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Behaviour of cyanidin-3-glucoside, β-lactoglobulin and polysaccharides nanoparticles in bulk and oil-in-water interfaces

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ABSTRACT

Particle size distributions as well the interfacial and rheological properties of the films at the oil/water interface were used to study the effect of the interacting system between β-lactoglobulin, cyanidin-3-glucoside and pectin or chitosan in buffer solutions. The particles obtained were smaller with cy-3-gluc-β-lg-pectin and had reduced polydispersity with cy-3-gluc-β-lg-chitosan. Based on time dependent surface pressure results, β-lg-pectin mixtures showed a slower increase at the beginning of the measurement, while β-lg-chitosan mixtures showed no differences with β-lg alone. Contrarily, dilatational properties increased for ternary chitosan mixtures, but they remained similar to the pure protein in ternary pectin mixtures. Cy-3-gluc interfacial properties were reduced by the presence of pectin and chitosan.

The interactions between cy-3-gluc and the biopolymers that have been selected in the present work resulted in a lower content of free polyphenol, reduced antioxidant properties as well as free β-lg. The impact of this effect was more relevant when pectin was used.

1. Introduction

Strawberries are a good source of anthocyanins and that have high antioxidant activity. Several studies have suggested that these compounds are beneficial to human health since several positive therapeutic effects have been proved (Bridle & Timberlake, 1997; Clifford, 2000). Additionally, due to consumer preferences as well as legislative action, there is a worldwide trend towards the development of natural food additives, and anthocyanins are natural pigments with low toxicity permitting their incorporation in many food systems (Brouillard, Chassaing, Isorez, Kueny-Stotz, & Figueiredo, 2010; Idham, Muhamad, & Sarmidi, 2012). Interactions between proteins and polyphenols have also shown to be of importance to understand astringency as a result of salivary proline rich proteins precipitation (Thongkaew, Gibis, Hinrichs, & Weiss, 2014). These interactions between proteins and polyphenols have been extensively studied in solution in order to identify their nature. It was found that they can bind each other by formation of multiple weak interactions (mainly hydrophobic) established between amino acid side chains and polyphenol aromatic rings, indicating that it is mainly a surface phenomenon. Sometimes these interactions could be complemented by hydrogen bonding, playing an important role in reinforcing and stabilizing the complexes (Carvalho, Póvoas, Mateus, & de Freitas, 2006; Charlton et al., 2002; de Freitas, Carvalho, & Mateus, 2003; de Freitas & Mateus, 2001; Kanakis et al., 2011; Scollary, Pásti, Kállay, Blackman, & Clark, 2012; Tang, Covington, & Hancock, 2003; Thongkaew et al., 2014; Viljanen, Kylli, Hubbermann, Schwarz, & Heinonen, 2005; Xiao et al., 2011). In terms of colloidal stability, the interactions between proteins and polyphenols can be divided into three stages: (i) several polyphenol molecules can bind to one protein molecule after the addition of polyphenol; (ii) if the amount of polyphenol is high enough, two peptide molecules are able to form polyphenol-coated dimers; (iii) more molecules are added and the complexes could form large particles that even precipitate (Diniz et al., 2008; Scollary et al., 2012). This behaviour has been observed for catechin, epicatechin, rutin and quercetin with bovine serum albumin (BSA) (Papadopoulou, Green, & Frazier, 2005) and for quercetin, rutin, hyperin and baicalin with HSA (Bi et al., 2004).

However, not only proteins are able to form complexes with polyphenols, but also polysaccharides, such as pectin, cyclodextrins, xanthan and arabic gum. Several studies demonstrated the

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ability of polysaccharides to bind to polyphenols and prevent or decrease polyphenol–protein interactions. These polysaccharides have an effect on electrostatic interactions with proteins in ternary mixtures (Carvalho et al., 2006; Le Bourvellec & Renard, 2012; Luck et al., 1994; Ozawa, Lilley, & Haslam, 1987; Thongkaew et al., 2014) and under low pH, polysaccharides like pectin stabilize milk proteins such as caseins and β-lg mainly via electrostatic forces (Kazmierski, Wicker, & Corredig, 2003).

Recent works have focused on the adsorption of anionic (carboxymethyl cellulose) and cationic (chitosan) polysaccharides on cellulose and subsequent adsorption of proteins with different charge densities (Mohan et al., 2013, 2014). The authors demonstrated that unspecific protein adsorption on chitosan is much higher than on cellulose. At its isoelectric point (pH = 4.7), BSA does not carry any net charges, leading to a lower solubility resulting in higher adsorption on all surfaces at pH 5. Above the isoelectric point, BSA is partially deprotonated concomitant with a negative net charge leading to higher solubility and subsequently lower protein deposition compared to pH 5 (Mohan et al., 2014).

Reports described that when BSA is adsorbed to a surface it can suffer elongation if the distortional forces become larger than the interaction energy within the protein (i.e., hydrogen bonding, electrostatic and hydrophobic interactions, etc.), the hydrophobic domains buried in the interior of the protein structure may be exposed leading to increased hydrophobicity leading to spontaneous protein adsorption (Roach, Farrar, & Perry, 2005, 2006). Increased surface roughness can be responsible for higher adsorbed amounts of protein apparently due to an increased surface area, as suggested by other authors as Molino, Higgins, Innis, Kapsa, and Wallace (2012) and Dolatshahi-Pirouz et al. (2008).

Polyphenols and polysaccharides can also form complexes, and the mechanisms would be similar to that described for proteins. The adsorption would be mediated by hydrogen bonding and hydrophobic interactions, being favoured by the existence of hydrophobic cavities (Renard, Baron, Guyot, & Drilleau, 2001; Williamson, Trevitt, & Noble, 1995). In fact, polysaccharides may have a suitable structure, composition, as well as a sufficient size and flexibility to be able to complex polyphenols. Conformational flexibility and hydrophobicity could strongly influence the retention of polyphenols by polysaccharides (Le Bourvellec, Bouchet, & Renard, 2005).

In dairy matrices, it is frequently observed the existence of interactions between milk proteins, polyphenols from fruit preparates and polysaccharides that are normally added as food stabilisers, which can influence the stability and structure of foods.

Fruit contains a large spectrum of phenolic components (Hartmann, Patz, Andlauer, Dietrich, & Ludwig, 2008; Oliveira, Almeida, & Pintado, 2014a) and carotenoids (Dalla Valle, Mignani, Spinardi, Galvano, & Ciappellano, 2007; Oliveira, Gomes, Alexandre, Almeida, & Pintado, 2014b), that contributes to their high antioxidant activity. Besides that they have protective effect against cardiovascular disease (Hertog et al., 1993, 1995) and reduction of digestive tract cancer risk (Bushman, 1998), protective effects against some types of cancer, age-related macular degeneration, and heart disease. Little is known about their *in vivo* free content and antioxidant capacity in the presence of dietary factors that may interact with phenolics interfering in their bioaccessibility (Argyri, Komaitis, & Kapsokefalou, 2006; Oliveira et al., 2015) and consequently reducing their biological properties.

Strawberry yoghurt is one of the most consumed dairy products around the world and they are a good source of anthocyanins that have high antioxidant activity. In previous studies we observed that bioaccessibility of the anthocyanins, decreased significantly possibly due to the interaction of main reactive protein in the yoghurt (β-lg) and polysaccharide (carrageenan) present in the fruit formulation (Oliveira et al., 2015). However, the impact and extent of interactions of these target molecules in model systems mixtures (anthocyanin, β-lg and polysaccharide) have not still been studied in detail. Thus, the aim of this work was to study the interactions between cy-3-gluc, β-lactoglobulin, and two different polysaccharides (pectin and chitosan) and evaluate the effect of the complexes formed in the interfacial properties at pH 4.0 as well as in the bioaccessibility of anthocyanin.

