Optimization of Enzyme-assisted Phenolic Reactions Applied to Thermal Stabilization of Collagen using Response Surface Methodology

by

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Abstract

Vegetable tannins used in tanning of hides and skins are limited by surface reactions as well as large molecular weights, reducing penetration into the skin and lowering the thermal stability or tanning effect. Investigation into the utilization of small phenolic compounds such as catechin to improve penetration with subsequent *in situ* enzyme-catalysed polymerization may provide a novel and alternative tanning agent. In this study, catechin was oxidized by enzymatic catalysis using laccase, with the polymerization confirmed by FT-IR and UHPLC. Tanning experiments were undertaken to measure the effect of laccase-catalysed polymerization of catechin in the thermal stabilization of collagen, *i.e.*, the change in shrinkage temperature between the treated and untreated sample of hide powder (ΔTs). A factorial design was subsequently used to study process parameters that may affect enzymatic reactions: temperature, substrate concentration, enzyme concentration and incubation period. The statistically significant variables were found to be temperature and incubation period, and were thus chosen to be studied further for process optimization using response surface methodology. Maximum ΔTs can be obtained for a temperature of 34.6°C and incubation period of 25 hours. This study demonstrates that the stabilization of collagen (ΔTs) is increased with the use of enzyme-assisted polymerization.

Introduction

Tanning chemically stabilizes hides and skins against bacterial attack, transforming the raw material into leather. The process of tanning increases the hydrothermal stability of leather, *i.e.*, the material's resistance to wet-heat in terms of the denaturation transition.[1] Hydrothermal stability is given by the measurement of shrinkage temperature, which is the temperature when the triple helices of collagen are denatured to a random coil structure.[2]

The importance of metal-free tanning is growing as environmental concerns lead the industry away from the use of chromium sulphate as a tanning agent. The technology currently applied for producing metal-free leather consists of using glutaraldehyde as a tanning agent. Glutaraldehyde, however, is not biodegradable. Furthermore, it makes wastewater treatment difficult, as glutaraldehyde is toxic to the microorganisms in activated sludge as well as inhibiting the biodegradation of other organic compounds in the effluent.[3] Therefore, the development of new technologies aimed at lower environmental impact processes is important and necessary.

The word 'tannin' is associated with natural vegetable extracts used for the tanning of leather. An early definition of vegetable tannins states that these substances have a molecular weight between 500 and 3000 Dalton and are water-soluble compounds that, besides the usual phenolic reactions, have other properties such as precipitating gelatin, alkaloids and other proteins.[4] Haslam[5] introduced the term 'plant polyphenol' to these mixed vegetable extracts, and included in this definition the low molecular weight phenolic compounds (non-tannins).

The gap zone of the collagen molecule, measuring approximately 1.4 nm in diameter and 40 nm in length consists in a void in the structure. The empty spaces facilitate the infiltration of polyphenol molecules, suggesting a

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possible site for the binding of vegetable tannin molecules.[6] The amino acid sequence in the gap zone is strongly hydrophobic, rich in alanine, proline, hydroxyproline and phenylalanine amino acids.[7] This is consistent with the theory of polyphenol complexation with proteins to be driven initially by hydrophobic effects, with hydrogen bonding as a secondary effect serving to re-enforce these initial interactions.[8] Tanning of collagen occurs due to the crosslinking of many peptide chains with the same polyphenol molecule; hence the molecule has to contain a suitable number of functional groups and must have at least 500 Da to promote bonding amongst the collagen chains.[9] Small phenolic molecules, having better mobility, may be absorbed faster and reach spaces between collagen molecules that would normally not be accessible to large molecules. The tanning capacity of these small molecules may be improved by performing polymerization *in situ*, once penetration has occurred into the hide/skin.

