

Penetration of the Polyflavonoids and Simple Phenolics: A Mechanistic Investigation of Vegetable Tanning

by

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Abstract

Mechanisms of tanning are the fundamental base for predicting and developing new tanning agents and technologies. However, many mechanistic questions remain for us, such as: How does the penetration happen? How do the phenolic compounds work individually? In this study, penetration processes of the tannin extracts were investigated with the Bayberry extract (prodelphenidins), the Larch extract (procyanidins) and the *Acacia Mangium* extract (prodelphenidin-procyanidin complexes) used as tanning agents. During the process, the tanning liquids were regularly sampled and the simple phenolics and polyflavonoids were quantified respectively with a Prussian blue assay, a conductivity analysis, an acid-butanol assay as well as a HPLC analysis. The results showed that, during tanning, the concentration of polyflavonoids in the tanning liquid decreased continuously. In contrast, for the simple phenolics, the concentration decreased at first and later increased. Therefore, penetration processes of the tannin extracts can be described as: in the beginning, both simple phenolics and polyflavonoids started to penetrate; then the simple phenolics completed their penetration first and reversibly bound with collagens; in the meantime, the polyflavonoids were still penetrating and gradually replacing those simple ones from collagen matrix; at the end of the tanning processes, most of the simple phenolics were replaced by the polyflavonoids, meanwhile they were migrated back into the tanning liquids. The results will provide valuable information to help understanding the mechanism of vegetable tanning.

Introduction

The vegetable tanning methods have been used for thousands of years. Because of their environmentally friendly properties, these methods are still being advocated in current industry and are considered as an important part of leather making.¹⁻³ In order

to help improving the vegetable tanning agents crafts, leather chemists put in massive amounts of efforts to study the mechanisms which age-related the tannin-collagen interactions.⁴⁻⁶

The hydrophobic-hydrogen bonding theory is widely accepted to describe the interactions between proteins and phenolics. This theory highlights the importance of both hydrophobic and hydrophilic sites of the tannin and protein molecules, meanwhile it is considered as the fundamental base to understand their interactions.⁷⁻⁸ Covington provided the “rigid, non-rigid” theory and deduced the relationships between structural and thermal properties.⁹ In this theory, not only the hydrogen bonds, but also the size and rigidity of the tannin molecules are all considered as determinants of the cross-links. Furthermore, it also explained the differences of shrinkage temperatures between vegetable tanned hides.

Since a collagen microfibril model was developed, computer-assisted modeling has become a very useful tool to visualize their interactions.¹⁰⁻¹² Brown prepared a type I collagen microfibril model which was consisted of 15 polypeptide chains of 315 amino acid residues each. After docking the typical phenolics (catechin, gallic acid, gallotannin etc.), some residues were identified as favorable binding sites, including proline, hydroxyproline, arginine, alanine, isoleucine as well as the gap region of the microfibril.¹³⁻¹⁵ These results are very comparable to the ones obtained with instruments like: X-ray diffraction, nuclear magnetic resonance (NMR), scanning electron microscope (SEM) etc., which also reinforced the hydrophobic-hydrogen bonding theory.¹⁶⁻¹⁷ These mechanisms are, of course, indispensable for the industry. However, due to the complexity and diversity of the tannin extracts, the mechanisms of vegetable tanning are still the most unknown ones for us.

As we know, tannin extracts are mainly composed of phenolics. For the condensed type, their phenolics can be subdivided into two parts: one is the simple phenolics (non-tannins) with

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molecular weight lower than 500Da, the other one is the polyflavonoids which have the molecular weight ranging from 500 to 3000Da.¹⁸ In accordance with their structural units, the condensed polyflavonoids can be subdivided into: procyanidin (formed by catechin/epicatechin), prodelphinidin (formed bygallo catechin), profisetinidin (formed byfisetinidin), prorobinetidin (formed byrobinetidinol), etc.¹⁹

The bayberry tannin extracts, *Acacia Mangium*, and larch tannin extracts are widely used in tanneries. They are famous for their outstanding performances on the fullness and penetration properties as well as the thermal and mechanical properties.²⁰⁻²² The polyflavonoids in these tannin extracts are mainly acidulated prodelphinidins, prodelphinidin-procyanidin complexes and procyanidins. Their simple phenolics are basically identified as flavan-3-ol monomers (catechin, epicatechin, gallo catechin, epigallo catechin, gallo catechin gallate), flavonols (such as: myricetin, quercetin and fisetin), flavanols (including: catechin, epicatechin, gallo catechin, epigallo catechin, gallo catechin gallate) and flavones (luteolin and apigenin) (Figure 1).²³⁻²⁵

