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Structure–Activity Relationships in the Hydrophobic Interactions of Polyphenols with Cellulose and Collagen

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Abstract: *Polyphenol interactions with both cellulose and collagen in the solid state have been studied by using chromatography on cellulose and by evaluating the hydrothermal stability of the polyphenol treated sheepskin collagen. Twenty-four polyphenolic compounds were studied, including seven glucose-based gallotannins, five polyalcohol-based gallotannins, and twelve ellagitannins. In the cellulose–polyphenols systems, the polyphenol's affinity to cellulose is positively correlated with their molecular masses, the number of galloyl groups, and their hydrophobicity (log*P*). The polyphenol treatment increased the hydrothermal stability of collagen samples, and such effects are also positively correlated with the molecular masses, total number of galloyl groups and the hydrophobicity of polyphenols. Ellagitannins showed much weaker interactions with both biopolymers than gallotannins having similar molecular mass, the same number of galloyl groups, and the same number of phenolic hydroxyl groups. It is concluded that, for the polyphenol interactions with both cellulose and collagen, (1) the galloyl group of polyphenols is the functional group; (2) the strength of interactions are positively correlated with molecular size, the number of galloyl groups and the hydrophobicity of polyphenols; (3) the hydrophobic interactions are of great significance; and (4) the interactions are strongly dependent on the flexibility of galloyl groups.* © 2003 Wiley Periodicals, Inc. Biopolymers 70: 403–413, 2003

Key words: *polyphenols; hydrolysable tannins; gallotannins; ellagitannins; collagen; cellulose; chromatography; hydrophobic interactions; shrinkage temperature; differential scanning calorimetry*

INTRODUCTION

Interactions between vegetable tannins and biopolymers, such as polysaccharides and proteins, are important in plant physiology,[1] food science,[2] biological activity of polyphenols,[3] chromatography of tannins,[4–8] and leather science.[9,10] Vegetable tannins are now more frequently referred to as plant polyphenols.

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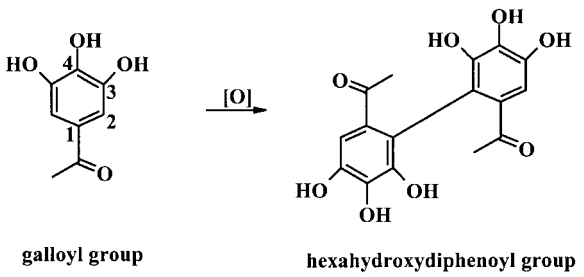

FIGURE 1 Structural fragments of hydrolysable tannins.

In food, the interaction is important in both nutritional and antinutritional aspects[2,11] due to the fact that biopolymers and polyphenols coexist in the food from the plant sources. More recently, many articles have been published on the bioactivities of plant polyphenols such as antioxidative,[12–16] antiinflammatory,[17] antimutegenesis,[18] antiviral activities,[19] and anticarcenogenesis.[20] In the leather tanning processes, the function of plant polyphenols have been realized and applied for a long time, tracing back to the ancient Egypt and China, which earned this group of phytochemicals the name *vegetable tannins.* Based on polyphenol–polysaccharide interactions, polysaccharides have been employed in chromatography of polyphenols.[4,5,21] For these reasons, plant polyphenols (vegetable tannins) have become the topic of numerous research studies in terms of their structure, activity, and applications.

According to their structural characteristics, plant polyphenols or tannins were, traditionally, classified into three groups, namely, condensed tannins, hydrolyzable tannins,[21] and complex tannins.[22,23] However, another group of tannins, phlorotannins, have also been reported recently.[24] In the modern organic chemistry context, the condensed tannins are more frequently referred to as proanthocyanidins[25] and they are essentially oligomeric derivatives of flavan-ols such as catechin and epicatechin. Hydrolysable tannins are esters of gallic acid (3,4,5-trihydroxybenzoic acid) and its derivatives (Figure 1). They can be subdivided into two categories, gallotannins,[6] which are esters of gallic acid, and ellagitannins, which, named after ellagic acid, are esters of hexahydroxydiphenoic acid[7,8,25] (Figure 1). Ellagitannins differ from gallotannins in which ellagitannins have intergalloyl C—C linkages probably resulting from oxidation coupling[7,8] of gallotannins as shown in Figure 1. The complex tannins have structural characteristics of both the above two classes in the same molecules.[22,23] The phlorotannins are polymeric forms of phloroglucinols (1,3,5-trihydroxbenzene) found in algal.[24]

Due to the widespread presence of tannins in the plant kingdom and their important functions in the aforementioned areas, the interactions between polyphenols and other biomolecules, such as carbohydrates,[26–28] proteins,[25,29,30] lipids,[31] and nuclei acids,[32] have been extensively studies in the solution states. It is now known that, in the solution state, polyphenol interactions with polysaccharides and proteins are chiefly hydrogen-bonding and hydrophobic interactions.[25–30] Both molecular size and the conformational flexibility of polyphenols are important factors affecting the strength of protein–polyphenol interactions.[25–30] The relative importance of the two modes of interactions is dependent on the structural characteristics of biopolymers and polyphenols.

In the solid state, however, the interactions between polyphenols and biopolymers were not thoroughly characterized although, in many cases, the polyphenol–biopolymer interactions are more relevant in this state. For example, in the leather tanning process, plant polyphenols (tannins) are used to treat collagen matrices so as to improve the hydrothermal stability and mechanical properties of the collagen–fiber matrix (leather).[9] The efficacy is essentially dependent on the interactions between the plant polyphenols (or tannins) and collagen, and subsequent formation of multiple-site cross-links between collagen molecules in the fibrils.[9] Given the long history of this tanning process, the mode of action of the polyphenol–collagen interactions remains poorly understood.