Polymerization of low molecular weight phenols can be carried out through oxidation using agents such as benzoyl peroxide, alkaline ferricyanide or CuCl/pyridine.[10] Some biological reactions that involve oxidative polymerization of phenolic compounds are catalyzed by enzymes, for example, the synthesis of lignin from peroxidase-catalyzed polymerization of monolignols.[11] Recently, research in the field of polymer science has explored the use of enzymes as an alternative environmentally-friendly catalyst, applying the principles of natural product synthesis in the production of synthetic polymers.[12-15]

An oxidizing enzyme that acts on mono- and polyphenols is laccase (p-diphenol: dioxygen oxidoreductase EC 1.10.3.2). It is a multi-copper enzyme that requires molecular oxygen to oxidize phenolic compounds by a radical-catalyzed reaction mechanism.[16] The oxidative effect of laccase produces free radicals that are prone to further reactions, forming dimers, oligomers or polymers that are covalently coupled by C-C, C-O or C-N bonds.[17]

A substrate subjected to oxidation by laccase is catechin, a monomeric flavan-3-ol present in green tea that, in conjunction with epicatechin, constitutes the repeated units of procyanidin tannins.[18] Catechin and procyanidins are known for their biological activities, acting as an aid for preventing diseases caused by oxidative effects, such as myocardial ischemia, in addition to presenting anticarcinogenic and antimutagenic activity.[19, 20]

The polymerization of catechin by laccase-catalyzed reaction was confirmed by Fourier-transform infrared spectroscopy (FT-IR) and phloroglucinolysis followed by ultra-high performance chromatography (UHPLC).[21] The aim of the study was to apply catechin to hide powder and perform the polymerization *in situ* using laccase as a catalyst. The effectiveness of the new application was evaluated by measuring the shrinkage temperature of collagen. A factorial design 2^4 (4 variables studied at 2 levels each)[22] was then used to study main effects and interactions of process parameters that may affect enzymatic reactions: temperature, incubation period, laccase concentration and catechin concentration. Central composite design and response surface methodology were applied in order to achieve the maximum increase in shrinkage temperature of hide powder.

Materials and Methods

Materials

Laccase from *Trametes versicolor* and +(-) catechin were purchased from Sigma-Aldrich (Poole, UK) and were of analytical grade. UHPLC solvents were HPLC grade and obtained from Fisher-Scientific (Loughborough, UK). All other chemicals used were of reagent grade and obtained from Fisher-Scientific (Loughborough, UK).

Hide powder was prepared from cattle hides at the Institute for Creative Leather Technologies (University of Northampton, Northampton, UK). Limed and fleshed hides were delimed, bated and acidified with acetic acid until pH 5.0. Excess water was removed mechanically. The hides were cut into 2-3 cm^2 pieces, and dehydrated using acetone. The specific gravity of the solvent is lowered as the water is removed; the dehydration is then continued until the specific gravity of the solvent remains the same. The pieces were kept in the oven at 40°C for 30 minutes to fully remove the solvent. The samples were then cut into smaller pieces (not larger than 1 cm^2) and ground to a relatively uniform particle size (less than 2 mm). Analysis and specifications of hide power are presented in Table I.

TABLE I
Analytical results and specifications of bovine hide powder.

Analysis	Specifications[a]	Analytical results
Ash content (%)	<0.5	0.38 ± 0.07
pH	5.0 – 6.5	5.87 ± 0.25
Onset temperature (Ts) (°C)	-	50.44 ± 0.90

[a]According to SLC131 – Specification of hide powder.[23]

Laccase-catalyzed Polymerization of Catechin

In order to analyze the products formed by laccase-catalysed oxidation of catechin by FT-IR and UHPLC and verify the occurrence of polymerization, incubation of laccase with catechin was performed to obtain oxidized catechin in a powder form, according to the following procedure. Catechin was dissolved in 3 mL of acetone and added to 10 mL of acetate buffer (pH 5.5) to produce a final concentration of 0.1 M. Laccase, at a final concentration of 50 mg/l, was added to the medium and the incubation was carried out at 30°C for 24 hours with an agitation speed of 100 rpm in a shaking water bath (Digital water bath OLS200, Grant Instruments, Cambridge, UK). A control was prepared by omitting the enzyme and following the same experimental conditions as the test sample. After the incubation period, the samples were freeze-dried to a powder form using an Alpha 2-4 LSC freeze-dryer (Martin Christ, Osterode, Germany), for 48 hours at -40°C and vacuum 0.250 mbar. A final drying stage of 30 minutes was performed at 5°C and vacuum 0.011 mbar.