In this study, bovine hides were processed with these tannin extracts, meanwhile the simple phenolics and the polyflavonoids in bayberry tanning liquids (BTL), *Acacia Mangium* tanning liquids (AMTL) and larch tanning liquids (LTL) were regularly tested and quantified with a Prussian blue assay, a conductivity analysis, a butanol-acid assay as well as a high-performance liquid chromatography (HPLC) analysis. Then the penetration processes of these phenolic compounds were compared and deduced accordingly. The results will provide valuable information for further understanding the mechanisms of vegetable tanning.

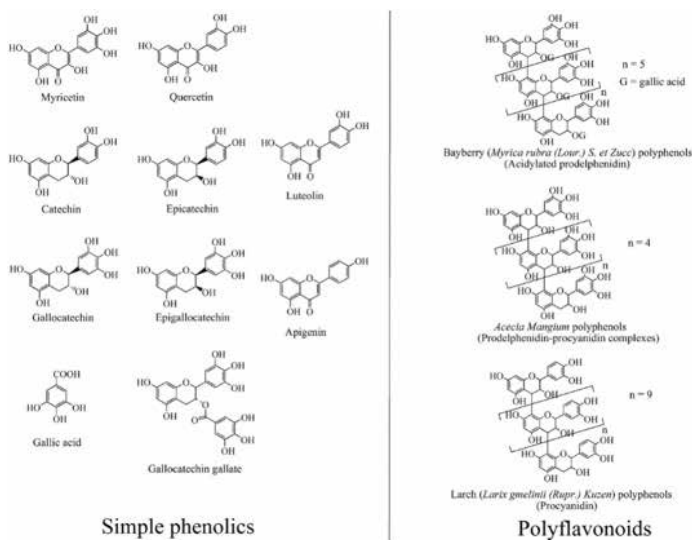


Figure 1. Typical structure of simple phenolics and polyflavonoids in the bayberry, *Acacia Mangium* and larch extracts.²³⁻²⁵

Experimental

Sample Preparation

The pickled hide (pH=2.0) was obtained through a conventional method. Chemicals used in the following procedure were based on the weight of the pickled hide. The hide was first neutralized to pH 6.5 with 10% sodium bicarbonate in the pickling liquor. It was then streaming washed with distilled water for 24hr and followed by a 400% float for 2hr. The washing was constantly repeated until the conductivity of the float was lower than 30 μ S/cm.

The following processes were carried out in a water bath shaker at 30°C with a shake speed of 60rpm, and the commercial bayberry tannin extract (71.80% of tannin content, Wuming vegetable tannin extracts factory, Guangxi, China), the commercial *Acacia Mangium* tannin extract (68.12% of tannin content, Wuming vegetable tannin extracts factory, Guangxi, China) and commercial larch tannin extracts (58.03% of tannin content, Yakeshi vegetable tannin extracts factory, Inner Mongolia) were separately used as tannin agents in different trials. The hides were cut into even pieces (1 \times 2cm) and about 50g hide pieces were tanned with 30% vegetable tannin extracts in 200% floats. 5mL of the BTL, AMTL and LTL were separately sampled in every 8 to 24hr meanwhile penetration was checked with a stainless knife.

Prussian Blue Assay

The Prussian blue tests basically preceded in accordance with Price's study but modified with Graham's research.^{26,27} BTL samples were diluted 1:1500 with distilled water, AMTL and LTL were prepared using a 1:500 and 1:2500 dilution respectively. 1mL diluted sample was mixed with 1mL potassium ferricyanide (0.02mol/L), then reacted with 1mL acid ferric trichloride (0.02M in 1M HCl) at 24 \pm 1°C for 15min. After 3mL phosphoric acid (6.02mol/L) were added, samples were kept standing for 2min. After that, 2mL gum Arabic was added then kept standing for another 3 min. Then the absorbance was immediately recorded at 700nm in 1 cm cells, using an UV-1900 spectrophotometer (Jinghua, Shanghai, China).

