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Use of DSC To Detect the Heterogeneity of Hydrothermal Stability in the Polyphenol-Treated Collagen Matrix

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The hydrothermal stability of the collagen matrixes treated with plant polyphenols (tannins) depends on not only the strength of the polyphenol−collagen interactions but also the distribution uniformity of polyphenolic molecules within the collagen fibrils. Traditional methods of uniformity tests rely heavily on the expertise of workers and are thus subjective. This paper describes a differential scanning calorimetry (DSC) study of the sheepskin collagen samples treated with hydrolyzable tannins, including two commercial tannins' extracts (chestnut and valonea), two pure ellagitannins (vescalagin and castalagin), and six synthetic gallotannins (di-galloyl-ethylene glycol (DGE), tri-galloyl-glycerol, tetra-galloyl-*meso*-erythritol, penta-galloyl-adonitol, penta-galloyl-glucose, and hexa-galloyl-ducitol). The collagen sample without polyphenol treatment and the sample treated with DGE showed a single sharp peak in their DSC thermogram with a full peak width at half height (fwhh) of 3−4 °C. The samples treated with other tannins all showed multiple peak DSC profiles with the fwhh of each peak at about 3−4 °C. These multiple peak profiles imply that in these polyphenol-treated samples, there is a distribution of collagen molecules having different hydrothermal stability. The results have demonstrated that DSC offers an objective method to detect the stability heterogeneity of collagen matrixes in the solid state, providing a useful tool for the leather industry to evaluate the uniformity of leather tanning.

KEYWORDS: Hydrolyzable tannins; collagen; DSC; hydrothermal stability; tanning uniformity; gallotannins; ellagitannins; leather tanning

INTRODUCTION

The essence of leather tanning is to introduce strong/stable cross-links between collagen molecules so that the hydrothermal and mechanical stability of collagen fibrils can be improved (*1*). The hydrothermal stability of collagen is traditionally evaluated by measuring the shrinkage temperature (T_s), at which a piece of leather sample starts shrinking, with a "shrinkage temperature" device (*2*). It was found that T_s and the shrinkage process are associated with the thermal transition of the collagen structure from triple helices to random coils (*1*, *3*). In principle, the tanning efficiency of a tanning agent is critically dependent on its ability to form interfibril cross-links with great strength and extensiveness (*1*, *2*). These cross-links are formed from the collective multiple site interactions between the tanning agents and the collagen protein, including hydrogen bonding, hydrophobic interactions, ionic interactions, and covalent bonds (*1*). In practice, however, uniformity of tanning is also essential for the thermal and mechanical properties of the resultant leather since poor uniformity results in some fraction of collagen fibrils insufficiently stabilized, hence creating the weakest link.

Therefore, two processes are vitally important to achieve a good leather tanning effect, good dispersion uniformity of the tanning agents across the collagen matrix (or the thickness of the skin), and strong/stable interactions (or fixation) with protein molecules. It is conceivable that uniform dispersion of tanning agents is the prerequisite for a uniform tanning process. Paradoxically, to achieve a uniform distribution, weak interactions are often required between the tanning agents and the proteins, so that they are not "fixed" on the surface of skin samples prior to achieving a uniform dispersion (*1*). In practice, parameters, such as pH, are used to adjust (or weaken) the collagen−tannin interactions at the early stage of the tanning process in order to ensure uniform dispersion of tannins (*1*).

The uniform dispersion across the sample thickness is traditionally monitored by inspecting the cross-section cut with

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coloration indicators. This method, however, heavily relies on (i) the experiences of tanners and (ii) the resolution of naked human eyes to length-scales and coloration; thus, this method is subjective. This method is also clearly based on the assumption that all components of the tanning agents diffuse into the collagen matrix with a similar rate. Unfortunately, this assumption is not valid in most cases since most of the tanning materials employed are mixtures having a wide range of molecular masses (size), affinity to collagen protein, and thus diffusivity across the skin/leather matrix. Therefore, the cross-section indication method is liable to produce a high probability of failure.

