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Study of carbohydrate influence on protein–tannin aggregation by nephelometry

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Abstract

The formation of polyphenol/BSA aggregates was measured by nephelometry; the amount of insoluble complexes increased with BSA concentration up to a maximum turbidity value but, in the presence of excess protein, a solubilization of the complexes was observed. The stoicheiometry of the complex at the point of maximum light scattering was calculated, yielding a molar ratio, tannin/BSA, of 7:1. Increase in ionic strength by NaCl addition decreased the amount of protein/tannin aggregates, suggesting that hydrophilic forces are the main driving forces in the complexation between BSA and condensed tannins. The influence of the following carbohydrate concentration on the interactions of BSA with procyanidin compounds was assayed using nephelometry: dextran, glucose, arabinogalactan, β-cyclodextrin, pectin, gum arabic, polygalacturonic acid and xanthan. Overall, carbohydrate concentration induced a solubilization of the protein/tannin complexes, with neutral and ionic polysaccharides displaying different behaviours in this process. Pectin, xanthan, polygalacturonic acid and gum arabic were much more effective in solubilizing the protein/tannin aggregates than glucose, dextran, β-cyclodextrin or arabinogalactan.

Keywords: Carbohydrates; Nephelometry; Grape seeds; Procyanidin; Tannins; BSA; Ionic strength

1. Introduction

Astringency is believed to be due to interactions between polyphenols and salivary proteins, resulting in insoluble aggregates that precipitate, obstructing the palate lubrication and causing a sensation of dryness and constriction. This phenomenon is supposed to involve hydrophobic effects and hydrogen bonding (Asano, Shinagawa, & Hashimoto, 1982; Charlton et al., 2002; Hagerman & Butler, 1981; Haslam, 1996; Luck et al., 1994; Oh, Hoff, Armstrong, & Haff, 1980; Sarni-Manchado, Cheynier, & Moutounet, 1999).

Polyphenol complexation with proteins has largely been studied in solution by NMR spectroscopy (Baxter, Lilley, Haslam, & Williamson, 1997; Murray, Williamson, Lilley, & Haslam, 1994), microcalorimetry (Beart, Lilley, & Haslam, 1985), enzyme inhibition (Goldstein & Swain, 1965) and by protein precipitation (Asquith & Butler, 1985, 1986; Bate-Smith, 1973; Bradford, 1976; Chapon, 1993; Chapon, Chollot, & Urion, 1961; Hagerman & Butler, 1978, 1980). Nephelometry was one of the first techniques used, but it has only recently been reintroduced to study the effect of polyphenol structure on its ability to bind proteins (Chapon, 1993; Chapon et al., 1961; de Freitas & Mateus, 2001, 2002). Nephelometry has proved to be a very attractive analytical technique since it gives direct measurements and is very sensitive and selective.

The interaction between tannins and proteins is affected by the medium composition, namely by the presence of other co-substrates such as polysaccharides (Haslam, 1998; Luck et al., 1994; McManus et al., 1985; Ozawa, Lilley, & Haslam, 1987).

Several studies have demonstrated the ability of some neutral and anionic polysaccharides to disrupt the binding of polyphenols to proteins (Ozawa et al., 1987). Pectin, gum arabic, carragenan, xanthan and gellan are effective inhibitors of protein precipitation whilst carob, guar and tara gum are not (Luck et al., 1994). This

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phenomenon probably results from the ability of polysaccharides to form a ternary complex protein/polyphenol/carbohydrate, which enhances solubility in an aqueous medium, or from the molecular association in solution between carbohydrates and polyphenols, competing with protein aggregation. Additionally, Haslam and co-workers proposed that some polysaccharides have the ability to develop a secondary structure in solution, forming hydrophobic pockets able to encapsulate and complex polyphenols (McManus et al., 1985; Ozawa et al., 1987).