Conductivity Test

The samples were diluted 1:100 with distilled water, then the conductivity of the liquids were tested with a DDS-307 conductivity tester (Jinghua science instrument, Shanghai, China) at 25°C.

Acid-butanol Assay

Samples were diluted 1:100 with distilled water. Then 1.0mL sample was mixed with 6.0mL acid butanol (5%(v/v) hydrochloric acid in n-butanol). After 75min of reaction in a boiling water bath, the samples were cooled with ice water bath and absorbance was recorded at 550nm in 1 cm cells, using an UV-1900 spectrophotometer (Jinghua, Shanghai, China).²⁸

HPLC Analysis

HPLC analysis was carried out based on Liao's research, but the condition was adjusted to obtain stable peak areas.²⁹⁻³¹ Samples were first diluted 1:10 with methanol (10% (v/v) methanol in distilled water) then filtered through a microfiltration membrane (450nm bore diameter). The HPLC analysis was performed with an Agilent series 1100 HPLC (Santa Clara, CA, USA) equipped with a diode array detector. The absorbance was recorded at 280nm. Separation was carried out at 25°C on a 5µm Agilent Eclipse XDB-C8 column (4.6×300mm) using a gradient of methanol (A) and 1.0% acetic acid in H₂O (B) with a 60min program: 0-20min, 10%A; 90%B; 20-21min, 10-30% A, 90-70% B; 21-40min, 30% A, 70% B; 40-41min, 30-50% A, 70-50% B; 41-60min, 50% A, 50% B. After that, the chromatogram was integrated with Origin 8.0 (Origin Lab Corporation, USA).

Results and Discussion

The Total Phenolics in the Tanning Liquids

The Prussian blue assay is a method that involves aredox reaction as well as a metallic complexation reaction. Since the phenolics are oxidized by Fe³⁺, the iron [III] hexacyanoferrate [II] chelates will be formed with Fe²⁺ meanwhile provide maximum absorbance at 720nm.^{26, 32} In this assay, the absorbance of the solution is positively related with the quantity of the phenolic compounds. In other words, the simple phenolics and polyflavonoids are all included in this reaction.

The quantities of the total phenolic compounds, were measured across a range of the whole vegetable tanning process, using Prussian blue assay. As we can see from the absorbency of these tannin liquids (Fig.2), similar patterns were shown on the results obtained with BTL, AMTL and LTL samples. At the beginning of the tanning processes, their absorbencies were 0.74, 1.53 and 0.45 (0h), however, after 16h of tanning, these values dropped to 0.32, 0.59 and 0.20 respectively. Quantities of the phenolic compounds were sharply decreased within the first 16h. After that, decreasing rates of these absorbencies became certainly slower, meanwhile the tannin extracts completed their penetrations around 40h to 56h. Soon afterwards, their absorbencies reduced to 0.23, 0.28 and 0.15 (56h to 64h) and reached their lowest values. Then, quantities of these phenolic compounds were gradually increased, while their absorbencies raised to 0.27, 0.33 and 0.22 at 112h.

The Simple Phenolics in the Tanning Liquids

The simple phenolic acid, tannic acid, gallic acid, etc. would be electrolytes in solution, but the majority of polyphenolic tannins would not.³³ Compared with the polyflavonoids, the simple phenolics have simpler structures and always provide lower protonation constants.³⁴⁻³⁵ In other words, simple phenolics can easily ionize and charge in a neutral or an acidic media. Thus,

the conductivity of a tanning liquids mainly contributed by these simple phenolics.

As it was shown in Figure 3, the conductivities of the BTL, AMTL and LTL were rapidly decreased to 546, 1176 and 711 µS/cm respectively, within the first 16h, from 680, 1247 and 812µS/cm (0h) at the beginning. Then the conductivities of these liquids decreased too their lowest values around 40 to 56h (518µS/cm, 1113µS/cm, and 665µS/cm,). Afterwards, their conductivities started to increase again, and reached 549µS/cm, 1152µS/cm and 687µS/cm at the end of the processes (112hr). Consequently, for the simple phenolics in BTL, AMTL and LTL samples, their quantities all followed a rule of decreasing firstly and then increasing with tanning process went on.