FT-IR

The samples obtained from the procedure described in Section 2.2, as well as catechin as received from the supplier, were analyzed using Fourier-transform infrared spectroscopy (FTIR-8400S, Shimadzu, Tokyo, Japan), coupled with in-compartment diamond Attenuated Total Reflectance (ATR) accessory (DuraSamplIR II, Smiths Detection, Watford, UK). Scanning was carried out at 4000 to 600 cm^{-1} at a nominal resolution of 4.0 cm^{-1} using 520 scans, against a background measurement. The samples were kept in a desiccator for 24 hours prior to analysis.

UHPLC – Phloroglucinolysis

The phloroglucinol solution consisted of 5 mg of phloroglucinol per ml of acidic ethanol (0.1M HCl in ethanol) prepared fresh prior to the start of the experiments. The acid cleavage/phloroglucinolysis was carried out with 4 mg of the polymerized catechin dissolved in 1 ml of the phloroglucinol solution and allowed to react at room temperature overnight. The solvent was evaporated under nitrogen and the residue was dissolved in 600 µl of distilled water. The solution was then extracted three times with ethyl acetate (600 µl per extraction). The three ethyl acetate fractions were combined and evaporated under nitrogen. The residue after evaporation was dissolved in 800 µl of 70% (v/v) methanol in ultrapure water obtained with a Direct-Q 5 UV system (Millipore, Watford, UK) and analyzed using a Dionex Ultimate 3000 apparatus (Thermo Scientific, Hemel Hempstead, UK). A reverse phase column, Accucore C18 150 x 2.1 mm, 2.6 µm particle size was used (Thermo Scientific, Hemel Hempstead, UK). Column temperature was kept at 25ºC. Mobile phase flow was 0.208 ml/min, injection of sample was 1.0 µl and the eluted compounds were monitored by a Diode Array detector at 280 nm. The solvent system was composed of 0.1% (v/v) trifluoroacetic acid and 5% (v/v) acetonitrile in water (solvent A), and 0.1% (v/v) trifluoroacetic acid in acetonitrile (solvent B). Total run was 25 minutes, performed with an isocratic flow of 100% solvent A for 1.2 minutes, followed by a gradient of 71.5% (A) and 28.5% (B) until 15.5 minutes, held isocratic at 71.5% (A) and 28.5% (B) until 17 minutes, and finished with an isocratic flow of 100% (A) from 17 to 25 minutes. Data was collected and analyzed using the software Chromeleon 7 (Thermo Scientific, Hemel Hempstead, UK).

The application of laccase-catalyzed polymerization of catechin to bovine hide powder Catechin of varying final concentrations of 40, 80 and 160 mM were first dissolved in 3 mL of acetone, and subsequently added to a medium composed of 500 mg hide powder and 25 mL acetate buffer (pH 5.5). The samples were then stirred for 30 minutes with a magnetic stirrer (Hot plate stirrer PC-351, Corning, New York, USA) to rehydrate the hide powder and allow the interaction of catechin with hide powder. The pH was measured with a pH meter Seven Multi (Mettler Toledo, Schwerzenbach, Switzerland) and adjusted to pH 5.5 ± 0.2 when necessary with 0.1 M NaOH. Laccase at a final concentration of 40 mg/L was added to the samples to initiate the reaction. One control sample for each concentration of catechin was prepared omitting the enzyme. The reaction mixtures were kept for 24 hours in a shaking water bath (Digital water bath OLS200, Grant Instruments, Cambridge, UK) at 25°C and 100 rpm. The enzymatic reaction was terminated with the addition of a sodium azide solution (final concentration 0.2 mM). At the end of the reaction, the samples were centrifuged (Heraeus Megafuge 16R, Thermo Scientific, Hemel Hempstead, UK) at 3075 *g* for 10 minutes, and washed twice with deionized water. After

TABLE II
Real and coded variables studied in the 2^4 factorial design.