In another example, cellulose has been employed as a stationary phase in chromatography of plant polyphenols for decades; the characteristics of the interactions between cellulose and polyphenols, which were only broadly described as "surface phenomena,"[27] remain insufficiently researched. A chromatographic study concluded that both hydrogen-bonding and hydrophobic interactions might be important for cellulose–tannin and collagen–tannin systems under certain circumstances.[33] While the approach was useful, the study suffered from the ambiguity due to employment of the tannins extracts which are complex mixtures.[33] Lack of defined molecular properties has also made it impossible to obtain any information on structure–activity relationships. A more recent study, aimed to provide background information for polyphenol interactions in the solution state, provided some useful evidence of cellulose–polyphenol interactions with both chromatographic data on cellulose and adsorption isotherm to cellulose acetate.[27] However, there is still a need for detailed investigation on both the nature of interactions and the relative merits of the hydrogen-bonding and hydrophobic interactions.

In order to understand more about the interactions between plant polyphenols (tannins) and biopolymers in the solid state, research has been carried out for

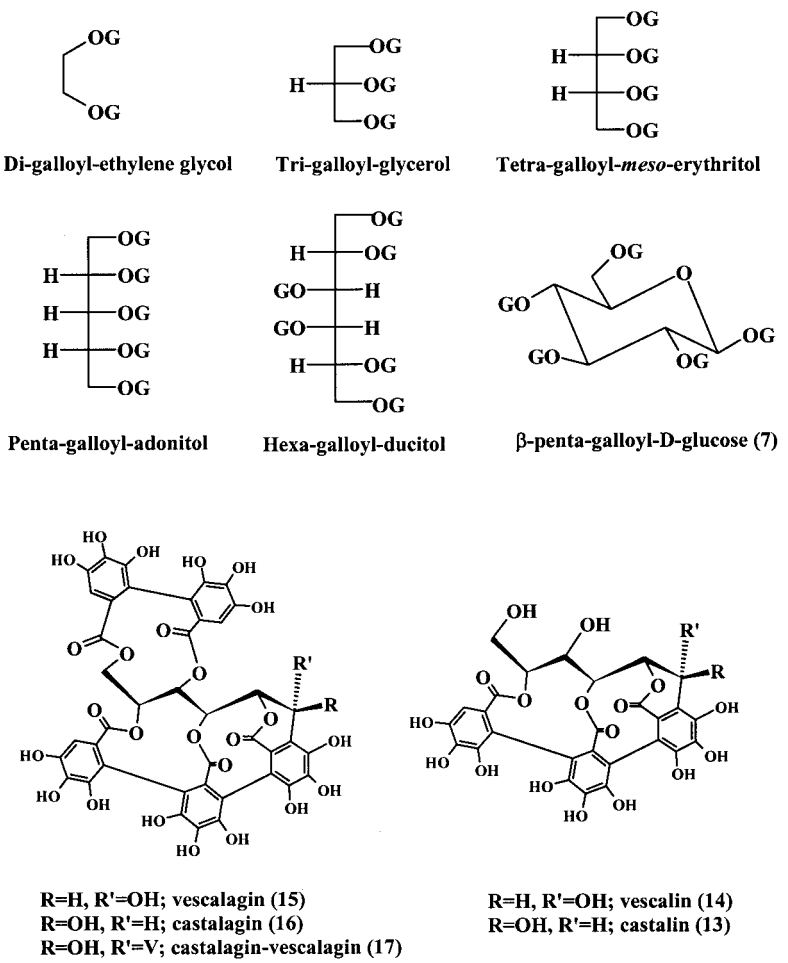

FIGURE 2 Some polyphenols (hydrolyzable tannins) used in this study.

both cellulose–polyphenol and collagen–polyphenol systems to ascertain (1) whether the hydroxyl groups or the aromatic moieties are functional groups of polyphenols, (2) whether hydrogen-bonding or hydrophobic interactions are more important for chromatographic mobility and collagen–polyphenol interactions (tanning process), and (3) the structure–activity relationships for the polyphenol interactions. This article reports some results on these aspects by employing the chromatographic method and by evaluating the polyphenol effects on the hydrothermal stability of collagen. Twenty-four tannins were employed, including seven glucose-based gallotannins, five polyalcohol-based gallotannins, and twelve ellagitannins (see Figure 2 for structural details).

EXPERIMENTAL

Materials

The precoated cellulose thin layer chromatography (TLC) plates were purchase from CAMLAB. Acetic acid, isobutanol, and anhydrous ferric chloride were all purchased from commercial sources without purification prior to use. Commercially used chestnut tannin extract was kindly supplied by Roy Williams Dickson Ltd. as a free sample in the form of "unsweetened." Vescalin, castalin, vescalagin, castalagin, and a dimeric ellagitannin, castalagin-vescalagin (Figure 2), were isolated from the chestnut-tannin extract as described previously.[5,34,35] Polyalcohol-based gallotannins (Figure 2) were synthesized according to a procedure described elsewhere,[36] including di-galloyl-ethylene glycol (DGE), tri-galloyl-glycerol (TGG), tetra-galloyl-*meso*-erythritol (TGE), penta-galloyl-adonitol (PGA), β-penta-galloyl-D-glucose (PGG), and hexa-galloyl-dulcitol (HGD). All the above polyphenolic compounds have been characterized using a combination of NMR spectroscopy and fast-atom bombardment mass spectroscopy as described previously.[34–36] Tannic acid was purchased from British Drug House (BDH).