Carbohydrate adsorption by polyphenols could be explained by cooperative hydrogen bonding between the oxygen atom of the carbohydrate and the phenolic hydroxyl group and by hydrophobic interactions. Electrostatic and ionic forces, which in general involve charged molecules, do not appear to be determinant since polyphenols (e.g. condensed tannins) have negligible negative charges (Vernhet, Pellerin, Prieur, Osmianski, & Moutounet, 1996).

The aim of this work is to provide insights of the process whereby polysaccharides inhibit grape seed procyanidin complexation and aggregation by bovine serum albumin (BSA).

2. Materials and methods

2.1. Reagents

BSA fraction V, (96% of purity), pectin from citrus fruit, xanthan gum and polygalacturonic acid (potassium salt) from citrus fruit (minimum 80%) were purchased from Sigma Chemical Co® (St Louis, MO, USA). Acacia gum and arabinogalactan were from Aldrich Chemical Co® (Dorset, UK). Dextran, from *Leuconostoc spp.* ($M_r \sim 40\,000$), and β-cyclodextrin (⩾99% purity) were from Fluka Chemie® (Buchs, Switzerland).

2.2. Grape seed extract

2.2.1. Fractionation of grape seed procyanidins

Condensed tannins were extracted from *Vitis vinifera* grape seed tissues with an ethanol/water/chloroform solution (1:1:2, v/v/v) using a blender (Ultra-Turrax) as described elsewhere (Darné & Madero-Tamargo, 1979). The 50% aqueous ethanol upper layer, containing polyphenols, was separated from the chloroform layer containing chlorophylls, lipids and other undesirable compounds. Ethanol was removed using a rotatory evaporator and the resulting aqueous solution, containing the polyphenolic compounds, was extracted with ethyl acetate, followed by precipitation with hexane, in order to obtain the procyanidin oligomers, according to the procedure described in the literature (Michaud, Lacaze, & Masquelier, 1971). The extract was firstly purified on a TSK Toyopearl HW-40(s) Gel column (100×10 mm i.d., with methanol 0.8 ml min^{-1} as eluent) in order to separate oligomeric and polymeric procyanidins (de Freitas, Glories, Bourgeois, & Vitry, 1998). The first fraction of 400 ml eluted, corresponding to the elution of catechin and oligomeric procyanidins, was collected, concentrated and applied in a similar column of greater length (100×25 mm i.d., with methanol 0.8 ml min^{-1} as eluent). The first 120 ml, corresponding to the elution of catechin monomers, were eliminated, and elution was followed over 10 h in order to elute the procyanidin oligomers. This last fraction was mixed with deionised water; the solvent was removed using a rotatory evaporator under reduced pressure at 30° and then freeze-dried.

LSI/MS analysis of the resulting solid revealed that it was composed essentially of procyanidin oligomers up to pentamers, together with several galloyl and digalloyl esters (de Freitas & Mateus, 2002): monomers, gallate ($[M+H]^+$ at $m/z=443$); dimers (579); dimers, gallate (731); trimers (867); dimers, digallate (883); trimers, gallate (1019); tetramers (1155); tetramers, gallate (1307); pentamers (1443); pentamers, gallate (1595). The mean molecular weight (g mol^{-1}) of the molecules in that fraction was estimated to be $M_w=1002$, by calculating the average of the different M_ws of each group of procyanidin identified.

2.3. Nephelometry

Nephelometry experiments were performed in a HACH 2100N Laboratory Turbidimeter. The optical apparatus was equipped with a tungsten-filament lamp with three detectors: a 90° scattered-light detector, a forward-scatter light detector and a transmitted light detector. Previous calibration was performed using a Gelex® Secondary Turbidity Standard Kit (HACH, Loveland, USA), which consisted of stable suspensions of a metal oxide in a gel.

