Metal Induced Degradation of Leather

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I dedicate my work to my beloved parents

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ABSTRACT

Large quantities of leather waste are generated from manufacturing processes and disposal of leather goods. Environmental directives encourage diversion of solid waste from landfills through viable recycling methods. Since leather is resistant to microbial degradation, direct recycling of leather waste through biodegradation methods are impractical.

In this research, the mechanism and potential application of metal induced oxidative degradation of leather were studied. Experimental work on the effects of retanning vegetable tanned samples with salts of the first row transition metals indicated the presence of unstable tannin-metal interactions in samples containing V(IV), Fe(II) and Cu(II) salts. Denaturation of collagen at ambient conditions was observed in leather samples treated with V(IV) and Fe(II) salts.

Autodegradation of leather samples containing Fe(II) and V(IV) ions occurred during at ambient conditions of storage. The process of degradation in the leathers was characterised in terms of the decline in hydrothermal stability, progressive lowering of tensile strength, loss of metal-tannin binding interactions, changes in the fibre structure and the occurrence of gelatinisation. Direct evidence on the reduction of V(V) by tannins was obtained using electron paramagnetic resonance spectroscopy.

Results of spectrophotometric studies of polyphenol-metal ion interaction in acidic media indicated that Fe(II) and V(IV) ions act as catalysts in the oxidation of tannins. To explain the phenomena of metal induced degradation, a mechanism involving redox cycling of the iron ions (Fe^{2+}/Fe^{3+}) and vanadium ions (VO^{2+}/VO_2^{+}) was proposed. In the semi-metal tanned leathers, Fe(II) and V(IV) species are oxidised by air, producing superoxide anion. Subsequently Fe(III) and V(V) are reduced back to the lower oxidation states by the *o*-diphenol moieties of tannins. In this way, the metal ions act as catalysts for the oxidation of tannins.

Autoxidation of Fe(II) and V(IV) species in aqueous media results in the formation of superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2). The subsequent formation of hydroxyl radicals ($OH^{\cdot}$) in the leathers, through metal catalysed decomposition of hydrogen peroxide (Fenton reaction), is considered to be the major cause for the observed oxidative denaturation of the fibrous collagen. An investigation on the oxidative effect of a dilute solution of Fenton reagent (0.2M H_2O_2, 0.03M Fe^{2+}) showed that hydroxyl radical mediated oxidation causes a rapid decomposition of the tanning structures and denaturation of the collagen in less than a day. Autodegraded semi-metal tanned leathers and Fenton-treated leathers showed increased degree of susceptibility to digestion by bacterial enzymes.

Based on the results of this research, it is recognised that Fenton reaction may be used as an efficient method of oxidative pre-treatment of leather waste for enhancing its biodegradability particularly in the context of recycling through composting and anaerobic digestion.

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Abbreviations

DSC	differential scanning calorimeter
T_s	shrinkage temperature
DPPH	2,2-diphenyl-1-picrylhydrazyl
SEM	scanning electron microscope
ΔT_s	change in shrinkage temperature
Y	synergy of hydrothermal stabilisation
ICLT	Institute for Creative Leather Technologies
IULTCS	International Union of Leather Chemists and Technologists societies
SLTC	Society of Leather Technologists and Chemist
ICP-OES	inductively coupled plasma – optical emission spectrometer
RSD	relative standard deviation
EPR	electron paramagnetic resonance
EPSRC	engineering and Physical Sciences Research Council
HAT	Hydrogen atom transfer reaction
ΔH	Enthalpy of shrinkage transition
ANOVA	Analysis of variance

Units

M	molarity concentration
mM	millimolar concentration
mg	milligram
g	gram
L	litre
mol	mole
mmol/g	mole per gram
mL	millilitre
µL	microliter
°C	degree celsius
J/g	joules per gram
nm	nanometer
mT	milliTesla
MPa	megapascal
Hz	hertz
% w/v	percent of weight (g) per 100 mL of solution
% w/w	percent in weights

CHAPTER 1

INTRODUCTION

1.1. The leather manufacturing process

Skins and hide are largely composed of proteins and water. The fibrous collagen, which is the essential protein component required for making leather, may constitute more than 80% of the dry weight of raw skins and hides. Keratin is also a fibrous protein. It is the main structural component of hair and the epidermis. The other fibrous protein, elastin, is a largely non-polar protein found in a relatively small quantities in skins and hides. The non-fibrous proteins of skins and hides are mainly globular proteins and glycoproteins. Fats, mineral salts and other components make up 3% of the weight (Sharphouse 1971). The average composition of fresh skins and hides are shown in Table 1.1.

Table 1.1 The average composition of fresh hide (Sharphouse 1971)

Chemical component	% (w/w)
Water	64
Collagen	29
Keratin	2
Elastin	0.3
Globular proteins and glycoproteins	1.7
Fats	2
Mineral salts	1

Skins and hides are putrescible due to the action of autolytic enzymes and bacteria. In leather manufacturing, the putrescible skins and hides are converted into non-putrescible and relatively permanent materials that have the desired properties for use. A series of chemical and mechanical treatments are carried out in the five major processes; *viz.* preservation, beamhouse process, tanning, post-tanning and finishing processes. A general outline of the operations carried out in each process is included in Appendix 1 and the main processes in the leather manufacturing are briefly described in Sections 1.2.1-4.

1.1.1. Preservation

The most common method of preventing putrefaction of skins and hides is by salting. Application of salt causes a reduction of the moisture content and an increase in ionic strength, thereby acting as a bacteriostat. Wet salted hides and skins can be stored for

months until processing. Other methods of temporary preservation include chilling and drying (Covington 2009).

1.1.2. The beamhouse process

The main purpose of the various operations in the beamhouse process is to purify the skins and hides into pelts that are mainly composed of hydrated collagen. Cleaning and uniform rehydration is accomplished in the soaking operation. In the subsequent liming operation, the hair is degraded in alkaline media using sodium sulfide and other chemicals. The liming operations also involves an extended treatment in alkaline media (pH 12.5-12.6) in which the non-collagen proteins and proteoglycans are solubilised by hydrolysis while triglycerides (fats) are solubilised by saponification. Further removal of the degraded components is achieved by deliming and an enzymatic treatment known as bating. Washing operations in between the different beamhouse operations enhance the removal the degraded non-collagen components. At the end of the beamhouse process, the skins and hides are acidified in saline solution in preparation for tanning (Heidemann 1993).

1.1.3. Tanning process

Tanning is the chemical treatment that transforms the hydrated collagen (pelt) into leather, which is non-putrescible. It results in a relatively permanent stabilisation against enzymatic action. The tanning process involves uniform absorption of chemicals and subsequent fixation through chemical interactions with collagen. A broad classification of the most commonly used tanning agents is shown in Table 1.2.

1.1.4. Post-tanning process

In the post tanning process additional tanning treatments (retanning), dyeing and fatliquoring treatments are carried out to modify the properties of the leather. Some of the desired properties may be physical attributes such as stiffness and fullness or aesthetic qualities such as colour and softness. Chemical properties such as resistance to perspiration and waterproofness may also be important in some type of leathers (Heidemann 1993).

Table 1.2 - Main classes of tanning agents used in leather manufacturing (Covington 2009)

Type	Commonly used tanning agents
Mineral salts	Salts of Cr(III) Al(III), Zr(IV) and Ti(IV)
Vegetable tanning agents	Aqueous extracts from plants (hydrolysable and condensed tannins)
Syntans	Sulfonated condensation products synthesised from phenol or naphthalene
Aldehydic tanning agents	Glutaraldhyde, other aldehydes and aldehydic crosslinkers, (*e.g.* oxazolidine)
Polymeric tanning agents	Acrylic, melamin-formaldehyde resins and others

1.1.5. Finishing process

Application of surface coating ensures uniformity of appearance and enhances the aesthetic appeal of the leather. Finish applications are also applied to enhance properties such as abrasion-resistance (Sharphouse 1971).

1.2. A general perspective on waste from leather industry

According to FAO (2013), the global leather production exceeds more than 14 billion square feet per annum of light leathers and 500,000 tonnes of heavy leather; the global production of leather footwear has reached 4.5 billion pairs per annum. Manufacturing of leather and leather products as well as disposal of leather goods generates large weight of solid waste. Studies on the mass balance (input-output analysis) of model leather manufacturing processes has shown that the production of a 220kg leather from one tonne of salted hide requires 450kg of a different of chemicals and approximately 40-50m^3 of water depending on the process (Buljan *et al.* 2000; Alexander *et al.* 1991). The different types of solid waste generated from processing of one tonne hide are shown in Table 1.3.

Some forms of untanned solid waste, not contaminated by process chemicals such as sodium sulfide, are valuable by-products that are amenable to processes for conversion to different products. Raw trimmings and limed splits; which are protein-rich materials, can be converted to gelatine and collagen hydrolysate fractions, that potentially have important applications in manufacturing biodegradable polymer films (Langmaier *et al.* 2006), ingredients of fertilizers and animal feed additives (Cabeza *et al.* 1998; Brown *et*

al. 1996) as well as retanning agents (Taylor *et al.* 2007). Similarly, the high triglyceride content in the waste generated from fleshing of soaked (rehydrated) skin and hides can be extracted through thermo-enzymatic processes to obtain tallow or olein for use in the production of soap, fatliquors and biodiesel (Priebe and Gutterres 2012; Taylor *et al.* 1989; Crispim *et al.* 2009; Colak *et al.* 2005). However, the high sulfide content in the waste generated from fleshing of limed pelt is a major obstacle for that hinders effective utilisation of the fat content (Ozgunay *et al.* 2007)

Table 1.3 Solid waste generated from leather manufacturing processes, estimated quantities based on processing of one tonne of salted hide (Buljan *et al.* 2000)

Sources	Type of solid waste	kg
Untanned waste (from the beamhouse processes)	• Fleshing waste (mainly subcutaneous tissue) • Untanned trimmings	400
Tanned waste or Leather waste (from the post tanning and finishing processes)	• Leather waste from thickness correction and surface levelling operations ○ unused split leather ○ shaving dust ○ buffing dust • Trimmings (unwanted leather pieces)	237

Due to the chemical stability created by tanning reactions, the different forms of leather waste (also referred to as tanned waste), shown in Table 1.3, that are generated from the post-tanning operations in leather manufacturing, are not readily susceptible to chemical or enzymatic hydrolysis, (Covington 2009). In the past, various techniques were developed to extract gelatine and collagen hydrolysate fractions from Cr(III) containing leather waste (CCLW). The main methods used to extract the valuable protein fractions from post-tanning waste (mainly shaving waste) include alkaline hydrolysis, thermal treatment, oxidative de-chroming with hydrogen peroxide and enzymatic digestion (Cabaza *et al.* 1998; Taylor *et al.* 1991; Catalina *et al.* 2010; Cot *et al.* 1991). However, these extraction techniques are not largely commercialised mainly due to the fact that most of the processes involve formation of secondary waste in the form of Cr(III) containing organic residue or dilute solution of Cr(VI) in the case of oxidative detanning.

Leather waste is also generated in large quantities from the manufacturing of leather products. The processes involve unavoidable leather waste due to the cutting operations that have to be carried out based on specific requirements of shapes and sizes of the leather components (Tatano *et al.* 2012). According to CTC(2000), about 57% of the total solid waste from the footwear factories is leather waste; similarly, large quantities of leather waste are also generated in the manufacturing of garments, gloves and upholstery. Particularly in the shoe industry, there have been attempts to reduced the solid waste by utilising leather and polymer waste through recycling in the form of composite materials known as 'bonded leather' and 'fibre-board' that are used for making insole leather (Diddi 2007; Dalla-Rossa 2012).

Disposal of used leather goods is also recognised to be a significant landfill burden; according to Staikos *et al.* (2006), the global footwear consumption has doubled every 20 years in the past 50 years. The quantity of solid waste from footwear consumption is estimated to be more than 1.2 million tonnes per annum in Europe alone, and much more elsewhere in the world. It is estimated that leather accounts for 25% (w/w) of the total solid waste generated from disposal of footwear (Rahimfard and Staikos 2007).

1.3. Waste management in the Leather and allied industries

Solid waste generation from the operations in the leather manufacturing process are unavoidable due to the nature of the raw material and the processes that are designed to meet specific criteria of physical properties (Reich 2007). As described in section 1.2, the beamhouse process is mainly a sequence of cleaning processes that inherently generates untanned waste composed of unwanted organic components. Similarly, the post-tanning process also involves necessary operations of trimming, thickness correction (shaving and splitting) and surface levelling (buffing).

In consideration of preferred priorities in accordance with the waste management hierarchy, various methods of best available techniques (BAT) of waste minimisation, reuse, recycling and recovery option (Shown in Table 1.4) have been recommended for use in the leather industry (Black *et al.* 2013). However, the wider implementation of

these options is hindered by the fact that, at small scale, the reuse and recycle options are not often feasible for individual tanneries due to high investment cost (EC-IPPC 2003).

Table 1.4 Best available techniques of waste management recommended for leather industry by the European commission – Integrated pollution prevention and control directive (Black *et al.* 2013)

Solid Waste	Uses as a by-product	Reuse after preparation on-site	Off-site Recycling	Recovery
Hair and Wool	– Filling material – Wool/textiles	– Protein hydrolysate	– Fertiliser	– Energy recovery
Raw trimmings	–	–	– Hide glue	– Energy recovery
Limed trimmings	– Collagen production	– Tallow – Technical grade gelatine	– Hide glue	
Fleshing waste	–	– Protein hydrolysate – Tallow	– Hide glue	– Energy recovery – Substitute fuel
Untanned splits	– Processed to leather – Production of sausage casings – Collagen production	– Technical grade gelatine – Protein hydrolysate	– Hide glue	–
Tanned splits and trimmings	– Roller-coated for use in patchwork, small leather goods, *etc.* – Collagen production	– Leather fibreboard and composite materials	– Protein hydrolysate	– Energy recovery
Shaving and buffing waste	–	–	– Protein hydrolysate	– Energy recovery

Due to the low level of implementation of the waste management options described in Table 1.4, the most common method of managing the solid waste from leather manufacturing is through disposal at landfill sites (Joseph and Nithiya 2007; Kanagarai *et al.* 2006). Solid waste disposal from leather industry has been a subject of concern in the leather industry. About 90% of leather production utilised Cr(III) salt for tanning (Reich 2007), there are concerns of environmental safety with regards to the disposal of leather waste, particularly in relation to pollution with Cr(VI), which is considered to be a carcinogen (Hertel 1986; Fishbein 1976). It is believed that Cr(VI) may be formed inside landfills and subsequently leached into the ground water (kolomaznik *et al.* 2008). In addition, the availability of landfill sites in the EU has decreased considerably in recent years. Environmental directives on landfill practices promote the diversion of waste from landfills towards through recycling and reuse options (DEFRA 2009).