This is exemplified in the tanning process with plant polyphenols (i.e., vegetable tannins (*1*)), in which plant polyphenol extracts are employed to treat prepared skin collagen so as to improve its hydrothermal and mechanical stability. The progress of this process is often monitored with visual inspection of the cross-section cut by either the coloration of tannins (brownish) or using ferric chloride solution (blue or dark blue coloration). Tannin extracts are mixtures of plant polyphenols with a range of molecular masses (*4*−*8*) (from a few hundred to a few thousand Daltons), thus a range of affinity to collagens and diffusivity. The small molecules, in particular, so-called "nontannins" (*1*), can achieve a uniform dispersion across the skin thickness much faster than the larger molecules due to their weaker affinity to collagens (*2*, *9*). However, they give the same coloration to the ferric chloride test (or any other chemical tests for this matter) as the larger tannin molecules (*6*). In such a case, while a positive ferric chloride test is a valid indication of uniform distribution of small molecules, it cannot necessarily indicate the state of distribution for the larger molecules, which are responsible for the tanning effects. Therefore, the method gives an overestimation to the dispersion uniformity for tannins.

Differential scanning calorimetry (DSC), which was widely used to study various aspects of chemical reactions such as thermally induced polymerizations, decompositions, and polycondensations, can also be employed to follow the phase changes or conformational transitions based on the measurements of the specific heat capacity of a sample (*10*). Therefore, the DSC method offers a much more objective and comprehensive way of evaluating the "thermal shrinkage" process of collagen (*11*−*13*). In this paper, we report some DSC results from studying the sheepskin collagen treated with the polyphenolic tannins including chestnut and valonea tannins extracts, two ellagitannins from chestnut extract (castalagin and vescalagin), and six synthetic gallotannins, 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) (see **Figure 1** for structures). The results will be discussed in the context of heterogeneity of the samples in their hydrothermal stability.

EXPERIMENTAL PROCEDURES

Materials. Commercially used chestnut tannin extract was kindly supplied by Roy Williams Dickson Ltd. as a free sample in the form of "unsweetened". Vescalagin and castalagin (**Figure 1**) were isolated from the chestnut tannin extract as described before (*5*, *7*, *14*). Polygalloyl esters (gallotannins, **Figure 1**) were synthesized according to a procedure described elsewhere (*15*), including DGE, TGG, TGE, PGA, PGG, and HGD.

Preparation of Sheepskin Collagen Samples. Commercially pickled sheepskins, from which noncollagen proteins were removed by treatment with base and relevant enzymes (*1*), were degreased with petroleum ether followed by washing with an 0.8% solution of nonionic detergent containing 10% NaCl. Following an extra washing with 10% NaCl solution to remove the excess detergent, the skin was drained on a "beam horse". The sample treated in this way consisted of mostly collagen proteins (*1*).