Variables		Level		
Real	**Coded**	**-1**	**0**	**1**
Temperature (°C)	X_1	25	30	35
Catechin concentration (mM)	X_2	40	60	80
Laccase concentration (mg/L)	X_3	5	12.5	20
Incubation period (h)	X_4	8	16	24

this procedure, the treated hide powder was kept for 48 hours in a controlled atmosphere at 23°C and 50% relative humidity, according to BS EN ISO 2419:2012 - Sample preparation and conditioning.[24] The hydrothermal stability was evaluated by the shrinkage temperature (Ts), measured using a differential scanning calorimeter DSC822e (Mettler Toledo, Schwerzenbach, Switzerland). Thermal analyses were undertaken of fully hydrated samples (5 mg ±1) placed in 40 µL aluminum pans and hermetically sealed. The temperature of the analysis ranged from 25 to 125°C with a heating rate of 5°C/min, under a nitrogen atmosphere. Shrinkage temperature (Ts) was taken as the onset. The results obtained from the samples were expressed in terms of increase in shrinkage temperature (ΔTs), according to Eq. (1).

ΔTs = Ts of treated hide powder on completion of experiment – Ts of untreated hide powder (1)

The software SPSS version 20 (International Business Machines Corp., USA) was used for the statistical analyses. Analysis of variance (ANOVA) was performed and the variables with a confidence level above 95% ($p \leq 0.05$) were considered as significant.

Factorial Design and Central Composite Design

Process parameters that may affect enzymatic reactions were studied for process optimization. Temperature, catechin concentration, laccase concentration and incubation period were varied according to Table II. The experimental procedure and measurement of shrinkage temperature was carried out as described in Section 2.5.

Statistical analyses were conducted using the software SPSS version 20 (International Business Machines Corp., USA). Analysis of variance (ANOVA) was performed and the variables with a confidence level above 95% ($p \leq 0.05$) were considered as significant.

A central composite design was carried out with the addition of star points for the variables found to be significant after statistical treatment of the 2^4 factorial.[25] The data obtained from the central composite design was fitted to a second-order model, shown in Eq. (2).[26]

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i<j}^{k} \beta_{ij} x_i x_j + \sum_{i=1}^{k} \beta_{ii} x_i^2 \quad (2)$$

Where y is the response variable, k is the number of variables, β_0 is the constant term, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is the interaction coefficient, x_i and x_j represent the variables.

Coefficient estimation for the mathematical model was calculated using the software SPSS version 20 (International Business Machines Corp., USA).

RESULTS AND DISCUSSION

Evaluation of Laccase-catalyzed Polymerized Catechin Using FT-IR and UHPLC

FT-IR analyses were performed on the product of laccase-assisted process and for the control product, which omitted the enzyme and was subjected to the same experimental procedure (Section 2.2). The analysis was also carried out for pure catechin, and the spectra are shown in Figure 1.

FT-IR analyses suggest that laccase-catalyzed oxidation of catechin results in the combination of monomers to form polymers. The absorbance for the product of laccase-catalyzed oxidation is lower in the range between 1600 and 1000 cm^{-1}, compared with pure catechin and the control, possibly due to the polymer's structure having more rigid groups than the monomer,[27] reducing the bond vibrations.

The control presents a peak at 1689 cm^{-1}, within the wavenumber range for quinones (1690 – 1675 cm^{-1}),[28] which indicates the possibility of oxidation due to the exposure to the experimental conditions, even without enzymatic action. The

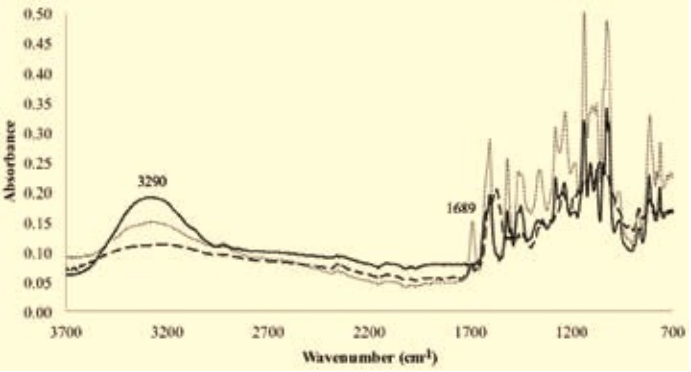

Figure 1. FT-IR spectra of pure catechin (——), control sample of catechin omitting the enzyme (·····), and catechin after the laccase-catalysed reaction (— — —).