Furthermore, the majority of the leather waste generated from leather products manufacturing and the end-of-life waste from leather goods are disposed at landfills (CTC 2000; Rahimfard and Staikos 2007). A trial carried out by Kolomaznik *et al.* (2008) to the produce protein hydrolysate fractions from end-of-life waste of leather products has shown that hydrolytic and thermo-enzymatic hydrolysis of leather requires a long process involving energy-intensive treatment steps.

Leather waste has also been considered as a good fuel, the heat energy from fluidised bed combustion of leather waste reaches 21 MJ/kg but its combustion may also release significant quantities of mono-nitrogen oxide gases or NO_x gases (Bahillo, 2005). The EU-waste incineration directive (DERFA 2010) imposes maximum emission limits on the quantities of pollutant gases that may be generated by incineration plants. On the other hand, since it is a protein-derived material, leather waste is a potential source of nitrogen-fertilizer. The potential of fertilizer production from leather was has been demonstrated in recycling trials that involved application of shaving waste as fertilizer after de-chroming treatments (Nogueira *et al.* 2010; Lima *et al.* 2010). In the case of other forms of leather waste, conversion into a nitrogen source (for fertiliser production) would require chemical decomposition and biodegradation in aerobic media *i.e.* composting.

1.4. Importance of the reversal of the effect of tanning

Leather is chemically stabilised to resist enzymatic and microbial degradation. Thus, the leather waste, originating from the manufacturing processes (Section 1.2) and disposal of leather goods, generally remains resistant to biodegradation (Covington 2009; Tatano *et al.* 2012). On the other hand, it is known that reversal of the stabilisation effect of tanning can be achieved through chemical treatments (detanning) or by thermal treatments. Dhayalan *et al.* 2007 has demonstrated that Cr(III) tanned leather samples that were detanned using oxalic acid and vegetable tanned leathers detanned using alkaline buffer showed show increased level of biodegradation by anaerobic digestion. According to Yagoub(2006), thermally denatured Cr(III)tanned leather show high level of biodegradation in anaerobic digestion experiment, nearly similar to untanned collagen

sample (hide powder). Nevertheless, the potential application of detanning and thermal pre-treatments have not been considered as feasible options at larger scale for application as part of treatment of leather waste via anaerobic biodegration (generation of biogas). This is because such treatments are likely to entail high cost in terms of chemical and energy consumption.

Leather waste is rich in nitrogen, the average nitrogen content in Cr(III) tanned leather is 10% w/w, while other non-Cr(III) tanned leathers e.g. wet-white leather or de-chromed leather contain upto 15% w/w of nitrogen (Lima *et al.* 2010). Despite the fact that leather waste has a potential application as a nitrogen source for making fertilizers, it is not biodegradable by aerobic micro-organisms under the normal conditions of a composting environment (Tatano *et al.* 2012; Ferriera *et al.* 2013). In order to facilitate recycling of leather waste through of biodegradation methods, *viz*. aerobic and anaerobic pathways; its susceptibility to enzymatic/microbial degradation has to be enhanced using efficient methods. In this regard, investigations into the mechanisms of reversal of tanning and degradation of leather are relevant.

1.5. General features of tanning reactions

All tanning treatments essentially result in the conversion of the putrescible pelt into a stable form that is resistant to the action of enzymes and bacteria. Tanning treatments result in permanent preservation of the putrescible collagen. Other effects of tanning reactions may include an increase in hydrothermal stability, change in colour and creation of distinct physical and aesthetic properties (Sharphouse 1971).

Untanned pelt dries into a semi-translucent hard and brittle mass in which fibres are stick together due to cohesive forces resulting in a non-porous structure. Tanning and post tanning processes introduce chemicals that occupy the inter-fibrilliar space and create a barrier to cohesive forces between individual fibres, as a result, leather dries to become a flexible and porous material. Hence, leather is characteristically different from parchment and vellum that are made from untanned pelts. Leather has a lower capacity

to absorb water as compared to untanned collagen and hence exhibits a reduced tendency of swelling (Reich 2007).

1.6. The permanence and stability of leather

Leather is a material required to maintain consistent functional properties during usage. Tanning reactions and other treatments in the post-tanning and finishing processes should be capable of producing effects that have a certain degree of permanence. Hence, the interaction between tanning agents and collagen should not be easily reversible under normal conditions of usage and storage (Reich 2007). Chemical treatments such as dehydration of untanned pelt with solvents are readily reversed by wetting and hence such treatments are not considered to be tanning treatments. Similarly, the old traditional practice known as tawing does not comply with the requirement of permanence of properties. Tawing involves treatment of wet pelts with potash alum salt (potassium aluminium sulfate) and organic ingredient (egg yolk, flour and other ingredients. Without further tanning treatment, tawed skins are not durable products because the aluminium can be dissolved out by water (Vest 1999).

Tanning is viewed as the formation a superamolecular structure of tanning chemicals around the collagen; this is referred to as the tanning matrix (Covington *et al.* 2008). The tanning matrix is characterised by a set of chemical interactions occurring between the collagen and tanning agents as well as interactions between the components of the tanning agents (Covington *et al.* 2011; Reich 2007). The tanning matrix and collagen in leather maintain their structural integrity only under normal conditions of usage and storage. Changes in the chemical environment of the leather may result in alteration in the tanning structure and potentially lead to degradation of the leather (Covington 2009; Haines 2006).

The effects of changes in the chemical environment on the stability and structural integrity of leather are reflected in lowering of the hydrothermal stability and changes in the physical properties of leathers (Chahine 2000; Larsen 1996; Heidemann 1993). Some of the changes that can lead to alterations of the composition and properties of

leather are exposure to extreme pH conditions causing hydrolytic reactions, partial removal of tanning agents by chemical reactions and presence of oxidative conditions (Florian 2006; Gustavson 1956; Covington 2009).

1.7. The chemistry of collagen

The collagen family comprises insoluble extracellular proteins characterised with the presence of triple helical domains in their structure. Collagens are the most abundant proteins in mammals, accounting for approximately 30% of the total protein mass (Kadler *et al.* 2007). There are 29 different type of collagens characterised so far, each having particularly functions, collagens types I, II, III, IV and IV are fibril-forming types. Collagen type I is the major fibrous protein found in skins, bones, ligaments and other internal organs (Ricard-Blum 2010). In this thesis, unless stated otherwise, the use of "collagen" refers to collagen type I.

1.7.1. Composition of collagen

Collagen is composed of 19 different amino acids, the largest amino acid constituents are glycine (33.53%), proline (11.97%) and hydroxyproline (11.18%). Hydroxyproline is found only in collagen, with a possible exception of elastin (Bentley and Hanson 1969, Bochicchio *et al.* 2013). The imino acids (proline and hydroxyproline) are the most important constituents in the formation of helical structure of collagen chains.

Table 1.5 Collagen Type I – content of the main amino acids crucial to the structure and reactivity (Reich 2007)

amino acid, symbol	amino acids per collagen molecule
Glycine (Gly)	1056
Tyrosine (Tyr)	6
Serine (Ser)	109
Tyrosine (Tyr)	6
Proline (Pro)	377
Hydroxyproline (Hyp)	352
Asparigine (Asn)	37
Glutamine (Gln)	81
Glutamic acid (Glu)	150
Aspartic acid (Asp)	97
Lysine (Lys)	100
Arginine (Arg)	159
Hydroxylysine (Hyl)	13
Histidine (His)	6

Because of the diversity of its amino acid composition (Table 1.5), collagen is capable of involving in different forms of chemical interactions (Reich 2007). The acidic (carboxylic) and the basic amino acids are most important in determining the isoelectric point of collagen and in the reaction of collagen with different tanning agents (Sharphouse 1971).

In the context of tanning, glutamic and asparitic acids are the main reactive sites for electrostatic interactions and coordinate-covalent bond formation with aqueous metal complexes (Covington 2009). The amino acids that contain OH groups (serine, threonine, and tyrosine) as well as the basic amino acids (lysine, argentine, hydroxylysine and histidine) are crucial reactive sites of collagen for binding of polyphenolic tanning agents through hydrogen bonding. Aldehydic crosslinkers (e.g. glutaraldehyde) form covalent bonds with the basic amino acid groups (Damink *et al.* 1995). In addition, non-polar amino acids and amino acids with aromatic side functional groups (e.g. tyrosine, phenylalanine) also play significant role in the binding of polyphenols and other non-polar structures through hydrogen bonding (Heidemann 1993; Haslam 1997)

1.7.2. Structure of collagen

1.7.2.1. Primary structure

The amino acid sequence of collagen shows trimetric pattern with every third residue being glycine. Chains of tripeptides of definite pattern of trimetric sequences make up the polypeptide of collagen known as α-chains. The trimetric amino acid sequence contains 350 repeating units of **Gly-X-Y**, where **X** and **Y** are different amino acids. About 10% of the **X** positions in the tripeptide of collagen α-chain are occupied by proline, while the 12% of the **Y** position are hydroxyproline (Bailey *et al.* 1998). The trimetric sequences of amino acids are found only in the helical domain of collagen, which accounts for 96% of the whole structure. The remaining portions of collagen are non helical regions, referred to as telopeptides. These regions are composed of globular polypeptides attached to the terminals of the helical domain and lack the regular **Gly-X-Y** pattern (Ricard-Blum 2010, Brinkmann *et al.* 2005).

1.7.2.2. Secondary structure

The frequent presence of the imino acids in tripeptides results in the restriction of rotation of the peptide chain since the nitrogen and the α-carbon are locked by the ring structures. In addition, the presence of **Gly-Pro-Hyp** tripeptides results in an angular twist in the tripeptide chain due to the steric repulsion between the pyrollidine rings; with every tripeptide, there is an average of 108° left handed angular twist, which is equivalent to one complete turn (360°) with every 3.33 tripeptides along the whole of the helix (shown in Figure 1.1). Longitudinally, the average axial rise per triplet is 0.86nm or 2.86nm for every coil (Bhattacharjee and Bansal *et al.* 2005; Ramachandran 1967)

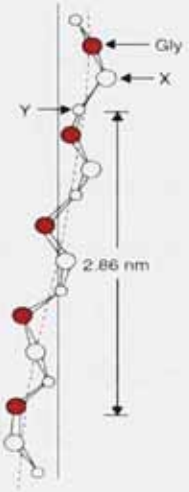


Figure 1.1 - Axial arrangement of tri-peptides (Gly-X-Y) into a twists chain with 2.86 nm axial rise per 3.33 tripeptides (Haines 2006)

1.7.2.3. Tertiary structure

Collagen is composed of three left-handed helical α-chains, two of them being identical (two α1 chain and one α2 chain). The three polypeptides (α-helices) form into right hand twisted strands to make up a 300 nm long rod-like triple helical structure as shown in Figure 1.2. In this arrangement, glycine invariably occupies the position nearest to the triple helical axis. The triple helix structure is stabilised by inter-chain hydrogen bonds formed between the amine group of glycine residue and the carbonyl group of the amino acid at the X position (Bhattacharjee and Bansal 2005).

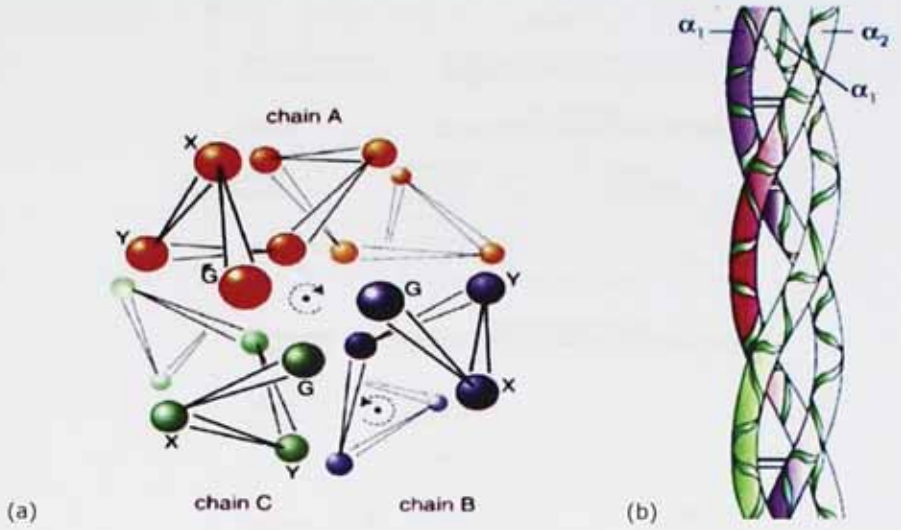


Figure 1.2 A diagrammatic representation of the structure of collagen showing (a) a cross-sectional view of the triple helical structure (b) the triple helical winding of the α-chains (Beck and Brodsky 1997)

1.7.2.4. Quaternary structure

Transmission electron microscopy (TEM) analysis of collagen fibrils that are stained with metals shows dark bandings at a periodic interval of 67 nm. Collagen molecules are 294 nm long, which is 4.4 times the periodic banding interval. This observation is the basis for theory that collagen molecules in fibrils are found longitudinally arranged adjacent to each other (as shown in Figure 1.3) with a displacement that is equivalent to a quarter of the length. This structure has been termed as quarter stagger array or D-interval structure (Orgel *et al.* 2001). In this structure, adjacent collagen molecules are overlap with 30 nm of their length and have an axial gap of 40 nm between the N-terminal of one and C- terminal of another tropocollagen.

