Structural Differences Between Commercial *Acacia Mangium* Tannin and Its Effluent

by

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Abstract

Recycling and reuse techniques provide an efficient way to overcome the environmental issues of the chrome tanning effluent. However, only few vegetable tanning related recycling techniques were developed due to the limited information about the effluent. In this study, a common vegetable tanning process was performed with a commercial *Acacia Mangium* tannin (CAMT) to produce *Acacia Mangium* tanning effluent (AMTE). The tannins in the AMTE and CAMT were characterized using Fourier transform infrared, nuclear magnetic resonance spectroscopy, matrix-assisted laser desorption/ ionization time of flight mass spectrometry, gel permeation chromatography and elemental analysis. The results showed that the stereochemistry and flavan-3-ol subunits of the tannins in the AMTE were the same with the CAMT. However the differences between the tannins in the AMTE and CAMT were presented in terms of the tannin category, molecular weight, and quantities of sulfo acid group. Tannins in the AMTE were mainly composed of procyanidin, while prodelphinidin and procyanidin content showed comparable in the CAMT. Average molecular weight of the AMTE was 1315Da, in contrast to an obviously higher value in the CAMT (2025Da). Meanwhile, higher sulfonic acid group content was also found in the AMTE. These differences were deduced in accordance with the collagen binding ability of the tannins. Results will be valuable for reuse and recycling of vegetable tanning effluent.

Introduction

The recycling technology is regarded as a very effective way to reduce effluent emissions and chemical consumptions, it also exhibits the advantages of decreasing the cost of leather manufacture.[1-3] Many chrome recycling technologies are developed based on the properties and components of chrome tanning effluent, such as membrane filtration, flocculation, sedimentation, ion exchange, ionization.[4,5] Moreover, some of the technologies were practically applied in the leather making process.[6,7] However, the recycling technologies for vegetable tanning were limited due to the insufficiency of detailed information about the vegetable tanning effluent.

Vegetable tannin has been widely used as an important tanning agent for centuries. Unfortunately, high chemical oxygen demand (COD) and high bio-chemical oxygen demand (BOD) are considered as important characters of the vegetable tanning effluent.[8] Meanwhile, the effluent also exhibits long-term negative environmental effects because of the toxicity for microorganisms.[9] Therefore, some technologies were developed to deal with these environmental issues. Cassano *et al*. reported a nanofiltration recycle method to enrich the tannins in tanning effluent, and reused the tannin into the tanning process.[10] Romero-Dondiz et al. developed a vegetable tanning effluent recycle method with ultrafiltration polymeric membrane to also recover the water in the effluent.[11]

Composition of effluent is the most important foundation, which must be known before developing any recycling technologies.[5] In other words, industrial application of recycling and reuse of the vegetable tanning effluent require complete understanding of the effluent nature and structure. However, such research work has not been explored in detail so far to the best of authors' knowledge. In this study, a common vegetable tanning process was performed with a commercial *Acacia Mangium* tannin (CAMT) to produce *Acacia Mangium* tanning effluent (AMTE). Then the tannins in the AMTE and the CAMT were characterized with a series of techniques, including Fourier transform infrared spectrum (FT-IR), matrix-assisted laser desorption/ ionization time of flight mass spectrometry (MALDI-TOF MS), nuclear magnetic resonance (^{13}C NMR), gel permeation chromatography (GPC), and an elemental analyzer. The differences between the tannins were studied, including the flavan-3-ol subunit constitutes, stereochemistry, tannin category, molecular weights, and quantity of sulfonic acid group.

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Manuscript received June 26, 2015, accepted for publication October 8, 2015.

