Enzymatic polymerisation *in situ* of depolymerised mimosa tannin applied to stabilisation of collagen

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I dedicate this thesis to my parents Carmen and Flavio, who even from afar, gave me all the love and support I needed to pursue this result. I love you both.

Dedico essa tese aos meus pais Carmen e Flavio, que mesmo à distância, me deram todo o amor e suporte que precisei na busca desse objetivo. Eu amo vocês.

“There is no such thing as applied science. There is only the application of science, which is very easy to anyone who is the master of the theory of it.”

Louis Pasteur

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Abstract

Vegetable tannins used in tanning of hides and skins are limited to surface reactions by their large molecular weights. The molecular weight reduces penetration into the skin and lowers the thermal stability or tanning effect. Investigation into the utilisation of small phenolic compounds such as catechin to improve penetration with subsequent *in situ* enzyme-catalysed polymerisation may provide a novel and alternative tanning agent. In this research, catechin was oxidised by enzymatic catalysis using laccase, with the polymerisation confirmed by FT-IR and UHPLC. Tanning experiments were undertaken to measure the effect of laccase-catalysed polymerisation of catechin in the thermal stabilisation of collagen, by monitoring the change in shrinkage temperature between the treated and untreated sample of hide powder (ΔTs). This study demonstrates that the stabilisation of collagen (ΔTs) is increased with the use of *in situ* enzyme-catalysed polymerisation.

Depolymerisation of condensed tannins is presented as an alternative source of low molecular weight phenolics to be applied in the stabilisation of collagen. In this research, mimosa tannin from the Black Wattle tree (*Acacia mearnsii*) was used in the depolymerisation process. Preliminary experiments on depolymerisation were undertaken using the methods laccase-mediator system and L-cysteine in mild acidic medium, both showing unsatisfactory results. Acid-catalysed depolymerisation followed by nucleophile addition is a common analytical method for determining the degree of polymerisation of proanthocyanidins. The acid-catalysed depolymerisation method was scaled-up, with the addition of pyrogallol as a nucleophile trapping agent, and powdered depolymerised mimosa was obtained. Data show an increase in the monomeric content and decrease in percentage of condensed tannins for the acid-catalysed process, indicating the potential of the depolymerisation of mimosa tannin in obtaining environmentally friendly sources of low molecular weight phenolic compounds for use in large scale/industrial applications.

The depolymerised product was applied in the stabilisation of hide powder and goat skin. Using hide powder, data show that the laccase-assisted polymerisation of the depolymerised mimosa reached higher values of ΔTs in comparison with the conventional process employing unmodified mimosa. Using goat skin, the new process achieved similar values of ΔTs as the conventional process. In order to obtain similar results for goat skin as obtained with hide powder, an optimisation may be carried out. Therefore, data obtained demonstrates the potential of the new route in vegetable tanning of leather.

List of Abbreviation

ΔTs: change in shrinkage temperature

ABTS: 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid)

ANOVA: analysis of variance

ATR: attenuated total reflectance

BCS: Basic chromium sulphate

cm: centimetre

D_2O: deuterated water

DMSO-d_6: hexadeuterodimethyl sulfoxide

FT-IR: Fourier Transform – Infrared Spectroscopy

g: gram

HOBt: 1-hydroxybenzotriazole

LCE: Leucocyanidin Equivalent

mg: milligram

ml: millilitre

µl: microlitre

n: number of independent experiments

nm: nanometre

PA: proanthocyanidin

PGG: pentagalloyl glucose

PRP: proline-rich proteins

RSM: response surface methodology

Spp.: species

Ts: shrinkage temperature

UK: United Kingdom

UV-Vis: ultraviolet - visible

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Chapter 1 Introduction

The growing concern about the environment is leading the chemical industry to the development of cleaner manufacture processes. In this context, the application of biotechnology arises as an alternative to conventional chemical processes, aiming at environmentally friendly production (Kawaguchi *et al.*, 2016). Biotechnological processes are often more expensive and the process conditions such as temperature and pH must be strictly controlled (Drauz *et al.*, 2012). Therefore, research covering biochemistry and biotechnology is essential to promoting knowledge in this field and enabling the application in an industrial scale.

The use of biochemistry and biotechnology is often related to the application of enzymes. Enzymes are proteins that act as catalysts. The catalytic action of an enzyme is very specific for each substrate (Burton, 2003). There is a narrow range of pH and temperature in which enzymes work, which means that the process must be carried out in controlled conditions (Saini, 2010). Enzymes are biodegradable and obtained from natural sources, such as fungi, bacteria and plants (Kudanga and Roes-Hill, 2014). Hence, the substitution of chemicals with enzymes results in more sustainable processes.

This research is based on the *in situ* enzymatic polymerisation of low molecular weight polyphenols in order to optimise leather tanning through facilitating penetration of tanning molecules and increasing crosslinking for the stabilisation of collagen molecules. An alternative source of low molecular weight phenolics is the depolymerisation of large molecular weight condensed tannins, such as mimosa extract. Mimosa is extracted from the Black Wattle tree, which has a cultivation cycle of 7 years (Santana *et al.*, 2015), an advantage when comparing with other sources of tannins such as quebracho from *Schinopsis* spp., which fully mature in 100 years (Roecklein and Leung, 1987).

1.1 Motivation

Hides and skins are subjected to degradation by microorganisms as soon as they are removed from the animal at the slaughterhouse (Sharphouse, 1983). The process of tanning stabilises collagen (the main protein in skin) against microorganism attack, transforming hides and skins into leather (Heidemann, 1993). 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 (Covington, 2009). 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, meaning the protein chain loses the secondary structure (Reich, 2007). Tanning increases the shrinkage temperature (Ts) of collagen, thereby raising the hydrothermal stability (Bienkiewicz, 1983). High hydrothermal stability is important to assure that the leather is able to resist all steps of production of manufactured goods. Nearly 90% of the world's leather production is tanned with basic chromium sulfate, which gives the leather a Ts of approximately 100°C or above, whereas the Ts for leather tanned with vegetable extracts such as mimosa, ranges between 80°C to 85°C in general (Covington, 2009).

The use of vegetable extracts for tanning of hides and skins dates from the prehistorical times (Van Driel-Murray, 2000). Vegetable tannins are limited by their surface reactions with collagen, as well as the large molecular weights, reducing penetration into the skin and so lowering the thermal stability or tanning effect. The vegetable tanning process has been overshadowed by cost-effective and more rapid tanning agents such as basic chromium sulfate (BCS). The use of chromium salts in tanning is being restricted by certain sectors such as the automotive upholstery, as the possibility of oxidation to Cr(VI) raises environmental concerns (Hedberg *et al.*, 2014). One technology currently applied for producing metal-free leather consists of using glutaraldehyde as a tanning agent (Covington, 1997). Glutaraldehyde, however, is not biodegradable and is toxic to the microorganisms in activated sludge, which makes wastewater treatment difficult, and may inhibit the biodegradation of other organic compounds in the effluent (Sun *et al.*, 2008). Therefore, the development of new technologies aimed at lower environmental impact processes is required.

Conventionally, plant polyphenols (vegetable tannins) are employed as extracts from natural products, such as leaves, barks and wood. The vegetable extracts, however, are highly reactive towards collagen and tend to react on the surface of the skin. Therefore, it may result in superficial tanning and poor penetration into the skin. The process must be carried out for

a prolonged period of time, in order to allow the complete penetration throughout the hide thickness (Covington, 1997). This increases the cost of leather production, one of the reasons why the process was replaced by combination and chromium tannage (Bienkiewicz, 1983). Hence, the application of vegetable extracts is limited.

1.2 Research aim

The research carried out was concerned with determining the potential of enzymes to create new products for tanning reactions. The path for achieving an efficient process, in a shorter period of time, consists in separating the tanning steps:

1. Breakdown of polyphenols, which facilitates the penetration of the small molecules into the hide structure without tanning;
2. Enzymatic polymerisation *in situ*, to transform the small non-tannins into larger tannins, thus facilitating tanning reactions with the collagen.

The overall aim of the research is to formulate and apply biotechnology in facilitating organic reactions for stabilising collagen, and to determine conditions for enzymatic catalysis of condensed tannins to improve tanning efficacy.

1.3 Overview of the thesis

This thesis is divided in 8 Chapters, according to the following description:

This introduction chapter presents the relevance of the thesis theme and motivation of this research.

Chapter 2 presents a literature review, describing relevant previous research as well as the theoretical background on the subject.

Chapter 3 describes the materials and analytical methods used to evaluate the experiments that support the conclusions obtained from the research.

Chapter 4 presents kinetic experiments using laccase as a catalyst, with different phenolic substrates. In this chapter, experiments confirming the free radical generation mechanism of laccase-catalysed reactions are described.

In Chapter 5 a study of the laccase-catalysed polymerisation of catechin applied to collagen stabilisation is presented.

Chapter 6 presents preliminary experiments on the depolymerisation of mimosa tannin, using laccase-mediator system and application of L-cysteine in a mild acidic medium. These methods demonstrated unsuitability for the application on stabilisation of collagen.

Chapter 7 describes the depolymerisation of mimosa tannin using acid cleavage with phloroglucinol and pyrogallol as nucleophile trapping agent, as well as the application of depolymerised tannins on hide powder and goat skin, and further laccase-assisted polymerisation aiming at increasing the thermal stabilisation of the collagen matrix.

The conclusion in Chapter 8 shows an overview of the results and their relevance. Furthermore, it presents suggestions for future research.

Chapter 2 Theoretical framework

2.1 Hides and Skins

Hides and skins are the largest organ in the animal body, playing a role in regulating body temperature, storage of lipids and water, and protection against the environment, such as bacterial attack. They also reflect body changes, such as age, sex, diet and health conditions.

Hides and skins present three distinct layers:

- Epidermis: the outermost layer of skin, which is in contact with the environment.
- Dermis: rich in collagen, the dermis is the basis of leather making (Bienkiewicz, 1993).
- Hypodermis: internal layer, rich in fat, connects the skin with the animal muscles.

Figure 2-1 illustrates the cross-section of hides. In beamhouse operations, the epidermis layer is removed in unhairing and the hypodermis layer is removed in the fleshing process. Hence, the dermis is the most important layer for the tanner, being the raw material for leather processing.

The dermis is divided into two layers: grain and corium. The grain is the upper layer and is composed of interwoven collagen fibres having a diameter of ≤ 5 μm (O'Leary and Attenburrow, 1996). This layer contains the hair follicles, and composes the leather surface after processing. The corium layer, located beneath the grain layer, is composed of thicker bundles of fibres, with a typical diameter of ≈ 100 μm (O'Leary and Attenburrow, 1996). The boundary between the layers is not precise, with a zone where the diameter of fibres has a non-uniform distribution.

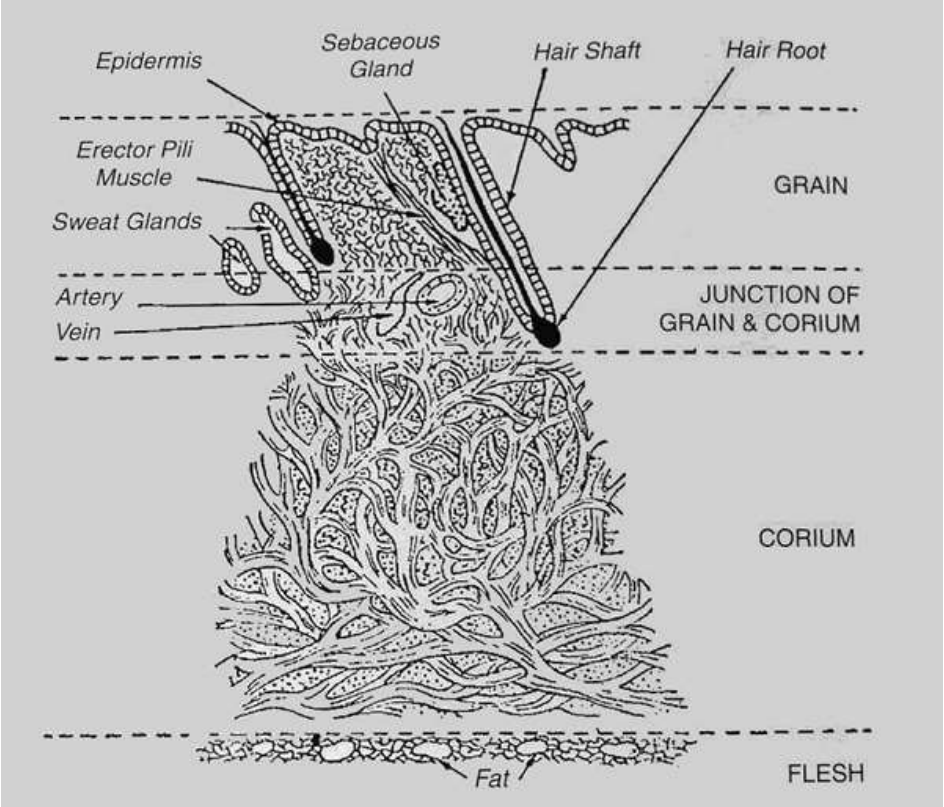


Figure 2-1: Cross-section of hides (Sharphouse, 1983).

The composition of hides and skins varies for different animals and breeds. The approximate composition of a freshly-flayed bovine hide is given in Table 2-1.

Table 2-1: Composition of hides (Sharphouse, 1983).

Component		***Composition (%w/w)***
Water		64
Protein	Elastin	0.3
	Collagen	29
	Keratin	2
	Albumens, globulins	1
	Mucins, mucoids	0.7
Fats		2
Mineral salts		0.5
Other substances		0.5

The keratin content of hides and skins can vary widely, depending on the amount of hair present. Collagen is the most important component for leather making, as the other substances are removed in leather processing.

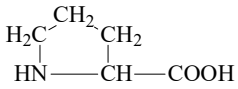
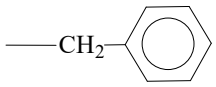
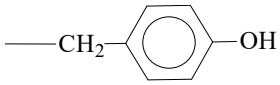
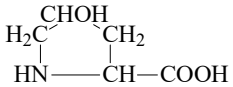
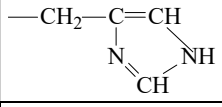
2.1.1 Collagen Structure

Collagen is characterised by a glycine residue at each third position of the amino acid sequence, and can be represented as $–(Gly–X–Y)_n–$. Often X is proline and Y hydroxyproline, making 12% of collagen triplets as –Gly–Pro–Hypro, 44% as –Gly–X–Hypro or –Gly–Pro–Y and 44% as –Gly–X–Y, where X and Y are other types of amino acids (Covington, 2009).

A unique feature of collagen is its high content in hydroxyproline. Both proline and hydroxyproline stabilise the individual chains in the triple helix due to their stereochemical restrictions (Brodsky and Ramshaw, 1997). Hydroxyproline plays an important role in the stabilisation of the triple helix structure, with participation of water molecules in hydrogen bonding between chains in the molecule and between molecules (Bella *et al.*, 1995). The small side chain of glycine contributes to the structure by allowing interaction between carboxylic and amino groups of adjacent strands, bringing the strands together (Guthrie-Strachan and Antunes, 2013).

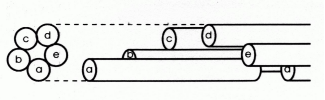
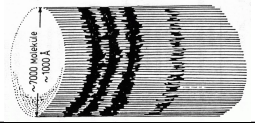
Table 2-2 shows the amino acids present in collagen molecules.

Table 2-2: Amino acids present in collagen molecules (adapted from Santos, 2006).

	Amino acid	***Radical –R***
Non-polar	Glycine	–H
	Alanine	$–CH_3$
	Valine	$–CH–(CH_3)_2$
	Leucine	$–CH_2–CH–(CH_3)_2$
	Isoleucine	$–CH–(CH_3)–CH_2CH_3$
	Proline	
	Phenylalanine	
	Methionine	$–CH_2–CH_2–S–CH_3$
Hydroxyl group	Serine	$–CH_2–OH$
	Threonine	$–CH(OH)–CH_3$
	Tyrosine	
	Hydroxyproline	
Amide	Asparagine	$–CH_2–CO–NH_2$
	Glutamine	$–CH_2–CH_2–CO–NH_2$
Acid	Aspartic acid	$–CH_2–COOH$
	Glutamic acid	$–CH_2–CH_2–COOH$
Basic	Lysine	$–CH_2–CH_2–CH_2–CH_2–NH_2$
	Arginine	$–CH_2–CH_2–CH_2–NH–C(NH)–NH_2$
	Histidine	
	Hydroxylysine	$–CH_2–CH_2–CH(OH)–CH_2–NH_2$

Fibrillar collagen is characterised by the assembly of three polypeptide chains (α chains) to form trimers with triple helical conformation and by the supramolecular assembly of trimers to form fibrils and fibres (Giudici *et al.*, 2003). Table 2-3 shows the structural hierarchy of collagen.

Table 2-3: Structural elements and hierarchy of collagen (adapted from Santos, 2006).

Structural element	***Dimensions***	***Structural hierarchy***
Amino acids		H_2N-CHR-COOH
Tripeptide		3 amino acids: Gly-X-Y
Polypeptide chain	1050 amino acids	$(Gly\text{-}X\text{-}Y)_n$
Collagen molecule (triple helix)	Length = 300 nm Diameter = 1.5 nm	3 polypetide chains: 2 α_1 and 1 α_2 
Microfibril	Diameter = 4 nm	5 collagen molecules 
Fibril	Diameter = 100-200 nm	≈ 7000 collagen molecules 
Fibril bundle	Diameter = 2000 nm	200-1000 fibrils
Fibres	20 μm in grain layer and 200 μm in corium layer	30-300 fibril bundles

The collagen fibre, when viewed through a microscope, exhibit a characteristic periodic banding pattern. The origin of this pattern is the quarter-staggered arrangement of individual molecules, producing alternating regions of protein density in the fibril, which explains the characteristic gap and overlap appearance of fibrils negatively contrasted for transmission electron microscopy (Holmes *et al.*, 2001). The repeating unit of the banding pattern is called a D-space or D-period, and a molecule is 4.4 D-space long with 0.6 D gap and 0.4 D overlap (Wess, 2008; Brown, 2009).

2.1.1.1 Denaturation of collagen

The three polypeptide chains that form the collagen molecule are coiled in a left-handed helix dominantly stabilised by periodic hydrogen bonds (Brodsky and Persikov, 2005). The collagen triple-helices associate laterally and longitudinally to form fibrils through covalent crosslinks. As the crosslinks increase, the thermal stability of the collagen increases. Thermal denaturation usually leads the hydrogen bonds breaking and induces the unfolding of the triple helix (Mu *et al.*, 2007).

Heat denaturation transforms collagen molecules into gelatin. If the triple-helical structure of the collagen molecule is destroyed by heat, the properties of the polypeptides change entirely, despite having the same chemical composition (Watanabe *et al.*, 1996). Therefore, introduction of chemical crosslinks such as plant polyphenols into the collagen molecules has been performed in order to stabilise the protein structure (Sionkowska *et al.*, 2010; Depalle *et al.*, 2015). Thermal stabilisation of collagen has been investigated using analytical techniques such as circular dichroism (CD) (Watanabe *et al.*, 1996; Gayatri *et al.*, 2001), nuclear magnetic resonance (NMR) (Rochdi *et al.*, 1999) and differential scanning calorimetry (DSC) (Miles *et al.*, 1998; Brown *et al.*, 2000; Miles *et al.*, 2005).

2.2 Leather processing

After the animal slaughtering, hides and skins are removed and must be transported to the tannery. During transportation and storage before the processing is initiated, preservation must be carried out in order to prevent deterioration. Salting is the most common preservative for hides and skins, as it is cheap, easy to apply and has a more lasting effect than other types of preservation (Santos, 2006). When the tannery is close to the slaughterhouse, and the processing will start on the same day, hides and skins may be preserved with biocides. The use of biocides is advantageous for not having salt emissions in the wastewater and not requiring extensive soaking. Other types of preservation are cooling, brine or pickling (Sharphouse, 1983).

The processing of leather is divided into four major stages: beamhouse, tanning, post-tanning and finishing.

2.2.1 Beamhouse

The objectives of beamhouse operations are to clean and open up the hide structure in order to prepare it for tanning. Unwanted components from hides and skins, that will not be part of the leather product, are removed in these preparatory stages. Beamhouse is constituted of chemical and mechanical operations, as follows (Adzet, 1985; Santos, 2006):

Soaking: removal of superficial grease, dirt and salt for salted cured hides, rehydrating and returning them to fresh state.

Unhairing/liming: removal of hair and non-protein constituents, employing an alkaline bath. The alkali promotes the swelling of hides, which facilitates fleshing and splitting, as well as opening up of the structure, preparing it for tanning.

Fleshing: mechanical removal of fat and flesh.

Splitting: division of hide into two layers: the upper layer is the grain, which has higher value and the lower layer is called split, used for making suede products.

Deliming: removal of lime and de-swelling of hides. A decrease in pH reached in deliming prepares the hides and skins for bating with the optimum pH for the enzyme action.

Bating: application of enzymes (proteases) to improve softness and complementing removal of hair and melanin.

Degreasing: removal of natural fats from deeper layers of hides, with addition of surfactants or enzymes (lipases), usually employed for hides and skins with a high fat content such as sheepskins.

Pickling: acidification of hides and skins, which decreases the astringency with tanning agents, providing even penetration of tanning products throughout the thickness.

2.2.2 Tanning

Tanning is the process that converts hides and skins into leather (Covington, 2009). According to Heidemann (1993), tanning is the stabilisation of the material, meaning it can no more undergo changes such as putrefaction, swelling or drying off to an inflexible solid mass. The drying of the water mechanically held between the fibres does not create the hardening effect. When the moisture content is lower than 25%, the drying affects the water that is chemically bound to the peptide, causing the collapsing of the fibres and stiffness of the skin (Sharphouse, 1983). The tanning process prevents this from happening, so the skin remains flexible when dry.

The determination of tanning efficacy can be made by evaluating the hydrothermal stability of leather, *i.e.* the resistance to wet heat. Hydrothermal stability is given by the measurement of shrinkage temperature. Shrinkage of collagen occurs when the triple helices of native collagen undergo a transition to coil structure (Reich, 2007).

2.2.2.1 Mineral tanning

Around 90% of leather produced globally is tanned with basic chromium sulfate (BCS), (Onem *et al.*, 2015). Chromium tanning is a versatile and fast process, and chromium-tanned leather can achieve a shrinkage temperature of over 120°C (Dewhurst, 1999). Chromium tanning raises environmental concerns, as 60-80% of BCS offer is absorbed, and all the salt (NaCl) used in the pickle process remains in the float, resulting in a chromium concentration in the spent float ranging between 2500 and 3000 mgCr/l and a Cl^- concentration of at least 20000 mg/l (Zhang *et al.*, 2016).

2.2.2.2 Vegetable tanning

The use of vegetable tannins, also called plant polyphenols, for stabilising collagen, is as old as the technology of using animal hides and skins (Covington *et al.*, 2005). According to Sharphouse (1983), the acid group of vegetable tannins may combine with the basic groups of collagen, and interact with the peptide groups *via* secondary interactions such as dipole or hydrogen bonds, displacing hydration water. As a result, on drying, the vegetable tannin

molecules prevent the fibres sticking together. More detail and information concerning vegetable tanning of leather may be found in Section 2.5.

2.2.2.3 Aldehydic tanning

Formaldehyde and glutaraldehyde combine with unionised basic groups of collagen. At higher pH values, these compounds undergo condensation reactions to form larger molecules that may crosslink between adjacent collagen molecules (Sharphouse, 1983).

Aldehydic tanning agents interact with collagen *via* covalent bonds. In a combination system of vegetable tannin-aldehyde-collagen, vegetable tannins mainly interact with collagen *via* multiple hydrogen bonds. If highly nucleophilic sites are present on tannin molecules, covalent cross-linkages can be formed between collagen and vegetable tannins *via* the bridge bond of aldehyde, resulting in a synergistic effect in increasing hydrothermal stability (Lu *et al.*, 2003).

2.2.2.4 Other tanning agents

Other agents may also be used in tanning of hides and skins, such as metal salts of titanium and aluminium, or synthetic tannin agents (syntans) (Bienkiewicz, 1983). Synthetic polymers (resins), such as melamine-formaldehyde resins, present a filling property and can contribute to the increase in hydrothermal stability (Covington, 2009).

2.2.3 Post-tanning

The tanned leather is considered a commodity, *i.e.*, it may be used to produce several articles. Each post-tanning operation (also referred as retanning) is directed to the article that will be produced, such as garment, shoe upper, upholstery, etc. The stages of post-tanning are explained as follow (Santos, 2006):

Sammying: squeezing the excess water from leather, leaving a moisture content of about 45-55% in weight.

Shaving: smoothing the thickness of the skin.

Wetting: rehydration of leather and removal of fibre powder that may be adhered from the shaving process.

Neutralisation: slightly increase of leather pH, depending on the type of article to be produced, in order to decrease reactivity with retanning, dying and fatliquoring agents, facilitating their penetration.

Retanning: addition of different tanning agents, such as syntans and vegetable tannins, in order to improve properties not achieved with chromium tanning.

Dyeing: application of dyes to change the colour of leather.

Fatliquoring: oils are applied to enhance softness, improve tensile strength and confer waterproofness to some types of leather.

Filling: addition of chemicals to make leather heavier and denser.

Fixation: lowering the pH below the isoelectric point, making the leather reactive to dyes and fatliquors, which will fix them in the fibre structure.

Setting: excess water removed.

Drying: decrease in moisture level.

Conditioning: adjusting the moisture level by adding water, to prepare the leather for softening.

Softening (Staking): physical softening of leather.

2.2.4 Finishing of leather

Finishing consists of coating and changing the surface of leather. It is related to the fashion appearance, but also conferring properties such as abrasion resistance, hydrophobicity, handle, amongst others.

Finishing consists of three coats: base coat, pigment coat and top coat. Depending on the leather produced, it is not necessary to apply all three coats (John, 1996). The finishing coats may be applied by brushing, padding, sponging, spraying, roller coating or curtain coating.

Other stages of finishing are described as follows:

Buffing: to correct the grain when necessary.

Impregnation: to improve grain firmness.

Milling: optional, to improve softness and modify the appearance of the grain.

Plating: conferring prints (surface patterns) to the leather.

Polishing: give the leather a two-tone effect, an antique effect or a semi-matt waxy gloss.

2.3 Plant polyphenols (vegetable tannins)

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 (Bate-Smith, 1962). Haslam (1989) introduced the term ‘plant polyphenol’ to these mixed vegetable extracts, and included in this definition are the low molecular weight phenolic compounds (non-tannins).

As suggested by Freundenberg in 1920, vegetable tannins can be classified as condensed or hydrolysable tannins, according to their structural types and chemical characteristics (Song, 2003). Table 2-4 shows sources for the main types of tannins.

Table 2-4: Sources of vegetable tannins (Haslam, 1989; Bhat *et al.*, 1998).

Hydrolysable tannins		**Condensed tannins**
Gallotannins	*Ellagitannins*	
• Chinese tannin (tannic acid) (galls, leaves of *Rhus semialata*) • Tara (fruit pods of *Caesalpinia spinosa*) • Turkish tannin (galls on wood of *Quercus infectoria*) • Sumac (leaves of *Rhus coriaria*)	• Wood of oak (*Quercus* spp.) • Chestnut (wood of *Castanea sativa*) • Myrobalan (fruit of *Terminalia chebula*)	• Tree bark and heart wood, such as Quebracho tannins (wood of *Schinopsis* spp.) • Wattle tannins from *Acacia* spp. • Bark tannins from pine (*Pinus* spp.), oak (*Quercus* spp.) and gaboon wood (*Aucoumea klaineana*) • Other sources are fruits and seeds such as grapes, apple, olives, beans, sorghum grains, carob pods, cocoa and coffee

Polyphenols are a diverse group of compounds ranging from simple molecules of low molecular weight to very complex molecules of high molecular weight. Due to the great diversity in their structure, the polyphenols have different properties, such as solubility and polarity, which enable different interactions with each other and with other molecules (Jakobek, 2015). Larger molecules have a greater number of hydroxyl groups, enabling a very large number of interactions with the environment such as crosslinking with proteins (De Freitas and Mateus, 2001).

2.3.1 Introduction to natural phenolics

A phenolic compound is characterised by the presence of one or more aromatic rings and one or more hydroxyl groups. These compounds are widely present in plants, ranging from simple phenols to polymerised substances such as tannins, and they are usually involved in defence against ultraviolet radiation or aggression by pathogens, parasites and predators, as well as contributing to the plants' colour (Dai and Mumper, 2010).

Polyphenolics are widely present in plant foods, such as vegetables, fruits, legumes, cereals, chocolate, and others, as well as beverages such as coffee, tea, wine and beer. They participate in the overall organoleptic properties of plant foods, for example, phenolics contribute to the astringency of wine and tea, because of the interaction between polyphenolics and proteins in saliva (Hagerman and Butler, 1981).

2.3.2 Simple phenols

Simple phenols consist of a single phenolic ring substituted with alcoholic, aldehydic or carboxylic acid groups. Resorcinol, catechol, pyrogallol, and phloroglucinol constitute the majority of phenolic moieties in polyphenol structures. Figure 2-2 shows the structure of simple phenols.

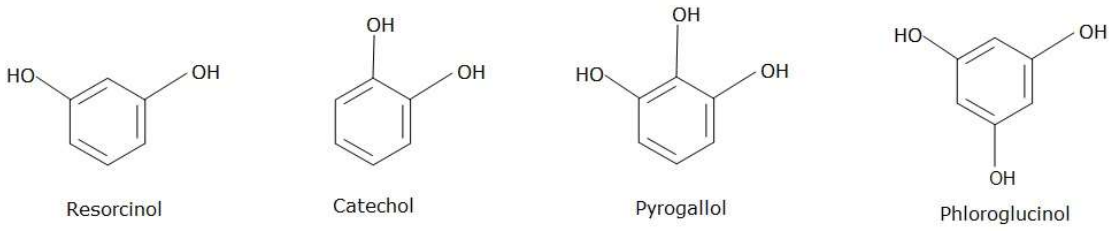
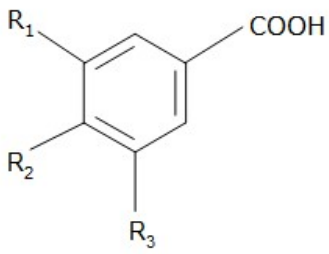


Figure 2-2: Simple phenols (Brielmann *et al.*, 2006).

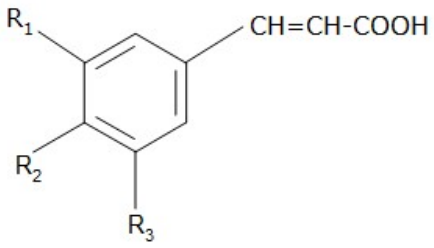
2.3.3 Phenolic acids

The predominant phenolic acids present in plants are hydroxybenzoic and hydroxycinnamic acids. Differences between their derivatives consist in the different patterns of hydroxylations and methoxylations of the aromatic rings. The structures of some of these compounds are shown in Figure 2-3.



	R_1	R_2	R_3
p-hydroxy	H	OH	H
Gallic	OH	OH	OH
protocatechuic	H	OH	OH

(a)



	R_1	R_2	R_3
p-coumaric	H	OH	H
Caffeic	OH	OH	H
Ferrulic	OCH_3	OH	H

(b)

Figure 2-3: Basic structure of (a) hydroxybenzoic and (b) hydroxycinnamic acid derivatives (Schuster and Herrmann, 1985).

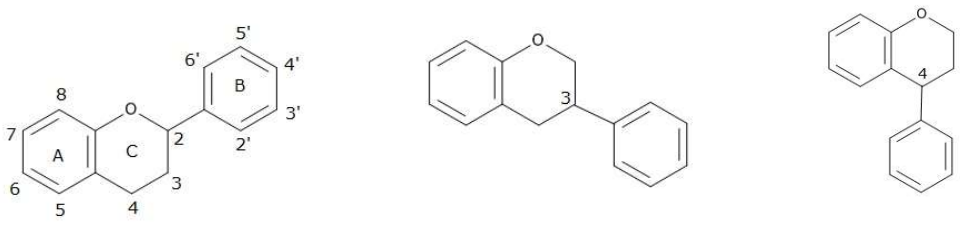
Hydroxybenzoic acids (Figure 2-3a) are found as forming units in complex structures such as hydrolysable tannins and lignins. These compounds are also present in the form of sugar derivatives (Schuster and Herrmann, 1985). One common hydroxybenzoic acid is gallic acid, which is a forming unit of gallotannins.

Hydroxycinnamic acids (Figure 2-3b) are usually found in foods of plant origin. Ferulic acid is commonly found in wheat, corn, rice, tomatoes, spinach, cabbage and asparagus; sources of caffeic acid are white grapes, white wine, olives, olive oil, spinach, cabbage, asparagus and coffee; p-coumaric acid sources are white grapes, white wine, tomatoes, spinach, cabbage and asparagus (Rice-Evans *et al.*, 1996).

2.3.4 Flavonoids

The term “flavonoid” describes the natural products that present a C_6-C_3-C_6 framework (phenylbenzopyran functionality), arranged in three rings, labelled as A, B and C. Depending

on how the aromatic ring is linked to the benzopyran moiety, this broad group may be divided into: flavonoids (2-phenylbenzopyrans); isoflavonoids, (3-benzopyrans); neoflavonoids (4-benzopyrans) (Marais *et al.*, 2006). The structures are shown in Figure 2-4.



(a) **(b)** **(c)**

Figure 2-4: (a) Flavonoids, (b) Isoflavonoids, (c) Neoflavonoids (Marais *et al.*, 2006).

Flavonoids can be divided into a variety of classes such as flavones (*e.g.*, flavone, apigenin, and luteolin), flavonols (*e.g.*, quercetin, kaempferol, myricetin, and fisetin), flavanones (*e.g.*, flavanone, hesperetin, and naringenin), and others (Kumar and Pandey, 2013). The general structures of different groups of flavonoids are shown in Figure 2-5. The various classes of flavonoids differ according to the saturation on the C-ring and degree of oxidation, while individual compounds within a class differ in the pattern of substitution of the A and B-rings (Middleton, 1998).

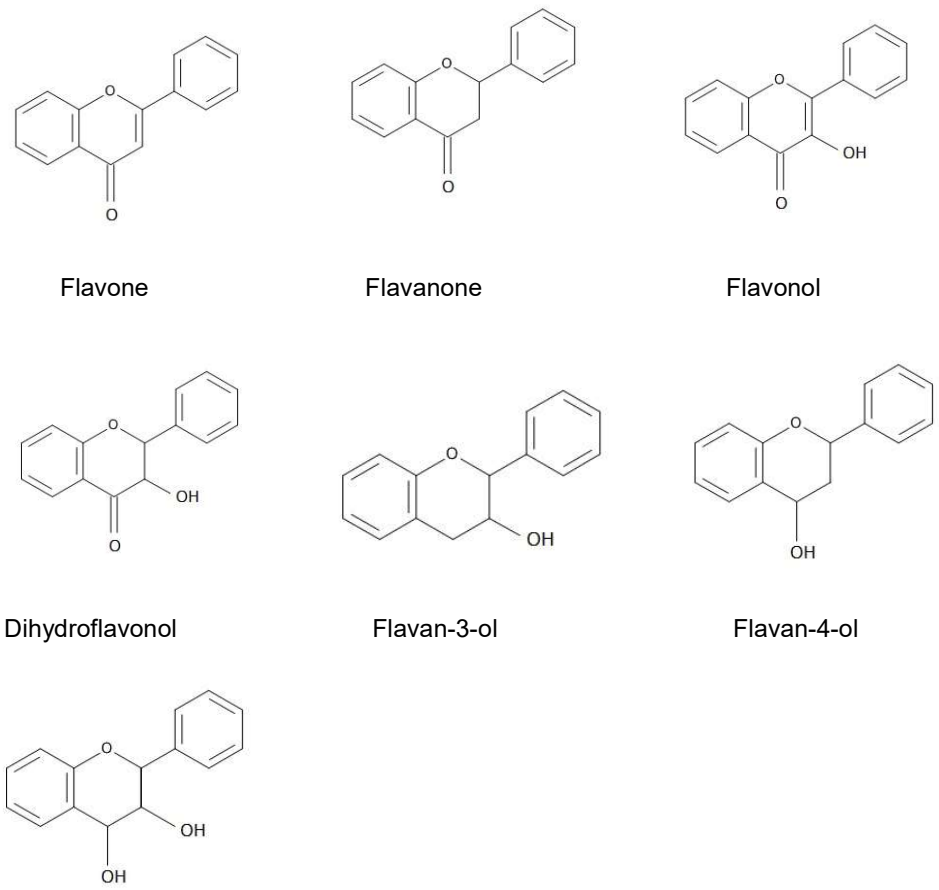


Figure 2-5: Types of flavonoids (Marais *et al.*, 2006).

Biosynthesis of flavonoids occur *via* condensation of phenylalanine-derived hydroxycinnamic acids and malonyl-moieties originating from phenylpropanoid metabolism, forming the B- and A-rings of flavonoids (Davies and Schwinn, 2005). Plant-derived products are the most important source of flavonoids in the human diet, as only plants are capable of flavonoid synthesis (de Villiers *et al.*, 2016). Flavonoids play several important roles in plants, including protection against stress factors such as ultraviolet radiation, harmful insects and animals as well as regulation of physiological and metabolic processes (Boudet, 2007).

2.3.4.1 Flavan-3-ols

Flavan-3-ols, also referred to as flavanols, are known by their action as antioxidants. The chemical nature of flavanols depends on their structural class, degree of hydroxylation, other substitutions and conjugations, and degree of polymerisation (Kelly *et al.*, 2002). These compounds constitute the building blocks of condensed tannins.

2.3.5 Hydrolysable tannins

Hydrolysable tannins are susceptible to hydrolysis by agents such as enzymes, hot water, alkali or acids. When the product of hydrolysis is gallic acid, these compounds are called gallotannins. If they are based on hexahydroxydiphenic acid, the hydrolysis yield ellagic acid and the tannin is referred as ellagitannin. (Haslam, 1998).

2.3.5.1 Gallotannins

Gallotannins, the simplest hydrolysable tannins, are formed by the structural unit of gallic acid as multiple esters with D-glucose (Haslam, 2007). Figure 2-6 illustrates the structure of gallic acid. The prototypical gallotannin is pentagalloyl glucose or PGG (β-1,2,3,4,6-Pentagalloyl-*O*-D-Glucopyranose) (Hagerman, 2002).

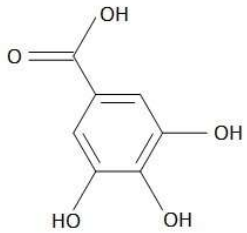
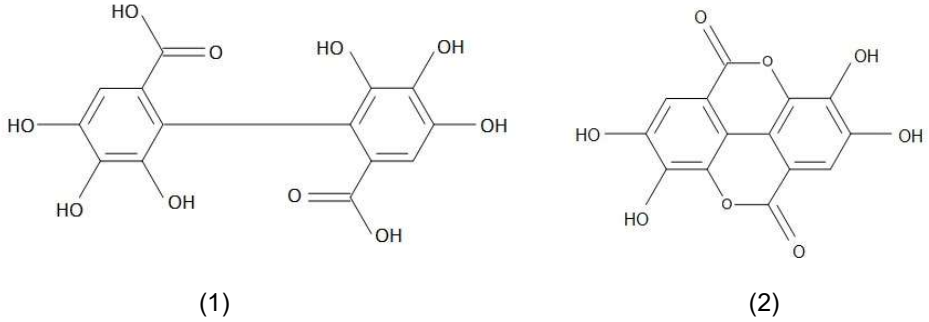


Figure 2-6: Gallic acid (Haslam, 1998).

2.3.5.2 Ellagitannins

Derivatives of hexahydroxydiphenic acid, the ellagitannins are formed by oxidative coupling of galloyl ester groups in a galloyl D-glucose ester (Haslam, 2007). Ellagitannins have a large structural variability due to the different possibilities for the linkage of hexahydroxydiphenic acid residues with the glucose moiety and particularly due to their tendency to form dimeric and oligomeric derivatives (Landete, 2011). The structures of hexahydroxydiphenic acid and ellagic acid are presented in Figure 2-7.



(1) (2)

Figure 2-7: Hexahydroxydiphenic acid (1) and ellagic acid (2) (Haslam, 1998).

2.3.6 Condensed tannins (proanthocyanidins)

Condensed tannins are constituted by oligomers and polymers of polyhydroxyflavan-3-ol monomer units (Porter, 1992). The terms proanthocyanidin (PA) and polyflavanoids are now used to describe condensed tannins (Lewis and Yamamoto, 1989). Proanthocyanidins occur widely in the plant kingdom and are considered the second most abundant group of natural phenolics after lignins (Arbenz and Averous, 2015).

The monomeric units that form condensed tannins are consisted of the flavan-3-ol ring system, as illustrated in Figure 2-8.

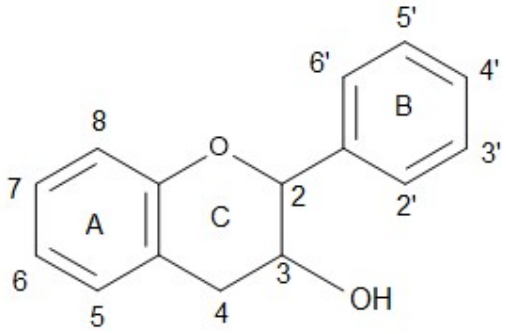


Figure 2-8: Flavan-3-ol ring system (adapted from Hagerman, 2011).

In the flavan-3-ol ring system, the hydroxyl in the 3-position in the central C-ring may be *cis* (epicatechin) or *trans* (catechin) with the B-ring, as shown in Figure 2-9.

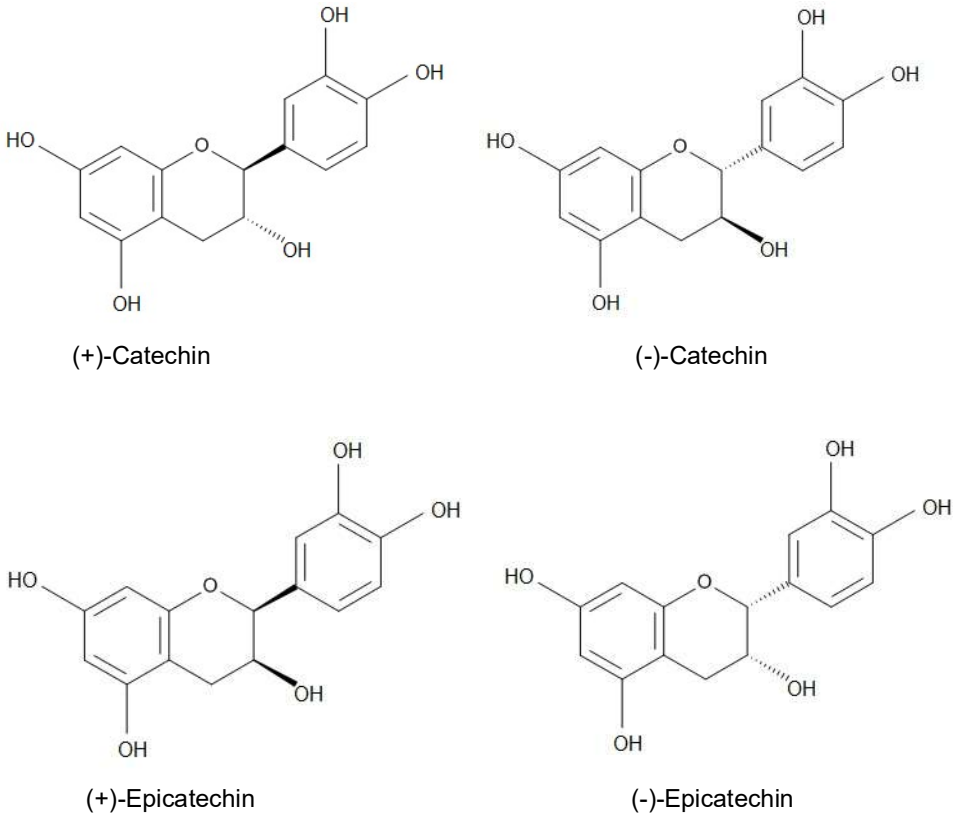


Figure 2-9: Stereochemistry in the flavan-3-ol ring system (Rinaldo *et al.*, 2010).

Catechin, a monomeric flavan-3-ol present in green tea, in conjunction with epicatechin, constitute the repeated units of procyanidin tannins (Jeong and Kong, 2004). 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 (Bordoni *et al.*, 2002; Kuroda and Hara, 1999).

The hydroxylation patterns in the A-ring and B-ring are important in determining the nature and rate of reactions that occur on the A- and B-rings and also play a role in the type of interflavanoid bonds (McGraw, 1989). The nomenclature of condensed tannins is based on the hydroxylation patterns of the A- and B-rings. Procyanidins and prodelphinidins have a phloroglucinolic A-ring structure, while profisetinidins and prorobinetinidins present A-rings with resorcinolic structure (Hemingway, 1989).

Table 2-5 presents the monomeric constituents of condensed tannins and the respective polymers formed. Vegetable extracts may as well present polymers with mixed monomeric structure. The molecular constitution of monomers for condensed tannin polymers are shown in Figure 2-10.

Table 2-5: Proanthocyanidins monomer units and their respective polymers (Porter, 1992).

Monomer	***Polymer***
Fisetinidol	Profisetinidin
Robinetinidol	Prorobinetinidin
Catechin	Procyanidin
Epicatechin	Procyanidin
Gallocatechin	Prodelphinidin
Epigallocatechin	Prodelphinidin

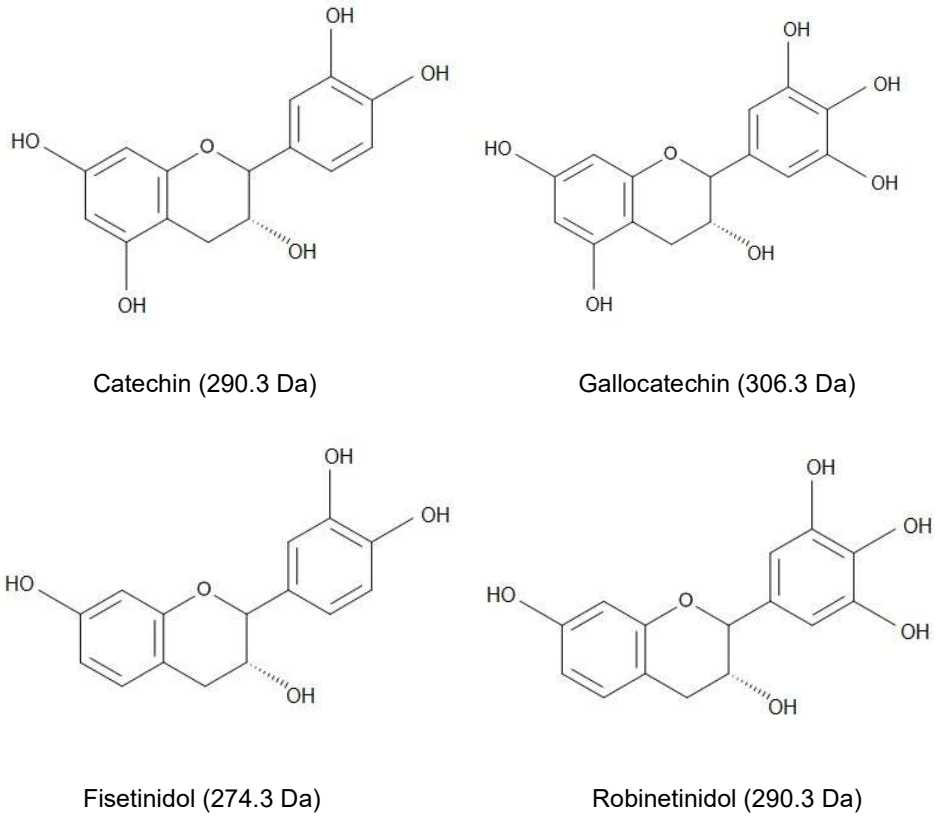


Figure 2-10: Flavan-3-ol structures of proanthocyanidins (Bianchi *et al.*, 2015).

The successive monomeric units in proanthocyanidins are linked via carbon-carbon bond between C-8 (linear) or C-6 (branched) of the terminal unit and C-4 of the extender unit (Hagerman, 2011). Figure 2-11 illustrates linear and branched polymers from flavan-3-ol monomers.

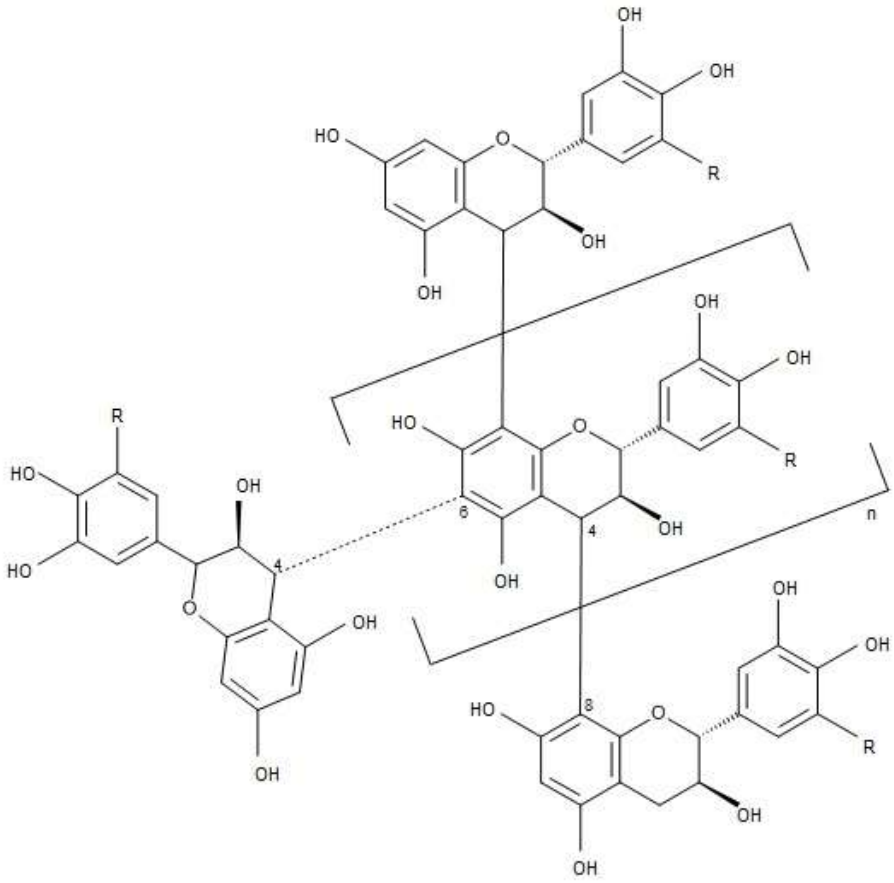


Figure 2-11: Branched (4 → 6) or linear (4 → 8) polymerised proanthocyanidins (Schofield *et al.*, 2001).