During biosynthesis and maturation of collagen, covalent crosslinks are formed in the micro-fibrils at the overlap segments between the non-helical and helical ends of adjacent collagen molecules. There are at least two intermolecular crosslinks of lysine residues at each overlap zone (Bailey and Paul 1998).

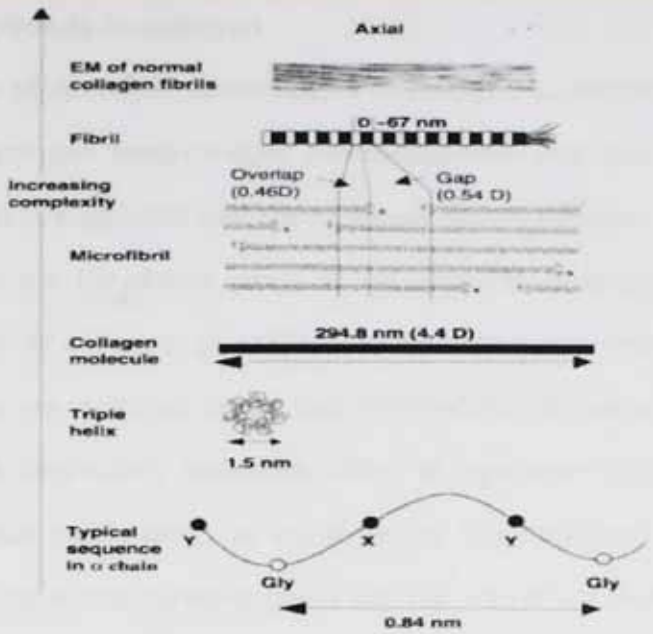


Figure 1.3 - Diagrammatic representation of the structural hierarchy of collagen (Orgel *et al.* 2001)

Fibrils may have diameter reaching 100 nm and contain thousands of micro-fibrils packed laterally in a regular pattern, an elementary fibre (Figure 1.4) consists of numerous fibrils twisted together (Heidemann 1993). The presence of a large number of crosslinks in the micro-fibril and fibril structures is an important factor in the biomechanical functions of the fibrous collagen (Eyre and Wu 2005).

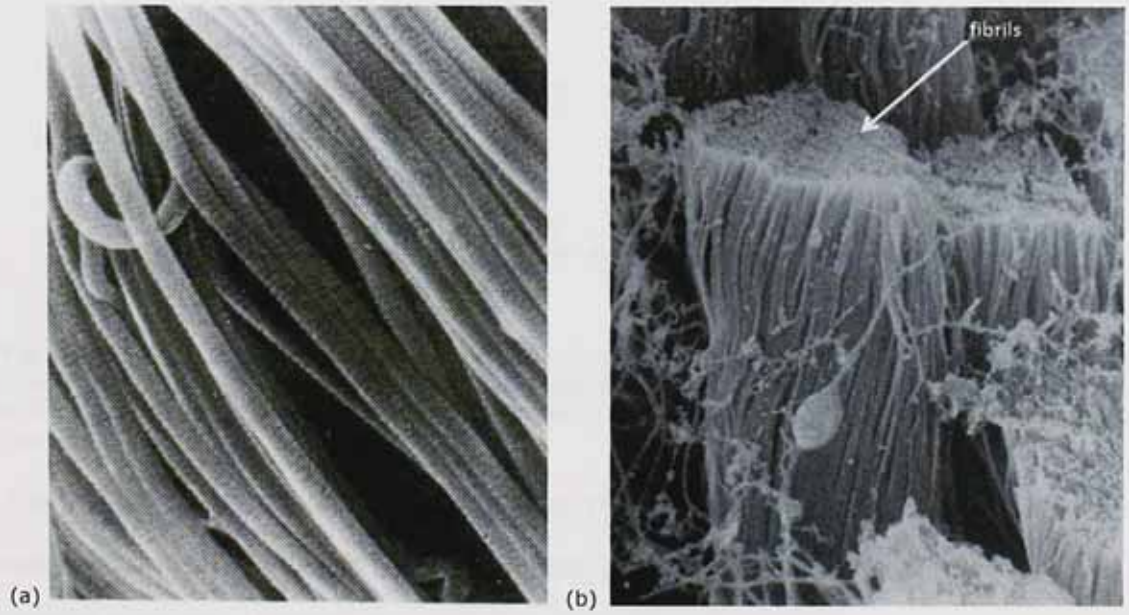


Figure 1.4 SEM micrographs showing (a) separate collagen fibrils and (b) cross-section of elementary fibre of collagen (Heidemann 1993)

1.7.3. Hydrogen bonds in collagen

The stability of the triple helical conformation of collagen is attributed to the repetitive water mediated hydrogen bond bridges (Ramachandran and Chandrasekharan 1968, Bella *et al.* 1995). It is suggested that the hydrogen bonds between the carbonyl groups of a collagen chain and the amine group of the adjacent chain occurs throughout the triple-helical domain of collagen. In addition to the direct inter-helical hydrogen bonds (Figure 1.5a), there are hydrogen bonds that are mediated by water molecule as shown in (Figure 1.5b). A particularly important effect of hydrogen bonds in stabilising the collagen triple helical confirmation is attributed to the formation of the intra-helical bonds *i.e.* between the amide carbonyl group and the –OH of the hydroxyproline that are mediated by water molecules (Figure 1.5a).

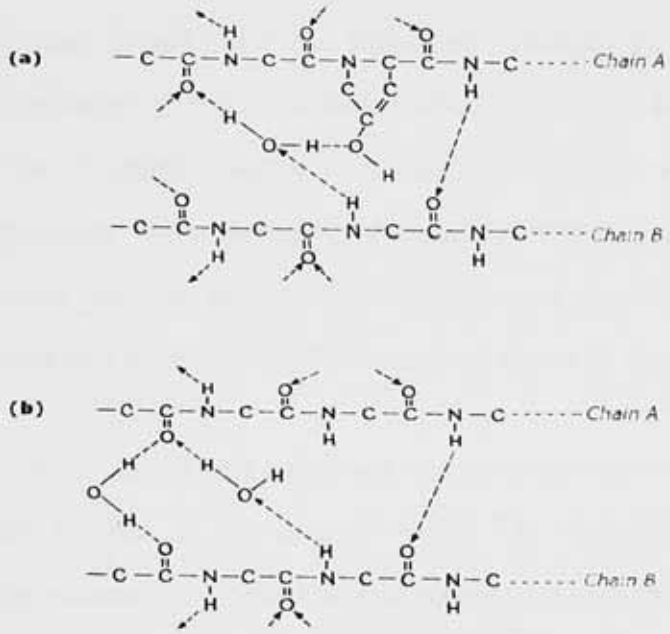


Figure 1.5. Models of hydrogen bond interactions in the collagen triple helix showing (a) intra-helical and inter-helical hydrogen bonds mediated by a water molecule involving hydroxyproline and (b) hydrogen bonds between collagen chains without involving hydroxyproline (Brodsky and Ramshaw 1997).

The presence of hydroxyproline moieties in the tripeptides enables the formation of the water-mediated intrahelical hydrogen bonds in the triple helical domain, as a result, the repetitive structure formed by water-hydroxyproline interactions is considered critical for the structural stability of the collagen (Brodsky and Ramshaw 1997). According to Mogilner *et al.* (2002), complete removal of water from collagen causes irreversible

changes in structural changes because of increased formation of molecular crosslinks. The widely accepted view is that water molecules are considered integral part of the collagen triple helical structure and hydroxyproline is a centre of the stabilisation of the triple helical confirmation (Privalov and Tiktopulo 1970; Bhattacharjee and Bansal 2005).

However, there are also differing views on the subject of collagen stabilisation. Engel *et al.* (1977) showed that collagen-like polypeptides prepared in non-aqueous solvents have triple helical structure and suggested that water molecules may not be critical for the stabilisation of the triple helical structure. Shoulders and Raines (2009) also suggested that the stabilisation effect of hydroxyproline may be related to inductive effects rather than hydrogen bonds.

1.7.4. Thermal denaturation of collagen

Denaturation of collagen occurs when the stabilising hydrogen bonds are disrupted by heat causing transformation of the triple helical structure into random coils. The role of hydroxyproline in the structural stability of collagen is also reflected in its influence on denaturation temperature. Different types of collagens and collagen-like polypeptides with relatively greater number of hydroxyproline content show greater denaturation temperature (Brodsky and Ramshaw 1997; Periscov *et al.* 2000; Burjanadze 1979).

According to Miles *et al.* (1995), the shrinkage transition is triggered at thermally labile regions of collagen located in the overlap region *i.e.* regions of collagen lacking hydroxyproline. The process is endothermic and occurs with reduction of volume of fibre structure or shrinkage. Finch and Ledward (1972) suggested that random coiling of collagen chains and shrinkage of fibres may be because of hydrophobic forces.

1.8. Tanning reactions and hydrothermal stabilisation

Due to the diversity in its amino acid composition, collagen is capable of binding with reactive groups of tanning agents through different forms of interactions. The basic amino groups can form covalent bonds with aldehydes, diisocyanates and other crosslinkers (Metz 2004; Damink *et al.* 1995a,b). The carboxylic groups form complexes

with metal ions through coordinate covalent bonds (Covington 1997). The peptide bond, amine groups and the hydroxyl groups in the side chain can engage in hydrogen bonding with vegetable tanning agents as well as some types of syntans (Reich 2007; Gustavson 1956). The chemical interaction of collagen with tanning agents may also involve hydrophobic and dipole interactions (Heidemann 1993; Covington 2011).

In an untanned pelt, the collagen macromolecule is surrounded by a supramolecular structure of water molecules forming a layer of hydration (Privalov and Tiktopulo 1970; Bella *et al.* 1995; Brodsky and Ramshaw 1997). A tanning reaction results in the partial displacement of the water structure of untanned collagen with a new structure composed of molecules of the tanning agent. With the reaction of tanning agents and the formation of superamolecular structural network by tanning agents, tanned collagen is said to be a chemically modified protein. One of the major effects of tanning is an increase in the hydrothermal stability, which is a property related to the degree of resistance of chemically modified collagen to the effect of heat in the presence of water (Reich 2007).

The effect of tanning with respect to hydrothermal stabilisation reflects the strength of the tanning matrix in counteracting the denaturation transition of the collagen in the leather. According to Covington *et al.* (2008), hydrothermal stabilisation effect of tanning is influenced by:-

- The strength of the interaction between tanning molecules and collagen,
- The degree of cross linking interactions between tanning agents,

The effectiveness of tanning is viewed in terms of the effect of the tanning matrix in creating an increased level of resistance to thermal denaturation. Hydrothermal stability is an important parameter that may be used to analyse the effectiveness of tannage as well as the stability of tanning structures in leather (Covington *et al.* 2011; Chahine 2000). It is measured in terms of the temperature at which the leather shrinks upon heating in the presence of water, this referred to as shrinkage temperature (T_s). Conventional measurements are carried out in accordance with a standard method described in the official methods of the Society of Leather Technologists and Chemists

(SLTC 2000). The method involves determination of the critical temperature at which leather of a known dimension shrinks when heated in water at 2°C per minute.

Shrinkage temperature of leather can also be determined using differential scanning calorimetric (DSC) measurement, which also gives an additional information on the energy related to the shrinkage transition (McClain 1972). Determination of the enthalpy of shrinkage transition can be used to analyse the structural integrity of collagen, a decrease in the enthalpy and shrinkage temperature of a sample indicates structural changes in the collagen molecule (Usha and Ramasami 2004). The occurrence of denaturation of the collagen in the leather can be detected (Chahine 2000). The shrinkage temperatures of that may be obtained by tanning pickled pelt with different tanning agents are shown in Table 1.6.

Table 1.6 Shrinkage temperature of leathers made by tanning using different tanning agents and combination of tannages (Covington 2009)

Tanning agents	Shrinkage temperature (T_s)
Basic Cr(III) sulfate	110-120
Other metal salts: Al(III), Ti(IV), Zr(IV)	65-85
Vegetable tanning agents	75-85
Phenolic syntans (replacement syntans)	70-80
Glutaraldehyde, oxazolidine	80-85
Combination tanning: vegetable tanning + Al(III)	105-115
Combination tanning: vegetable tanning + oxazolidine	105-115

Combination of the effects of different tanning agent is an important aspect related to hydrothermal stabilisation. Leather manufacturing process often involves two or more steps of tanning treatments. The interaction between the successive tanning treatments may affect the hydrothermal stability of the leather (Covington *et al.* 2011) A significant increase in the shrinkage temperatures are observed when vegetable tanned leathers are retanned with metal salts or aldehydic crosslinkers, as indicated in Table 1.6.

1.8.1. Tanning with metal salts

With the exception of Cr(III) sulfate, tanning pickled collagen pelt with transition metal salts (sulfates and chlorides) results in shrinkage temperature of 70°C and below. The reactivity of metal complexes towards collagen was relatively greater only near the precipitation point (Lampard 2000). Amongst the transition metals, only Cr(III), Ti(IV), Fe(III) and Zr(IV) are used for in tanning (Heidemann 1993). The basic chemical interaction between the collagen and aqueous metal complexes is based on the electrostatic attraction and complex formation with the carboxylate side chain groups of collagen (Covington 1987).

1.8.1.1. Tanning with chromium(III)

When a Cr(III) sulfate is dissolved in water, an acidic pentaqua-sulfato-Cr(III) complex is formed (Figure 1.6a) at pH lower than 2.0. With increase of pH, this complex undergoes hydrolysis, initially forming the sulfate complexed monohydroxy complex (Figure 1.6b) that subsequently forms the olated (oxy-bridged) binuclear Cr(III) complex (Figure 1.6c) Further increase of pH above 3.5 leads to increased formation trimeric and tetrameric aqua Cr(III) complexes (Imer and Vernali 2000; Indubala and Ramaswami 1973).