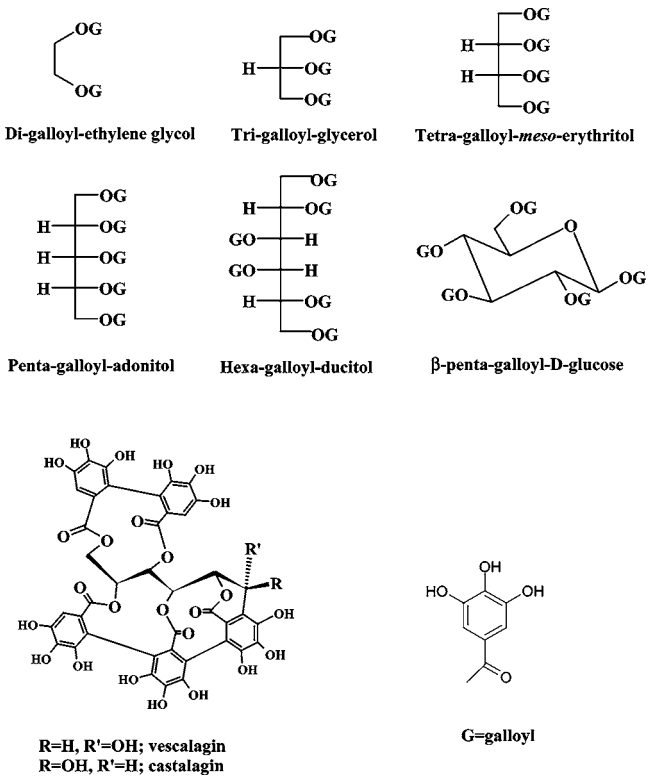

Figure 1. Structures of tannins employed in this study.

Treatment of Collagen Samples with Polyphenols (Vegetable Tannins). In laboratory-scale tanning drums, the above skin samples were put in 10% NaCl (0.8 g per gram sample) and drummed for 10 min. Then, the polyphenols (0.05 g per gram 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 standing overnight. After the samples were drummed for another 1 h the following day, the samples were dried in the air under cover of (blue) laboratory tissue.

DSC Measurements. The above treated samples were wet back with distilled water, respectively, for 24 h followed with removal of the excess water with laboratory tissue. The water content of the samples was 50−70% (g water per g fully hydrated sample). The wet leather samples (including both grain and reticular layers (*1*)), 10−20 mg, were sealed, respectively, into aluminum pans, and their DSC thermograms were recorded on a DSC calorimeter (Mettler TC 10A) with a temperature increment of 5 °C per min. As a comparison, the shrinkage temperature of the samples was also measured in water with a conventional T_s device by observing the onset of the sample shrinkage. The T_s values of both the leather samples (including both grain layer and reticular layer) and the reticular layers (alone) were measured.

RESULTS AND DISCUSSION

The uniformity of the tanning process was initially monitored by inspection of the coloration of the cross-section cut using $FeCl_3$ solution. As soon as the skin samples treated with the commercial chestnut and valonea extracts showed good cross-section uniformity of tannins' dispersion (dark blue coloration), the tanning process was stopped and the samples were studied with the DSC method.

In principle, DSC measures the difference in the heat-flow into a sample and a reference as a function of temperature, giving a so-called thermogram (**Figure 2**). In practice, a number of unique temperatures (*10*) can be recorded as onset (T_i), extrapolated onset (T_E), peak (T_P), and recovery (T_R) temperatures as shown in **Figure 2**. It is known that the shrinkage

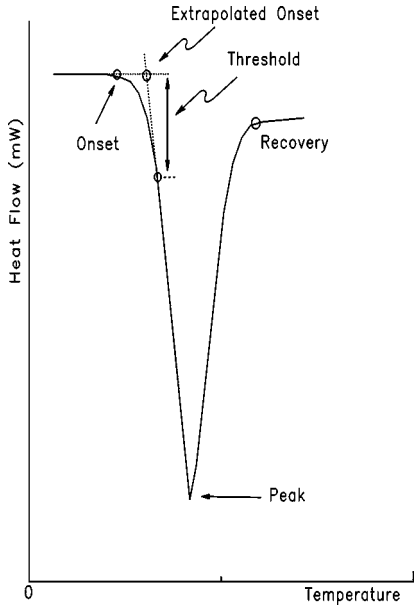

Figure 2. Typical DSC thermogram in which the unique temperatures were defined as follows: the onset (T_i) is the temperature at which the thermogram begins to depart from the baseline; the extrapolated onset (T_E) is defined as the temperature at the intersection of the baseline and the tangent line to the curve at the point that differs from the baseline by a specified threshold value (1 mW); the peak temperature (T_P) is the temperature of reversal of the curve; and the recovery temperature (T_R) is the temperature at which the thermogram returns to either the initial or a different baseline (*10*).

temperature of collagen (T_s) is normally associated with the onset temperature in the DSC profiles (*3*, *11*). From the thermogram, many other parameters of a phase transition can also be obtained for thermodynamics, such as the specific heat values (C_P) and enthalpy changes (ΔH), and for kinetics, such as the activation energy, E_a, and the order of the reaction (*10*). In addition, the rate of reaction or degree of conversion as a function of time under isothermal conditions can also be evaluated. These can provide comprehensive measurements of the leather shrinkage process. However, these are beyond the objectives of this study.