The shape of the polymeric structure of polyphenols is determined by the hydroxylation pattern of the monomers. The presence of a hydroxyl in the 5-position in procyanidins and prodelphinidins restricts the ability of the ring system to react at the 6-position, therefore, the polymers tend to be linear (Covington, 2009).

2.3.6.1 Anthocyanidins and anthocyanins

Anthocyanidins, in contrast with flavanols, carry a positive charge in the central ring structure (Hou *et al.*, 2004). These structures are present in plants as glycosidic compounds (bonded to a sugar moiety). The glycoside form of anthocyanidins are called anthocyanins (Castañeda-Ovando *et al.*, 2009). Figure 2-12 shows the most common anthocyanidins structures.

Anthocyanins are water soluble pigments that confer colour to many fruits and vegetables such as red grapes, red cabbages and berries (Hou *et al.*, 2004). Anthocyanins, as opposed to other flavanols, strongly absorb visible light, conferring an infinite variety of colours upon the media in which they occur (Brouillard, 1982).

Anthocyanins and anthocyanidins are known by their biological activities, including anticarcinogenic (Wang and Stoner, 2008), anti-inflammatory (Patel *et al.*, 2013) and vasodilatory (Xu *et al.*, 2007). The positive effects of these compounds on health have been attributed to the antioxidant and antiradical activity of anthocyanins. Two mechanisms have been proposed to describe the radical scavenging activity of phenolic compounds: the hydrogen-atom transfer mechanism, where the antioxidant donates a hydrogen atom to the active radical and produces a stable phenoxyl radical in one step; and the single electron transfer mechanism, where an electron and a proton are transferred in two consecutive steps to produce first a radical cation then the phenoxyl radical, respectively (Ali *et al.*, 2016).

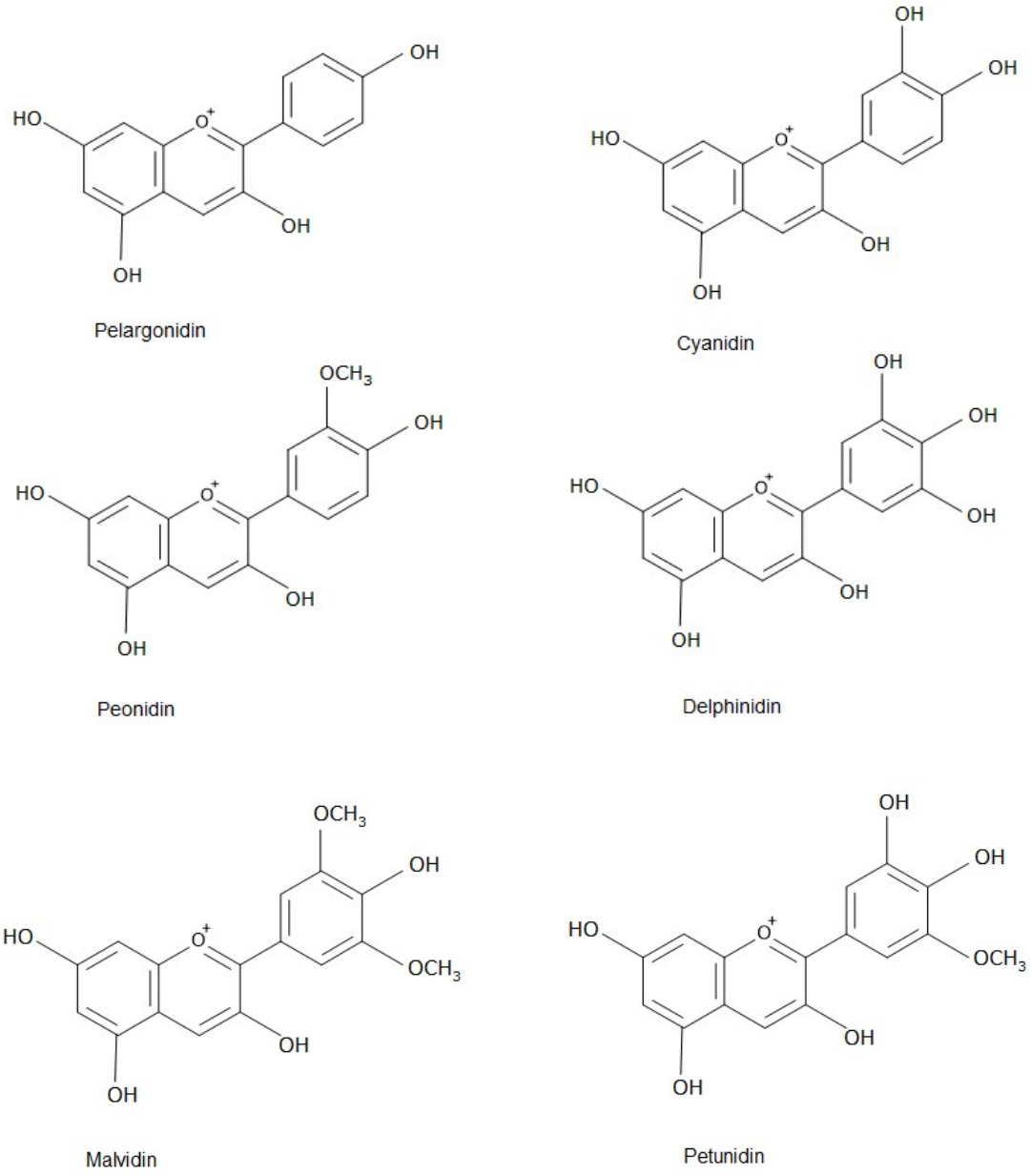


Figure 2-12: Common anthocyanidins found in plants (Brouillard, 1982).

2.3.7 Extraction of tannins

The extraction of tannins from vegetable substances is usually carried out in solution. The industrial method is based on the extraction of tannins in boiling water. The process is described by Arbenz and Averous (2015):

- Wood shavings are loaded in autoclaves, which run in counter-current. Each autoclave operate at a temperature range of 50-110°C, maximum pressure 0.8 bar, time of 6-10 hours and a ratio of water/wood equal to 2-2.4 in mass. The solution obtained by this process contains 4-5% of tannins;
- Tannin is cleared by decantation;
- The tannin solution is concentrated by evaporation under vacuum to a final concentration of 40-50% in weight;
- The concentrated solution is reduced to dry powder by atomisation until 90-96% dry mass.

Other solvents such as ethanol, methanol, acetone, and mixtures of these solvents may be used to increase extraction yield (Prior *et al.*, 2001). Other methods of extraction such as microwave-assisted extraction (Pan *et al.*, 2003), ultrasound-assisted extraction (Toma *et al.*, 2001), supercritical fluid extraction (Yesil-Celiktas *et al.*, 2008) have been developed to improve the tannin extraction efficiency.

2.3.8 Mimosa tannin

Mimosa tannins are extracted from the bark of the Black Wattle tree (*Acacia mearnsii*). The extract is a source of tannins for leather manufacture, waterproof wood adhesives, amongst others. Mimosa extracts can also be applied as inhibitors of electrochemical corrosion of steel in acidic media (Martinez, 2003). South Africa and Brazil are the largest producers of mimosa tannin (Bergman and Kessler, 2015).

Venter *et al.* (2012) have studied the chemical composition of commercial mimosa extract. The authors used electrospray mass spectrometry to establish that the flavan-3-ol based oligomers present in mimosa tannins consist of a starter unit which is either catechin or gallocatechin, angularly bonded to fisetinidol or predominantly robinetinidol extender units.

Studies on the molecular weight of mimosa extract using ^{13}C NMR were carried out by Thompson and Pizzi (1995), showing that the mimosa tannin presents molecular weight between 1343 – 1406 Da, and a degree of polymerisation of 4.90. The molecular weight of mimosa is within the range of 500-3000 Da, which means mimosa tannin has a high tanning content and high reactivity with collagen (Bienkiewicz, 1983).

2.4 Crosslinking reactions between polyphenols and collagen

Conventionally, plant polyphenols are employed in leather tanning as extracts from natural products, such as leaves, barks and wood. The vegetable tannin, however, tend to react on the surface of the skin, due to the high reactivity towards collagen (Covington, 1997). Therefore, superficial tanning and poor penetration into the skin may occur. The process must be carried out in diluted baths, for a prolonged period of time, in order to allow the complete penetration throughout the hide/skin thickness (Covington, 1997). The very slow penetration of the large molecules of vegetable tannins makes the saturation of the hides with this tanning agent taking longer than tannages with small molecules, such as metal salts.

To be considered as a tannin, the molecular weight of polyphenol molecules must range between 500 and 3000 Da (Bienkiewicz, 1983). Low molecular weight phenols do not stabilise collagen, due to their deficient capacity of crosslinking with protein chains. Large molecules have also a deficient tanning action, due to penetration difficulty.

According to Haslam (1997), vegetable tannage takes place especially in the gap zones of the collagen structure. This zone of approximately 1.4 nm in diameter and 40 nm in length represents a void in the structure, which facilitates the infiltration of polyphenol molecules. This relates to the importance of an effective opening-up of fibre structure that occurs during liming, when the complete removal of hyaluronic acid and dermatan sulphate is essential for making this zone accessible to vegetable tannin molecules. The importance of an effective opening-up was confirmed by the work of Kanth *et al.* (2009), which developed a pickle-free tanning process with application of proteolytic enzymes to improve the exhaustion of vegetable tannins. The authors measured an exhaustion of 95% of tannins, a 10% increment in comparison with the conventional process.

The amino acid sequence in the gap zone is strongly hydrophobic, rich in alanine, proline, hydroxyproline and phenylalanine amino acids (Fraser and Trus, 1986). 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 (Haslam, 1998).

Acidity plays an important role in the reaction between collagen and polyphenolic species. Protein and tannin are attracted by hydrogen bonding and dipole interactions. Acidic conditions of pH between 3 and 4 provide a rapid and quite firm binding. The speed of binding slows down at a pH above 6 and the tan solutions become darker (Heidemann, 1993).

2.5 Enzymes

Enzymes are globular proteins that act as biological catalysts for certain chemical reactions. The reactants of enzyme-catalysed reactions are called substrates and each enzyme catalyses a particular reaction with a particular substrate, which makes enzymatic reactions specific in character. Table 2-6 presents classes of enzymes according to their catalytic specificity.

Table 2-6: Classes of enzymes (Drauz *et al.*, 2012).

Enzyme class	***Catalytic activity***
Oxidoreductases (E.C. 1)	Catalyse redox processes (electron transfer)
Transferases (E.C. 2)	Catalyse the transfer of atom groups between molecules. *E.g.* transaminase
Hydrolases (E.C. 3)	Catalyse hydrolysis (reaction between substrate and water)
Lyases (E.C. 4)	Addition of molecular groups to double bonded molecules
Isomerases (E.C. 5)	Catalyse isomerisation reactions

2.5.1 Kinetics of enzymatic reactions

For any reaction, the appearance of a product with time may be represented in a graph showing the product concentration ([P]) versus time (t). The rate of the reaction at any time (t) will be the slope of the curve at that point. At the start of the reaction the rate is constant and it decreases as the reaction proceeds due to decreasing in substrate concentration.

The initial velocity (V_0) depends on the initial concentration of the reactants. For a single reactant, the velocity of a first-order reaction is proportional to the reactant concentration: V = k[R]. For the initial time, then: $V_0 = k[R_0]$. Likewise, a second order reaction for a single substrate has $V_0 = k[R_0]^2$ and a zero-order reaction presents $V_0 = k$ (Palmer, 1981).

The enzyme combines with the substrate during catalysis and then undergoes a further step to release the products. This is illustrated by the Michaelis-Menten mechanism for a single substrate reaction, as shown in Equation (2-1).

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \qquad \textbf{(2-1)}$$

The Michaelis-Menten equation for a one-substrate enzyme-catalysed reaction is shown in Equation (2-2).

$$\boldsymbol{v_0} = \frac{\boldsymbol{d[P]}}{\boldsymbol{dt}} = \frac{\boldsymbol{Vmax[}}{\boldsymbol{Km+[S]}} \qquad \textbf{(2-2)}$$

Equation (2-2) demonstrates the quantitative relationship between the initial velocity V_0, the maximum velocity V_{max}, and the initial substrate concentration [S], all related through the Michaelis constant K_m (Chaplin and Bucke, 1990). When the enzymatic reaction exhibits a hyperbolic dependence of V_0 on [S], it is said to follow the Michaelis-Menten kinetics. In this case, K_m = [S] when $V_0 = ½ V_{max}$ (Nelson and Cox, 2013).

2.5.2 Oxidising enzymes

Oxidoreductive enzymes catalyse the transformation of phenols through oxidative coupling reactions with production of polymeric products by self-coupling or cross-coupling with other molecules (Ghoul and Chebil, 2012). Chemical polymerisation of low molecular weight phenols can be carried out through oxidation using agents such as benzoyl peroxide, alkaline ferricyanide or CuCl/pyridine (Kobayashi and Higashimura, 2003). Some biological reactions that involve oxidative polymerisation of phenolic compounds are catalysed by enzymes, for example, the synthesis of lignin from peroxidase-catalysed polymerisation of monolignols (Gross, 2008). 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 (Uyama and Kobayashi, 2002; Gross *et al.*, 2010; Ragupathy *et al.*, 2012; Mespouille *et al.*, 2014).

Oxidases are enzymes that react with molecular oxygen, and peroxidases react with peroxide, producing a reactive intermediate that react further with reducing substrates (Burton, 2003). Polyphenol oxidase (monophenol monooxygenase EC 1.14.18.1), also called tyrosinase, has a dinuclear copper centre, which enables the cleavage of a dioxygen molecule, resulting in the substrate oxidation by the oxygen-copper complex. The reaction catalysed by PPO can be divided into two steps: first the phenol is hydroxylated and the catechol formed is then oxidised to an *o*-quinone (Burton, 2003), as shown in Figure 2-13.

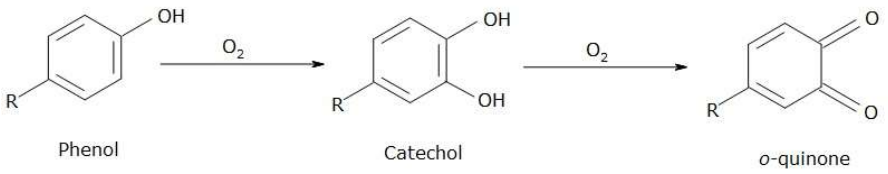


Figure 2-13: PPO-catalysed hydroxylation of phenol and oxidation of catechol (Burton *et al.*, 1998).

Catechol oxidase (*o*-diphenol: oxygen oxidoreductase EC 1.10.3.1) reacts with diphenols such as catechol, forming benzoquinones. Laccase (*p*-diphenol: dioxygen oxidoreductase EC 1.10.3.2) is a multi-copper enzyme that acts on mono- and polyphenols, using molecular oxygen to oxidise these compounds by a radical catalysed reaction mechanism (Fernandez-Sanchez, 2002). Tyrosinase and catechol oxidase have *o*-diphenol activity, while laccase has

ortho- and *para*-diphenol activity, but generally more affinity with the second group (Madhavi and Lele, 2009).

2.5.3 Laccase

The oxidative effect of laccase produces free radicals that are prone to react further non-enzymatically, forming dimers, oligomers or polymers covalently coupled by C-C, C-O or C-N bonds (Claus, 2004). Furthermore, laccase may enable the crosslinking of certain biopolymers that present phenolic moieties susceptible to oxidation (Selinheimo, 2008). Figure 2-14 illustrates a simplified mechanism of the laccase oxidative effect.

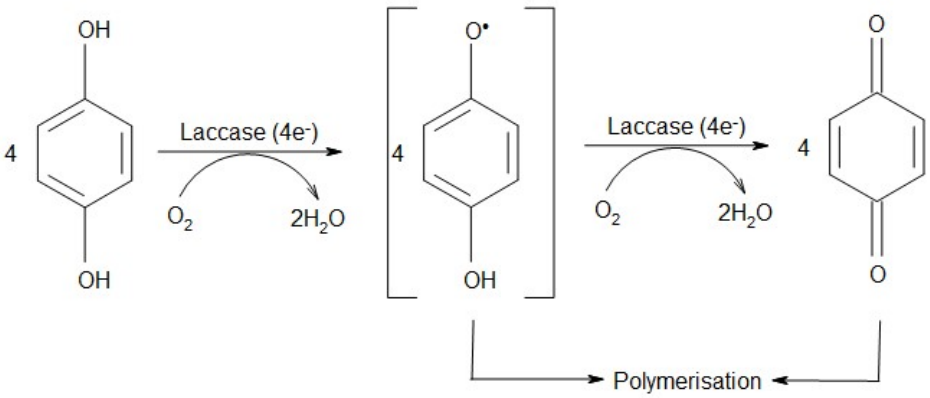


Figure 2-14: Oxidative effect of laccase (adapted from Osma *et al.*, 2010).

Enzymatic oxidation presents several advantages in comparison to chemical oxidation, including specificity in the catalytic effect, reactions carried out in mild conditions, and moreover, the biodegradability of enzymes (Burton, 2003). Laccase is an oxidative enzyme applied to several biotechnological processes, such as detoxification of industrial effluents, especially from paper, textile and petrochemical industries (Madhavi and Lele, 2009). This enzyme can be also used as cleaning agent for certain water purification systems, in bioremediation and even as ingredient in cosmetics, replacing H_2O_2 as oxidising agent in hair dyes (Couto and Herrera, 2006).

Fungi are the major laccase producers especially ascomycetes, deuteromycetes and basidiomycetes (Senthivelan *et al.*, 2016). Laccase-substrate affinity changes from one producing organism to another. *Neurospora crassa* laccase effectively oxidises *para*- and *ortho*-diphenols, while *Pyricularia oryzae* prefers phloroglucinol. Laccases from *Cerrena unicolor* and *Trametes versicolor* are able to oxidise *meta*-substituted phenols, but the degree of oxidation varies (Madhavi and Lele, 2009). Laccase from *Cerrena unicolor* prefers *para*-substituted phenols, while *Trametes versicolor* laccase oxidises *ortho*-substituted phenols to great extent (Madhavi and Lele, 2009).

Laccase activity can be monitored by spectrophotometric measurements of the oxidised products (Johannes and Majcherczyk, 2000). Aktas and Tanyolac (2003) monitored the oxidation of catechol by measuring oxygen consumption. The authors developed an interactive kinetic model as a function of catechol and dissolved oxygen, and the model presented fair correlation to the experimental data.

In this research, laccase was applied in the oxidation of phenolic compounds aiming at polymerisation. The formation of products were monitored spectrophotometrically and the laccase activity was quantified through absorbance measurements.

2.6 Research aims

The specific aims of this research study are:

(a) To determine the optimum phenolic substrate for the enzyme laccase and to elucidate the mechanism of the laccase catalytic action on the chosen substrate.

(b) To demonstrate the potential of laccase-catalysed oxidation of catechin in increasing the thermal stabilisation of collagen, in order to provide an alternative method of vegetable tanning.

(c) To develop a process of depolymerisation of mimosa extracts, and to test the suitability of the depolymerised product on the application of laccase-assisted increase in thermal stabilisation of collagen.

(d) To improve the tanning efficacy of hide powder and goat skin, using a 2-step modified tanning process: firstly the absorption of depolymerised mimosa tannin, and secondly the laccase-catalysed polymerisation of the phenolic molecules and crosslinking with collagen.

Chapter 3 Materials and Methods

3.1 Materials

3.1.1 Chemicals

Laccase from *Trametes versicolor,* catechol, resorcinol, (+)-catechin, HOBt, sodium azide, phloroglucinol, pyrogallol, ascorbic acid and Folin-Ciocaulteu reagent were purchased from Sigma-Aldrich (Poole, UK). All other chemicals were obtained from Fisher-Scientific (Loughborough, UK). Ultrapure water was obtained with a Direct-Q 5 UV system (Millipore, Watford, UK).

3.1.2 Hide powder

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 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). The hide powder was stored under a controlled atmosphere at 23°C and 50% relative humidity, according to BS EN ISO 2419 (British Standards Institute, 2012). Table 3-1 shows the analytical results and specifications of the hide powder used in this study.

Table 3-1: Analytical results and specifications of hide powder. Analytical results are expressed as the mean ± standard deviation of triplicate measurements. All samples of hide powder used in this study were derived from the same batch.

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

[a]According to SLC131 (Society of Leather Technologists and Chemists, 1996).

3.1.3 Mimosa tannin

Mimosa tannin (Mimosa ME) was obtained from Forestal Mimosa (Reading, UK). Results of analysis for characterisation of the mimosa extract are presented in section 6.4.1.

3.1.4 Goat skin

Experiments on skins were carried out using a goat skin. The recipe for processing the goat skin from salted to pickled is detailed in Table 3-2.

Table 3-2: Recipe for processing goat skin from salted to pickled.

Process	*Amount (% w/w)*	*Chemicals*	*Temperature (°C)*	*Time (min)*	*Observations*
Dirt soak	500	Water	25		
	0.5	De-greasing/wetting agent			
	0.5	Enzyme soaking aid			
	0.25	Bactericide		60	
					Drain
Main soak	300	Water	25		
	0.5	De-greasing/wetting agent			
	0.5	Enzyme soaking aid			
	0.25	Bactericide		240	
					Drain
Fleshing					
Liming	300	Water	25		
	1	Hydrated lime			
	2	Sodium sulfide		30	
	2	Hydrated lime			
	2	Sodium sulfide		120	+ run 5 min/h overnight
					Drain
Washing	500	Water	25	10	
					Drain
De-liming	100	Water	25		
	1	Ammonium chloride		60	
	1	Ammonium chloride	37	60	
Bating	1	Bate powder		60	
					Drain
Washing	500	Water	20	10	
					Drain
Pickle	100	Water	25		
	10	Salt			Baume = 6
	0.8	Formic acid (1:10)			Final pH = 4.2
	0.4	Sulfuric acid (1:10)		60	
					Leave stationery for 24 hours

The commercial chemicals used in the recipe described in Table 3-2 are listed as follows:

- De-greasing/wetting agent: Pastosol BZ (Trumpler GmbH & Co. KG, Worms, Germany).
- Enzyme soaking aid: Trupowet PH (Trumpler GmbH & Co. KG, Worms, Germany).
- Bactericide: Preventol Z-L (Lanxess AG, Köln, Germany).
- Bate powder: Oropon ON2 (TFL SpA, Castelfranco di Sotto, Italy).

After processing, the pickled goat skin was characterised according to its moisture content and shrinkage temperature. Analytical results are shown in Table 3-3.

Table 3-3: Characterisation of goat skin. Analytical results are expressed as the mean ± standard deviation of triplicate measurements. All samples of goat skin used in this study were derived from the back region of the same skin.

Analysis	*Analytical Results*
Moisture content (%)	48.63 ± 0.35
Shrinkage temperature - onset (Ts) (°C)	59.56 ± 0.41

3.2 Analytical methods

3.2.1 Ultraviolet-visible spectroscopy (UV-Vis)

Spectroscopy studies of flavonoids have long been used for determining their structure. The typical spectrum for flavonoids shows one maximum in the range of 240-285 nm, determined by the A-ring, and absorption on the 300-550 nm related to the B-ring. All flavonoids show a maximum at the range determined by the A-ring, thus this is less useful to provide information on structure identification. Flavanols usually do not have absorption in the B-ring range, whereas anthocyanins show a peak around 450-560 nm and flavones and flavonols around 300-380 nm (Santos-Buelga *et al.*, 2003).

For the UV-Vis analyses, spectrum scanning was collected at 200 nm/min, ranging from 190 to 800 nm, using UV-Vis Spectrophotometer Genesys 10S (Thermo Scientific, Hemel Hempstead, UK). The solutions were read in a quartz cuvette with a 1 cm cell path.

3.2.2 Fourier Transform – Infrared Spectroscopy (FT-IR)

Fourier-Transform Infrared Spectroscopy (FT-IR) is an analytical technique that provides information about the molecular structure of a compound through the spectrum associated with its molecular vibrations (Wolkers, 2009). FT-IR analyses were carried out using FTIR-8400S (Shimadzu, Tokyo, Japan), coupled with in-compartment diamond Attenuated Total Reflectance (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 (self-indicating silica gel) for 24 hours prior to analysis.

3.2.3 Thin Layer Chromatography (TLC)

Analyses using thin layer chromatography (TLC) were performed to separate different molecular weight compounds from the polyphenol mixtures. The analyses were carried out using plates of aluminium sheets coated with 0.20 mm layer of silica 60 Å, specific pore volume 0.75 ml/g, particle size 5-17 µm, with fluorescent indicator for UV light at 254 nm (Alugram SIL G/UV_{254}, Macherey-Nagel, Düren, Germany). Each sample was dissolved in acetone (3 mg/ml) and a drop was laid 1 cm from the bottom of the TLC plate and left to dry. The separation was carried out in a hermetically closed TLC chamber, where the solvent system was previously left overnight to ensure saturation of the atmosphere inside the chamber. The solvent system for separation was constituted of toluene-acetone-formic acid (3:3:1). According to Karchesy *et al.* (1989), a visual pattern of the molecular weight distribution of procyanidin mixtures can be obtained with this solvent system, and retention factor (R_f) values were found to be in reverse order of molecular weight. The compounds were visualised under UV light at 254 nm and the ones that are easily oxidised, such as catechin, appear as brown spots after the plate is dried. Retention factor (R_f) was calculated as the distance travelled by the compound divided by the distance travelled by the solvent.

3.2.4 Tannin content – Absorption on hide powder

Tannin content was analysed by absorption on hide powder, according to the method SLC 116 – Determination of non-tannin constituents (Society of Leather Technologists and Chemists, 1996). The method is based on the gravimetric determination of non-tannins after absorption of tannins by the hide powder, and the tannin content is determined by difference (100 - %non-tannin - %moisture - %insolubles).

3.2.5 Separation of tannins and non-tannins by column chromatography

Column chromatography was used for separating larger polyphenols (tannins) from smaller molecules (non-tannins). The packing material used was Sephadex LH-20 beads (GE Healthcare, Little Chalfont, UK), which accomplish a general separation of larger from smaller polyphenols, based mainly on polarity. Lower molecular weight compounds, such as sugars, organic acids, phenolic acids, anthocyanins and flavanol monomers, were eluted with an ethanol solution in water (Cheynier and Fulcrand, 2003), while tannins were adsorbed by the polar Sephadex gel. The more polar components, with higher molecular weight (tannins) were then eluted with aqueous acetone.

Separation of tannin and non-tannin fraction with column chromatography on Sephadex LH-20 was carried out according to the following procedure. The Sephadex beads (25 g) were mixed with 100 ml of 85% (v/v) ethanol solution in ultrapure water and mixed gently to promote the swelling of the beads. The mixture was left to decant and the supernatant particles were removed. The slurry was then poured into the glass column (4 cm diameter). The beads were extensively washed with 85% (v/v) ethanol in ultrapure water. The polyphenol solution (0.1 g/l) was loaded into the column, and the non-tannin fraction was eluted with the 85% (v/v) ethanol in ultrapure water solution. Each 4 ml of eluate was monitored by UV-Vis absorbance at 280 nm, and the elution with the ethanol solution was carried out until the absorbance reached the baseline. The tannin fraction was absorbed by the stationary phase and was visible as a brown coloured band. The absorbed tannins were then recovered with 70% (v/v) acetone in ultrapure water until the Sephadex was white and the eluate was colourless. Each fraction was evaporated under vacuum at 30°C using a rotary evaporator (Rotavapor R-114 and Waterbath B-481, Büchi, Flawil, Switzerland; Speedivac ES35, Edwards High Vaccum Limited, Crawley, UK) near dryness and redissolved in 20 ml of ultrapure water to be freeze-dried to powder using an Alpha 2-4 LSC freeze-dryer (Martin Christ, Osterode, Germany), for 24 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. The powder obtained was weighed and the percentage (w/w) in dry basis was determined for the ethanol eluted fraction (non-tannins) and for the acetone eluted fraction (tannins).

3.2.6 Total phenolic content

Total phenolic content was analysed based on ISO 14502-1 (International Organization for Standardization, 2005), with a few modifications, as the extraction step was not performed. The samples were prepared by weighing 5 mg ±1 of the substance to be analysed, transferring to a 100 ml volumetric flask and the volume completed with deionised water. From this solution, 1 ml was used to the analysis and the methodology followed the ISO 14502-1 (International Organization for Standardization, 2005). A calibration curve using gallic acid was constructed for each analysis, and the results were expressed as µg of gallic acid/mg of dried substance.

3.2.7 Ethyl acetate partition

The ethyl acetate partition method allows the determination of the fraction soluble in ethyl acetate present in a vegetable extract sample. Similarly to the chromatography on Sephadex LH-20, it is a separation based on polarity. The less polar substances such as sugars, organic acids and monomeric and oligomeric proanthocyanidins are solubilised by the ethyl acetate, whilst the more polar such as large polyphenols, remain in the aqueous phase (Cheynier and Fulcrand, 2003).

The total soluble content is determined by evaporating 25 ml of a 0.4 g/l of tannin solution after filtration with a Whatmann n.1 filter paper (Fisher-Scientific, Loughborough, UK). Another 25 ml of this solution is shaken with 25 ml of ethyl acetate in a separating funnel. The aqueous layer goes to another funnel and is shaken with fresh 25 ml of ethyl acetate. The procedure is repeated three times. From the aqueous layer, 20 ml is evaporated to dryness until constant weight and 5/4 of this weight is subtracted from the total soluble of 25 ml. The percentage of solubles in ethyl acetate is related to the percentage of non-tannins in the sample.

3.2.8 Shrinkage temperature

Tanning degree was evaluated by the shrinkage temperature (Ts). Measurements presented in Chapter 5 were made using the differential scanning calorimeter DSC822e (Mettler-Toledo,

Schwerzenbach, Switzerland). Subsequent measurements were performed in the DSC 2 (Mettler-Toledo, Schwerzenbach, Switzerland). Data was analysed using the STARe software (Mettler-Toledo, Schwerzenbach, Switzerland). Thermal analyses were undertaken from fully hydrated samples (5 mg ±1) placed in 40 µl aluminium 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 in the thermograph. The results obtained from the samples were expressed in terms of change in shrinkage temperature (∆Ts), according to Equation (3-1).

∆Ts = Ts of treated hide powder on completion of experiment – Ts of untreated hide powder **(3-1)**

Miles *et al.* (2005) studied the thermal stability of rat-tail tendon at different levels of hydration, for native collagen and crosslinked with malondialdehyde, glutaraldehyde and hexamethylene diisocyanate. Each showed a linear relationship between hydration level and denaturation temperature, up to the fully hydrated fibre, in which case the denaturation temperature remained constant no matter the content of excess water. The authors concluded that the thermal stability is increased by the reduced hydration and the effect of drawing collagen molecules together provided by crosslinking. At low hydrations occurs a collapse of the gap-overlap fibre structure. At high levels of hydration, the enthalpy of denaturation on dry basis was independent of hydration (Miles and Ghelashvili, 1999). Therefore, in order to provide a true estimation of hydrothermal stability, the measurement of shrinkage temperature was undertaken from fully hydrated samples.

3.2.9 Phloroglucinolysis - Preparative method for UHPLC

Due to the polymeric characteristic of polyphenols, these compounds can be characterised by their molecular size. The degree of polymerisation (DP) corresponds to the number of flavan-3-ol units in condensed tannins. It is referred to the molecular weight, which is related to the tannin capacity of binding (or crosslinking) with proteins. A method based on the acid-catalysed cleavage of the interflavanyl bonds can be used to determine the DP of a polyphenol. The acid cleavage liberates the terminal units as monomeric flavanols, whereas the extender units are released as carbocations. When a nucleophile is added to the reaction

media, they combine with the carbocations, forming adducts that are derived from the extension units of the initial molecule. The molar ratio between the adduct and the terminal unit allows the calculation of average degree of polymerisation (DPn) (Guyot *et al.*, 1999). Usually, the nucleophiles employed to combine with the acid cleavage products are: phloroglucinol, in a method called phloroglucinolysis; or benzyl mercaptan, in a method called thiolysis.

The sample preparation for UHPLC analysis was adapted from Hagerman (2002). The phloroglucinol solution consisted of 5 mg of phloroglucinol per ml of acidic ethanol (0.1 M HCl in ethanol) prepared fresh prior to the start of the experiments. The acid cleavage/phloroglucinolysis was carried out with 4 mg of sample 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 was dissolved in 800 µl of 70% (v/v) methanol in ultrapure water in a 2 ml vial and analysed using an UHPLC system following the method described in Section 3.2.10.

3.2.10 UHPLC analysis – reversed phase

The UHPLC analyses were carried out using a Dionex Ultimate 3000 apparatus (Thermo Scientific, Hemel Hempstead, UK). A reversed 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 analysed using the software Chromeleon 7 (Thermo Scientific, Hemel Hempstead, UK).

3.2.11 Acid butanol assay

Analysis of the percentage of condensed tannins were undertaken using the acid butanol assay. The procedure was described by Ndhlala *et al.* (2006) based on the work of Porter *et al.* (1986).

A solution of 0.2 g/l of sample in 70% (v/v) acetone was prepared, and 500 µl of this solution was added to a screw cap tube, with 3 ml of the butanol:HCl reagent (95:5 v/v), followed by 100 µl of the ferric reagent (2% ferric ammonium sulfate in 2 M HCl). The tubes were vortexed and placed in a beaker containing boiling water for 60 minutes. A blank was prepared without heating, and the absorbance was read at 550 nm in a Genesys 10S spectrophotometer (Thermo Scientific, Hemel Hempstead, UK). The condensed tannins content (% dry matter) as leucocyanidin equivalent was calculated according to Equation (3-2).

$(A_{550\ nm} \times 78.26 \times \text{dilution factor}) / (\%\ \text{dry matter})$ **(3-2)**

The equation considers the extinction factor ($E^{1cm}_{1\%}$) of leucocyanidin to be 460, as described by Porter *et al.* (1986).

In this assay, the colour is produced by the releasing of anthocyanins due to the acid-catalysed cleavage of the interflavanyl bond. This means that the analyses detect from dimers to polymers. The vanillin assay described in Section 3.2.12 detects the monomers from the terminal units of the polymeric molecule. The data from both assays provide information about the average size of the polyphenol molecules. Figure 3-1 demonstrates the sections of the polyphenol molecule that reacts in the butanol and vanillin assays.

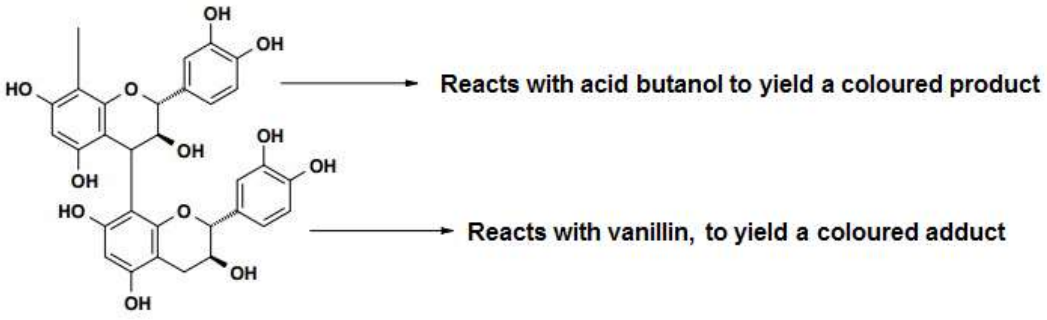


Figure 3-1: Sections of polyphenol molecule that reacts in the butanol and vanillin assays (Hagerman, 2002).

3.2.12 Vanillin assay

The vanillin assay was carried out according to the procedure described by Hagerman (2002). Vanillin reacts with the terminal units, forming a coloured adduct that can be detected spectrophotometrically at 500 nm, as shown in Figure 3-2.

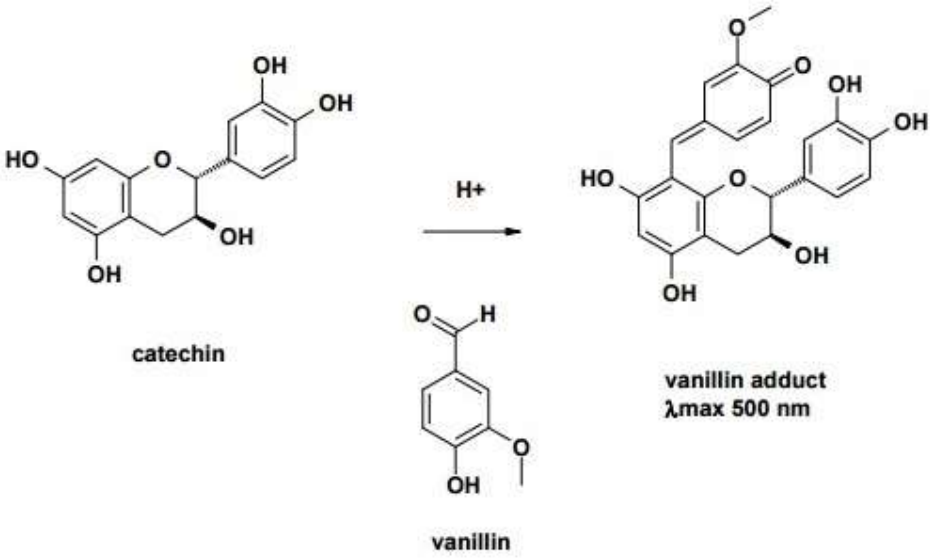


Figure 3-2: Reaction of vanillin and the terminal unit (catechin) of a polyphenol molecule (Hagerman, 2002).

For the assay, a solution of 2 g/l of each sample in methanol was prepared, and 1 ml of this solution was added to a screw cap tube. A calibration curve using catechin as standard was prepared for concentrations of 0, 2, 4, 6, 8 and 10 g/l. The tubes were placed in a water bath (Digital water bath OLS200, Grant Instruments, Cambridge, UK) at 30ºC. Each sample and standard was prepared twice. To the first set of standards and samples (blanks), 5 ml of a 4% HCl in methanol (v/v) solution was added in 1.0 minute intervals. To the second set of standards and samples, 5 ml of the vanillin solution (0.5 g of vanillin in 4% HCl in methanol (v/v)) was added in 1.0 minute intervals. The samples were left in the water bath for exactly 20 minutes and the absorbance was read at 500 nm in a Genesys 10S spectrophotometer (Thermo Scientific, Hemel Hempstead, UK). Results are expressed in catechin equivalents (mg catechin/g dry sample).

3.2.13 Colour measurement

Colour measurements were conducted using a spectrophotometer Konica Minolta Colour Measurement Meter CM-2600d with calibration cap CM-A145 (Konica Minolta Sensing, Tokyo, Japan), under the conditions of CIE 10° standard observer angle and CIE D_{65} light source, according to International Commission on Illumination (2004). The change in colour (ΔE) was calculated using the CIE (Commission Internationale de l'Eclairage) 1976 formula (Equation 3-3), having as reference a sample of untreated hide powder.

$$\Delta E = \sqrt{\Delta L^2 + \Delta a^2 + \Delta b^2} \quad \textbf{(3-3)}$$

Where *L* represents the clarity (axis black-white), *a* represents the chromatic component green-red, and *b* represents the chromatic component blue-yellow.

3.2.14 Light stability

The stability to light was assayed by placing the hide powder samples into a closed chamber (SDL, Stockport, UK), shown in Figure 3-3. The samples were exposed to the lamp Philips ML 500 W E40 mixed light for 58 hours. After the period, the colour of the samples was measured according to the procedure described in Section 3.2.13.

Figure 3-3: Chamber for light stability test.

3.2.15 Nuclear Magnetic Resonance (NMR)

The NMR analyses were carried out using a Bruker Ascend 400 MHz Avance III HD Nanobay spectrometer (Bruker, Switzerland) equipped with a 5 mm BBO probe. Standard 1D ^{1}H NMR pulse sequences were used and spectra were acquired in deuterated methanol (CD_3OD) and DMSO-d_6 at 298K. The spectra were calibrated to 3.31 ppm for CD_3OD and 2.50 ppm for DMSO-d_6, using the residual solvent peaks and the chemical shifts are reported relative to the solvent. The spectral width was kept at 8000 Hz.

Solid state ^{13}C NMR experiments were carried out using 4 mm diameter zirconia rotors (Bruker, Karlsruhe, Germany); ^{13}C NMR spectra were obtained with a 500 MHz Bruker AVANCE III HD 11.7 Tesla standard bore spectrometer equipped with a dual channel broad band probe at a magic angle spinning rate of 14 kHz, frequencies of 500 MHz (^{1}H) and 125 MHz (^{13}C) and standard cross polarization (CP) MAS techniques (^{1}H $\pi/2$ pulse length 2.5 μs, ^{1}H–^{13}C cross polarisation contact time 2.5 ms, broadband TPPM15 decoupling sequence during signal acquisition at a ^{1}H field strength of 120 kHz, recycle time 2 s, typical number of

scans accumulated per spectrum *ca.* 3000). Chemical shifts were referenced to the downfield signal of solid adamantane at 38.30 ppm.

Integration of chemical shift signals were carried out using either Spinworks 4 (Winnipeg, Canada) or Topspin 3.2 (Bruker BioSpin, 2013).

3.2.16 Statistical analysis

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.

Post Hoc analysis for comparison between subjects was carried out using the Tukey's HSD (honest significant difference) test. Different letters were assigned for data presenting significant difference ($p \leq 0.05$) and the same letter was assigned for data presenting no significant difference ($p > 0.05$).

Chapter 4 Kinetics of laccase-catalysed reactions of monomeric phenols

4.1 Introduction

Laccase is a multi-copper oxidising enzyme that acts on mono- and polyphenols. It requires molecular oxygen to oxidise phenolic compounds by a radical-catalysed reaction mechanism (Fernandez-Sanchez *et al.*, 2002). 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 (Claus, 2004).

The enzymatic oxidative polymerisation of phenols is a polycondensation reaction. According to Ghoul and Chebil (2012), the polycondensation reaction may be divided into four steps:

1) Formation of the phenoxy radicals, controlled by enzyme kinetics;
2) The phenoxy radicals form dimers by recombination. Essentially all phenols are converted to dimers at the beginning of the reaction;
3) Radical transfer from an oxidised monomer to a dimer;
4) Chain growth by alternating radical transfer and recombination.

The step of radical transfer from an oxidised monomer to an oligomer regenerates the phenol monomer. The phenol monomer may suffer another oxidation by the enzyme, initiating new radical transfer reactions. When the reaction of recombination or radical transfer by the phenoxy radical is not fast enough, it may lead to the formation of quinone structures.

The resonance structure for the phenoxide ion permits the electrons to rearrange themselves from one structure to the next. The acid strength of phenols can be enhanced by electron-withdrawing substituents at the *ortho*- and *para*- positions of the ring. (Macomber, 1996; Bansal, 2003). Bark *et al.* (2011) studied the oxidation of catechin in the presence and absence of air. The authors suggest that the oxidation of catechin is carried out in the catechol moiety (*o*-phenol), as shown in Figure 4-1. To confirm the findings, the authors carried out

oxidation of phenol and β-naphthol in water, resulting in neither difference in the UV-Vis spectra nor change in colour.

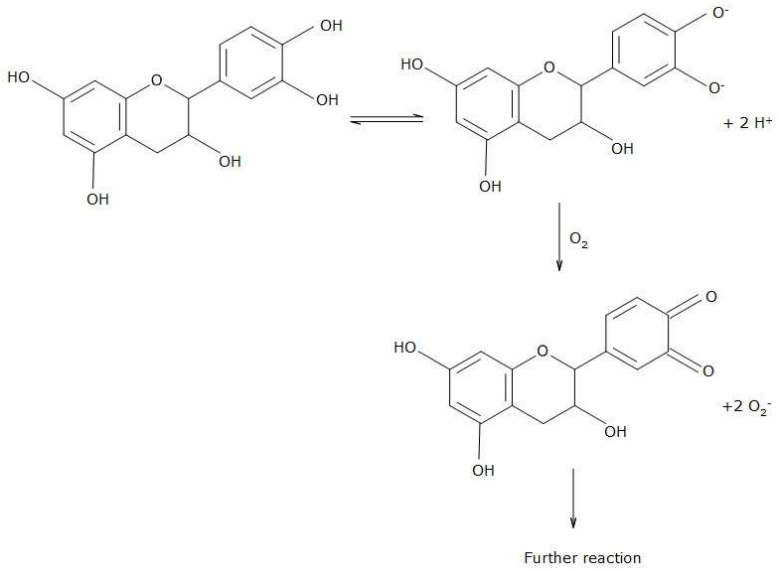


Figure 4-1: Catechin oxidation reaction proposed by Bark *et al.* (2011).

For the oxidative polymerisation of catechin, the reaction involves formation of *o*-quinone, an unstable intermediate, followed by its coupling (Uyama and Kobayashi, 2006). Flavonoid polymers may be obtained by oxidative polymerisation catalysed by enzymes such as laccase, polyphenol oxidase and peroxidase (Burton, 2003). The linkage on the polymerisation may take place through C-C bond formation, which might be expected to occur between two B-rings or by linkage of an A-ring to a B-ring (Haslam, 1998). Kurisawa *et al.* (2003) demonstrated through FT-IR analysis that polymerisation of catechin by laccase occurred *via* C-C bonds.

Catechin oxidation produces dimers referred as dehydrodicatechin A and dehydrodicatechin B4, as shown in Figure 4-2 (Haslam, 1998). Dehydrodicatechin B4 presents C-C linkage and is colourless, whilst dehydrodicatechin A suffers oxidation and forms a quinone structure, presenting a yellow colour (Guyot *et al.*, 1996). The mechanism of formation of dimers during the laccase-catalysed oxidation of catechin showed in Figure 4-2 demonstrates the rearrangement that the phenolic moieties undergo during oxidation, leading to the formation of quinones. Quinones absorb light at the visible range (340 – 480 nm) (Torreggiani *et al.*,

2008), thus it is possible to detect the product of the laccase-catalysed reaction at this wavelength range.

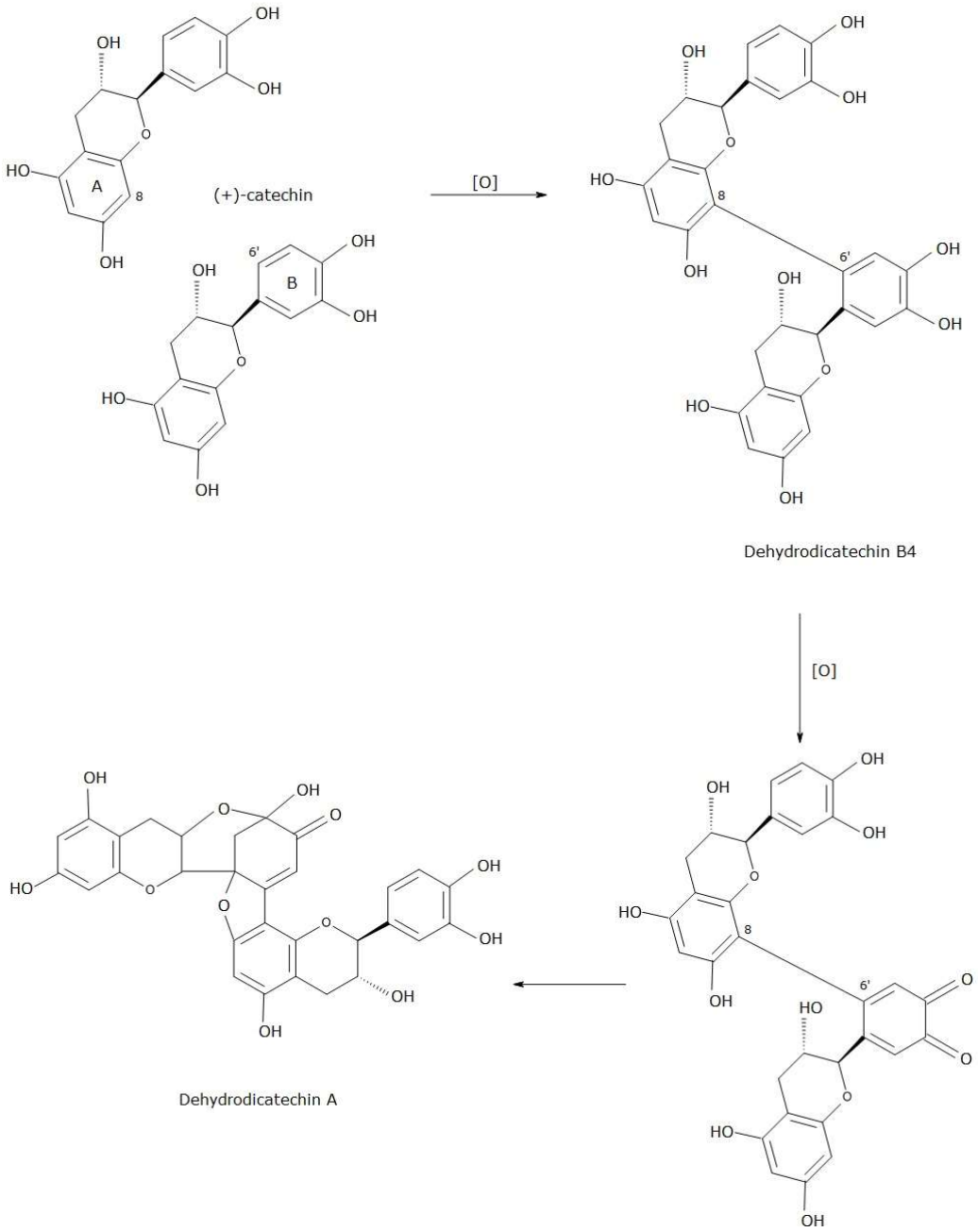


Figure 4-2: Oxidation of catechin and formation of dimers with quinone structure (adapted from Lopez-Serrano and Barcelo, 2001; Haslam, 1998).

Enzyme kinetics offers details about how reaction rate changes in response to changes in experimental parameters, providing information on enzyme mechanisms (Nelson and Cox, 2013). Kinetic studies for the laccase-catalysed oxidation of small phenolic species were carried out as exploratory experiments in order to compare the enzymatic action on different phenolic moieties. Catechol, pyrogallol, resorcinol, phloroglucinol and catechin were the chosen substrates for this preliminary study, providing information on how the enzyme acts on compounds with different configurations of the phenolic ring and number of hydroxyls.

Radical scavenging experiments using ascorbic acid were undertaken in order to confirm the mechanism of free radical generation and quinone formation for the laccase-catalysed oxidation of catechin. Sodium azide, a true laccase inhibitor (Johannes and Majcherczyk, 2000), was applied in a kinetic study in order to compare the results of inhibition with the free radical scavenging experiments.

For catechin to be applied in collagen stabilisation, the oxidation initiated by laccase should result in polymerisation, as the phenolic molecules need to possess an adequate number of phenolic hydroxyls to ensure effective crosslinking between collagen molecules. The molecular mass of catechin is 290 Da. Therefore, according to Bienkiewicz (1983), this compound does not have the minimum size required to be considered as a tannin. In order to verify if the laccase-catalysed oxidation of catechin resulted in polymerisation, Fourier-transform infrared spectroscopy (FT-IR), thin layer chromatography (TLC) and phloroglucinolysis followed by ultra-high performance chromatography (UHPLC) analyses were carried out. Analytical methods are described in Chapter 3.