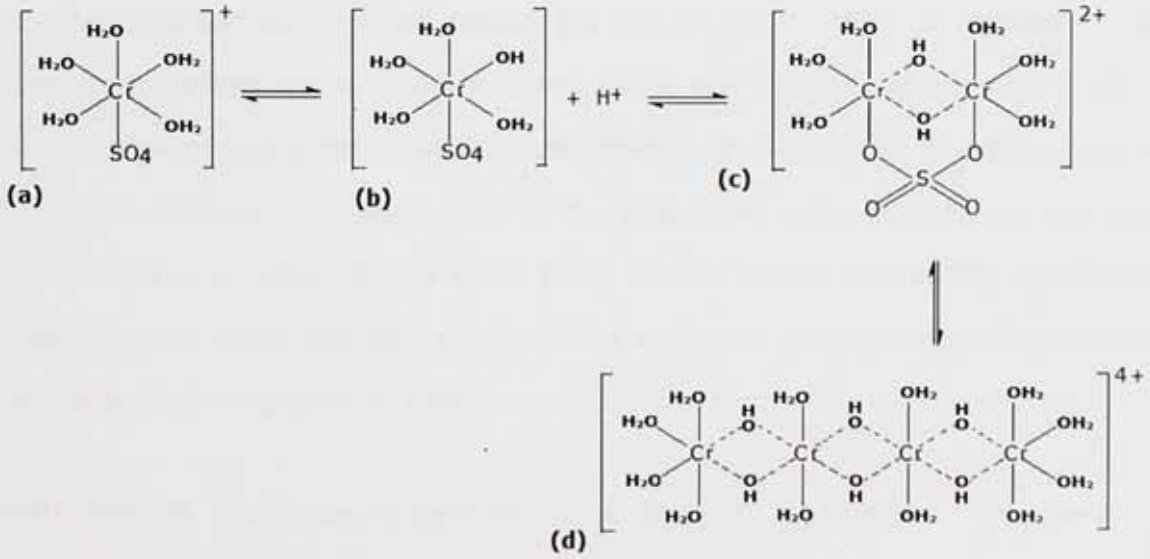


Figure 1.6. Structures of aqueous Cr (III) complexes in acidic medium showing (a) the monomeric sulfato Cr(III) complex, (b) the monomeric species (sulfatohydroxo-Cr(III) complex), (c) oxy-bridged μ-dihydroxy-μ-sulfato-di Cr(III) complex and (d) linear trimeric Cr(III) complex (Covington 2009).

During tanning of pickled skins and hides, more than 25% of the aspartic acid (pKa 3.8) and glutamic acid (pKa 4.1) side chains are ionised above pH 3.5 (Covington 2009). Hence reactions take place between the carboxylate groups and the soluble Cr(III) complexes. One of the unique features in the chemistry of Cr(III) tanning is that the pH zone for the formation of the reactive oligomeric Cr(III) species (*i.e.* pH 2.7-4.0), matches with that of the ionisation of the carboxyl groups of collagen.

Analysis of Cr(III) tanned leather using EXAFS (extended X-ray absorption fine structure) technique showed that the tanning matrix is predominantly composed of linear tetrameric Cr(III) species (Covington *et al.* 2001). The highest level of hydrothermal stability is achieved by tanning collagen with Cr(III) sulfate, rather than with Cr(III) chloride or other salts. It is assumed that, the sulfate ion may act as a bridge between the oligomeric Cr(III) complexes, resulting in the formation of increased size of supramolecular units in the tanning structure (Reich 2007; Covington *et al.* 2008).

1.8.1.2. Tanning with aluminium(III)

In acidic medium below pH 2.5, the hexa-aqua complex $[Al(H_2O)_6]^{3+}$ species hydrolyses into the binuclear oxy bridged species $[Al_2(OH)_2(H_2O)_8]^{4+}$. With an increase in pH extensive hydrolysis results in precipitation due to the formation of polycation species having 13 aluminium atoms. Complete precipitation of polyhydroxy Aluminium species may occur below pH 4.0, particularly in the absence of complexation with non-aqua ligands (Stol *et al.* 1976; Shriver *et al.* 1994). During tanning with Al(III), carboxylate ligands such as citrate are used to reduce the degree of hydrolysis and allow reaction with collagen (Covington *et al.* 1987).

Al(III)-collagen interaction is believed to be more of electrostatic than coordinate covalent complexation (Brown 2005; Covington 2009), shrinkage temperature of Al(III) tanned pelt may reach 80°C by tanning in presence of polycarboxylic ligands (Covington *et al.* 1997). The use of Al(III) salt as a solo tanning agent is not common. However, it

has been used as the most preferred for retanning vegetable tanned leathers in the production of semi-Al(III) tanned leathers (Vitolo *et al.* 2003; Slabbert 1981) .

1.8.1.3. Tanning with titanium(IV)

Aqueous Ti(IV), which is in the form of titanyl ion $[TiO]^{2+}$in acidic media undergoes extensive hydrolysis leading to the formation of the polycation $[(TiO)_8(OH)_{12}]^{4+}$ that polymerises further and precipitates at pH 3 (Einaga and Komatsu 1981). Hence in absence of other ligands, tanning with Ti(IV) salts may be hampered by precipitation. Tanning can be carried out at pH 5-6 in the presence of polycarboxylate ligands. Leather made by tanning with Ti(IV) salt is characterised with properties related to deposition of polytitanyl species in the fibre structure, typically whiteness and 'fullness' in handle. Shrinkage temperatures of 75-85°C have been achieved by titanium(IV) tanning in the presence of polycaboxylic salts (Peng *et al.* 2007; Covington 1987). Ti(IV) salts have been used for making white leather and orange coloured semi-Ti(IV) tanned leathers (i.e. vegetable tanned leathers retanned with Ti(IV) salts) that are known to have high resistance to perspiration (Van Benschoten 1985).

1.8.2. Vegetable tanning agents

Plants produce and accumulate different types of secondary metabolites including polyphenolic compounds known as tannins. The biological functions of these metabolite are related to the defence mechanism of the plant (Beart *et al.* 1985). Tannins are also considered as renewable resources, extensively used in tanning, adhesive manufacturing and formulation of anti-corrosive coatings (Pizzi 2008).

Bate-Smith and Swain (1962) have defined tannins as *"water soluble phenolic compounds having molecular weight 500-3000 g/Mol and, besides giving the usual phenolic reactions, they have special properties such as the ability to precipitate alkaloids, gelatine and other protein".*

The definition of tannins in relation to molecular weight is based on characterisation of astringency or the relative capacity of different phenolic compounds to precipitate

gelatine from a dilute solution. Polyphenolic compounds with molecular weights greater than 3000 generally have low solubility, while those with molecular weight less than 500 are considered to be non-astringent (Hagermann 1992).

However, in phytochemical literature, water insoluble natural polyphenols are often referred to as tannins (Takekawa and Mutsumoto 2012; Taira 1995; Khanbabaee and Van Ree 2001). The more inclusive term 'polyphenols' is commonly used interchangeably with 'tannins' (Haslam 1998). Based on their structural feature and chemical properties, tannins are broadly classified as (i) hydrolysable tannins, which are galloyl and hexahydroxydiphenoyl esters and their derivatives, (ii) condensed tannins (oligomeric proanthocyanidins) and (iii) phlorotannins (oligomers of phloroglucinol). The first two groups are the most common and important groups with respect to vegetable tanning (Haslam 1999).

1.8.2.1. Hydrolysable tannins

As their name indicates, hydrolysable tannins are composed of compounds that are prone to hydrolytic breakdown in warm solutions at both acidic and alkaline media. The chemical structures of hydrolysable tannin, also known as 'pyrogallol' tannins (Sharphouse 1971). They have chemical structures that typically consists a saccharide centre esterified with the aromatic carboxylate species bearing pyrogallol groups or 1,2,3 trihydroxybenzene (Haslam 1998). The aromatic carboxylate groups of hydrolysable tannins may be gallic, di-gallic, ellagic or chebulic esters attached to the D-glucose centre (Figure 1.7).

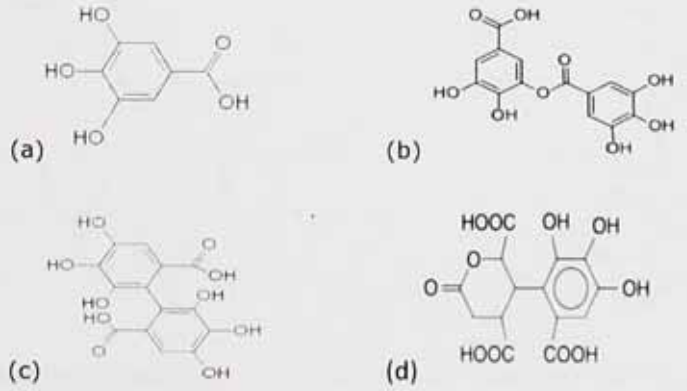


Figure 1.7. Aromatic carboxylic moieties found in the structures of various hydrolysable tannins (a) gallic acid unit gallolyl, (b) di-gallic acid or di-gallolyl group, (c) ellagic acid and (d) chebulic acid

Depending on the structure of the ester units, hydrolysable tannins are classified as gallotannins and ellagitannins. Gallotannins are composed of gallic or di-gallic esters of D-glucose. Compounds like pentagalloloy glucose (Figure 1.8a) are precursors in the biosynthesis of larger gallotannins such as tannic acid (Figure 1.8b). Ellagitannins are mainly esters of ellagic acid; some ellagitannins undergo oxidative rearrangement and to form chebulic esters from ellagic units (Okuda *et al.* 1993). Ellagitannins having chebulic esters include chebulagic acid (Figure 1.9b), which is the main tannin compound in myrabalan tannin (Okuda and Ito 2011) and castalagin (Figure 1.9a) is a major constituent of chestnut extract (Tang *et al.* 1992a). Hydrolysis of the ester links of gallotannins and ellagitannins in acidic media yields gallic acid and ellagic acid, respectively (Haslam 1998).

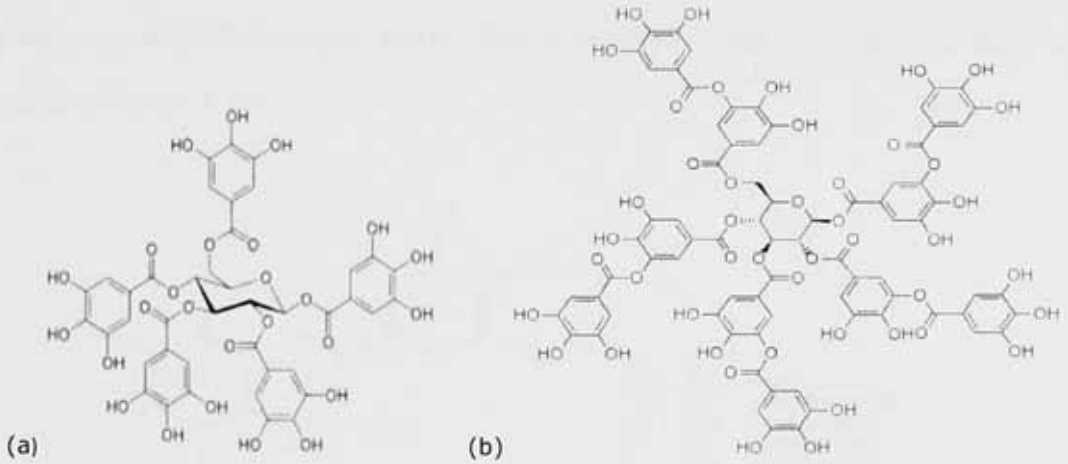


Figure 1.8. Examples of gallotannin compounds, (a) 1,2,3,4,6 penta-galloly-O-glucose, an ingredient of sumac tannin (b) tannic acid, with five depside linked di-gallolys units

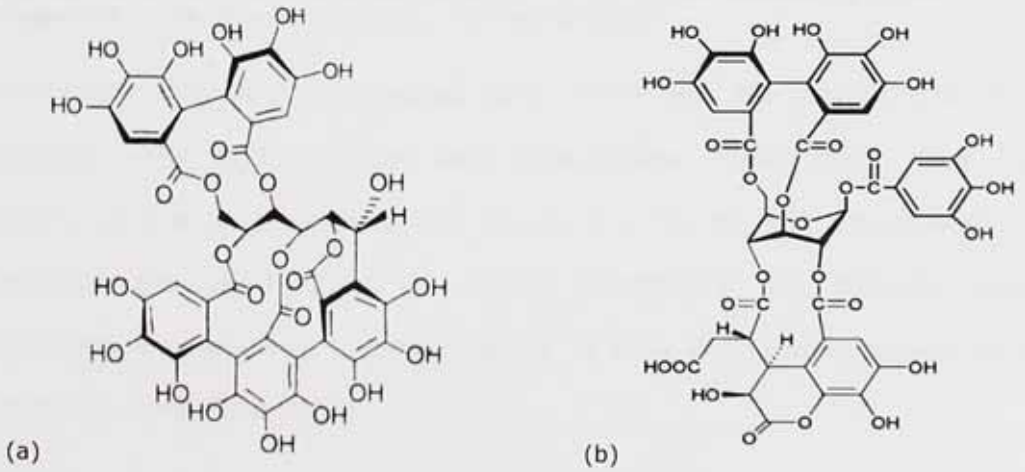


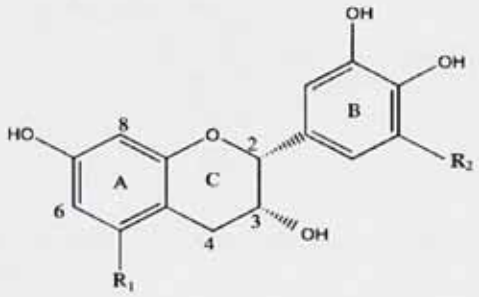
Figure 1.9. Examples of ellagitannins (a) structure of castalagin having ellagic ester units and (b) structure of chebulagic acid with ellagic, chebulic and gallolyl esters (Okuda *et al.* 2011; Hagerman 2011)

The most common types of hydrolysable tannins used in the leather manufacturing include :-

- Chestnut tannin, extract from the wood of *Castanea Sativa,*
- Myrabalan tannin from fruits of myrabalan tree, *Terminalia Chebula,*
- Sumactannin from the leaves of sumac shrub, *Rhus Coriaria,*
- Tara tannin from the fruits of tara tree, *Caesalpinia Spinosa.*

1.8.2.2. Condensed tannins

Condensed tannins or proanthocyanidins are oligomers of flavol-3-ol linked together by C-C bonds. They are commonly referred to as 'catechol' tannins in leather industry because the most common types of condensed tannin compounds contain the catechol group (dihydroxybenzene) as their B-ring as shown (Heidemann 1993). However, in some of the condensed tannin compounds, the B-ring is a pyrogallol group (1,2,3-trihydroxybenzene) (Schnofield 2001). The monomeric units of condensed tannins are described in Figure 1.10



R_1	R_2	Class
OH	H	Proanthocyanidin
OH	OH	Prodelphinidin
H	H	Profisetinidin
H	OH	Prorobinetinidin

Figure 1.10. Flavonoid structural model condensed tannin compounds having aromatic A-ring and B-rings attached to the alicyclic ring C-ring (Schnofield 2001)

The monomeric units in the condensed tannin differ with the position and degree of hydroxylation. The profistetinidine and procyanidine types don't have hydroxy substitution at the R_2 position of the B-ring (*i.e.* R_2=H), the monomeric units of procyanidines are catechin and its isomer epicatechin. The prodelphinidine and prorobinetinidine types have 'pyrogallol group' as their B-ring and they are known as gallocatechins (Schnofield 2001).