Experimental

Sample Preparation

The bated hide (pH=4.0) was obtained through a conventional method. The chemicals used in the following procedure were based on the weight of the limed hide and the experiment was carried out as follows in a stainless steel drum. The hide was first washed in a 400% float for 2hr, then pickled with 7% sodium chloride, 0.5% formic acid and 1.2% sulfuric acid for 4hr. Then the pH of the float was determined to be 2.3. After this step, the hide was neutralized to pH 4.0 with 6% sodium thiosulfate for 1hr. The hide was then tanned with 30% CAMT for 48h at 25°C. After penetrated completely, the hide was continuing soaked in the float for 12hr. Then, AMTE was collected in effluent. The total phenolics content in AMTE was determined as 196g/L with the Prussian blue method, meanwhile the condensed tannin content was determined to be 27g/L with butanol/HCL assay.[12,13]

Both the AMTE and CAMT solution (32.74g/L, referenced with the total solid content of the AMTE) were filtered with a medium speed filter paper, then dialyzed against distilled water to remove simple phenolics and salts. The glucose was removed by gel filtration on Sephadex LH-20 (GE, American) with 50% ethanol and 50% water, then tannins were obtained with 50% acetone and 50% water. After the solvents were removed by evaporation and a lyophilization successively, the AMTE and CAMT samples were prepared.

FT-IR Spectroscopy

The AMTE and CAMT samples (about 2 mg) were mixed with KBr (100mg) and grinded to a powder (2μm diameter), before 60mg of the mixtures were pressed to form pellets. The spectra of the samples were obtained using a Nicolet iS10 FT-IR instrument (Thermo Scientific, America), with a wavenumber range of 500– 4000 cm^{-1} at a resolution of 4 cm^{-1}.

MALDI-TOF MS spectroscopy

Analyses were performed on an Autoflex III MALDI-TOF MS (Bruker daltonics, Switzerland), which was equipped with a N_2 laser (337 nm). Samples were analyzed in the positive ion reflection mode, with a laser pulse of 3ns. Acceleration and reflection voltage were set to 20.0 and 23.0 kV respectively. Based on the previous reports, samples were dissolved in acetone (4 mg/mL), and mixed with 2,5-dihydroxy benzoic acid (DHB) at a ratio of 1:3 (v: v), NaCl was then added to enhance the ion formation process.[14,15] The mixtures were applied onto a stainless steel target and dried at room temperature, subsequently MALDI-TOF MS analyses were carried out.[16,17]

^{13}C NMR Analysis

NMR spectra were obtained on a AV II-400MHz spectrometer (Bruker, Switzerland), operating at 297K. The solvent used was acetone-d_6 and D_2O mixture (acetone-d_6: D_2O=90:10, v:v).

GPC Analysis

The AMTE and CAMT were dissolved in dimethyl formamide (DMF) and subjected to GPC analysis. An HLC-8320 Gel permeation chromatography (TOSOH Corporation, Japan) equipped with a differential refraction detector, a combination of a TSK gel and a Super AWM-H column were used to measure the molecular weight. DMF as a mobile phase was pumped into the column with flow rate of 0.6ml/min at 25°C. A serious of polymethylmethacrylate was used as standard markers. Content of each peak was calculated by equation (1).

$$\text{Content of fraction (\%)} = 100 \times \text{Peak area/Total area} \quad (1)$$

Elemental Analysis

A EuroVector EA 3000 elemental analyzer (LEEMAN Labs, America) was used to obtain the content of C, H, N, and S in the tannins; then the sulfonyl content was quantified according to equation (2).

$$\text{F:S (mol:mol)} = [C\% \div (12 \times 15)]/(S\% \div 32) \quad (2)$$

F:S is the molar ratio of flavan-3-ol unit: sulfonic acid group; C% is the weight percentage of carbon; S% is the weight percentage of sulfur; 12 is the molar mass of carbon; 32 is the molar mass of sulfur; 15 is the number of carbon atoms in a flavan-3-ol unit.