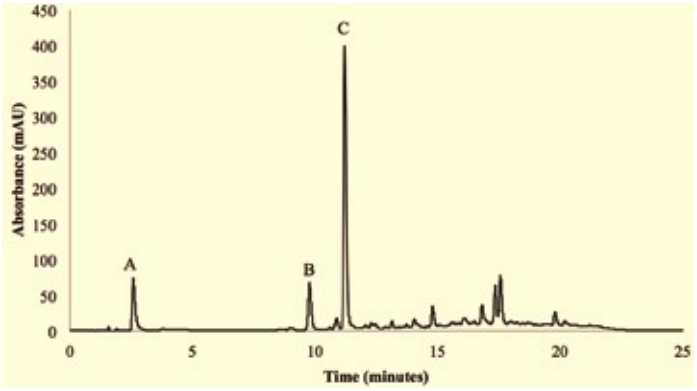

Figure 2. UHPLC chromatogram of a sample of catechin oxidized by laccase, after the process of phloroglucinolysis. The identified peaks correspond to: phloroglucinol (A); catechin-phloroglucinol adduct (B); catechin (C).

peak for the enzyme-catalyzed product at 1689 cm^{-1} (quinone) has lower absorption than the peak for the control, indicating that, due to the catalytic effect of the enzyme producing free radicals rapidly, the quinones possibly suffered further reactions, potentially forming polymeric structures. As expected, untreated catechin does not show any peak in the quinone region.

The phenolic H-bonded O-H stretch peak at 3290 cm^{-1} [28] is present for all samples. The peak for the oxidation product is broader than the O-H peak of pure catechin. This may indicate the increase in intramolecular weaker H-bonds for the polymer in comparison with the stronger intermolecular H-bonds for the monomer.[29] The higher absorbance in the O-H peak for pure catechin is related to the higher density of the bond vibration, indicating that the phenolic moieties in the control and the oxidation product may have suffered transformations, into quinones or polymerization.

Figure 2 shows the UHPLC chromatogram of a sample of catechin oxidized by laccase after the process of phloroglucinolysis.

The compound with a retention time of 2.59 minutes corresponds to the unreacted phloroglucinol. The catechin detected at 11.21 minutes was not only related to terminal units of the polymer being released, but also possibly unreacted catechin present in the sample. For this reason, it is not possible to estimate the degree of polymerization. The chromatogram shows the appearance of a peak for the adduct catechin-phloroglucinol with a retention time of 9.77 minutes. This adduct indicates the breakage of the interflavanyl bond in the polyphenol molecule and further reaction with phloroglucinol,[21] suggesting that the free radicals combined to form at least dimers, and possibly oligomers and polymers.

Investigation into the effect of Laccase-catalyzed Process for Varying Concentrations of the Substrate Catechin on the Stabilization of Hide Powder

The effect of enzyme on various concentrations of catechin on the stabilization of bovine hide powder was measured and compared with the control (no enzyme) (Figure 3).

The analysis of variance indicated a significant difference between the control and the test sample, for all concentrations studied: $F(1,12) = 187.151$, $p<0.001$. *Post Hoc* analysis (Turkey's range test) for catechin concentration indicated a significant difference between 40 mM and 80 mM ($p = 0.036$), and between 40 mM and 160 mM ($p = 0.020$). No significant differences between 80 mM and 160 mM of catechin were found ($p = 0.949$). For subsequent research, a maximum concentration of 80 mM of catechin was used in the factorial design of experiments, as presented in the following section.