The monomeric flavonoid units are coupled to form oligomeric and polymeric structures mainly between the 4 and 8 positions of adjacent flavonoid monomers giving a linear chain (Figure 1.11). However, in some condensed tannins the coupling may occur also at the flavonoid positions of 4 and 6 giving a branched structure (Porter 1992). The degree of polymerisation in water-soluble condensed tannin could reach up to 6 monomeric units (Butler *et al.* 1982). However, Guyot *et al.* (2000) have shown that condensed tannins from methanolic extracts of freeze dried fruits may contain 2-10 monomers.

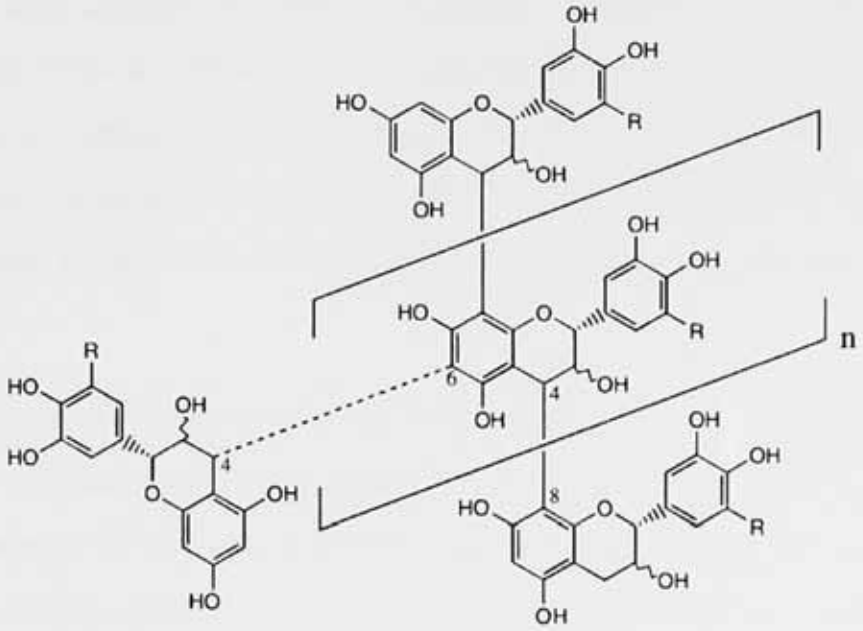


Figure 1.11 - A structural model of linear and branched polymeric chains in condensed tannins (Porter 1992).

Some of the condensed tannins used in the leather industry include:-

- Mimosa tannin extracts from the bark of *Acacia sp.*
- Quebracho tannin, extract from wood of *Schinopsis sp.*
- Gambier tannin extract from the leaves and twigs of *Uncaria sp.*
- Hemlock tannin extract from the bark of *Tsuga sp.*

1.8.2.3. Interaction of vegetable tannins with collagen

Tannins react with protein and cause precipitation of gelatin from solution. The reaction of tannins with proteins in aqueous solution is influenced by amino acid composition, isoelectric point, size and structure of the tannin. The main forms of protein-tannin interaction in solution are considered to be hydrogen bonding and hydrophobic

interaction (Hagerman 1992; Oh *et al.* 1980). Gustavson(1956) observed that the binding of tannins to collagen is similar over a wide pH range of 2-8 and the binding is reversible by treatment with hydrogen bond breakers (*e.g.* urea). The indication is that hydrogen bonding is the main form of interactions and electrostatic interactions do not play significant role. Whereas, a study by Madhan *et al.* (2001) carried out using molecular modelling of the interaction of collagen-like polypeptide with gallic acid, suggested that electrostatic interactions might also play an important role in the binding of tannins with collagen. A similar investigation by Brown *et al.* (2011) using a gallocatechin compound indicated that hydrogen bonds and hydrophobic interactions are the main tannin-collagen binding forces and the potential role for binding of tannins at the gap region of collagen was also highlighted. Haslam (1997) has also supported the idea of fixation of tannins at the gap region of collagen primarily through hydrophobic interaction.

The complete mechanism of tannin-collagen interaction is not fully understood. However, based on known facts from various experiments, it is assumed that formation of multiple hydrogen bonding between tannin molecules and functional groups of collagen (as shown in Figure 1.12) may be the main form of interaction responsible for the tanning effect of polyphenols (Covington 1997).

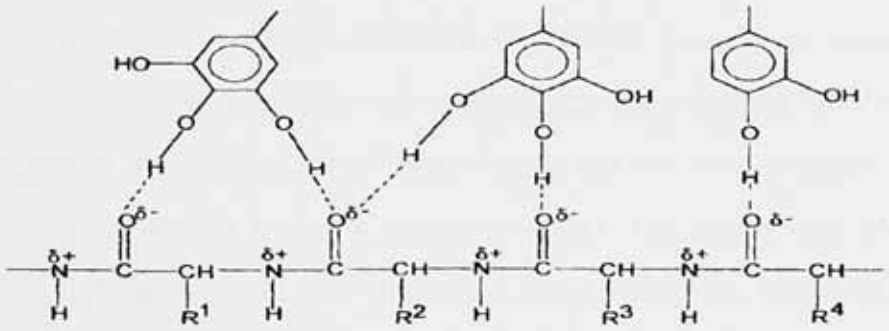


Figure 1.12. Model of the interaction between plant polyphenol and collagen peptide bond (Covington 1997)

It is generally accepted that vegetable tanning interactions occur throughout the collagen molecule with the peptide bond and functional groups in the amino acid side

chain. So far, there seems to be no compelling evidence to consider complete localisation of tannin-interactions at the gap region of collagen structure.

1.8.2.4. Properties of vegetable tanned leathers

Vegetable tanning may be used as sole tanning agents or in conjunction with other tannages. Tannins have the property of filling the inter-fibre space and hence give leathers increased degree of stiffness and fullness. Vegetable tanning agents are often used as retanning agents to modify the properties of Cr(III) tanned leather (Sharphouse 1971). Combination tannage using metals, mainly Al(III) and Ti(IV), can also be applied to impart desired properties such as colour, increase in shrinkage temperature and enhancement of resistance to perspiration (Vitolo *et al.* 2003; Van Benschoten 1985).

Tanning pickled pelts with hydrolysable tanning agents results in shrinkage temperatures in the range of 75-80°C, slightly lower than that of leathers tanned with condensed tannins (75-80°C). The greater shrinkage temperature of condensed tannins could be related to the the larger size of the oligomeric condensed tannin molecules (Covington 2008). Oxidation of condensed tannins may form quinone moieties in the structure (Hathway 1957), quinone moieties of oxidised tannins may also react with the collagen amino groups forming covalent crosslinks (Heidemann 1993).

1.8.3. Deterioration of vegetable tanned leathers

Vegetable tanned leathers are generally stable, the tanning reaction are non-reversible. However, deterioration and degradation of vegetable tanned leather of historical or aged leathers has been a commonly observed phenomenon and has been a subject of concern for conservators and leather chemists (Thomson 2006). The degradation of vegetable tanned leather is reflected in physical weakening, change of colour, formation of surface cracks, reduced flexibility, weak fibre, powdery surface and lowering of shrinkage temperature (Larsen 1996). Analysis of the chemical properties of most of degraded vegetable leathers also shows increased acid content, increased sulfate content, and dissolution of degraded tannin components (Wouters *et al.* 1996). A review of the mechanisms of degradation of aged vegetable tanned leathers by Florian (2006) indicates that hydrolysis and oxidation are the principal mechanisms of the process.

Other factors, namely temperature and moisture, also play an important role in enhancing the physical degradation of the leather.

1.8.3.1. Hydrolytic degradation

Sulfuric acid in leather, originating from the adsorption of sulfur dioxide from industrial air pollution, it is believed to be the most common cause of hydrolytic damage in age-old leathers. In presence of light, the sulfur dioxide is oxidised to sulfur trioxide that is subsequently hydrolysed by the moisture in the leather to form sulphuric acid. In addition, other organic acids or mineral acids already existing in the leather may also enhance hydrolytic degradation (Florian 2006).

Increased acidity in the leather may cause changes in the tannin structures and hydrolytic breakdown of the peptide chain of the collagen (Figure 1.13). Acid-deterioration (hydrolytic degradation) of vegetable tanned leathers is characterised by disintegration of fibres, lose of strength and lowering of shrinkage temperature (Stambolov 1989; Haines 1991).

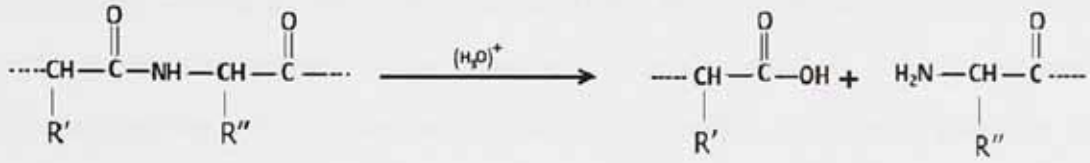


Figure 1.13 Schematic representation of hydrolytic cleavage of the peptide chain of collagen in vegetable tanned leathers undergoinFlod-deterioration (Haines 1977)

1.8.3.2. Oxidative degradation in vegetable tanned leathers

According to Haines (1977), sulfur dioxide and nitrogen oxides from atmospheric pollution react with tannins and unsaturated hydrocarbons (*e.g.* fats) in the leather to form oxidative species such as ozone (O_3), nitrogen dioxide (NO_2), peroxyacetylnitrate ($CH_3[CO]OONO_2$), and oxygen-derived free-radical species. As a result, oxidative cleavage the peptide chain of collagen may occur as described in Figure 1.14.

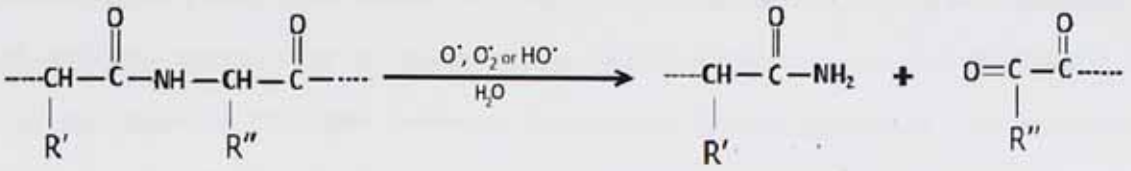


Figure 1.14 Schematic representation of oxidative cleavage of the peptide chain of collagen in aged vegetable tanned leathers exposed to oxygen derived free radicals in the presence of water (Haines 1977).

The oxidation of fatliquors and tannins may lead to formation of hydrogen peroxide and oxygen radicals (Florian 2006). In various investigations including artificial-ageing tests, it was concluded that hydrolytic and oxidative degradation of vegetable tanned leather may occur simultaneously in acid media (Stambolov 1969; Florian 2006). Degradation of collagen during deterioration of leather occurs to a greater extent in warmer and moist conditions (Larsen 1996)

1.8.3.3. The role of metal ions in leather deterioration

Transition metals that may undergo univalent redox reactions, can participate as catalysts in the oxidation reaction in vegetable tanned leathers (Florian 2006). Metal ions such as Fe^{2+}, Cu^{2+} and Mn^{2+} are capable of catalysing the oxidation of sulfur dioxide into sulfur trioxide (Zuo *et al.* 2005; Bradt *et al.* 1995; Berglund *et al.* 1995) and enhance acid-deterioration of leather. Some metal ions may also catalyse the autoxidation of polyunsaturated fatty acids in leather and increase formation of hydrogen peroxide (Mills *et al.* 1999). Earlier studies of artificial ageing test have also demonstrated that oxidative deterioration is enhanced by the presence of trace quantities of iron and cobalt (Cheshire 1946). Later studies by Phillips (1954) and Deasy (1967) showed that, during leather degradation under conditions of artificial ageing, iron catalysed decomposition of hydrogen peroxide (Fenton reaction shown in equation 1.1) may results in the formation hydroxyl radicals through the Fenton reaction, leading to degradation of the collagen.

$$Fe^{2+} + H_2O_2 + H^+ \longrightarrow Fe^{3+} + HO^\bullet + H_2O$$

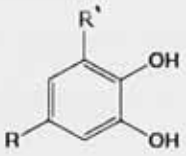
$$Fe^{3+} + H_2O_2 \longrightarrow Fe^{2+} + HOO^\bullet + H^+$$

Equation 1.1 The Fenton reaction – a catalytic decomposition of H_2O_2 in the presence of iron ions resulting in the formation of hydroxy radical ($HO^\bullet$) and superoxyl radical ($HOO^\bullet$) (Phillips 1954).