Results and Discussion

FT-IR Spectroscopic Characterization

The basic structural information of samples were obtained through FT-IR analysis and shown in Figure 1. The bands and peaks arose from –OH stretching vibration (3384 cm^{-1}), C–H stretching in aromatic methoxyl groups (2943 cm^{-1}), aromatic skeleton vibrations (1619 $cm^{-1}$and 1508 cm^{-1}), C–H deformation and aromatic ring vibration (1453 cm^{-1}), C–O stretching in aromatic ring (1312 cm^{-1} and 1214 cm^{-1}), C–O stretching of methoxyl groups (1033 cm^{-1}) conformed that the prepared samples were tannins.[18,19]

MALDI-TOF MS Analysis

MALDI-TOF MS has been used extensively to elucidate the complexity of the vegetable tannin. It is able to provide the

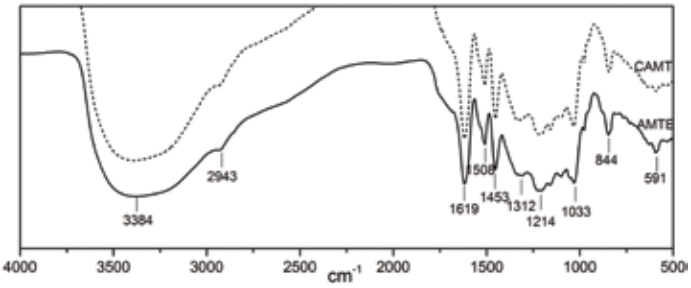

Figure 1.. FT-IR spectra of the AMTE and CAMT samples.

structural information of the flavan-3-ol subunits that compose the tannins. The MALDI-TOF MS spectra of tannins with molecular weights ranging between 500 and 3000Da, are shown in Figure 2. An obvious repetitive pattern of peaks was shown in the spectrum of AMTE and CAMT. Intervals of 288Da, 272Da and 304Da were observed between the peaks with strongest signals. Based on the result obtained by Hoong and Behrens, the intervals of 272Da, 288Da, and 304Da in the MALDI-TOF MS spectrum indicated the presence of (epi) afzelechin, (epi)fisitinidol, (epi)catechin, (epi)robinetinidol and (epi)gallocatechin subunits (Figure 3).[20,21] The presence of these subunits was also confirmed by the 16Da intervals which indicated the molar mass difference of the subunits.

Number of repeating units in each peak was calculated using equation (3).

$$[M+Na]^+ = 23.0 + 2.0 + 272.0A + 288.0B + 304.0C \quad (3)$$

23.0 is the molar mass of Na; 2.0 is the two hydrogen in the end groups; The A is the number of (epi)afzelechin or (epi) fisetinidol units; The B is the number of (epi)catechin or (epi) robinetinidol units; The C is the number of (epi)gallocatechin units.

The results represented the oligomers from dimers to decamers (see TABLE I). It must be noted that, there is no direct relationship between the concentration of an oligomer and the signal intensity, which makes quantitation with MALDI-TOF MS problematic. [22,23] However, a similar subunit composition was still revealed according to the m/z of the peaks (TABLE I), in other words the tannins in AMTE and CAMT were composed of same flavan-3-ol subunits.

^{13}C NMR Spectra

NMR analysis is considered to be the most powerful tool for the tannin characterization. Detailed structural information, including the composition of subunits, average polymerization degree, and stereochemistry, can be obtained through the NMR of carbon atoms in the A ring, the B ring, and the pyranoid C ring (Figure 4.). [24,25]

Based on the NMR study of model compounds and isolated oligomers presented by Czochanska, the typical NMRs of the tannins were identified as follows: NMRs at 146ppm were ascribed to the C–3' and C–5' in the prodelphinidin (PD) B–ring, while signals at 145ppm represented the typical indicator for the procyanidin (PC), 70–90ppm was sensitive to the stereochemistry of the C–ring, the C3 in terminal units had their chemical shift around 67–68 ppm, while NMRs at 73 ppm was attributed to the C–3 in extension units.[26]