Figure 3 shows the DSC profiles of the sheepskin collagen samples treated with the commercial chestnut and valonea extracts, vescalagin and castalagin, which are two major ellagitannins in both the chestnut (*7*, *16*) and the valonea extracts (*17*, *18*). It is apparent that the vescalagin- and castalagin-treated samples showed multiple peak DSC profiles whereas the samples from both tannins' extracts showed a "single" peak in their DSC profiles. This multiple peak pattern has been observed (*12*) previously but not discussed. In the textbooks (*1*), it has been explained that tannins' extracts are polyphenolic mixtures having a range of molecular masses (e.g., chestnut, 170−1890 Da (*7*)), where the small molecules act as a "buffer", so that tannins can diffuse into the collagen matrix more uniformly (*1*). However, this cannot fully explain the above observations.

Because a peak in the DSC profile shows a unique population of molecules with a defined thermal stability (or transitional properties), the multiple peaks indicate that there is more than one fraction of collagen molecules in terms of their thermal transitional properties. Therefore, **Figure 3** appeared to show that the pure compound vescalagin- and castalagin-treated samples have more than one fraction of collagen, in terms of their thermal stability, whereas the tannins' extracts, which are complex mixtures, produced homogeneous samples. This is

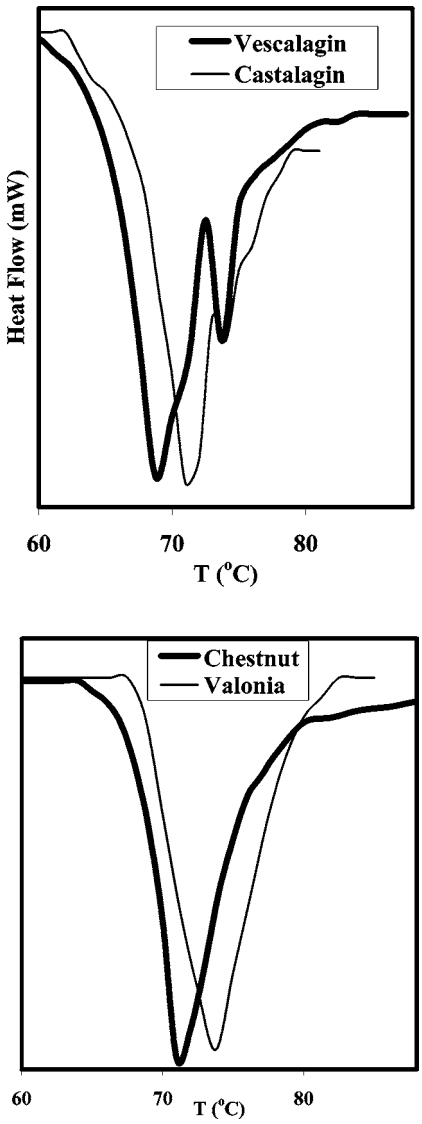

Figure 3. DSC profiles of collagen samples treated with chestnut, valonea tannins' extracts, vescalagin, and castalagin.

clearly not true. A close inspection revealed that the DSC profile of the vescalagin-treated sample has three resolvable peaks with T_i values of 60, 66, and 68 °C, respectively. The first peak is similar to that of untreated collagen (**Figure 4**), and the other two peaks occurred at higher temperatures. Similar conclusions can be drawn for the castalagin-treated sample. Therefore, it can be concluded that these samples showed a distribution of collagen molecules having different hydrothermal stability across the thickness of the collagen matrix (or skin). On the other hand, the samples treated with both chestnut and valonea tannin extracts showed broad profiles.