Haslam (1998) also hypothesised that the principal mechanism for the slow degradation of vegetable leather may be related to the oxidative effect of iron ions (Fe^{2+}/Fe^{3+}) and the formation of hydrogen peroxide in leather; it was suggested that subsequent collagen degradation may be the result of the oxidative action of the hydroxyl radicals generated *via* the Fenton reaction.

1.9. Tannin-metal interactions in leather

One of the basic chemical properties of tannins is the formation of chelate complexes with metal ions. This interaction is often characterised by colour formation and precipitation of metal complexes (McDonald *et al.* 1996; Bark *et al.* 2012). With respect to the formation of tannin-metal complexes, the catechol (1,2 dihydroxybenzene) and the pyrogallol (1,2,3-trihydroxybenzene) moieties are the most important structural parts of tannins (Slabbert 1992). These metal chelating moieties of tannin compounds (Figure 1.15) are referred to as *o*-diphenol groups (Porter 1992).



R denotes the link to the C-ring of flavonoid structure (in condensed tannins) or the glycoside ester link (in hydrolysable tannins)

R'=H in the case of catechol moieties and **R'= OH** in the case of pyrogallol moieties

Figure 1.15 A structural model of the metal chelating *o*-diphenol groups of tannin compounds of tannins (Slabbert 1992)

According to Sykes *et al.* (1980), the pyrogallol moieties form more stable complexes with metals as compared to the catechol groups. All hydrolysable tannin compounds have multiple pyrogallol groups as shown in the structure of tannic acid (Figure 1.16b); hence, they are generally more reactive to metal ions. Condensed tannins having a pyrogallol type B-ring of the flavonoid structure (Figure 1.16a), *i.e.* prorobinetinidines and prodelphinidins, are also considered to be more reactive to metal ions as compared to those having catechol type B-ring structure *i.e.* procyanidins and profisetinidines.

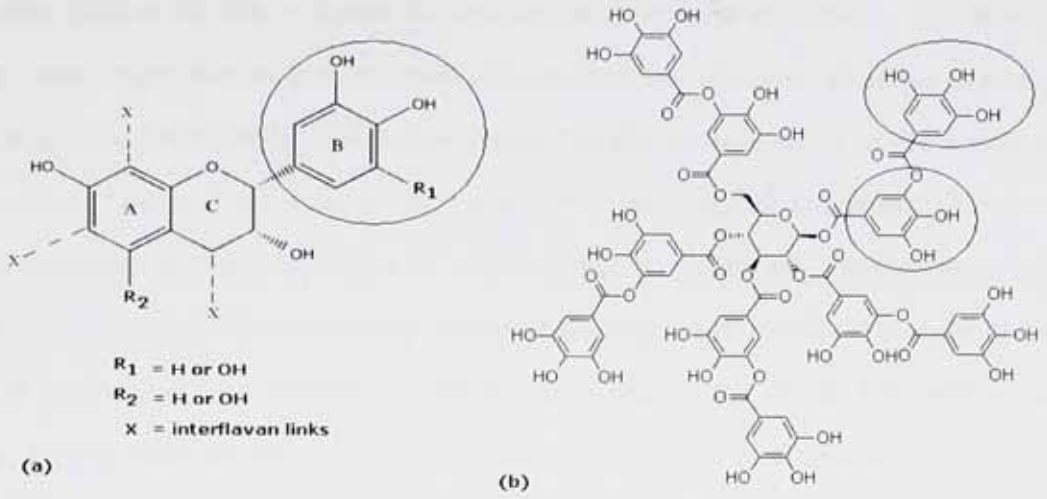


Figure 1.16 The metal chelating site of tannins (*o*-diphenol groups) in the (a) flavonoid structure of condensed tannins and (b) in hydrolysable tannins (*e.g.* tannic acid)

The nature of tannin-metal complex formation may involve a charge transfer mechanism (Figure 4.17) in which the two hydrogen ions are displaced from the *o*-diphenol group by the metal ion when chelate complexes are formed. A potentiometric study by Slabbert (1992) indicates that, stability of the tannin-metal complexes is inversely related to their primary pK_a values of the chelating groups, increased degree of deprotonation or formation of phenolate units, may favour chelation of metal ions.

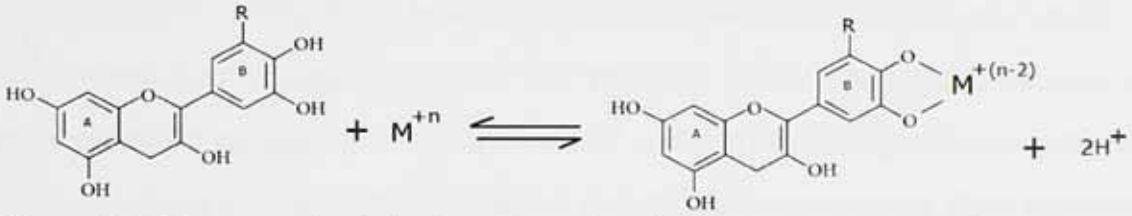


Figure 1.17 Tannin-metal complex formation with a flavonoid structure through ligand to metal charge transfer mechanism (Slabbert 1992)

The pK_a of primary ionisation *o*-diphenol groups in various polyphenolic compounds varies in the the range of 6.7-9.3 depending on the type of aromatic substituent. Catechol groups (Figure 1.15, R=R'=H) and pyrogallol groups (Figure 1.15, R=H, R'=OH) have pk_a values of 9.37 and 9.05 respectively. For secondary ionisations, the pK_a values of most *o*-diphenols are greater than 11.0 (Jovanovic *et al.* 1994; Porter 1992).

A greater portion of the polyphenols are not ionised in acidic media, therefore the tannin-metal interaction in this condition may involve the formation of dative bonds with the metal being the acceptor (Covington 2009). On the other hand, it is also known that the presence of metal ions such as Al^{3+} and Fe^{3+} in solutions of polyphenol compounds causes lowering of the pKa values of the *o*-diphenol groups and hence increase the degree of ionisation of the *o*-diphenol groups (Kumamato *et al.* 2001). The type of metal ion also determines the stability of tannin-metal complexes. Metal ions with greater charge density, such as Al(III), V(IV), Fe(III) and Ge(IV) form stable tannin complexes in acid media resulting in precipitation at pH 3.0-3.6. In contrast, metals, such as Pb(II), Zn(II), Co(II), and Ni(II) precipitate as tannin complexes at higher pHs, above 6.0 (Slabbert 1992).

1.9.1. Mechanism of semi-metal tanning

Treatment of vegetable tanned leathers with salts of as Al(III), Ti(IV) metal raises the shrinkage temperature to 110°C and above (Slabbert 1981). Retanning of vegetable tanned leather with metal salts is a form of combination tanning that is referred to as semi-metal tanning, analogous to the semi-alum or semi-Al(III) tanning process (Covington *et al.* 1981). It has been suggested that semi-Al(III) tanning may be used as an alternative to tanning with Cr(III) salt (Slabbert 1981). Semi-Al(III) tannage has been developed for making shoe upper leather of T_s>110°C using the hydrolysable tara tannin (Vitolo *et al.* 2003). Other transition metal salts may also bring about increase in the shrinkage temperature when used to retan vegetable tanned leathers (Kallenberger 1984). Semi-metal tanning using Ti(IV) salts, *i.e.* semi-Ti(IV) tanning, has been used for making perspiration-resistant leathers (Thomson 1981; Van Benschoten 1985).

In combination tanning using metals and vegetable tanning agents, the order of tanning is an important aspect. Retanning of metal tanned leather with vegetable tanning agents generally imparts lower shrinkage temperature as compared to that of vegetable tanned leather retanned with metal salt (Kallenberger and Hernandez 1984; Covington 1997). Concerning the mechanism of semi-metal tanning, Slabbert (1981) initially suggested

that aluminium ion reacts with the already bound tannin molecules and forms a covalent link with the carboxyl groups of collagen as shown in Figure 1.18.

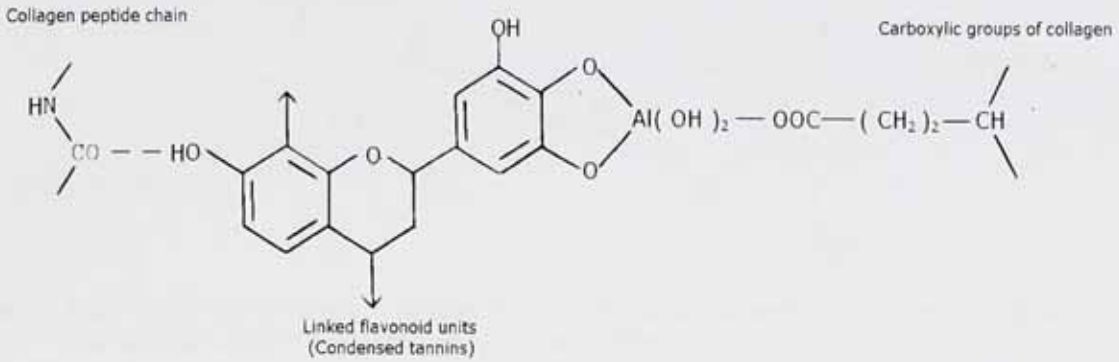


Figure 1.18. Slabbert's model of collagen—Al(III)—tannin interaction in mimosa-Al(III) tanned leather (Slabbert 1981).

Slabbert(1992) assumed that further crosslinking of the tanning interaction by the tannin-Al(III)-collagen interaction, involving Al(III)-collagen covalent bonds, was responsible for the increase in the shrinkage temperature of semi-Al(III) leather. The proposed model of semi-metal tanning interaction lacked evidence in relation to the suggested formation of collagen-Al(III) covalent bonds.

However, it was also known that Al(III) does not exhibit a stable interaction with collagen, as observed with the reversibility of Al(III) tannage (Covington *et al.* 1987). An investigation by Kallenberger and Hernandez (1983) also showed that modified hide powder samples with blocked carboxyl groups, *i.e.* by methylated hide powder, showed semi-metal tanning reaction and there was no difference between the shrinkage temperature of the samples made using the modified (methylated) and unmodified hide-powder samples. The conclusion was that carboxyl groups of collagen do not take part in the chemical reactions of semi-metal tanning. Hence, the Al(III)-collagen interaction was found to be unimportant in the semi-metal tanning reactions.

In addition it was also shown that transition metals such as Ni(II) and Co(II), that are known to have a weak interaction with carboxyl groups of collagen in acidic media can significantly increase the shrinkage temperature of the vegetable tanned leathers (Kallenberger and Hernandez 1984). Based on these experimental results, it is believed that the increase in shrinkage temperature because of semi-metal tanning may originate

from complexation of metal ion by polyphenols, as shown in Figure 1.19 (Kallenberger and Hernandez 1984; Covington and Lampard 2004).

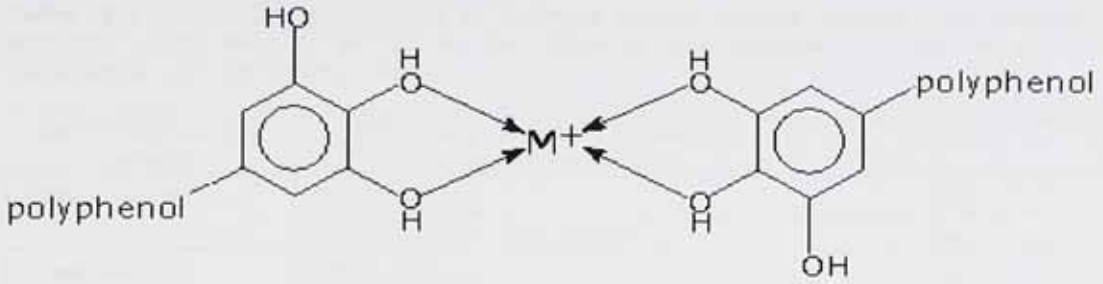


Figure 1.19. Proposed schematic representation of the principal semi-metal tanning interaction in semi-metal tanned leather (Covington and Lampard 2004)

Complexation of the metal centre during semi-metal tanning creates crosslinks in the polyphenolic matrix resulting in the formation of a reinforced tanning structure around the collagen (Tang *et al.* 1992). The resulting rigidity of structure poses greater resistance to unravelling of the collagen macromolecule during the shrinkage transition. As a result, the shrinkage temperature of semi-metal tanned samples is raised considerably as compared to that of the vegetable tanned leather (Vitolo *et al.* 2003)

The main tannin-metal interaction in semi-metal tanned leathers is chelate complexation of metal centres by *o*-diphenol moieties of the tannin molecules. However, hydrothermal stabilisation is considered the result of a set of inter-related chemical interactions that essentially involves-

- collagen-metal interaction (electrostatic interaction and complex formation),
- collagen-tannin interaction (mainly hydrogen bonding)
- tannin-metal interactions (chelate complex formation)

Amongst these, the collagen-metal interactions may have comparatively less significant contribution towards the overall effect as compared to that of metal-polyphenol complexation (Kallenberger and Hernandez 1983).

1.9.2. Transition metals in semi-metal tanning

The results of semi-metal tanning experiment by Kallenberger and Hernandez (1984) shown that the hydrothermal stabilities of the semi-metal tanned samples made with transition metals are not dependent on the interaction of the metal ions with collagen.

Another observation from the results (Table 1.7) is the significant variation of shrinkage temperatures in relation to the absence or presence of a citrate salt.