2.4. Nephelometric study of procyanidin/BSA interactions

The experimental conditions correspond to those described in the literature (de Freitas & Mateus, 2001, 2002). Procyanidins were dissolved in ethanol/water (12% v/v), acetate buffer solution (0.1 M, pH 5.0). Two mililitres of the solution of procyanidin oligomers (0.10 mM) were introduced in a turbidimetric cell for each assay. Following this, different volumes of an aqueous BSA solution (0.30 mM) were added to each tube (20, 40, 60, 100, 140, 180 and 220 μl) and the mixture was mixed in a vortex and stored at room temperature. Haze formation was monitored during 60 minutes after addition of BSA, until stabilization was achieved. This pro-

cedure allowed the determination of the amount of protein needed to react with the procyanidin oligomers. Under these conditions, the control solutions showed that the solvent did not precipitate proteins in the absence of tannins and no precipitate was observed in the polyphenol solutions without any protein, even with higher concentrations than those used in the experiments. All experiments were performed in quadruplicate.

The influence of ionic strength on haze formation was determined by changing NaCl concentration (0, 0.1, 0.2, 0.4 and 0.5 M) in the procyanidin solution (0.10 mM). The mixture was allowed to stand for at least 30 min before the addition of 80 μl of an aqueous BSA solution (6.1×10^{-3} mM).

2.5. Influence of carbohydrates on procyanidin/BSA interaction

Procyanidin solutions 0.10 mM (ethanol/water, 12% v/v, acetate buffer, pH 5.0) were prepared with different concentrations of carbohydrates. The mixture was allowed to stand for at least 30 min before the addition of 80 μl of an aqueous BSA solution (6.1×10^{-3} mM).

It is important to note that, under the experimental conditions described, control experiments practically did not induce any polyphenol/carbohydrate precipitation in the absence of protein. In general, the carbohydrates studied and BSA were co-soluble, especially at low carbohydrate concentration. In some cases (pectin, arabinogalactan, β-cyclodextrin, dextran) and when the concentration of carbohydrate was high, the procyanidin–carbohydrate solution became slightly cloudy and, in this case, these reference values were subtracted from the one obtained after protein addition, to suppress this interference.

3. Results and discussion

3.1. Evaluation of protein–tannin interactions

The interaction between condensed tannins and BSA was directly determined in solution by measuring the amount of tannin/BSA insoluble complexes, using the nephelometric technique. When both components were mixed, the amount of insoluble aggregates, dispersed in solution, increased quickly up to a maximum value, which thereafter remained practically unchanged. This behaviour is apparently characteristic of the interaction between condensed tannins and BSA, and the maximum level of precipitate depends on the relative concentration of tannins and proteins but also on the pH, ionic strength and solvent composition (Calderom, Van Buren, & Robinson, 1968). The pH was chosen at a value of 5.0 (acetate buffer), where BSA has been shown to be stable and to have maximum ability to bind and precipitate condensed tannins as described previously (de Freitas & Mateus, 2001).

Preliminary experiments were performed, aiming to establish the stoicheiometry of the tannin/BSA aggregates. A weak concentration of tannins (0.10 mM) was chosen in order to obtain results in a range of 0–10 NTU units, where the light absorption is negligible, the refractive index of solvated particles is small and practically identical to that of the solution, and in this case, the intensity of the scattered light is proportional to the concentration of dispersed insoluble aggregates (Chapon, 1993). Fig. 1 shows the influence of BSA concentration on the amount of insoluble aggregates formed. The tannin/BSA aggregates dispersed in solution increased with the addition of protein up to a maximum, after which no further precipitation occurred. From this point, the progressive addition of BSA caused a re-solubilization of the insoluble aggregates. This phenomenon has already been referred to by other authors (Siebert, 1999; Siebert, Troukhanova, & Lynn, 1996; Luck et al., 1994; Haslam & Lilley, 1988) and could be explained by the reversibility of the tannin/protein aggregate formation and by the changing stoicheiometry of the complex formed, resulting from changes of the polyphenol/protein ratio.