4.2 Aims and objectives

The aim of this study was to determine the optimum substrate for the enzyme laccase and to elucidate the mechanism of the laccase catalytic action on the chosen substrate.

The objectives of this study were:

- To undertake preliminary kinetic experiments to compare the enzyme action on the product formation for different monomeric phenols: catechin, catechol, resorcinol, phloroglucinol and pyrogallol;
- To carry out free radical scavenging and inhibition studies in order to elucidate the mechanism of laccase-catalysed oxidation of phenols;
- To analyse the product obtained by laccase-catalysed oxidation of catechin, to verify if polymerisation has occurred.

4.3 Experimental procedure for the study of laccase-catalysed polymerisation of monomeric phenols

4.3.1 Kinetic studies

The kinetic parameters for laccase-catalysed polymerisation of phenols were determined by measuring the appearance of the oxidised products using spectrophotometric determination. For each substrate studied, it is necessary to find a wavelength at which only the product or the substrate has absorbance, without interference of any other reaction components (Sorouraddin *et al.*, 2010). Spectrum scanning was measured from a 1 mM solution of each phenolic compound in acetate buffer (pH 5.5) at 25°C. Laccase for a final concentration of 25 mg/l was added, and the spectrum analysed after 30 minutes. The procedure and parameters of the spectrum scanning measurement are described in Section 3.2.1.

After the determination of the wavelength at which is the maximum absorbance for the product of laccase-catalysed oxidation of catechin, catechol, resorcinol, phloroglucinol and pyrogallol, the kinetic studies were performed in a UV-Vis Spectrophotometer Genesys 10S (Thermo Scientific, Hemel Hempstead, UK). Substrate concentrations were 0.1, 0.2, 0.3, 0.5, 0.8 and 1 mM, in sodium acetate buffer (pH 5.5). The total reaction volume was 2 ml, cell path 1 cm

and the temperature was kept at 25°C. The final concentration of laccase was 25 mg/l. A blank omitting the enzyme was prepared for each phenol concentration, in which the enzyme was substituted by deionised water. Absorbance was read at the maximum wavelength of each phenolic compound at 15 seconds intervals for 25 minutes. Laccase activity unit is defined as the change in absorbance of 0.001 absorbance unit per minute (Suparno, 2005). Experiments were conducted in triplicate measurements.

4.3.2 Free radical scavenging

The absorbance of catechin oxidation by laccase in the presence of ascorbic acid was measured for different concentrations of ascorbic acid in the reaction medium. The final concentrations of ascorbic acid solution in the medium prior to starting the enzymatic oxidation reaction were 0.1, 0.2, 0.3, 0.5, 0.8 and 1 mM. The final concentrations of laccase and catechin were 25 mg/l and 1 mM, respectively. The addition of ascorbic acid shifted the wavelength of maximum absorbance from 450 nm to 395 nm. Therefore, the absorbance was monitored at 395 nm. Experiments were conducted in triplicate measurements.

Experiments when ascorbic acid was added after starting the enzymatic oxidation were performed in order to determine if interaction with the oxidation products occurs. The reaction medium was composed of catechin (final concentration 1 mM) and laccase (final concentration 25 mg/l). The oxidation reaction was initiated with laccase, with 500 µl of an ascorbic acid solution (final concentration 1.75 mM) added after 2 minutes, 4 minutes and 8 minutes. Absorbance was monitored at 450 nm and 395 nm. An additional experiment was performed with deionised water, replacing the ascorbic acid solution, added after 8 minutes of reaction, to determine if the decrease in absorbance occurred due to dilution or interaction of ascorbic acid with the oxidation products.

4.3.3 Enzyme inhibition with sodium azide

Studies of enzyme inhibition with sodium azide were performed by reading the absorbance values at 450 nm each 15 seconds for 25 minutes, for catechin concentrations of 0.1, 0.2, 0.3,

0.5, 0.8 and 1 mM, in sodium acetate buffer (pH 5.5). The total reaction volume was 2 ml, cell path 1 cm and the temperature 25°C. The final concentration of laccase was 25 mg/l. Catechin prepared in sodium acetate buffer (pH 5.5) was pipetted into the cuvette for each final concentration needed, along with 2 mM sodium azide solution in acetate buffer (pH 5.5). The volume was adjusted to 2 ml with acetate buffer. A blank omitting the enzyme was prepared for each catechin concentration, in which the enzyme was substituted by deionised water. Experiments were conducted in triplicate measurements.

Experiments when sodium azide was added after starting the enzymatic oxidation were carried out to compare the behaviour of the reaction medium with the ascorbic acid experiments. Reaction medium was composed of catechin (final concentration 1 mM) and laccase (final concentration 25 mg/l). The oxidation was started with laccase, and 500 µl of sodium azide solution (final concentration 2 mM) added after 2 minutes, 4 minutes and 8 minutes of reaction. Absorbance was monitored at 450 nm.

4.3.4 Laccase-catalysed polymerisation of catechin

In order to evaluate if polymerisation occurred after laccase-catalysed oxidation of catechin, an experiment was performed aiming at obtaining a powder of polymerised catechin for analysis. Catechin was first dissolved in 3 ml of acetone and added to 10 ml of acetate buffer (pH 5.5) to 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 and 24 hours under agitation of 100 rpm in a shaking water bath (Digital water bath OLS200, Grant Instruments, Cambridge, UK). A control was prepared 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 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. The samples obtained by the laccase-catalysed polymerisation of catechin were analysed using TLC, FT-IR and phloroglucinolysis/UHPLC, following the methodologies described in Chapter 3.

4.4 Results and discussions

4.4.1 UV-Vis spectra of monomeric phenols before and after laccase-catalysed oxidation reaction

Spectrum scanning was performed for each phenol studied in order to obtain the optimum wavelength for the product formation. Figure 4-3 shows the spectra for phenols before and after oxidation catalysed by laccase.

All flavonoids and polyflavanols exhibit a similar pattern in UV-Vis absorption spectra, showing a peak of maximum absorption around 280 nm (Santos-Buelga *et al.*, 2003). UV-Vis spectroscopy is a useful tool to monitor changes in the flavonoid structure such as oxidation and formation of quinones, which show a yellow colour and absorb in the range of 340 – 480 nm (visible) (Torreggiani *et al.*, 2008). According to Figure 4-3, all phenolic compounds before oxidation present a peak of maximum absorption around 280 nm, whilst after incubation with laccase, the spectra for all compounds showed also absorbance in the quinone range (340 – 480 nm), not present in the non-oxidised phenolic compound. Therefore, the wavelengths of 395 nm for catechol, 350 nm for pyrogallol, 450 nm for catechin and 320 nm for phloroglucinol were used to monitor the reaction kinetics for each respective compound.

The laccase-catalysed oxidation of the substrate resorcinol has not resulted in spectrum changes during the time studied. This indicates the non-formation of quinones during the oxidation reaction. The *meta*- position of the hydroxyl in the phenolic ring of resorcinol makes its quinone structure non-stable by resonance, as illustrated in Figure 4-4 (Macomber, 1996). Therefore, the kinetic parameters for resorcinol oxidation were not determined. The higher activity of laccase on *o*-phenols like catechol and pyrogallol suggests that the oxidation of catechin occurs especially in the B-ring (*o*-phenol), instead of in the A-ring (*m*-phenol), which is consistent with the reaction proposed by Bark *et al*. (2011) illustrated in Figure 4-1.

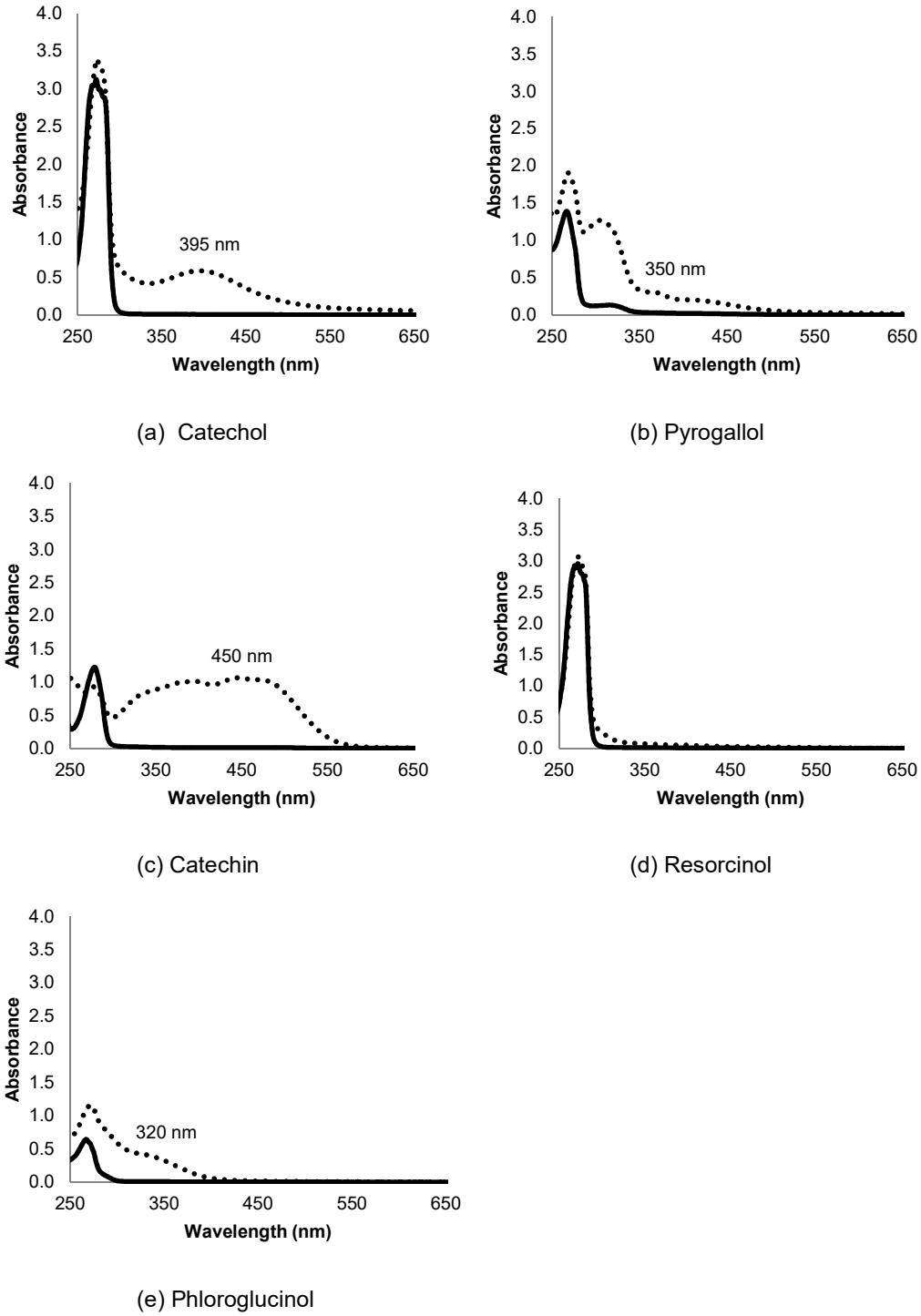


Figure 4-3: UV-vis spectra for phenolic compounds and the oxidised product by the enzyme laccase (substrate concentration 1 mM, laccase concentration 25 mg/l, temperature 25°C, pH 5.5): phenolic compound (—), oxidised product after 30 min reaction (···).

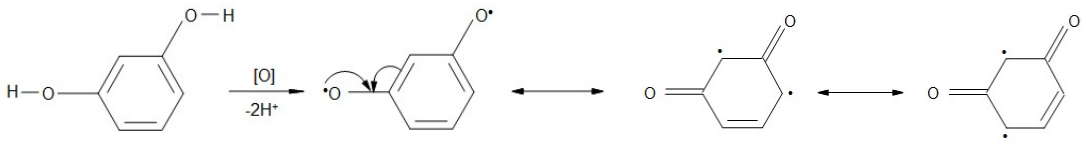


Figure 4-4: Oxidation of resorcinol (Macomber, 1996).

4.4.2 Kinetic studies of laccase-catalysed oxidation of simple phenols

The Michaelis–Menten theory defines the relationship between initial velocities and substrate concentrations, providing information on the kinetics of enzyme-catalysed reactions. Initial velocities are determined by linear regression of initial data points of the progress curves at different substrate concentrations (Bezerra *et al.*, 2013). The Michaelis-Menten equation explains the observation that the enzyme reaction rate reaches a plateau at saturating substrate concentrations, as a result of the formation of an enzyme–substrate complex (Goldbeter, 2013).

Figure 4-5 shows that laccase-catalysed oxidation for the phenolic species studied obeys the Michaelis-Menten kinetics. The Michaelis-Menten graph for phloroglucinol is shown in Figure 4-6, in order to facilitate the visualisation, as the laccase activity with this substrate was considerably lower than for catechin, catechol and pyrogallol. For this reason, the kinetic parameters for phloroglucinol were not calculated.

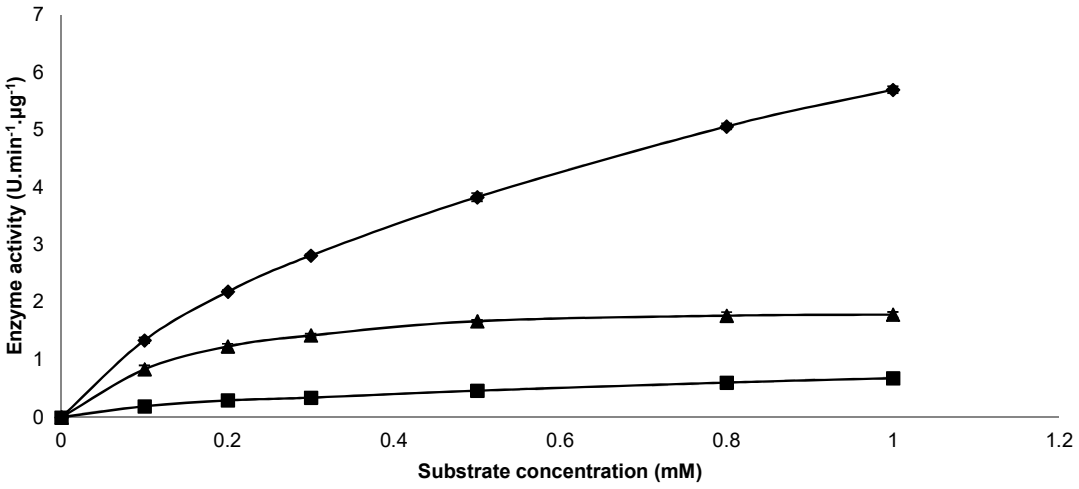


Figure 4-5: Variation in laccase activity with substrate concentration for catechin (♦), pyrogallol (▲) and catechol (■), at 25°C, pH 5.5 and laccase concentration of 25 mg/l. Error bars denote the standard deviation of triplicate measurements (standard deviations were smaller than the markers used for the graph).

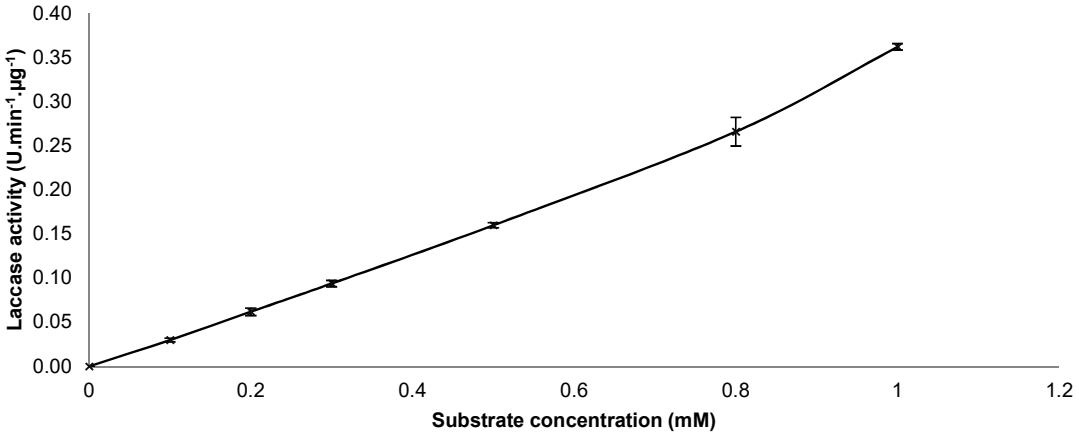


Figure 4-6: Variation in laccase activity with substrate concentration for phloroglucinol, at 25°C, pH 5.5 and laccase concentration of 25 mg/l. Error bars denote the standard deviation of triplicate measurements.

Figure 4-5 shows that the laccase activity for catechol is lower, compared to pyrogallol and catechin. For pyrogallol, a small concentration of this substrate was sufficient for the maximum activity to be achieved, in comparison with catechin and catechol. Catechin showed the

highest activity, and it is possible to observe that the maximum velocity was not achieved for the concentrations studied, suggesting that the enzyme may catalyse higher concentrations of the substrate catechin.

The low activity of laccase with phloroglucinol (benzene-1,3,5-triol) and resorcinol (benzene-1,3-diol), both *meta*-phenols, confirms the information given by Madhavi (2009), that laccases act mostly on *para*- and *ortho*- substituted phenols. Babu *et al.* (2012) stated that *ortho*-substituted compounds (guaiacol, *o*-phenylenediamine, caffeic acid, catechol, dihydroxyphenylalanine, protocatechuic acid, gallic acid and pyrogallol) were better substrates for laccase than *para*-substituted compounds (*p*-phenylenediamine, *p*-cresol, hydroquinone) and the lowest rates were obtained with *meta*-substituted compounds (*m*-phenylenediamine, orcinol, resorcinol and phloroglucinol). This indicates that the oxidation of catechin occurs in the B-ring (*o*-phenol) instead of in the A-ring (*m*-phenol), corroborating with the reaction proposed by Bark *et al.* (2011), shown in Figure 4-1.

The kinetic parameters: enzyme-substrate affinity (K_m) and maximum initial velocity (V_{max}) were calculated for catechin, catechol and pyrogallol, using the Lineweaver-Burk inversion of the Michaelis-Menten equation, illustrated in Figure 4-7.

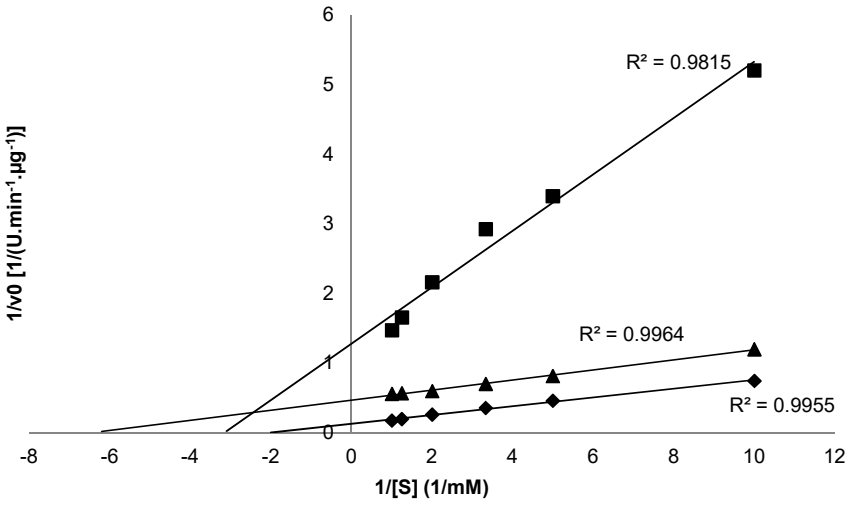


Figure 4-7: Lineweaver-Burk plot for catechin (♦), pyrogallol (▲) and catechol (■), based on the data shown in Figure 4-5.

With the Lineweaver-Burk inversion of the Michaelis-Menten equation, it is possible to fit the data to a line and calculate the enzyme-substrate affinity (K_m) and the maximum initial velocity (V_{max}) from the equation obtained. This is particularly useful when the maximum velocity is not reached for the substrate concentrations studied, as observed for catechin in Figure 4-5. The calculated kinetic parameters V_{max} and K_m are presented in Table 4-1.

Table 4-1: Kinetic parameters for laccase-catalysed oxidation of catechin, catechol and pyrogallol, at 25°C, pH 5.5 and laccase concentration of 25 mg/l. Results are expressed as average ± standard deviation of triplicate measurements.

Substrate	***K_m (mM)***	***V_{max} ($U.min^{-1}.\mu g^{-1}$)***
Catechin	0.49 ± 0.01	7.74 ± 0.91
Catechol	0.31 ± 0.02	0.80 ± 0.03
Pyrogallol	0.18 ± 0.09	2.28 ± 0.35

In terms of enzyme-substrate affinity, given by the K_m value, the data shown in Table 4-1 demonstrate that, for the substrates studied, pyrogallol has higher affinity for the enzyme laccase. This means that a lower concentration of pyrogallol is needed to saturate the enzyme. Catechin showed lower affinity compared to catechol and pyrogallol, presenting, however, the highest maximum initial velocity. This indicates a higher enzyme activity and catalytic effect for this substrate. For this reason, catechin was chosen as the substrate for further studies. Moreover, catechin, being a flavan-3-ol, constitutes a monomeric unit for proanthocyanidins (condensed tannins). This makes the polymerisation of catechin a viable route in the polymerisation of tannins to be applied in collagen stabilisation.

4.4.3 Free radical scavenging of catechin oxidised by laccase

Laccase couples the four-electron reduction of dioxygen to water with oxidation of the substrate (Burton, 2003). The substrate is oxidised by one electron, creating a free radical species (Claus, 2004). Free radicals are unstable and very reactive molecules that have an unpaired electron in the outer orbit (Fang *et al.*, 2002). Antioxidant compounds, also called free radical scavengers, are defined as ‘a substance that, when present at low concentrations compared with those of an oxidisable substrate, significantly delays or prevents oxidation of

that substrate' (Halliwell, 1995). Brand-Williams *et al.* (1995) studied the antioxidant activity of various substances on the free radical form of the compound 2,2-diphenyl-1-picrylhydrazyl (DPPH). The authors measured a very rapid reaction of ascorbic acid with the radical DPPH•, monitored by UV-Vis spectroscopy. Figure 4-8 shows the mechanism of the free radical scavenging action of ascorbic acid with DPPH.

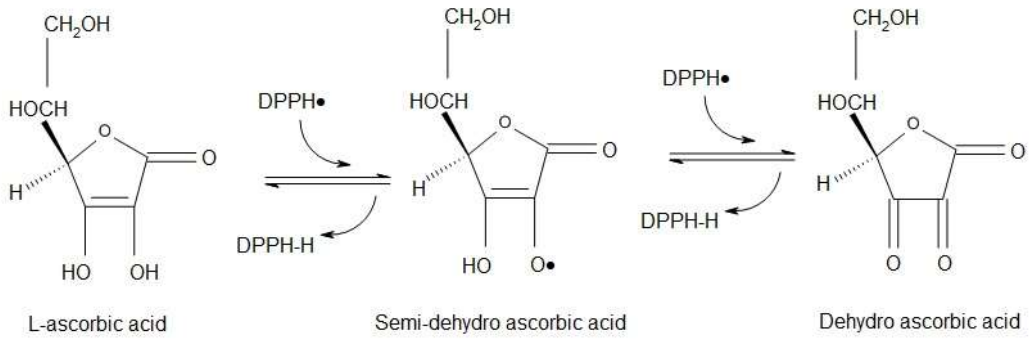


Figure 4-8: Mechanism of the free radical scavenging action of ascorbic acid with the substrate 2,2-diphenyl-1-picrylhydrazyl (DPPH) (adapted from Brand-Williams *et al.*, 1995).

Data obtained from experiments aimed at investigating the effect of ascorbic acid in the laccase-catalysed oxidation of catechin are shown in Figures 4-9 to 4-11. Figure 4-9 presents the change in absorbance over time for different concentrations of ascorbic acid in the reaction media of catechin and laccase.

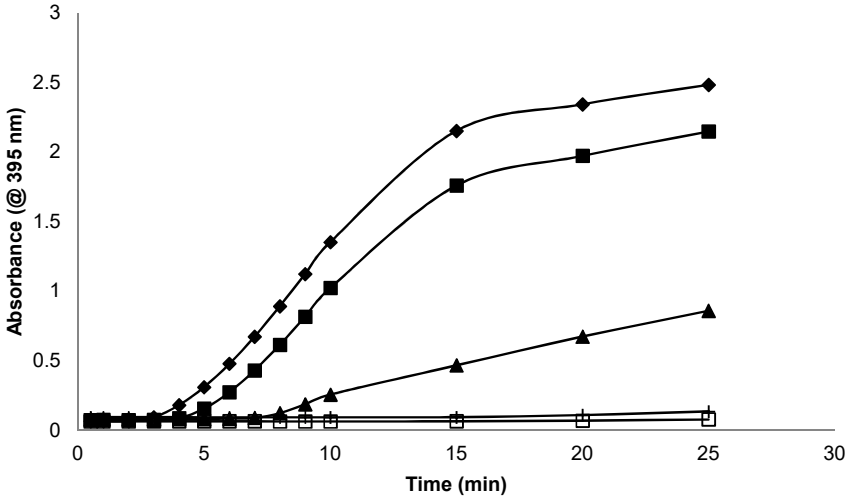


Figure 4-9: Absorbance *versus* time at 395 nm for different concentrations of ascorbic acid: 0.1 mM (♦), 0.2 mM (■), 0.5 mM (▲), 0.8 mM (+) and 1 mM (□), at 25°C, pH 5.5, catechin concentration 1mM and laccase concentration 25 mg/l.

Low concentrations of ascorbic acid of 0.1 and 0.2 mM delayed the start of the reaction and slowed down the initial rate. The reaction then proceeded, as shown by the increasing absorbance readings at 395 nm. An intermediate concentration of 0.5 mM showed a slower rate for the increase in absorbance. For concentrations of 0.8 and 1 mM of ascorbic acid, the absorbance did not increase during the assessed period, suggesting the non-formation of products. These data show that the free radical scavenging action of ascorbic acid prevents the formation of oxidation products, indicating that the generation of free radicals may be necessary for the reactions to occur.

Based on the results, experiments were performed with the addition of an ascorbic acid solution in a sufficient concentration to act as a free radical scavenger, after initiation of the enzymatic oxidation. The absorbance was monitored at 450 nm, which is the wavelength of maximum absorbance for the oxidised product from the laccase-catalysed reaction with catechin. The same procedure was also undertaken at 395 nm, which is the wavelength of maximum absorbance of the medium composed by catechin, laccase and ascorbic acid. Figure 4-10 and Figure 4-11 show the data obtained in the experiments.

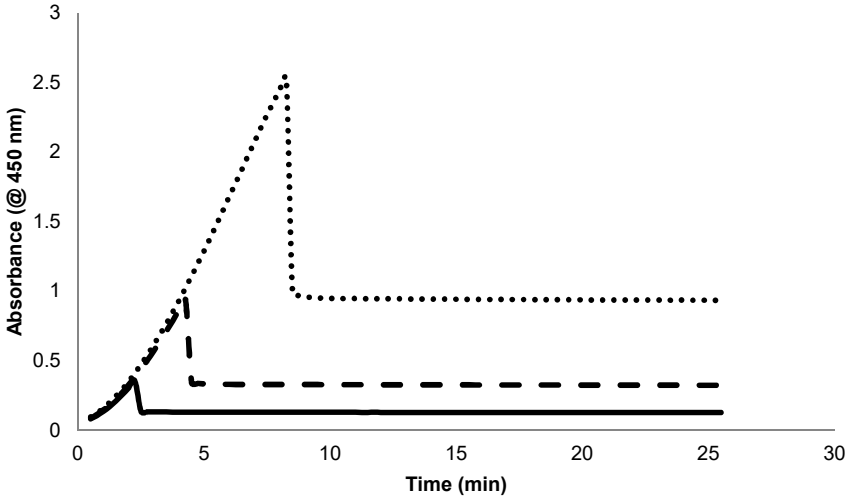


Figure 4-10: Absorbance *versus* time at 450 nm for catechin (1 mM) and laccase (25 mg/l), temperature 25°C, pH 5.5, with addition of ascorbic acid (1.75 mM) after 2 min (—), 4 min (- - -) and 8 min (···) reaction.

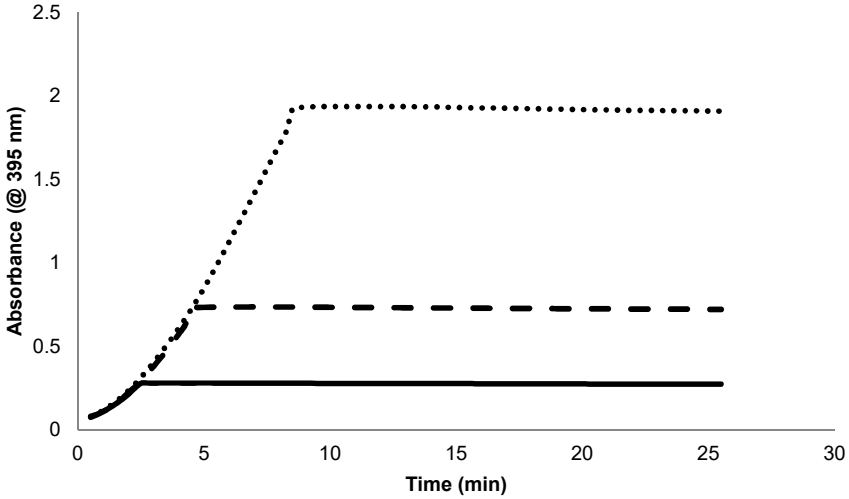


Figure 4-11: Absorbance *versus* time at 395 nm for catechin (1 mM) and laccase (25 mg/l), temperature 25°C, pH 5.5, with addition of ascorbic acid (1.75 mM) after 2 min (—), 4 min (- - -) and 8 min (···) reaction.

At 450 nm there was an instant reduction in absorbance when the ascorbic acid solution was added and no further increase was observed after the addition (Figure 4-10), for all addition

times. At 395 nm (Figure 4-11), before the addition of ascorbic acid, the absorbance increased more slowly than at 450 nm (Figure 4-10), as 395 nm is not the wavelength of maximum absorbance for the reaction medium that does not contain ascorbic acid. When the ascorbic acid solution was added, the absorbance remained constant, indicating that the wavelength of maximum absorbance indeed shifted from 450 to 395 nm (closer to the limit between UV and visible).

The enzymatic oxidation of polyphenols to coloured compounds is a field of great interest in food science. The market of fresh-cut fruit faces the challenge of browning, which is caused by the action of polyphenol oxidase (PPO) in the presence of oxygen, oxidising the *o*-dihydroxy groups of polyphenols to the corresponding *o*-quinones (Lunadei *et al.*, 2011). Ascorbic acid has been applied to increase shelf life of fresh-cut fruits, retarding the browning effect (Li-Qin *et al.*, 2009). Osman *et al.* (2007) studied the effect of ascorbic acid on the formation of catechin dimers by laccase, showing that at higher concentrations the effect of ascorbic acid as a free radical scavenger inhibited the oxidation reaction, most likely reducing the *o*-quinone to polyphenol. The decrease in absorbance showed in Figure 4-10 and the shifting of the wavelength of maximum absorption (Figure 4-11) suggests the occurrence of reversibility of the quinone to polyphenol, corroborating with the work of Osman *et al.* (2007) and Li-Quin *et al.* (2009).

In order to determine if the decrease in absorbance occurred as a function of dilution or interaction of ascorbic acid with the oxidation products, experiments with the addition of deionised water instead of ascorbic acid solution were performed at 450 nm and 395 nm and the data are shown in Figure 4-12.

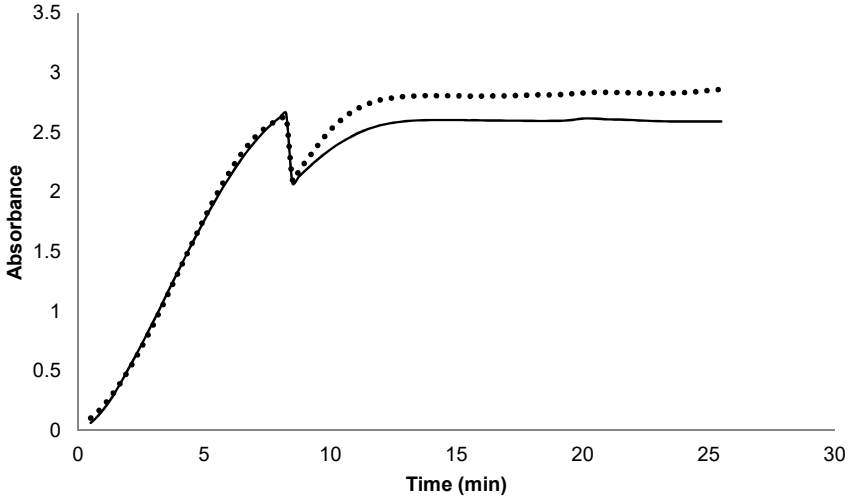


Figure 4-12: Absorbance *versus* time for catechin (1 mM) and laccase (25 mg/l), temperature 25°C, pH 5.5, with addition of deionised water after 8 min reaction: at 450 nm (—) and at 395 nm (···).

The dilution had an effect in decreasing the absorbance, however, the absorbance continued to increase with the formation of products, showing a different behaviour from data shown in Figures 4-10 and 4-11. This result indicates that the decrease in absorbance after addition of ascorbic acid was not a result of dilution.

4.4.4 Enzyme inhibition with sodium azide

Additional experiments were carried out to determine if the effect of ascorbic acid could be inhibiting the enzyme, rather than acting as a free radical scavenger. In these experiments, sodium azide, an enzyme inhibitor of laccase (Johannes and Majcherczyk, 2000), was added to the reaction media. Results are shown in Figures 4-13 to 4-15.

The Michaelis-Menten graph for the reaction in a medium with sodium azide is shown in Figure 4-13. The results obtained for the non-inhibited medium of catechin and laccase, shown previously in Figure 4-5, are also presented in Figure 4-13 for illustration purposes.

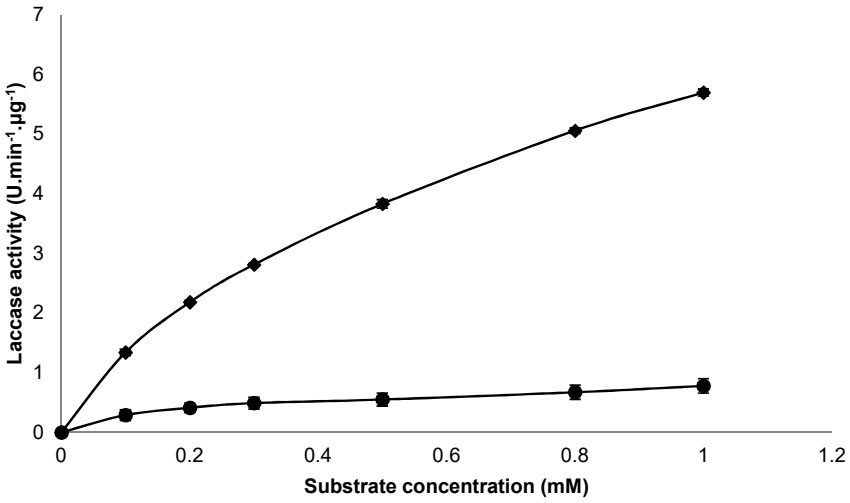


Figure 4-13: Variation in laccase activity with substrate (catechin) concentration without sodium azide (♦), with 2 mM sodium azide (●), at 25°C, pH 5.5 and laccase concentration of 25 mg/l. Error bars denote the standard deviation of triplicate measurements (standard deviations were smaller than the markers used for the graph).

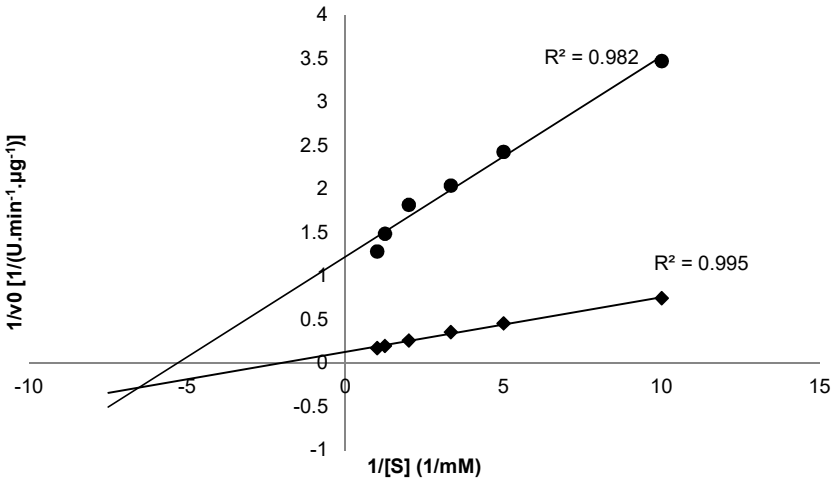


Figure 4-14: Lineweaver-Burk plot for catechin without sodium azide (♦), with 2 mM sodium azide (●), based on the data shown in Figure 4-13.

The Lineweaver-Burk plot (Figure 4-14) shows that the effect of sodium azide on the enzymatic reaction is a type of non-competitive mixed inhibition, in which the inhibitor may bind to either the enzyme, or to the enzyme-substrate complex (Nelson and Cox, 2013).

Figure 4-15 presents the data obtained in an experiment similar to the radical scavenging with ascorbic acid (Figure 4-10), where the test solution is added after the laccase-catalysed oxidation has started.

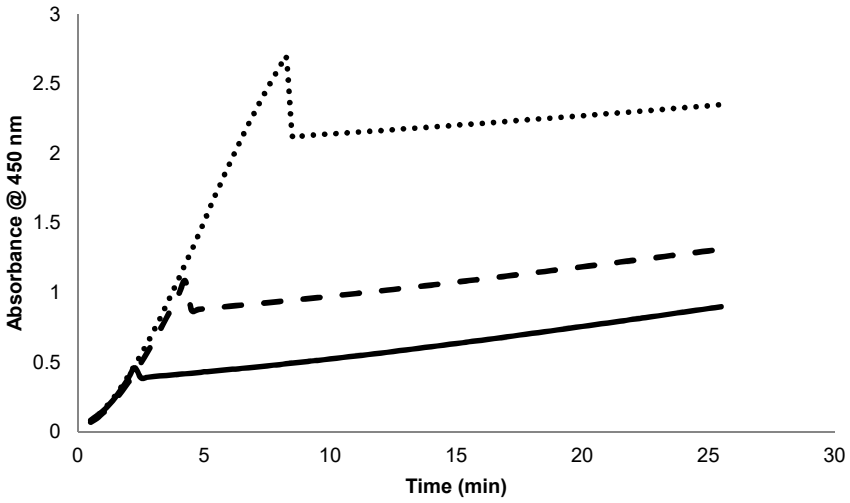


Figure 4-15: Absorbance *versus* time at 450 nm for catechin (1 mM) and laccase (25 mg/l), temperature 25°C, pH 5.5, with addition of sodium azide (2 mM) after 2 min (—), 4 min (- - -) and 8 min (···) reaction.

Figure 4-15 shows a different behaviour when sodium azide is added, in comparison to ascorbic acid (Figure 4-10). After the addition of ascorbic acid, the absorbance lowered instantly and no further increase in absorbance was observed. Differently, the absorbance after the addition of sodium azide was lowered but continued to increase slowly, indicating that the reaction was slowed down without stopping completely. The shape of the figure obtained in this study (Figure 4-15) was similar to the one reported by Johannes and Majcherczyk (2000), who added a solution of 1 mM of sodium azide to a medium containing laccase (0.017 U/ml) and ABTS (5 mM) in acetate buffer (pH 5.0), and monitored the oxidation of ABTS to $ABTS^{+\bullet}$ at 420 nm. The authors determined that sodium azide is a true inhibitor for laccase. Therefore, the ascorbic acid was not acting as an inhibitor, and the generation of free radicals is part of the mechanism of laccase-catalysed oxidation of catechin.

4.4.5 Analyses of the polymerised product of laccase-catalysed oxidation of catechin

The polymerised product of laccase-catalysed oxidation of catechin, the control obtained with the process described in Section 4.3.4, as well as pure catechin were analysed using FT-IR and TLC. The polymerised product was also analysed by phloroglucinolysis followed by reversed phase UHPLC, in order to verify if interflavanyl bonds between monomeric units were present, indicating the polymerisation.

4.4.5.1 FT-IR analyses

Fourier transform infrared spectroscopy analyses were performed to verify structural changes in the product of the laccase-catalysed process and in the control product, both obtained by the method described in Section 4.3.4. The analysis was also carried out for pure catechin and the spectra are shown in Figure 4-16.

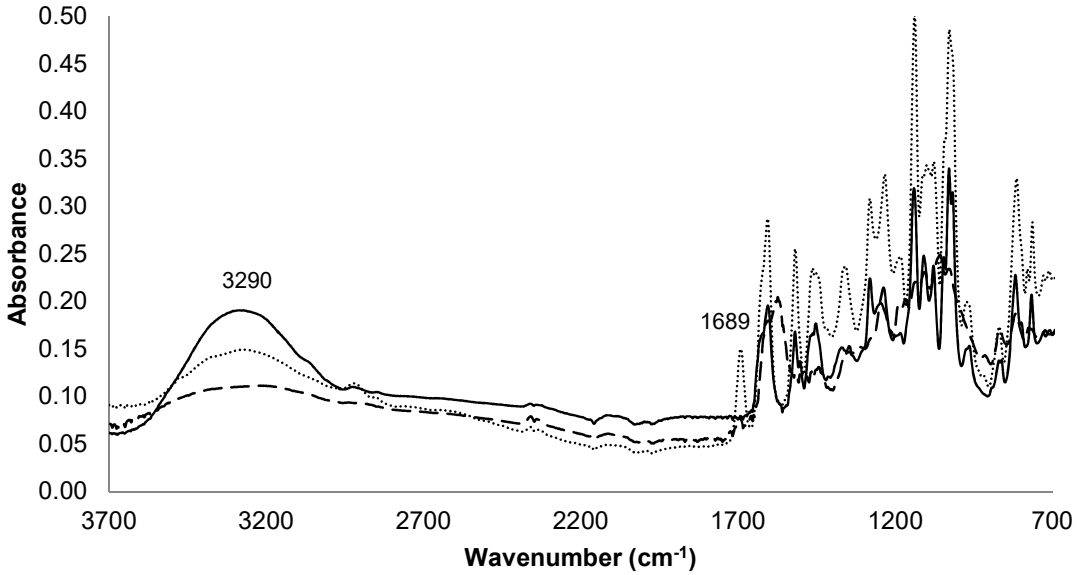


Figure 4-16: FT-IR spectra of pure catechin (——), control sample of catechin omitting the laccase (·····), and catechin incubated with laccase (— — —).

FT-IR analyses suggest that laccase-catalysed oxidation of catechin results in the combination of monomers to form polymers. The intensity for the product of laccase-catalysed 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 (Xuejiao *et al.*, 2013), reducing the bond vibrations.

The control presents a peak at 1689 cm^{-1}, within the wavenumber range for quinones (1690 – 1675 cm^{-1}) (Coates, 2000), which indicates the possibility of oxidation due to the exposure to the experimental conditions, even without the enzymatic action. As expected, untreated catechin does not show any peak in the quinone region, as it was not subjected to the experimental conditions. The peak for the enzyme-catalysed product at 1689 cm^{-1} (quinone) has a lower absorption than 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.

The phenolic H-bonded O-H stretch peak at 3290 cm^{-1} (Coates, 2000) 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 (He *et al.*, 2004).

4.4.5.2 Thin Layer Chromatography (TLC) analysis

Thin Layer Chromatography (TLC) analysis was carried out according to the procedure described in Section 3.2.3, for the control, oxidised samples obtained by the method described in Section 4.3.4, as well as for pure catechin.

After visualisation of the spots under UV light and measurement of the distance from the starting point, the retention factor (Rf) was calculated, and results are presented in Table 4-2.

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Table 4-2: Retention factor (R_f) calculated based on thin layer chromatography analyses, for pure catechin, control and polycatechin.

Sample	***R_f***
Pure catechin	0.78
Control	0.78
Polycatechin	0.69

TLC is a qualitative analysis that can indicate changes in structure through the distance travelled by the sample (retention factor). According to Karchesy *et al.* (1989), with the solvent system used (methodology described in Section 3.2.3), compounds can be compared in terms of relative sizes, as the higher the retention factor, the lower is the molecular weight. Data on Table 4-2 show that the control travels the same distance as the pure catechin. The test sample incubated with the enzyme, however, has a lower retention factor, interacting more with the stationary phase of the plate. This indicates that the test sample has larger molecules than the pure catechin and the control, as a result of the polymerisation occurring due to the enzymatic oxidation.

4.4.5.3 UHPLC – Phloroglucinolysis

The phloroglucinolysis method described in Section 3.2.9 followed by reversed phase UHPLC (Section 3.2.10) were applied to confirm the polymerisation indicated by the FT-IR and TLC analysis. Figure 4-17 shows the chromatogram of a sample of catechin oxidised by laccase after the process of phloroglucinolysis.

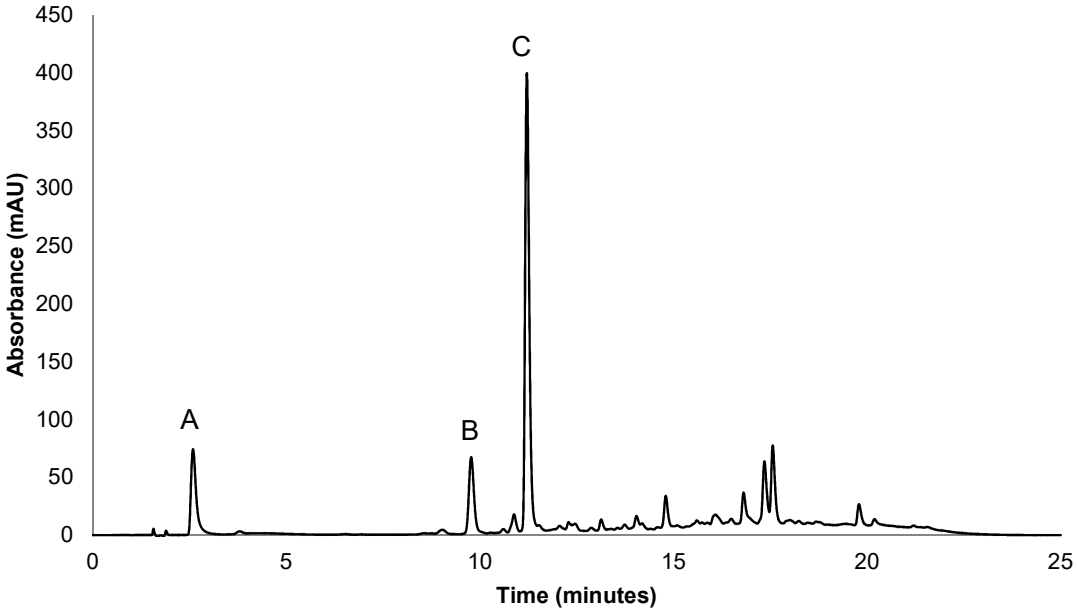

Figure 4-17: UHPLC chromatogram of a sample of catechin oxidised by laccase, after the process of phloroglucinolysis (described in section 3.2.9). The identified peaks correspond to: phloroglucinol (A), catechin-phloroglucinol adduct (B), and catechin (C).

The peak at 2.59 minutes (peak A) corresponds to the unreacted phloroglucinol. The catechin detected at 11.21 minutes (peak C) was not only related to terminal units being released, but also possibly unreacted catechin present in the sample. The chromatogram shows the appearance of a peak for the adduct catechin-phloroglucinol at 9.77 minutes (peak B). This adduct indicates the breakage of the interflavanyl bond between monomers in the polyphenol molecule and further reaction with phloroglucinol (Cheynier and Fulcrand, 2003), suggesting that the free radicals combined to form at least dimers, and possibly oligomers and polymers. It was not possible to estimate the degree of polymerisation using the chromatogram showed in Figure 4-17, as it considers the ratio of the concentration of the adduct formed with the breakage of interflavanyl bonds to the concentration of terminal units released. Seeing that catechin was used as a substrate for the polymerisation, it is not possible to determine the exact amount of catechin released as terminal units, as the presence of unreacted catechin is highly probable.

The analyses showing polymerisation of catechin confirm the findings of Kurisawa *et al.* (2003). The authors performed the polymerisation of catechin with laccase from

Myceliophthora, and characterised the polymer obtained using FT-IR, UV-Vis and ESR (electron spin resonance).

4.5 Summary

Kinetic studies to compare the catalytic effect of laccase on different monomeric phenols showed that the incubation of catechin as a substrate for laccase presented the highest activity when compared to catechol, pyrogallol, phloroglucinol and resorcinol. Catechin was thus the chosen monomeric phenol source for further experiments. The low velocity in laccase-catalysed oxidation presented by the *meta*- phenols, phloroglucinol and resorcinol, suggested that laccase acts on the B-ring of catechin (*o*-phenol), instead of on the A-ring (*m*-phenol).

Free radical scavenging experiments with ascorbic acid and inhibition studies with sodium azide confirmed the mechanism of free radical generation and formation of quinone structure as part of the catalytic effect of laccase on catechin. The quinones conferred a yellow colour to the product, and the addition of ascorbic acid after starting the incubation showed a reversibility in the colour formation. This effect can be explored in further work, where the polymerisation and reversibility to phenol can be monitored to an optimal, for applications in which the colour development must be avoided.

Structure analysis using FT-IR, TLC and UHPLC demonstrated that the free radicals formed by the interaction of the substrate catechin with the enzyme laccase combined to form polymers. The polymeric characteristic of the product formed by the enzymatic reaction was further explored on the application of laccase-catalysed polymerisation of catechin on the stabilisation of collagen.

Chapter 5 Laccase-catalysed polymerisation of catechin applied to the stabilisation of collagen

5.1 Introduction

Vegetable tanning occurs due to the crosslinking of many peptide chains in the collagen structure 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 (Bienkiewicz, 1983). Figure 5-1 illustrates the interaction of the hydroxyl moieties of a polyphenol molecule with collagen residues (Covington, 2009).

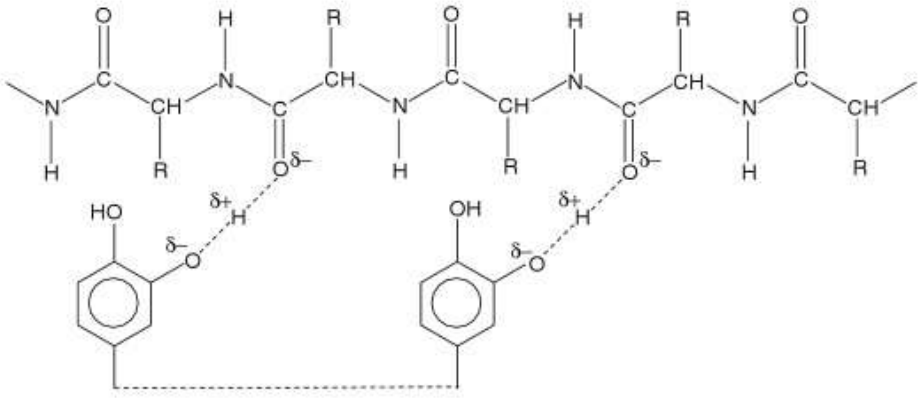

Figure 5-1: Interaction of a polyphenol molecule with collagen residues (Covington, 2009).