2. Material and methods

2.1. Reagents list

The 2,2-azinobis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS), acetic acid, florisil 60–100 mesh, sodium acetate, sodium carbonate, fluorescein, 2,2′-azo-bis-(2-methylpropionamidine)-dihydrochloride (AAPH) and 6-hydroxy-2,5,7,8-tetramethylbroman-2-carboxylic acid (trolox) were purchased from Sigma–Aldrich (Sintra, Portugal). Folin-Ciocalteu's reagent and potassium persulfate from Merck (Algés, Portugal).

Pure compounds of ascorbic acid, β-lactoglobulin and high molecular weight chitosan (HMW) (624 kDa; >75% of deacetylation) were obtained from Sigma–Aldrich (Sintra, Portugal), whereas cy-3-gluc was purchased from Extrasynthése (Lyon, France).

The pectin from citrus peel type DF (70% esterification) was provided by CP Kelco (Lille Skensved, Danmark).

2.2. Protein–polyphenol–polysaccharide mixtures

The acetate buffer (0.1 M; pH 4) was used to dissolve under agitation (400 rpm) cy-3-gluc and β-lg powders at room temperature, while pectin and chitosan were heated at 70 °C for 30 min to assist the dissolution. The pH in each solution was adjusted at pH 4 with HCl (1 N).

All the samples were prepared at final concentrations of 0.02 wt% for polyphenols, 0.3 wt% for protein and 0.38% for polysaccharides. All the mixed systems were prepared at 400 rpm for 30 min at room temperature and finally stored 24 h at 4 °C. Samples were directly used for Dynamic Light Scattering (DLS), dynamic interfacial tension and surface dilatational properties.

One part of the mixed systems was filtered by 3 kDa filters for total antioxidant activity measurements (ABTS, ORAC) and free polyphenol quantification by HPLC–DAD. For the determinations of free β-lg, sample mixtures were filtered by 100 kDa filters to isolate the complexes formed.

2.3. Dynamic light scattering and Z-potential measurements

Particle size analysis were determined one day after the preparation of the samples using a Dynamic Light Scattering (DLS) instrument (Zetasizer Nano-ZS, Malvern Instruments, Worcestershire, UK) provided with a He-Ne laser (633 nm) and a digital correlator, Model ZEN3600. Measurements were carried out at a fixed scattering angle of 173°. Samples were contained in a disposable polystyrene cuvette (von Staszewski, Pizones Ruiz-Henestrosa, & Pilosof, 2014a).

The zeta potential of the solutions was measured using the laser Doppler velocimetry (LDV) technique (measurement range from 5 nm to 10 mm). In this technique a voltage was applied across a pair of electrodes placed at both ends of a cell containing the particle dispersion. Samples temperature was set at 25 °C and the assays were carried out in duplicate (von Staszewski et al., 2014a).

The zeta potential (z) was calculated by using the Dispersion Technology Software provided by Malvern according to Henry's equation (Eq. (1)):

$$\xi = \frac{3\eta}{2\varepsilon F(\kappa a)}\mu e \quad (1)$$

where ξ is the zeta potential of the sample, μe is the electrophoretic mobility, ε is the dielectric constant of water, $F(\kappa a)$ is the function of the dimensionless parameter κa, which was determined to be 1.5 according to Smoluchowski approximation that is usually used when the radius of particle is much larger than the Debye length of the electric double layer. The particles sizes are reported as the average and standard deviation of ten readings made on a sample (Martinez, Pizones Ruiz-Henestrosa, Carrera Sánchez, Rodríguez Patino, & Pilosof, 2013; Pizones Ruiz-Henestrosa, Martinez, Carrera Sánchez, Rodríguez Patino, & Pilosof, 2014; Von Staszewski, Jagus, & Pilosof, 2011).

2.4. Dynamic interfacial properties

Time-dependent surface pressure (π) and interfacial dilatational rheology of the adsorbed β-lg/polyphenols/polysaccharides films were determined at the o/w interface with an automatic pendant drop tensiometer PAT-1 (Sinterface Technologies, Berlin, Germany).

The surface active impurities present in the commercial sunflower oil were removed according to Bahtz et al. (2009) by mixing it with Florisil 60–100 mesh for several days and finally filtered. All the glass materials were properly cleaned using a mixture of ammonium persulfate and sulphuric acid to eliminate all the possible surface active contaminants that could interfere in the measurements and rinsed with Millipore water.

A drop of the solution is formed at the tip of a capillary (volume: 12 μL), immersed in a cell filled with the purified oil. Measurements were done until the adsorption equilibrium was reached (around 180 min). The surface tension (γ) was calculated through the analysis of the droplet profile by applying the Young–Laplace equation to accurately (±0.1 mN/m) determine the surface tension (Labourdenne et al., 1994). The surface pressure is $\pi = \gamma_0 - \gamma$, where γ_0 is the value of the interfacial tension of the subphase (24 mN/m) and γ the interfacial tension of the solution at each time (θ) (Pizones Ruiz-Henestrosa et al., 2014; von Staszewski et al., 2014a). All the experiments were performed at 20 °C and two measurements have been done for each system.

The computer controlled dosing system allows to control a constant volume of the drop during the measurement and also to induce area deformations. In order to determine the surface dilatational properties it was used a method that involved a periodic automatically controlled, sinusoidal interfacial compression and expansion performed by decreasing and increasing the drop volume at the desired amplitude and angular frequency. The dilatational rheology experiments were carried out during the formation of the adsorption layer. Oscillations at a frequency of 0.05 Hz were performed and each perturbation consisted of six oscillations cycles followed by 10 min constant interfacial area recording. The amplitude of the oscillations was 3% of the initial drop volume in order to guarantee that the rheological parameters are independent of the amplitude. The surface area perturbations lead to a respective harmonic surface tension response. The data obtained were analysed using the Fourier transformation, obtaining the dilatational rheological parameters of the interfacial layer, namely the interfacial elasticity and viscosity.

The surface dilatational modulus (E), derived from the change in the surface tension induced by a small change in the surface area, is a complex term that is built up by a storage part E', representing the real part of the term and a loss part E", describing the imaginary part of the modulus (Eq. (2)):

$$E(i\omega) = E'(\omega) + iE''(\omega) \quad (2)$$

where $E' = \omega$ (interfacial elasticity) and $E''/\omega = \eta$ is the interfacial viscosity ($\omega = 2\pi f$, which is the angular frequency of the generated area variations) (Berthold, Schubert, Brandes, Kroh, & Miller, 2007; Pizones Ruiz-Henestrosa et al., 2014).

2.5. Antioxidant activity measurements by ABTS and ORAC

The free radical-scavenging activity was determined by 2,2-azinobis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) method as described by Giao et al. (2007). To oxidize the colourless ABTS to the blue-green, ABTS radical cation (7 mmol/L) was mixed with potassium persulfate (2.45 mmol/L) and kept for 12–16 h at room temperature in the dark. The ABTS solution was diluted with water to an absorbance of 0.70 (±0.02) at 734 nm. After the addition of 1.0 mL ABTS solution to 10 μL of the sample it was made the analysis of the absorbance after 6 min. The inhibition (%) of the sample was then compared with a standard curve made from the corresponding readings of ascorbic acid solutions at different concentrations (0.02–0.50 mg/mL) and the results were expressed as mg ascorbic acid equivalents/mL.