TLC Chromatography

TLC of tannins was conducted with (A) 6% aqueous acetic acid solution and (B) isobutanol–acetic acid–water (14:1:5) as solvents. Visualization of TLC was achieved by fluores-

cence under a UV/h (256 nm) lamp or spraying saturated KIO_3 solution or 0.03% ethanolic solution of $FeCl_3$ as appropriate. Some paper chromatographic data in the literature were used as R_f values of polyphenols on cellulose without correction, including those for β-1,2,6-tri-galloyl-glucose **(3),**[6] β-1,2,4,6-tetra-galloyl-glucose **(5),**[6] β-2,3,4,6-tetra-galloyl-glucose **(6),**[21] β-1-galloyl-2,3:4,6-bis-hexahydroxydiphenoyl-glucose **(9),**[7] β-1-galloyl-2,4:3,6-bis-hexahydroxydiphenoyl-glucose **(10),**[8] β-2,3-di-galloyl-4,6-hexahydroxydiphenoyl-glucose **(11),**[7] β-2,3:3,6-bis-hexahydroxydiphenoyl-glucose **(12),**[7] and β-2,3-hexahydroxydiphenoyl-glucose **(18).**[7]

Hydrophobicity of Polyphenols

The values of the partition coefficients (*P*) of tannins in *n*-octanol–water were taken from a previous study[37] and log*P* values were used as a measurement of the hydrophobicity.

Preparation of Sheepskin Collagen Samples

The samples were prepared from the commercially pickled sheepskins in the same way as described previously.[10] To describe the process briefly: noncollagen proteins have been removed by treatment with base and relevant enzymes.[9] The samples were degreased with petroleum ether followed with washing with a 0.8% solution of nonionic detergent containing 10% NaCl. Following an extra washing with 10% NaCl solution to remove the excess detergent, the samples were drained overnight and their pH were measured as that of the liquor. The resultant skin samples, consisting of mostly collagen protein, were used as solid collagen samples.[9]

Treatment of Collagen Samples with Polyphenols

In laboratory-scale tanning drums, the above collagen samples were put in 10% NaCl (0.8 g solution/g sample) and drumming mixed for 10 min. Polyphenols (0.05 g/g skin sample) in the form of freeze-dried powder were added into the drums respectively in three portion steps over 2 h. The sample was drummed for additional 5 h and then left stand for overnight. After drumming for another 1 h following day, the samples were removed from the aqueous bath and dried in the air under cover of laboratory tissue. For the same mass concentration for the polyphenols, the molar concentration of the polyphenols used decreases as their molecular weight increases. However, since the galloyl groups are responsible for the interactions, their molar concentration will be more relevant to interactions than the concentration of compounds. For all the compounds used in this study, the amount of galloyl groups is about 0.27 mmol except in vescalin (0.24 mmol) and in castalin (0.24 mmol).

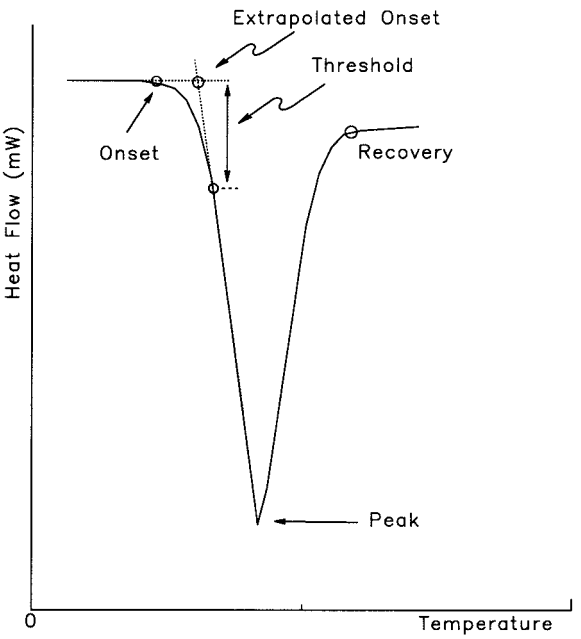

FIGURE 3 A typical DSC thermogram.

Differential Scanning Calorimetric Measurements

The above-treated samples were measured as previously described[10] with some modifications. To assure uniformity of the sample treatment, only the reticular layer of the skin/leather samples, 10–20 mg, was sealed respectively into aluminium pans and their differential scanning calorimetry (DSC) thermogram were recorded on a DSC calorimeter (Mettler TC 10A) with a temperature increment of 5°C/min. A number of unique temperatures were recorded as onset (T_i), extrapolated onset (T_E), peak (T_P), and recovery (T_R) temperatures, as shown in Figure 3. Since these temperatures were not defined consistently in the literature, the definition from a previous study is adopted here.[10,38] The onset (T_i) is defined as the temperature at which the thermogram begins to depart from the baseline. This temperature is consistent with the "shrinkage temperature" (T_s).[39,40] The extrapolated onset (T_E) is defined as the temperature at the intersection of the baseline and the tangent line to the curve at the point, which differs from the baseline by a specified threshold values (1 mW). The peak temperature (T_P), as defined elsewhere, is the temperature of reversal of the curve. The recovery temperature (T_R) is the temperature at which the thermogram returns to either the initial or a different baseline. The measurements of these temperatures offer a systematic evaluation of the hydrothermal stability of the polyphenol treated collagen samples.