Catechin, although a small phenolic molecule, can increase the shrinkage temperature of collagen. Madhan *et al.*[30] applied catechin to rat tail tendon collagen, at 27°C, 24 hours and no agitation. For 5 mM of catechin, the authors measured a ΔTs of 6.5°C in relation to native collagen; for 10 mM the ΔTs was 8°C and for 20 mM, the ΔTs was 9°C. These findings are coherent with the data obtained for the control (omitting the enzyme), where catechin was applied to hide powder under the conditions of 25°C, 24 hours and 100 rpm. A ΔTs of 9.68°C was obtained for 40 mM of catechin; a ΔTs of 9.70°C was obtained for 80 mM of catechin and ΔTs of 11.21°C for 160 mM of catechin.

Condensed tannins, such as mimosa and quebracho extracts, increase the shrinkage temperature of hides and skins by 20 to 25°C.[31] The increase in shrinkage temperature of hide powder using the enzyme-assisted process was found to be 20.65°C for 80 mM of catechin and 19.62°C for 160 mM of catechin, reaching similar ΔTs values obtained by the application of condensed tannins. This demonstrates the potential of using biotechnology in creating tanning reactions for stabilization of collagen.

Factorial Design and Central Composite Design to Optimize Process Parameters of Laccase-assisted Stabilization of Bovine Hide Powder Using Catechin as a Substrate

The results of the factorial design to study the influence of variables temperature, catechin concentration, laccase concentration and incubation period on the shrinkage temperature of bovine hide powder are shown in Table III.

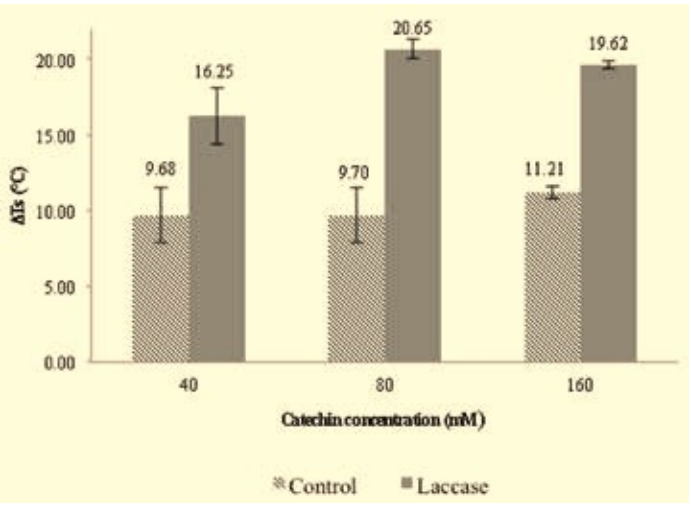

Figure 3. Comparison of the effect of laccase (40 mg/L) with a control (no enzyme), for varying concentrations of catechin, on the ΔTs of hide powder. The reaction conditions were 25°C, pH 5.5±0.2 and 24 hours. Error bars denote the standard deviation (n=3).

The analysis of variance was carried out to estimate the main effects and interaction effects. Results indicated a significant effect for temperature: $F(1,2) = 64.207$, $p = 0.015$, and incubation period: $F(1,2) = 24.234$, $p = 0.039$. Analysis also revealed a significant interaction between the laccase concentration and incubation period: $F(1,2) = 22.022$, $p = 0.043$. The effect of the interaction of laccase concentration and incubation period may be observed as higher values of ΔTs are obtained by increasing the incubation period, even for the lower concentration of enzyme. Therefore, in order to minimize the use of enzyme, laccase concentration was kept constant at 5 mg/L with optimization achieved by varying the temperature and incubation period, whilst maintaining a constant catechin concentration at 40 mM.

A central composite design was then undertaken with the variables temperature and incubation period. Experimental matrix and results obtained for the increase in shrinkage temperature of hide powder are shown in Table IV.

TABLE III
Experimental matrix for the factorial design 2^4 and response variable expressed as change in shrinkage temperature (ΔTs).