The oxygen radical absorbance capacity (ORAC-FL) assay was based on that proposed by Contreras, Hernández-Ledesma, Amigo, Martín-Álvarez, and Recio (2011). The reaction was carried out at 40 °C in 75 mM phosphate buffer (pH 7.4) and the final assay mixture (200 μL) containing fluorescein (70 nM), AAPH (14 mM), and antioxidant [Trolox (9.98×10^{-4}–7.99×10^{-3} μmol/mL) or the sample (at different concentrations)]. The fluorescence was recorded during 137 min (104 cycles). A FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany) with 485 nm excitation and 520 nm emission filters was used. The equipment was controlled by the FLUOstar Control software version (1.32 R^2) for fluorescence measurements. Black polystyrene 96-well microplates (Nunc, Denmark) were used. AAPH and Trolox solutions were prepared daily and fluorescein was diluted from a stock solution (1.17 mM) in 75 mM phosphate buffer (pH 7.4). All the reaction mixtures were prepared in duplicate and at least three independent measurements were performed for each sample. Final ORAC-FL values were expressed as mg of Trolox equivalent/mL.

2.6. HPLC–DAD analysis

2.6.1. Apparatus

Waters Alliance (Waters Series 600, Mildford MA, USA) high-pressure liquid chromatographer, equipped with a Waters 996 PDA detector. The absorption spectra were recorded through a diode array detector (Waters, Mildford MA, USA) at wavelengths ranging from 200 to 600 nm in 2 nm intervals. Absorbance was measured at 510 nm.

2.6.2. Chromatographic procedures

Separation of the phenols was accomplished on a reverse phase Symmetry® C18 column (250 × 4.6 mm i.d. 5 μm particle size and 125 Å pore size) with a guard column (Symmetry® C18). The mobile phase was composed by solvent A, water/methanol/formic acid (92.5:5:2.5 v/v/v) and solvent B, methanol/water (94:6 v/v), and the program began with a linear gradient starting at: 0 to 30% B in 10 min 30 to 50% B in 10 min 50 to 0% B in 5 min and kept at 0% B during 5 min. Flow rate was 0.75 mL/min, the oven temperature was set as 25 °C and the injection volume was 50 μL.

For the quantification of β-lactoglobulin the mobile phase A was composed by water and 1% trifluoroacetic acid (TFA) and mobile phase B included acetonitrile with 1% TFA. The program began with

a linear gradient starting at: 0 to 50% B in 10 min and from 50 to 0% B in 10 min and kept at 0% B during 5 min.

The flow rate was 0.8 mL/min, the oven temperature was set as 30 °C and the injection volume was 50 μL. Photodiode array detector (Waters, Mildford, MA, USA) spectra were measured over the wavelength range 200–600 nm in steps of 2 nm and the runs were monitored at 220 nm. Results were expressed as micrograms per millilitres.

2.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.00 for Windows. Normality of the data distribution was tested by Kolmogorov–Smirnov method.

Statistical significance values of the groups' means were made by one-way analysis of variance with Tukey post hoc test. The statistical analyses performed were considered significant when $P < 0.05$. The correlations analysis made were considered for Spearman correlation coefficient with $P < 0.05$.

3. Results and discussion

3.1. Size distribution and electric charge of particles

Strawberry yoghurt is one of the most consumed dairy products around the world. In previous studies we observed that bioaccessibility of the anthocyanins decreased significantly possibly due to the interaction between the main reactive protein in the yoghurt (β-lg) and the polysaccharides (carrageenan) present in the fruit formulation (Oliveira et al., 2015).

In order to reproduce some of the real conditions, protein concentration (β-lg 3 mg/mL) as well as anthocyanin (cy-3-gluc 0.2 mg/mL) and the polysaccharides (pectin and chitosan 3.8 mg/mL) used in the experiments were close to those values detected in commercial strawberry yoghurt formulations (Oliveira et al., 2015). The cy-3-gluc content reported in fresh strawberry was ca. 3–7 mg/100 g (Garcia-Viguera, Zafrilla, & Tomás-Barberán, 1998; Nyman & Kumpulainen, 2001; Wang, Zheng, & Galletta, 2002).

Polysaccharides are present in food products as stabilisers agents at concentrations that could vary between 2 and 7 mg/mL (Girard, Turgeon, & Gauthier, 2002). Ionic polysaccharides are usually more reactive and that is the reason why one anionic (pectin) and a cationic polysaccharide (chitosan) were selected in this study. It is also known that the content of β-lg in natural yoghurt is ca. 3 mg/mL (Farrell et al., 2004; Jovanovic, Barac, Macej, Vucic, & Lacnjevac, 2007).

3.1.1. Cy-3-gluc, β-lg and pectin systems

Fig. 1 shows the particle size distributions, measured by DLS, of β-lg alone and also in the mixtures with cy-3-gluc and pectin. The results showed that β-lg solutions when at pH 4 presented one predominant population with a diameter particle size at 6.5 nm and a minor population with higher particle size at ca. 255 nm (Fig. 1A). The predominant peak observed probably corresponds to monomers and dimers as it was reported by Martinez, Farías, and Pilosof (2010) when working with protein solutions at a concentration at 4 wt% and pH 3.5 where it was showed a bimodal distribution with a predominant lower size peak at 5 nm.

The second peak (only observed in the plot of the intensity size particle distribution) may correspond to octamers as described by Gottschalk, Nilsson, Roos, and Halle (2003) for β-lg solutions in the pH range 3.7–5.2. When looking to the intensity graphic, the mixture of β-lg with cy-3-gluc presented a new population with a relevant intensity value at 712 nm (Fig. 1C); however, the volume size distribution (Fig. 1D) revealed that this population did not represent a significant percentage of the total volume.

The intensity size distributions of the pectin solution (Fig. 1E) presented only one population with a maximum diameter value at 1281 nm. The molecular weight (MW) for this citrus pectin ranged in between 145 and 180 kDa (Morris et al., 2008) and according to the hydrodynamic diameter, calculated by the DLS software, should be close to 22 nm. This difference was observed due to the fact that pectin molecule has a strong tendency to self-associate in free salt solutions forming aggregates between 700 and 2000 nm (Lima, Soldi, & Borsali, 2009). The pectin-β-lg mixture showed an intensity size distribution with a single peak at 396 nm (Fig. 1G). The complexation of the protein molecules with pectin reduces the internal repulsion of the pectin molecules leading to a smaller hydrodynamic radius (Ganzevles, Cohen Stuart, Vliet, & de Jongh, 2006). Stronger interactions between β-lg and low methylated pectin at pH 4.5 originated mainly from ionic and hydrogen bonds, was reported by Girard et al. (2002) and Zimet and Livney (2009). At this pH the ester groups of pectin can form hydrogen bonds with the hydroxyl, amine, phenyl and carboxylic groups of β-lg (Girard et al., 2002). The formation of biopolymer complexes between β-lg and beet pectin (83 °C, 15 min, pH 5) was also reported by Jones, Decker, and McClements (2009).

The interaction of pectin with cy-3-gluc resulted in a population with a diameter size of 458 nm (Fig. 1I), while in the volume size particle distribution two populations were noticeable at 24 and 615 nm (Fig. 1J). This result could be evidence that the populations, when analysing the pectin solutions, could be broken down by the presence of the polyphenol and it was then formed two smaller populations. Buchweitz, Speth, Kammerer, and Carle (2013) described the existence of a strong interaction between anthocyanins and pectin from apple or citrus. Moreover, they reported an improvement in anthocyanins' stability during the storage due to the protective effect of the negatively charged carboxyl groups from pectin. Padayachee et al. (2012) also reported the formation of weak bonds between anthocyanins and pectin at the level of the components of the cell wall. Fernandes, Brás, Mateus, and De Freitas (2014), using other kind of polysaccharides, showed that the oxygen atoms of the glycosidic linkages of β-cyclodextrin were important in the interactions with the hydroxyl groups of cy-3-*O*-gluc leading to the formation of hydrogen bonds. Additionally some authors have also reported that covalent bonds can also be formed between phenolic acids and polysaccharides (Jakobek, 2015; Le Bourvellec et al., 2005).