The Polyflavonoids in the Tanning Liquids

In the acidic and alcoholic medium, the condensed polyflavonoids can be degraded, then yield anthocyanidins and provide a red color, while the simple phenolics are not able to participate in this degradation process.³⁶ Therefore, the condensed polyflavonoids can be specifically quantified using the acid-butanol assay; meanwhile the absorbance cannot be influenced by the simple phenolics.^{28,37}

The results of acid-butanol assay are shown on Figure 4. Absorbencies of the BTL, AMTL and LTL samples were all found to be sharply decreased from 0 to 16hr. Like the results obtained with Prussian blue and conductivity assays, these absorbencies remained decreasing, but the decreasing rates became slower after the first 16h. However, the lowest absorbencies were not shown around 60h, these absorbencies were found to keep decreasing across the whole tanning processes, moreover, the increasing trends were not shown.

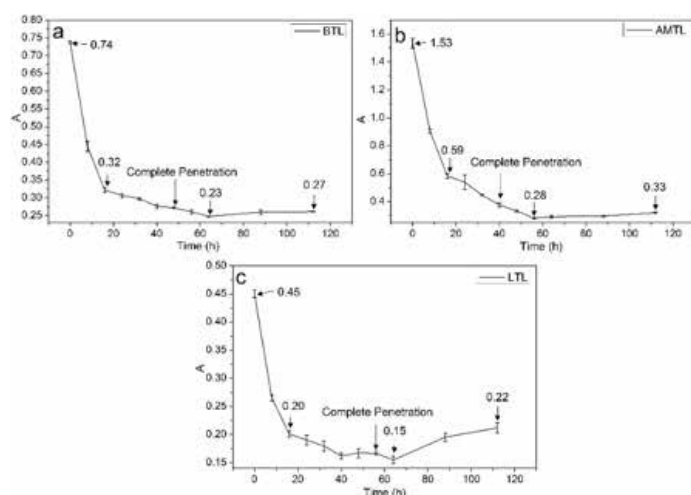


Figure 2. The absorbance of (a) BTL, (b) AMTL and (c) LTL samples tested with Prussian blue assay.

The Results of the HPLC Analysis

The reverse phase HPLC analysis is commonly used to identify and quantify the phenolic compounds. Normally, an acidified methanol-water or acetonitrile-water mobile phase will be applied on the column, the simple phenolics and polyflavonoids will gradually be eluted with the increasing polarity of the mobile phase.³⁸⁻⁴⁰ For the tanning liquid samples taken from different time points, the phenolic compounds were separated into different peaks with different retention times (Figure 5). In accordance with Liao's studies, these peaks were assigned to simple phenolics and polyflavonoids separately;^{29,31} meanwhile areas of these peaks were also integrated and shown on Table I.

Areas of these peaks were found to be constantly changing during the whole vegetable tanning process (Table 1). Therefore, both simple phenolics and polyflavonoids were engaged in the whole tanning processes. At the beginning (0hr), areas of the simple phenolics were 204.87 (BTL), 31.30 (AMTL) and 28.58 (LTL). As the processes went on, their quantities started to decrease and reached their lowest values around 40h to 56h (BTL=109.05, 56hr; AMTL=18.88, 48hr; LTL=19.91, 40hr). After these tannin extracts completed their penetration, it began to increase and reached obvious higher values at 112hr (BTL=113.81; AMTL=24.76; LTL=26.41). On the contrary, the quantities of the polyflavonoids showed a different pattern. Areas of those polyflavonoids in the BTL, AMTL and LTL samples were 580.30, 338.43 and 579.23 respectively (0hr). Afterwards, these values kept decreasing as the time went on, quantities of the polyflavonoids only showed their lowest values at the end of the tanning processes (BTL=80.00, 112hr; AMTL=35.40, 112hr; LTL=133.30, 112hr).

The Penetration Process of the Simple Phenolics and the Polyflavonoids

The tanning liquids were regularly sampled and assessed by a series of methods, where simple phenolics and polyflavonoids

