, the higher RP of $F_{0.15}$-$F_{0.25}$ films may be due to the higher solubility of active components favored by PEG.

As for RP, an increase in PEG concentration produced an increase in BBI (Fig. 4C), indicating that the high molecular weight protein components are the most active. These results are in agreement with those found by Pedroche *et al.*[41] for *Brassica carinata* hydrolysates. In that work, the antioxidant effect decreased with decreasing peptide size, achieving the lowest value of BBI for peptide fractions with 500 Da.

In general, it was observed that PEG affected the mechanism by which proteins exert their antioxidant effect, probably inducing changes in protein secondary structure, affecting the accessibility of the protein or polypeptides to the radicals or the electron transfer.

Among other bioactive properties, antimicrobial properties were investigated. Due to BSG-PC has oligopeptides (Fig 1D), antibacterial and antifungal activities could be expected,[42] but results showed that both BSG-PC and films did not inhibit the growing of assayed bacterial strain and fungus. Also, gelatin films did not present antibacterial properties. However, the addition of multi-wall carbon nanotubes allowed obtaining gelatin films effective against to both gram-positive and gram-negative bacteria.[43]

4. Conclusion

The formulations $F_{0.10}$ and $F_{0.15}$ appear to be the most promising, balancing the plasticizer content and the properties of the films (mechanical, water-barrier and antioxidant properties). This work offers new insights into a better understanding of how properties of BSG-PC films are linked with changes in protein structure induced by PEG. Moreover, BSG proteins could be a cheap alternative for the preparation of biodegradable films since the low cost of BSG. In addition, BSG-PC films exhibited some antioxidant activity, which could be considered as an advantage for active food packaging.

5. Acknowledgements

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6. Declaration of conflict of interest

The authors declare there are no conflicts of interest.

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Legends to Figures

Fig. 1 SDS-PAGE electrophoresis with β-mercaptoethanol of **A)** Molecular mass markers; **B)** BSG; and **C)** BSG-PC**; D)** Protein profile of BSG-PC determined by FPLC.

Fig. 2 BSG-PC films made at pH 2, 8 and 11, using Polyethylene glycol-400 (PEG) and glycerol as plasticizers.

Fig. 3 Mechanical properties measured in tensile test: **A)** Tensile strength (MPa); **B)** Elastic modulus (MPa); **C)** Elongation at break (%) of BSG-PC films prepared at pH 2 and different PEG concentrations ($F_{0.05}$, $F_{0.10}$, $F_{0.15}$, and $F_{0.20}$). Means are shown in bars. Different letters in bars mean significant differences ($p < 0.05$) among samples. **D)** ATR-FTIR spectra of F_0 and $F_{0.10}$ films. F_0 - $F_{0.20}$ indicate ratios of PEG/ BSG-PC for each film formula.

Fig. 4 Antioxidant capacity of BSG-PC films prepared at pH 2 with different PEG concentrations (F_0, $F_{0.05}$, $F_{0.10}$, $F_{0.15}$, $F_{0.20}$, and $F_{0.25}$) evaluated by: **A)** ABTS*+ assay; **B)** reducing power (RP); **C)** β-carotene bleaching inhibition assay (BBI). Different letters mean significant differences ($p < 0.05$) among samples. F_0 - $F_{0.25}$ indicate ratios of PEG/ BSG-PC for each film formula.