Siebert (1999, Siebert et al., 1996) has proposed a model to explain this behaviour: proteins are supposed to have a number of sites to which polyphenols can bind. Polyphenols, on the other hand, are supposed to have a number of ends that can bind to proteins. When the concentration of polyphenol ends is equal to the number of protein binding sites, a large network is formed, corresponding to the largest complexes and resulting in maximum light scattering. When a large excess of protein is present, each polyphenol molecule should be able to bridge some protein molecules, but it

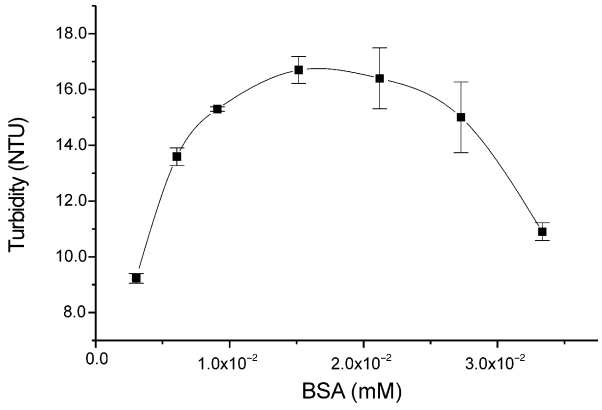

Fig. 1. Influence of BSA concentration on its ability to form insoluble aggregates with oligomeric tannins (0.10 mM) in ethanol (12% v/v) aqueous buffer solution at pH 5.0. All results are means of four replicates.

is unlikely that there would be sufficient excess polyphenols to bridge between these complexes. This would result in small aggregates and less light scattering.

It was possible to estimate the stoicheiometry of the insoluble complex from the relative concentration of tannin and BSA corresponding to the maximum of insoluble tannin/protein aggregates. When the concentration of tannins was about 6.6 times that of the BSA, the maximum of aggregates was obtained, indicating a molar ratio of polyphenol/BSA of 7:1.

Hagerman, Rice, and Ritchard (1998) obtained a molar ratio of polyphenol/BSA of 20:1 at pH 4.9 for a complex between procyanidin dimer B1, epicatechin-(4-8)-catechin, and BSA. This procyanidin has a molecular weight of 578, which is approximately half of the average molecular weight of the oligomers used in this experiment. Therefore, this result appears to agree with the stoicheiometry referred to, taking into account the different molecular weights.

In the following experiments an amount of BSA slightly below the stoicheiometric quantities was adopted in order to ensure that all the protein complexes with polyphenols.

3.2. Influence of ionic strength on the protein–tannin interactions

The influence of ionic strength on the amount of insoluble aggregates formed between protein (6.1×10^{-3} mM) and tannin (0.10 mM) was studied at pH 5.0 (Fig. 2). Several assays were performed with different ionic strengths adjusted by addition of sodium chloride from 0.07 (corresponding to the ionic strength resulting from the buffer ions) to 0.5. The insoluble aggregates decreased regularly with the increase in ionic strength. The BSA precipitation by tannins was very sensitive to ionic strength, with an 80% decrease in precipitate when the ionic strength increased from 0.07 to 0.5.

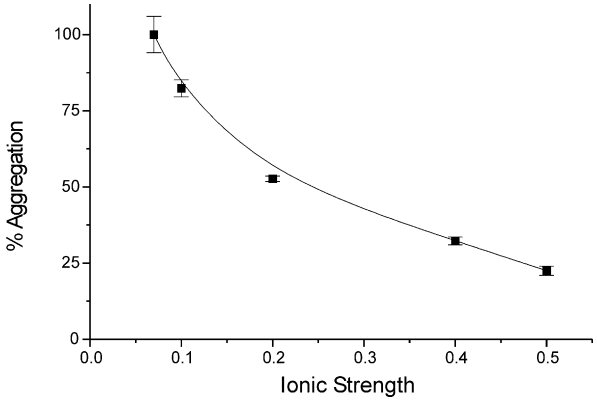

Fig. 2. Influence of ionic strength on the formation of insoluble aggregates between BSA (6.1×10^{-3} mM) and oligomeric procyanidins (0.10 mM) in the water/ethanol 12% (v/v) buffer solution at pH 5.0. All results are means of four replicates.