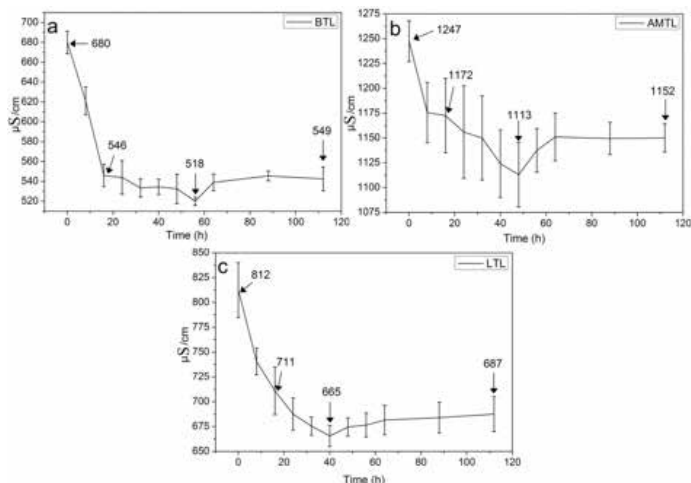


Figure 3. The conductivities of (a) BTL, (b) AMTL and (c) LTL samples.

were quantified, both in combination and separately. Some phenomena were observed, their penetration processes were deduced as follows accordingly.

(1) At the beginning of the tanning process (0-16hr), both simple phenolics and polyflavonoids penetrated rapidly due to the high osmotic pressure across the hides. In the meantime, concentrations of these phenolics reduced sharply in the tanning liquids.

(2) As the tanning processes went on (16-64hr), penetration of these compounds became slower. Their concentrations were kept decreasing in the tanning liquids but with lower rates. After that, the concentrations of the simple phenolics reached their lowest values. In the meantime, concentrations of the polyflavonoids were still decreasing and the lowest values were not detected until the end of the tanning process. Based on the previous research, the micelles which are composed of the simple phenolics provide smaller volumes in an aqueous solution, and this colloid chemical property benefits their penetration process.^{20,22} In addition, the polyflavonoids have higher reactivity and astringency which is able to hinder their penetration through hide.⁴¹⁻⁴² Therefore, in the present study, the simple phenolics completed their penetration and arrived the central part of the hide prior to the polyflavonoids, meanwhile their concentrations reduced to the lowest values in the tanning liquids.

(3) As we know, the simple phenolics and polyflavonoids were defined as "non-tannins" and "tannins" because of their difference in astringency. Compared with the simple phenolics, the polyflavonoids have more hydroxyl groups on their surface areas, meanwhile they are able to form more hydrogen bonds as well as stronger non-bonded interaction forces in the collagen-phenolics reactions.⁴³⁻⁴⁴ Furthermore, once the polyflavonoids are combined with collagens, these complexes will provide more

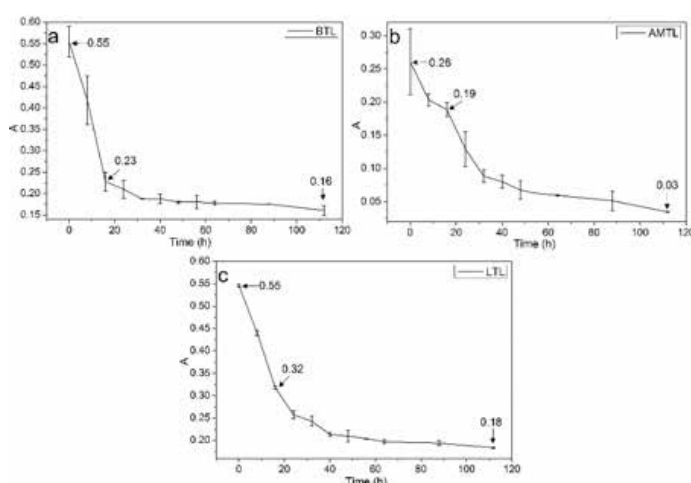


Figure 4. The absorbance of (a) BTL, (b) AMTL and (c) LTL samples tested with acid-butanol assay.