Fig. 1K shows that the ternary mixtures of pectin-cy-3-gluc-β-lg presented a particle size population of a similar size (396 nm) to that observed for the mixture of pectin-β-lg (Fig. 1G), which may denote that the protein and pectin interact more strongly among them than with the polyphenol. The hydrogen bond interactions between anthocyanins sugars at the position 3 of the heterocyclic ring, the OH groups of the B ring and the planarity of the molecule are characteristics that contribute to the formation of the π–π bonds between the anthocyanins and adsorbents like lignin (Mazzaracchio, Pifferi, Kindt, Munyaneza, & Barbiroli, 2004). These authors also observed for model systems that β-lg has an adsorption effect on anthocyanins at pH 3.0 due to the hydrogen bond, the π–π hydrophobic and the electrostatic type interactions.

The β-lg presented a low net charge ($\xi = 7.41$ mV) at pH 4.0 because of the proximity to its isoelectric point (Kinsella & Morr, 1984) (Table 1). Pectin presented a negative charge of −23.04 mV (Table 1). Electrostatic attractions and further hydrophobic interactions may take place between β-lg and pectin, as they are oppositely charged. The values of *Z*-potential of this protein-polysaccharide mixture has an intermediate value ($\xi = -18.31$ mV) that is ranged in between those values obtained for the individual components suggesting that the anionic polysaccharide actually bound to the

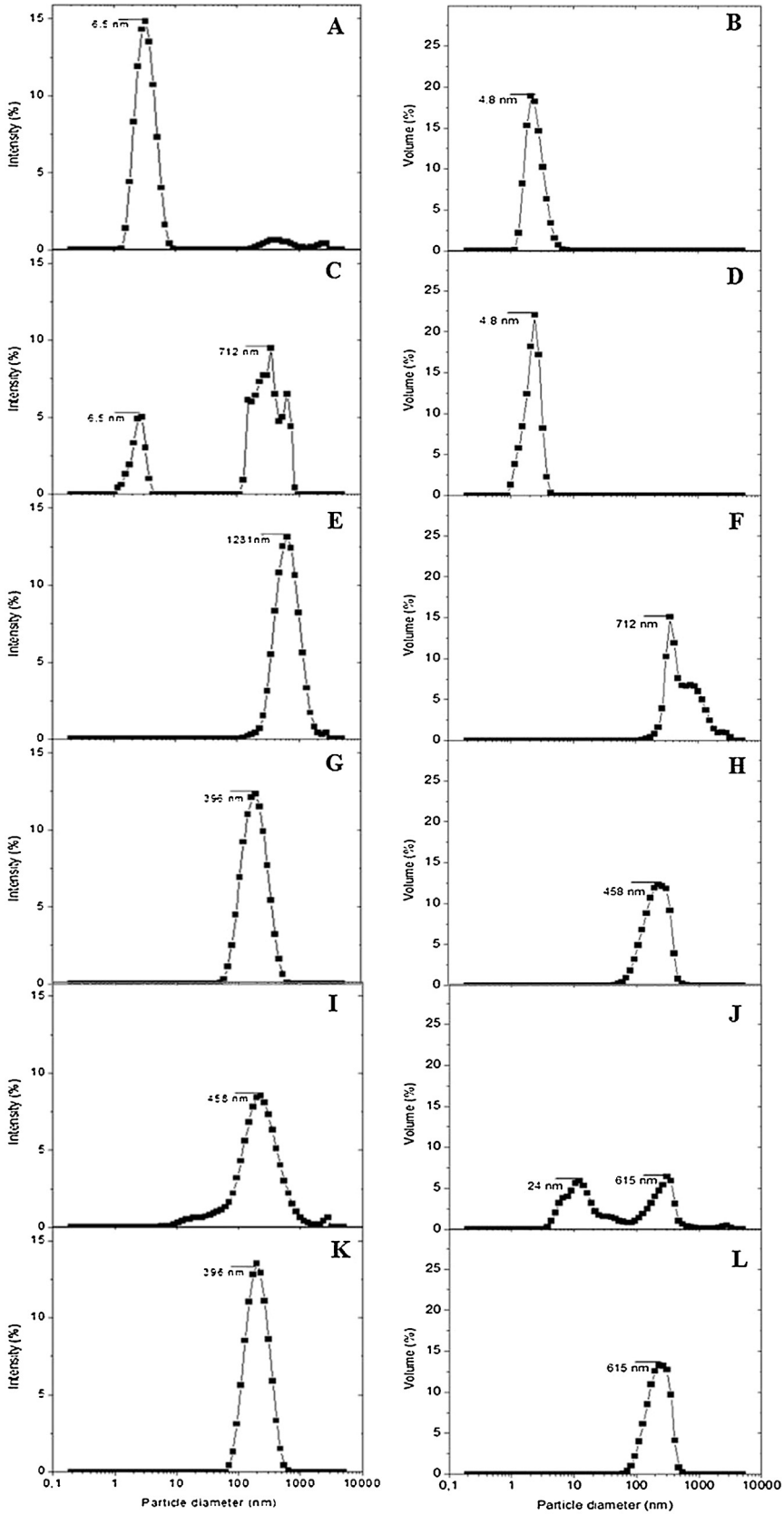

Fig. 1. Intensity (A, C, E, G, I) and volume particle size distribution (B, D, F, H, J) measured for β-lg alone and in mixture systems with cy-3-gluc and pectin at pH 4.0 (sodium acetate buffer) and at 23 °C. β-lg (A and B); β-lg-cy-3-gluc (C and D); pectin (E and F); β-lg-pectin (G and H); pectin-cy-3-gluc (I and J); β-lg-pectin-cy-3-gluc (K and L).

Table 1
Electrical charge (ξ-potential) of pectin, β-lactoglobulin and cy-3-gluc and their mixtures after 24 h.

Sample	ξ Potential (mV)
β-lg	7.41 ± 1.17^{a}
β-lg + Cy-3-gluc	13.05 ± 2.05^{a}
Pectin	-23.04 ± 1.31^{d}
Pectin + Cy-3-gluc	-14.29 ± 1.61^{b}
Pectin + β-lg	-18.31 ± 0.81^{c}
Pectin + β-lg + Cy-3-gluc	-18.23 ± 0.62^{c}

Different letters represent significant differences ($P < 0.001$) between lines.

cationic molecules of β-lg (Table 1). At this pH the interactions of cy-3-gluc with β-lg or pectin increased the positive charge (β-lg) or decreased the negative charge (pectin), pointing out an interaction of the polyphenol with both biopolymers (Table 1). Despite the potential interactions of cy-3-gluc with both molecules at this pH, the ternary mixtures of pectin-β-lg-cy-3-gluc showed a similar charge similar than the pectin-β-lg complexes that may indicate that the protein and pectin interact more strongly among them than with the polyphenol, as it was indicated previously.

3.1.2. Cy-3-gluc, β-lg and chitosan systems

The HMW chitosan presented a multimodal size intensity distribution (Fig. 2E). However, it can be deduced from the volume size distribution that it only predominates one population with a maximum value at 4.2 nm (Fig. 2F). The MW of chitosan is 624 kDa and the particles with a size at around 47 nm may be expected to exist when considering the information from the DLS software. However, the polydispersity reflects the tendency of this biopolymer to self-associate in solution (Fan, Annous, Beaulieu, & Sites, 2008) forming populations with smaller particle sizes than expected. Although it was different the intensity size distribution in a mixture of β-lg-chitosan than that for chitosan alone, no changes were detected in the volume size distribution, as they showed the same peak (Fig. 2H). The mixture of cy-3-gluc-chitosan presented one population with a maximum size volume of 18.17 nm (Fig. 2J). The increase in the particle diameter size could be related with the formation of complexes. Chang, Choi, and No (2000) described that chitosan presented the highest binding capacity at pH 3.0, causing the complexion of the flavylium cation form of the anthocyanins with the amino groups of chitosan.