Low molecular weight polyphenols, referred as non-tannins, have a deficient tanning effect due to the lacking of proper number of phenolic moieties to ensure crosslinking between collagen molecules. Conversely, the tannin molecule cannot penetrate or poorly penetrates into the rawhide in the tanning process if it is too large (Mavlyanov *et al.*, 2001). Reactive tannins combine very rapidly with the hide and skin substance, causing full or over-tannage on the surface before the corium is fully tanned (Sharphouse, 1983).

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 polymerisation *in situ*, once penetration has occurred into the hide/skin. Investigation into the utilisation of small phenolic compounds to improve penetration with subsequent *in situ* enzyme-catalysed polymerisation may provide a novel and alternative tanning agent.

The use of oxidative enzymes to create collagen crosslinking was studied by Pretzler *et al.* (2012). The authors have applied laccase and tyrosinase to catalyse crosslinks on collagen molecules through oxidation of tyrosine residues. The study demonstrated some ability of the enzymes in promoting crosslinking, despite the low number of tyrosine residues in collagen. The authors suggested the addition of small phenolic molecules to assist the crosslinking process in order to enhance the mechanical resistance of the produced gel. Therefore, laccase-catalysed reactions of polyphenols and collagen have the potential to be applied in the development of collagenic biomaterials.

Catechin, having a molecular weight of 290 Da, presents a lower capacity of binding with collagen, thereby the penetration into the hide/skin is facilitated when compared to larger molecules. The results of laccase-catalysed oxidation of catechin presented in Chapter 4 demonstrated the occurrence of polymerisation, hence the catalytic effect of laccase on catechin may be explored to increase tanning reactions and consequently the hydrothermal stability of collagen.

Experiments using hide powder were undertaken to determine the effect of laccase-catalysed polymerisation of catechin in the thermal stabilisation of collagen. Thermal stabilisation was evaluated by the change in shrinkage temperature between the treated and untreated sample of hide powder (ΔTs). In order to evaluate the effect of process parameters in the increase in shrinkage temperature, a factorial design 2^k was used to study process temperature, substrate concentration, enzyme concentration and incubation time. The statistically significant variables were studied further for the optimisation of ΔTs, using response surface methodology. Darkening of the hide powder samples subjected to the enzymatic process was observed; therefore, in addition to the measurement of shrinkage temperature, the samples were also evaluated regarding the change in colour (ΔE) calculated in relation to untreated hide powder.

Application of laccase-catalysed polymerisation of catechin on goat skin was undertaken. In a first step, the absorbance of catechin was monitored for different times of penetration. The enzymatic process was carried out following the optimised conditions as determined through response surface methodology.

5.2 Aims and objectives

The aim of this study was to demonstrate the potential of laccase-catalysed oxidation of catechin in increasing the thermal stabilisation of collagen, in order to provide an alternative method of vegetable tanning.

The objectives of this study were:

- To apply catechin to hide powder and perform the polymerisation *in situ* using laccase as a catalyst, and subsequently measuring the effect of the enzyme-induced reactions on the shrinkage temperature of collagen;

- To study the effect of variables temperature, incubation time, laccase concentration and catechin concentration on the change in shrinkage temperature (ΔTs) and colour change (ΔE) of hide powder, using design of experiments;

- To optimise the change in shrinkage temperature (ΔTs) of hide powder using central composite design;

- To monitor the absorption of catechin into goat skin by measuring the remaining solids in the float and apply the optimised conditions for the enzymatic process.

5.3 Design of experiments

5.3.1 Introduction

Statistical methods are useful to determine if a variation in the response variable is due to the variation of process parameters or is related exclusively to experimental error. The first phase of the experimental design consists in determining the important factors. This information may be obtained through experience or by executing a fractional design, with which it is possible to study several factors at a time. Full factorial design of experiments is used to evaluate the influence of process parameters in the response variable, as well as the interactions amongst them, using the minimum number of experiments. A mathematical model may be obtained from the experimental data by applying least squares modelling. Response surface methodology (RSM) is used to determine the minimum or maximum for a response variable.

5.3.2 Terminology

The terms used in design of experiments are described according to the following (Bezerra *et al.*, 2008; Lundstedt *et al.*, 1998):

- Experimental domain: defined by the minimum and maximum limits of the studied variables, giving the experimental field that is investigated.
- Independent variables or factors: experimental variables that can be independently changed, and may affect the response. These variables can be continuous, such as temperature, pH and time; or discrete, such as type of solvent.
- Response or dependent variables: the measured values of the results from the experiments. These are usually related to the quality characteristics that the experiments aim to enhance, *e.g.* the degree of tanning, given by the measurement of the shrinkage temperature. The dependent variables could be also focused in reducing costs or waste emissions, *e.g.* minimising time of processing to save energy or maximising a chemical reaction.
- Levels of a variable: different values set for the independent variables. For example, temperature can be studied at 20, 30 and 40ºC.
- Experimental design: set of experiments defined by the different combinations of levels for the factors studied.

- Residual: the difference between the experimental and the calculated results for a determined set of conditions. The lower the residual values, the better the mathematical model fits the experimental data.
- Repetition: performing more than one experiment under the same experimental conditions. The repetition allows the determination of the response variability due to the experimental error. If the effect of a factor is significant, it must be higher than the experimental error. In order to decrease time and cost, the repetitions may be carried out at one experimental condition. For a 2-level factorial design, if the repetition is carried out at the centre point, it also can provide information about the linearity of the response variation with the factors.
- Main effect of a factor: average change in response when the factor level is changed from level -1 to 1.
- Interaction amongst factors: the interaction is given when the response depends of a second factor, as the level of another factor is changed.

5.3.3 Conducting the experimental design

According to Montgomery (2009) and Anthony (2003), the sequence for an effective design of experiments is described as the following:

1. Establishment of the objective. This may be, for example, an increase in quality or decrease in cost for a product. All hypotheses may be established by a team “brainstorming”, aiming at gather the most information possible.

2. Selection of the response variable. The response variable must provide useful information about the process.

3. Choice of factors, levels and range. Design factors are the ones selected to be studied in the experiment. Held-constant factors are the ones that may affect the response, but were chosen not to be studied, and therefore are held at a specific level. The noise factors are divided into controllable and uncontrollable: controllable are the ones that may be set, such as batches of raw material; and when an uncontrollable factor cannot be set, but can be measured, such as relative humidity, analysis of covariance may be used and this factor is treated as a covariate.

After the design factors are selected, the levels and the range over each factor will be varied must be defined. If the process is not well known, a factor screening using fractional design may be used. Cause-and-effect diagrams are also helpful to organise the information gathered in the pre-experimental stage.

4. Choice of experimental design. This step requires the consideration of sample size or number of replicates and selection of the run order for the experimental trials. It is important that the trials are conducted in a randomised way to minimise the uncontrollable factors effect.

5. Performing the experiment. The process must be carefully monitored to ensure experimental validity.

6. Statistical analysis of the data. Statistical methods are used to ensure the conclusions are objective. Data is analysed with hypothesis testing and confidence interval estimation procedures. It is also helpful to adjust the data to an equation (empirical model) that establishes the relationship between the response variables and the significant design factors.

7. Conclusions and recommendations.

5.3.4 Analysis of variance (ANOVA)

Analysis of variance (ANOVA) is a method of accounting sources of variation from the total variation in an experiment, used to determine if the factors' main effects and/or interactions amongst factors are significant (Krishnaiah and Shahabudeen, 2012). For the validity of the statistical tests, three assumptions are commonly incorporated into the formulas (Turner and Thayer, 2001):

- The observations are independent, *i.e.*, each observation is not correlated with any other observation. It can be obtained by randomising the execution of the experiments;
- The observations are normally distributed;
- Homogeneity of variances.

5.3.5 Full factorial design

A full factorial design consists of studying all possible combinations of levels for all factors. For a design studying *k* factors at 2 levels, the total number of experiments is 2^k (Antony, 2003). The 2^k factorial design can only produce a linear model. If the effect of the variables is quadratic, more design points must be added (Tabachnick and Fiddell, 2007).

5.3.6 Central composite design

The screening of the factors studied, using full factorial or fractional factorial design, is the first step of multivariate optimisation. After determining the significant factors, the optimum operation conditions are attained by using more complex experimental designs such as Doehlert matrix, central composite designs and three-level designs such as the Box-Behnken design (Ferreira *et al.*, 2007).

Central composite designs allow testing linear and quadratic effects. In this design, each independent variable has five levels, which is considered the prototype of the response surface design (Tabachnick and Fiddell, 2007). The two-level full or fractional factorial design is combined with additional points (axial points or star points) and at least one point at the centre of the experimental region, in order to fit quadratic polynomials (Ferreira *et al.*, 2004). The distance of the star points from the centre point is given by $\alpha = 2^{n/4}$, where n is the number of factors (Vicente *et al.*, 1998). Therefore for two factors, $\alpha = 1.414$. Figure 5-2 shows a representation of the factorial design with the addition of star points for two factors.

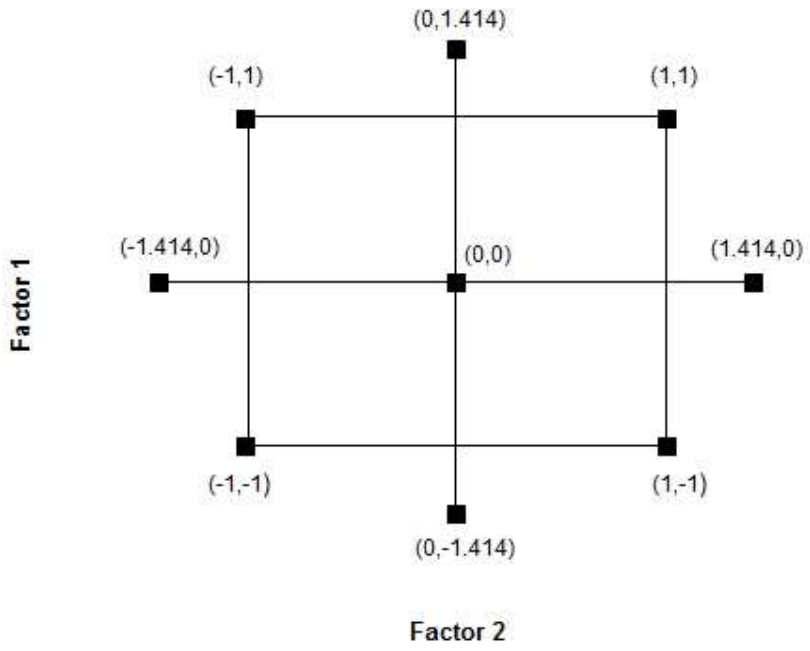

Figure 5-2: Representation of a central composite design for 2 factors (adapted from Hibbert, 2007).

Equation (5-1) was used for calculating the real value of the variables for each coded value (Bezerra *et al.*, 2008).

$$x_i = \frac{(z_i - z_{0i})}{\Delta z_i} \quad \textbf{(5-1)}$$

Where x_i is the coded value of the independent variable (IV), z_i is the real value of the IV, z_{0i} is the value of the IV in the centre point and Δz_i is the step change value (distance between the real value of the IV in the centre point and the real value of the IV in the superior or inferior level).

5.3.7 Regression analysis and response surface methodology

Regression analysis is a method of investigating functional relationships amongst variables (Chatterjee and Hadi, 2012). Relating the variables to a mathematical equation may be useful for predicting values of some variables from knowledge of other variables, even under certain restrictions (Draper and Smith, 1998).

A linear relationship between the response variable *y* and *k* independent variables is given by Equation (5-2) (Seber and Lee, 2003).

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \ldots + \beta_k x_k + \varepsilon \quad \textbf{(5-2)}$$

Where ε is a random error component, β_0 is the intercept and β_1 to β_k are the model parameters. The method of least squares used for parameter estimation is based on minimising the sum of the squares of the residual, *i.e.*, minimising the difference between the real value of the dependent variable and the value calculated by the model (Montgomery *et al.*, 2012).

When using a central composite design to gather data, a second-order polynomial can be fitted, and the relationship between the response and the independent variables can be visualised either with a three-dimensional plot or a contour plot. By analysing the response surface, it is possible to estimate where the point (or points) of optimal condition is, if inside the experimental region, or if it tends towards the edges, which would require additional experiments (Bezerra *et al.*, 2008).

5.4 Experimental procedure

5.4.1 Exploratory experiments for laccase-catalysed polymerisation of catechin on 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 x *g* for 10 minutes, and washed twice with deionised water. After 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 (British Standards Institute, 2012). Tanning degree was evaluated by the shrinkage temperature (Ts), measured using differential scanning calorimetry, according to the method described in Section 3.2.8.

5.4.2 Study of process parameters and optimisation of shrinkage temperature of hide powder using laccase-catalysed polymerisation of catechin

Process parameters that may affect enzymatic reactions were studied in order to optimise the process. Temperature, catechin concentration, laccase concentration and incubation time were varied according to Table 5-1.

For each sample, catechin for the required final concentrations according to Table 5-1 was first dissolved in 3 ml of acetone, and then added to a medium composed of 500 mg hide powder and 25 ml acetate buffer (pH 5.5). The samples were stirred in a magnetic stirrer (Hot plate stirrer PC-351, Corning, New York, USA) for 30 minutes to rehydrate the hide powder and absorb the catechin, and then the pH was measured with a pH meter Seven Multi (Mettler Toledo, Schwerzenbach, Switzerland) and adjusted to 5.5 ± 0.2 when necessary with 0.1 M NaOH. The adequate amount of laccase for the required final concentrations according to Table 5-1 was then added to the samples to initiate the polymerisation. The reaction mixtures were kept for the varied temperature and time according to Table 5-1 in a shaking water bath (Digital water bath OLS200, Grant Instruments, Cambridge, UK) at 100 rpm. The enzymatic reaction was terminated with 5 ml of a sodium azide solution (1 mM). At the end of reaction, the samples were centrifuged (Heraeus Megafuge 16R, Thermo Scientific, Hemel Hempstead, UK) at 3075 x *g* for 10 minutes, and washed twice with deionised water. The tanned hide powder was dried for 48 hours in a controlled atmosphere at 23°C and 50% relative humidity, according to BS EN ISO 2419 (British Standards Institute, 2012), and the

tanning degree evaluated and measured according to the method described in Section 3.2.8. Colour measurement was conducted according to the procedure described in Section 3.2.13.

Table 5-1: Real and coded variables studied in the 2^4 factorial design.

Parameters		***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 time (h)	X_4	8	16	24

5.4.3 Application of laccase-catalysed polymerisation of catechin on goat skin

Goat skin was processed from salted to pickled according to the procedure described in Section 3.1.4. For each gram of goat skin (dry basis), 25 ml of deionised water and catechin for a final concentration of 40 mM was added. A control was prepared, omitting the enzyme. The samples were shaken at 25°C and 100 rpm, in a shaking water bath (Digital water bath OLS200, Grant Instruments, Cambridge, UK). Absorption tests were carried out at 8, 16, 24 and 48 hours. The absorption was calculated by the difference between added catechin and total solid content in the remaining solution. The enzymatic reaction was carried out only at the optimum time for maximum absorption. Laccase at a final concentration of 10 mg/l in acetate buffer (pH 5.5) was added to the test sample to initiate the reaction. The test and control samples were then incubated for 25 hours in a shaking water bath (Digital water bath OLS200, Grant Instruments, Cambridge, UK) at 34.6°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 skin pieces were washed twice with deionised water. After this procedure, the pieces were kept for 48 hours in a controlled atmosphere at 23°C and 50% relative humidity, according to BS EN ISO 2419 (British Standards Institute, 2012). Tanning degree was measured using differential scanning calorimetry, according to the method described in Section 3.2.8.

5.5 Results and discussion

5.5.1 Exploratory experiments for laccase-catalysed polymerisation of catechin on hide powder

Exploratory experiments were undertaken in order to evaluate whether the enzymatic process had any impact in increasing tanning reactions, *i.e.*, in increasing the shrinkage temperature of collagen. Hide powder was used in these experiments, as the diffusion through the thickness step is eliminated, resulting in less variability.

Data for the change in shrinkage temperature (ΔTs) obtained for the application of various concentrations of catechin on hide powder, comparing the control without enzyme and the test sample using laccase are shown in Figure 5-3.

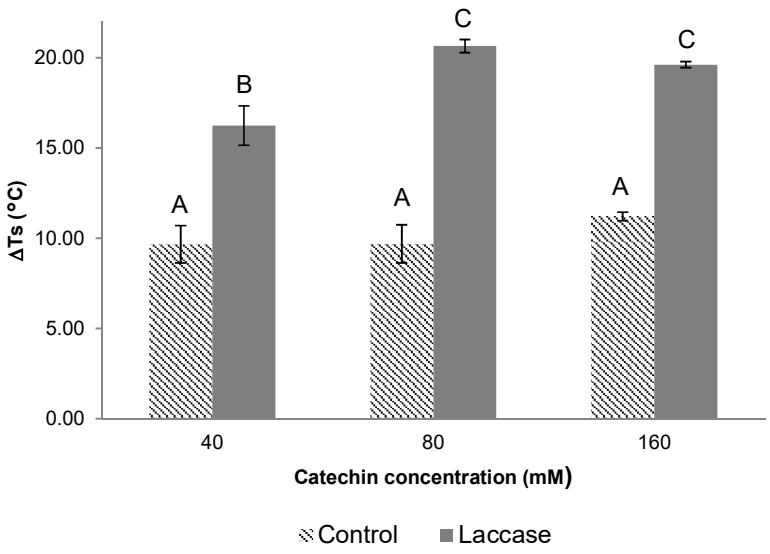

Figure 5-3: Comparison of the effect of laccase (40 mg/l) with a control (no enzyme), for varying concentrations of catechin, on ΔTs of hide powder. The reaction conditions were 25°C, pH 5.5±0.2 and 24 hours. Error bars denote the standard error of the mean (n=3). Means with different letters (A, B, C) are significantly different (Tukey's HSD, $p<0.05$).

Analysis of variance indicated a significant difference between the control and test sample, for all concentrations studied: $p<0.001$. *Post Hoc* analysis (Tukey's HSD) for catechin concentration between samples with the addition of laccase (test samples) 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 difference between 80 mM and 160 mM of catechin was 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 Section 5.5.2.

Catechin, although a small phenolic molecule, can interact with collagen and impart some level of increase in the shrinkage temperature. Madhan *et al.* (2005) 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 (enzyme was absent), where catechin was applied to hide powder under the conditions of 25°C, 24 hours and 100 rpm and 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 (Covington, 1997). 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 values of ΔTs obtained by the application of condensed tannins. This demonstrates the potential of using biotechnology in creating tanning reactions for stabilisation of collagen.

For illustration purposes, Figure 5-4 shows the differential scanning calorimetry (DSC) thermographs for the samples treated with laccase (40 mg/l) and catechin concentrations of 40, 80 and 160 mM.

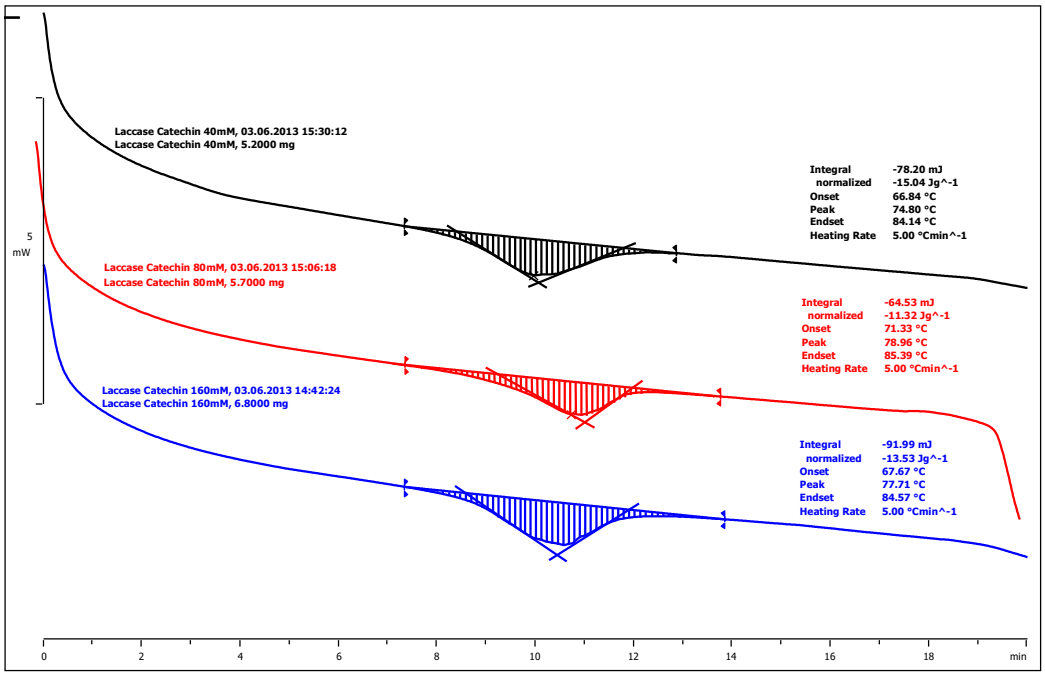

Figure 5-4: Differential scanning calorimetry thermographs for the comparison of laccase-catalysed polymerisation of catechin on Ts of hide powder, for catechin concentrations of 40, 80 and 160 mM. The reaction conditions were 25°C, pH 5.5±0.2, laccase concentration 40 mg/l and 24 hours.

In order to verify if a higher concentration of laccase would be necessary for polymerising higher concentrations of catechin, experiments were carried out with increased laccase concentrations of 80 and 120 mg/l to be compared with the concentration of 40 mg/l applied in the previous experiment, for the highest concentration of catechin (160 mM). Figure 5-5 presents the results.

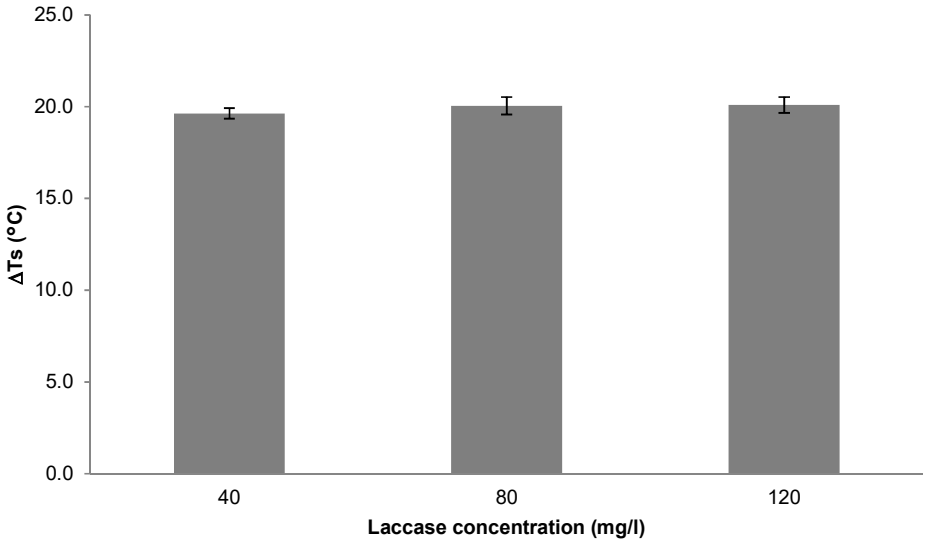

Figure 5-5: Effect of varying concentrations of laccase on the increase in shrinkage temperature (ΔTs) of hide powder reacted with catechin (160 mM), at 25°C, pH 5.5±0.2 and 24 hours reaction time. Error bars denote the standard error of the mean (n=3).

Statistical analysis of variance indicated there was not a significant difference in applying increasing concentrations of laccase: p=0.359. Statistical analysis confirms what can be observed in Figure 5-5, that a concentration higher than 40 mg/l of laccase does not increase the shrinkage temperature significantly. Considering that increasing the concentration of enzyme did not result in further increase in shrinkage temperature, even for a catechin concentration of 160 mM, the higher level of laccase concentration chosen to be studied in a factorial design of experiments was 20 mg/l, in order to minimise the use of the enzyme.

The samples of hide powder treated with the enzyme showed a darker colour when compared to the samples omitting the enzyme. This occurs due to the initial free radicals formed in the laccase-catalysed oxidation of phenols being usually unstable. These free radicals may undergo another oxidation, producing quinones that also participate in the polymerisation (Thurston, 1994). Quinones are coloured components: *p*-quinones are often yellow, and *o*-quinones are usually orange or red (Haslam, 1989). Therefore, the product of oxidative polymerisation of phenols present colour derived from the presence of unreacted quinone groups in the final polymeric structures (Haslam, 1998). For this reason, colour measurements were undertaken in further experiments.

5.5.2 Study of process parameters and optimisation of laccase-catalysed polymerisation of catechin on hide powder

The results acquired with preliminary experiments showed the potential of laccase-catalysed polymerisation of catechin in increasing the shrinkage temperature of hide powder, *i.e.*, promoting the stabilisation of the collagen matrix. In order to obtain information on how process parameters influence the increase in shrinkage temperature, a factorial design 2^k was used. The experimental error was estimated by running three independent repetitions on the central point of the experimental design. Temperature, substrate concentration, enzyme concentration and incubation time are amongst the factors known to influence enzyme activity (Saini, 2010). Therefore, these variables were chosen to be studied.

Due to the colour conferred to hide powder by the enzymatic process, colour measurements were undertaken and the change in colour (ΔE) was calculated for each sample, in comparison with an untreated sample of hide powder, according to the procedure described in Section 3.2.13. Measured data for 'L', 'a' and 'b' chromatic components are presented in Appendix 1. Experimental matrix and response variables are displayed in Table 5-2.

Table 5-2: Experimental matrix for the factorial design 2^4 and response variables expressed as change in shrinkage temperature (ΔTs) and change in colour (ΔE).

Run	Coded				Temperature (°C)	Catechin concentration (mM)	Laccase concentration (mg/l)	Incubation time (h)	ΔTs (°C)	ΔE
	X_1	X_2	X_3	X_4						
1	-1	-1	-1	-1	25	40	5	8	16.18	30.86
2	1	-1	-1	-1	35	40	5	8	18.50	23.75
3	-1	1	-1	-1	25	80	5	8	13.68	20.29
4	1	1	-1	-1	35	80	5	8	17.95	22.28
5	-1	-1	1	-1	25	40	20	8	18.90	23.58
6	1	-1	1	-1	35	40	20	8	21.11	33.43
7	-1	1	1	-1	25	80	20	8	17.12	21.99
8	1	1	1	-1	35	80	20	8	19.82	28.20
9	-1	-1	-1	1	25	40	5	24	18.56	26.39
10	1	-1	-1	1	35	40	5	24	21.39	38.38
11	-1	1	-1	1	25	80	5	24	17.72	37.19
12	1	1	-1	1	35	80	5	24	20.07	27.49
13	-1	-1	1	1	25	40	20	24	16.98	35.13
14	1	-1	1	1	35	40	20	24	19.65	46.68
15	-1	1	1	1	25	80	20	24	18.85	46.64
16	1	1	1	1	35	80	20	24	21.76	45.66
17	0	0	0	0	30	60	12.5	16	18.97	36.85
18	0	0	0	0	30	60	12.5	16	18.41	36.37
19	0	0	0	0	30	60	12.5	16	19.71	38.55

Analysis of variance was carried out to estimate the main effects and interaction effects of the process parameters on the response variables. Table 5-3 shows the significance of each process parameter.

Table 5-3: Statistical analysis for the effect of process parameters studied in the 2^k factorial on the ΔTs of hide powder. Difference is statistically significant when $p \leq 0.05$.

Parameter	*p-value*	*Significant?*
Temperature	0.015	Yes
Catechin concentration	0.125	No
Laccase concentration	0.097	No
Incubation time	0.039	Yes

For the change in shrinkage temperature (ΔTs), the effects of process temperature ($p = 0.015$), and incubation time ($p = 0.039$) were found to be significant. Statistical analysis also revealed a significant interaction between laccase concentration and incubation time ($p = 0.043$). The effect of the interaction of laccase concentration and incubation time may be observed as higher values of ΔTs are obtained by increasing incubation time, even for the lower level of enzyme. Therefore, in order to minimise the use of enzyme, laccase concentration was kept constant at 5 mg/l with optimisation achieved by varying process temperature and incubation time, whilst maintaining a constant catechin concentration at 40 mM.

Considering the response variable change in colour, all parameters studied showed a significant impact in the ΔE, possibly because all parameters impact on the amount of quinone groups formed, as the colour is given by the quinones generated after oxidation. A disadvantage of the enzymatic process is its limitation to be applied in light coloured leather production. Alternatively, this effect can be explored in the production of dark coloured leather, aiming at minimising the amount of dye needed.

Vegetable tanned leather usually present poor light fastness, undergoing darkening when exposed to light (Ozgunay, 2008). Pizzi *et al.* (2004) studied the variation in colour of leather due to prolonged exposure to UV light, measured using electron spin resonance (ESR) and colorimetry. According to the authors, the darkening reaction is due to the photo oxidation and formation of quinones on the phenolic structure of the vegetable tannin.

As the enzymatic process induces the formation of quinones and the darkening of hide powder was observed and measured, experiments on light stability were carried out according to the procedure described in Section 3.2.14. Change in colour was calculated between the measurements of chromatic components 'L', 'a' and 'b' before and after the exposure to light, for each sample. ASTM D3134 (ASTM International, 2003) describes standard practice for establishing colour tolerance. The colour tolerance is usually determined by the customer. ASTM D3134 (ASTM International, 2003) presents an example of colour tolerance, ranging from ΔE 1.0 as a tight tolerance to 3.0 as a more liberal. The change in colour obtained between the samples prior to exposure to light and after exposure to light were within the range of 1.80 and 2.97 (Data shown in Appendix 1). This indicates that, since the oxidation of the phenolic moieties of catechin has already occurred due to the action of laccase, the effect of exposure to light on oxidation and colour change was very low. Therefore, dark coloured metal-free leather can be obtained with the enzymatic process without presenting the further change in colour resulting from the application of vegetable tannins.

5.5.3 Optimisation of ΔTs

Considering the effect of variables temperature and incubation time on the change in shrinkage temperature (ΔTs), further experiments aiming at maximising ΔTs were carried out. A central composite design was then undertaken with the variables: temperature and incubation time. Experimental matrix and results obtained for the change in shrinkage temperature of hide powder are shown in Table 5-4.

Table 5-4: Experimental matrix for the central composite design and response variable expressed as change in shrinkage temperature (ΔTs).

Run	*Coded*		*Temperature (°C)*	*Incubation time (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

The data obtained with the central composite design (Table 5-3) was fitted to a second-order model. Parameter estimation was carried out using the software SPSS version 20 (International Business Machines Corp., USA), and the resulting mathematical model is expressed in Equation (5-3). Statistical significance of the equation was confirmed using F–test. The regression model was highly significant ($p<0.001$) with a coefficient of determination: $R^2 = 0.93$.

$$\Delta Ts = -7.117 + 1.376X_1 + 0.298X_4 - 0.021X_1^2 - 0.008X_4^2 + 0.003X_1X_4 \quad \textbf{(5-3)}$$

Figure 5-6 shows the response surface graph obtained from Equation (5-3).

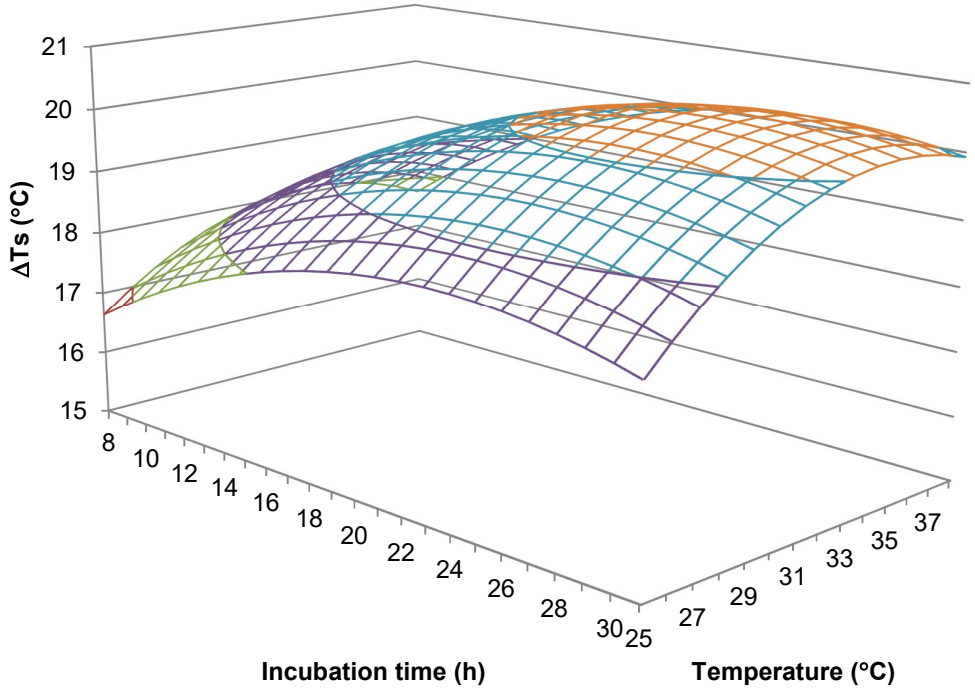

Figure 5-6: Response surface graph for the change in shrinkage temperature (ΔTs) of bovine hide powder as a function of incubation time and process temperature, calculated according to Equation (5-3).

From the curvature shown in Figure 5-6 it is possible to observe that the mathematical model presents a maximum point, after which further increase in process temperature and/or incubation time results in lower values for ΔTs.

The maximum point is obtained when the first derivative equals to zero for each variable, as shown in Equation (5-4).

$$\frac{\partial \Delta Ts}{\partial x_1} = 0 \;\; ; \;\; \frac{\partial \Delta Ts}{\partial x_4} = 0 \qquad \textbf{(5-4)}$$

The optimum point at which the increase in shrinkage temperature is maximum corresponds to temperature of 34.6°C and incubation time of 25 hours. Higher temperatures will most likely

contribute to the denaturation of the enzyme, and a longer incubation time will not increase the ΔTs further, suggesting that the substrate is no longer available for the enzymatic reaction.

5.5.4 Application of laccase-catalysed polymerisation of catechin on goat skin

The application of the enzymatic polymerisation of catechin on goat skin followed two steps. First, it was necessary to ensure the catechin absorption. Subsequently, the enzyme was applied to initiate the polymerisation *in situ*. Tanning efficiency was monitored by measuring the change in shrinkage temperature (ΔTs).

Figure 5-7 shows the percentage of catechin absorbed by the skin, calculated by difference between added catechin and total solids in the float after completion of the experiment.

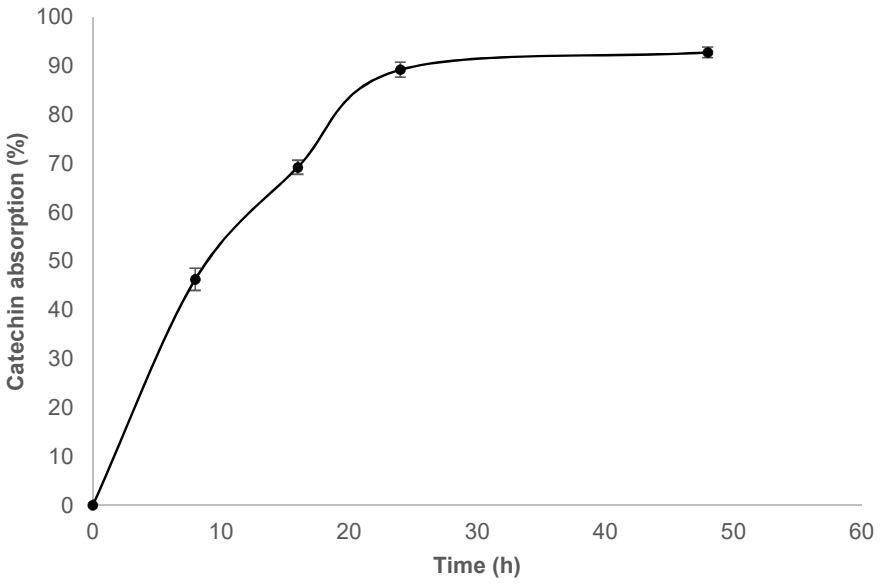

Figure 5-7: Percentage of absorption of catechin by goat skin (w/w), calculated by difference between added catechin and total solids in solution after 8, 16, 24 and 48 hours, at 25°C, pH 4.5 and catechin concentration 40 mM. Error bars denote the standard error of the mean (n=3).

Figure 5-7 shows that a considerable absorption (46% w/w) occurs within 8 hours of processing. After 16 hours of processing, 69% of the catechin offer was absorbed, with a further increase to 89% for 24 hours of agitation. After this period, the increase in absorption is negligible. Therefore, 24 hours is the ideal time to ensure catechin penetration occurs into the skin.

The enzymatic process was then applied to the goat skin after 24 hours for absorption of catechin. Table 5-5 shows the change in shrinkage temperature (ΔTs) for the control (enzyme absent) and for the enzymatic process, in relation to pickled goat skin. The characterisation of the pickled goat skin is shown in Table 3-3.

Table 5-5: Change in shrinkage temperature (ΔTs) for the control and laccase-catalysed process, using goat skin, after 24 hours for catechin penetration (40 mM), process carried out at 34.6°C, 25 hours, pH 5.5. Results are expressed as mean ± standard error of the mean (n=3).

Sample	***ΔTs (°C)***
Control	9.74 ± 1.51
Laccase-catalysed	15.73 ± 1.17

The ΔTs data obtained for the application of laccase-catalysed polymerisation of catechin in goat skin shows similar values for the control in comparison with the application on hide powder (Figure 5-3). The test sample ΔTs, however, resulted lower than the values achieved with hide powder, indicating that the enzyme possibly requires a higher incubation time to fully penetrate the skin thickness. Nevertheless, the higher shrinkage temperature obtained with the test sample in comparison with the control demonstrates the potential of applying the enzyme laccase in increasing the tanning capacity of catechin, a low molecular weight flavan-3-ol compound in increasing the thermal stability of goat skin.

5.6 Summary

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).

A factorial design applied to determine the influence of temperature, substrate concentration, enzyme concentration and incubation time on the change in shrinkage temperature and change in colour showed:

- The increase in shrinkage temperature is influenced by the process temperature and incubation time in comparison with catechin and laccase concentrations;
- All variables influence the change in colour, as the colour is conferred by the formation of quinones during the laccase-catalysed oxidation of catechin.

Light fastness test after the enzymatic process showed a small change in colour, as the oxidation of catechin and formation of quinones had already occurred due to the enzymatic action. This result represents a potential for the process to be used in dark coloured leather.

A central composite design was applied to optimise the change in shrinkage temperature, with variation of process temperature and incubation time. The optimum reaction conditions to maximise ΔTs of hide powder were found to be 34.6°C and 25 hours with the application of response surface methodology.

The application on goat skin showed that absorption of catechin requires at least 24 hours to be completed. The increase in shrinkage temperature for the enzymatic process showed the potential of the laccase-catalysed polymerisation of catechin to be applied in the stabilisation of goat skin.

The results shown in this chapter were used to further explore the laccase-catalysed polymerisation of depolymerised tannins applied to collagen stabilisation.

Chapter 6 Preliminary experiments on the depolymerisation of mimosa tannin

6.1 Introduction

The study presented in Chapter 5 demonstrated the potential of laccase-catalysed polymerisation of catechin, a monomeric flavan-3-ol, in the stabilisation of collagen, evidenced by the increase in shrinkage temperature obtained using the enzymatic process. An issue that may affect the industrial application of this process is the source of small or monomeric phenolic substances. Catechin is found mainly in green tea, which by being a food product may cause the alternative process not to be financially competitive compared to the conventional process. The utilisation of polyphenols from sources such as green tea, apple and grapes means a direct competition with the food chain. The sources of renewable raw materials have to prove their effectiveness in terms of ecological, economic, and social performance against their fossil-based competitors and against other renewable raw materials sources (Ulber *et al.*, 2011). Therefore, sources of tannins outside the food chain may be more advantageous.

Depolymerisation of condensed tannins is presented as an alternative route in obtaining small phenolic molecules. Mimosa extracts, obtained from the bark of the Black Wattle tree (*Acacia mearnsii*), are widely used for tanning and retanning of leather (Slabbert, 1992). Mimosa extract is industrially available and not used as a source of dietary polyphenols. Mimosa is extracted from the Black Wattle tree, which has a cultivation cycle of 7 years (Santana *et al.*, 2015). It is an advantage when comparing with other sources of tannins, such as quebracho from *Schinopsis* spp., for example. The quebracho tree does not develop significant amounts of tannins until they reach 40-50 years, being fully mature in 100 years (Roecklein and Leung, 1987). Therefore, depolymerisation of mimosa extracts has the potential to be applied in producing low molecular weight phenolics for the chemical industry.

Studies on depolymerisation of phenolic polymers are mostly focused on the breakdown of lignin. Lignin is a highly-branched, three dimensional biopolymer, based on the repetition of three different phenylpropane units, linked together by C–C or ether bonds (Yang *et al.*, 2015).

Many functional groups (carbonyl, phenolic or aliphatic hydroxyls, carboxyl, etc.) can be found in different proportions of lignin, and the molecular weight can extend from thousand to several tens of thousands (El Mansouri and Salvadó, 2007). Lignin and condensed tannins are both constituted of phenolic polymers.

Depolymerisation of lignin may be applied for obtaining fossil-fuel like aromatic compounds (Xu *et al.*, 2014). Studies on the depolymerisation of lignin have been done in order to convert lignin into small molecules for fuels using pyrolysis or gasification methods (Wang *et al.*, 2013). Laccase-mediator system is another process applied for degradation of lignin (Call and Mücke, 1997). The process consists in degrading the phenolic compounds using the enzyme laccase in conjunction with another compound called the mediator. The mediator acts as the substrate, being transformed into free radicals by laccase. The free radical form of the mediator then reacts non-enzymatically with the phenolic compound, causing its degradation (Morozova *et al.*, 2007). Figure 6-1 shows a simplified mechanism of the laccase-mediator system. High molecular weight biopolymers such as lignin, cellulose and starch can be oxidised by the mediator, as the steric issues that hinder the direct interaction between enzyme and polymer are overcome by the action of the redox mediator (Kunamneni *et al.*, 2008). Nutsubdize *et al.* (1998), applied the compound 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) as the mediator for laccase-assisted depolymerisation of lignin.

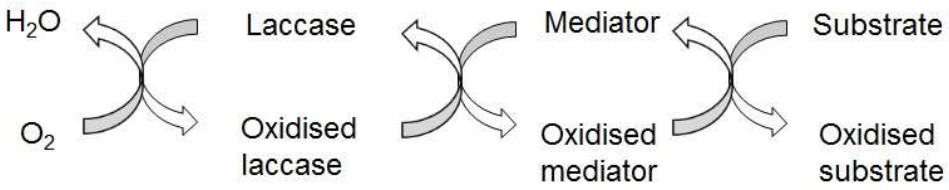

Figure 6-1: Simplified mechanism of laccase-mediator system (adapted from Wells *et al.*, 2006).

Moldes *et al.* (2004) studied the degradation of phenol red dye by laccase. Using laccase alone, the authors observed polymerisation instead of degradation. By adding a mediator (1-hydroxybenzotriazole: HOBt), the dye was completely degraded. When laccase acts alone it catalyses the removal of a hydrogen atom from the hydroxyl group by one electron abstraction to form free radicals capable of undergoing further laccase-catalysed oxidation or non-enzymatic reactions such as polymerisation. When the mediator is incorporated, the free

radical is responsible for the oxidation reactions of the dye, avoiding the polymerisation reactions due to its high redox potential (Moldes *et al.*, 2004). The application of laccase together with low molecular weight redox-mediator compounds can be used in order to generate a desired worn appearance on denim, by bleaching indigo blue dye (Viswanath *et al.*, 2014).

Other methods have been used in the depolymerisation of polyphenols. Studies on the depolymerisation of procyanidins from grape seeds have been carried out by Torres *et al.* (2002). The authors have undertaken an acid treatment of grape polyphenols with hydrochloric acid in methanol, in the presence of L-cysteine. The reaction yielded cysteinyl-flavan-3-ol conjugates, to be applied as antioxidants (Torres *et al.*, 2002). Figure 6-2 illustrates the cleavage of the interflavanyl bond and reaction of the extension units with L-cysteine, yielding terminal units as catechin, and adducts as 4β-(S-cysteinyl)catechin. Fujii *et al.* (2007), based on the work of Torres *et al.* (2002), have obtained proanthocyanidin-L-cysteine complexes in a mild acidic medium using ascorbic acid.

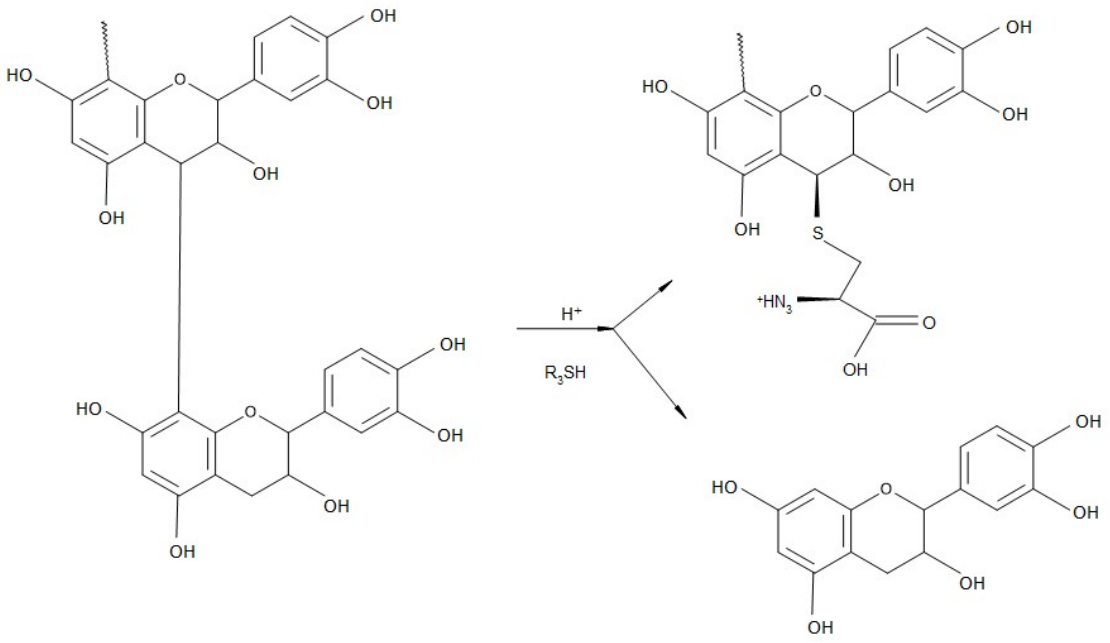

Figure 6-2: Interflavanyl bond cleavage of procyanidin in the presence of acid and L-cysteine, yielding terminal units as catechin and 4β-(S-cysteinyl)catechin (adapted from Torres *et al.*, 2002).

This Chapter presents preliminary experiments aiming at the depolymerisation of mimosa tannin. The laccase-mediator system was tested, using Benzotriazol-1-ol (also called 1-hydroxybenzotriazole or HOBt) as the mediator. The method described by Fujii *et al.* (2007), aiming at the depolymerisation of polyphenols in mild acidic medium in presence of L-cysteine was adapted and tested on mimosa tannin. The laccase-mediator system method revealed to be unsuitable for the application in increasing the shrinkage temperature of hide powder. The depolymerisation in mild acidic medium in presence of L-cysteine has not showed a significant effect in depolymerising mimosa tannin. The results obtained in this Chapter, however, led to adaptations in the methodology that improved the results obtained with acid-catalysed depolymerisation of mimosa, presented in Chapter 7.

6.2 Aims and objectives

The aim of this study is to depolymerise mimosa extracts, and test the suitability of the depolymerised product on the application of laccase-assisted increase in thermal stabilisation of collagen.

The objectives of this study are:

- To apply laccase-mediator system on the depolymerisation of mimosa extract, aiming at decreasing the tannin content;
- To apply L-cysteine in a mild acidic medium on the depolymerisation of mimosa extract, aiming at decreasing the tannin content;
- To apply the depolymerised product on hide powder with subsequent polymerisation with laccase, and measurement of the tanning effect through the change in shrinkage temperature.

6.3 Experimental procedure

6.3.1 Laccase-mediator system

For depolymerisation using laccase-mediator system, 2 g of mimosa tannin was added to a 250 ml Erlenmeyer flask, with 100 ml of a 5 mM solution of HOBt in acetate buffer (pH 5.5), along with the enzyme (final concentration: 5 mg/l). The open flask was agitated for 48 hours in a shaking water bath (Digital water bath OLS200, Grant Instruments, Cambridge, UK) at 30ºC and 100 rpm. After incubation, the product was freeze-dried to powder using an Alpha 2-4 LSC freeze-dryer (Martin Christ, Osterode, Germany), for 72 hours at -40°C and vacuum 0.250 mbar. A final drying stage of 45 minutes was performed at 5°C and vacuum 0.011 mbar. Control samples were prepared as follows: control 1 omitted the enzyme, control 2 omitted the mediator (HOBt) and control 3 omitted both the enzyme and mediator, *i.e.*, a solution of mimosa in acetate buffer (pH 5.5).

6.3.2 Depolymerisation with L-cysteine in mild acidic medium

The procedure for depolymerisation using L-cysteine in mild acidic medium was adapted from Fujii *et al.* (2007). Mimosa tannin (4 g) was added to a 250 ml Erlenmeyer flask, with 40 ml of a 6 mM solution of ascorbic acid in ultrapure water, along with L-cysteine (final concentration: 25 mM). The flask was agitated for 24 hours in a shaking water bath (Digital water bath OLS200, Grant Instruments, Cambridge, UK) at 45ºC and 100 rpm. The solution was then extracted twice with 25 ml of ethyl acetate for each extraction. The samples were centrifuged (Heraeus Megafuge 16R, Thermo Scientific, Hemel Hempstead, UK) at 3075 x *g* for 10 minutes. The supernatants for both extractions were collected and combined for each sample, and the ethyl acetate was evaporated under vacuum at 30°C using a rotary evaporator (Rotavapor R-114 and Waterbath B-481, Büchi, Flawil, Switzerland; Speedivac ES35, Edwards High Vaccum Limited, Crawley, UK). The solid was mixed with 10 ml of deionised water and freeze-dried to powder 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. Control samples were prepared

as follows: control 1 omitted L-cysteine, control 2 omitted the ascorbic acid and control 3 omitted both the ascorbic acid and L-cysteine, *i.e.*, a solution of mimosa in ultrapure water.