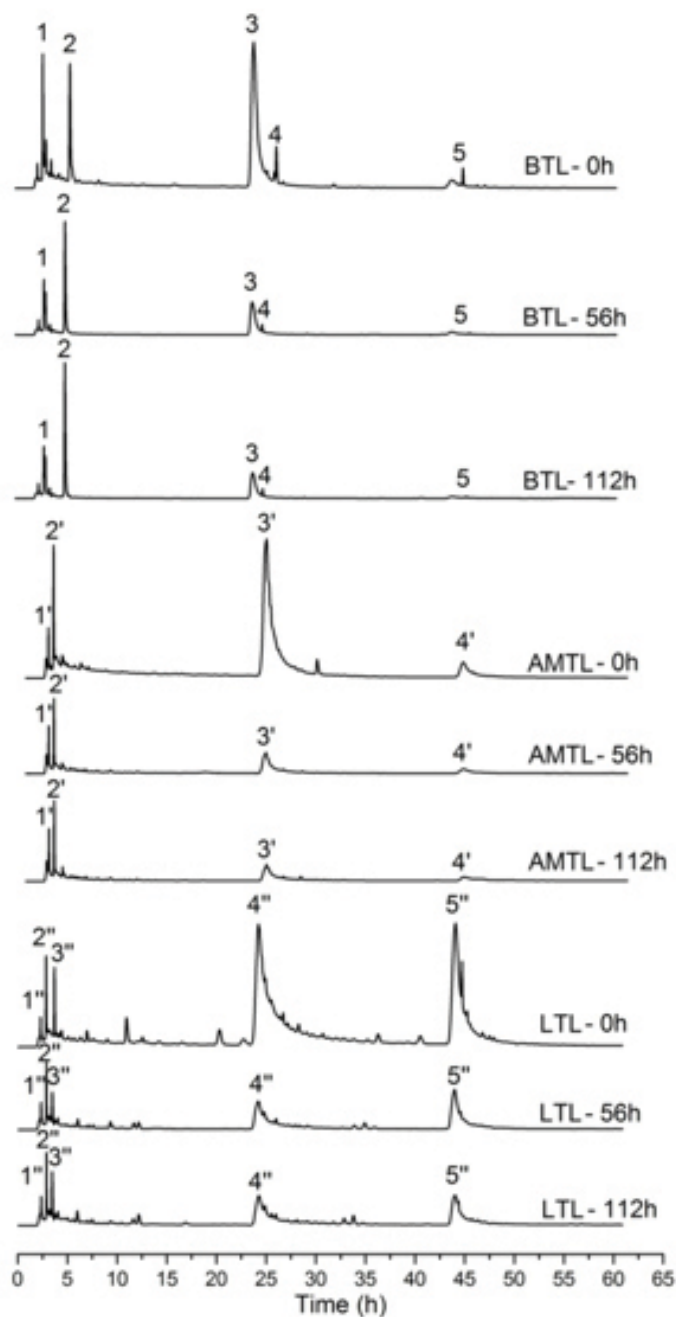


Figure 5. The chromatogram of the BTL, AMTL and LTL samples.

stable properties than those ones formed by simple phenolics.^{22,45,46} Therefore, after the simple phenolic completed their penetration (40-56h), the polyflavonoids were still penetrating meanwhile replacing those simple phenolics which are reversibly bound on collagens. Then these simple phenolics were exuded into the tanning liquid to balance the osmotic pressure. As a consequence, concentration of the simple phenolics were gradually increased.