Table 1.7 Shrinkage temperature of mimosa tanned leather treated with different transition metal salts at pH 5.0 in the presence and absence of a sodium citrate Kallenberger and Hernandez (1984)

semi-metal tanned samples	Retanning chemicals applied on mimosa tanned leather	T_s, °C
mimosa-Ni(II)	Ni(II) sulfate	107
	Ni(II) sulfate + plus citrate	89
mimosa-Co(II)	Co(II) chloride	112
	Co(II) chloride + plus citrate	95
mimosa-Mn(II)	Mn(II) acetate	90
	Mn(II) acetate + plus citrate	90
mimosa-Mo(VI)	Sodium molybdate	69
	Sodium molybdate + plus citrate	79
mimosa-Fe(III)	Fe(III) chloride	101
	Fe(III) chloride + plus citrate	111

The presence of citrate with the semi-Ni(II) and semi-Co(II) samples resulted in weakening of the crosslinking interactions as observed in the decline of shrinkage temperature. The opposite was observed in the case of the semi-Fe(III) samples. The effect of citrate on the divalent and trivalent ions appears to be different, change in the ligand environment of the metal may affect the stability of metal-tannin complex . The result indicated that the hydrothermal stability of semi-metal tanned samples is influenced by a complex set of interactions including the ligand environment of the metal.

1.9.3. Metal induced degradation of vegetable tanned leathers

As shown in Table 1.6, the shrinkage temperature of the mimosa tanned leather was significantly lowered after treatment with molybdate $(MoO_4)^{2-}$, this indicated the reaction between the tannins and the oxy-anion $(MoO_4)^{2-}$ caused breaking of crosslinks in the tanning matrix. This change most likely occurred as a direct result of oxidation of tannins in the leather by the oxy-anion of the hexavalent molybdenum. Compounds and complexes containing molybdenum (VI) are known oxidising agents (Greenwood *et al.* 1989). In another study, Lampard (2000) also observed that a semi-V(IV) leather prepared by treating myrabalan tanned leather (T_s 71°C) with V(IV) sulfate (myrabalan-

V(IV) leather) showed an increase in hydrothermal stability to 95°C. However, after months of storage at ambient condition, the shrinkage temperature lowered to 60°C and the leather became significantly weaker during storage at ambient conditions.

Based on the observation of the metastability of the myrabalan-V(IV) leather, Covington (2009) suggested that, understanding of the mechanism of the observed V(IV) induced degradation of leather may be useful in developing tanning methods for production of gradually deteriorating leathers, that may eventually be biodegradable. It was assumed that the leather that undergoes a slow V(IV) induced degradation, might be serviceable for a definite period (i.e. depending of the rate of degradation) and eventually become vulnerable to the action of enzymes as a result decomposition of the tanning matrix. This concept is similar to the application of metal catalysed degradation of the so-called oxo-degradable plastics, which contain trace quantities of photocatalytic metals, such as Fe(II), Co(II), Cr(III) and Mn(II), that are added to enhance degradation (Wiles *et al.* 2006).

In the oxo-degradable plastics, metal induced photoxidation reactions in the polymer structure leads to fragmentation in natural environment (Sipinene and Rutherford 1992). There have been claims of biodegradability and compostability of some oxo-degradable plastics after fragmentation (Stranger-Johannessen 1979; Chiellini *et al.* 2003) based on tests that demonstrated the growth of microorganisms the polymer surface. However, recent studies (Thomas *et al.* 2010; Roy *et. al.* 2011) have shown that, metal catalysed photoxidation of the oxo-degradable polymers leads to physical fragmentation of the polymers but not necessarily degradation by microorganisms.

Unlike plastics, leather has a complex composition and heterogeneous structure; hence, degradation by metal catalysed oxidation may have different features. In this research, the interaction of vanadium and other transition metals with untanned collagen and vegetable tanned leather were investigated. The relative permanence of metal-tanned and semi-metal tanned samples was evaluated experimentally. The mechanisms of

metal induced degradation and the susceptibility of the physico-chemically degraded leathers to the action of bacterial enzymes were studied.

1.10. Description of the research project

The research included literature review and practical work on general aspects of the chemistry of tanning interactions, particular aspects of tannin-metal interactions related to complex formation and redox interactions, mechanisms of degradation of vegetable tanned leather, the Fenton reaction and enzymatic degradability of oxidatively degraded leathers. The aim and objectives of the research project are stated in the following sections (Section 1.10.1-2)

1.10.1. Aim of the project

To study the rate, extent and mechanism of metal induced degradation in leather, characterise the degree of susceptibility of degraded leathers to enzymatic hydrolysis and there by identify chemical methods that can be applied for treatment of leather waste in the context of enhancing the its recyclability.

1.10.2. Objectives of the project

- To evaluate the tanning reactions of transition metals with collagen and vegetable tanned collagen; analyse the permanence and stability of the tanning interactions (Chapter 2)
- To characterise the properties of vegetable tanned leathers containing Fe(II) and V(IV) ions and the process of degradation in terms of the changes in the physical and chemical properties of the leathers (Chapter 3)
- To elucidate the mechanism of metal-induced degradation of vegetable tanned leathers by studying oxidation of polyphenol compounds in aqueous solution and evaluating the effect of V(IV) and Fe(II) on the oxidation reactions (Chapter 4)
- To investigate the effect of hydroxyl radicals on the hydrothermal stability and physical properties of different types of leathers and analyse the applicability of Fenton reaction (Fe^{2+}/H_2O_2) as a method for rapid degradation of leathers (Chapter 5)
- To study the enzymatic degradability of leather samples that have undergone metal-induced degradation and Fenton-oxidised leather samples using bacterial enzymes namely, collagenase and subtilisin (Chapter 6)
- To elucidate the mechanism of metal catalysed oxidative degradation of leather based on results of the research and to propose the potential application of hydroxyl radiated oxidative treatment (via Fenton reaction) as an oxidative pre-treatment method for use on leather waste, for the purpose of enhancing its biodegradability in composting or anaerobic digestion. (Chapter 7)

CHAPTER 2

THE STABILITY OF METAL TANNED AND SEMI-METAL TANNED COLLAGEN

2.1. Introduction

Transition metal ions have empty or partially filled d-orbitals and hence engage in complex formation with the *o*-diphenol moieties of tannin compounds. According to the semi-metal tannin mechanism suggested by Covington and Lampard (2004), semi-metal tanning interactions are multiple coordinate-covalent bonds formed between polyphenols and metal ions. Slabbert (1992) suggests that the tannin-metal interaction involves charge transfer complex formation, in which the hydrogen ion from the chelating hydroxy groups the of *o*-diphenol groups will be substituted by metal ion. It is known that the formation of tannin-metal complex results in a more cross linked tanning structure. Subsequently, semi-metal tanned leathers show greater hydrothermal stability as compared to metal tanned and vegetable tanned leathers (Kallenberger and Hernandez 1983; Slabbert 1992; Covington *et al*. 2008).

The most common semi-metal tanned leathers, semi-Al(III) are known for their greater hydrothermal stability (T_s>110°C) and resistance to deterioration under artificial ageing conditions (Larsen 1996; Thomson 2006). In contrast, semi-metal tanning experiments carried out with other metals; indicates that certain tannin-metal interactions might lead to lowering of T_s of leathers as described in Chapter 1, Section 1.9. The permanence of tannin interactions depends on the stability of *tannin-metal*, *metal-collagen* and *collagen-tannin* interactions. In vegetable tanned leather *collagen-tannin* interactions, i.e. mainly hydrogen bonds, are not readily reversed under normal conditions of storage. Hence, the increase in T_s during semi-metal tanning as well as the lowering of T_s observed in some semi-metal tanned leathers, e.g. semi-V(IV) leathers, may be related to the *metal-collagen* or *tannin-metal* interactions.

In previous studies, Kallenberger and Hernandez (1984) and Lampard (2000) have demonstrated that several transition metals may exhibit semi-metal tanning, resulting an increase in T_s when used in retanning vegetable tanned leathers. However, the permanence of the semi-metal tanning interactions has not been explored in detail. In

this chapter, comparative experiments carried out on the properties of a range of metal-tanned and semi-metal tanned collagen are described. Tanned samples were prepared using hide powder (collagen). The tanning properties of transition metals were investigated and the permanence of tanning interaction was evaluated by periodic analysis of samples that were stored at a controlled conditions.

2.2. Materials and methods

2.2.1. Chemicals

Glacial acetic acid (99% w/w), anhydrous sodium acetate, anhydrous sodium bicarbonate, sodium chloride, acetone solvent, nitric acid (70% w/w), sulfuric acid (98% w/w), perchloric acid (70% w/w), standard solutions of the first row transition metals (1000 ppm), and sulfate salts of metals (Table 2.1) were obtained from Fisher-Scientific Ltd. (UK).

Table 2.1. The first-row transition metal sulfate salts used for preparation of metal tanned and semi-metal tanned samples.

metal ion	Molecular formula of the salt
Al^{3+}	$Al_2(SO_4)_3.16H_2O$
Ti^{4+}	$Ti(SO_4)_2$. 15% w/w solution
V^{4+}	$VOSO_4.5H_2O$
V^{5+}	NH_4VO_3 (anhydrous)
Cr^{3+}	$CrK(SO_4)_2.12H_2O$
Mn^{2+}	$MnSO_4.4H_2O$
Fe^{2+}	$FeSO_4.7H_2O$
Fe^{3+}	$Fe_2(SO_4)_3$
Co^{2+}	$CoSO_4.7H_2O$
Ni^{2+}	$Ni(SO_4).6H_2O$
Cu^{2+}	$CuSO_4.5H_2O$
Zn^{2+}	$ZnSO_4.7H_2O$

2.2.2. Vegetable tanning extracts

A hydrolysable tanning agent (Myrabalan extract) was sourced from SilvaTeam Spa. (Italy) and a condensed tanning agent, (Mimosa ME) was obtained from Forestal Co. Ltd (UK).

2.2.3. Hide powder

The hide powder used in the experiments was prepared according to the method of Brown *et al.* (2010). A piece of wet salted hide (100cm X 100cm) was processed to the

bated stage according to a conventional process. The bated hide was repeatedly washed with excess water and then immersed in a 0.5% solution of acetic acid for 48 hours to equilibrate the pH to 5.5. The wet pelt was then cut to small strips and dehydrated completely using acetone solvent. Then the strips were removed from the solvent, dried at room temperature, ground and stored in sealed plastic bags.

2.2.4. Analytical instruments

2.2.4.1. Differential scanning calorimeter (DSC)

A differential scanning calorimeter, Mettler-Toledo 822e (Mettler-Toledo GmbH., Switzerland), was used to determine the shrinkage temperature (T_s) and enthalpy of shrinkage transition (ΔH) of samples. Standard aluminium pans (40μL) were used for all measurements. The calorimeter was calibrated with indium metal (*i.e.* fusion transition:156.6±0.3°C and enthalpy -28.45±0.5 J/g). A constant flow of nitrogen gas (0.2 L/min) was applied during all measurements to regulate heat flow in the oven. The thermographs of heat flow against temperature were analysed using Mettler-Toledo STARe software.

2.2.4.2. Inductively coupled plasma - optical emission spectrometer (ICP-OES)

A thermo-ICAP 6000 ICP-OES (Thermo Scientific Ltd., Hamel hempstead, UK) was used for the analysis of the metal contents by measuring the intensities of emission from the elements in argon plasma. Thermo-ITEVA software was used to control parameters of measurement and analyse the data. All measurements were carried out with the following set up of parameters: 15 L/min of plasma argon flow, 0.5 L/min of auxiliary argon flow, sample flash rate of 4 mL/min and sample flow rate 1.3 mL/min.

2.2.4.3. pH meter

A Multi-Seven pH meter (Mettler-Toledo GmbH., Switzerland) was used, calibration were done using phthalate, phosphate and carbonate buffers of pH 4.0, pH 7.0 and 9.2, respectively.

2.2.5. Procedures

Hide powder to be used in the experiments was prepared and characterised by determining the pH-value and ash content according to standard methods. Hide powder samples were tanned separately with the different metal salts shown Table 2.1 and the vegetable tanning agents described in Section 2.2.2. A range of semi-metal tanned samples were also prepared by retanning the vegetable tanned hide powders different metal salts.

The tanning effect of the transition metals with hide powder and vegetable tanned hide powder was evaluated by determination of T_s using DSC. The metal content of the different semi-metal tanned samples were analysed by ICP-OES. To evaluate the permanence of tanning interactions, samples were stored in a conditioned room (21°C and 65% RH) and periodic measurements of hydrothermal stability were carried out.

2.2.5.1. Analysis of hide powder

The ash contents of the hide powder were determined in accordance with the official methods of analysis, "Determination of ash in hide powder", SLC-127 (SLTC 2000). A hide powder sample was dried in oven (110°C) for 12 hours and cooled in a desiccator, triplet portions of the hide powder (each weighing 2.5g) were transferred to platinum crucibles of known weights, after ignition at 600°C for 2 hours the samples were cooled, weight differences were recorded and the average. The average ash contents were calculated. The pH of the hide powder was measured using the official method of SLTC,"Determination of pH of hide powder" SLC-131 (SLTC 2000).

2.2.5.2. Preparation of metal tanned hide powder samples

Dry hide powder samples (2g) were rehydrated with 20 mL of 5% NaCl solution in 100 mL flasks and equilibrated over 24 hours to pH 2.6-2.8 using 0.1M acetic acid. Calculated quantities of the metal salts (Table 2.1), equivalent to 100mg of the metal, were dissolved in 10 mL of 0.01M of hydrochloric acid and stirred into the separate samples. After an hour of stirring the pH was adjusted to 5.0 using 0.1M sodium

bicarbonate solution, then the samples were heated at 35°C for another 1 hour. Finally, the tanned samples were filtered out, then dried and stored at 21°C and 65% RH.

2.2.5.3. Preparation of vegetable tanned hide powder samples

Separate batches of hide powder (5g) were rehydrated for 12 hours with 50 mL of 5%(w/v) NaCl solution and adjusted to pH 6.5. Tanning was carried out with a total of 0.5g of tannin powder, equivalent to 10% w/w on the weight of the dry hide powder samples. The tannin powder was pre-dissolved in water in 1:10 ratio and stirred into the hide powder samples in four equal aliquots at 30 minute intervals. Then the pH was adjusted to 4.0 and the tanned hide powder samples were filtered and dried at room temperature.