Table 1. Amino acid profile of BSG protein concentrate

Amino Acids	**Groups**	g kg^{-1} protein	Eq kg^{-1} protein	**Eq Eq $total^{-1}$ (%)**
Asp + Glu	**Acid side chain**	165.72 ± 0.09	1.18	15.7
Lys	**Basic side chain**	63.07 ± 0.38	0.43	20.5
Arg		65.70 ± 0.33	0.38	
His		114.11 ± 0.77	0.74	
Met	**S-containing side chain**	27.12 ± 1.37	0.18	4.2
Cys		16.54 ± 0.07	0.14	
Gly	**Small neutral side chain**	4.45 ± 0.10	0.06	21.6
Thr		44.05 ± 0.17	0.37	
Ser		53.24 ± 0.23	0.51	
Ala		61.52 ± 0.07	0.69	
Pro	**Large hydrophobic side chain**	58.24 ± 1.75	0.51	38.0
Tyr		43.75 ± 0.28	0.24	
Val		56.81 ± 0.29	0.48	
Ile		46.94 ± 0.25	0.36	
Leu		100.63 ± 0.52	0.77	
Phe		78.12 ± 0.43	0.47	

Table 2. Susceptibility to water and appearance of BSG-PC films prepared at pH 2 and different PEG concentrations.

Films	Water susceptibility			Appearance		
	Moisture content (g kg^{-1})	**Water Solubility (g kg^{-1})**	**Water vapor permeability (g H_2O Pa^{-1}.s^{-1} m^{-1}) x 10^{-10}**	**Thickness (µm)**	**ΔE***	**Opacity (AU mm^{-1})**
F_0	95.6 ± 3.7^{a}	nd	nd	95.60 ± 3.02^{d}	62.47 ± 0.26^{a}	11.52 ± 0.24^{b}
$F_{0.05}$	95.4 ± 2.4^{a}	704.0 ± 6.7^{a}	nd	83.10 ± 3.26^{a}	61.44 ± 1.15^{a}	12.58 ± 0.29^{c}
$F_{0.10}$	97.0 ± 2.0^{a}	715.1 ± 21.3^{a}	8.5 ± 0.3^{a}	106.84 ± 2.89^{e}	66.45 ± 0.92^{c}	9.83 ± 0.21^{a}
$F_{0.15}$	120.5 ± 4.3^{b}	774.7 ± 18.8^{b}	10.7 ± 0.3^{b}	88.30 ± 2.91^{c}	65.33 ± 0.64^{c}	12.90 ± 0.22^{cd}
$F_{0.20}$	119.7 ± 1.5^{b}	885.8 ± 20.1^{c}	11.6 ± 0.3^{b}	85.81 ± 2.52^{b}	63.48 ± 0.25^{b}	13.02 ± 0.21^{d}
$F_{0.25}$	148.1 ± 4.0^{c}	897.8 ± 11.7^{c}	nd	85.57 ± 2.28^{b}	62.15 ± 0.81^{a}	15.63 ± 0.21^{e}

		b

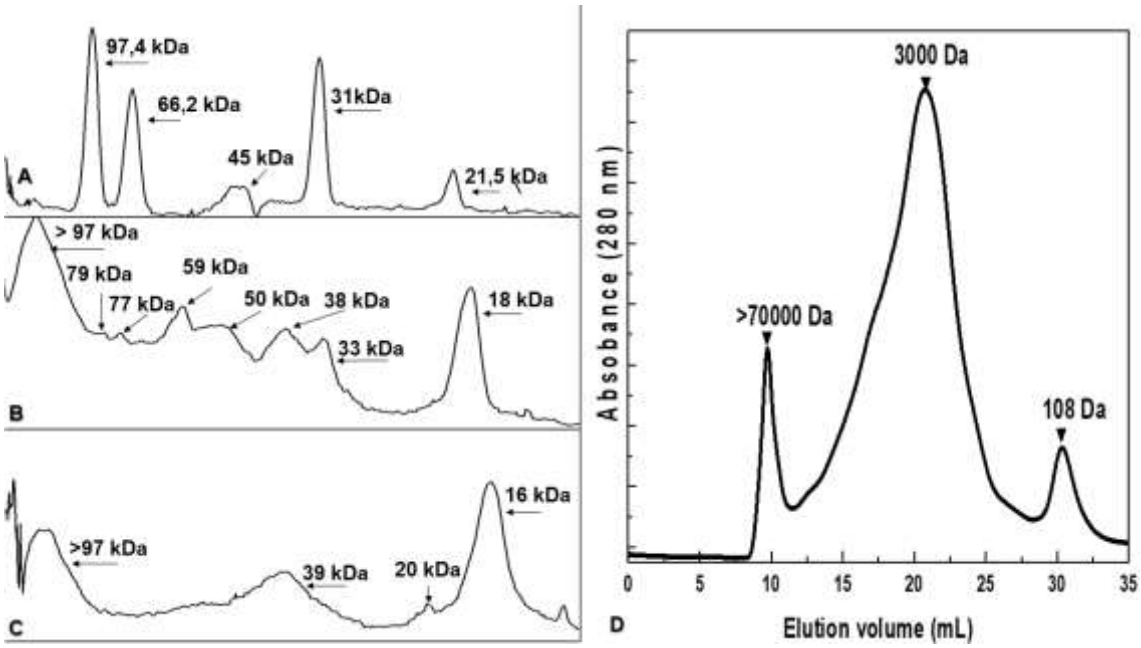
Values are expressed as mean ± standard deviation. Different letters in the same column mean significant differences between samples ($p<0.05$); F_0 - $F_{0.25}$ indicate g PEG g $^{-1}$BSG-PC for each film formula; nd: not determined.