This result appears to contradict some reports where the augmentation of the ionic character of the medium decreases the protein/polyphenols solubility (Kawamoto & Nakatsubo, 1997; Luck et al., 1994; Siebert et al., 1996). Nevertheless, the experimental conditions studied in these reports were different and the polyphenols used were mainly gallotannins, which are supposedly less polar than condensed tannins (Hagerman et al., 1998). Therefore the driving forces between gallotannin and protein interactions are supposed to be essentially hydrophobic.

From the present results, it seems evident that the aggregation phenomena between condensed tannins and BSA are importantly affected by ionic factors, suggesting that hydrophilic interactions are dominant over hydrophobic ones, as proposed by Hagerman et al. (1998). These latter, are supposed to result from the interaction between the aromatic rings and the carbon–hydrogen skeleton of the pyranic ring of condensed tannins with proteins. In general, procyanidins are weak acids (pK_a 9–10), indicating practically no negative charges in its structure at pH 5.0 (Vernhet et al., 1996). Two possible explanations could be the adsorption or fixation of the ions, mainly chloride anions, from the solution directly onto the tannin hydrophobic surface, restraining their interactions and especially the hydrogen-bond cross-linking, or the ion fixation directly onto the tannin/BSA complex surface, giving rise to an ionic monolayer with the resulting complex becoming more hydrophilic than the protein/tannin itself, leading to solubilization.

3.3. Influence of carbohydrates on protein–tannin interactions

3.3.1. General

The protective effect of different carbohydrates against tannin precipitation by BSA was evaluated by measuring the decrease of the solution turbidity with the progressive addition of carbohydrate. The ionic strength was set at 0.07, without any NaCl addition, as it corresponds to the maximum tannin ability to form insoluble aggregates with BSA.

Several assays were performed with the progressively increased concentrations of several carbohydrates, until practically no changes in the amount of insoluble tannin/protein aggregates were observed (Fig. 3a–c). In general, the presence of the carbohydrates in solution restrained the formation of insoluble aggregates. The only exception was observed for dextran, for which the amount of aggregates precipitated tended to remain constant or to slightly increase. Other authors have already described the tendency of the polyphenol/protein aggregates to re-solubilize in the presence of carbohydrate (Luck et al., 1994; Ozawa et al., 1987).

Overall, the % of insoluble aggregates decreased linearly with the addition of the carbohydrates at the lowest