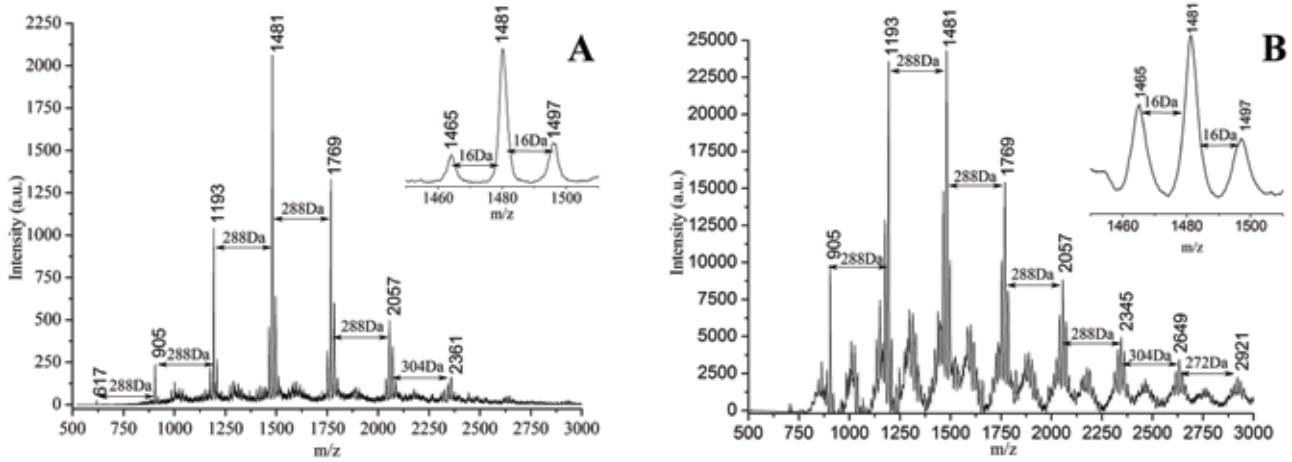

Figure 2. MALDI-TOF MS spectra of the tannins in the (A) AMTE and (B) CAMT.

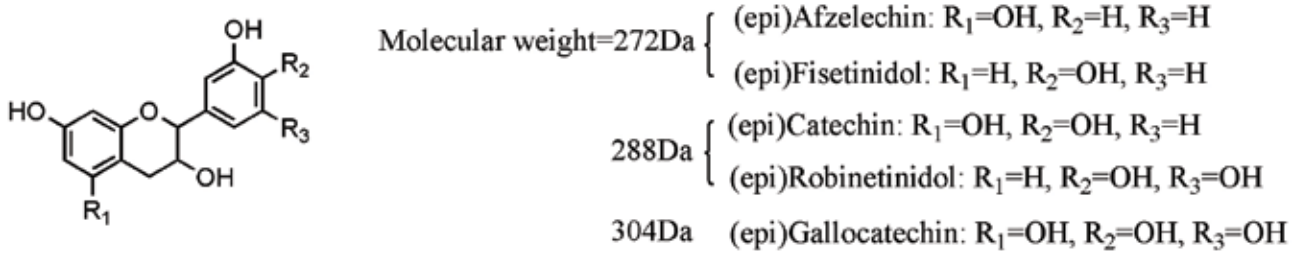

Figure 3. Subunits of the tannins.

TABLE I
Distributions of the tannin oligomers from the MALDI-TOF MS spectra of the AMTE and CAMT.

Oligomer	A*	B**	C***	Calculated	Experimental	Present (yes) or not present (no) in the spectra	
						AMTE	CAMT
Dimer	0	1	1	617	617	yes	no
	0	2	1	905	905	yes	yes
Trimer	0	1	2	921	921	no	yes
	0	4	0	1177	1177	yes	yes
Tetramer	2	0	2			yes	yes
	0	3	1	1193	1193	yes	yes
	1	1	2			yes	yes
	1	0	3	1209	1209	yes	yes
	2	1	2	1465	1466	yes	yes
Pentamer	1	3	1			yes	yes
	0	4	1	1481	1481	yes	yes
	1	2	2			yes	yes
	0	3	2	1497	1497	yes	yes
	1	1	3			yes	yes
	0	6	0	1753	1753	yes	yes
Hexamer	1	4	1			yes	yes
	0	5	1	1769	1769	yes	yes
	1	3	2			yes	yes
	0	4	2	1785	1785	yes	yes
	1	2	3			yes	yes
	0	7	0	2041	2041	yes	yes
Heptamer	1	5	1			yes	yes
	0	6	1	2057	2057	yes	yes
	1	4	2			yes	yes

Table I continued on following page.