Run	Coded				Temperature (°C)	Catechin concentration (mM)	Laccase concentration (mg/l)	Incubation period (h)	ΔTs (°C)
	X_1	X_2	X_3	X_4					
1	-1	-1	-1	-1	25	40	5	8	16.18
2	1	-1	-1	-1	35	40	5	8	18.50
3	-1	1	-1	-1	25	80	5	8	13.68
4	1	1	-1	-1	35	80	5	8	17.95
5	-1	-1	1	-1	25	40	20	8	18.90
6	1	-1	1	-1	35	40	20	8	21.11
7	-1	1	1	-1	25	80	20	8	17.12
8	1	1	1	-1	35	80	20	8	19.82
9	-1	-1	-1	1	25	40	5	24	18.56
10	1	-1	-1	1	35	40	5	24	21.39
11	-1	1	-1	1	25	80	5	24	17.72
12	1	1	-1	1	35	80	5	24	20.07
13	-1	-1	1	1	25	40	20	24	16.98
14	1	-1	1	1	35	40	20	24	19.65
15	-1	1	1	1	25	80	20	24	18.85
16	1	1	1	1	35	80	20	24	21.76
17	0	0	0	0	30	60	12.5	16	18.97
18	0	0	0	0	30	60	12.5	16	18.41
19	0	0	0	0	30	60	12.5	16	19.71

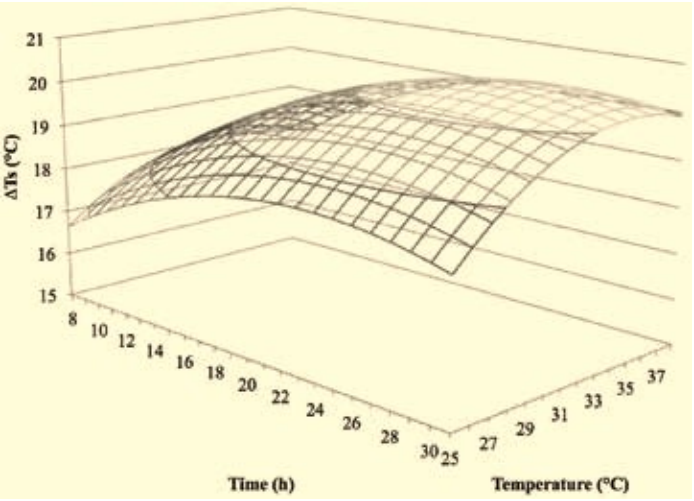

Figure 4. Response surface graph for the increase in shrinkage temperature (ΔTs) of bovine hide powder as a function of incubation period and temperature, calculated according to Eq. (3).

Data was fitted to a second-order model given by the equation showed in Eq. (3). The statistical significance of the equation was confirmed using the F-test. The regression model was highly significant ($p<0.001$) with a coefficient of determination: $R^2 = 0.928$.

$$\Delta Ts = -7.117 + 1.376X_1 + 0.298X_4 - 0.021X_1^2 - 0.008X_4^2 + 0.003X_1X_4 \quad (3)$$

Figure 4 shows the response surface graph obtained from Eq. (3).

The maximum point is obtained when the first derivate equals to zero. The optimum point in which the increase in shrinkage temperature is maximum corresponds to a temperature of 34.6°C and incubation period of 25 hours. Higher temperatures most likely contribute to the denaturation of the enzyme, and a longer incubation period will not increase the ΔTs further, suggesting that the substrate is no longer available for the enzymatic reaction.

TABLE IV
Experimental matrix for the central composite design and response variable expressed as change in shrinkage temperature (ΔTs).

Run	Coded		Temperature (°C)	Incubation period (h)	ΔTs (°C)
	X_1	X_4			
1	-1	-1	25	8	16.18
2	1	-1	35	8	18.50
3	-1	1	25	24	18.56
4	1	1	35	24	21.39
5	-1.414	0	23	16	18.13
6	0	-1.414	30	4.7	17.40
7	1.414	0	37.1	16	19.50
8	0	1.414	30	27.3	20.29
9	0	0	30	16	19.96
10	0	0	30	16	19.63
11	0	0	30	16	19.75
12	0	0	30	16	19.65

CONCLUSION

Experiments using bovine hide powder as a model substrate for leather making confirmed the modification of the catechin structure with laccase, enabling tanning-type reactions. This was demonstrated with a higher ΔTs obtained with the enzyme-assisted process, compared with samples where no enzyme was used (control). The optimum reaction conditions to maximize the shrinkage temperature of hide powder were found with the application of response surface methodology.

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