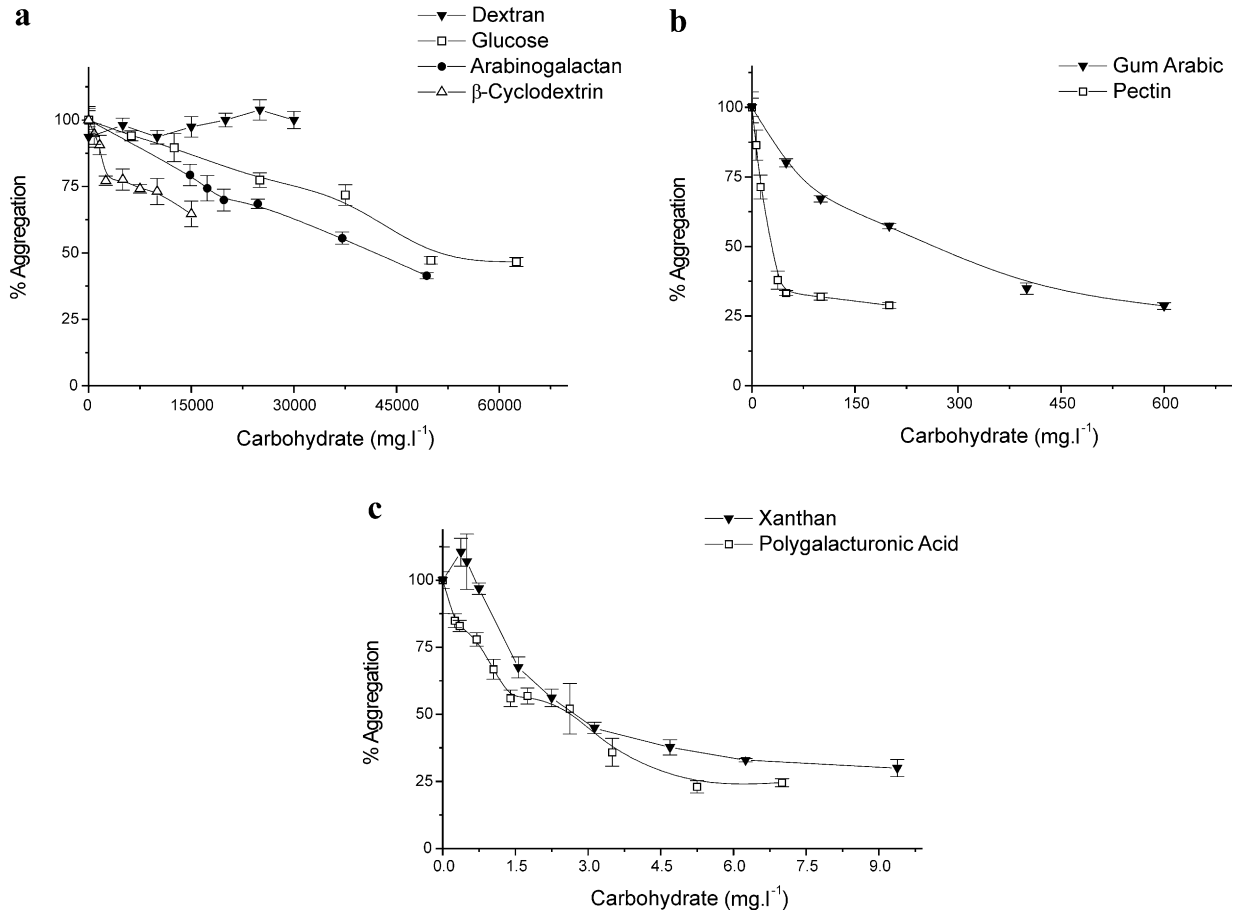

Fig. 3. Influence of carbohydrate concentration on the ability of oligomeric procyanidins (0.10 mM) to form insoluble aggregates with bovine serum albumin (BSA: 6.1×10^{-3} mM) in the ethanol/water 12% (v/v) buffer solution, pH 5.0. All results are means of four replicates.

concentrations, allowing the calculation of the slopes of the linear regressions. These "decrease constants" represent the solubilization constants (K_S) of the tannin/BSA insoluble aggregates by the carbohydrate (Table 1). K_S, expressed in NTU per unit of concentration (mg l^{-1}) of carbohydrate, depends greatly on the carbohydrate structure. In fact, carbohydrates must have a suitable structure and composition (ionic character), as well as a sufficient size and flexibility to be able to complex polyphenols.

Table 1
Solubilization constants (K_S) of the tannins/BSA insoluble aggregates by carbohydrates

	K_S (NTU/mg l^{-1})	r^2
Glucose	0.77	0.98
Arabinogalactan	1.5	1.00
β-Cyclodextrin	5.9	0.99
Gum arabic	329	0.98
Pectin	1610	0.98
Xanthan gum	30 219	0.98
Polygalacturonic acid	24 225	0.94

3.3.2. Neutral carbohydrates

The concentration of a neutral carbohydrate, such as glucose, β-cyclodextrin or arabinogalactan needed to reduce the % of aggregation is much higher when compared to the other carbohydrates studied, which are supposed to have an ionic character. This result confirms the low affinity of neutral carbohydrates for complex polyphenols, as recently reported by other authors (Haslam, 1998; Luck et al., 1994).