Table I continued.

	1	3	3	2073	2073	yes	yes
	0	8	0	2329	2329	yes	yes
Octamer	1	6	1			yes	yes
	0	7	1	2345	2345	yes	yes
	0	6	2	2361	2361	yes	yes
	0	8	1	2633	2633	no	yes
Nonamer	1	6	2			no	yes
	0	7	2	2649	2649	no	yes
	3	1	5			no	yes
	0	7	2	2665	2665	no	yes
	0	10	0	2905	2905	no	yes
Decamer	1	8	1			no	yes
	0	9	1	2921	2921	no	yes
	0	8	2	2937	2937	no	yes

*A is the afzelechin / epiafzelechin / fisetinidol / epifisetinidol (272Da); **B is the catechin / epicatechin / robinetinidol / epirobinetinidol (288Da); ***C is the gallocatechin /epigallocatechin (304Da).

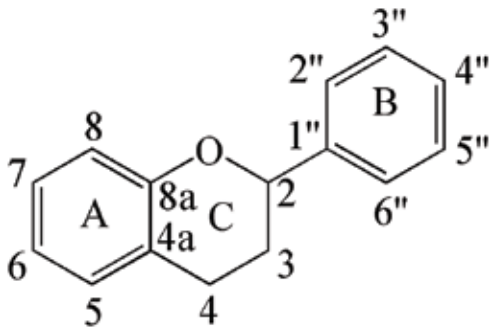

Figure 4. Structure and numbering scheme of the tannin unit.

^{13}C NMR spectra of the tannins in the AMTE and CAMT are shown in Figure 5. The NMRs at 146.29ppm and 144.52ppm were found in the AMTE, while 146.04ppm and 145.41ppm resonances were presented in the CAMT spectra. It indicated both PC and PD were the component of the tannins. This conclusion also confirms the MALDI-TOF MS analysis. However, the relative lower intensity of 146.29ppm resonance revealed that the AMTE were mainly composed of PC (Figure 5A). In contrast, NMRs showed a comparable intensity at 146.04ppm and 145.41ppm, indicating the PC and PD presented comparable quantity in the CAMT. This result was also revealed from the absence of other PD resonance in Figure 5B.

Figure 5A had a resonance line at 63.94ppm, which was designated to the C–3 in the terminal unit of AMTE. In the spectrum of CAMT, 63.96ppm and 70.68ppm were attributed to the NMRs of terminal and extending units, respectively (Figure 5B). Theoretically, these signals have identical T_1 and NOE values, which means the average polymerization degree could be calculated by integrating these signals. Unfortunately, in the case of the spectra presented here, the signal-to-noise ratio is too low for such quantification. However, the NMRs of the AMTE still revealed a molecular weight lower than that of the CAMT, by considering the relative intensity of the C-3 NMR lines.

Resonance at 73.22ppm, 80.30ppm (Figure 5A), and the 80.66ppm, 73.23ppm (Figure 5B) indicated both –*cis* and –*trans* stereoisomers were shown in the AMTE and CAMT. The signal assignment was performed according to the previous literatures, as reported by Davis and Behrens, and the results were summarized in TABLE II.[27,28]

GPC Analysis

GPC is a useful method for calculating the average molecular weight of tannin, meanwhile it allows an analysis of molecular weight distributions. The GPC column elutes tannins by the size of the molecule. Consequently, the oligomers with higher molecular weights (molecular size) presented a shorter retention time, and vice versa.[29,30]