RESULTS AND DISCUSSION

Interactions of Polyphenols and Cellulose

In chromatography, the retardation factors (*Rf*) are inversely correlated to the eluate's affinity to the

stationary phase; therefore, Rf^{-1} and the *capacity factor S* defined as, $Rf^{-1} = S + 1$, offer excellent descriptors for the interactions between the eluates and the stationary phase. Since the polyphenol's affinity to cellulose is the basis of the polyphenol separation on paper chromatography and the cellulose thin layer chromatography (TLC) the availability of the *Rf* values for polyphenols offers an excellent opportunity to obtain useful information on cellulose–polyphenol interactions. Based on some of these *Rf* data and the absorption isotherm, a preliminary study of the polyphenol–cellulose and polyphenol–cellulose acetate systems has already suggested that both hydrogen-bonding and hydrophobic interactions are probably of importance.[6] However, the detailed analysis on the interactions, the relative merits of the interactions, and the structure–activity relationships have not been carried out so far.

It is conceivable that, in order to discuss the relative importance of the hydrogen-bonding and hydrophobic interactions in a given system, it is essential to take into consideration that both interactions are dependent on the solvent systems employed.[41] For example, in hydrophilic solvents, hydrophobic interactions is favored whereas hydrogen bonding is favored in the more hydrophobic solvents.[41]

For chromatography of polyphenols on cellulose, two solvent systems[21] were frequently employed—namely, solvent A, 6% aqueous acetic acid solution, and solvent B, isobutanol–acetic acid–water (14:1:5), and the *Rf* values were designated as Rf_A and Rf_B respectively. Clearly, solvent A is similar to water in hydrophilicity and solvent B is more hydrophobic. In fact, paper chromatography and cellulose TLC developed with solvent B gives similar *Rf* values for polyphenols to those developed with the upper layer of butanol–acetic acid–water (5:1:4), providing some indications of the strength of the hydrophobicity of solvent B. Since the hydrophilic solvent A primarily interrupts hydrogen bonding whereas the hydrophobic solvent B affects hydrophobic interactions to a greater extent, a small value for Rf_A or great value for Rf_B will be an indication for strong hydrophobic interactions. Conversely, a great value for Rf_A and a small value for Rf_B indicate strong hydrogen bonding. For the same token, a great value for S_A or small value for S_B can be used as an indicator for the strength of hydrophobic interactions between polyphenols and cellulose.

Table I shows a collection of the *Rf* (and *S*) values for 24 gallotannins and ellagitannins studied here, from which a number of observations can be made. First, a linear correlation is clearly evident (Figure 4A) between the gallotannin's affinity to cellulose ($\log Rf_A^{-1}$) and the number of galloyl groups (N_G). A similar, though less steep, correlation is also observable for ellagitannins **(12–19)** between $\log Rf_A^{-1}$ and N_G (Figure 4A). This is in good agreement with observations in previous studies,[21,27] and implies that the galloyl group is the major functional group in the cellulose–tannin interactions. The galloyl group possesses three hydroxyl groups, which are potentially important for hydrogen bonding, and an aromatic moiety, which is important for hydrophobic interactions. Therefore, further clarification is necessary on whether the hydrogen-bonding or hydrophobic interactions are more important in the cellulose–polyphenol interactions.

If the interactions between a polyphenol molecule and cellulose are mainly hydrogen bonding, the Rf_A value will be greater than Rf_B due to stronger competition of solvent A to the hydrophilic sites than solvent B. Conversely, solvent B affects the hydrophobic interactions to greater extent, reducing the affinity to cellulose (S_B); thus the Rf_B has a greater value if hydrophobic interactions are of greater improtance. For glucose-based gallotannins (1–7, Table I), Rf_A is greater than Rf_B only when the number of galloyl groups is less than two. This implies that the hydrophobic interactions are dominant when the number of galloyl groups is more than two. For the other polyalcohol-based gallotannins (DGE-HGD in Table I), small Rf_A values suggest that hydrophobic interactions are of greater significance. In both cases, however, a clear correlation is evident between the strength of hydrophobic interactions, indicated by S_A, and the number of galloyl groups (N_G). This seems to suggest, therefore, that hydrophobic interactions are of greater significance between the cellulose and galloyl groups. The glucose-based molecules had a "switch-over" probably due to the roles played by the free sugar hydroxyl groups.

With solvent A, the affinity (S_A) of the glucose-based gallotannins **(1–7)** to cellulose increases dramatically with the rise of the number galloyl groups (N_G), whereas the affinity in solvent B (S_B) decreases steadily as a function of the increase of galloyl groups. For the polyalcohol-based gallotannins, similar correlations are observable between S_A values and N_G, while there is little affinity to cellulose in solvent B. This supports the notion that galloyl groups are the functional groups and implies the hydrophobic interactions are of greater importance in gallotannin–cellulose interactions. For ellagitannins **(12–19)** in solvent A, a steady but positive correlation is evident between their affinity to cellulose (S_A) and the number of galloyl groups. In contrast to the gallotannins, a weak but positive correlation is also observable between the S_B and N_G. This together with the greater S_B

Table I Chromatographic Data for the Polyphenols on Cellulose[g]