On the contrary, the ternary mixtures presented one population with the same volume size of the particles (Fig. 2L) than pure chitosan (Fig. 2F) or pure β-lg (Fig. 2B), showing that interactions of cy-3-gluc with chitosan are not so relevant when β-lg is present in the mixture.

When analysing the values of ξ pure chitosan presented a positive charge of 33.38 mV (Table 2). The *Z*-potential of chitosan was not modified in the presence of cy-3-gluc or β-lg. However, the ternary mixture chitosan-β-lg-cy-3-gluc showed less charge ($\xi = 20.46$ mV) as compared with chitosan alone ($P < 0.05$) (Table 2). Thus, some of the positively charged functional groups of chitosan and β-lg could be neutralized by the negatively charged polyphenol (Zhang & Kosaraju, 2007).

Table 2
Electrical charge (ξ-potential) of chitosan, β-lactoglobulin and cy-3-gluc and their mixtures after 24 h.

Sample	ξ Potential (mV)
β-lg	7.41 ± 1.17^{c}
β-lg + Cy-3-gluc	13.05 ± 2.05^{cb}
Chitosan	33.38 ± 4.00^{a}
Chitosan + Cy-3-gluc	30.59 ± 3.09^{a}
Chitosan + β-lg	33.17 ± 2.43^{a}
Chitosan + β-lg + Cy-3-gluc	20.46 ± 4.90^{b}

Different letters represent significant differences ($P < 0.001$) between lines.

3.2. Interactions at the oil/water interface

The surface pressure (π) time evolution of β-lg, cy-3-gluc and their mixtures with pectin or chitosan at the o/w interface are shown in Figs. 3–5. These assays were performed to confirm if the complexes are indeed formed and to show how these particles behave at the o/w interface. The polysaccharides, pectin and chitosan, presented no interfacial activity (data not shown). The fast π increment for β-lg solution at the beginning of the experiment is related to the β-lg adsorption (Fig. 3A) (von Staszewski, Pizones Ruiz-Henestrosa, & Pilosof, 2014b). However, it was also visible that cy-3-gluc expresses interfacial activity (Fig. 5A).

The mixture of cy-3-gluc and β-lg presented almost no differences in the values of the surface pressure when compared to pure β-lg (Fig. 3A). Nevertheless, the rate of the migration of the protein to the interface in the presence of pectin was slowed down, although it was reached similar values of surface pressure than that for pure protein at the end of the experiment (Fig. 3C). This fact agrees with the formation of β-lg-pectin complexes, as indicated previously, as compared to pure β-lg (Fig. 1G). Thus, a possible explanation for the change in the adsorption kinetics could be that the larger size of these complexes could make slower the diffusion of these components to the o/w interface. Ganzevles et al. (2006) indicated that the complexation of β-lg with low methoxyl pectin at pH 4.5 can slow down the kinetics of the π development at the air/water interface. Ducel, Richard, Popineau, and Boury (2005) also reported a decrease in the interfacial tension, at the o/w interface, for coacervates obtained with arabic gum and pea globulin at pH 3.5. Several reasons were pointed out for the delay in the increase of π: (i) decreases in the quantity of protein molecules available for a direct adsorption; (ii) complexes diffuses much more slowly than the free state; (iii) the attachment of the bound protein to the interface may be hindered by the presence of the surrounding polysaccharide molecules and (iv) an increase of the viscosity due to the presence of a polysaccharide (Ganzevles et al., 2006).

The ternary mixture of β-lg with cy-3-gluc and pectin presented a higher interfacial activity than pure β-lg, where the value of π at the end of the adsorption increased from 12.5 to 13.5 mN/m (Fig. 3E).

The mixtures of chitosan and β-lg showed a slightly faster adsorption to the o/w interface than β-lg alone (Fig. 4C), however, the interfacial behaviour of the ternary mixture system (β-lg-cy-3-gluc-chitosan) was similar to that of pure β-lg (Fig. 4E). Guzey and McClements (2006) reported that the turbidity of the mixed β-lg–chitosan solution at pH 4 was less than that of the pure β-lg solution, suggesting the formation of a soluble complex by electrostatic attractions between the negatively charged groups remaining in the β-lg surface and the positive charge groups on the chitosan. Hong and McClements (2007) also reported the adsorption by electrostatic attraction between chitosan and β-lg on coated lipid droplets from pH 4.5 to 7.5. So, the interactions reported previously from the DLS results (size particle distribution and ξ values) affect in the interfacial behaviour observed for this ternary mixture.

The cy-3-gluc showed interfacial activity by itself (surface pressure around 11.5 mN/m), which was an unexpected result (Fig. 5A). As seen above, when cy-3-gluc interacts with β-lg the value of π was similar to that of the protein, revealing that the protein dominates the adsorption to the o/w interface. Mixtures of cy-3-gluc with pectin (Fig. 5C) and chitosan (Fig. 5E) presented a significant reduction of the interfacial activity.

With respect to the interfacial rheology, it is showed in Fig. 3 the variation of the surface dilatational modulus (*E*) of β-lg and its mixtures with cy-3-gluc and pectin with time. It can be seen that the film elasticity of this ternary mixture was practically the same than