The width of DSC peaks is an indication to the sharpness of the thermal transition and therefore the distributions of the collagen population with distinct thermal stability. It is conceivable that a broader peak is an indication of broader distribution of collagen molecules having a different thermal stability. When the difference of the thermal stability of collagen fractions reaches a certain level, the DSC profile starts to show the resolved multiple peaks. Therefore, the peak width was evaluated for these collagen samples by measuring the full width at half height (fwhh). The tannin extracts treated samples showed a peak width (5−6 °C, **Figure 3**) almost twice as great as that of each individual peak (∼3 °C) in the thermogram of the vescalagin-treated sample (**Figure 3**). There can be two pos-

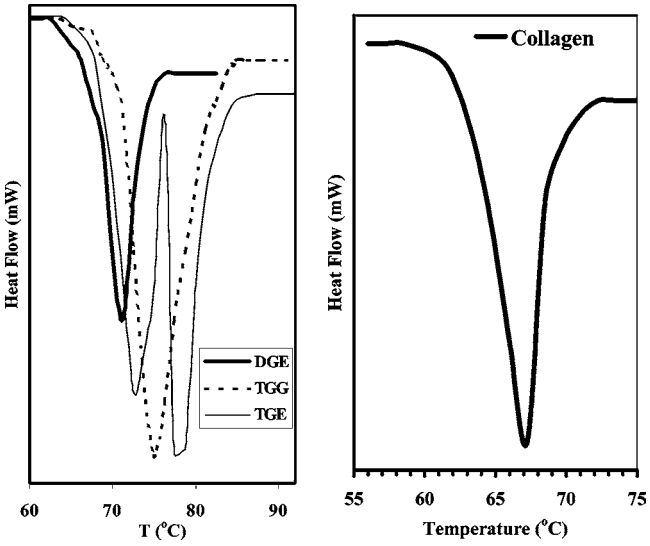

Figure 4. DSC profiles of collagen samples treated with small synthetic gallotannins (below 740 Da); collagen, without treatment.

sibilities for this: (i) collagens treated by different tannins have marked differences in their thermodynamics and (ii) the peak of the valonea and chestnut sample is a sum of some overlapping peaks. If the first explanation was true, one would expect markedly different interaction mechanisms for the tannins in the chestnut and valonea extracts as compared to that for vescalagin and castalagin, thus different molecular characteristics for valonea and chestnut tannins. This is not true and, in fact, is contrary to the findings that (i) valonea (*17*, *18*) and chestnut (*5*, *7*, *16*) tannins are ellagitannins with similar structure features to those of vescalagin and castalagin and (ii) vescalagin and castalagin are two major components in these two extracts. Therefore, it is likely that the broad peak profiles for chestnut and valonea are due to a distribution of collagen molecules with different thermal stabilities.

Similar observations can be made for the collagen samples treated with gallotannins, namely, DGE, TGG, and TGE (**Figure 4**). The DGE-treated sample showed a single peak with fwhh of 3.5 °C, consistent with that for the untreated collagen (**Figure 4**) and the individual peak width observed in the vescalagin-treated collagen. The TGG-treated sample showed, although a single peak, a broader profile with a fwhh of 7.5 °C, twice as broad as in the DGE profile. This implies that in the TGG-treated sample, a distribution is present for the collagen molecules with different hydrothermal stabilities. The TGE-treated sample showed two clearly resolved peaks in its DSC profile with the peak width of 3−4 °C for each peak. This is in good agreement with the observations made for the ellagitannins in the earlier discussion.

To extend the study to gallotannins with higher molecular masses, sheepskin collagen samples were also treated with three other gallotannins, namely, PGA, PGG, and HGD, with molecular masses of 912, 940, and 1094 Da, respectively. **Figure 5** showed that all of these pure compounds give rise to multiple peak DSC profiles for the treated collagen samples. For example, PGG, also found widespread in nature (*19*), had three peaks in its treated sample. The individual peak widths of them are in the region of 3−4 °C, implying three collagen populations with distinct hydrothermal properties. The PGA-treated sample showed two peaks in its profile, and the low temperature peak has a width of 3−4 °C while the other peak showed a width of 6−7 °C. This suggests that the high temperature peak is an overlapping "double peak" profile. The HGD-treated sample showed a much broader profile.