6.3.3 Analysis of the products obtained with depolymerised processes

The products obtained from the depolymerisation process using laccase-mediator system were analysed by tannin content and column chromatography. Samples obtained by depolymerisation with L-cysteine in mild acidic medium were analysed with acid butanol assay (condensed tannin content) and vanillin assay (catechin equivalent). The methods are described in Chapter 3.

6.3.4 Application on hide powder

Tanning experiments were performed with 500 mg hide powder and 165 mg of tannin product in dry basis added to 25 ml sodium acetate buffer (pH 5.5). The samples were stirred with a magnetic stirrer (Hot plate stirrer PC-351, Corning, New York, USA) for 30 minutes to rehydrate the hide powder and absorb the tannin product, and then the pH was measured with a pH meter Seven Multi (Mettler Toledo, Schwerzenbach, Switzerland) and adjusted to 5.5 ± 0.2 when necessary with 0.1 M NaOH. Laccase for a final concentration of 5 mg/l was added to the samples to start polymerisation. One control sample for each tanning product was prepared, omitting the enzyme. The reaction mixtures were kept for 25 hours in a shaking water bath (Digital water bath OLS200, Grant Instruments, Cambridge, UK) at 34.6°C and 100 rpm. The enzymatic reaction was terminated with 5 ml of a sodium azide solution (1 mM). At the end of reaction, the samples were centrifuged (Heraeus Megafuge 16R, Thermo Scientific, Hemel Hempstead, UK) at 3075 x *g* for 10 minutes, and washed twice with deionised water. 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 (British Standards Institute, 2012). Tanning degree was measured using differential scanning calorimetry, according to the method described in section 3.2.8. The results were expressed in terms of change in shrinkage temperature (ΔTs), according to Equation (3-1).

6.3.5 Kinetic study of laccase inhibition by mimosa

Inhibition studies were performed similarly to the procedure described in Section 4.3.3, using catechin as the substrate. A non-inhibited study was performed as a control, by reading the absorbance values at 450 nm over time, for catechin concentrations of 0.1, 0.2, 0.3, 0.5, 0.8 and 1 mM, in sodium acetate buffer (pH 5.5). The total reaction volume was 2 ml, cell path 1 cm and the temperature was kept at 25°C. The final concentration of laccase was 25 mg/l. A blank omitting the enzyme was prepared for each catechin concentration, in which the enzyme was substituted by deionised water. In order to evaluate the inhibition effect of mimosa, for each catechin concentration, 500 µl of a mimosa solution in sodium acetate buffer (pH 5.5) to a final concentration of 100 ppm was added to the reaction media prior to the addition of laccase.

6.4 Results and discussions

6.4.1 Characterisation of commercial mimosa tannin

The commercial mimosa tannin used in this study was fully characterised in order to obtain a reference for the analysis of depolymerised products. Tannin content was analysed according to the standard method SLC 116 (Society of Leather Technologists and Chemists, 1996). Separations based on polarity (column chromatography using Sephadex LH-20 and ethyl acetate partition) were performed as a comparison, dividing the fractions obtained as tannins and non-tannins. The procedures of the analytical methods are given in Chapter 3.

Tannin content may as well be analysed with the standard method BS EN ISO 14088 (British Standards Institute, 2012). The BS EN ISO 14088 method relies on the same principle of the SLC 116 method, of determining gravimetrically the non-tannins after absorption of tannins by hide powder. In the BS EN ISO 14088 method, a filter bell is packed with hide powder and the tanning solution is passed through siphoning action. It differs from the SLC 116 method, as in this, the hide powder is shaken and thus it is unaffected by differences in packing the bell. Inefficient packing may result in “channels”, not allowing the minimum contact of the tanning solution with the packed hide powder. Another source of error faced by both methods is the solubilisation of soluble matter from the hide powder into the non-tannin fraction. Both

methods rely on the previous chroming of hide powder in order to decrease the soluble content. In the filter bell method (BS EN ISO 14088), the hide powder must be dry to allow the packing; and to discount the soluble matter that may be contained in the hide powder, the first 30 ml of exhausted solution is discarded. In the shake method, the hide powder is freshly chromed before the analysis and is washed three times with de-ionised water, which allows the removal of any remaining soluble matter. Due to these differences, the SLC 116 method was chosen aiming at reducing possible sources of error.

Table 6-1 shows the results for tannin, non-tannin, moisture and insoluble content according to the column chromatography, ethyl acetate partition and SLC 116 (Society of Leather Technologists and Chemists, 1996) methods.

Table 6-1: Characterisation of commercial mimosa tannin according to different methods of analysis. Results are expressed as the average ± standard deviation of triplicate measurements.

Method	*Tannin (%)*	*Non-tannin (%)*	*Moisture (%)*	*Insolubles (%)*
Column Chromatography (CC)	60.92 ± 2.53	31.05 ± 2.07	7.96 ± 0.10	0.07 ± 0.01
Ethyl acetate partition	59.11 ± 0.74	33.38 ± 0.60	7.44 ± 0.68	0.07 ± 0.01
SLC 116	64.94 ± 0.81	27.44 ± 0.66	7.55 ±0.41	0.07 ± 0.01
Manufacturer specification (typical results)	68.3	25.0	6.5	0.2

The data obtained for tannin content with the analytical methods used in this study were slightly lower than the typical results given by the manufacturer, which uses the SLC 116 (Society of Leather Technologists and Chemists, 1996) method. This can be explained by the slightly higher moisture content found in the mimosa sample used in this research, as it was stored in a controlled atmosphere at 23°C and 50% relative humidity. The tannin content is calculated by difference (100 - %non-tannin - %moisture - %insolubles), therefore a higher moisture content means a lower tannin content. The differences found between methods are expected, as each method relies on different interactions due to polarity (column chromatography and ethyl acetate partition) and reactivity (absorption in hide powder).

Total phenolic content was measured for the fractions obtained after separation with column chromatography on Sephadex LH-20, as well as for the commercial mimosa tannin. The higher molecular compounds (tannins) were eluted in the acetone fraction, whilst the low molecular weight compounds were eluted in the ethanol fraction. Data for total phenolic content of the powders obtained after freeze-drying acetone (tannin) and ethanol (non-tannin) fractions, and for the commercial mimosa tannin are presented in Table 6-2.

Table 6-2: Total phenolic content of commercial mimosa tannin and of the fractions obtained from column chromatography analysis of mimosa tannin in μg gallic acid/g dry sample. Acetone fraction is rich in tannins; the non-tannin components are presented in the ethanol fraction. Results are expressed as the average ± standard deviation of triplicate measurements.

Total phenolic content – dry basis (μg gallic acid/g dry sample)	
Mimosa	66.26 ± 3.3
Acetone fraction (column chromatography)	86.14 ± 4.7
Ethanol fraction (column chromatography)	11.29 ± 2.4

Data shown in Table 6-2 demonstrate that the acetone fraction (rich in tannins) concentrate a higher proportion of the phenolic content of mimosa. The phenolic compounds eluted in the ethanol fraction are the low molecular weight molecules. This demonstrates the higher content of phenolic polymers in the mimosa extract, which contributes to its high astringency towards collagen.

6.4.2 Laccase-mediator system (LMS)

The depolymerisation with laccase-mediator system was evaluated through the tannin content method (SLC116) and column chromatography with Sephadex LH-20.

6.4.2.1 Tannin content (SLC 116)

The analysis using SLC 116 (Society of Leather Technologists and Chemists, 1996) method measures gravimetrically the substances that are not absorbed by hide powder (non-tannins).

The tannin content is then calculated by difference (100 - %non-tannin - %moisture - %insolubles). Therefore, the results are in wet basis, as the higher the moisture, the lower is the tannin content of an extract. Data obtained for tannin content analysis are expressed in Figure 6-3.

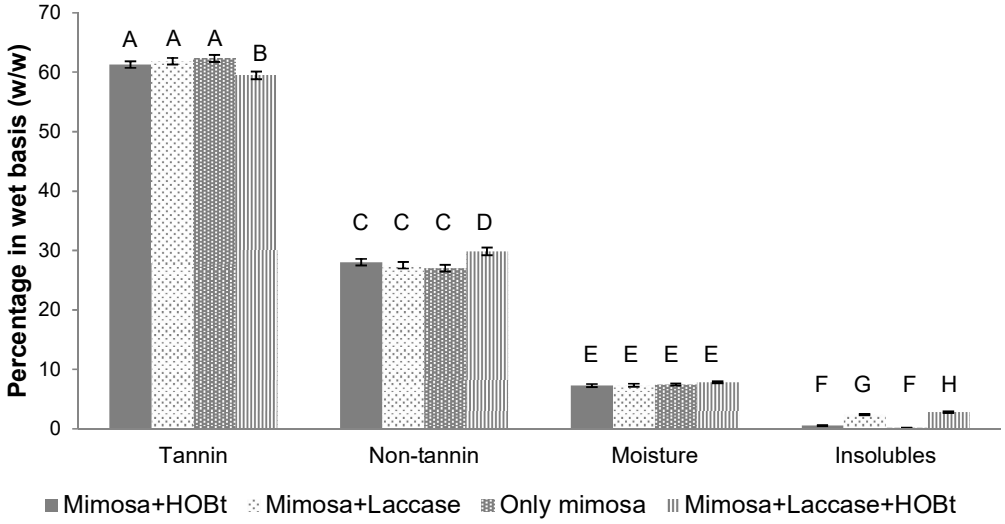

Figure 6-3: Tannin content for samples subjected to laccase-mediator system process. Controls are mimosa+HOBt, Mimosa+laccase and only mimosa; test sample was comprised of laccase+HOBt+mimosa. Error bars denote the standard error of the mean (n=3 in duplicate). Means with different letters (A, B, C, D, E, F, G, H) are significantly different (Tukey's HSD, $p<0.05$).

In order to reduce variability due to moisture content, the samples were kept in a controlled environment, at 23°C and 50% relative humidity. Figure 6-3 shows that the moisture content for all samples are not statistically different. Therefore, the moisture content of samples does not interfere in the difference of tannin content amongst samples.

As shown in Figure 6-3, the control samples (Mimosa + HOBt, mimosa + laccase and only mimosa) have not presented significant difference in terms of tannin and non-tannin content. The test sample, however, shows tannin and non-tannin contents statistically different from the controls, being the tannin content of the test sample lower than the controls, and the non-tannin content higher than the controls. One of the reasons for the lower tannin content is that the insoluble content of the test sample is statistically higher than the controls. The control

mimosa + laccase also presented a higher insoluble content than the controls mimosa + HOBt and only mimosa, albeit lower than the test sample. This indicates that the enzyme was interacting with the mimosa tannin, possibly a precipitation was occurring.

6.4.2.2 Column Chromatography

The products obtained with the laccase-mediator system process were also analysed with column chromatography using Sephadex LH-20 as stationary phase. The ethanol solution elutes the lower molecular weight compounds, whereas the higher molecular substances (tannins) are adsorbed by the Sephadex LH-20 beads. The tannins are then eluted with an acetone solution. The percentage in mass (dry basis) recovered in each fraction (ethanol and acetone) for the test samples and controls are shown in Figure 6-4. Samples were filtered prior to the analyses; therefore, the insoluble content was not considered. Percentage of mass recovered in ethanol and acetone fractions were calculated in relation to the total mass recovered, according to the Equations (6-1) and (6-2).

$$\%\boldsymbol{Ethanol\ fraction} = \frac{\boldsymbol{Dry\ mass\ of\ substances\ eluted\ with\ ethanol\ solution}}{\boldsymbol{Total\ dry\ mass\ recovered\ after\ column\ chromatography}} \times \mathbf{100} \quad \textbf{(6-1)}$$

$$\%\boldsymbol{Acetone\ fraction} = \frac{\boldsymbol{Dry\ mass\ of\ substances\ eluted\ with\ acetone\ solution}}{\boldsymbol{Total\ dry\ mass\ recovered\ after\ column\ chromatography}} \times \mathbf{100} \quad \textbf{(6-2)}$$

Data obtained from separation with column chromatography using Sephadex LH-20 for test sample and controls are presented in Figure 6-4.

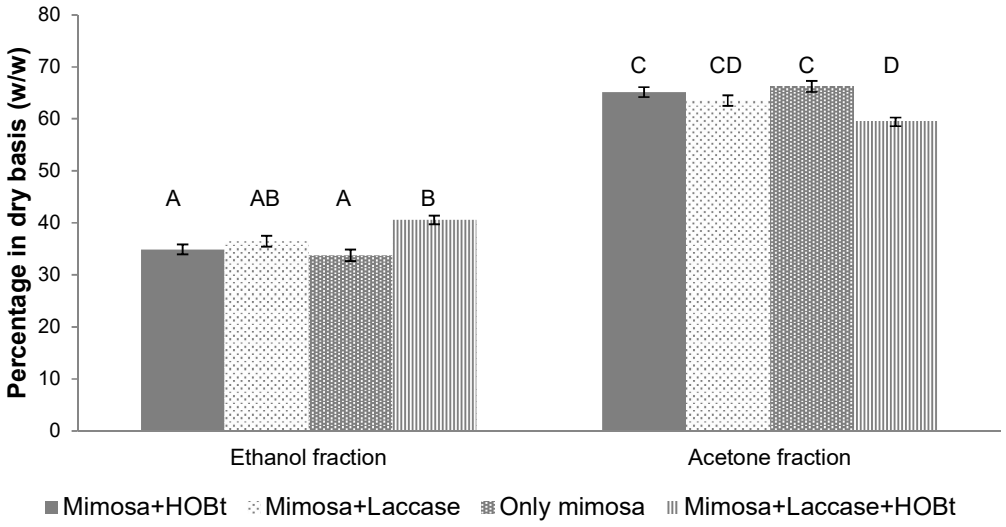

Figure 6-4: Fractionation using Sephadex LH-20. The acetone fraction is rich in tannins, and non-tannins are eluted in the ethanol fraction. Controls are mimosa+HOBt, mimosa+laccase and only mimosa; test sample was comprised of laccase+HOBt+mimosa. Error bars denote the standard error of the mean (n=3 in duplicate). Means with different letters (A, B, C, D) are significantly different (Tukey's HSD, $p<0.05$). When both letters are used, *e.g.* AB, the mean is not significantly different from both A and B.

Considering the percentage of mass eluted for both the ethanol and acetone fractions was calculated in relation to the total mass recovered, instead of the initial mass loaded into the column, the sum of percentages of ethanol fraction and acetone fraction is 100. Consequently, the statistical significance for both fractions follows the same pattern.

Data in Figure 6-4 shows that the control samples (Mimosa + HOBt, mimosa + laccase and only mimosa) were not significantly different in terms of separation with Sephadex LH-20. The control mimosa + laccase was also not significantly different from the test sample. The test sample, however, showed statistically difference from controls mimosa + HOBt and only mimosa, being the ethanol fraction for the test sample higher, and the acetone fraction lower. This indicates a lower tannin content and higher non-tannin content for the test sample, in comparison with the controls.

The total phenolic content was analysed for the controls and test sample, and also for the ethanol and acetone fractions obtained after column chromatography, as the non-tannins eluted by ethanol with Sephadex LH-20 may not necessarily be phenolic compounds. Data for the total phenolic content for controls and test samples are presented in Figure 6-5.

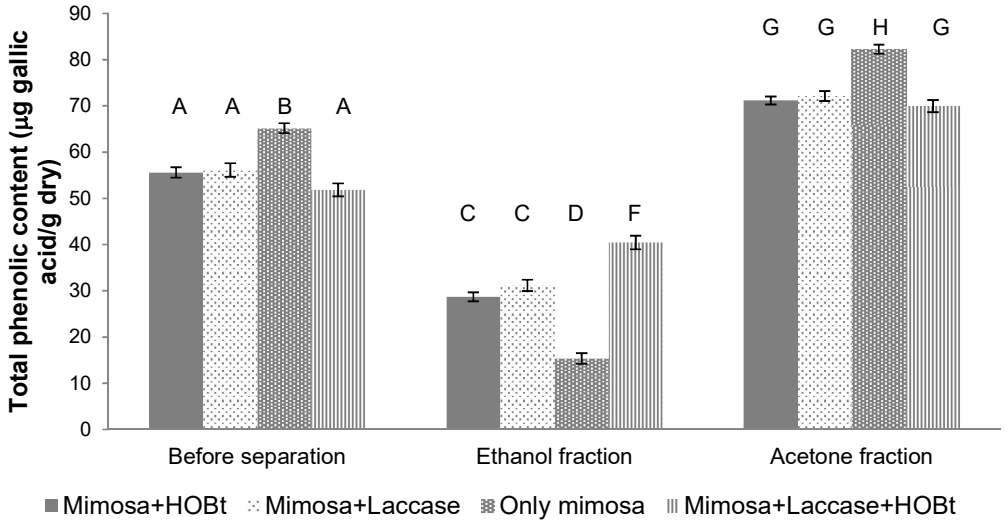

Figure 6-5: Total phenolic content of the original samples and fractions obtained with column chromatography using Sephadex LH-20 in µg gallic acid/g dry sample. Acetone fraction is rich in tannins; the non-tannin components are presented in the ethanol fraction. Error bars denote the standard error of the mean (n=3 in duplicate). Means with different letters (A, B, C, D, E, F, G, H) are significantly different (Tukey's HSD, $p<0.05$).

The total phenolic content presented in Figure 6-5 shows that for the depolymerisation products before column chromatography, the control with only mimosa was significantly higher than the other samples. This may be explained as for the other samples there was addition of other compounds, and the phenolic content is measured based on the dry mass of sample.

The control sample with only mimosa also show a significant lower phenolic content for the ethanol fraction and a significant higher phenolic content for the acetone fraction. These data are similar to the data obtained for the unprocessed mimosa, showed in Table 6-2. Therefore, it is possible to conclude that the process itself does not impart any major modification on the mimosa extract.

The test sample showed a significantly higher phenolic content in the ethanol fraction and lower phenolic content in the acetone fraction. Both data from Figures 6-4 and 6-5 suggest that part of the low molecular compounds eluted were in fact phenolic. This indicates, therefore, that a higher amount of phenolic non-tannins was obtained with the laccase-mediator process.

Due to the higher non-tannin content found for the test sample in comparison with the controls, the products were applied in experiments with hide powder, and the data obtained is presented in Section 6.4.2.3.

6.4.2.3 Application on hide powder

The product obtained after laccase-mediator system was applied in the stabilisation of collagen, according to the procedure described in Section 6.3.4. Figure 6-6 illustrates the procedure and samples generated by the application of control and depolymerised mimosa products obtained with the laccase-mediator system. Data on the change in shrinkage temperature of hide powder are presented in Table 6-3.

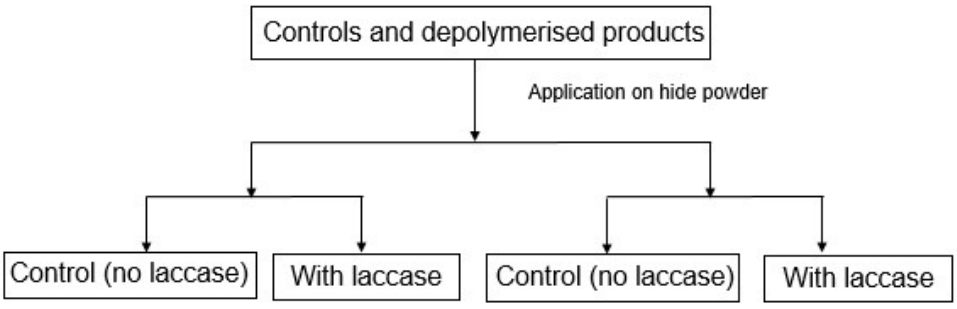

Figure 6-6: Scheme for the application of control and test products obtained with laccase-mediator system on hide powder, with and without addition of laccase.

Table 6-3: Change in shrinkage temperature (ΔTs) for hide powder treated with products obtained with the laccase-mediator system. Each product was applied with and without addition of laccase to the medium containing hide powder. Results are expressed in mean ± standard error of the mean (n=3 in duplicate).

Controls and test sample subjected to the laccase-mediator system process	***Conditions for application on hide powder***	***Change in shrinkage temperature ΔTs (℃)***
Mimosa + HOBt	No laccase	19.11 ± 0.96
Mimosa + HOBt	With laccase	9.81 ± 1.17
Mimosa + laccase	No laccase	17.28 ± 0.71
Mimosa + laccase	With laccase	10.65 ± 0.71
Only mimosa	No laccase	19.78 ± 0.89
Only mimosa	With laccase	13.19 ± 0.40
Mimosa + laccase + HOBt (Test sample)	No laccase	17.06 ± 0.63
Mimosa + laccase + HOBt (Test sample)	With laccase	10.61 ± 0.89

The analysis of variance indicated a significant difference between the conditions of only product and with laccase, for all products: $F(1,24) = 301.8$, $p<0.001$. The values of shrinkage temperature, however, were the opposite of what was expected, as the application of laccase resulted in a lower change in shrinkage temperature in comparison with the samples omitting the enzyme. *Post Hoc* analysis (Tukey's HSD) for each sample indicated a significant difference between the control only mimosa and all other samples: mimosa + HOBt ($p = 0.016$), mimosa + enzyme ($p = 0.003$) and test ($p = 0.002$). No significant differences were found between the other samples.

A possible explanation for the lower shrinkage temperature of the samples using laccase is that the enzyme, being a protein, is binding with the mimosa tannin. This means that possibly the mimosa tannin is actually inhibiting the enzyme. For this reason, inhibition studies were carried out, similarly to the study presented in Chapter 4, which tested the inhibitory effect of sodium azide on the laccase-catalysed oxidation of the substrate catechin.

6.4.2.4 Kinetic study of laccase inhibition by mimosa

The presence of tannins in beverages such as wine, beer and tea confers a desired characteristic of astringency, perceived as a dryness feeling to the palate. The astringency is

a result of the precipitation of proteins present in saliva by crosslinking of these proteins with the tannin molecules (Haslam, 2007). Moreover, the polyphenolic compounds of tannins are capable of binding to digestive enzymes and dietary proteins, reducing the digestibility of plants consumed by herbivores (Robbins *et al.*, 1987).

According to Haslam (1989), the inhibition of enzymes by phenolic compounds may occur by precipitation of the enzyme protein, by the formation of soluble but inactive enzyme-inhibitor complexes and/or by complexation with the substrate(s) to confer reduced reactivity in the enzyme reaction. In the case of enzymes that have as substrate another protein, such as proteases, the inhibition may be due to the polyphenol complexation with either the enzyme itself or with the substrate, in which case the polyphenol may protect the substrate against the enzymatic reaction (Mole and Waterman, 1986).

The data for shrinkage temperature showed in Table 6-3 suggests that mimosa tannin was possibly precipitating the enzyme laccase. In order to determine if the tannin was binding to the laccase and consequently inhibiting the enzyme action, inhibition studies using catechin as a substrate were undertaken. The Michaelis-Menten and Lineweaver-Burk graphs for evaluation of the inhibitory effect of mimosa on laccase are presented in Figures 6-7 and 6-8 respectively.

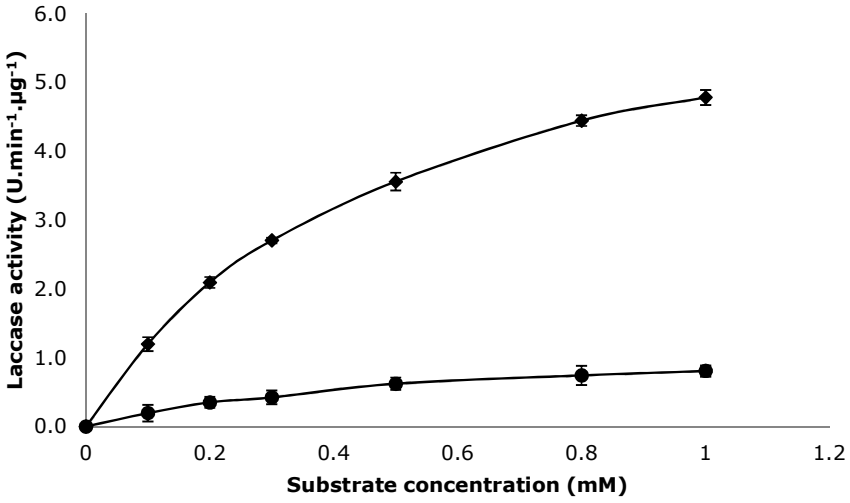

Figure 6-7: Michaelis-Menten graph for the variation in laccase activity with substrate (catechin) concentration without inhibition (♦), with 100 ppm mimosa (●), at 25°C, pH 5.5 and laccase concentration of 25 mg/l. Error bars denote the standard deviation (n=3).

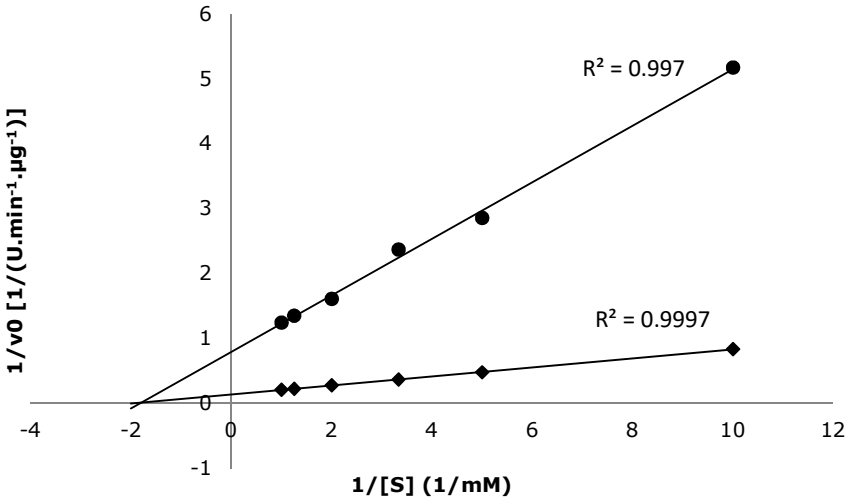

Figure 6-8: Lineweaver-Burk plot for catechin without inhibition (♦), with 100 ppm mimosa (●), based on the results showed in Figure 6-7.

Data shown in Figures 6-7 and 6-8 indicate that the laccase activity for the reaction medium containing mimosa is lower than the activity data for the medium of catechin solution without addition of mimosa. The Michelis-Menten parameter maximum velocity (V_{max}) is lower for the inhibited medium in comparison with the medium omitting the mimosa tannin, whilst the enzyme-substrate affinity (K_m) is similar for both media. The data presented in Figures 6-7 and 6-8 indicate a type of non-competitive inhibition, in which the binding of the inhibitor does not prevent the binding of the substrate, but inactivates the enzyme so no products are formed (Berg *et al.*, 2002). In this type of inhibition, the inhibitor may bind with either the enzyme or the enzyme-substrate complex. For the inhibition by mimosa, the tannin will most possibly bind with the amino acid side chains of the enzyme, whilst the substrate catechin will be attracted to the active sites.

6.4.2.5 Separation with ethyl acetate

Data presented in Section 6.4.2.4 demonstrated the inhibitory effect of mimosa with the enzyme laccase, as the tannin molecules were binding and precipitating the enzyme, resulting in a lower catalytic effect. For this reason, tannins must be separated from low molecular

weight phenolic molecules before the application on hide powder. Therefore, a solvent extraction using ethyl acetate was performed, and two fractions were obtained: an aqueous fraction rich in tannins and an ethyl acetate fraction rich in non-tannins. The separation was performed for the control 'only mimosa' and the test sample. Figure 6-9 illustrates the procedure and samples obtained for the extraction with ethyl acetate, in order to facilitate the understanding of Table 6-4.

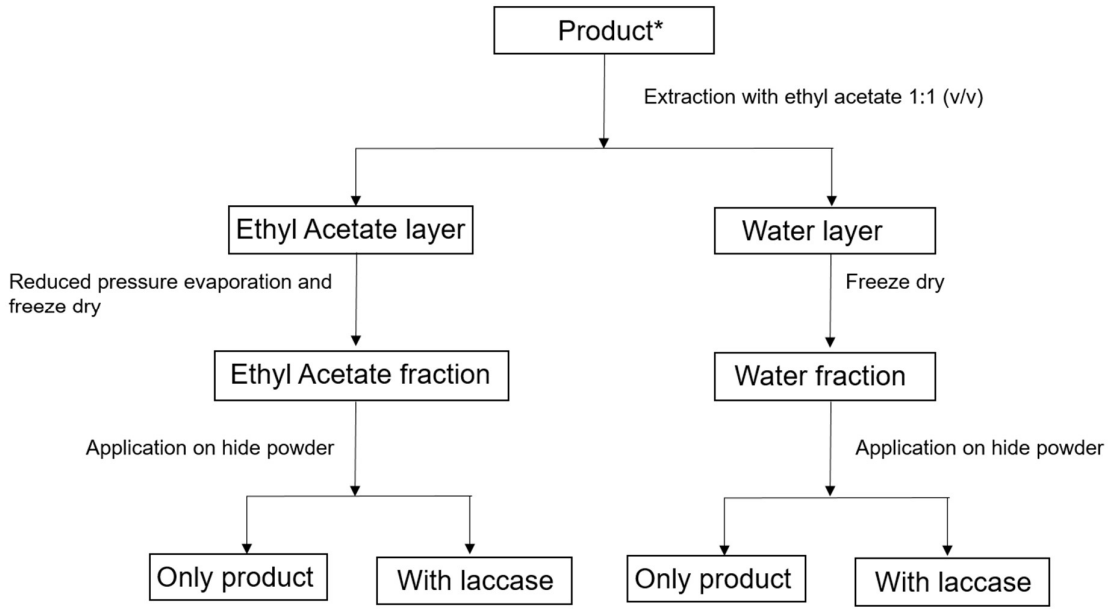

Figure 6-9: Scheme for the extraction with ethyl acetate. (*) Products were the control sample 'Only mimosa' and 'Test sample', each subjected to the procedure illustrated here.

Table 6-4 shows the change in shrinkage temperature (ΔTs) of hide powder treated with both fractions obtained after extraction with ethyl acetate. Each sample was applied to hide powder with and without addition of laccase.

Table 6-4: Change in shrinkage temperature (ΔTs) for hide powder treated with only mimosa and test sample (laccase-mediator system) fractions after solvent extraction (water/ethyl acetate). Each product was applied with and without addition of laccase to the medium containing hide powder. Results are expressed in mean ± standard error of the mean (n=3 in duplicate).

Fractions of product obtained from the laccase-mediator system treatment of mimosa after separation with ethyl acetate	***Conditions for application on hide powder***	***Increase in shrinkage temperature ΔTs (℃)***
Only mimosa – ethyl acetate fraction	No laccase	17.17 ± 0.53
Only mimosa – ethyl acetate fraction	With laccase	19.58 ± 0.71
Only mimosa – water fraction	No laccase	19.56 ± 0.89
Only mimosa – water fraction	With laccase	12.81 ± 0.86
Mimosa+laccase+HOBt – ethyl acetate fraction	No laccase	18.23 ± 0.65
Mimosa+laccase+HOBt – ethyl acetate fraction	With laccase	11.52 ± 0.72
Mimosa+laccase+HOBt – water fraction	No laccase	16.11 ± 0.55
Mimosa+laccase+HOBt – water fraction	With laccase	9.83 ± 0.92

For the sample containing only mimosa, data on the increase in shrinkage temperature (ΔTs) shows that the water fraction after solvent extraction presented a lower shrinkage temperature for the laccase-assisted stabilisation of hide powder samples in comparison with the sample omitting the enzyme. Differently, the ethyl acetate fraction sample with the application of laccase showed a slightly higher ΔTs in comparison with the sample omitting the enzyme. Therefore, the extraction with ethyl acetate demonstrated to be effective in preventing the enzyme inhibition.

For the test sample, however, even after separation of tannins and non-tannins, the application of laccase resulted in lower shrinkage temperature for the hide powder samples subjected to the enzyme incubation, for both water and ethyl acetate fraction, in comparison with the samples omitting the enzyme. One possible reason for the lower shrinkage temperature is that the mediator may be present in both phases, hindering the enzymatic reaction. Therefore, in order to determine if the mediator was present in the samples, UHPLC analyses were carried out, and the chromatograms are showed in Figure 6-10.

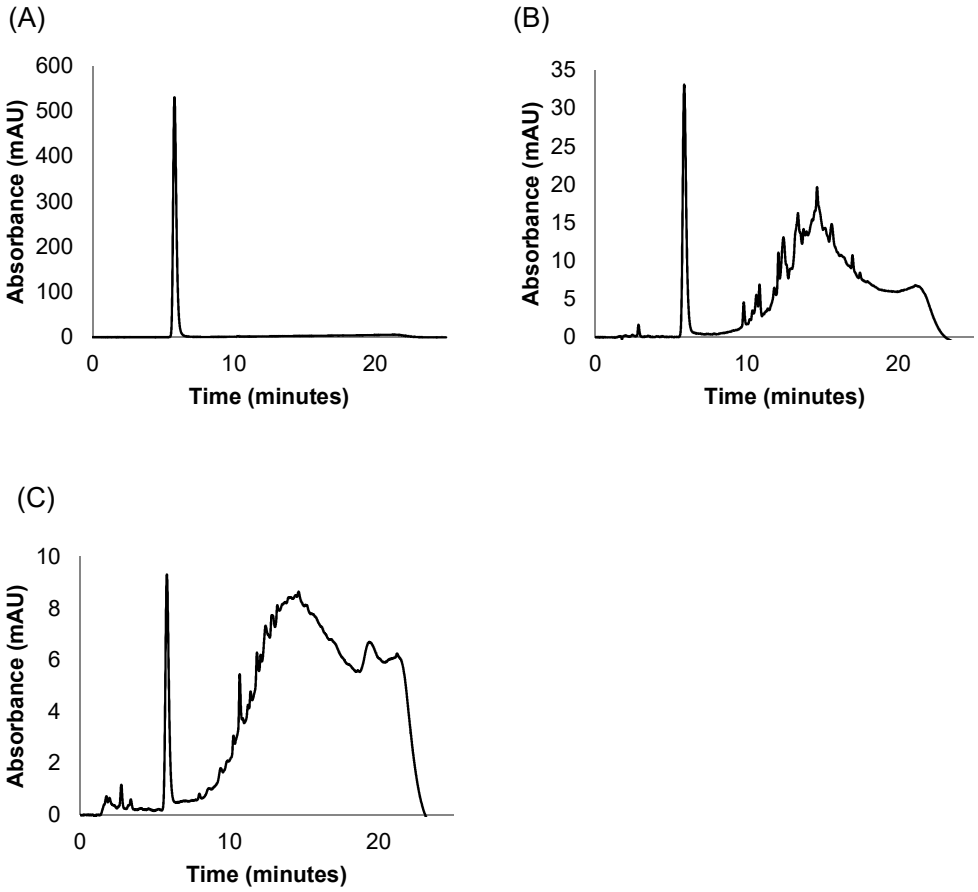

Figure 6-10: UHPLC chromatograms of: HOBt (A); mimosa subjected to laccase-mediator system with HOBt extracted with ethyl acetate (B); mimosa subjected to laccase-mediator system with HOBt remaining substances in aqueous phase (C).

As shown in Figure 6-10, after separation with ethyl acetate, the mediator is present in both phases. Therefore, the presence of the mediator is possibly hindering the enzymatic reactions. For this reason, it can be concluded that the laccase-mediator system is not feasible for the application of enzyme-assisted phenolic reactions on the stabilisation of collagen.

6.4.2.6 Summary and discussion about the suitability of laccase-mediator system as a depolymerisation process

The tannin content and separation with column chromatography analyses showed an increase in non-tannins and decrease in the tannin content for the test sample subjected to the laccase-mediator system in comparison with controls, supported by the total phenolic content showing a higher degree of small phenolics eluted with the ethanol fraction by the test sample. Studies on hide powder stabilisation using the products obtained with the laccase-mediator process, as well as a control with unprocessed mimosa, showed that the application of laccase with mimosa resulted in a lower shrinkage temperature, in comparison with the sample omitting the enzyme. These results indicated that possibly the enzyme was binding with the mimosa tannin, as the main characteristic of tannins is binding with proteins.

The binding of mimosa and laccase was confirmed by inhibition studies, which showed a lower activity of the enzyme laccase with catechin when mimosa was added to the reaction medium. In order to only obtain lower molecular weight phenolics, an extraction with ethyl acetate was then performed for the test sample and the control with only mimosa. Results on shrinkage temperature showed that for the only mimosa sample, the fraction extracted with ethyl acetate resulted in a higher shrinkage temperature for the hide powder sample in which the enzyme was applied, in comparison with the sample omitting the enzyme. An opposite result was obtained from the aqueous fraction, confirming that the enzyme was possibly binding with the tannins present in the fraction, lowering the shrinkage temperature. For the test sample, a lower shrinkage temperature was obtained with the application of laccase, for both hide powder samples with and without the application of laccase. The mediator was found to be present in both phases, which was possibly hindering the stabilisation reactions with collagen. In conclusion, for the reasons stated, this is not a suitable method for the application intended, which is the stabilisation of collagen.

6.4.3 Depolymerisation with L-cysteine in mild acidic medium

Depolymerisation of mimosa extract using the laccase-mediator system demonstrated that the process is not adequate for the intended purpose, which is obtaining low molecular phenolic molecules for the application of *in situ* laccase-catalysed polymerisation in the

stabilisation of collagen. Therefore, experiments using acid cleavage with addition of L-cysteine were carried out.

Data presented previously (Figures 6-7 and 6-8) show that due to the inhibition effect as a result of tannins in mimosa extract binding to the enzyme, the depolymerised small phenolics must be separated from the large molecules, in order to avoid precipitation of the enzyme laccase when applying the *in situ* polymerisation to the stabilisation of collagen. An extraction with ethyl acetate was then performed after the depolymerisation process, to obtain a product rich in low molecular weight phenols. The analytical methods used to evaluate the depolymerisation using laccase-mediator system are not entirely adequate in evaluating the ethyl acetate extracted product. Analysis of tannin content with SLC 116 is a gravimetric measurement of non-tannins after tannin absorption by hide powder, whilst the separation using Sephadex LH-20 chromatography relies on a similar interaction to the separation with ethyl acetate. Therefore, other analytical methods were sought in order to provide a more effective measurement of the depolymerisation process. The methods chosen to monitor the depolymerisation process were the analysis of condensed tannin content (% leucocyanidin equivalent) and catechin equivalent (mg catechin/g dry sample). Condensed tannin content is analysed using the acid butanol assay and catechin equivalent is analysed using the vanillin assay, and the analytical procedures are described in Sections 3.2.11 and 3.2.12 respectively.

6.4.3.1 Condensed tannin content and catechin equivalent

The colour developed in the acid-butanol assay, used for measuring the condensed tannin content, is produced by the release of anthocyanidins and is due to the acid-catalysed cleavage of the interflavanyl bond (Porter *et al.*, 1986). The vanillin assay, used to obtain the catechin equivalent, is based on the formation of an adduct of vanillin and the monomers released from the terminal units of the polymeric molecule. As shown in Figure 6-11, the results from both assays provide information about the average size of the polyphenol molecules.

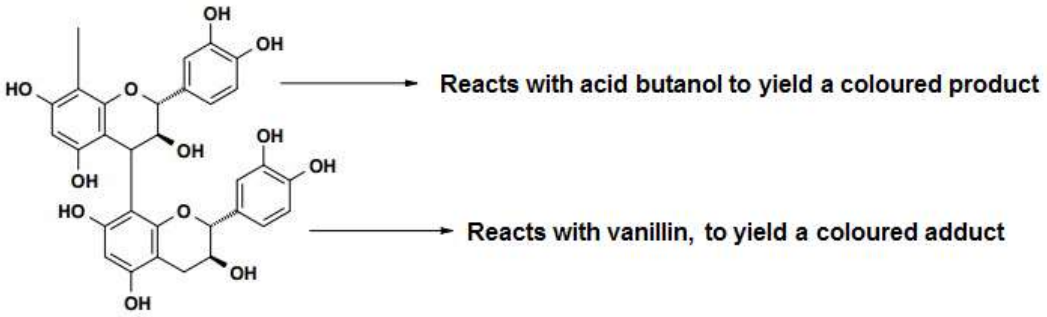

Figure 6-11: Sections of a polyphenol molecule that reacts either with acid butanol or vanillin (Hagerman, 2002).

Figures 6-12 and 6-13 present data obtained with the analysis of condensed tannin content and catechin equivalent, respectively, for samples subjected to the acid cleavage with addition of L-cysteine.

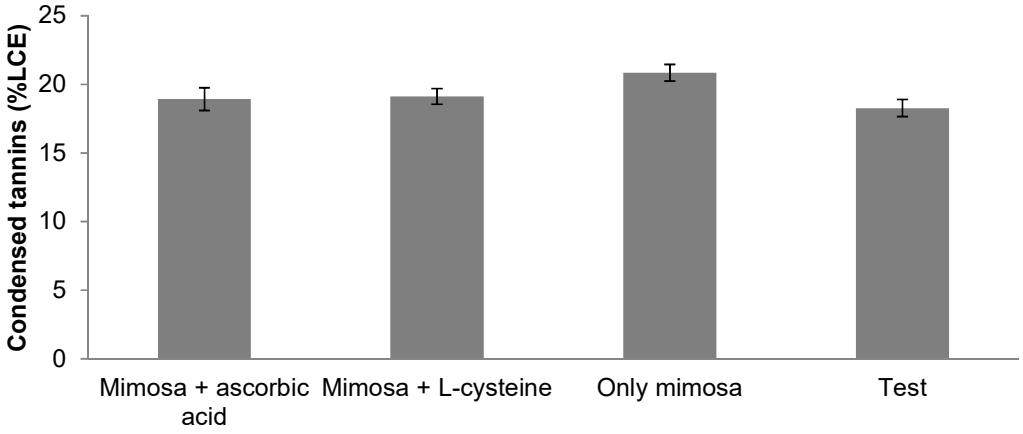

Figure 6-12: Condensed tannin content (% Leucocyanidin equivalent) for controls and test sample after depolymerisation with L-cysteine in mild acidic medium. Controls are mimosa+ascorbic acid, mimosa+L-cysteine, and only mimosa; test sample was comprised of mimosa+ascorbic acid+L-cysteine. Error bars denote the standard error of the mean (n=3 in duplicate).

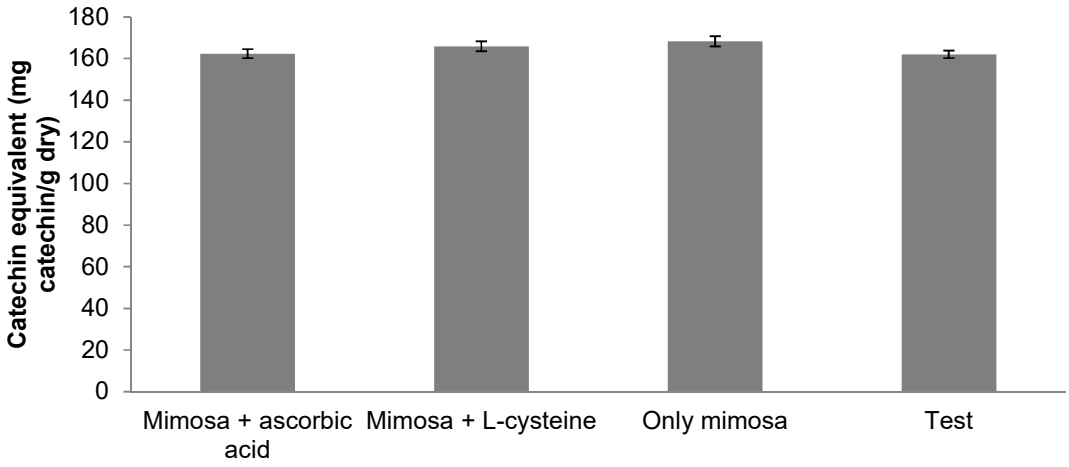

Figure 6-13: Catechin equivalent content (mg catechin/g dry sample) for controls and test sample after depolymerisation with L-cysteine in mild acidic medium. Controls are mimosa+ascorbic acid, mimosa+L-cysteine, and only mimosa; test sample was comprised of mimosa+ascorbic acid+L-cysteine. Error bars denote the standard error of the mean (n=3 in duplicate).

For the condensed tannin content (Figure 6-12), the analysis of variance showed that there was not a significant difference between the controls and the test sample: $F(3,12) = 2.714$, $p = 0.115$. No significant difference was found in *Post Hoc* analysis (Tukey's HSD) for comparison between subjects. Analysis of variance for catechin equivalent (Figure 6-13) presented similar results, showing that there was not a significant difference between the controls and the test sample: $F(3,12) = 1.842$, $p = 0.218$. No significant difference was found in *Post Hoc* analysis (Tukey's HSD) for comparison between subjects. The statistical analysis, therefore, demonstrate that the test sample and controls all statistically presented the same results.

The experimental conditions of this test were adapted from the work of Fujii *et al.* (2007). The authors' objective was to find a dietary source of polyphenols with antioxidant properties, performing the depolymerisation in order to increase digestibility. In the work of Fujii *et al.* (2007), the depolymerisation was carried out for grape seeds polyphenols, and the separation of the monomers and oligomers was performed in a different process, as the authors used column chromatography (cation exchange and reversed phase HPLC). Considering that the objective of the work presented in this thesis is to develop an industrial source of small phenolics for large scale applications, a more viable method of separation was needed, hence the choice for ethyl acetate extraction. It is possible that chromatographic separations would

yield a lower molecular weight product, but the cost would be much higher in comparison with ethyl acetate extraction and the process would not be competitive in relation to traditional technologies.

An optimisation was not undertaken, as the nucleophile L-cysteine was already applied in excess, and the process was carried out at a temperature of 45°C. Polyphenols present a low thermal stability, undergoing side reactions such as oxidation and degradation in high temperatures (Kyi *et al.*, 2005; Ross *et al.*, 2011; Pascariu *et al.*, 2014). Therefore, it is unlikely that higher content of low molecular weight phenolics is obtained by changing process conditions and maintaining the separation with ethyl acetate.

6.5 Summary

Preliminary experiments were undertaken in order to establish a process for obtaining depolymerised mimosa extracts. Two methods were tested: laccase-mediator system, currently applied for the depolymerisation of lignin; and application of L-cysteine in mild acidic medium.

In the course of the research of depolymerisation with laccase-mediator system, it was determined that the mimosa tannin was binding with the enzyme laccase, hindering the enzymatic action. The application of the products obtained with the laccase-mediator system in the stabilisation of hide powder showed a decrease in the stabilisation effect. Extraction with ethyl acetate was efficient in separating low molecular weight phenolics, however it was not efficient in separating the mediator from the product. Therefore, the use of laccase-mediator system was not suitable for the application intended, which is the stabilisation of collagen.

Depolymerisation using L-cysteine in a mild acidic medium demonstrated similar results for condensed tannin content and catechin equivalent for the controls and test sample. This means that either the depolymerisation was not occurring, or a different method of separation was required, as ethyl acetate extraction was used to separate the low molecular weight phenolics. Therefore, this method was discarded as a route for obtaining large-scale depolymerised mimosa extract.

As a result of the reasons previously described, the aims and objectives of this Chapter were not achieved, as both methods studied have not demonstrated to be suitable for the application intended. The research on these methods, however, showed the need to perform a solvent extraction for the separation of low molecular weight phenolic molecules, as well as the development of additional analytical methods aiming at evaluating the depolymerisation of tannins. Therefore, these results were used as a foundation for further experiments.

Due to the preliminary results obtained with the experiments shown in this Chapter not being satisfactory in obtaining a depolymerised product from mimosa tannin, further work on the depolymerisation of mimosa using acid cleavage with addition of a nucleophile was undertaken and the results are presented in Chapter 7. Pyrogallol was chosen as a nucleophile agent, as it can participate in stabilisation reactions with collagen, eliminating the necessity of an additional separation step. Madhan *et al.* (2004) have studied the role of small phenolics on the stabilisation of collagen, and found that rat tendon tail (RTT) collagen fibres treated with pyrogallol showed an increase in shrinkage temperature of 4.5°C in comparison with native RTT collagen.

Chapter 7 Acid-catalysed depolymerisation of mimosa tannin and application on laccase-catalysed stabilisation of collagen

7.1 Introduction

Chapter 6 presented the study of laccase-mediator system, and L-cysteine in mild acidic medium, in the depolymerisation of mimosa tannin. Laccase-mediator system was not suitable for the application in the stabilisation of collagen. Application of L-cysteine in mild acidic medium has not shown significant increase in small phenolic content for the test sample. Therefore, a new method for the depolymerisation of mimosa tannin needed to be developed.

Acid-catalysed depolymerisation of condensed tannins was developed as an analytical technique to determine the degree of polymerisation of proanthocyanidins (Rigaud *et al.*, 1991; Labarbe *et al.*, 1999). The reaction mechanism consists in the cleavage of the proanthocyanidin in the protonated medium, releasing carbocations and terminal units (monomeric flavan-3-ols), followed by nucleophilic condensation and formation of an adduct that can be analysed by HPLC (Hagerman, 2002; Cheynier, 2006).

In the analytical method, the most used nucleophile agents are: phloroglucinol in a method called phloroglucinolysis; and benzyl mercaptan in a method called thiolysis. Matthews *et al.* (1997) have studied both methods with the analysis of *P. sitchensis* and *P. pinaster* bark tannins, concluding that benzyl mercaptan was the preferred reagent, as it presented a reaction yield approximately four times higher than the reaction yield obtained with phloroglucinol. For this reason, thiolysis is still applied, despite the unpleasant smell and lachrymatory characteristics of benzyl mercaptan (Schofield *et al.*, 2001). Phloroglucinol, on the contrary, has the advantage of being odourless and it does not require special handling (Kennedy and Jones, 2001). The acid cleavage followed by reaction with phloroglucinol (phloroglucinolysis) is illustrated in Figure 7-1.