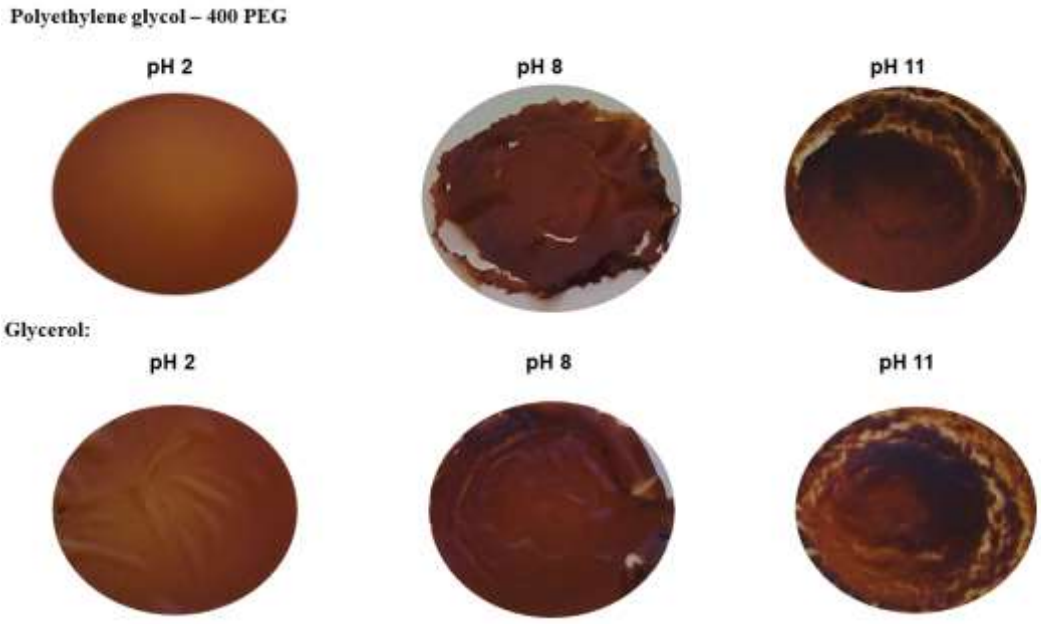
Table 3. Percentage of secondary structure segments of total area of amide I band (ATR-FTIR) from BSG-PC films prepared at pH 2 and different PEG concentrations.