The association of the lower molecular weight carbohydrates, glucose and β-cyclodextrin, with tannins is supposed to be primarily a surface effect, resulting from a co-operative hydrogen-bonding interaction between the hydroxyl groups of these carbohydrates and the phenolic hydroxyl groups of tannins. Low molecular weight polysaccharides are too small to effectively cross-link polyphenols and restrain their association with proteins. With increasing concentration, these carbohydrate molecules adsorb onto the tannin surface (associative interaction) and the complex protein/polyphenol/carbohydrate is solvated by the hydrophilic character of the carbohydrate components, leading to their solubilization. The ability of β-cyclodextrin to

encapsulate and complex small molecules inside its hydrophobic structure (Ozawa et al., 1987; Saenger, 1980) does not seem to be effective in this case probably because of the relatively high M_w of the tannin molecules.

Dextran is not sufficiently soluble and steric effects might limit the number of available binding sites to readily fit polyphenols. Neutral polysaccharides usually have a relatively low solubility due to the existence of a large number of hydrogen bonds, which stabilize intrachain and interchain interactions (Rinaudo, 2001). The slight increase of the % of aggregates could be explained by an increase of the tannin/BSA aggregate size, resulting from the adsorption of some dextran molecules, which would not be enough to dissolve the complex.

3.3.3. Ionic carbohydrates

The anionic carbohydrates such as pectin, xanthan, polygalacturonic acid and gum Arabic, are more effective, suggesting again that hydrophilic interactions are dominant, as previously concluded for the influence of the ionic strength.

Pectin is less effective, in the decrease of the % of aggregates, than xanthan or polygalacturonic acid, which have a higher ionic character. Effectively, xanthan has a structure consisting of a linear β-D-glucose "backbone" with a charged trisaccharide side chain attached every second main chain glucose residue, and polygalacturonic acid consists of a linear (1-4)-poly-α-D-galacturonic acid polymer whereas pectin is a chain-like polymer consisting of α-D-galacturonic structural units with its carboxyl groups partially esterified with methanol, which reduces its polarity.

The gum arabic structure consists of an arabinogalactan-type polysaccharide with some galacturonic acid residues. It occurs as a weakly acidic molecule, which could explain the lower value of the K_S than for the other ionic carbohydrates.

Besides the formation of soluble ternary complexes, another explanation for the differences of behaviour between carbohydrates could be their ability to develop a gel-like structure in solution, able to encapsulate polyphenols and prevent their interaction with proteins, as suggested for xanthan and polygalacturonic acid (Haslam, 1998; Ozawa et al., 1987). Xanthan is believed to form a gel-like network by lateral association of ordered chain sequences (Norton, Goodal, Frangou, Morris, & Rees, 1984) and the resulting pores would have an adequate size to incorporate the relatively low molecular weight tannins.

The unusual slight increase of aggregate formation observed when xanthan was present in small concentrations could be explained by an increase in aggregate size, resulting from the adsorption of insufficient molecules to dissolve the complex, as mentioned for dextran. Alternatively, at these concentration ranges (0–0.7 mg l^{-1}) it could be that the number of xanthan molecules is insufficient to develop a secondary gel-like structure able to encapsulate polyphenols.

4. Conclusion

The natural loss in astringency observed during fruit maturation has been attributed to the depolymerization of pectin of the cell wall in water-soluble fragments, which would compete with salivary proteins when tasting (Goldstein & Swain, 1963; Haslam & Lilley, 1988; Ozawa et al., 1987). This work clearly demonstrates the ability of pectin to disrupt the tannin/protein complex aggregates. Some beverages, such as wines, have a relatively important level of structural polysaccharides, such as water-soluble pectin fragments (e.g. rhamnogalacturonans I and II, arabinogalactan II), which probably lead to a corresponding decrease in the perception of astringency (Riou, Vernhet, Doco, & Moutounet, 2002).

Besides the sweetish properties of some small carbohydrate molecules, present at high levels in foods and beverages, such as fructose (fruit juice) and glucose (Port wines), the results reported herein indicate to a possible additional contribution for reducing the astringent response of polyphenols, as was already proposed for pectins.

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