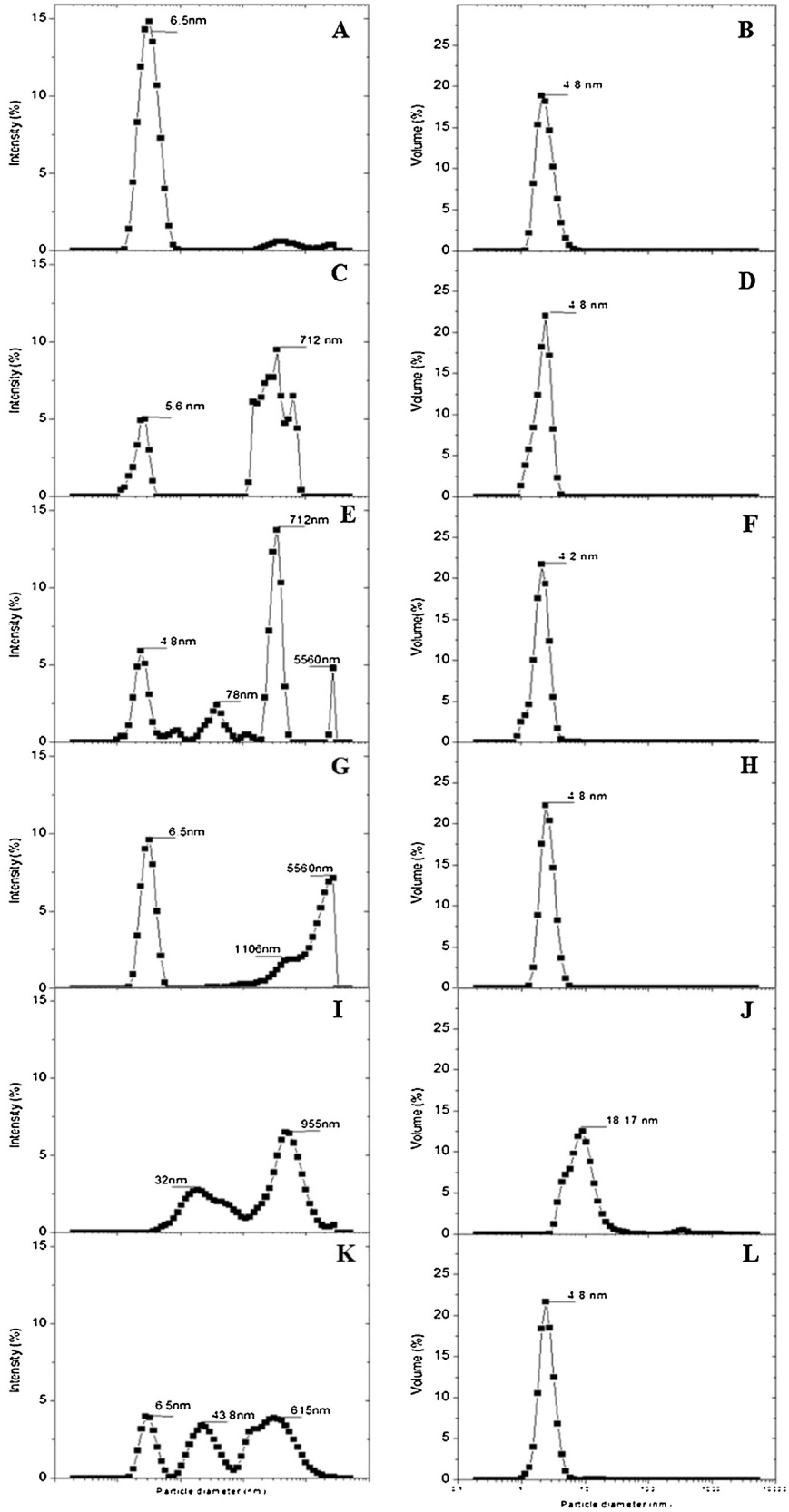

Fig. 2. Intensity (A, C, E, G, I, K) and volume particle size distribution (B, D, F, H, J, L) measured for β-lg alone and in mixture systems with cy-3-gluc and high molecular weight chitosan at pH 4.0 (sodium acetate buffer) at 23 °C. β-lg (A and B); β-lg-cy-3-gluc (C and D); high molecular weight chitosan (E and F); β-lg-high molecular weight chitosan (G and H); high molecular weight chitosan-cy-3-gluc (I and J); β-lg-high molecular weight chitosan-cy-3-gluc (K and L).

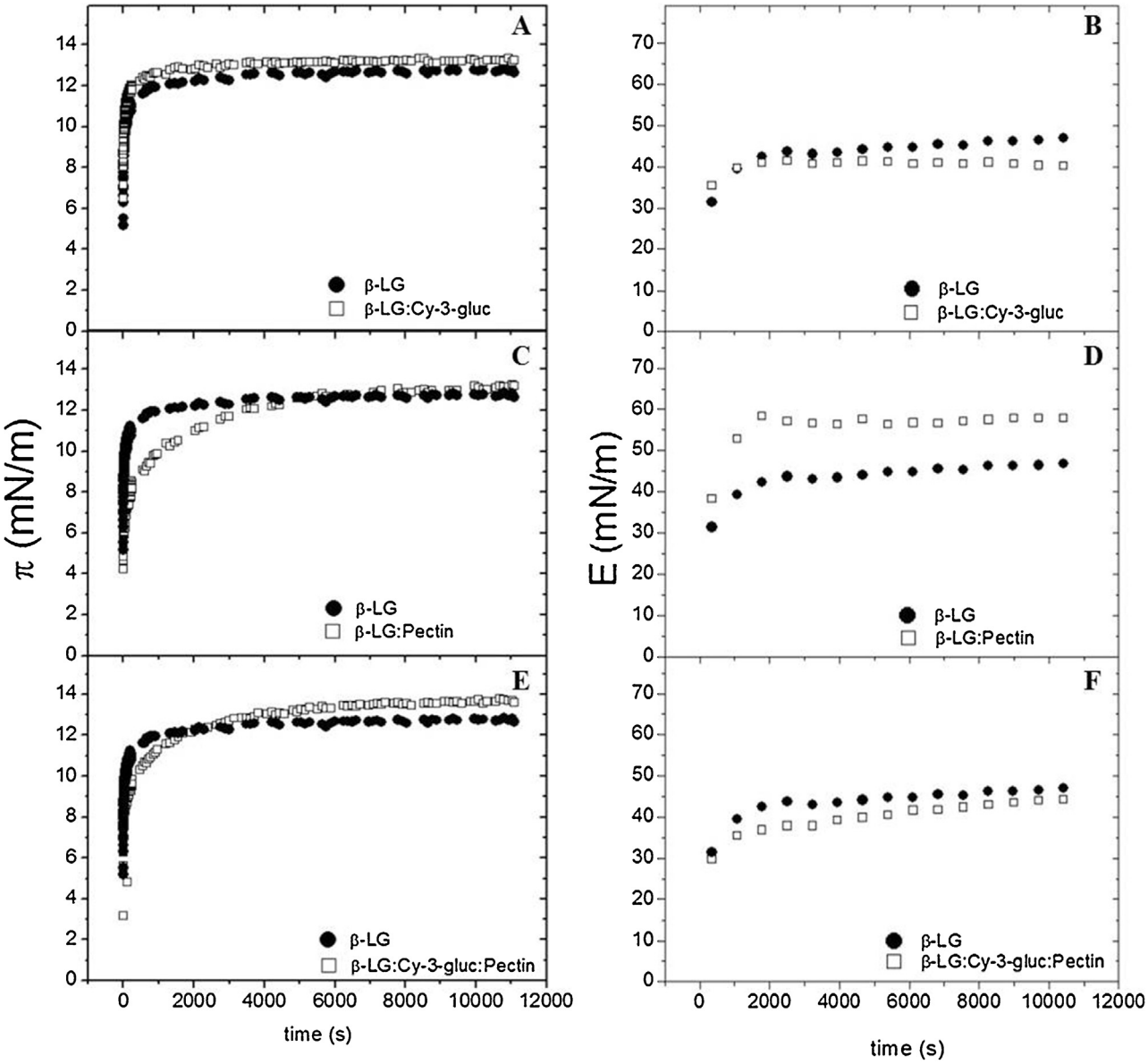

Fig. 3. Time evolution of surface pressure and dilatational modulus for β-lg alone and in mixture system with cy-3-gluc and pectin at pH 4.0 (sodium acetate buffer) at 23 °C.

that for pure β-lg (~45 mN/m), indicating that the protein dominates this parameter (Fig. 3F). The presence of pectin promoted an increase of the elasticity of the β-lg interfacial film from ~45 to ~60 mN/m (Fig. 3D). Pectin molecules formed complexes with β-lg, as seen in previous section, and once the complexes were inserted at the interface, the film became more elastic (higher values of *E*).

Fig. 4 shows the values of *E* of β-lg interfacial films and its mixtures with cy-3-gluc (Fig. 4B) and chitosan (Fig. 4D). Ternary mixture of chitosan, cy-3-gluc and β-lg formed a slightly more elastic interfacial film than pure β-lg (Fig. 4F).

Fig. 5 shows the surface dilatational modulus (*E*) of the cy-3-gluc interfacial film and the mixtures with the biopolymers. There is an increment in the values of *E* when mixing cy-3-gluc and the protein (Fig. 5B). However, the interactions between cy-3-gluc with pectin or chitosan decreased the elasticity of the cy-3-gluc interfacial films. Regarding the interfacial rheological properties, it was observed no differences between cy-3-gluc and cy-3-gluc-pectin mixtures (Fig. 5D), and a decrease of *E* from ~25 to ~10 mN/m when mixing cy-3-gluc with chitosan (Fig. 5F).