	M_t	N_G	Rf_A	Rf_B	S_A	S_B	log*P*
Glucose-based gallotannins							
6-galloyl-glucose (**1**)	332	1	0.72[a]	0.22[a]	0.39	3.55	−1.89
1,6-di-galloyl-glucose (**2**)	484	2	0.45[a]	0.42[a]	1.22	1.38	−0.33
1,2,6-tri-galloyl-glucose (**3**)	636	3	0.30[b]	0.35[b]	2.33	1.86	0.61
1,2,3,6-tetra-galloyl-glucose (**4**)	788	4	0.10[a]	0.48[a]	9.00	1.08	1.56
1,2,4,6-tetra-galloyl-glucose (**5**)	788	4	0.11[b]	0.45[b]	8.09	1.22	1.00
2,3,4,6-tetra-galloyl-glucose (**6**)	788	4	0.21[c]	0.60[c]	3.76	0.67	1.30
1,2,3,4,6-penta-galloyl-glucose (**7**)	940	5	0.06[a]	0.56[a]	15.67	0.79	2.20
Other polyols-based gallotannins							
DGG[d]	366	2	0.12	0.85	7.33	0.07	—
TGG[d]	548	3	0.06	0.83	15.67	0.08	—
TGE[d]	730	4	0.02	0.86	49.00	0.07	—
PGA[d]	912	5	0.01	0.82	99.00	0.09	—
HGD[d]	1094	6	0.01	0.88	99.00	0.06	—
Galloyl ellagitannins							
1,2,3-tri-galloyl-4,6-hhdp-glc (**8**)	938	5	0.35[a]	0.37[a]	1.86	1.70	1.00
1-galloyl-2,3:4,6-bis-hhdp-glc (**9**)	936	5	0.28[e]	0.26[e]	2.57	2.85	−1.07
1-galloyl-2,4:3,6-bis-hhdp-glc (**10**)	936	5	0.41[f]	0.25[f]	1.44	3.00	−0.72
2,3-di-galloyl-4,6-hhdp-glc (**11**)	786	4	0.48[e]	0.40[e]	1.08	1.50	—
2,3:4,6-bis-hhdp-glc (**12**)	784	4	0.56[e]	0.15[e]	0.78	5.67	−2.01
2,3-bis-hhdp-glc (**18**)	482	2	0.69[e]	0.16[e]	0.45	5.25	—
4,6-bis-hhdp-glc (**19**)	482	2	0.71[a]	0.17[a]	0.41	4.88	—
Ellagitannins							
Vescalin (**14**)	632	3	0.65[a]	0.05[a]	0.54	19.00	—
Castalin (**13**)	632	3	0.60[a]	0.08[a]	0.67	11.50	—
Vescalagin (**15**)	934	5	0.50[a]	0.05[a]	1.00	19.0	−1.77
Castalagin (**16**)	934	5	0.42[a]	0.05[a]	1.38	19.0	—
Castalagin-vescalagin (**17**)	1850	10	0.35[a]	0.03[a]	1.86	32.23	—

[a] data from Ref. 5.
[b] Paper chromatography data from Ref. 6.
[c] Paper chromatography data from Ref. 21.
[d] Data from Ref. 36.
[e] Paper chromatography data from Ref. 7.
[f] Paper chromatography data from Ref. 8.
[g] hhdp: Hexahydroxydiphenoyl, glc: *D*-glucose, DGE: di-galloyl-ethylene glycol; TGG: tri-galloyl-glycerol; TGE: tetra-galloyl-*meso*-erythritol; PGA: penta-galloyl-adonitol; HGD: hexa-galloyl-dulcitol.

values than S_A suggest that hydrogen bonding is also important in the hydrophilic solvent, although hydrophobic interactions present some contributions to the ellagitannin's affinity to cellulose.

Furthermore, ellagitannins **(8–19)** has a much greater Rf_A values than gallotannins having the same number of galloyl groups (Table I). Therefore, the introduction of intergalloyl linkages has a detrimental effect on the hydrophobic interactions between polyphenols and cellulose. In fact, Rf_A values increase dramatically with the increase of the number of intergalloyl linkages; in contrast, the Rf_B values had an apparent decrease. For example (Figure 4B), penta-galloyl-glucose **(7)** has an Rf_A of 0.06, introduction of one intergalloyl bond results in the increase of Rf_A to 0.35 and more intergalloyl bonds eventually lead to the increase of Rf_A to 0.5 (Figure 4B). Rf_B values decreased from 0.56 to 0.05 when the number of the inter-galloyl linkages increased from 0 to 3. Similar observations can be made by comparing data for glucose-based tannins with four galloyl groups, such as **6, 11,** and **12,** and tannins with three galloyl groups such as **3, 13,** and **14.** This is consistent with the observations made for the polyphenol–biopolymers interactions in aqueous solution,[25–30] and has been attributed to the molecular flexibility restrictions induced by the intergalloyl linkages. This is also supportive to the notion that hydrophobic interactions are important in this system since hydrophobic interactions are more critically dependent on the orientations of the aromatic rings than the hydrogen bonding, which involves hydroxyl groups.