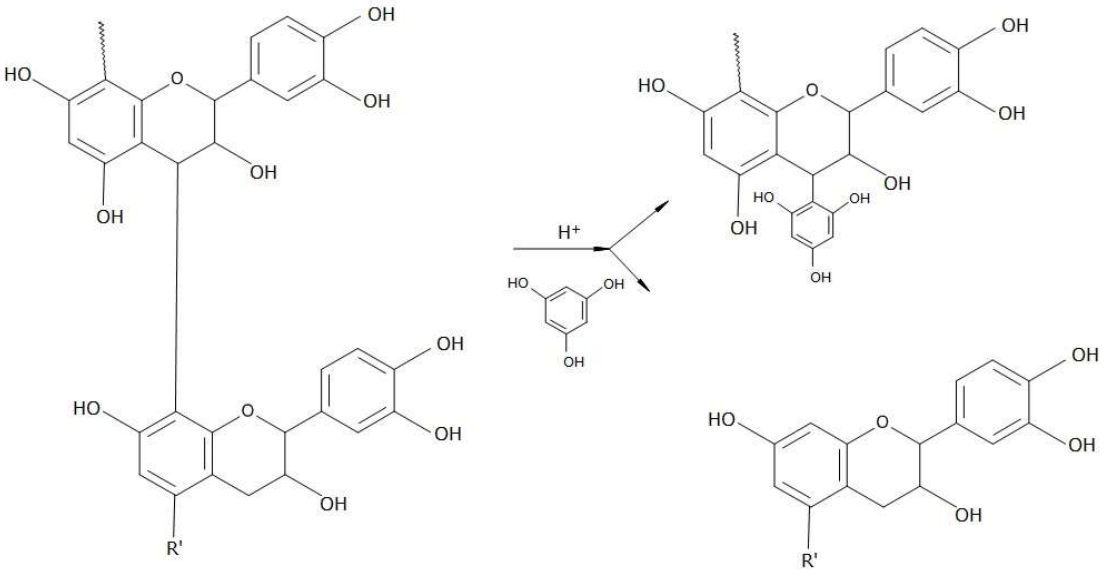

Figure 7-1: Phloroglucinolysis reaction (Schofield *et al.*, 2001).

In this Chapter, the scaling-up of the analytical method was applied in the depolymerisation of mimosa tannin obtained from the bark of *Acacia mearnsii.* The following methods were tested and compared, in order to obtain the most suitable method possible:

- Acid-catalysed depolymerisation without nucleophile;
- Acid-catalysed depolymerisation using phloroglucinol as nucleophile trapping agent;
- Acid-catalysed depolymerisation with phloroglucinol as nucleophile trapping agent, followed by extraction with ethyl acetate.
- Acid-catalysed depolymerisation with pyrogallol as nucleophile trapping agent, followed by extraction with ethyl acetate.

Benzyl mercaptan was not tested, due to the reasons described previously.

The depolymerised products were applied to hide powder and goat skin, followed by laccase-catalysed polymerisation. The stabilisation effect on collagen was determined by the measurement of the change in shrinkage temperature (ΔTs), as described in Section 3.2.8.

7.2 Aims and objectives

The aims of this study are to develop a process of depolymerisation of mimosa tannin, and to improve the tanning efficacy of hide powder and goat skin.

The objectives of this study are:

- To apply acid-catalysed cleavage of the interflavanyl bonds followed by nucleophile trapping in the depolymerisation of mimosa tannin;
- To optimise the process of depolymerisation of mimosa extracts;
- To apply the depolymerised product on hide powder and goat skin with subsequent polymerisation with laccase, and measurement of the tanning effect by the change in shrinkage temperature.

7.3 Experimental procedure

7.3.1 Acid-catalysed depolymerisation

For the acid-catalysed depolymerisation experiments, 5 g of commercial mimosa tannin was dissolved in 40 ml of a 0.1 M HCl in ethanol solution. The mixture was left to react without agitation, at room temperature, for 24 hours. A control sample was prepared by dissolving 5 g of commercial mimosa tannin in 40 ml of ethanol. After reaction, the ethanol was evaporated under vacuum at 30°C using a rotary evaporator (Rotavapor R-114 and Waterbath B-481, Büchi, Flawil, Switzerland; Speedivac ES35, Edwards High Vaccum Limited, Crawley, UK). The solid was mixed with 20 ml of deionised water and freeze-dried to powder 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.

7.3.2 Acid-catalysed depolymerisation with addition of a nucleophile (phloroglucinol)

A solution of 0.1 M of phloroglucinol was prepared in acidic ethanol solution (0.1 M HCl in ethanol). Mimosa tannin (5 g) was dissolved in 40 ml of the phloroglucinol solution and left to react without agitation, at room temperature, for 24 hours. A control sample was prepared by dissolving 5 g of mimosa tannin in 40 ml of a solution of 0.1 M of phloroglucinol in ethanol. After reaction, the ethanol was evaporated under vacuum at 30°C using a rotary evaporator (Rotavapor R-114 and Waterbath B-481, Büchi, Flawil, Switzerland; Speedivac ES35, Edwards High Vaccum Limited, Crawley, UK). The solid was mixed with 20 ml of deionised water and freeze-dried to powder 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.

7.3.3 Acid-catalysed depolymerisation with addition of phloroglucinol, followed by ethyl acetate extraction

A solution of 0.1 M of phloroglucinol was prepared in acidic ethanol solution (0.1 M HCl in ethanol). Mimosa tannin (5 g) was dissolved in 40 ml of the phloroglucinol solution and left to react without agitation, at room temperature, for 24 hours. A control sample was prepared by dissolving 5 g of mimosa tannin in 40 ml of a solution of 0.1 M of phloroglucinol in ethanol. After reaction, the ethanol was evaporated under vacuum at 30°C using a rotary evaporator (Rotavapor R-114 and Waterbath B-481, Büchi, Flawil, Switzerland; Speedivac ES35, Edwards High Vaccum Limited, Crawley, UK). The solid was mixed with 20 ml of deionised water and extracted twice with 25 ml of ethyl acetate for each extraction. The samples were centrifuged (Heraeus Megafuge 16R, Thermo Scientific, Hemel Hempstead, UK) at 3075 x *g* for 10 minutes. The supernatant for both extractions were collected and combined for each sample, and the ethyl acetate was evaporated under vacuum at 30°C using a rotary evaporator (Rotavapor R-114 and Waterbath B-481, Büchi, Flawil, Switzerland; Speedivac ES35, Edwards High Vaccum Limited, Crawley, UK). The solid was mixed with 10 ml of deionised water and freeze-dried to powder 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.

7.3.4 Acid-catalysed depolymerisation with addition of pyrogallol, followed by ethyl acetate extraction

A solution of 0.1 M of pyrogallol was prepared in acidic ethanol solution (0.1 M HCl in ethanol). Mimosa tannin (5 g) was dissolved in 40 ml of the pyrogallol solution and left to react without agitation, at room temperature, for 24 hours. A control sample was prepared by dissolving 5 g of mimosa tannin in 40 ml of a solution of 0.1 M of pyrogallol in ethanol. The mixtures were left for reaction at room temperature, for 24 hours. After reaction, the ethanol was evaporated under vacuum at 30°C using a rotary evaporator (Rotavapor R-114 and Waterbath B-481, Büchi, Flawil, Switzerland; Speedivac ES35, Edwards High Vaccum Limited, Crawley, UK). The solid was mixed with 20 ml of deionised water and extracted twice with 25 ml of ethyl acetate for each extraction. The samples were centrifuged (Heraeus Megafuge 16R, Thermo Scientific, Hemel Hempstead, UK) at 3075 x *g* for 10 minutes. The supernatant for both extractions were collected and combined for each sample, and the ethyl acetate was evaporated under vacuum at 30°C using a rotary evaporator (Rotavapor R-114 and Waterbath B-481, Büchi, Flawil, Switzerland; Speedivac ES35, Edwards High Vaccum Limited, Crawley, UK). The solid was mixed with 10 ml of deionised water and freeze-dried to powder 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.

7.3.5 Optimisation of acid-catalysed depolymerisation with addition of pyrogallol as nucleophile

The effect of solvent was tested by replacing the ethanol with ultrapure water and following the same procedure described in Section 7.3.4.

The temperature effect was studied by following the procedure described in Section 7.3.4, except that instead of carrying out the reaction at room temperature, the samples were kept in a water bath (Digital water bath OLS200, Grant Instruments, Cambridge, UK) at 25°C, 35°C and 45°C.

7.3.6 Application on hide powder

Tanning experiments were performed with 500 mg hide powder and 165 mg of tannin product in dry basis added to 25 ml sodium acetate buffer (pH 5.5). The samples were stirred with a magnetic stirrer (Hot plate stirrer PC-351, Corning, New York, USA) for 30 minutes to rehydrate the hide powder and absorb the tannin product, and the pH was measured with a pH meter Seven Multi (Mettler Toledo, Schwerzenbach, Switzerland) and adjusted to 5.5 ± 0.2 when necessary with 0.1 M NaOH. Laccase for a final concentration of 5 mg/l was added to the samples to start polymerisation. One control sample for each tanning product was prepared, omitting the enzyme. The reaction mixtures were kept for 25 hours in a shaking water bath (Digital water bath OLS200, Grant Instruments, Cambridge, UK) at 34.6°C and 100 rpm. The enzymatic reaction was stopped with 5 ml of a sodium azide solution (1 mM). At the end of reaction, the samples were centrifuged (Heraeus Megafuge 16R, Thermo Scientific, Hemel Hempstead, UK) at 3075 x *g* for 10 minutes, and washed twice with deionised water. 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 (British Standards Institute, 2012). Tanning degree was evaluated by the shrinkage temperature (Ts), measured using differential scanning calorimetry, according to the method described in Section 3.2.8.

7.3.7 Application on goat skin

Goat skin was processed from salted to pickled according to the procedure described in Section 3.1.4. For each gram of goat skin (dry basis), 25 ml of deionised water and 500 mg of tannin in dry basis were added. The samples were shaken at 25°C and 100 rpm, in a shaking water bath (Digital water bath OLS200, Grant Instruments, Cambridge, UK), for 24 hours. The solution was drained and 25 ml of a solution of laccase at a final concentration of 10 mg/L in acetate buffer (pH 5.5) was added to the test sample to initiate the reaction. The control sample was prepared in 25 ml of acetate buffer (pH 5.5). The test and control samples were then incubated for 25 hours in a shaking water bath (Digital water bath OLS200, Grant Instruments, Cambridge, UK) at 34.6°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 skin pieces were washed twice with deionised water. After this procedure, the pieces were kept for 48 hours in a controlled atmosphere at 23°C and 50% relative humidity,

according to BS EN ISO 2419 (British Standards Institute, 2012). Tanning degree was evaluated by the shrinkage temperature (Ts) of samples, measured using differential scanning calorimetry, according to the method described in Section 3.2.8.

7.4 Results and discussion

7.4.1 Acid-catalysed depolymerisation

Preliminary experiments on acid-catalysed depolymerisation were undertaken without addition of a nucleophile. The products obtained were analysed using the tannin content method SLC116 (Society of Leather Technologists and Chemists, 1996), following the procedure described in Section 3.2.4. Figure 7-2 shows the data obtained for the control and test samples.

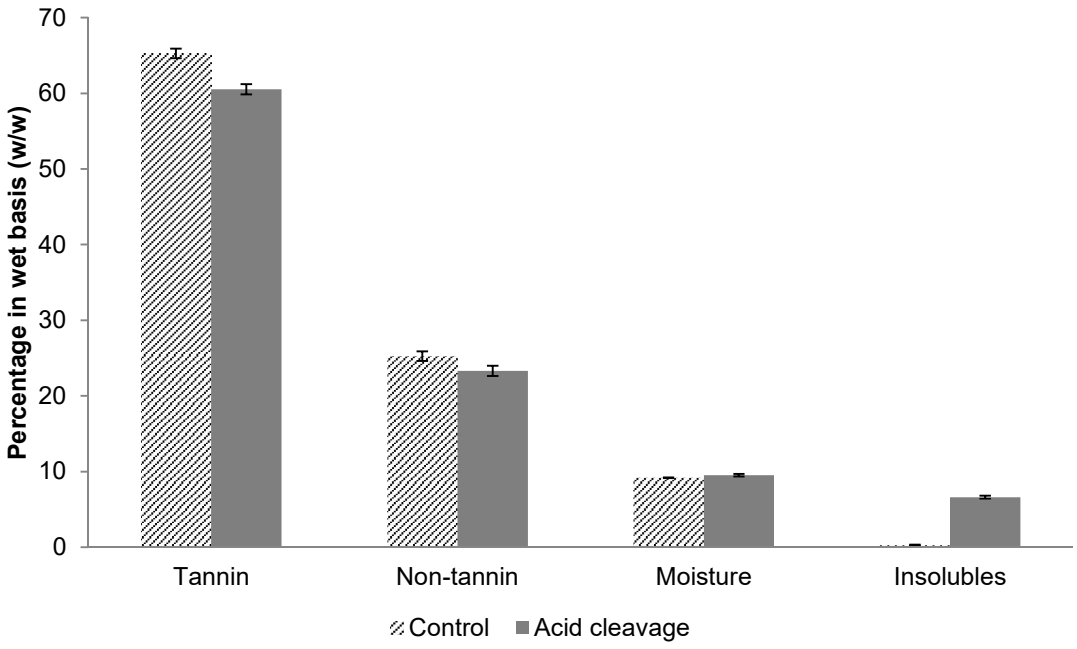

Figure 7-2: Tannin, non-tannin, moisture and insoluble content for control and test samples subjected to acid-catalysed depolymerisation, according to the method described in Section 7.3.1. Error bars denote the standard error of the mean (n=3 in duplicate).

Data from Figure 7-2 showed that non-tannin and moisture contents were not significantly different between control and test samples (*t*-test, p = 0.102 for non-tannin content and p = 0.285 for moisture content). The tannin content for the test sample was significantly lower than the tannin content for the control (*t*-test, p = 0.038). The insoluble content, on the contrary, was significantly higher for the test sample in comparison with the control (*t*-test, p < 0.001). The tannin content is calculated by difference (100 - %non-tannin - %moisture - %insolubles). Therefore, a higher insoluble content means a lower tannin content. The lower tannin content for the test sample in comparison with the control was due to an increase in the insoluble content, rather than an increase in non-tannins, as intended.

According to Foo and Karchesy (1989), acid-induced transformations of proanthocyanidins gave products that reflected cleavage of the interflavanyl bond followed by secondary reactions of anthocyanidin formation, rearrangement of the interflavanyl bond or self-condensation. This means that the anthocyanidins produced by the cleavage of the interflavanyl bond may combine and form phlobaphenes or red tannins, which are substances of high molecular weight (>3000 Da), usually insoluble in water (Arbenz and Averous, 2015). Figure 7-3 illustrates the acid-catalysed rearrangement of a proanthocyanidin in the presence of a strong acidic medium.

The acid cleavage in the absence of a nucleophile increased considerably the content of insoluble matter in water, indicating that self-condensation was occurring after depolymerisation. Sealy-Fisher and Pizzi (1992) minimised phlobaphene formation during extraction of pine tannins with the addition of a nucleophile (urea). Therefore, the nucleophile agent is essential for obtaining monomeric and/or oligomeric phenolics. For this reason, in order to avoid the formation of phlobaphenes, a nucleophile, phloroglucinol, was added to react with the anthocyanidins formed by the acid cleavage, preventing further condensation.

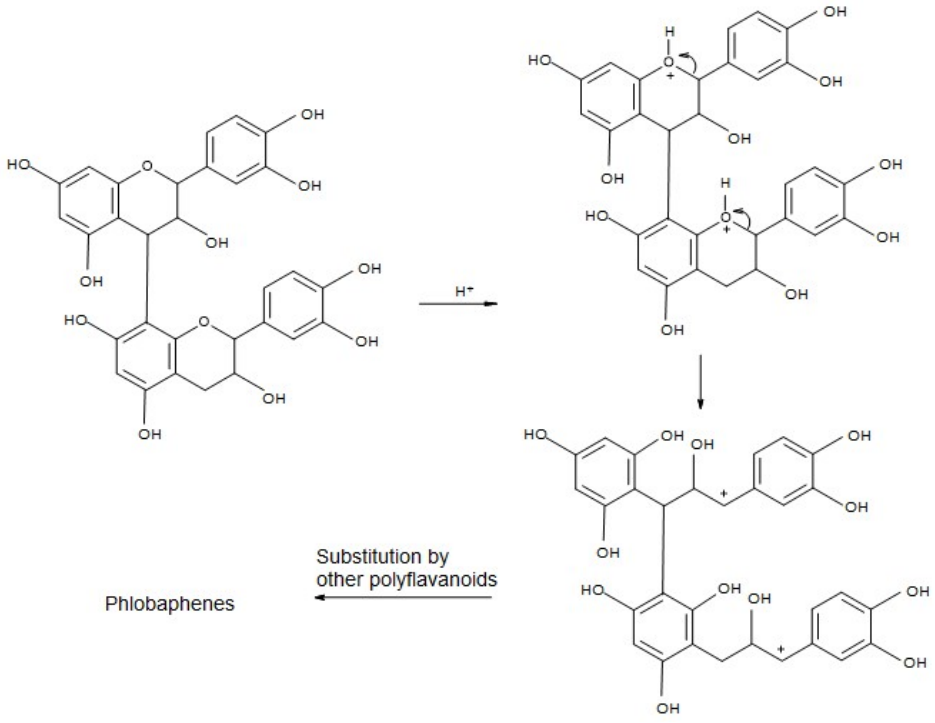

Figure 7-3: Acid condensation by hydrolysis of the heterocycles and formation of phlobaphenes (Arbenz and Averous, 2015).

7.4.2 Acid-catalysed depolymerisation with addition of phloroglucinol as nucleophile agent

The products obtained from the acid-catalysed depolymerisation with phloroglucinol as nucleophile trapping agent were analysed according to tanning content using the method SLC 116 (Society of Leather Technologists and Chemists, 1996). Data are shown in Figure 7-4.

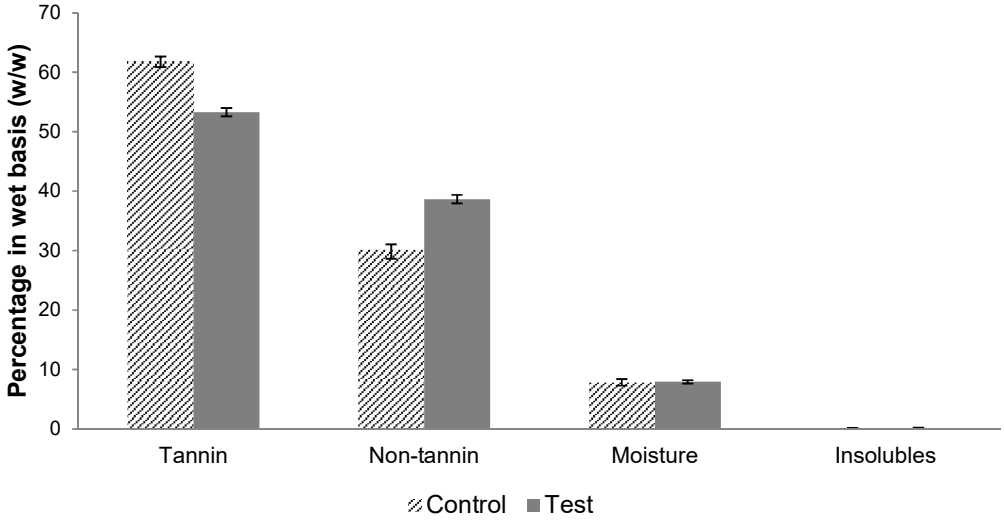

Figure 7-4: Tannin, non-tannin, moisture and insoluble content for control and test samples subjected to acid cleavage with addition of pholoroglucinol, according to the method described in Section 7.3.2. Insoluble content values do not show due to scale, and the results were 0.10%±0.04 for control and 0.15%±0.02 for test sample. Error bars denote the standard error of the mean (n=3 in duplicate).

Data presented in Figure 7-4 showed that moisture content and insoluble content were not significant different between control and test samples (*t*-test, p = 0.671 for moisture content and p = 0.322 for insoluble content). The results for insoluble content demonstrate that, differently from the data showed in Figure 7-2, the addition of phloroglucinol averted the increase of insoluble content by trapping the carbocations and preventing the condensation reactions and formation of phlobaphenes.

Tannin and non-tannin content showed a significant difference between control and test sample. The non-tannin content for the test sample was significantly higher than the non-tannin content for the control (*t*-test, p = 0.01). As tannin content is calculated by difference (100 - %non-tannin - %moisture - %insolubles), it presented the same statistical result as the non-tannins. This means that the phloroglucinolysis process was producing smaller molecules.

Due to results of mimosa inhibition showed in Section 6.4.2.4, an additional step of extraction with ethyl acetate was performed for the process of acid-catalysed depolymerisation.

7.4.3 Acid-catalysed depolymerisation with addition of phloroglucinol, followed by ethyl acetate extraction

An additional step of extraction with ethyl acetate was performed in order to separate larger polyphenols and minimise the inhibition of laccase when applying to collagen stabilisation. As stated in Chapter 6, Section 6.4.3, the analytical method SLC 116 (Society of Leather Technologists and Chemists, 1996), based on the gravimetric measurement of non-tannins after tannin absorption by hide powder, is not adequate to provide information about the depolymerised product after extraction with ethyl acetate. Therefore, in this section, the depolymerisation process was monitored *via* analysis of condensed tannin content (% leucocyanidin equivalent) and catechin equivalent (mg catechin/g dry sample). Data in Table 7-1 show the condensed tannin content (% Leucocyanidin equivalent) and catechin equivalent (mg catechin/g dry sample) for mimosa extract, control and test samples subjected to acid-catalysed depolymerisation with addition of phloroglucinol. Mimosa extract was analysed as received from the supplier; the control sample was subjected to the same procedure as the test sample, except it was carried out in the absence of acid; and the test sample underwent the acid-catalysed depolymerisation with phloroglucinol.

Table 7-1: Condensed tannin content (% Leucocyanidin equivalent) and catechin equivalent (mg catechin/g dry sample) for mimosa extract, control and test samples. Data shown as average ± standard error of the mean. Mimosa: n=1 in triplicate; control and test samples: n=3 in duplicate.

Samples	***Condensed tannins (%LCE)***	***Catechin equivalent (mg catechin/g dry sample)***
Mimosa	37.47 ± 0.53	386.34 ± 1.83
Control – absence of acid	20.52 ± 0.66	166.74 ± 3.83
Test – acid catalysed	16.91 ± 0.65	257.96 ± 3.94

As explained in Section 6.4.3.1, the results from condensed tannin content and catechin equivalent content provide information about the average size of the polyphenol molecule. Catechin equivalent gives the monomer content, and condensed tannins is related to the number of interflavanyl bonds.

The control and test samples were subjected to an extraction with ethyl acetate as part of processing, in order to obtain a fraction rich in lower molecular weight polyphenols. The less polar substances, such as monomeric and oligomeric proanthocyanidins are solubilised by

the ethyl acetate, whilst the more polar molecules, such as large polyphenols, remain in the aqueous phase (Cheynier and Fulcrand, 2003). The large polyphenols remaining in the aqueous phase were discarded after the extraction with ethyl acetate. Data for mimosa, in comparison with the control (Table 7-1) show higher values for condensed tannin content and catechin equivalent. The mimosa sample was analysed prior to any processing, therefore, as the larger molecular weight polyphenols were separated on processing for the control and test samples, it was expected that unprocessed mimosa would show higher readings for both condensed tannins and catechin equivalent.

The comparison between the control and test sample (Table 7-1) demonstrates that the test sample, subjected to acid-catalysed process, presented lower condensed tannin content and higher catechin equivalent content than the control sample, in which the acid was absent. This indicates that the polyphenols present in the test sample have lower molecular weight, demonstrating the action of the acid-catalysed process in the depolymerisation of mimosa. These data suggest the potential of the acid-catalysed depolymerisation of mimosa tannin as a method of obtaining low molecular weight phenolic compounds.

7.4.3.1 Application on hide powder

Control and test samples obtained with the acid-catalysed depolymerisation method using phloroglucinol, followed by ethyl acetate extraction, were applied in the stabilisation of hide powder, followed by the application of laccase for *in situ* polymerisation. Unmodified mimosa was also used for comparison. Data are presented in Figure 7-5.

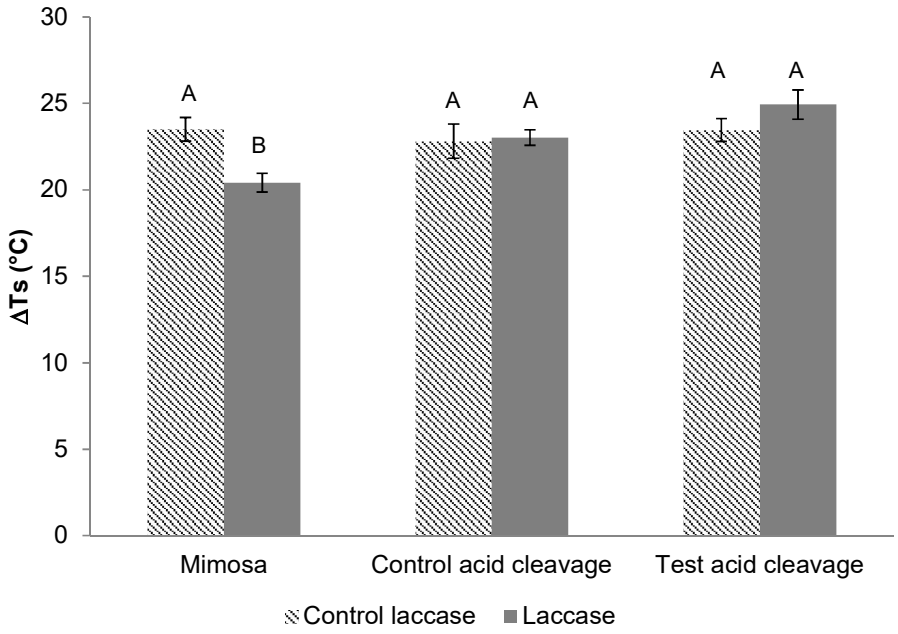

Figure 7-5: Change in shrinkage temperature (ΔTs) of hide powder, tanned with unmodified mimosa extract, control and test samples obtained with the process described in Section 7.4.3. Error bars denote the standard error of the mean (n=3 in duplicate). Means with different letters (A,B) are significantly different (Tukey's HSD, $p<0.05$).

According to Figure 7-5, data for mimosa shows that the enzyme application resulted in lower shrinkage temperature in comparison with the control. This corroborates with results presented on Chapter 6, concluding that mimosa inhibits the laccase by biding with the enzyme.

The increase in shrinkage temperature for the control acid cleavage showed that there was not a significant difference between the sample omitting the enzyme (control laccase) and the sample that underwent the enzyme reaction (laccase). Both results for the control acid cleavage (laccase and control laccase) also showed no significant difference from the sample using mimosa and omitting the enzyme (control laccase for mimosa). This was not expected, as the acid cleavage control sample underwent extraction with ethyl acetate, meaning only smaller polyphenols were applied. For the mimosa sample, the tannin offer was higher, as no extraction was undertaken, thus a higher shrinkage temperature was expected for this sample. The effect of inhibition observed in the mimosa sample was not present in the control acid cleavage. Nevertheless, the polymerisation effect (between samples laccase and control

laccase) for the control acid cleavage on the hide powder stabilisation was not significant as well.

The test acid cleavage showed a slightly higher shrinkage temperature for the sample subjected to the laccase reaction in comparison with the sample omitting the enzyme (control laccase). This difference, however, was not statistically significant. A possible explanation for this result is that small phenolics can also participate in the stabilisation reactions with collagen. The increase in shrinkage temperature could have been boosted by the polymerisation of remaining phloroglucinol. Results shown in Chapter 4, Sections 4.4.1 and 4.4.2, showed that laccase has a low activity on *meta*-phenols such as phloroglucinol. Although phloroglucinol has an excellent nucleophilic character, the application of the depolymerised product on the laccase-assisted stabilisation of collagen may benefit from the use of an alternative nucleophilic trapping agent.

The conjugation of one of the two unshared pairs of electrons from the oxygen atom of the hydroxyl group with the benzene ring corresponds to the positive electron-donor mesomeric effect. This phenomenon extends the electron delocalisation and confers a partial negative charge on the carbons in *ortho* and *para* position with respect to the hydroxyl group. The presence of hydroxyl groups in *meta* position (resorcinol and phloroglucinol) enables the accumulation of electron density at C2, C4 and C6, thus increasing the nucleophilic character (Cheynier *et al.*, 2010). Figure 7-6 shows a comparison amongst phloroglucinol, catechol and pyrogallol, regarding their nucleophilicity character.

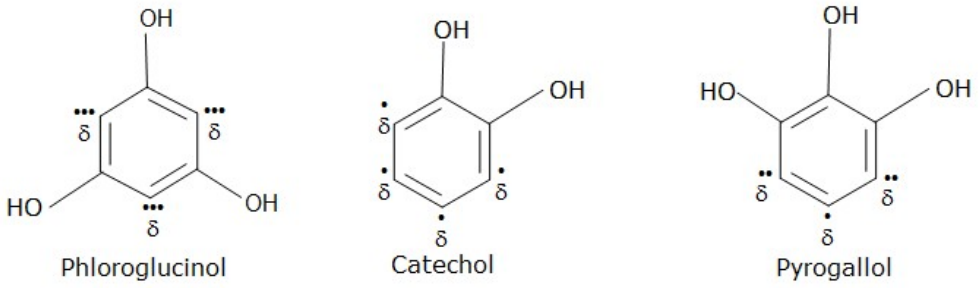

Figure 7-6: Nucleophilicity of phloroglucinol, catechol and pyrogallol (Ribereal-Gayon *et al.*, 2006).

As seen in Figure 7-6, the electron density on the carbons adjacent to the hydroxyls in pyrogallol confers a better nucleophilicity character to this compound, in comparison with catechol. In terms of reactivity with laccase, the kinetic parameters presented in Table 4-1 (Chapter 4) showed that pyrogallol presents a higher maximum velocity and enzyme-substrate affinity with laccase, in comparison with catechol. Madhan *et al.* (2004) have studied the role of small phenolics (catechol, resorcinol and pyrogallol) on the stabilisation of collagen, and found that rat tendon tail (RTT) collagen fibres treated with pyrogallol showed an increase in shrinkage temperature of 4.5°C in comparison with native RTT collagen. Resorcinol, a *meta*-phenol as phloroglucinol, exhibited the least resistance against both heat and collagenase. For the reasons stated above, experiments using pyrogallol as a nucleophile agent were undertaken, as pyrogallol can participate in stabilisation reactions with collagen, even without the polymerisation action of laccase.

7.4.4 Acid-catalysed depolymerisation with addition of pyrogallol as nucleophile

The process of acid cleavage of mimosa tannin was carried out with pyrogallol as nucleophile, aiming at improving the laccase-assisted stabilisation of collagen. Bordiga *et al.* (2013) have applied pyrogallol as a nucleophile agent to the analysis of proanthocyanidins extracted from grape seeds, and compared the results of pyrogallol and phloroglucinol as a trapping agent. The authors found that the reaction yield, when using pyrogallol at 30°C, was comparable to the reaction yield with phloroglucinol at 50°C, thus it was possible to reduce the reaction temperature maintaining a similar yield. Lower reaction temperature is an advantage when working with polyphenols, as to minimise side reactions (Ross *et al.*, 2011).

Figures 7-7 and 7-8 show a comparison between acid cleavage using phloroglucinol and pyrogallol, in terms of condensed tannin content and catechin equivalent, respectively.

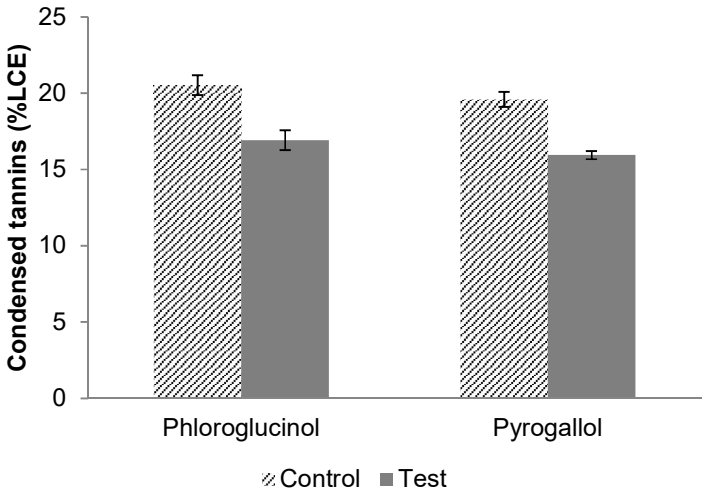

Figure 7-7: Condensed tannin content (% Leucocyanidin equivalent) for control and test samples with the depolymerisation process carried out using phloroglucinol and pyrogallol as nucleophile trapping agent. Error bars denote the standard error of the mean (n=3 in duplicate).

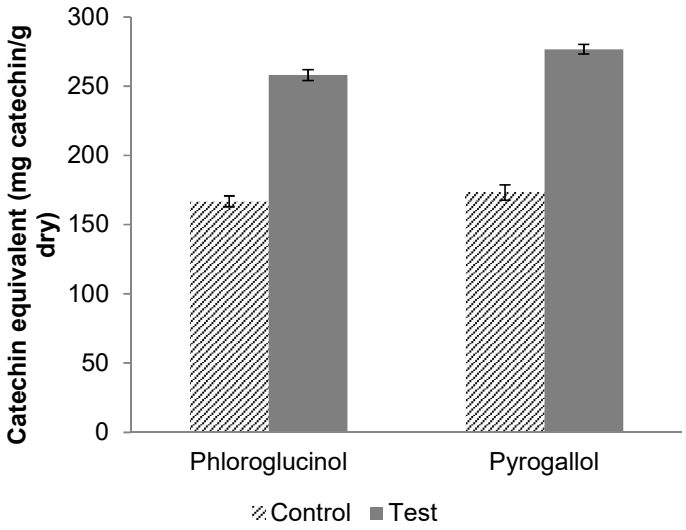

Figure 7-8: Catechin equivalent content (mg catechin/g dry sample) for control and test samples with the depolymerisation process carried out using phloroglucinol and pyrogallol as nucleophile trapping agent. Error bars denote the standard error of the mean (n=3 in duplicate).

Data from Figures 7-7 and 7-8 show a lower condensed tannin and a higher catechin equivalent content for the process using pyrogallol in comparison with phloroglucinol. The difference, however, was not statistically significant (*t*-test, p = 0.128 for condensed tannin

content and p = 0.469 for catechin equivalent). These results, therefore, showed that pyrogallol has a similar effect in depolymerisation as phloroglucinol.

7.4.4.1 Characterisation of products obtained with acid-catalysed depolymerisation of mimosa with addition of pyrogallol

The control and test samples subjected to the procedure described in Section 7.3.4, as well as pure pyrogallol and unmodified mimosa, were characterised using thin layer chromatography (TLC) and nuclear magnetic resonance (NMR).

Thin Layer Chromatography of pyrogallol, mimosa, control and test sample is presented in Figure 7-9.

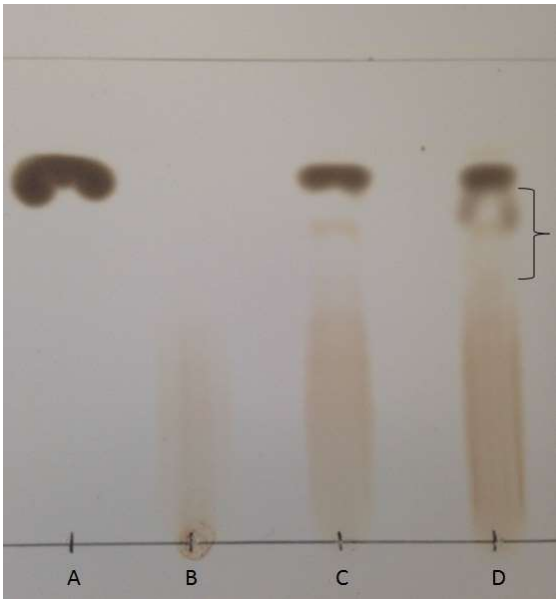

Figure 7-9: TLC analysis of pyrogallol (A), unmodified mimosa (B), control (C) and test sample (D). Analysis made following procedure described in Section 3.2.3.

For the solvent system used (methodology described in Section 3.2.3), the retention factor (R_f) of phenolic compounds can be considered as inversely proportional to their molecular weight. As Figure 7-9 shows, pyrogallol (A), the lower molecular weight molecule, travel a

longer distance within the stationary phase. Unmodified mimosa (B) interacts more with the stationary phase due to the higher molecular weight. For the comparison between control and test samples (C and D respectively), it is possible to notice that the test sample presents a distribution of lower molecular weight compounds that are not present in the control samples (marked area in Figure 7-9). This results corroborate with the data for condensed tannins and catechin equivalent analyses, confirming the presence of lower molecular weight compounds for the test sample in comparison with the control and mimosa samples.

^{13}C solid state and ^{1}H NMR analyses were carried out in order to elucidate structural changes due to the depolymerisation process, according to the methodology described in Section 3.2.15. The ^{1}H NMR spectra for mimosa tannin, acquired in deuterated methanol, is presented in Figure 7-10.

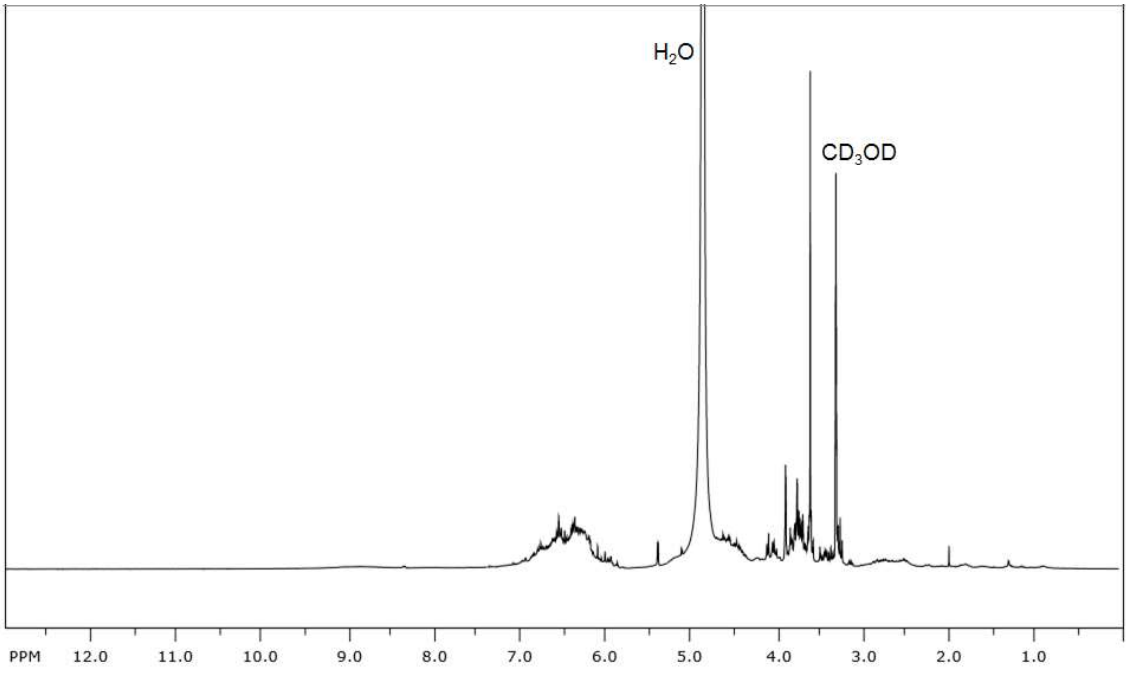

Figure 7-10: ^{1}H NMR of mimosa tannin (400 MHz, CD_3OD).

In the ^{1}H NMR spectrum for mimosa tannin (Figure 7-10), the solvent peak (deuterated methanol – CD_3OD) is shown at 3.31 ppm, while the signal at 4.87 ppm is due to the water

present in the sample. Signals in the range between 6.5 and 7.5 ppm correspond to the tannin B-ring protons, while the signals between 3.2 and 5.6 correspond to the C-ring protons (Guyot *et al.,* 1999). This spectrum shows no signals in the region of 11 – 13 ppm, indicating the absence of free acids such as gallic acid (Duval and Averous, 2016). Solvents commonly used in ^{1}H NMR such as CD_3OD and D_2O, however, may exchange the Deuterium with the –OH groups from the acids, making the acid signals seem absent. DMSO-d_6, on the contrary, does not exchange Deuterium with the acid hydroxyl protons. Therefore, in order to confirm the absence of free acids, the ^{1}H NMR spectrum for mimosa tannin was run in DMSO-d_6 as well, as shown in Figure 7-11, confirming the absence of peaks in the region of 11 – 13 ppm.

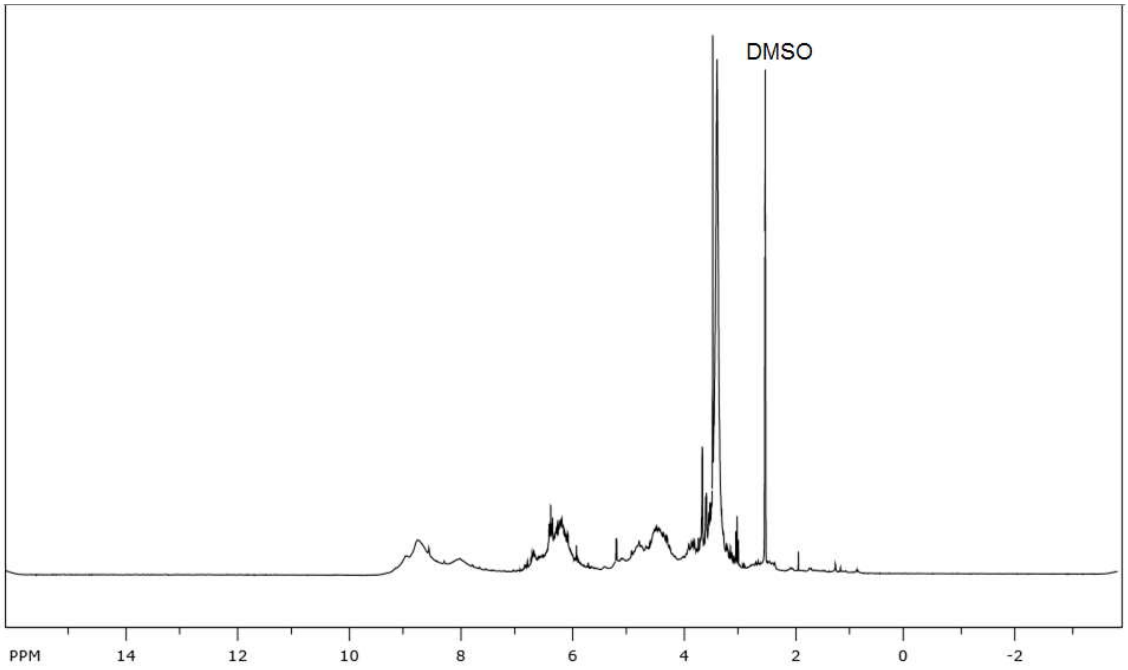

Figure 7-11: ^{1}H NMR of mimosa tannin (400 MHz, DMSO-d_6).

Figure 7-12 presents the ^{1}H NMR spectrum for pyrogallol, in deuterated methanol.

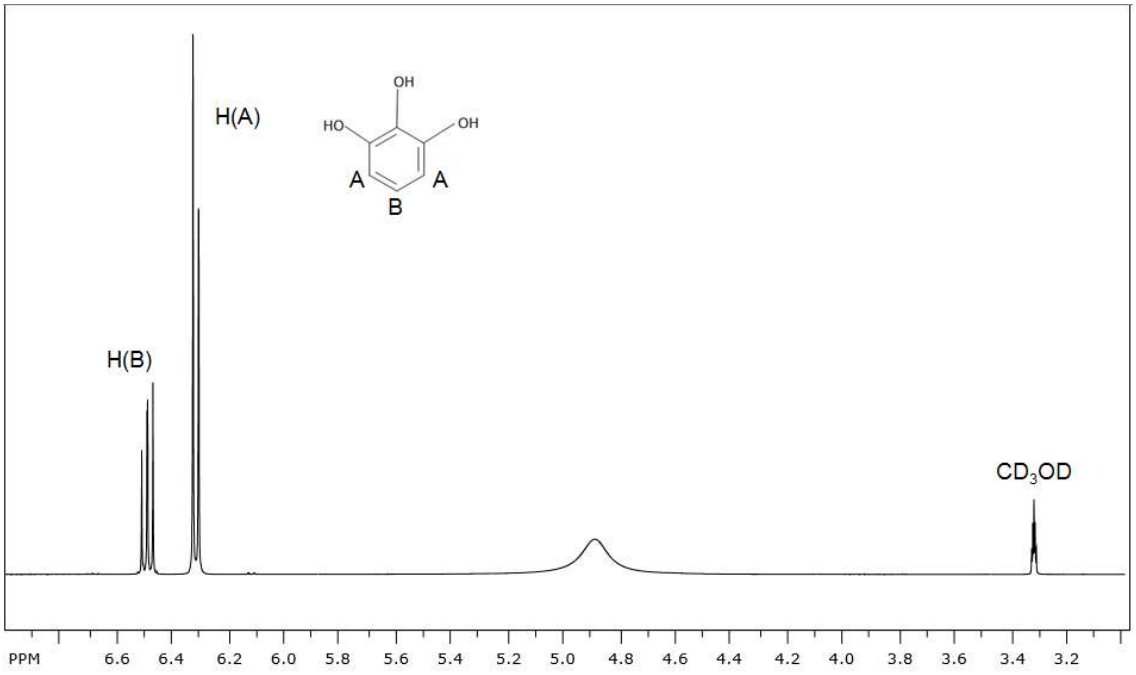

Figure 7-12: ^{1}H NMR of pyrogallol (400 MHz, CD_3OD).

The characteristic signals pertaining to the pyrogallol aromatic protons are clearly observed as a doublet at 6.25 and as a triplet at 6.42 ppm, corresponding to the H(A) and H(B) protons, respectively, as shown in Figure 7-12. This spectrum was used for comparison with the spectra generated for the depolymerisation control and test samples.

As explained before, the deuterated methanol may exchange deuterium with the hydroxyl protons, which means their signals are not observed in the spectrum. The spectrum acquired for the sample dissolved in DMSO-d_6, presented in Figure 7-13, shows the hydroxyl protons.

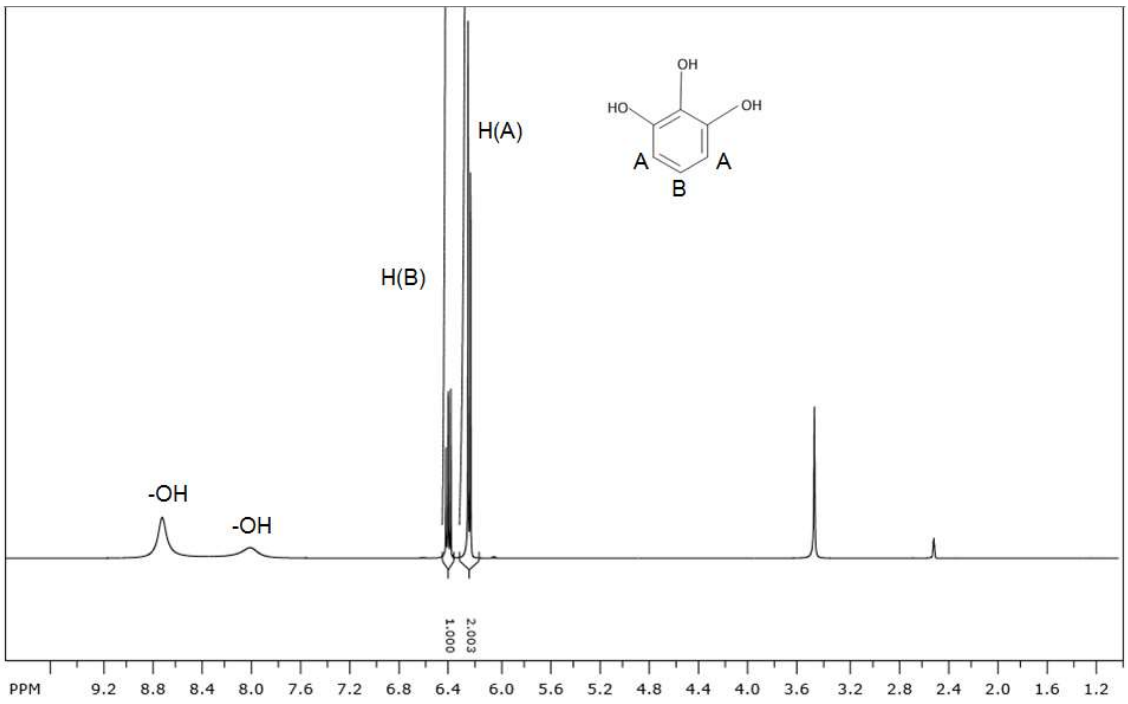

Figure 7-13: ^{1}H NMR of pyrogallol (400 MHz, DMSO-d_6).

Figure 7-13 shows the integration of signals at 6.25 and 6.42 as 2.0 and 1.0, confirming the assignment of these signals to the 2 protons H(A) and 1 proton H(B), respectively. The broad singlets observed at 8.02 and 8.72 ppm correspond to the OH functional groups of the pyrogallol.

Figures 7-14 and 7-15 present the ^{1}H NMR spectrum in deuterated methanol for the control and test samples, respectively.

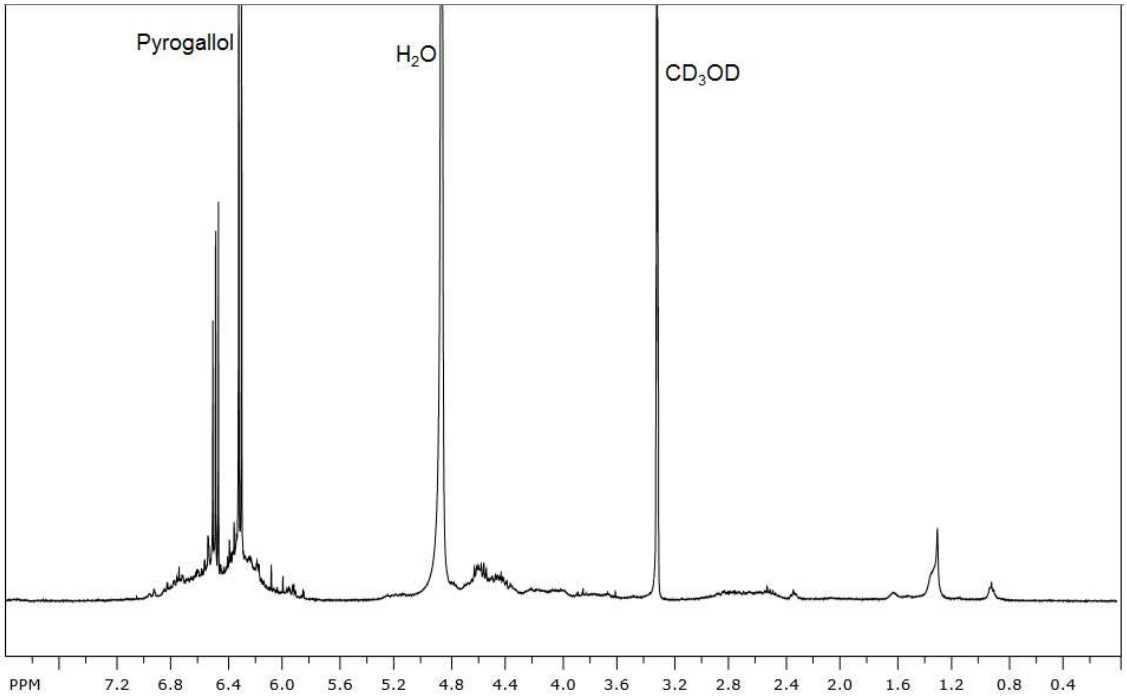

Figure 7-14: ^{1}H NMR of depolymerisation control sample (400 MHz, CD_3OD).