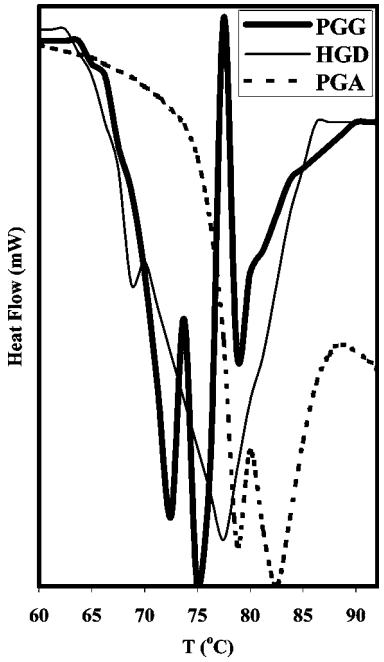

Figure 5. DSC profiles of collagen samples treated with medium-sized gallotannins (910–1100 Da).

Table 1. Shrinkage Temperature (T_s) Values from a Conventional T_s Device in Water

compds	mass (Da)	T_s (°C)[a]	T_s (°C)[b]	P (%)[c]
DGE	366	63.0	63.0	100
TGG	548	62.0	71.5	70
TGE	730	64.5	71.5	70
PGA	912	65.0	76.0	50
PGG	940	63.0	77.0	50
HGD	1094	63.0	71.5	20
castalagin	934	63.0	65.5	90
vescalagin	934	63.0	65.0	90

[a] T_s measured from whole leather samples. [b] T_s measured from the reticular layer. [c] Penetration percentage across the thickness of the leather sample indicated by $FeCl_3$ solution at the cross-cut.

Among the pure tannins, only DGE-treated collagen had a single peak profile. For tannins with a molecular size as small as TGG (548 Da), however, their treated collagen samples start showing heterogeneity. It is also apparent that the heterogeneity of collagen matrix increases with the increase of molecular mass of the polyphenols (or tannins), consistent with the observations (*2*, *13*) that the strength of polyphenol−collagen is positively correlated with the mass and number of galloyl groups of tannins. Therefore, the heterogeneity observed in this work is related to poor tannins diffusivity across the thickness of skin matrix, resulting from the tannin−collagen interactions.

Table 1 shows T_s values obtained from the conventional method. It is apparent that T_s values of whole samples and reticular layers alone, in which it is easier for tannins to diffuse, are noticeably different. The T_s values of the whole sample are lower since the thermally activated sample shrinkage occurs as soon as the "weakest link" described earlier is affected. Consequently, there is no way that the heterogeneity of the treated samples can be assessed with the conventional method. In contrast, the DSC method enables all fractions of collagen with different thermal stabilities to be assessed.

To sum up, the collagen samples treated by two commercial tannin extracts (chestnut and valonea), two ellagitannins, castalagin and vescalagin, and five synthetic gallotannins showed multiple components in terms of hydrothermal stability, implying poor uniformity in tannins' distribution in the collagen matrix. With the conventional shrinkage temperature device (*2*), it is the fraction of proteins with the lowest hydrothermal stability that is measured. Therefore, the tanning efficiency of a tanning material is underestimated and tannins are used inefficiently. However, on the basis of the heat capacity changes of the samples during the thermally activated denaturation process, the DSC method offers an objective and reliable way of evaluating the tanning uniformity. This allows measurements of all peaks with appropriate peak deconvolution methods, which is standard with the modern calorimeters. Although demonstrated with gallotannins, this method is applicable universally to all tanning processes. Additional information is also readily accessible such as thermal dynamics and kinetics of the collagen denaturation. This provides indirect information about the interactions between tannins and collagen and hence the mechanistic aspects.

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