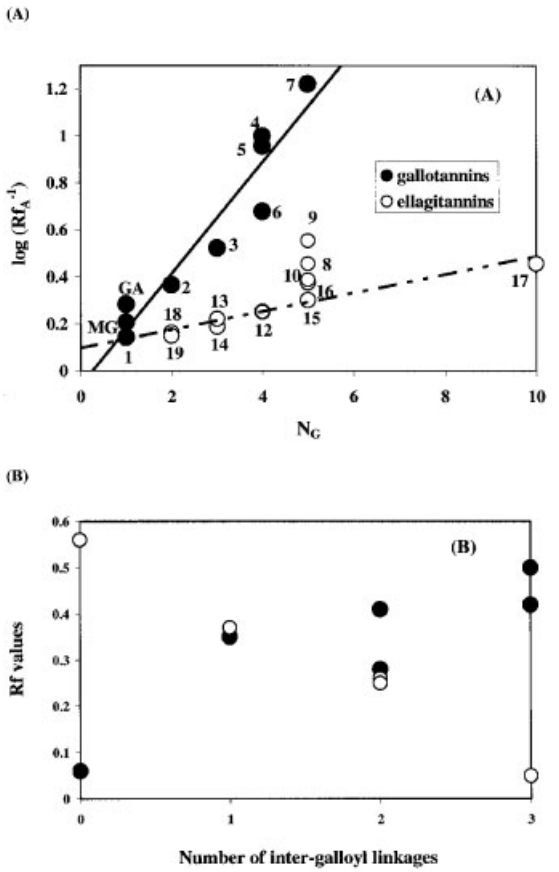

FIGURE 4 *Rf* values of polyphenols. (A) The log(Rf_A^{-1}) vs the number of galloyl groups; GA: gallic acid; MG: methyl gallate. Solid line: equated data for gallotannins (**1–7,** GA, MG) from log(Rf_A^{-1}) = 0.237*N_G − 0.0612, correlation coefficient, r^2, 0.914. Dashed line: equated data for ellagitannins (**12–19**) from log(Rf_A^{-1}) = 0.039*N_G + 0.097, correlation coefficient, r^2, 0.880. (B) *Rf* values vs the number of intergalloyl linkages for polyphenols having five galloyl groups; dots: Rf_A; circle: Rf_B.

Moreover, if the hydrophobic interactions are dominantly important, the affinity of polyphenols to cellulose is expected to correlate with the hydrophobicity of polyphenols. This is indeed the case; the Rf_A is inversely correlated with the log*P* values[37] (Table I). A linear relationship is evident (Figure 5) between the log (Rf_A^{-1}) and the log*P* values of these compounds including both ellagitannins and gallotannins. Therefore, the hydrophobic interactions for both gallotannins and ellagitannins are the governing force for polyphenol–cellulose interactions in a solvent as hydrophilic as 6% acetic acid solution. The dominance of the hydrophobic interactions start decreasing when not enough unrestricted galloyl groups are present or their hydrophobic interactions were substantially hindered by restricting the flexibility of galloyl groups.

Polyphenol–Collagen Interactions

It is not as easy to study the polyphenol interactions with collagen chromatographically as with cellulose. However, it is well known that the polyphenol treatment leads to improved hydrothermal stability of collagen, and this improvement is associated with the interactions between polyphenol and collagen molecules; stronger interactions yield high stability for the collagen matrices.[9,42] Therefore, the hydrothermal stability of collagen can be measured as an indirect evaluation of polyphenol–collagen interactions.

In classical literature, the hydrothermal stability of collagen is evaluated by measuring the shrinkage temperature (*Ts*)[9,42] since a solid collagen (skin) sample undergoes a sharp thermal transition, leading to a "length-shrinkage" of the samples. This shrinkage is easily detectable by a "shrinkage temperature" device[42] or even naked eyes. On the molecular level, such a shrinkage process is associated with the thermally activated conformational transition of collagen protein from coiled coils to random coils.[9] DSC provides a better way to monitor this denaturation process by measuring the difference in heat flow into a sample and a reference as a function of temperature. Apart from the onset temperature (T_i), which is consistent with *Ts,*[39,40] DSC measures some more temperatures[10,38,42] such as extrapolated temperature (T_E), peak temperature (T_P), and recovery temperature (T_R), thus offering an excellent approach to evaluate the hydrothermal stability of the polyphenol-treated collagen systematically.

For both gallotannins and ellagitannins, the plot of shrinkage temperature (*Ts*) for the polyphenol-treated

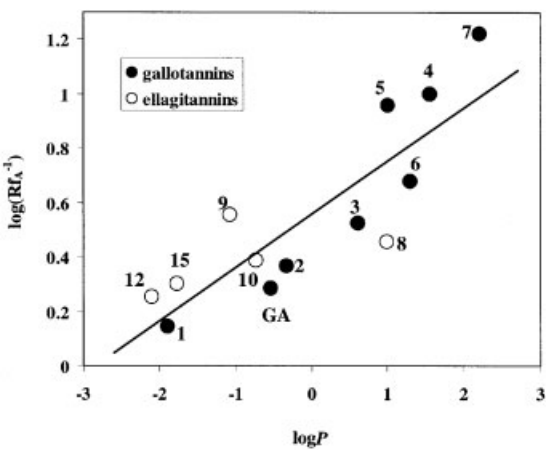

FIGURE 5 Rf_A values as a function of the partition coefficients (*P*) in *n*-octanol–water for polyphenols; GA: gallic acid; MG: methyl gallate. Solid line: equated data from log(Rf_A^{-1}) = 0.196*log*P* + 0.559; correlation coefficient, r^2, 0.727.