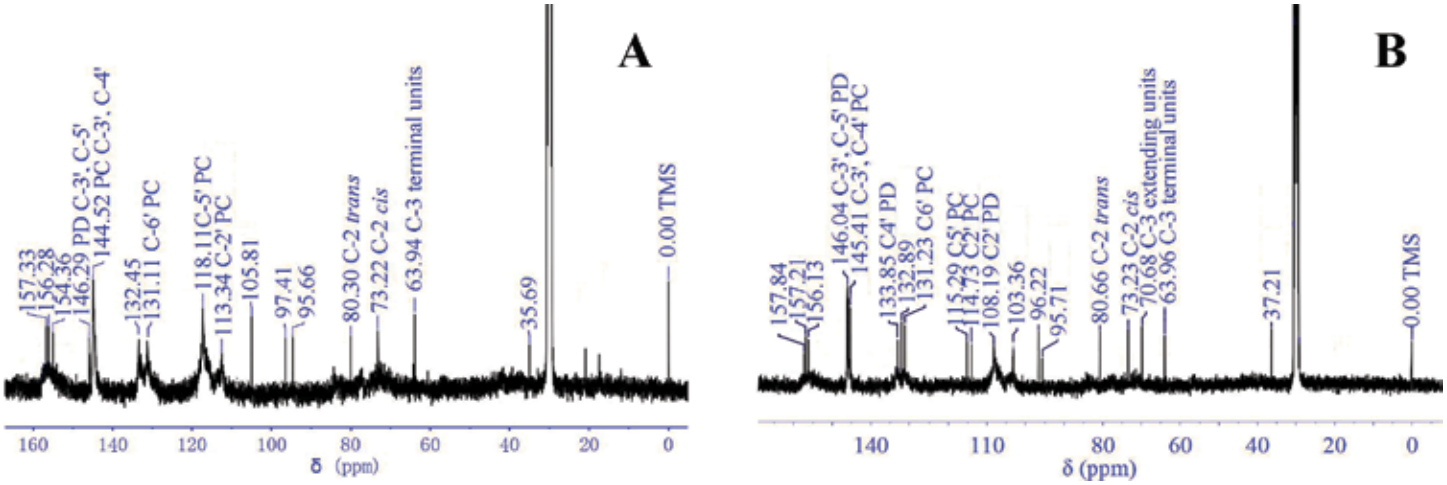

Figure 5. ^{13}C NMR spectrum of the tannins in the (A) AMTE and (B) CAMT.

TABLE II
^{13}C NMR chemical shift data for the tannins in the AMTE and CAMT.

Sample	**C-2(trans)**	**C-2(cis)**	**C-3(terminal)**	**C-3(extending)**	**C-4**
AMTE	80.30	73.22	63.94	none	35.69
CAMT	80.66	73.23	63.96	70.68	37.21
Sample	**C-4a**	**C-5**	**C-6**	**C-7**	**C-8**
AMTE	105.81	156.28	97.41	157.33	95.66
CAMT	103.36	157.21	96.22	157.84	95.81
Sample	**C-8a**	**C-1'**	**C-2' (PC)**	**C-2' (PD)**	**C-3' (PC)**
AMTE	154.36	132.45	113.34	none	144.52
CAMT	156.13	132.89	114.73	108.19	145.41
Sample	**C-3' (PD)**	**C-4' (PC)**	**C-4' (PD)**	**C-5' (PC)**	**C-5' (PD)**
AMTE	146.29	144.52	none	118.11	146.29
CAMT	146.04	145.41	133.85	115.29	146.04
Sample	**C-6' (PC)**				
AMTE	131.11				
CAMT	131.23				

There were obvious differences between the GPC chromatograms of the tannins in the AMTE and CAMTE (Figure 6). Tannins in the AMTE were subdivided into three fractions (a, b, and c) in accordance with shape of the peaks. The retention times of the fractions were: (a) 8.91–10.42 min; (b) 10.42–11.89min; and (c) 12.21–12.86min.