3.3. Quantification of free cyanidin-3-glucoside and β-lactoglobulin

Table 3 shows the quantification of free cy-3-gluc and free β-lg, after 24 h of incubation, as determined by HPLC–DAD. The antioxidant properties (ABTS and ORAC methods) were also assessed. Cy-3-gluc free content decreased 5 and 76% after 24 h incubation with β-lg and pectin, respectively (Table 3). Padayachee et al. (2012) reported ionic interactions between anthocyanins and pectin on purple carrot juice concentrate at pH 4. Mechanisms for polyphenol/polysaccharide associations would be similar to those for proteins, being the adsorption mediated by hydrophobic interactions and reinforced by hydrogen bonding (Le Bourvellec & Renard, 2011; McManus et al., 1985; Tang et al., 2003). From the flavonoid perspective, these interactions are modulated by characteristics such as degree of polymerization, molecular flexibility, number of external hydroxyl groups, or number of terminal galloyl groups. On the other hand, it is known that pectin has the ability to develop a gel-like network, forming hydrophobic pockets able to encapsulate procyanidins (Le Bourvellec et al., 2005).

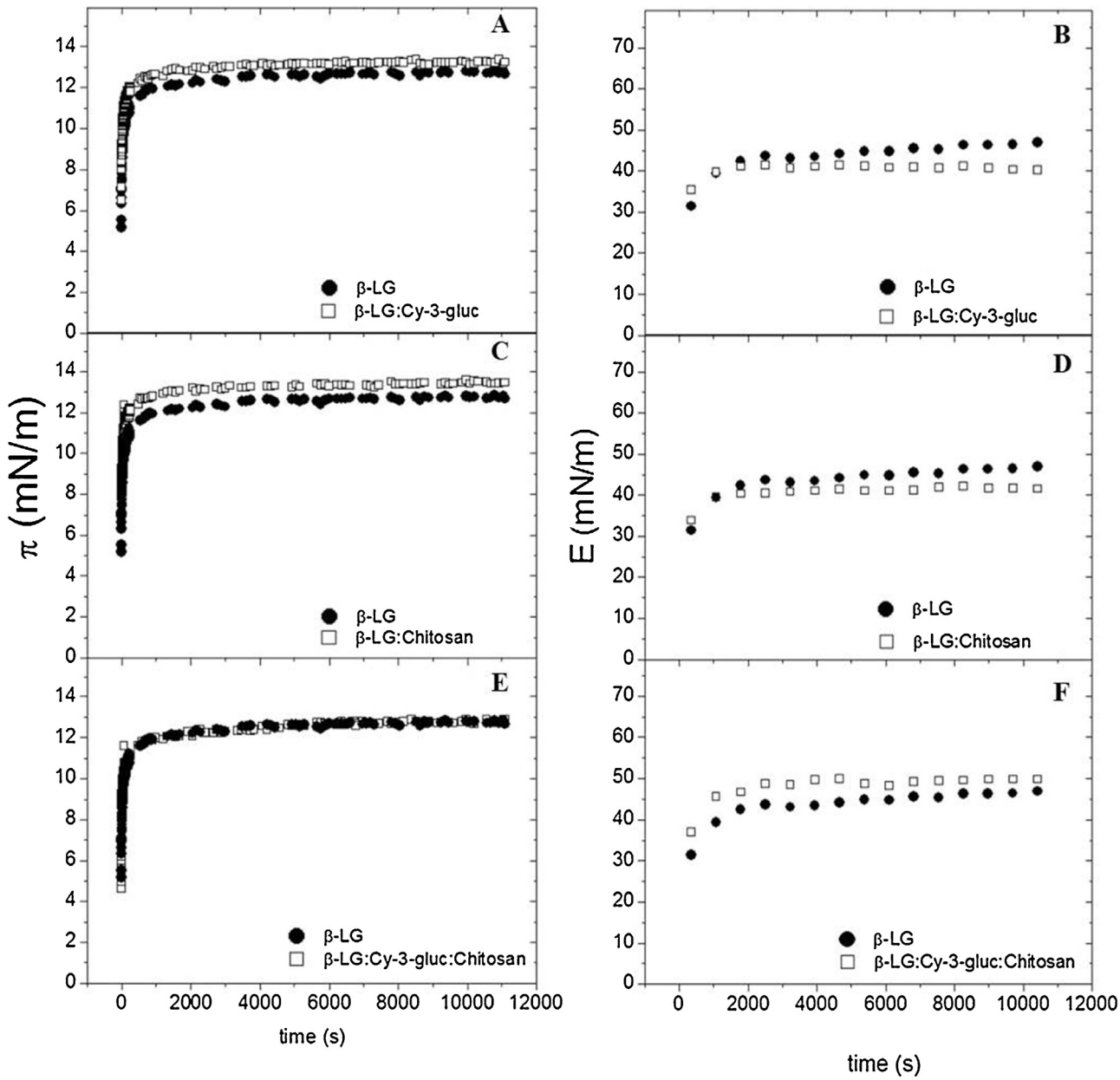

Fig. 4. Time evolution of surface pressure and dilatational modulus for β-lg alone and in mixture system with cy-3-gluc and high molecular weight chitosan at pH 4.0 (sodium acetate buffer) at 23 °C.

Considering other polysaccharides such as cellulosic supports, polyphenol retention would be primarily a surface phenomenon due to hydrophobic interactions (Ozawa et al., 1987). When analysing the ternary mixtures of cy-3-gluc-pectin-β-lg, the content of free cy-3-gluc still remains 32% lower than cy-3-gluc alone (Table 3).

The presence of protein affected polyphenol and pectin interactions by decreasing them, revealing evidences of competitive interaction between cy-3-gluc and β-lg for pectin. Ionic polysaccharides such as arabic gum, cyclodextrins, pectins and other polygalacturonic acids, such as xanthan gum, dextrans, arabinogalactans and glucose have been assessed according to their ability

Table 3
Antioxidant activity (ABTS and ORAC) and HPLC–DAD quantification of cy-3-gluc in the presence of β-lg and polysaccharides (pectin and chitosan).

Sample	ABTS (mg Ascorbic acid/mL)	ORAC (mg Trolox/mL)	HPLC–DAD (μg/mL)
Cy-3-gluc	0.283 ± 0.001^{a}	2.00 ± 0.11^{a}	185.588 ± 1.103^{a}
Cy-3-gluc + β-lg	0.289 ± 0.003^{a}	1.97 ± 0.09^{a}	176.709 ± 1.581^{b}
Cy-3-gluc + pectin	0.062 ± 0.002^{b}	0.47 ± 0.06^{b}	44.380 ± 0.288^{b}
Cy-3-gluc + chitosan	0.282 ± 0.006^{a}	2.26 ± 0.13^{a}	176.261 ± 1.47^{b}
Cy-3-gluc + β-lg + pectin	0.246 ± 0.005^{b}	0.74 ± 0.06^{b}	125.883 ± 0.596^{b}
Cy-3-gluc + β-lg + chitosan	0.279 ± 0.001^{a}	1.97 ± 0.30^{a}	168.909 ± 0.232^{b}

Different letters represent significant differences ($P < 0.001$) between free polyphenol and interactions with polysaccharides and β-lg.