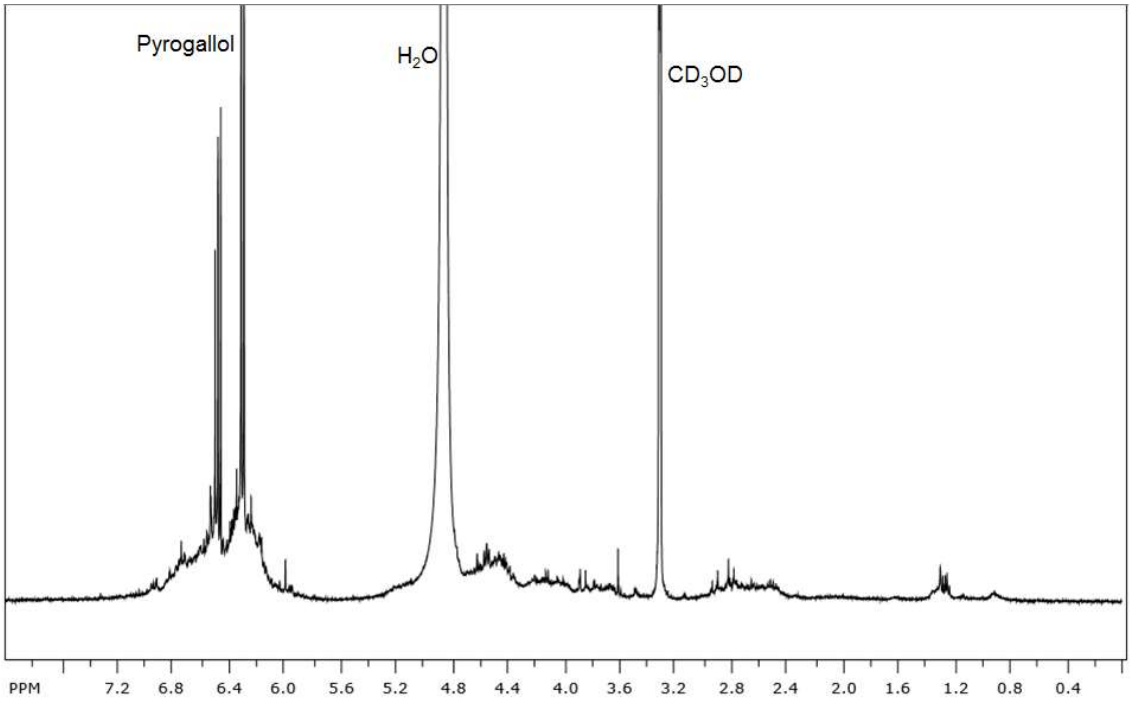

Figure 7-15: ^{1}H NMR of depolymerisation test sample (400 MHz, CD_3OD).

In Figures 7-14 and 7-15, it can be observed that the pyrogallol peaks at 6.25 and 6.42 ppm are prominent in the samples. This is caused by the greater solubility of pyrogallol in methanol, as compared to mimosa. Nevertheless, a comparison with the integrated peaks may provide information on the biding of pyrogallol with the carbocations released during the acid cleavage.

Guyot *et al.* (1999) used ^{1}H NMR to determine the degree of polymerisation of apple procyanidins, using the relationship between the signal area for H4 terminal protons, between 2.4 and 3.0 ppm, and signal area for the H6/H8 protons, between 5.8 and 6.5 ppm. The interflavanyl bond between monomeric units binds the C4 of one unit and the C6 (branched) or C8 (linear) of another unit (as shown in Figure 7-16). Hence, the integration ratio (H6+H8)/(H4 terminal) increases with the degree of polymerisation. The method described, however, was not suitable for determining the degree of polymerisation for the control and test samples, because the pyrogallol signals overlap with the H6 and H8 protons of the A-ring.

In order to overcome solubility difference and avoid overlapping of solvent peaks, in addition to CD_3OD, the NMR measurements were also carried out in DMSO-d_6 and in a 1:1 mixture of D_2O:Acetone-d_6 (data not shown). Unfortunately, the pyrogallol peaks were still shown to be prominent for all solvents tested. Alternatively, the pyrogallol may be removed from the samples using purification techniques, such as chromatography, or the NMR analyses may be run in solid state. As the presence of residual pyrogallol is potentially beneficial to the application of the depolymerised product in the stabilisation of collagen, carrying out analyses of ^{13}C NMR in solid state was preferred.

Solid state ^{13}C NMR spectra of amorphous materials results in broad lineshapes, due to a variety of reasons such as anisotropic interactions, in stark contrast to the sharp peaks obtained with solution state NMR. Cross polarisation (CP) is a technique used to enhance sensitivity in solid state NMR, which makes use of the polarisation transfer from abundant nuclei (such as ^{1}H) to dilute or rare nuclei (such as ^{13}C) in order to improve signal to noise ratio and reduce waiting time between successive experiments (Hediger *et al.*, 1995).

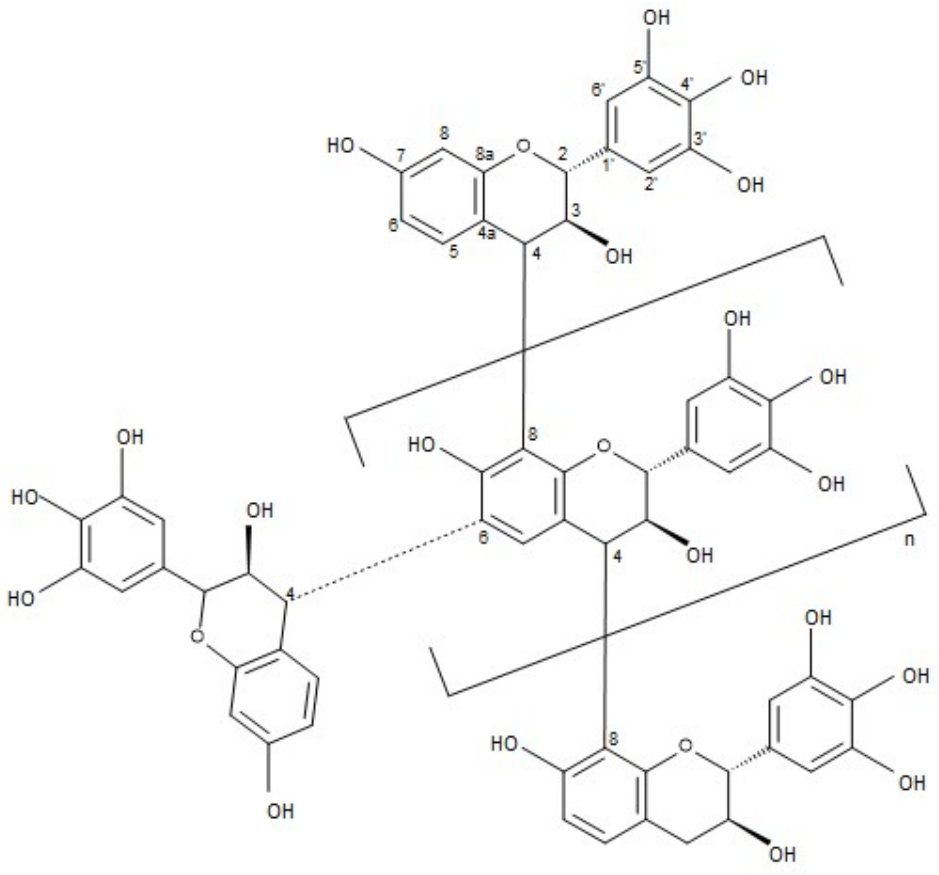

Figure 7-16: Chemical structure of prorobinetinidin (main constituent of mimosa tannin) (adapted from Covington *et al.*, 2005).

Based on the research of Reid *et al.* (2013), experiments aiming at selecting an optimal cross polarisation (CP) time were carried out. In order to validate the use of signal intensities as a measure of molecular abundance, it is usual to explore signal intensity as a function of CP time. The aim is to identify an ideal CP time for all signals at which the increase in signal intensity due the CP process predominates over a decrease in intensity due to competing relaxation processes (Reid *et al.*, 2013). Figure 7-17 shows several spectra acquired from mimosa tannin at different CP times. Figure 7-18 presents a plot of the intensity of certain signals *vs.* CP time.

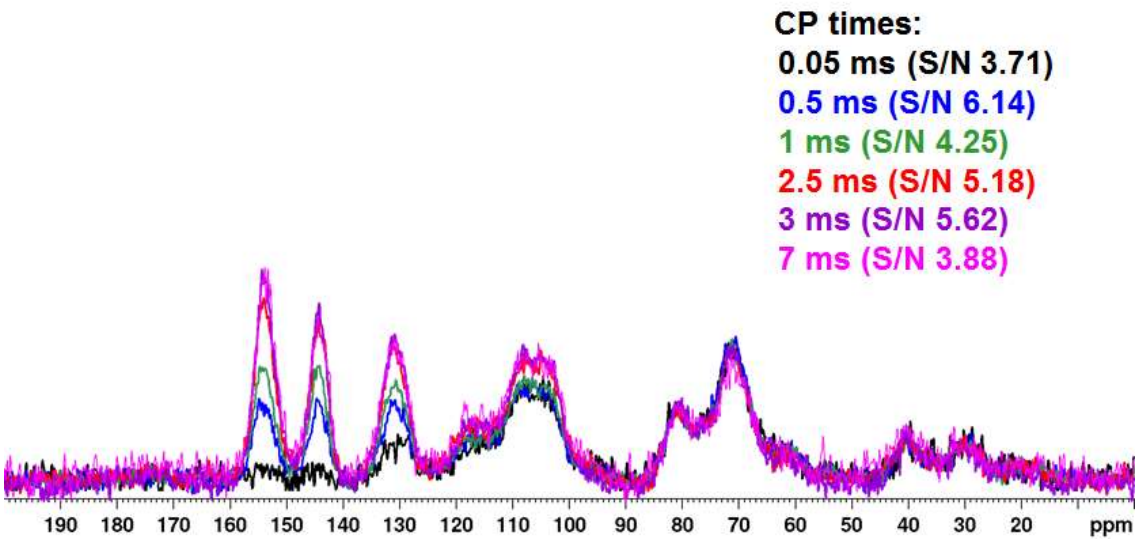

Figure 7-17: ^{13}C NMR spectra for mimosa tannin, acquired at different CP times (at 125 MHz).

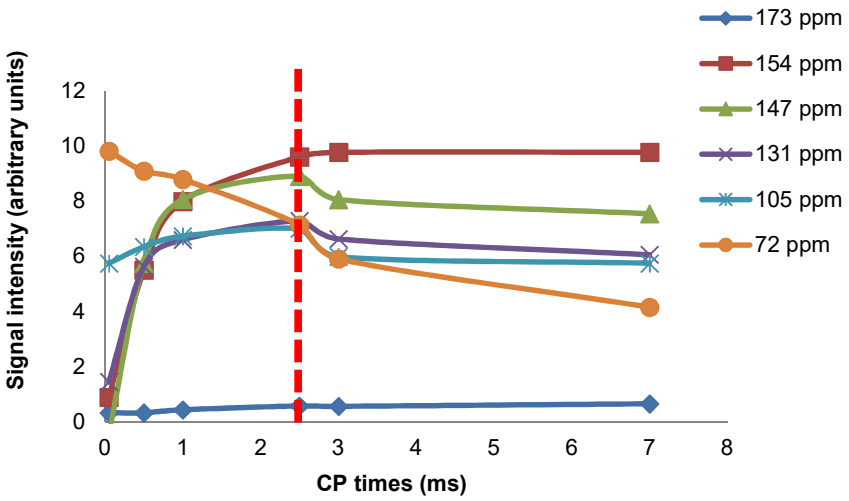

Figure 7-18: Signal intensities *vs.* CP times for mimosa tannin, at different chemical shifts.

Characteristic signals which are predominantly due to tannins at 131 ppm, 147 ppm and 154 ppm attain their maximum intensity at *ca.* 2.5 ms. In order to define the relative intensities of

the key tannin signals, 2.5 ms was chosen as the optimal CP time. The same value was also obtained by Reid *et al.* (2013).

^{13}C CP-MAS NMR analyses were then carried out on the mimosa, pyrogallol, control and test samples. Figure 7-19 presents the ^{13}C NMR spectrum for pyrogallol, while Figure 7-20 presents the ^{13}C NMR spectra for mimosa, control and test samples.

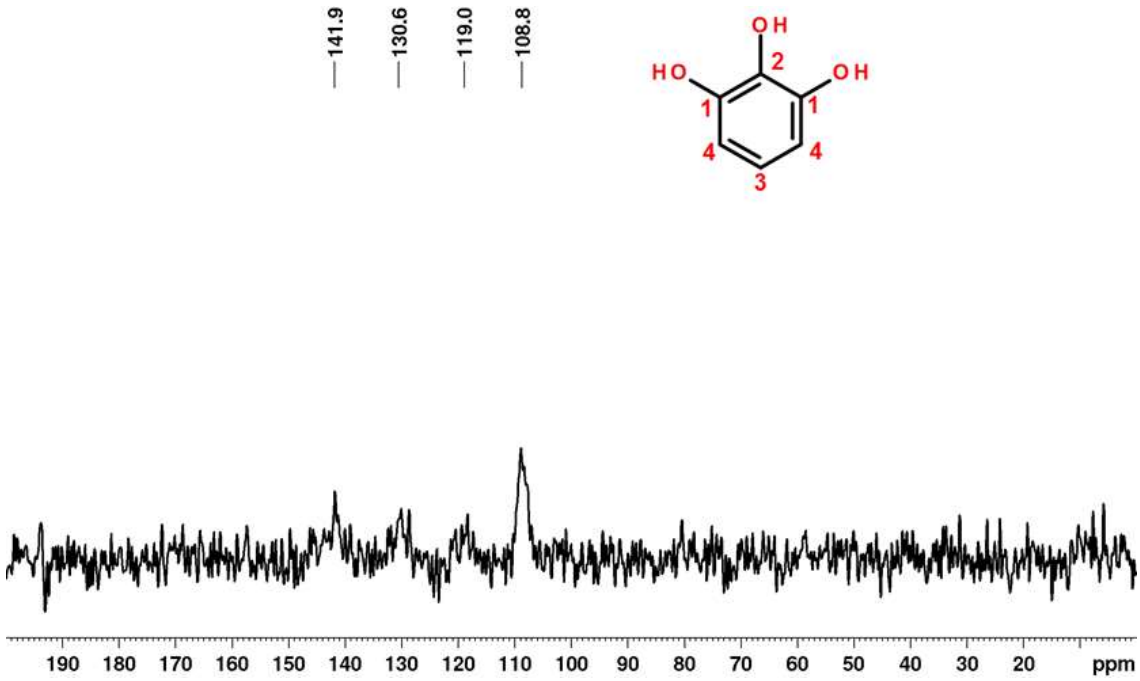

Figure 7-19: : ^{13}C CP-MAS solid state NMR spectrum for pyrogallol (500 MHz).

The signals shown in Figure 7-19 correspond to the following numbered carbons in the pyrogallol structure: C1: 141.9 ppm; C2: 130.6; C3: 119.0 ppm; C4: 108.8 ppm.

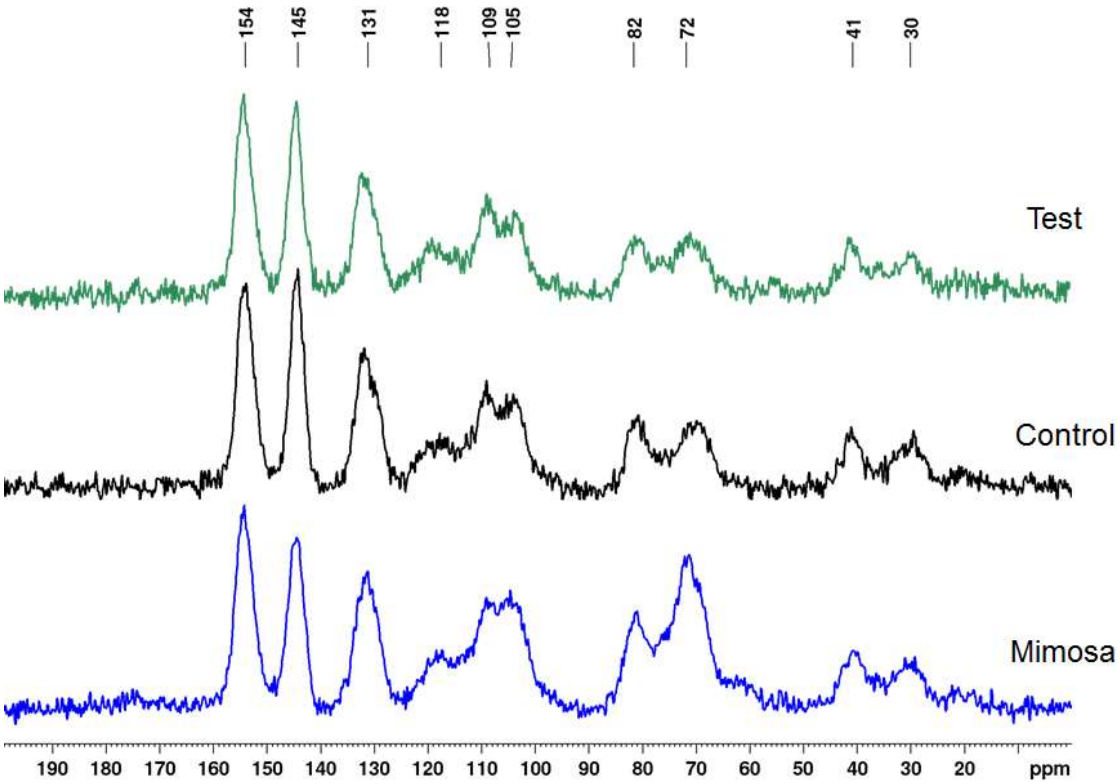

Figure 7-20: ^{13}C CP-MAS solid state NMR spectrum for unmodified mimosa; control and test samples subjected to acid cleavage with addition of pyrogallol (500 MHz).

As opposed to the ^{1}H NMR, ^{13}C NMR in the solid state shows that, under the identical conditions that were used to acquire the spectra, the signal to noise ratio for the samples were 17:1 for mimosa; 25:1 for control; 20:1 for test sample; and 4:1 for pyrogallol. The lower signal to noise ratio for pyrogallol demonstrates the lower intensity of its spectrum in comparison with the tannin samples. It is possible, therefore, to compare the samples submitted to the depolymerisation process with the unmodified mimosa tannin without the interferences experienced by the presence of pyrogallol in solution state NMR.

Table 7-2 presents the assignment of peaks to each carbon for mimosa tannin, as numbered in Figure 7-16 (Thompson and Pizzi, 1995; Hoong *et al.*, 2010; Reid *et al.*, 2013).

Table 7-2: Solid state ^{13}C CP-MAS NMR assignment of the signals obtained for mimosa tannin (Thompson and Pizzi, 1995; Hoong *et al.*, 2010; Reid *et al.*, 2013) and relative intensities (% of total area). Carbon numbers are given according to Figure 7-16.

Carbon number	*Chemical shift (ppm)*	*Relative intensities (% of total area)*		
		Mimosa	**Control**	**Test**
C4 internal	30	1.947	3.232	2.838
C4 terminal	41	3.507	3.720	3.508
C3	72	15.209	6.535	6.533
C2	82	7.929	6.748	5.523
C4-C8	105	10.692	8.063	10.229
C4-C6	109	11.878	12.720*	14.361*
C4a C2' C6' C5	118	7.376	10.691	9.474
C1' C4'	131	14.540	17.080	16.592
C3' C5'	145	12.119	15.357	15.885
C7 C8a	154	14.804	15.855	15.056

*Overestimated value, as the signal for C6 overlaps the signal for pyrogallol.

The signals at 82 and 72 ppm, attributed to C2 and C3, overlap the signals attributed to sugar moieties, located in the region between 60 and 90 ppm (Reid *et al.*, 2013). Figure 7-20 shows that unmodified mimosa presents peaks with higher intensity in this region, which may indicate that the samples subjected to the extraction with ethyl acetate (depolymerisation control and test samples) result in a lower sugar content.

For unmodified mimosa, the relative intensity of the C4-C6 signal is slightly higher than for C4-C8, showing the presence of branched interflavanyl bonds between C4 and C6. This is consistent with prorobinetinidin tannins, as the absence of a hydroxyl in the C5 position facilitates the polymerisation in the C6 position (Covington, 2009). For control and test samples, the overlapping of the pyrogallol signal with the C4-C6 signal overestimates the relative intensity of this peak.

7.4.4.2 Effect of solvent and temperature on the depolymerisation of mimosa tannin

As the acid-catalysed process shows potential in depolymerising the mimosa extract, the study was followed by an investigation of the solvent and temperature effect in condensed

tannin content, catechin equivalent and concentration of pyrogallol, measured by UHPLC, which indicates the consumption of pyrogallol in the depolymerisation reaction.

The characteristics of a solvent, such as polarity, have a strong influence in organic reactions (Reichardt and Welton, 2011). Alcoholic solvents such as ethanol and methanol are usually applied in the acid-catalysed depolymerisation of proanthocyanidins (Hagerman, 2002; Cheynier, 2006; Bordiga *et al.*, 2012). In order to verify if the alcoholic solvent could be replaced by water, tests were also carried out in aqueous medium.

Data obtained for the comparison of the depolymerisation process carried out in water and ethanol are presented in Figures 7-21 to 7-23.

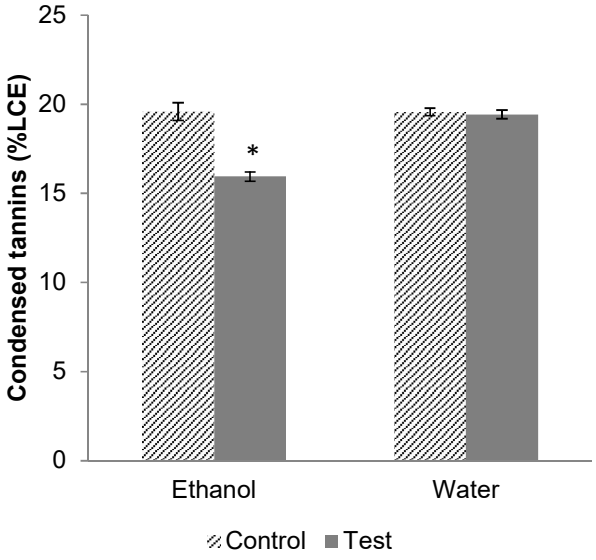

Figure 7-21: Condensed tannin content (% Leucocyanidin equivalent) for control and test samples with the depolymerisation process carried out in ethanol and water. Error bars denote the standard error of the mean (n=3 in duplicate). The (*) symbol indicates a significant difference (*t*-test, p=0.046).

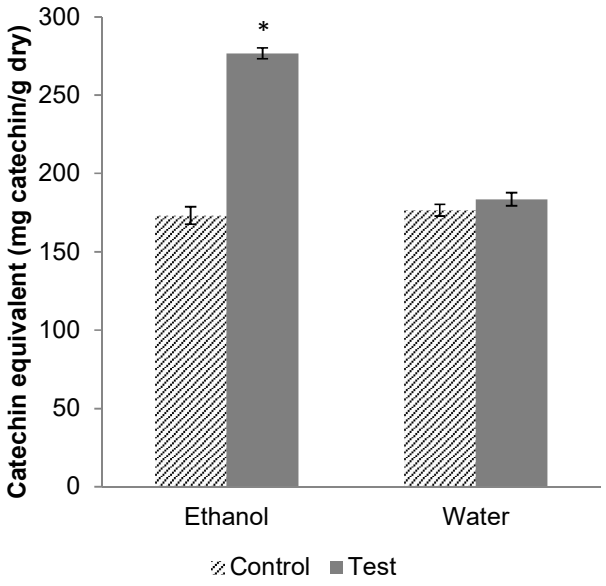

Figure 7-22: Catechin equivalent content (mg catechin/g dry sample) for control and test samples with the depolymerisation process carried out in ethanol and water. Error bars denote the standard error of the mean (n=3 in duplicate). The (*) symbol indicates a significant difference (*t*-test, p=0.026).

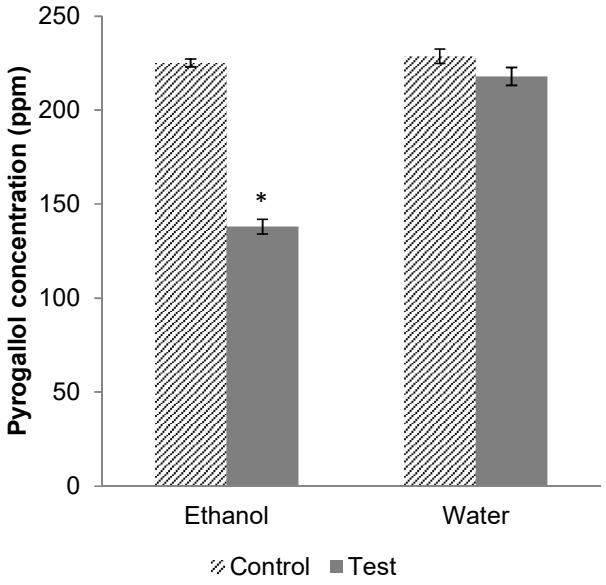

Figure 7-23: Pyrogallol concentration (ppm), analysed with UHPLC, for control and test samples with the depolymerisation process carried out in ethanol and water. Error bars denote the standard error of the mean (n=3 in duplicate). The (*) symbol indicates a significant difference (*t*-test, p=0.023).

Figure 7-21 shows that the condensed tannin content is significantly decreased by the depolymerisation process, from 19.59% for the control to 15.94% for the test sample, when ethanol is used as solvent. When the process is carried out in water, the condensed tannin content is not significantly different between control and test samples. When ethanol was used as solvent, the catechin equivalent is significantly higher for the test sample (276.62 mg catechin/g) in relation to the control (173.14 mg catechin/g) (Figure 7-22). There was no significant difference between control and test when the process was conducted in water. UHPLC analyses for the determination of residual pyrogallol (Figure 7-23) showed that the pyrogallol concentration was significantly lower for the test sample (137.99 ppm) in comparison with the control (225.12 ppm), when ethanol was used as a solvent. This indicates that pyrogallol was being consumed due to the acid cleavage reaction. No significant difference was found between test and control when the solvent used was water.

The mechanism of proanthocyanidin depolymerisation consists of releasing terminal units as flavan-3-ol monomers and extension units as intermediate carbocations, followed by the nucleophilic addition, forming a stable adduct (Tarascou *et al.*, 2010). According to Beart *et al.* (1985), the rate-determining step in acid-catalysed depolymerisation of proanthocyanidins is the initial protonation. Data regarding the comparison between solvents indicate that the solvation effect of water in the intermediate carbocation released by the acid cleavage is possibly hindering the nucleophilic addition. The solubility of phenolic compounds is slightly lower in water than in ethanol (Cuevas-Valenzuela *et al.*, 2014), which may also have contributed to the lower yield. Kennedy and Jones (2001) studied the phloroglucinolysis of grape proanthocyanins in methanol, and the authors determined that with a 20% addition of water to methanol, total formation of phloroglucinol adduct was reduced by 13%, confirming that the reaction yield is higher in an alcoholic medium. For this reason, ethanol was used as a solvent for subsequent experiments.

Temperature is a parameter that affects reaction rates. In order to verify if a higher yield in the depolymerisation may be achieved, tests were conducted with increasing temperature. The low thermal stability of polyphenols means that, when subjected to higher temperatures, they may undergo side reactions such as oxidation and degradation (Kyi *et al.*, 2005; Ross *et al.*, 2011; Pascariu *et al.*, 2014). For this reason, the tested temperatures of depolymerisation were 25°C, 35°C and 45°C.

Analysis of condensed tannin content, catechin equivalent and pyrogallol concentration, for the process carried out at 25°C, 35°C and 45°C, are shown in Figures 7-24 to 7-26.

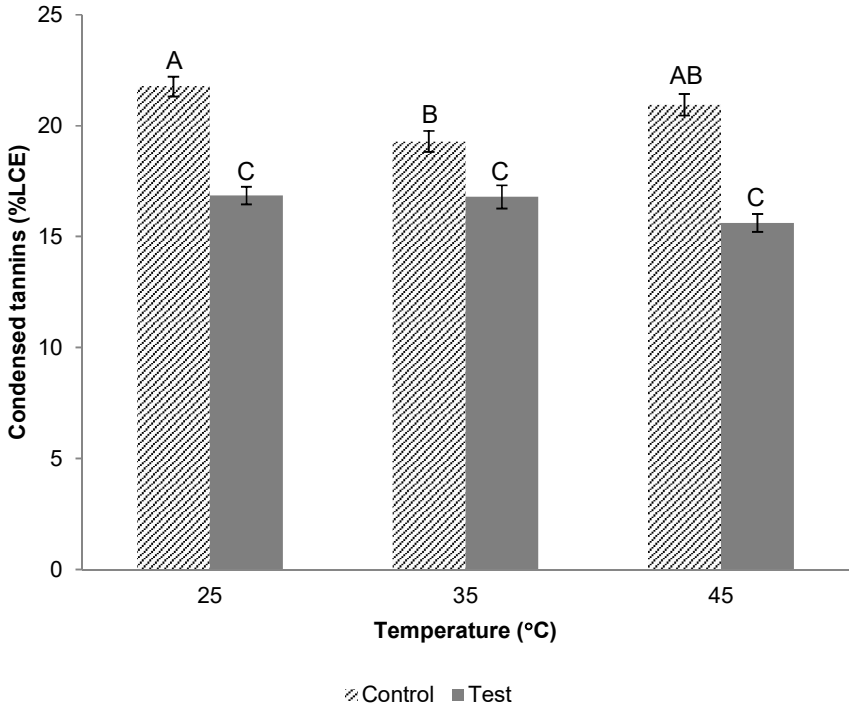

Figure 7-24: Condensed tannin content (% Leucocyanidin equivalent) for control and test samples with the depolymerisation process carried out at 25°C, 35°C and 45°C. Error bars denote the standard error of the mean (n=3 in duplicate). Means with different letters (A, B, C) are significantly different (Tukey's HSD, $p<0.05$). When both letters are used, *e.g.* AB, the mean is not significantly different from both A and B.

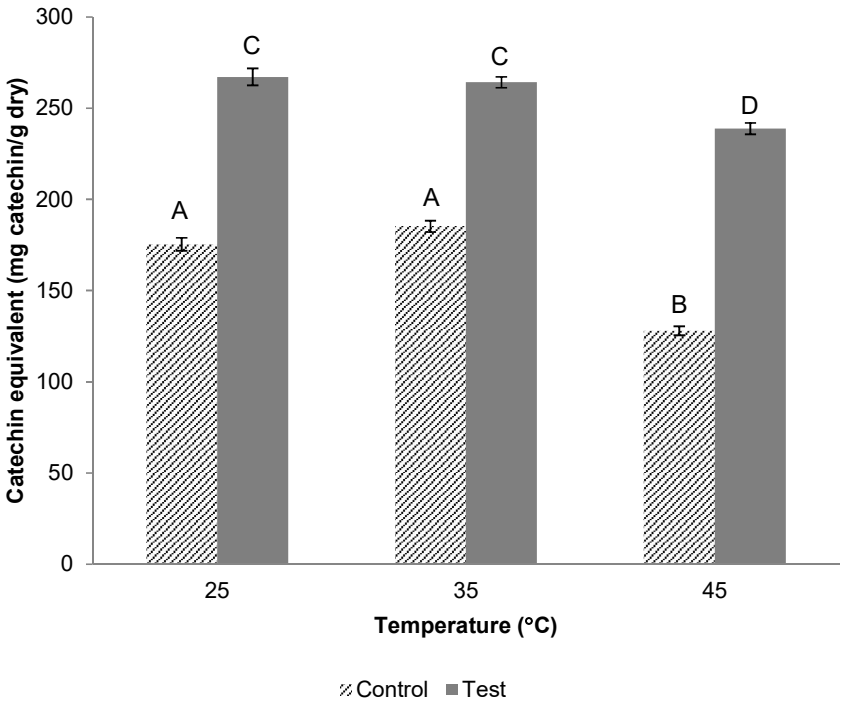

Figure 7-25: Catechin equivalent content (mg catechin/g dry sample) for control and test samples with the depolymerisation process carried out 25°C, 35°C and 45°C. Error bars denote the standard error of the mean (n=3 in duplicate). Means with different letters (A, B, C, D) are significantly different (Tukey's HSD, $p<0.05$).

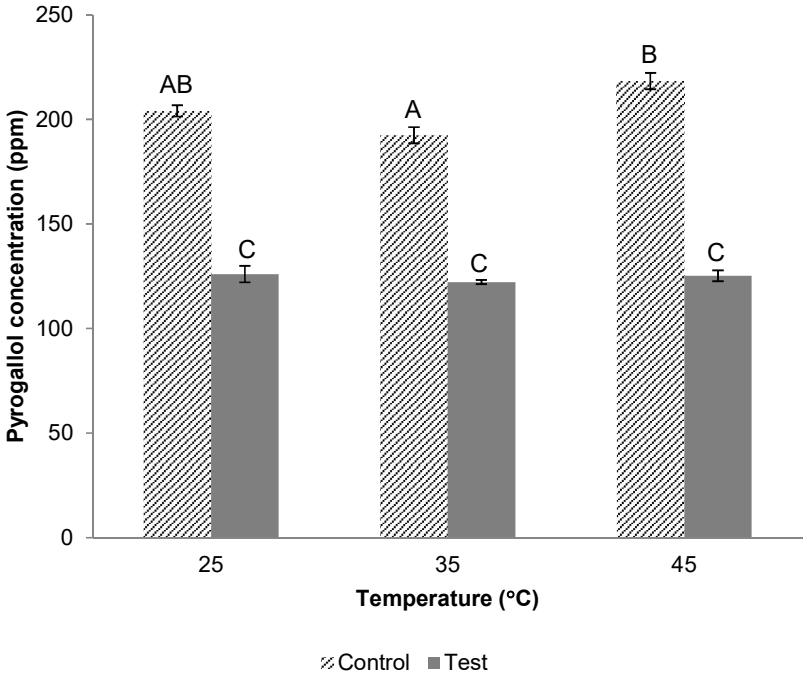

Figure 7-26: Pyrogallol concentration (ppm), analysed with UHPLC, for control and test samples with the depolymerisation process carried out 25°C, 35°C and 45°C. Error bars denote the standard error of the mean (n=3 in duplicate). Means with different letters (A, B, C) are significantly different (Tukey's HSD, $p<0.05$). When both letters are used, *e.g.* AB, the mean is not significantly different from both A and B.

For all analytical data (condensed tannin content, catechin equivalent and pyrogallol concentration), analysis of variance indicated a significant difference between the control and the test sample, for all temperatures studied ($p<0.001$). Regardless of temperature, the acid-catalysed process showed a decrease in polymeric proanthocyanidins, in comparison with the control, indicated by the lower condensed tannin content (Figure 7-24) and higher catechin equivalent (Figure 7-25) obtained with this process. The lower pyrogallol concentration (Figure 7-26) for the test samples indicate that its consumption is related to the formation of carbocations due to the breakage of the interflavanyl bond.

Data from the vanillin assay to measure the catechin equivalent (Figure 7-25) showed a significantly lower content in catechin when the depolymerisation was carried out at 45°C, for both test and control, upon comparison of the data obtained for the process carried out at 25°C and 35°C. According to Hernes and Hedges (2000), the terminal units are less stable to higher temperature than the adducted extender units. This indicates that, at higher

temperature, side reactions and/or degradation may be occurring in the monomeric units released by the acid cleavage.

When used as a method for determining the degree of polymerisation, ambient temperature is generally used for the acid-catalysed depolymerisation (Koupai-Abyazani, 1992; Hagerman, 2002). In order to standardise the process to a controllable temperature, 25°C can be used, as the increase in temperature to 35°C has not resulted in yield improvement, and further increase results in possible degradation of the monomers. Industrial processing in mild temperatures represent lower energy consumption, being an advantage in terms of economic and environmental perspectives.

7.4.4.3 Application on hide powder

The depolymerised products were applied to hide powder, followed by laccase-catalysed polymerisation and the process was evaluated through the change in shrinkage temperature (ΔTs).

Figure 7-27 present data for the depolymerised products obtained *via* acid cleavage in ethanol and water, applied in the stabilisation of hide powder. A sample omitting the enzyme was prepared for each product (control laccase).

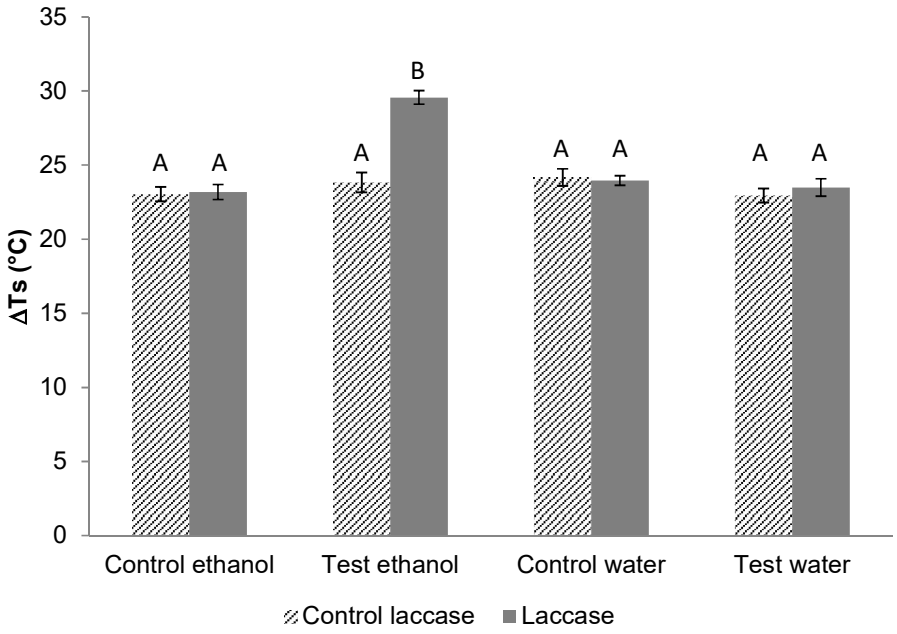

Figure 7-27: Change in shrinkage temperature (ΔTs) of hide powder, tanned with control and test samples subjected to acid cleavage with addition of pyrogallol carried out in ethanol and water. For each tanning product, a control omitting the enzyme and a sample containing the enzyme laccase were prepared. Error bars denote the standard error of the mean (n=3 in duplicate). Means with different letters (A, B) are significantly different (Tukey's HSD, $p<0.05$).

Data from Figure 7-27 show that the depolymerisation product controls, obtained in ethanol and water, have not presented statistically significant differences in shrinkage temperature, both for the samples omitting the enzyme (control laccase) and for the samples subjected to the enzymatic reaction.

Test sample obtained in water showed that values of shrinkage temperature were not statistically different from all other samples. This result corroborates with results for condensed tannin and catechin equivalent, which showed similar data to the control obtained with ethanol.

Test sample obtained in ethanol was statistically higher than the control, showing that the smaller molecules extracted with ethyl acetate (see Figure 7-22) were being polymerised by laccase, thus increasing the thermal stabilisation of hide powder. The average increase in shrinkage temperature obtained with the test sample subjected to polymerisation with laccase was 29.56°C, which is higher than usual values of 20 to 25°C obtained with vegetable tanning (Covington, 1997).

7.4.4.4 Application on goat skin

The laccase-catalysed polymerisation of depolymerised mimosa was also applied in tanning of goat skin. Figure 7-28 presents the change in shrinkage temperature (ΔTs) for control and test samples subjected to acid cleavage with addition of pyrogallol carried out in ethanol and 25°C, for samples omitting the enzyme (Control laccase), and samples incubated with laccase (Laccase).

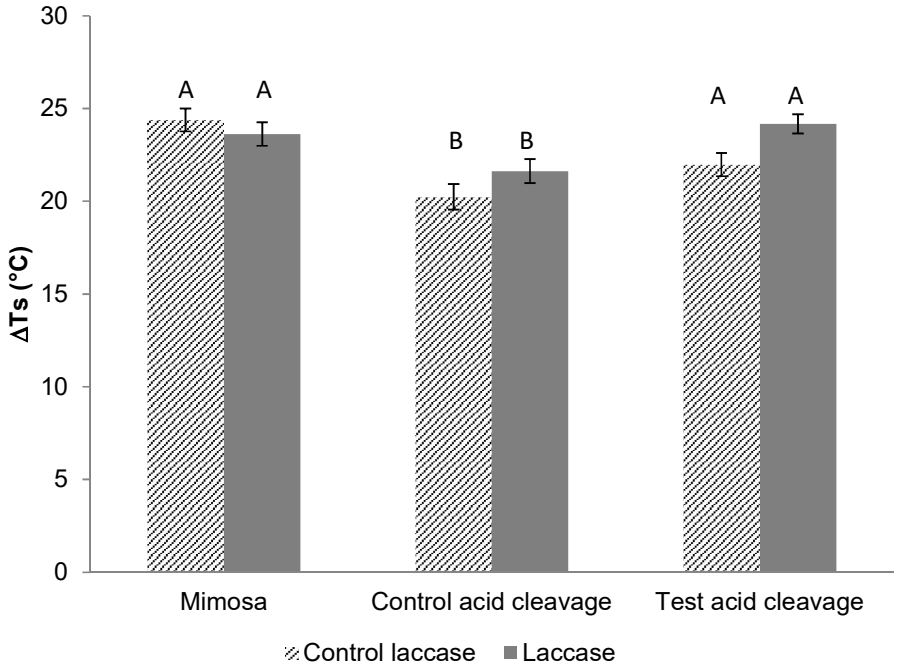

Figure 7-28: Change in shrinkage temperature (ΔTs) of goat skin, tanned with control and test samples subjected to acid cleavage with addition of pyrogallol carried out in ethanol and 25°C. For each tanning product (control and test), a control omitting the enzyme and a sample containing the enzyme laccase were prepared. Error bars denote the standard error of the mean (n=3 in duplicate). Means with different letters (A, B) are significantly different (Tukey's HSD, $p<0.05$).

Data presented in Figure 7-28 show that, for all products (mimosa, control acid cleavage and test acid cleavage), the difference between samples omitting the enzyme (Control laccase) and subjected to incubation with laccase (Laccase) was not statistically significant ($p = 0.088$).

For the application of unmodified mimosa, the sample which the enzyme was applied (Laccase), presented a slightly lower shrinkage temperature than the sample omitting the

enzyme (Control laccase). This difference, nevertheless, was not statistically significant. The control acid cleavage showed the lowest values for shrinkage temperature in comparison with unmodified mimosa and test acid cleavage. For the acid cleavage test sample, the sample subjected to incubation with laccase (sample laccase) showed slightly higher ΔTs in comparison with the sample omitting the enzyme (sample control laccase), although the difference was not statistically significant. The test sample subjected to the acid cleavage and laccase incubation (sample laccase) showed similar results in comparison with unmodified mimosa omitting the enzyme (sample control laccase), which demonstrates that the process of laccase-catalysed polymerisation of depolymerised mimosa achieves similar values of shrinkage temperature as the conventional process.

The experiments on goat skin show the potential of the laccase-catalysed polymerisation of depolymerised mimosa tannin on the stabilisation of collagen. The enzymatic process, however, must be further optimised in order to achieve similar results in comparison with hide powder.

7.5 Summary

Preliminary experiments using acid cleavage with addition of phloroglucinol showed that the process was effective in the depolymerisation of mimosa tannin. The depolymerised product, however, showed a small impact in the stabilisation of collagen. In order to increase the effect of laccase-catalysed polymerisation of the depolymerised product applied to the stabilisation of collagen, pyrogallol was used as nucleophile trapping agent in the acid cleavage depolymerisation of mimosa tannin.

Data indicates the potential of scaling-up production of depolymerised mimosa tannin *via* acid-catalysed process in the presence of pyrogallol, indicated by the decrease in condensed tannin content and increase in monomeric content for the acid-catalysed process, in comparison with a control. Depolymerisation of condensed tannins represents a new route in obtaining monomeric phenols for industrial applications, such as the production of adhesives and resins. The reaction was influenced by the solvent, as the depolymerisation effect was significant in ethanol and not significant when water was used. In terms of the reaction temperature, data show that the process may be carried out at mild temperature (25°C), representing low energy consumption. The lower molecular weight phenolic molecules

obtained with the depolymerisation of mimosa tannin may be presented as a prospective substitute of phenolics from other sources, such as petrochemical.

Application of depolymerised mimosa on the laccase-assisted stabilisation of hide powder and goat skin showed the potential of a new biotechnological route for a time effective process of vegetable tanning.

Chapter 8 Conclusions

This research presents a step forward in the direction of developing new processes for leather tanning (stabilisation of collagen). A more time-effective process for organic tanning has the potential to replace the use of metal compounds such as chromium in the stabilisation of hides and skins. This study represents an advance towards reducing the environmental impact of leather production.

Enzymatic catalysis showed that a non-tannin phenolic compound (catechin) can be transformed into a polyphenol with capacity of promoting crosslinks between collagen molecules. The environmental advantage of a biotechnological process expands the applicability to areas beyond the tanning of leather, such as the production of collagenic biomaterials. Biomaterials made from collagen are applied in tissue engineering as to function as cell scaffolds to replace native collagen. Usually aldehyde crosslinking agents are used, therefore, the laccase-catalysed polymerisation of catechin can be presented as an alternative crosslinking agent.

The original idea of the research was the search for an enzymatic method of depolymerisation of condensed tannins. As lignin has a similar chemical constitution as vegetable tannins, the laccase-mediator system, applied to the degradation of lignin was tested. The method showed unsatisfactory results, as the enzyme laccase was being inhibited by the mimosa extract, *i.e.*, the mimosa extract was binding to the enzyme.

Although the interflavanyl bond between building blocks of condensed tannins is stable, the method of acid-catalysed depolymerisation with the addition of a nucleophile showed that low molecular weight phenolics can be produced from high molecular weight tannins. Depolymerisation of condensed tannins represents a new route in obtaining monomeric phenols for industrial applications, such as the production of adhesives and resins. Phloroglucinol and pyrogallol can be used as nucleophile agent in the depolymerisation of mimosa, although pyrogallol is the preferred reagent for the application on leather tanning.

The production of low molecular weight phenolics showed that other methods for analysing tannin content had to be sought. The current methods used to evaluate tannin content in vegetable extracts used in leather tanning, which are based in tannin absorption by hide powder, showed not to be appropriate for samples with a low tannin content. The methods rely on outdated theories and simple apparatus. With the advances in polyphenol chemistry and modern analytical techniques, more reliable results on tannin content can be obtained.

8.1 Suggestions for further research

Other solvents can also be applied in experiments aiming at optimising the solvent extraction of low molecular weight phenols. Solvents such as diethyl ether and methyl ethyl ketone can be used and the extraction yield compared with the extraction using ethyl acetate.

The process of mimosa depolymerisation, presented in this research in a laboratory scale, can be scaled-up to a pilot scale. The scale-up can provide further information on the financial viability of the process.

Further studies on the application of depolymerised mimosa in leather tanning can be carried out. The depolymerised mimosa extract can be applied in combination tanning with aldehyde compounds such as oxazolidine, aiming at exploring the synergetic effect of polyphenol-aldehyde tanning (Covington, 2009). Covington *et al.* (2005) have applied combination tanning of green tea extract and oxazolidine to hide powder, having found an increase in shrinkage temperature of 33°C for the combination tanning in comparison with the green tea extract alone. This shows the potential of facilitating penetration of organic tanning agents into the hide/skin thickness achieving similar thermal stabilisation results as chromium tanning.

Further analytical work can be undertaken, aiming at achieving a deep understanding of the molecular conformation of the depolymerised mimosa extract. The analyses may be carried out by performing chromatographic fractionation using size exclusion beads, aiming at separating the different molecular weight molecules. The fractions may be analysed using NMR, which enables the study of the polyphenol stereochemistry and its influence in the reactions with collagen and/or with aldehydic crosslinkers.

An optimisation of the laccase-catalysed polymerisation of depolymerised mimosa *in situ* may be carried out for goat skins and bovine hides. The colour conferred by the quinones generated by the laccase-catalysed polymerisation may be explored as a pre-dyeing treatment.

References

ADZET, J.M.A., 1985. *Quimica tecnica de teneria.* Igualada: Romanya/Valls.

AKROUM, S., HAADI, M.L. and LALAOUI, K., 2009. Fungal tannase degrading condensed tannins of *Camellia sinensis* and measure of the enzyme activity on quebracho. *Middle-east Journal of Scientific Research,* **4**, pp. 237-241.

AKTAŞ, N. and TANYOLAÇ, A., 2003. Kinetics of laccase-catalyzed oxidative polymerization of catechol. *Journal of Molecular Catalysis B: Enzymatic,* **22**(1–2), pp. 61-69.

ALI, H.M., ALMAGRIBI, W. and AL-RASHIDI, M.N., 2016. Antiradical and reductant activities of anthocyanidins and anthocyanins, structure–activity relationship and synthesis. *Food Chemistry,* **194**, pp. 1275-1282.

ANTONY, J., 2003. *Design of experiments for engineers and scientists.* Oxford: Elsevier Butterworth-Heinemann.

ARBENZ, A. and AVEROUS, L., 2015. Chemical modification of tannins to elaborate aromatic biobased macromolecular architectures. *Green Chemistry,* **17**(5), pp. 2626-2646.

ASTM INTERNATIONAL, 2003. *Standard Practice for Establishing Color and Gloss Tolerances.* ASTM D3134. West Conshohocken: ASTM.

BABU, P.R., PINNAMANENI, R. and KOONA, S., 2012. Occurrences, Physical and Biochemical Properties of Laccase. *Universal Journal of Environmental Research and Technology,* **2**(1), pp. 1-13.

BANSAL, R.K., 2003. *Textbook of organic chemistry.* New Delhi: New Age International.

BARK, K.M., YEOM, J.E., YANG, J.I., PARK, C.H. and PARK, H.R., 2011. Spectroscopy studies on the oxidation of catechin in aqueous solution. *Bulletin of the Korean Chemical Society,* **32**(9), pp. 3443-3447.

BATE-SMITH, E.C. and SWAIN, T., 1962. Flavonoid compounds. In: H.S. MASON and A.M. FLORKIN, eds, *Comparative Biochemistry.* Vol. 3. New York: Academic Press, pp. 705-809.

BAUSCHER, M. and MANTELE, W., 1992. Electrochemical and Infrared-Spectroscopic Characterization of Redox Reactions of p-Quinones. *Journal of Physical Chemistry,* **96**, pp. 11101-11118.

BEART, J.E., LILLEY, T.H. and HASLAM, E., 1985. Polyphenol interactions. Part 2. Covalent binding of procyanidins to proteins during acid-catalysed decomposition: Observations on some polymeric proanthocyanidins. *Journal of the Chemical Society, Perkin Transactions 2,* (9), pp. 1439-1443.