The tannins in the CAMT were also subdivided into three fractions (a', b' and c') which were referenced with the AMTE chromatogram. The areas of these fractions were calculated to reveal the molecular weight distributions of the tannins.

The fraction with the shortest retention time in the AMTE chromatogram (peak a) comprised 58.7% of the total tannins, the corresponding fraction in the CAMT (peak a') comprised 80.9%. In contrast, 8.4% of the AMTE tannins consisted of the fraction with longest retention time (peak c), but the equivalent fraction (peak c') in the CAMT consisted just 2.3%.

The result indicated that compared with the CAMT, the AMTE was mainly composed of low molecular weight tannins. Meanwhile, the average molecular weight (Mn) of tannins in AMTE was 1315Da. Meanwhile the CAMT showed a higher value (2025Da) (Figure 6). It was also comparable to the result obtained from ^{13}C NMR analysis.

The subunits of tannins in AMTE and CAMT were (epi) afzelechin (272Da), (epi)fisetinidol (272Da), (epi)catechin (288Da), (epi)robinetinidol (288Da), and (epi)gallocatechin (304Da) (see TABLE I). Therefore, average polymerization degrees of the AMTE and CAMT was 4.8 and 7.4 respectively in terms of the (epi)afzelechin or (epi)fisetinidol subunits, in contrast to 4.3 and 6.7 respectively, for the (epi)gallocatechin subunits. In other words, the polymerization degree of the AMTE was lower than the CAMT, no matter which subunit was used for calculation.

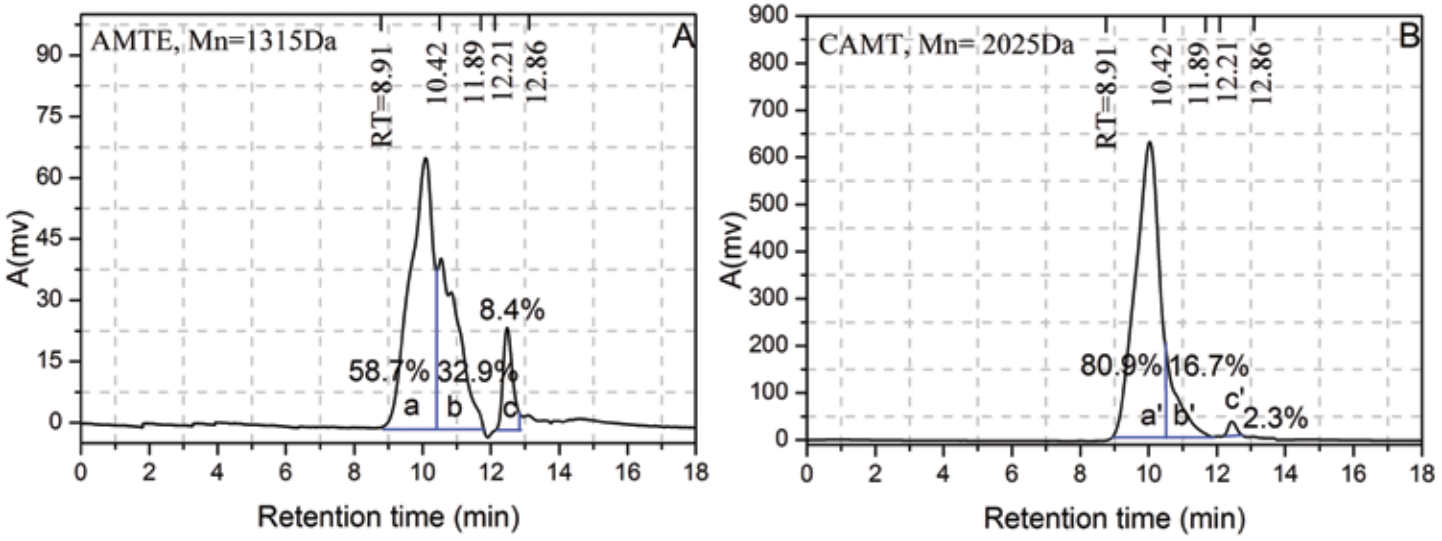

Figure 6. GPC chromatograms of the tannins in the (A) AMTE and (B) CAMT.