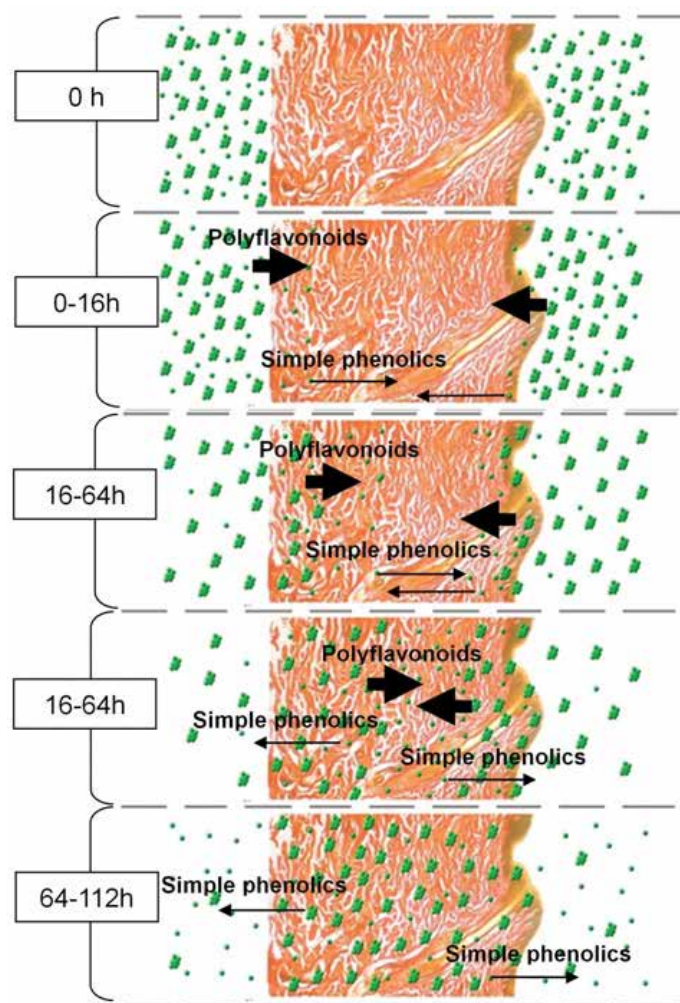


Figure 6. Penetration process of the simple phenolics and the polyflavonoids.

(4) After 64h of the penetration, concentrations of the simple phenolics were still gradually increasing while the polyflavonoids were decreasing. It indicated that the replacing between polyflavonoids and simple phenolics were still proceeding until the end of the tanning processes. After tanning processes were finished (112h), most of the simple phenolics were remained in the liquids, and the polyflavonoids were irreversibly bound with the collagens. This deduction was also supported by our previous studies.⁴⁷⁻⁴⁸ The penetration process of the simple phenolics and polyflavonoids were demonstrated on Figure 6.

Conclusion

The condensed tannin extracts (Bayberry tannin, prodelphenidins; Larch tannin, procyanidins; *Acacia Mangium* tannin extract, prodelphenidin-procyanidin complexes) were individually applied on the vegetable tanning. Compositions of

Table I
Peak areas of the simple phenolics and polyflavonoids in the HPL Chromatograms.

	BTL		AMTL		LTL	
	Simple phenolics (peak 1+ peak 2)	Polyflavonoids (peak 3+ peak 4+ peak 5)	Simple phenolics (peak 1'+ peak2')	Polyflavonoids (peak 3'+ peak4')	Simple phenolics (peak 1''+ peak 2'' + peak 3'')	Polyflavonoids (peak 4''+ peak 5'')
0hr	204.87±21.42	580.30±31.42	31.30±5.21	338.43±46.79	28.58±4.86	579.23±43.61
8hr	151.46±13.40	251.52±21.33	25.35±3.46	159.35±10.60	25.85±3.17	401.88±48.33
16hr	122.54±11.20	179.33±17.66	19.69±3.15	103.25±17.31	24.84±0.69	248.51±37.24
24hr	121.59±14.61	170.47±12.31	19.63±3.37	92.44±12.77	22.84±3.01	244.84±33.17
32hr	121.22±12.22	156.90±15.78	20.04±2.61	78.90±9.40	21.96±2.00	233.49±16.53
40hr	119.66±13.49	140.91±14.32	19.28±1.19	63.26±9.18	19.91±2.43	173.43±26.41
48hr	112.42±8.66	112.54±6.65	18.88±3.82	59.65±6.87	21.65±2.38	149.69±13.42
56hr	109.05±8.54	111.90±17.52	20.57±2.15	45.10±7.00	23.97±3.27	141.82±17.55
64hr	127.12±11.21	104.80±6.31	22.60±1.92	39.86±5.21	23.18±2.64	140.93±12.61
88hr	130.87±15.31	89.45±7.21	23.14±0.68	38.22±4.40	24.24±1.34	137.27±15.45
112hr	133.81±7.64	80.00±7.28	24.76±2.79	35.40±2.42	26.41±2.60	133.30±12.07

the tanning liquids were continuously tested across the whole vegetable tanning processes. The results showed that in the first 16h, concentrations of all phenolic compounds were sharply decreased in the tanning liquids; when the tanning processed to 16-64h, concentrations of the simple phenolics decreased to their minimum values, then gradually increased to higher values. However, concentrations of the polyflavonoids were kept decreasing until the end of the tanning process; After 64h of the tanning, concentrations of the polyflavonoids were still gradually decreasing while the simple phenolics were increasing.

Based on these results, penetration of the tannin extracts were deduced and concluded as: In the beginning of vegetable tanning, penetration of both simple phenolics and polyflavonoids proceeded quickly; then the simple phenolics completed their penetration prior to polyflavonoids, while the polyflavonoids were still penetrating and gradually replacing those simple phenolics from collagen matrix; at the end of the tanning, most of the simple phenolics were replaced by the polyflavonoids, the simple phenolics squeezed out from the hides.

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