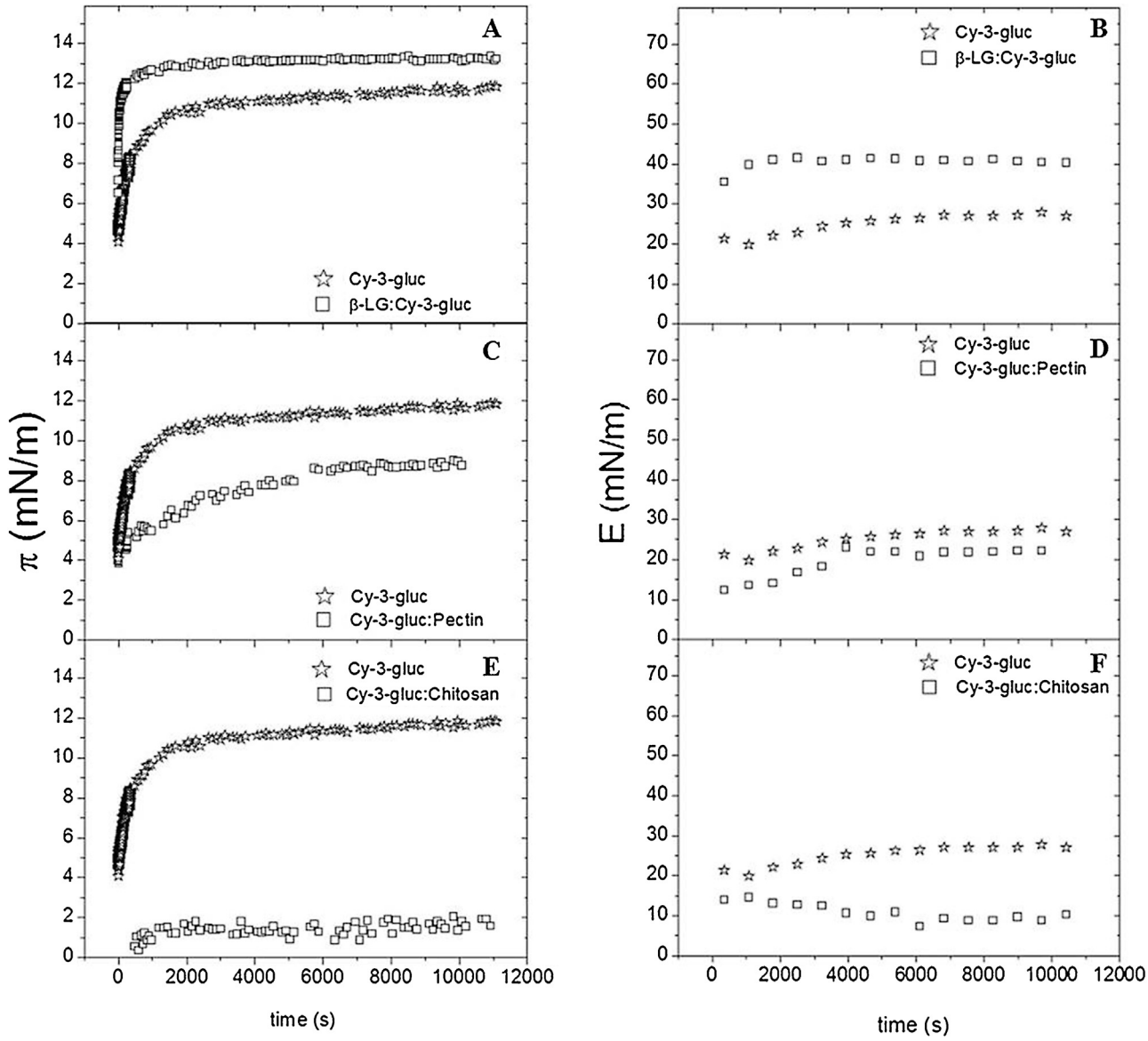

Fig. 5. Time evolution of surface pressure and dilatational modulus for cy-3-gluc alone and in mixture system with pectin and high molecular weight chitosan at pH 4.0 (sodium acetate buffer) at 23 °C.

to disrupt procyanidin–protein interactions (Carvalho et al., 2006; de Freitas et al., 2003; Mateus, Carvalho, Luís, & de Freitas, 2004; Soares, Gonçalves, Fernandes, Mateus, & de Freitas, 2009). Gum arabic can compete directly with proteins for non-specific tannin-binding, preventing the formation of aggregates (Scollary et al., 2012).

When cy-3-gluc was mixed with chitosan a decrease of 5% in free cy-3-gluc content was observed. In the ternary mixture cy-3-gluc-chitosan-β-lg the free content of cy-3-gluc decreased 9%. Thus, the interaction within the ternary mixture would be a week interaction since free cy-3-gluc was not so strongly affected (Table 3). Similar results were observed by Jing et al. (2011), who observed a decrease of 14% in free anthocyanins from radish extracts when treated with chitosan at pH 4 for 3 h. Chitosan may favour ionic interactions with flavonoids due to its naturally cationic character. Quercetin has been entrapped in chitosan particles for inclusion in aqueous media and protection against degradation (Smith & Markham, 1998; Zhang, Yang, Tang, Hu, & Zou, 2008). The complexation process between polyphenols and chitosan can be reversible or irreversible. Polyphenols and chitosan, by the development of non-covalent forces, can form soluble complexes that may aggregate and precipitate from solution. The whole process is however reversible, and under suitable conditions the precipitated complex may be re-dissolved (Popa, Aelenei, Popa, & Andrei, 2000).

In all the interactions a clear reduction on antioxidant capacity was observed for pectin mixtures (Table 3). The cy-3-gluc antioxidant activity decreased by 78% (ABTS) and 76% (ORAC) when mixed with pectin, while in the ternary mixture decreased 13% (ABTS) and 63% (ORAC) (Table 3). These results are in accordance with HPLC–DAD quantification, where the ternary mixtures allowed obtaining a higher quantity of free cy-3-gluc. The antioxidant capacity of cy-3-gluc-chitosan-β-lg mixtures presented no differences when compared with cy-3-gluc alone. Similar results were observed by Zhang and Kosaraju (2007) who obtained chitosan nanoparticles loaded with quercetin that maintain the antioxidant capacity as measured by DPPH.

The correlations between antioxidant activity and cy-3-gluc content measured through HPLC–DAD were analyzed according the Spearman correlation since data do not follow the Gaussian distribution. All the interactions presented positive correlation, where

Table 4
β-Lactoglobulin quantification by HPLC–DAD in the presence of cy-3-gluc and polysaccharides (pectin and chitosan).

Sample	HPLC–DAD (mg/mL)	Bound %
β-lg	4.41 ± 0.54^{a}	–
β-lg + Cy-3-gluc	2.84 ± 0.33^{c}	36
β-lg + pectin	3.40 ± 0.46^{b}	23
β-lg + chitosan	0.97 ± 0.13^{f}	78
β-lg + Cy-3-gluc + pectin	2.15 ± 0.51^{d}	51
β-lg + Cy-3-gluc + chitosan	1.01 ± 0.20^{e}	77

Different letters represent significant differences ($P < 0.001$) between free protein and in interactions with polysaccharides and β-lg. Bound percentage was represented as compared with the content of pure β-lg.

the antioxidant activity measured through ABTS and ORAC presented a correlation of 0.77 and 0.70 respectively with cy-3-gluc content measured through HPLC–DAD. The results revealed a good relation between antioxidant capacity and the concentration of cy-3-gluc available after the interactions.

The free content of β-lg decreased 36, 23 and 51% after incubation with cy-3-gluc, pectin and both components, respectively (Table 4). Even more reduction was observed with chitosan (78%) and in ternary mixtures of β-lg-cy-3-gluc-chitosan (77%).

Conformational changes in β-lg were reported for a complex model system with β-lg, acacia gum and quercetin at pH 4.2. The protein showed β-sheets loss because of the exposed hydrophobic aminoacids involved in the non-covalent complexation with acacia gum, which were increased by the presence of quercetin, favouring the protein–polysaccharide interaction (Aberkane et al., 2012).

4. Conclusion

Interactions between cy-3-gluc, pectin and β-lg were observed and have led to a decrease in its free content and consequently its antioxidant capacity. The β-lg forms complexes with chitosan resulting in lower quantity of free protein than that observed for mixtures with pectin. These interactions have an impact in the protein film formed at the o/w interface. Chitosan and pectin allowed to obtain a more elastic and stable interfacial film without interferences in the interfacial pressure.

In food systems, fruit polyphenols interact with matrix components like proteins and polysaccharides decreasing polyphenols availability, however, they could be protected from degradation, through the interactions with proteins and polysaccharides.

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