TABLE III
Element component of tannins in AMTE and CAMT.

sample	C	H	N	S	F:S
AMTE	20.43	3.32	0.54	0.63	5.7
CAMT	51.47	6.03	0.46	1.28	7.1

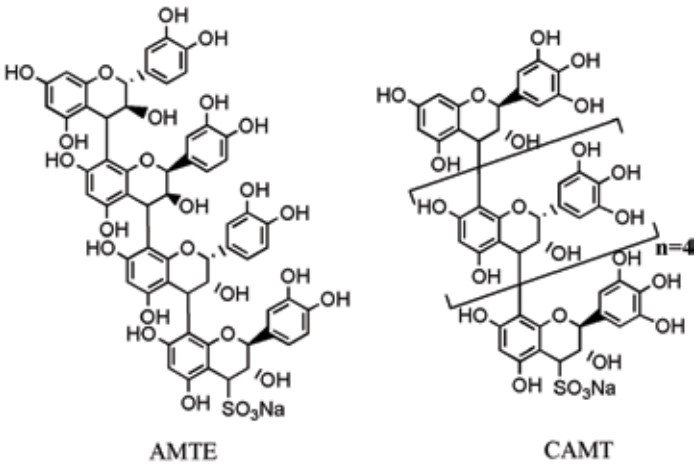

Figure 7. Typical structures of the tannins in AMTE and CAMTE.

Element Component of the Tannins

Sulfonation was considered to be a very important step of the commercial tannin making process, which enhanced the water solubility, while joining the sulfonic acid group to the tannins. The quantities of the C and S atoms were shown in TABLE III, the result demonstrated that the F:S value in the AMTE and CAMT were 5.7 and 7.1. The values indicated that the AMTE contained more sulfonic acid groups, compared with those in the CAMT. Based on the results obtained from above analyses, the typical structure of the tannins in the AMTE and CAMT were illustrated in Figure 7.

The hydrophobic–hydrophilic co-effect theory is generally accepted for illustrating the binding interaction between tannins and proteins (collagen).[31,32] Based on the Sarni-Manchado's research, binding ability and reliability are positively related to the molecular weight of the tannins. In addition, if the tannins are excessively provided, protein combine preferentially with higher molecular weight tannins.[33]Also, positive relation between –OH groups content and binding energy was provided by McManus.[34] It indicated that the more the –OH groups were presented, the easier the binding was occurred. Inverse correlation between quantities of sulfonyl groups and bonding activities was already showed in the previous literature.[35]

Therefore, the tannins with higher molecular weight and more hydroxyl groups (prodelphinidins) in the CAMT were previously bound to the collagen during the vegetable tanning process. On the contrary, the ones presented poor binding ability, in terms of a lower molecular weight, less hydrogen groups (procyanidins) and more sulfonic acid group were remained in the AMTE.

Conclusions

The tannins in the AMTE and the CAMT were composed of same flavan-3-ol subunits and presented same stereochemistry. However the tannins also exhibited a lot of differences, including the category of the tannins, molecular weight, and quantities of sulfo acid group. The tannins in the AMTE were mainly composed of procyanidins, while both procyanidins and prodelphinidins were detected in the CAMT. The polymerization degree and the average molecular weight of AMTE was lower than the CAMT, meanwhile there is higher sulfo group quantity on the tannins in the AMTE than CAMT. Reasons for these structural differences were deduced in accordance with the poor collagen binding activity of the tannins. All of the results will contribute to recycling technologies related vegetable tanning effluent in leather making.

Acknowledgement

Authors wish to thank to the Doctoral Scientific Fund Project of the Ministry of Education of China (No. 20130181130009) and the National Science Foundation of China (No. 21576171) for finance support.

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