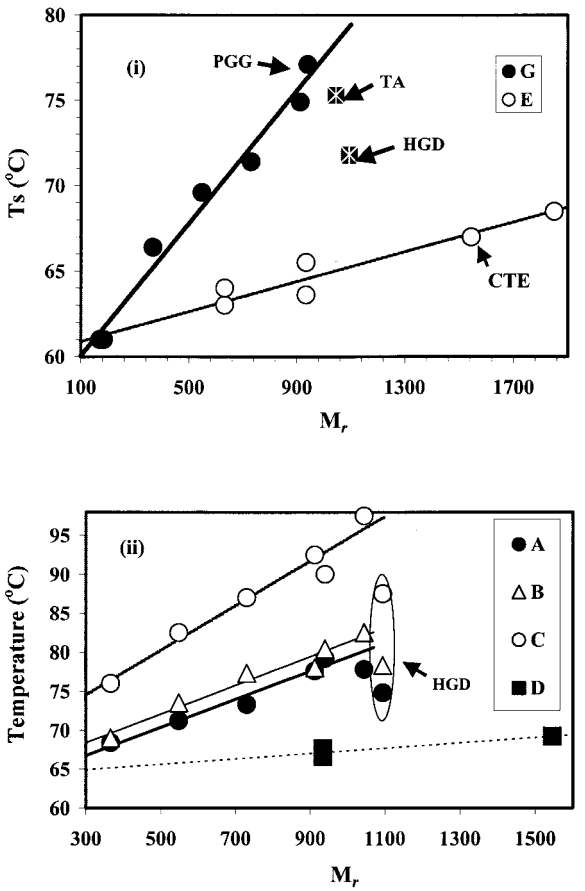

FIGURE 6 Hydrothermal stability of polyphenol-treated collagen vs the molecular masses of polyphenols. The fitted data indicated by straight lines are tabulated in Table II. PGG: penta-galloyl-glucose; HGD: hexa-galloyl-ducitol; TA: tannic acid; CTE: chestnut tannin extract; (i) *Ts*, G: gallotannins, E: ellagitannins; (ii) A-T_E, gallotannins, B-T_P, gallotannins; C-T_R, gallotannins; D-T_E, ellagitannins.

collagen (Figure 6i) showed a positive linear correlation with their molecular masses (M_r); a positive linear correlation is also clearly evident (Figure 7i) between the *T*s and the total number of galloyl groups (N_g) in the molecules. Such correlations can be expressed as

$$Ts = T_0 + K^*X \quad (1)$$

Where T_0 is the intercept reflecting the *Ts* of collagen without polyphenol treatment; *K,* the gradient, is an indicator for the efficacy (or tanning efficiency) of polyphenols. *X* is the variable related to the molecular properties such as molecular mass and the number of galloyl groups (N_G). The correlation parameters for both the gallotannins and ellagitannins are tabulated in Table II.

Correlation between *Ts* and N_G suggests that galloyl group is responsible for the effects of polyphenols on the thermal stability of treated collagen. Such effects depend on their molecular masses (or size) and the number of galloyl groups. This is similar to the situations of other polyphenol–biopolymer interactions in aqueous solution[25–30] and to the polyphenol–cellulose interactions discussed in the previous section. Additionally, the ellagitannin-treated samples had much lower *K* values than the gallotannin-treated ones; thus gallotannins are superior in association with collagen. This suggests that the introduction of intergalloyl covalent linkages decreases the tanning efficiency probably due to restrictions to the flexibility of galloyl groups.[29,30] It is also interesting to note that *Ts* values for ellagitannins and gallotannins converged at where gallic acid and methyl gallate are. This implies that the polyphenols, having molecular mass as great as 200 Dalton, start showing tanning properties.

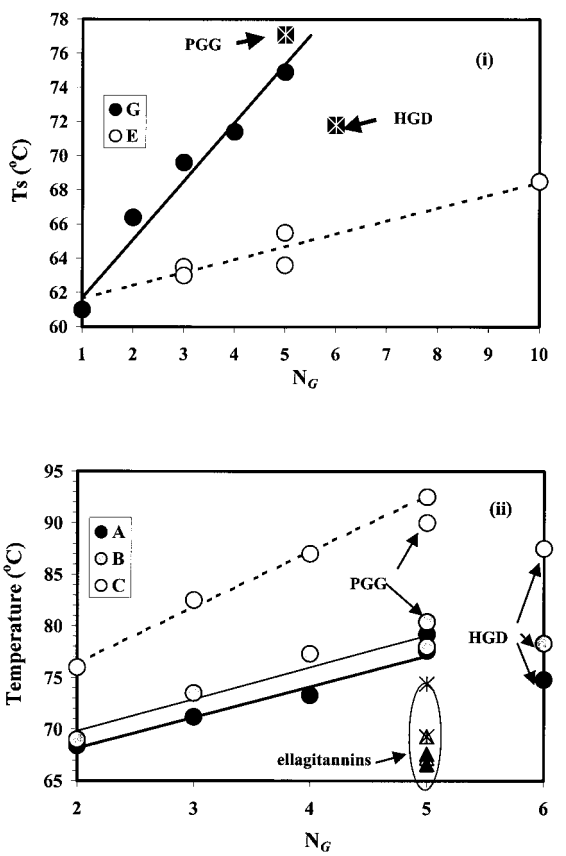

FIGURE 7 Hydrothermal stability of polyphenol-treated collagen vs the number of galloyl groups in polyphenols. The fitted data indicated by straight lines are tabulated in Table II. PGG: penta-galloyl-glucose; HGD: hexa-galloyl-ducitol; (i) G: gallotannins, E: ellagitannins, (ii) A-T_E, gallotannins, B-T_P, gallotannins; C-T_R, gallotannins.

Table II Correlation Parameters for the Polyphenol-Treated Collagen Samples

	Gallotannins	Ellagitannins
Ts vs M_r		
T_o	59.5	60.9
K	0.0175	0.0039
r^2	0.991	0.981
Ts vs N_G		
T_o	59.5	61.4
K	3.17	0.68
r^2	0.991	0.987
T_E vs M_r		
T_o	60.9	—
K	0.0191	—
r^2	0.989	—
T_E vs N_G		
T_o	60.9	—
K	3.53	—
r^2	0.989	—

[a] T_o and K were intercepts and gradients as defined in Eq. (1); r^2 is a correlation coefficient.