Films	α-helix (%)*	β–turns (%)*	β-sheets parallel (%)*	Random coils (%)*	Antiparallel β-sheets (%)*	α-helix/β-structure (flexibility)
F_0	20.49 ± 2.49[a]	13.29 ± 2.64[b]	3.38 ± 1.62[a]	11.44 ± 7.50[a]	10.47 ± 6.46[a]	6.81 ± 2.37[b]
$F_{0.05}$	20.35 ± 2.24[a]	8.04 ± 0.86[a]	6.37 ± 0.66[b]	14.84 ± 4.78[a]	14.54 ± 4.34[a]	3.24 ± 0.72[a]
$F_{0.10}$	24.90 ± 1.32[b]	11.43 ± 4.10[ab]	5.37 ± 1.67[ab]	6.88 ± 6.45[a]	14.56 ± 2.09[a]	5.06 ± 2.10[ab]
$F_{0.15}$	19.74 ± 2.82[a]	8.14 ± 2.32[a]	5.59 ± 0.51[ab]	14.85 ± 2.09[a]	9.94 ± 3.30[a]	3.52 ± 0.20[a]
$F_{0.20}$	17.85 ± 0.06[a]	14.58 ± 0.52[b]	6.97 ± 1.15[b]	9.15 ± 7.90[a]	15.95 ± 2.58[a]	3.06 ± 1.19[a]

*Percentage of each protein secondary structure was calculated as the ratio of its deconvoluted peak area to the total area of the amide I band. Values are expressed as mean ± standard deviation. Different letters in the same column mean significant

differences between samples ($p<0.05$); F_0 - $F_{0.25}$ indicate g PEG g $^{-1}$BSG-PC for each film formula.





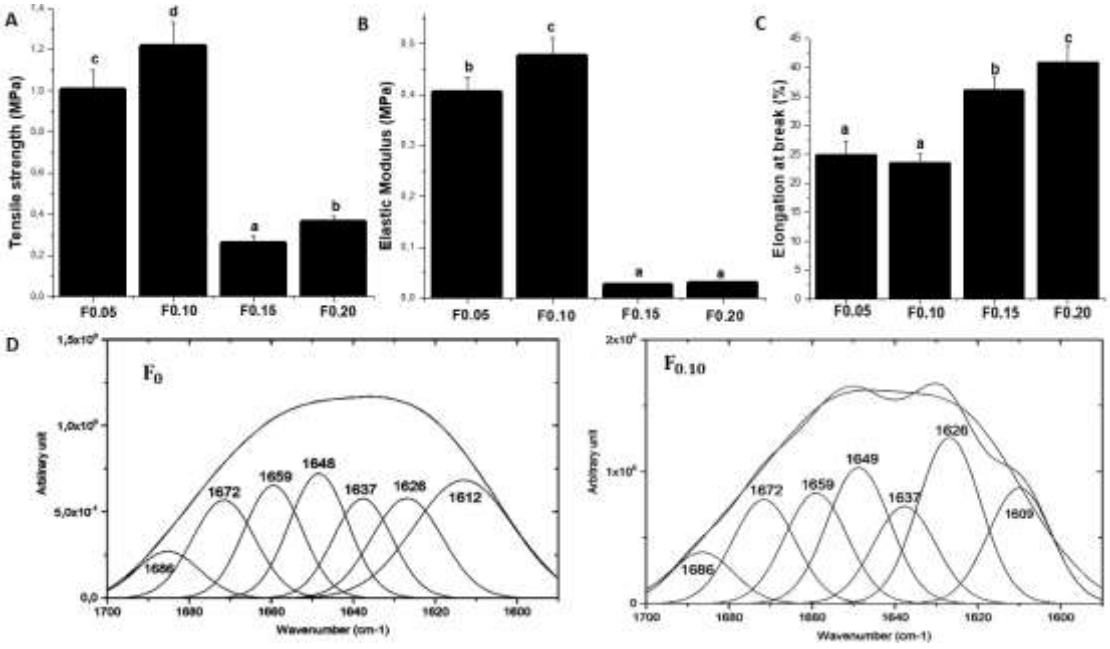


Fig. 4