BELLA, J., BRODSKY, B. and BERMAN, H.M., 1995. Hydration structure of a collagen peptide. *Structure,* **3**(9), pp. 893-906.

BENYAHYA, S., AOUF, C., CAILLOL, S., BOUTEVIN, B., PASCAULT, J.P. and FULCRAND, H., 2014. Functionalized green tea tannins as phenolic prepolymers for bio-based epoxy resins. *Industrial Crops and Products,* **53**, pp. 296-307.

BERG, J.M., TYMOCZKO, J.L. and STRYER, L., 2002. *Biochemistry.* 5th edn. New York: W. H. Freeman.

BERGMAN, J.A. and KESSLER, M.R., 2015. Monomers and resulting polymers from biomass. In: J. CLARK and F. DESWARTE, eds, *Introduction to chemicals from biomass.* 2nd edn. Chichester: John Wiley & Sons, pp. 157-193.

BEZERRA, M.A., SANTELLI, R.E., OLIVEIRA, E.P., VILLAR, L.S. and ESCALEIRA, L.A., 2008. Response surface methodology (RSM) as a tool for optimization in analytical chemistry. *Talanta,* **76**(5), pp. 965-977.

BEZERRA, R.M.F., FRAGA, I. and DIAS, A.A., 2013. Utilization of integrated Michaelis–Menten equations for enzyme inhibition diagnosis and determination of kinetic constants using Solver supplement of Microsoft Office Excel. *Computer methods and programs in biomedicine,* **109**(1), pp. 26-31.

BHAT, T.K., BHUPINDER, S. and SHARMA, O.P., 1998. Microbial degradation of tannins – A current perspective. . *Biodegradation,* **9**, pp. 343-357.

BIANCHI, S., KROSLAKOVA, I., JANZON, R., MAYER, I., SAAKE, B. and PICHELIN, F., 2015. Characterization of condensed tannins and carbohydrates in hot water bark extracts of European softwood species. *Phytochemistry,***120**, pp. 53-61.

BIENKIEWICZ, K.J., 1983. *Physical chemistry of leather making.* Malabar: Robert E. Krieger.

BOND, T., LEWIS, J.R., DAVIS, A. and DAVIES, A.P., 2003. Analysis and purification of catechins and their transformation products. In: C. SANTOS-BUELGA and G. WILLIAMSON, eds, *Methods in polyphenol analysis.* Cambridge: The Royal Society of Chemistry, pp. 229-313.

BORDIGA, M., COÏSSON, J.D., LOCATELLI, M., ARLORIO, M. and TRAVAGLIA, F., 2013. Pyrogallol: an Alternative Trapping Agent in Proanthocyanidins Analysis. *Food Analytical Methods,* **6**, pp. 148-156.

BORDONI, A., HRELIA, S., ANGELONI, C., GIORDANO, E., GUARNIERI, C., CALDARERA, C.M. and BIAGI, P.L., 2002. Green tea protection of hypoxia/reoxygenation injury in cultured cardiac cells. *Journal of Nutritional Biochemistry,* **13**, pp. 103-111.

BOUDET, A., 2007. Evolution and current status of research in phenolic compounds. *Phytochemistry,* **68**(22–24), pp. 2722-2735.

BRAND-WILLIAMS, W., CUVELIER, M.E. and BERSET, C., 1995. Use of a free radical method to evaluate antioxidant activity. *LWT - Food Science and Technology,* **28**(1), pp. 25-30.

BRIELMANN, H.L., SETZER, W.N., KAUFMAN, P.B., KIRAKOSYAN, A. and CSEKE, L.J., 2006. Phytochemicals: The Chemical Components of Plants. In: L.J. CSEKE, A. KIRAKOSYAN, P.B. KAUFMAN, S. WARBER, J.A. DUKE and H.L. BRIELMANN, eds, *Natural Products from Plants.* 2nd edn. Boca Raton: Taylor & Francis, pp. 1-42.

BRITISH STANDARDS INSTITUTE, 2012. *Leather — Chemical tests — Quantitative analysis of tanning agents by filter method.* BS EN ISO 14088. Brussels: European Committee for Standardization.

BRITISH STANDARDS INSTITUTE, 2012. *Leather — Physical and mechanical tests — Sample preparation and conditioning.* BS EN ISO 2419. Brussels: European Committee for Standardization.

BRODSKY, B. and PERSIKOV, A.V., 2005. Molecular Structure of the Collagen Triple Helix. *Advances in Protein Chemistry,* **70**, pp. 301-339.

BRODSKY, B. and RAMSHAW, J.A.M., 1997. The collagen triple-helix structure. *Matrix Biology,* **15**(8–9), pp. 545-554.

BROUILLARD, B., 1982. Chemical Structure of Anthocyanins. In: P. MARKAKIS, ed, *Anthocyanins as Food Colors.* New York: Academic Press, pp. 1-40.

BROWN, E.M., 2009. Collagen - A Natural Scaffold for Biology and Engineering. *Journal of the American Leather Chemists Association,* **104**(8), pp. 275-285.

BROWN, E.M., FARRELL, H.M. and WILDERMUTH, R.J., 2000. Influence of Neutral Salts on the Hydrothermal Stability of Acid-Soluble Collagen. *Journal of protein chemistry,* **19**(2), pp. 85-92.

BURTON, S.G., 2003. Oxidizing enzymes as biocatalysts. *TRENDS in Biotechnology,* **21**(12), pp. 543-549.

BURTON, S.G., BOSHOFF, A., EDWARDS, W. and ROSE, P.D., 1998. Biotransformation of phenols using immobilised polyphenol oxidase. *Journal of Molecular Catalysis B: Enzymatic,* **5**, pp. 411-416.

CALL, H.P. and MÜCKE, I., 1997. History, overview and applications of mediated lignolytic systems, especially laccase-mediator-systems (Lignozym®-process). *Journal of Biotechnology,* **53**(2–3), pp. 163-202.

CANALS, T., MORERA, J.M., COMBALIA, F. and BARTOLI, E., 2011. Infrared spectroscopy (FTIR and NIR) possibilities to determine the tanning degree of the vegetable leather. *XXXI IULTCS Congress* 2011.

CASTANEDA-OVANDO, A., PACHECO-HERNÁNDEZ, M.D.L., PÁEZ-HERNÁNDEZ, M.E., RODRÍGUEZ, J.A. and GALÁN-VIDAL, C.A., 2009. Chemical studies of anthocyanins: A review. *Food Chemistry,* **113**(4), pp. 859-871.

CHAPLIN, M.F. and BUCKE, C., 1990. *Enzyme Technology.* Cambridge: Cambridge University Press.

CHATTERJEE, S. and HADI, A.S., 2012. *Regression analysis by example.* 5th edn. Hoboken: John Wiley & Sons.

CHEYNIER, V., 2006. Flavonoids in wine. In: O. ANDERSEN and K.R. MARKAM, eds, *Flavonoids: Chemistry, biochemistry and applications.* Boca Raton: CRC Press, pp. 263-318.

CHEYNIER, V. and FULCRAND, H., 2003. Analysis of polymeric proanthocyanidins and complex polyphenols. In: C. SANTOS-BUELGA and G. WILLIAMSON, eds, *Methods in polyphenol analysis.* Cambridge: The Royal Society of Chemistry, pp. 284-313.

CHEYNIER, V., SCHNEIDER, R., SALMON, J. and FULCRAND, H., 2010. Chemistry of Wine. In: H. LIU and L. MANDER, eds, *Comprehensive Natural Products II.* Oxford: Elsevier, pp. 1119-1172.

CLAUS, H., 2004. Laccases: structure, reactions, distribution. *Micron,* **35**, pp. 93-96.

COATES, J., 2000. Interpretation of Infrared Spectra, a Practical Approach. In: R.A. MEYERS, ed, *Encyclopedia of Analytical Chemistry.* Chichester: John Wiley & Sons Ltd., pp. 10815-10837.

COLLIGHAN, R.J., LI, X., PARRY, J., GRIFFIN, M. and CLARA, S., 2004. Transglutaminases as tanning agents for the leather industry. *Journal of the American Leather Chemists Association,* **99**(7), pp. 293-298.

COUTO, S.R. and HERRERA, J.L.T., 2006. Industrial and biotechnological applications of laccases: A review. *Biotechnology Advances,* **24**, pp. 500-513.

COVINGTON, A.D., 2009. *Tanning chemistry: The science of leather.* Cambridge: The Royal Society of Chemistry.

COVINGTON, A.D., 2008. Quo vadit chromium? The future directions of tanning. *Journal of the American Leather Chemists Association,* **103**(1), pp. 7-23.

COVINGTON, A.D., 1997. Modern tanning chemistry. *Chemical Society Reviews,* pp. 111-126.

COVINGTON, A.D., EVANS, C.S. and SUPARNO, O., 2003. A new application of biotechnology in leather dyeing. *Proceeding PubSci AEIF,* **3**(1), pp. 18.

COVINGTON, A.D., LILLEY, T.H. and SONG, L., 2005. Collagen and polyphenols: New relationships and new outcomes. Part 1. Flavonoid reactions for new tanning processes. *Journal of the American Leather Chemists Association,* **100**(9), pp. 325-335.

COVINGTON, A.D. and SHI, B., 1998. High stability organic tanning using plant polyphenols. Part 1. The interactions between vegetable tannins and aldehydic crosslinkers. *Journal of the Society of Leather Technologists and Chemists,* **82**, pp. 64-71.

CUEVAS-VALENZUELA, J., GONZÁLEZ-ROJAS, A., WISNIAK, J., APELBLAT, A. and PÉREZ-CORREA, J.R., 2014. Solubility of (+)-catechin in water and water-ethanol mixtures within the temperature range 277.6–331.2 K: Fundamental data to design polyphenol extraction processes. *Fluid Phase Equilibria,* **382**, pp. 279-285.

DAI, J. and MUMPER, R.J., 2010. Review Plant Phenolics: Extraction, Analysis and Their Antioxidant and Anticancer Properties. *Molecules,* **15**, pp. 7313-7352.

DAVIES, K. and SCHWINN, K., 2005. Molecular Biology and Biotechnology of Flavonoid Biosynthesis. CRC Press, pp. 143-218.

DE FREITAS, V. and MATEUS, N., 2001. Structural Features of Procyanidin Interactions with Salivary Proteins. *Journal of Agricultural and Food Chemistry,* **49**(2), pp. 940-945.

DE VILLIERS, A., VENTER, P. and PASCH, H., 2016. Recent advances and trends in the liquid-chromatography–mass spectrometry analysis of flavonoids. *Journal of Chromatography A,* **1430**, pp. 16-78.

DEPALLE, B., QIN, Z., SHEFELBINE, S.J. and BUEHLER, M.J., 2015. Influence of cross-link structure, density and mechanical properties in the mesoscale deformation mechanisms of collagen fibrils. *Journal of the Mechanical Behavior of Biomedical Materials,* **52**, pp. 1-13.

DESAI, S.S., TENNALI, G.B., CHANNUR, N., ANUP, A.C., DESHPANDE, B.P. and MURTUZA, A., 2011. Isolation of laccase producing fungi and partial characterization of laccase. *Biotechnology, Bioinformatics and Bioengeneering,***1**(4), pp. 543-549.

DEWHURST, J., 1999. Chrome tanning - A tanner's view. In: M.K. LEAFE, ed, *Leather Technologists Pocket Book.* Withernsea: The Society of Leather Technologists and Chemists, pp. 45-56.

DRAPER, N.R. and SMITH, H., 1998. *Applied regression analysis.* 3rd edn. Hoboken: John Wiley & Sons.

DRAUZ, K., GROGER, H. and MAY, O., 2012. *Enzyme catalysis in organic synthesis.* 3rd edn. Darmstadt: Wiley-VCH.

DUVAL, A. and AVEROUS, L., 2016. Characterization and Physicochemical Properties of Condensed Tannins from Acacia catechu. *Journal of Agricultural and Food Chemistry,* **64**(8), pp. 1751-1760.

EL MANSOURI, N. and SALVADÓ, J., 2007. Analytical methods for determining functional groups in various technical lignins. *Industrial Crops and Products,* **26**(2), pp. 116-124.

FANG, Y., YANG, S. and WU, G., 2002. Free radicals, antioxidants, and nutrition. *Nutrition,* **18**(10), pp. 872-879.

FERNANDEZ-SANCHEZ, C., TZANOV, T., GUBITZ, G.M. and CAVACO-PAULO, A., 2002. Voltammetric monitoring of laccase-catalysed mediated reactions. *Bioelectrochemistry,* **58**, pp. 149-156.

FERREIRA, S.L.C., BRUNS, R.E., FERREIRA, H.S., MATOS, G.D., DAVID, J.M., BRANDAO, G.C., DA SILVA, E.G.P., PORTUGAL, L.A., DOS REIS, P.S., SOUZA, A.S. and DOS SANTOS, W.N.L., 2007. Box-Behnken design: An alternative for the optimization of analytical methods. *Analytica Chimica Acta,* **597**(2), pp. 179-186.

FERREIRA, S.L.C., DOS SANTOS, W.N.L., QUINTELLA, C.M., NETO, B.B. and BOSQUE-SENDRA, J.M., 2004. Doehlert matrix: a chemometric tool for analytical chemistry—review. *Talanta,* **63**(4), pp. 1061-1067.

FOO, L.Y. and KARCHESY, J.J., 1989. Chemical nature of phlobaphenes. In: R.W. HEMINGWAY and J.J. KARCHESY, eds, *Chemistry and significance of condensed tannins.* New York: Plenum Press, pp. 109-118.

FRASER, R.D.B. and TRUS, B.L., 1986. Molecular Mobility in the Gap Regions of Type 1 Collagen Fibrils. *Bioscience Reports,* **6**(2), pp. 221-226.

FUJII, H., NAKAGAWA, T., NISHOKA, H., SATO, E., HIROSE, A., UENO, Y., SUN, B., YOKOZAWA, B. and NONAKA, G., 2007. Preparation, Characterization, and Antioxidative Effects of Oligomeric Proanthocyanidin-L-Cysteine Complexes. *Journal of Agricultural and Food Chemistry,* **55**(4), pp. 1525-1531.

GAYATRI, R., SHARMA, A.K., RAJARAM, R. and RAMASAMI, T., 2001. Chromium(III)-Induced Structural Changes and Self-Assembly of Collagen. *Biochemical and biophysical research communications,* **283**(1), pp. 229-235.

GHOUL, M. and CHEBIL, L., 2012. Enzymatic Polymerization of Phenolic Compounds by Oxidoreductases. In: S.K. SHARMA, ed, *SpringerBriefs in Molecular Science: Green Chemistry for Sustainability.* Springer, pp. 1-47.

GIUDICI, C., VIOLA, M., TIRA, M.E., FORLINO, A. and TENNI, R., 2003. Molecular stability of chemically modified collagen triple helices. *FEBS letters,* **547**(1–3), pp. 170-176.

GOLDBETER, A., 2013. Oscillatory enzyme reactions and Michaelis–Menten kinetics. *FEBS letters,* **587**(17), pp. 2778-2784.

GROSS, G.G., 2008. From lignins to tannins: Forty years of enzyme studies on the biosynthesis of phenolic compounds. *Phytochemistry,* **69**(18), pp. 3018-3031.

GROSS, R.A., GANESH, M. and LU, W., 2010. Enzyme-catalysis breathes new life into polyester condensation polymerizations. *Trends in biotechnology,* **28**(8), pp. 435-443.

GUTHRIE-STRACHAN, J. and ANTUNES, A.P., 2013. Overview: Collagen and tanning. In: R. DANIELS and W. LANDMAN, eds, *The framework for leather manufacture.* Liverpool: World Trades Publishing Limited, pp. 118-125.

GUYOT, S., LE GUERNEVE, C., MARNET, N. and DRILLEAU, J.F., 1999. Methods for determining the degree of polymerization of condensed tannins: A new ^{1}H-NMR procedure applied to cider apple procyanidins. In: G.G. GROSS, R.W. HEMINGWAY and T. YOSHIDA, eds, *Plant Polyphenols 2: Chemistry, biology, pharmacology, ecology.* New York: Kluwer Academic.

GUYOT, S., VERCAUTEREN, J. and CHEYNIER, V., 1996. Structural determination of colourless and yellow dimers resulting from (+)-catechin coupling catalysed by grape polyphenoloxidase. *Phytochemistry,* **42**(5), pp. 1279-1288.

HAGERMAN, A.H., 2002. *Tannin Handbook.* Available: http://www.users.miamioh.edu/hagermae/. [06/03 (2013)].

HALLIWELL, B., 1995. How to characterize an antioxidant: an update. *Biochemical Society Symposium,* **61**, pp. 73-101.

HASLAM, E., 2007. Vegetable tannins – Lessons of a phytochemical lifetime. *Phytochemistry,* **68**, pp. 2713-2721.

HASLAM, E., 1998. *Practical polyphenolics: from structure to molecular recognition and physiological action.* Cambridge: Cambridge University Press.

HASLAM, E., 1997. Vegetable tannage: where do the tannins go? *Journal of the Society of Leather Technologists and Chemists,* **81**(2), pp. 45-51.

HASLAM, E., 1989. *Plant polyphenols: vegetable tannins revisited.* Cambridge: Cambridge University Press.

HE, Y., ZHU, B. and INOUE, Y., 2004. Hydrogen bonds in polymer blends. *Progress in Polymer Science,* **29**(10), pp. 1021-1051.

HEDBERG, Y.S., LIDÉN, C. and ODNEVALL WALLINDER, I., 2014. Correlation between bulk- and surface chemistry of Cr-tanned leather and the release of Cr(III) and Cr(VI). *Journal of hazardous materials,* **280**, pp. 654-661.

HEDIGER, S., MEIER, B.H. and ERNST, R.R., 1995. Adiabatic passage Hartmann-Hahn cross polarization in NMR under magic angle sample spinning. *Chemical Physics Letters,* **240**(5–6), pp. 449-456.

HEIDEMANN, E., 1993. *Fundamentals of leather manufacturing.* Darmstadt: Eduard Roether KG.

HEMINGWAY, R.W., 1989. Structural variations in proanthocyanidins and their derivatives. In: R.W. HEMINGWAY and J.J. KARCHESY, eds, *Chemistry and significance of condensed tannins.* New York: Plenum Press, pp. 83-107.

HERNES, P.J. and HEDGES, J.I., 2000. Determination of Condensed Tannin Monomers in Environmental Samples by Capillary Gas Chromatography of Acid Depolymerization Extracts. *Analytical Chemistry,* **72**, pp. 5115-5124.

HIBBERT, D.B., 2007. *Quality assurance in the analytical chemistry laboratory.* New York: Oxford University Press.

HOLMES, D.F., GILPIN, C.J., BALDOCK, C., ZIESE, U., KOSTER, A.J. and KADLER, K.E., 2001. Corneal collagen fibril structure in three dimensions: Structural insights into fibril assembly, mechanical properties, and tissue organization. *Proceedings of the National Academy of Sciences,* **98**(13), pp. 7307-7312.

HOONG, Y.B., PIZZI, A., MD. TAHIR, P. and PASCH, H., 2010. Characterization of Acacia mangium polyflavonoid tannins by MALDI-TOF mass spectrometry and CP-MAS ^{13}C NMR. *European Polymer Journal,* **46**(6), pp. 1268-1277.

HOU, D., FUJII, M., TERAHARA, N. and YOSHIMOTO, M., 2004. Molecular Mechanisms Behind the Chemopreventive Effects of Anthocyanidins. *Journal of Biomedicine and Biotechnology,* **2004**(5), pp. 321-325.

IKEDA, R., SUGIHARA, J., UYAMA, H. and KOBAYASHI, S., 1996. Enzymatic Oxidative Polymerization of 2,6-Dimethylphenol. *Macromolecules,* **29**, pp. 8702-8705.

INTERNATIONAL ORGANIZATION FOR STANDARDIZATION, 2005. *Determination of substances characteristic of green and black tea — Part 1: Content of total polyphenols in tea — Colorimetric method using Folin-Ciocalteu reagent.* ISO 14502-1. Geneva: ISO.

JAKOBEK, L., 2015. Interactions of polyphenols with carbohydrates, lipids and proteins. *Food Chemistry,* **175**, pp. 556-567.

JEONG, W. and KONG, A.T., 2004. Biological Properties of Monomeric and Polymeric Catechins: Green Tea Catechins and Procyanidins. *Pharmaceutical Biology,* **42**, pp. 84-93.

JOHANNES, C. and MAJCHERCZYK, A., 2000. Laccase activity tests and laccase inhibitors. *Journal of Biotechnology,* **78**(2), pp. 193-199.

JOHN, G., 1996. *Possible defects in leather production. Definitions, causes, consequences, remedies and types of leather.* Hemsbach: Druck Partner Rubelmann GmbH.

KANTH, S.V., VENBA, R., MADHAN, B., CHANDRABABU, N.K. and SADULLA, S., 2009. Cleaner tanning practices for tannery pollution abatement: Role of enzymes in eco-friendly vegetable tanning. *Journal of Cleaner Production,* **17**(5), pp. 507-515.

KARCHESY, J.J., BAE, Y., CHALKER-SCOTT, L., HELM, R.F. and FOO, L.Y., 1989. Chromatography of proanthocyanidins. In: R.W. HEMINGWAY and J.J. KARCHESY, eds, *Chemistry and significance of condensed tannins.* 1st edn. New York: Plenun Press, pp. 139-151.

KAWAGUCHI, H., HASUNUMA, T., OGINO, C. and KONDO, A., 2016. Bioprocessing of bio-based chemicals produced from lignocellulosic feedstocks. *Current opinion in biotechnology,* **42**, pp. 30-39.

KELLY, E.H., ANTHONY, R.T. and DENNIS, J.B., 2002. Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. *Journal of Nutritional Biochemistry,* **13**(10), pp. 572-584.

KENNEDY, J.A. and JONES, G.P., 2001. Analysis of Proanthocyanidin Cleavage Products Following Acid-Catalysis in the Presence of Excess Phloroglucinol. *Journal of Agricultural and Food Chemistry,* **49**, pp. 1740-1746.

KHURI, A.I. and MUKHOPADHYAY, S., 2010. Response surface methodology. *Wiley Interdisciplinary Reviews: Computational Statistics,* **2**(2), pp. 128-149.

KOBAYASHI, S. and HIGASHIMURA, H., 2003. Oxidative polymerization of phenols revisited. *Progress in Polymer Science,* **28**(6), pp. 1015-1048.

KOUPAI-ABYAZANI, M.R., MCCALLUM, J. and BOHM, B.A., 1992. Identification of the constituent flavanoid units in sainfoin proanthocyanidins by reversed-phase high-performance liquid chromatography. *Journal of Chromatography A,* **594**(1–2), pp. 117-123.

KRISHNAIAH, K. and SHAHABUDEEN, P., 2012. *Applied design of experiments and Taguchi methods.* 1st edn. New Delhi: PHI Learning Private Limited.

KUDANGA, T. and ROES-HILL, M., 2014. Laccase applications in biofuels production: current status and future prospects. *Applied Microbiology and Biotechnology,* **98**(15), pp. 6525-6542.

KUMAR, S. and PANDEY, A.K., 2013. Chemistry and Biological Activities of Flavonoids: An Overview. *The Scientific World Journal,* **2013**, pp. 1-16.

KUNAMNENI, A., CAMARERO, S., GARCÍA-BURGOS, C., PLOU, F.J., BALLESTEROS, A. and ALCALDE, M., 2008. Engineering and Applications of fungal laccases for organic synthesis. *Microbial Cell Factories,* **7**(32).

KURISAWA, M., CHUNG, J.E., UYAMA, H. and KOBAYASHI, S., 2003. Laccase-catalyzed synthesis and antioxidant property of poly(catechin). *Macromolecular Bioscience,* **3**, pp. 758-764.

KURNIAWATI, S. and NICELL, J.A., 2008. Characterization of *Trametes versicolor* laccase for the transformation of aqueous phenol. *Bioresource Technology,* **99**, pp. 7825-7834.

KURODA, Y. and HARA, Y., 1999. Antimutagenic and anticarcinogenic activity of tea polyphenols. *Mutation Research,* **436**, pp. 69-97.

KYI, T.M., DAUD, W.R.W., MOHAMMAD, A.B., WAHID SAMSUDIN, M., KADHUM, A.A.H. and TALIB, M.Z.M., 2005. The kinetics of polyphenol degradation during the drying of Malaysian cocoa beans. *International Journal of Food Science & Technology,* **40**(3), pp. 323-331.

LABARBE, B., CHEYNIER, V., BROSSAUD, F., SOUQUET, J. and MOUTOUNET, M., 1999. Quantitative Fractionation of Grape Proanthocyanidins According to Their Degree of Polymerization. *Journal of Agricultural and Food Chemistry,* **47**(7), pp. 2719-2723.

LANDETE, J.M., 2011. Ellagitannins, ellagic acid and their derived metabolites: A review about source, metabolism, functions and health. *Food Research International,* **44**(5), pp. 1150-1160.

LEWIS, N.G. and YAMAMOTO, E., 1989. Tannins - Their place in plant metabolism. In: R.W. HEMINGWAY and J.J. KARCHESY, eds, *Chemistry and Significance of Condensed Tannins.* New York: Plenum Press, pp. 23-46.

LI, K., GENG, X., SIMONSEN, J. and KARCHESY, J., 2004. Novel wood adhesives from condensed tannins and polyethylenimine. *International Journal of Adhesion and Adhesives,* **24**(4), pp. 327-333.

LI-QIN, Z., JIE, Z., SHU-HUA, Z. and LAI-HUI, G., 2009. Inhibition of browning on the surface of peach slices by short-term exposure to nitric oxide and ascorbic acid. *Food Chemistry,* **114**(1), pp. 174-179.

LÓPEZ-SERRANO, M. and ROS BARCELÓ, A., 2001. Reversed-phase and size-exclusion chromatography as useful tools in the resolution of peroxidase-mediated (+)-catechin oxidation products. *Journal of Chromatography A,* **919**(2), pp. 267-273.

LU, Z., LIAO, X. and SHI, B., 2003. The reaction of vegetable tannin-aldehyde-collagen: A further understanding of vegetable tannin-aldehyde combination tannage. *Journal of the Society of Leather Technologists and Chemists,* **87**(5), pp. 173-178.

LUNADEI, L., GALLEGUILLOS, P., DIEZMA, B., LLEÓ, L. and RUIZ-GARCIA, L., 2011. A multispectral vision system to evaluate enzymatic browning in fresh-cut apple slices. *Postharvest Biology and Technology,* **60**(3), pp. 225-234.

LUNDSTEDT, T., SEIFERT, E., ABRAMO, L., THELIN, B., NYSTROM, A., PETTERSEN, J. and BERGMAN, R., 1998. Experimental design and optimization. *Chemometrics and Intelligent Laboratory Systems,* **42**(1–2), pp. 3-40.

MACOMBER, R., 1996. *Organic chemistry - volume I.* Sausalito: University Science Books.

MADHAN, B., RAGHAVA RAO, J., SUBRAMANIAN, V., UNNI NAIR, B. and RAMASAMI, T., 2004. Role of phenolics in the stabilization of collagen. *Journal of the American Leather Chemists Association,* **99**(4), pp. 157-163.

MADHAN, B., SUBRAMANIAN, V., RAO, J.R., NAIR, B.U. and RAMASAMI, T., 2005. Stabilization of collagen using plant polyphenol: Role of catechin. *International journal of biological macromolecules,* **37**(1–2), pp. 47-53.

MADHAVI, V. and LELE, S.S., 2009. Laccase: Properties and applications. *Bioresources,* **4**(4), pp. 1694-1717.

MARAIS, J.P.J., DEAVOURS, B., DIXON, R.A. and FERREIRA, D., 2006. The stereochemistry of flavonoids. In: E. GROTEWOLD, ed, *The science of flavonoids.* Ohio: Springer, pp. 1-46.

MARTINEZ, S., 2003. Inhibitory mechanism of mimosa tannin using molecular modelling and substitutional adsorption isotherms. *Materials Chemistry and Physics,* **77**(1), pp. 97-102.

MATTHEWS, S., MILA, I., SCALBERT, A., POLLET, B., LAPIERRE, C., HERVE DU PENHOAT, C.L.M., ROLANDO, C. and DONNELLY, D.M.X., 1997. Method for Estimation of Proanthocyanidins Based on Their Acid Depolymerization in the Presence of Nucleophiles. *Journal of Agricultural and Food Chemistry,* **45**(4), pp. 1195-1201.

MAVLYANOV, S.M., ISLAMBEKOV, S.Y., ISMAILOV, A.I., DALIMOV, D.N. and ABDULLADZHANOVA, N.G., 2001. Vegetable tannin agents. *Chemistry of Natural Compounds,* **37**(1), pp. 1-24.

MCGRAW, G.W., 1989. Reactions at the A-ring of proanthocyanidins. In: R.W. HEMINGWAY and J.J. KARCHESY, eds, *Chemistry and significance of condensed tannins.* New York: Plenum Press, pp. 227-248.

MESPOUILLE, L., COULEMBIER, O., KAWALEC, M., DOVE, A.P. and DUBOIS, P., 2014. Implementation of metal-free ring-opening polymerization in the preparation of aliphatic polycarbonate materials. *Progress in Polymer Science,* **39**(6), pp. 1144-1164.

MIDDLETON, E.J., 1998. Effect of plant flavonoids on immune and inflammatory cell function. *Advances in Experimental Medicine and Biology,* **439**, pp. 175-182.

MILES C. A., AVERY, N.C., RODIN, V.V. and BAILEY, A.J., 2005. The Increase in Denaturation Temperature Following Cross-linking of Collagen is Caused by Dehydration of the Fibres. *Journal of Molecular Biology,* **346**, pp. 551-556.

MILES, C.A., KNOTT, L., SUMNER, I.G. and BAILEY, A.J., 1998. Differences between the thermal stabilities of the three triple-helical domains of type IX collagen. *Journal of Molecular Biology,* **277**(1), pp. 135-144.

MOLDES, D., LORENZO, M. and SANROMAN, M.A., 2004. Degradation or polymerisation of phenol red dye depending to the catalyst system used. *Process Biochemistry,* **39**, pp. 1811-1815.

MOLE, S. and WATERMAN, P.G., 1986. Tannic acid and proteolytic enzymes: Enzyme inhibition or substrate deprivation? *Phytochemistry,* **26**(1), pp. 99-102.

MONTGOMERY, D.C., 2009. *Design and analysis of experiments.* 7th edn. Hoboken: John Wiley & Sons, Inc.

MONTGOMERY, D.C., PECK, E.A. and VINING, G.G., 2012. *Introduction to linear regression analysis.* 5th edn. Hoboken: John Wiley & Sons.

MOROZOVA, O.V., SHUMAKOVICH, G.P., SHLEEV, S.V. and YAROPOLOV, Y.I., 2007. Laccase–Mediator Systems and Their Applications: A Review. *Applied Biochemistry and Microbiology,* **43**(5), pp. 523-535.

MU, C., LI, D., LIN, W., DING, Y. and ZHANG, G., 2007. Temperature induced denaturation of collagen in acidic solution. *Biopolymers,* **86**(4), pp. 282-287.

NDHLALA, A.R., KASIYAMHURU, A., MUPURE, C., CHITINDINGU, K., BENHURA, M.A. and MUCHUWETI, M., 2007. Phenolic composition of *Flacourtia indica*, *Opuntia megacantha* and *Sclerocarya birrea*. *Food Chemistry,* **103**(1), pp. 82-87.

NELSON, D.L. and COX, M.M., 2013. *Lehninger Principles of Biochemistry.* 6th edn. New York: W. H. Freeman and Company.

NONIER, M.F., VIVAS, N., VIVAS DE GAULEJAC, N. and FOUQUET, E., 2005. Development of direct size-exclusion chromatography separation for the determination of molar mass of native procyanidins in the phenolate form. *Journal of Chromatography A,* **1089**, pp. 263-269.

NUTSUBDIZE, N.N., SARKANEN, S., SCHMIDT, E.L. and SHASHIKANTH, S., 1998. Consecutive polymerisation and depolymerisation of kraft lignin by *Trametes cingulata*. *Phytochemistry,* **49**, pp. 1203-1212.

O'LEARY, D.N. and ATTENBURROW, G.E., 1996. Differences in strength between the grain and corium layers of leather. *Journal of Materials Science,* **31**(21), pp. 5677-5682.

ONEM, E., GULUMSER, G., RENNER, M. and YESIL-CELIKTAS, O., 2015. High pressure vegetable tanning of sheepskins using supercritical carbon dioxide. *The Journal of Supercritical Fluids,* **104**, pp. 259-264.

OSMA, J. F., TOCA-HERRERA, J. L. and RODRIGUEZ-COUTO, S., 2010. Uses of Laccases in the Food Industry. *Enzyme Research*, Article ID 918761, pp. 1-8.

OSMAN, A.M., WONG, K.K.Y. and FERNYHOUGH, A., 2007. The laccase/ABTS system oxidizes (+)-catechin to oligomeric products. *Enzyme and microbial technology,* **40**(5), pp. 1272-1279.

OZGUNAY, H., 2008. Lightfastness properties of leathers tanned with various vegetable tannins. *Journal of the American Leather Chemists Association,* **103**, pp. 345-351.

PALMER, T., 1981. *Understanding enzymes.* Chichester: Ellis Horwood Limited.

PAN, X., NIU, G. and LIU, H., 2003. Microwave-assisted extraction of tea polyphenols and tea caffeine from green tea leaves. *Chemical Engineering and Processing: Process Intensification,* **42**(2), pp. 129-133.

PASCARIU, S., POP, I., ALBU, A., 2014. Degradation Degree of Polyphenols Depending on the Drying Temperature of the Grape Pomace. *Bulletin of University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca.Animal Science and Biotechnologies,* **71**(2).

PATEL, K., JAIN, A. and PATEL, D.K., 2013. Medicinal significance, pharmacological activities, and analytical aspects of anthocyanidins 'delphinidin': A concise report. *Journal of Acute Disease,* **2**(3), pp. 169-178.

PIZZI, A., SIMON, C., GEORGE, B., PERRIN, D. and TRIBOULOT, M.C., 2004. Tannin antioxidant characteristics in leather versus leather light stability: models. *Journal of Applied Polymer Science,* **91**, pp. 1030-1040.

PIZZI, A., 2008. Chapter 8 - Tannins: Major Sources, Properties and Applications. In: M.N.B. GANDINI, ed, *Monomers, Polymers and Composites from Renewable Resources.* Amsterdam: Elsevier, pp. 179-199.

PORTER, L.J., 1992. Structure and chemical properties of the condensed tannins. In: R.W. HEMINGWAY and P.E. LAKS, eds, *Plant Polyphenols.* New York: Plenum Press.

PORTER, L.J., HRSTICH, L.N. and CHAN, B.G., 1986. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. *Phytochemistry,* **25**, pp. 223-230.

PRETZLER, M., JUS, S., STACHEL, I., MEYER, M. and GUBITZ, G.M., 2012. Cross-linking of collagen with laccases and tyrosinases. *5th Freiberg Collagen Symposium.* 2012.

PRIOR, R.L., LAZARUS, S.A., CAO, G., MUCCITELLI, H. and HAMMERSTONE, J.F., 2001. Identification of Procyanidins and Anthocyanins in Blueberries and Cranberries (*Vaccinium* Spp.) Using High-Performance Liquid Chromatography/Mass Spectrometry. *Journal of Agricultural and Food Chemistry,* **49**(3), pp. 1270-1276.

RAGUPATHY, L., ZIENER, U., DYLLICK-BRENZINGER, R., VON VACANO, B. and LANDFESTER, K., 2012. Enzyme-catalyzed polymerizations at higher temperatures: Synthetic methods to produce polyamides and new poly(amide-co-ester)s. *Journal of Molecular Catalysis B: Enzymatic,* **76**(0), pp. 94-105.

REICH, G., 2007. *From collagen to leather - The theoretical background.* Ludwigshafen: BASF Service Center.

REICHARDT, C. and WELTON, T., 2011. *Solvents and solvent effects in organic chemistry.* 4th edn. Weinheim: Wiley-VHC Verlag.

REID, D.G., BONNET, S.L., KEMP, G. and VAN DER WESTHUIZEN, J.H., 2013. Analysis of commercial proanthocyanidins. Part 4: Solid state ^{13}C NMR as a tool for in situ analysis of proanthocyanidin tannins, in heartwood and bark of quebracho and acacia, and related species. *Phytochemistry,* **94**, pp. 243-248.

RIBEREAU-GAYON, P., GLORIES, Y., MAUJEAN, A. and DUBOURDIEU, D., 2006. *Handbook of Enology - Volume 2.* 2nd edn. Chichester: John Wiley & Sons.

RICE-EVANS, C.A., MILLER, N.J. and PAGANGA, G., 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology and Medicine,* **20**(7), pp. 933-956.

RIGAUD, J., PEREZ-ILZARBE, J., DA SILVA, J.M.R. and CHEYNIER, V., 1991. Micro method for the identification of proanthocyanidin using thiolysis monitored by high-performance liquid chromatography. *Journal of Chromatography A,* **540**, pp. 401-405.

RINALDO, D., BATISTA JR, J.M., RODRIGUES, J., BENFATTI, A.C., RODRIGUES, C.M., SANTOS, L.C.D., FURLAN, M. and VILEGAS, W., 2010. Determination of catechin diastereomers from the leaves of *Byrsonima species* using chiral HPLC-PAD-CD. *Chirality,* **22**, pp. 726-733.

ROBBINS, C.T., MOLE, S., HAGERMAN, A.E. and HANLEY, T.A., 1987. Role of Tannins in Defending Plants Against Ruminants: Reduction in Dry Matter Digestion? *Ecology,* **68**(6), pp. 1606-1615.

ROCHDI, A., FOUCAT, L. and RENOU, J.P., 1999. Effect of thermal denaturation on water-collagen interactions: NMR relaxation and differential scanning calorimetry analysis. *Biopolymers,* **50**(7), pp. 690-696.

ROECKLEIN, J.C. and LEUNG, P., 1987. *A profile of economic plants.* New Brunswick: Transaction Books.

ROSS, C.F., HOYE, J., CLIFFORD and FERNANDEZ-PLOTKA, V.C., 2011. Influence of Heating on the Polyphenolic Content and Antioxidant Activity of Grape Seed Flour. *Journal of Food Science,* **76**(6), pp. C884-C890.

SAINI, B.L., 2010. *Introduction to biotechnology.* 1st edn. New Delhi: University Science Press.

SANTANA, G.S., KNICKER, H., GONZÁLEZ-VILA, F.J., GONZÁLEZ-PÉREZ, J.A. and DICK, D.P., 2015. The impact of exotic forest plantations on the chemical composition of soil organic matter in Southern Brazil as assessed by Py–GC/MS and lipid extracts study. *Geoderma Regional,* **4**, pp. 11-19.

SANTOS, L.M.D., 2006. *Influence of fatliquor properties and process variables of fatliquoring in soft leather production*, Universidade Federal do Rio Grande do Sul.

SANTOS-BUELGA, C., GARCIA-VIGUEIRA, C. and TOMAS-BARBERAN, F.A., 2003. On-line identification of flavonoids by HPLC coupled to diode array detection. In: C. SANTOS-BUELGA and G. WILLIAMSON, eds, *Methods in Polyphenol Analysis.* Cambridge: The Royal Society of Chemistry, pp. 92-127.

SCHOFIELD, P., MBUGUA, D.M. and PELL, A.N., 2001. Analysis of condensed tannins: a review. *Animal Feed Science and Technology,* **91**, pp. 21-40.

SCHUSTER, B. and HERRMANN, K., 1985. Hydroxybenzoic and hydroxycinnamic acid derivatives in soft fruits. *Phytochemistry,* **24**(11), pp. 2761-2764.

SEALY-FISHER, V.J. and PIZZI, A., 1992. Increased pine tannins extraction and wood adhesives development by phlobaphenes minimization. *Holz als Roh- und Werkstoff,* **50**(5), pp. 212-220.

SEBER, G.A.F. and LEE, A.J., 2003. *Linear regression analysis.* 2nd edn. Hoboken: John Wiley & Sons.

SELINHEIMO, E., 2008. *Tyrosinase and laccase as novel crosslinking tools for food biopolymers*, Helsinki University of Technology.

SENTHIVELAN, T., KANAGARAJ, J. and PANDA, R.C., 2016. Recent trends in fungal laccase for various industrial applications: An eco-friendly approach - A review. *Biotechnology and Bioprocess Engineering,* **21**(1), pp. 19-38.

SHARPHOUSE, J.H., 1983. *Leather technician's handbook.* 2nd edn. Northampton: Leather Producers' Association.

SIONKOWSKA, A., SKOPINSKA-WISNIEWSKA, J., GAWRON, M., KOZLOWSKA, J. and PLANECKA, A., 2010. Chemical and thermal cross-linking of collagen and elastin hydrolysates. *International journal of biological macromolecules,* **47**(4), pp. 570-577.

SLABBERT, N., 1992. Leather Manufacture with Wattle Tannins. In: R.W. HEMINGWAY and P.E. LAKS, eds, *Plant Polyphenols.* New York: Plenum Press, pp. 1021-1031.

SOCIETY OF LEATHER TECHNOLOGISTS AND CHEMISTS, 1996. *Specification of hide powder.* SLC 131. Northampton: SLTC.

SOCIETY OF LEATHER TECHNOLOGISTS AND CHEMISTS, 1996. *Determination of non-tannin constituents.* SLC 116. Northampton: SLTC.

SONG, L., 2003. *Basic studies of the cross-linking reaction of collagen with oxazolidine and plant polyphenols*, University College Northampton.

SOROURADDIN, M.H., AMINI, K., NASERI, A., VALLIPOUR, J., HANAEE, J. and RASHIDI, M.R., 2010. A new multi-wavelength model-based method for determination of enzyme kinetic parameters. *Journal of Biosciences,* **35**(3), pp. 395-403.

SUN, D., HE, Q., ZHANG, W., WANG, Y. and SHI, B., 2008. Evaluation of environmental impact of typical leather chemicals. Part II. Biodegradability of organic tanning agents by activated sludge. *Journal of the Society of Leather Technologists and Chemists,* **92**(2), pp. 59-64.

SUPARNO, O., 2005. *Phenolic reactions for leather tanning and dyeing*, University of Northampton.

TABACHNICK, B. and FIDDELL, L., 2007. *Experimental Design Using ANOVA.* Belmont: Thomson.

TARASCOU, I., SOUQUET, J.M., MAZAURIC, J.P., CARRILLO, S., COQ, S., CANON, F., FULCRAND, H. and CHEYNIER, V., 2010. The hidden face of food phenolic composition. *Archives of Biochemistry and Biophysics,* **501**(1), pp. 16-22.

TERS, T., KUNCINGER, T. and SREBOTNIK, E., 2009. Carboxylic acids used in common buffer systems inhibit the activity of fungal laccases. *Journal of Molecular Catalysis B: Enzymatic,* **61**(3–4), pp. 261-267.

THOMPSON, D. and PIZZI, A., 1995. Simple ^{13}C-NMR methods for quantitative determinations of polyflavonoid tannin characteristics. *Journal of Applied Polymer Science,* **55**, pp. 107-112.

THURSTON, C.F., 1994. The structure and function of fungal laccases. *Microbiology,* **140**, pp. 19-26.

TOMA, M., VINATORU, M., PANIWNYK, L. and MASON, T.J., 2001. Investigation of the effects of ultrasound on vegetal tissues during solvent extraction. *Ultrasonics Sonochemistry,* **8**(2), pp. 137-142.

TORREGGIANI, A., JURASEKOVA, Z., SANCHEZ-CORTES, S. and TAMBA, M., 2008. Spectroscopic and pulse radiolysis studies of the antioxidant properties of (+)catechin: metal chelation and oxidizing radical scavenging. *Journal of Raman Spectroscopy,* **39**, pp. 265-275.

TORRES, J.L., LOZANO, C., JULIÀ, L., SÁNCHEZ-BAEZA, F.J., ANGLADA, J.M., CENTELLES, J.J. and CASCANTE, M., 2002. Cysteinyl-flavan-3-ol Conjugates from Grape Procyanidins. Antioxidant and Antiproliferative Properties. *Bioorganic & medicinal chemistry,* **10**(8), pp. 2497-2509.

ULBER, R., SELL, D. and HIRTH, T., 2011. *Renewable raw materials: New feedstocks for the chemical industry.* Weinheim: Wiley-VCH.

UYAMA, H. and KOBAYASHI, S., 2005. Enzymatic Synthesis and Properties of Polymers from Polyphenols. *Advances in Polymer Science,* **194**, pp. 51-67.

UYAMA, H. and KOBAYASHI, S., 2002. Enzyme-catalyzed polymerization to functional polymers. *Journal of Molecular Catalysis B: Enzymatic,* **19–20**(0), pp. 117-127.

VAN DRIEL-MURRAY, C., 2000. Leatherwork and skin products. In: P.T. NICHOLSON and I. SHAW, eds, *Ancient Egyptian materials and technology.* Cambridge: Cambridge University Press, pp. 299-319.

VENTER, P.B., SENEKAL, N.D., KEMP, G., AMRA-JORDAAN, M., KHAN, P., BONNET, S.L. and VAN DER WESTHUIZEN, J.H., 2012. Analysis of commercial proanthocyanidins. Part 3: The chemical composition of wattle (Acacia mearnsii) bark extract. *Phytochemistry,* **83**, pp. 153-167.

VICENTE, G., COTERON, A., MARTINEZ, M. and ARACIL, J., 1998. Application of the factorial design of experiments and response surface methodology to optimize biodiesel production. *Industrial Crops and Products,* **8**(1), pp. 29-35.

VISWANATH, B., RAJESH, B., JANARDHAN, A., KUMAR, A.P.K. and NARASIMHA, A., 2014. Fungal Laccases and Their Applications in Bioremediation. *Enzyme Research,* **2014**, pp. 1-21.

WAITE, T., 1992. Tannin analysis - A 130 year problem. *Journal of the Society of Leather Technologists and Chemists,* **76**(6), pp. 187-194.

WANG, H., TUCKER, M. and JI, Y., 2013. Recent Development in Chemical Depolymerization of Lignin: A Review. *Journal of Applied Chemistry*, pp. 1-9.

WANG, L. and STONER, G.D., 2008. Anthocyanins and their role in cancer prevention. *Cancer letters,* **269**(2), pp. 281-290.

WATANABE, K., NAKAGAWA, J., EBIHARA, T. and OKAMOTO, Y., 1996. Structural effects of cross-linking reagents on triple-helix reformation of intramolecularly cross-linked collagen. *Polymer,* **37**(7), pp. 1285-1288.

WELLS, A., TERIA, M. and EVE, T., 2006. Green oxidations with laccase–mediator systems. *Biochemical Society transactions,* **34**(2), pp. 304-308.

WESS, T.J., 2008. Collagen Fibrillar Structure and Hierarchies. In: P. FRATZL, ed, *Collagen: Structure and Mechanics.* New York: Springer, pp. 49-74.

WILLIAMS, V.M., PORTER, L.J. and HEMINGWAY, R.W., 1983. Molecular weight profiles of proanthocyanidin polymers. *Phytochemistry,* **22**(2), pp. 569-572.

WOLKERS, W.F., 2009. Biomedical FTIR spectroscopy of lipids. In: A. BARTH and P.I. HARIS, eds, *Biological and biomedical infrared spectroscopy.* 1st edn. Amsterdam: IOS Press, pp. 272-287.

XU, C., ARANCON, R.A.D., LABIDI, J. and LUQUE, R., 2014. Lignin depolymerisation strategies: towards valuable chemicals and fuels. *Chemical Society Reviews,* **43**(22), pp. 7485-7500.

XU, Y.C., LEUNG, S.W.S., YEUNG, D.K.Y., HU, L.H., CHEN, G.H., CHE, C.M. and MAN, R.Y.K., 2007. Structure–activity relationships of flavonoids for vascular relaxation in porcine coronary artery. *Phytochemistry,* **68**(8), pp. 1179-1188.

XUEJIAO, S., RUBING, B., YA, Z., QIANG, W., XUERONG, F., JIUGANG, Y., LI, C. and PING, W., 2013. Laccase-Catalyzed Oxidative Polymerization of Phenolic Compounds. *Applied Biochemistry and Biotechnology,* **171**(7), pp. 1673-1680.

YANG, W., KENNY, J.M. and PUGLIA, D., 2015. Structure and properties of biodegradable wheat gluten bionanocomposites containing lignin nanoparticles. *Industrial Crops and Products,* **74**, pp. 348-356.

YESIL-CELIKTAS, O., OTTO, F., GRUENER, S. and PARLAR, H., 2009. Determination of Extractability of Pine Bark Using Supercritical CO_2 Extraction and Different Solvents: Optimization and Prediction. *Journal of Agricultural and Food Chemistry,* **57**(2), pp. 341-347.

ZHANG, C., LIN, J., JIA, X. and PENG, B., 2016. A salt-free and chromium discharge minimizing tanning technology: the novel cleaner integrated chrome tanning process. *Journal of Cleaner Production,* **112,** pp. 1055-1063.

Appendix 1 Data of colour measurements

Data of colour measurements (*L*, *a* and *b*), and calculated ΔE in relation to the reference sample, for samples as described in Table 5-2.

Run	*L*	*a*	*b*	ΔE
Reference	80.00	1.50	13.81	-
1	34.31	18.26	33.10	30.86
2	33.90	19.60	33.94	23.75
3	37.45	18.79	32.21	20.29
4	33.18	19.14	33.47	22.28
5	34.53	19.72	37.61	23.58
6	33.99	18.99	36.66	33.43
7	32.21	20.10	35.52	21.99
8	36.56	21.26	37.73	28.20
9	34.30	18.68	36.73	26.39
10	36.28	19.28	37.65	38.38
11	33.81	19.25	33.98	37.19
12	35.97	19.28	34.28	27.49
13	25.78	15.84	25.48	35.13
14	24.62	15.22	24.76	46.68
15	30.49	18.44	33.29	46.64
16	32.24	16.27	36.45	45.66
17	30.31	19.18	34.45	36.85
18	33.81	20.74	36.76	36.37
19	35.67	20.85	38.32	38.55

Data of colour measurements (*L*, *a* and *b*), and calculated ΔE for samples before and after exposure to light (light stability test).

Run	*L*	*a*	*b*	ΔE
1	36.28	17.50	31.67	2.55
2	33.40	17.73	32.17	2.62
3	35.99	17.34	30,68	2.56
4	33.57	18.26	31.58	2.12
5	34.54	18.39	35.28	2.68
6	34.13	16.39	33.51	2.85
7	33.07	19.04	33.73	2.09
8	34.38	20.40	36.29	2.75
9	34.31	17.98	34.98	1.88
10	34.95	18.06	36.18	2.33
11	34.55	17.87	33.08	1.80
12	34.66	18.21	32.15	2.72
13	27.40	15.07	27.76	2.90
14	26.73	15.18	26.85	2.97
15	33.15	17.50	34.20	2.96
16	30.56	15.94	34.94	2.28
17	33.11	18.55	33.83	2.94
18	33.63	18.52	33.96	2.18
19	34.97	19.42	36.65	2.31