Apart from the *Ts,* which is a descriptor of the onset of the collagen denaturation, T_E, T_P and T_R can also be collectively employed as descriptors for the "shrinkage" process. The plot of T_E, T_P, and T_R as a function of the polyphenols' molecular mass (Figure 6ii) and the number of galloyl groups (Figure 7ii) showed that (1) for both gallotannins and ellagitannins, there is a positive correlation between these temperatures and the number of galloyl groups; (2) for ellagitannins possessing the same number of galloyl groups, the treated samples had lower overall thermal stability (Figure 6ii and 7ii) than gallotannins-treated ones. This is supportive to the notion that the galloyl group is the functional group and the gallotannins interact with collagen more strongly than ellagitannins.

It is particularly interesting to note that tannic acid (a mixture of gallotannins) and chestnut tannin extracts (a mixture of ellagitannins) both showed consistence in the *Ts* vs M_r plot (Figure 6i) with gallotannins and ellagitannins respectively, although both of their average molecular masses were estimated from a melting-point-depression method.[43]

The only exception among all polyphenols is HGD. HGD-treated collagen has lower temperatures than expected. The exact reason is unknown, although this is probably due to poorer uniformity of its distribution in the collagen matrix, resulting from stronger interactions with collagen.[10]

Therefore, the following conclusions can be drawn immediately from the above observations: (1) *Ts* alone can be a good indicator for the effects of polyphenols on the thermal stability of treated collagen; (2) the effects of polyphenols were dependent on the molecular mass (size) and the number of galloyl groups in the polyphenols molecules; (3) gallotannins showed more potent effects than ellagitannins having similar molecular mass and number of galloyl groups; (4) the polyphenol interactions with collagen is parallel to their interactions with cellulose, being in broad agreement with the findings in an early study.[33]

The next question to answer is whether the hydrogen-bonding or hydrophobic interactions are more important to the effects of polyphenols on the hydrothermal stability of collagen. From Figures 6 and 7, it is apparent that the introduction of the intergalloyl linkages, such as in ellagitannins, caused a strong and detrimental decrease of the *Ts* of treated collagen, indicating that freedom of galloyl groups is vitally important. For example, vescalagin **(15)** and castalagin **(16),** containing five restricted galloyl groups, achieved a *Ts* much lower than penta-galloyl-glucose and even lower than DGE does; the dimeric ellagitannin, castalagin-vescalagin **(17),** having 10 restricted galloyl groups, only achieved a *Ts* similar to that DGE does, which have only two free galloyl groups. Since the hydrophobic interactions of gallotannins are more critically dependent on the orientation of the aromatic rings than hydrogen bonding, these results suggest that hydrophobic interactions are more important for tannins to increase the hydrothermal stability of collagen.

If this is true, there will be a positive correlation between Ts and the partition coefficients of polyphenols, P (or log$P$), which is a measure of their hydrophobicity, or between *Ts* and log (Rf_A^{-1}) since log$P$ is linearly correlated with log (Rf_A^{-1}) (Figure 5). Although the log$P$ values for most of the polyphenols used in collagen treatment are not available, it is indeed the case that a plot of Ts vs log (Rf_A^{-1}) shows an unambiguous positive-correlation for both glucose-based polyphenols, including gallotannins and ellagitannins, and polyalcohol-based gallotannins (Figure 8). The difference between the glucose-based tannins and polyalcohol-based ones probably results from the steric effects.

To sum up, in both polyphenol–cellulose and polyphenol–collagen systems in the solid state, galloyl groups are responsible for polyphenol–

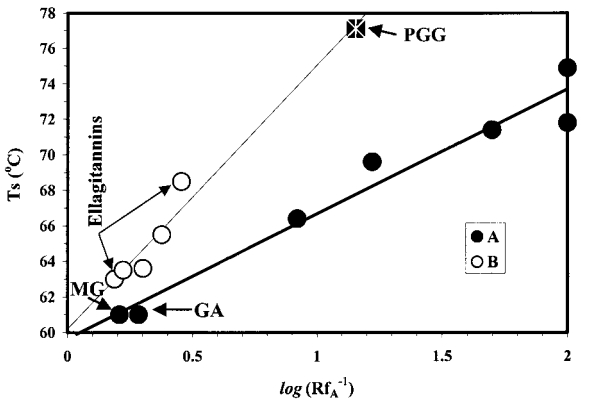

FIGURE 8 *Ts* of polyphenols-treated collagen vs $\log(Rf_A^{-1})$ of the polyphenols. A: polyalcohol-based gallotannins; GA: gallic acid; MG: methyl gallate; PGG: 1,2,3,4,6-penta-galloyl-glucose; solid line: equated data from $Ts = 7.0*\log(Rf_A^{-1}) + 59.7$, correlation coefficient, r^2, 0.958. B: glucose-based ellagitannins; solid line: equated data from $Ts = 14.9*\log(Rf_A^{-1}) + 60.2$, correlation coefficient, r^2, 0.975.

biopolymer interactions and hydrophobic interactions are of great importance. When gallotannins have more than two galloyl groups, the hydrophobic interactions are dominant and, for ellagitannins, the intergalloyl linkages severely restricted the flexibility of galloyl groups, reducing the hydrophobic interactions. When the number of such linkages is increased to a certain level, the dominance of hydrophobic interactions is detrimentally compromised. Success of chromatographic separation of these tannins on cellulose relies on their interactions and the hydrophobicity of the solvents employed. The effects of polyphenols on the hydrothermal stability of collagen depend on the number of galloyl groups and molecular size. The introduction of intergalloyl C—C bonds restricts the flexibility of the galloyl groups, thus hindering the collagen–polyphenol hydrophobic interactions. There is a clear parallel between the polyphenol–cellulose and polyphenol–collagen interactions.

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