2.2.5.4. Preparation of semi-metal tanned hide powder samples

Dried vegetable tanned hide powder samples, each weighing 1g, were rehydrated with 15 mL of 5% (w/v) NaCl solution in 50 mL flasks for 2 hours and adjusted to pH 2.8-3.0 using 0.1 M acetic acid. Then 5 mL of 0.1 M solution of the metal ion was stirred into each sample, stirring was continued for 1 hour. The samples were heated at 35°C for 1 hour and the pH was adjusted 5.0±0.1 by drop wise addition of 0.5M sodium bicarbonate solution. Then the samples were filtered, rinsed with 5 mL of deionised water and dried at 21°C and 65% RH. Control samples of vegetable tanned hide powder were processed in the same way but without addition of metal salt.

2.2.5.5. Determination of metal content

The metal content in the semi-metal tanned samples was determined in accordance with the IULTCS official method of analysis "Chemical determination of metal content in leather" IUC-27/ISO- 17072-1:2011 (IULTCS 2011). Dry samples weighing 0.25 g were placed in 25 mL digestion tubes and digested with 5 mL of a ternary acid digestion matrix of concentrated acids (nitric acid : perchloric acid : sulfuric acid, volume ratio 3:1:1). The digestion was carried out for 2 hours at 105 °C in a thermostatic digestion block. After cooling to room temperature, analyte solutions of each sample were

prepared by diluting the digested solution to 50 mL in volumetric flasks using deionised water. Standard solutions of each of the metal (0, 5, 10, 15, 20 mg/L) were prepared in deionised water. Triplet measurements of the calibration solutions, the analyte solutions and a blank solution of the digestion matrix were carried out using ICP-EOS at the characteristic optical emission wavelengths of the elements.

The calibration curves of the emission intensities against the gradient concentration showed correlation coefficient above 0.99 in all cases in all cases. The concentrations in each analyte solutions were determined using the calibration curve obtained at particular wavelengths. The RSD (relative standard deviation) of concentrations obtained at the different optical emission wavelengths of each metal were less than 1%. The average metal concentration was determined from the results of triplet measurements. The values of metal concentration in the hide powder samples in units of mmol/g were calculated as follows:-

$$M_c = \frac{P \times V}{A \times W}$$

Where :-

P is the measured concentration of metal in mg/L in analyte solutions
V is the volume of the analyte solution (0.05 L),
W is the dry weight of samples (0.25 g)
A mass of a milllimole of the metal in mg/mMol

2.2.5.6. Analysis of the hydrothermal stability

Measurements of the temperature and enthalpy of shrinkage were carried out based on the method of Chahine (2000). Samples were fully rehydrated with deionised water for two hours. After removal of excess water by blotting with filter paper, wet samples (5-10mg) were placed flat at the base of standard aluminium pan (40μL) and hermetically sealed. Measurements were carried out using DSC by scanning from 20 to 140°C at the heating rate of 5°C/min with an empty sample pan being used as reference. After completion of the measurements, the dry weight of the samples was measured after heating samples at 110°C oven for 60 minutes to remove all remaining moisture. From the thermographs of the measurements, the on-set temperature and enthalpy of the shrinkage transition were determined and the normalised values of enthalpy were calculated based on dry weight of the measured samples.

2.3. Results and Discussion

2.3.1. Hide powder

The characteristics of the hide powder prepared in this study (shown in Table 2.2) comply with the requirements of pH and ash content stated in the SLTC official methods (SLTC 2000). The average T_s hide powder is also similar to the values T_s of untanned collagen mentioned in literature *i.e.* 58-60°C (Reich 2007; Tang *et al.* 2003; Bozec *et al.* 2011).

Table 2.2. Characteristics of the hide powder used in the experiments

parameter	Expected values	Average values
pH value	5.0-5.5	5.46
Ash content (%w/w)	< 0.3 %	0.28 %
T_s, °C	60°C (±2)	59.5 °C

2.3.2. Vegetable tanned hide powder

The hide powder samples that were tanned with 10% of myrabalan and mimosa tanning powders showed T_s of 71.5°C and 79.5°C, respectively. As described in Chapter 1 (Section 1.6), condensed tannins are oligomeric structures of flavonoid monomeric units linked in linear or branched geometry through C-C bonds (Porter 1992). The structure makes up a relatively larger interlinked polyphenols as compared to hydrolysable tannins, which are composed of discrete polyphenolic compounds (Okuda *et al.* 1991). Hence, in the vegetable tanned leathers, the mimosa tannin forms a relatively more rigid matrix than the myrabalan tannin.

2.3.3. Shrinkage temperatures of the metal tanned samples

As shown in Figure 2.1, the metal tanned hide powder samples have T_s that are lower than 75°C, with the exception of the Cr(III) tanned sample. The samples tanned with Al(III), Ti(IV) and V(IV) have T_s values between 70-73°C, tanned samples as compared to the other samples. The results shown in Figure 2.1 are similar to those reported by fKallenberger and Hernandez (1984) and Lampard (2000). The effect of Mn(II) and Zn(II)

in terms of hydrothermal stabilisation appears to be almost negligible, the T_s of the collagen samples tanned with these metals was raised only by 1-2°C. The Co(II), Ni(II) and Cu(II) tanned samples showed increase in Ts by 6-7°C by tanning with In these cases, the tanning effect of the metals may be considered as non-existent because the collagen-metal interactions are most likely to be labile electrostatic attractions.

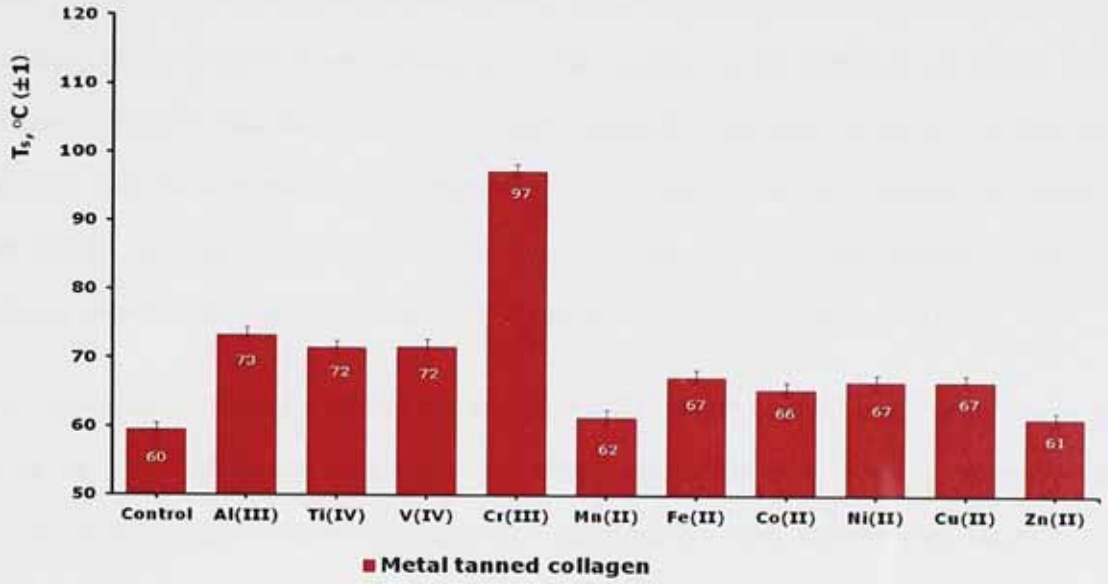


Figure 2.1 Shrinkage temperature (T_s) hide powder samples tanned with metals salts

As described in Chapter 1 (Section 1.8), hydrolysis of the metal ions and subsequent formation of oligomeric metal-complex species important factor that influences the interaction of the metal ions with collagen and resulting hydrothermal stabilisation. The tanning effect of transition metal complexes is relatively greater when tannage is carried out to at pH values close to the precipitation point of the metal hydroxides (Heidemann 1993; Covington 2009). The primary hydrolysis of the metal ions and the acid-hydrolysis constant (K_a) is represented as:-

$$M^{n+}(H_2O)_6 \rightleftharpoons M^{(n-1)+}(H_2O)_5(OH) + H^+ \quad ; \quad K_a = \frac{[M^{(n-1)+}(H_2O)_5(OH)]\ [H^+]}{[M^{n+}(H_2O)_6]}$$

In acidic media, Ti(IV) occurs in the form of titanyl ion $(TiO)^{2+}$ which also extensively hydrolyses with pK_a value lower than 1. Similarly V(IV) also occurs in oxycation form known as vanadyl ion $(VO)^{2+}$ which hydrolyses with of pK_a 6.0. Al(III) and Cr(III) ions

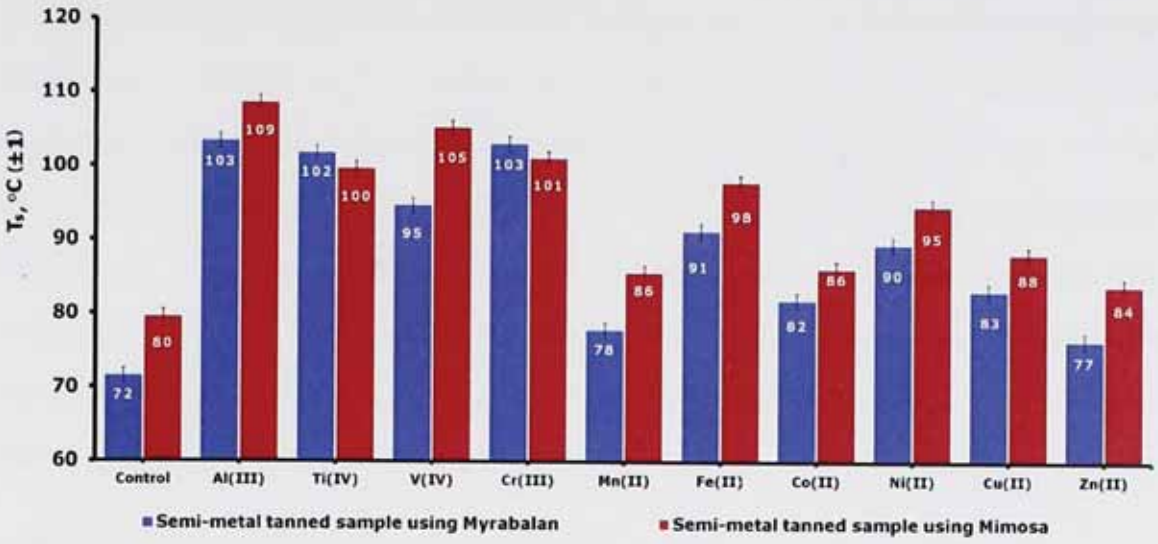


Figure 2.2 – Shrinkage temperature (T_s) of semi-metal tanned samples prepared from mimosa and myrabalan tanned hide powders by retanning with transition metal salts.

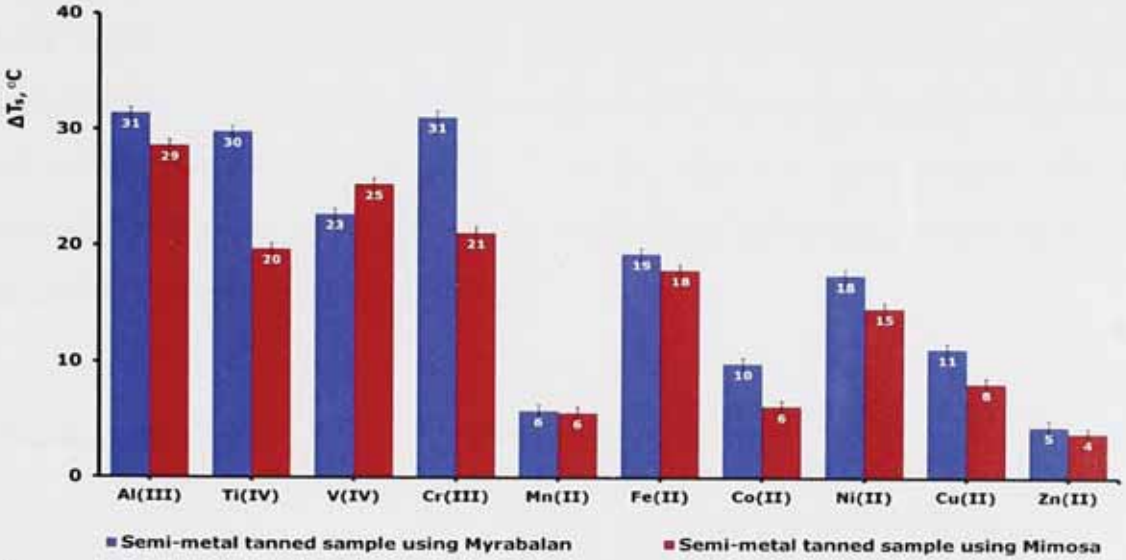


Figure 2.3. Net change in shrinkage temperature (ΔT_s) as a result of retanning of vegetable tanned hide powder samples with transition metals.

The mimosa based semi-metal tanned samples of Al(III), Ti(IV), V(IV) and Cr(III) showed T_s values of 100°C and above, showing rise of T_s by 21-29°C. The highest T_s of all semi-metal tanned samples was that of the mimosa-Al(III) sample (109°C). Similar to the case of the myrabalan samples, the mimosa based semi-metal tanned samples of Mn(II), Co(II),Cu(II) and Zn(II) showed relatively lower rise in T_s as compared to that of the mimosa tanned control (T_s 80°C).

Except with the case of the semi-V(IV) tanned samples, the myrabalan based semi-metal tanned samples have shown slightly greater net increase of T_s (Figure 2.3) as

compared to the corresponding samples of mimosa bases semi-metal tanned samples. This may be related to the greater concentration of the pyrogallol groups in the myrabalan tannins resulting in the formation of relatively more stable tannin-metal complexes (Sykes *et al.* 1980). Most of the transition metal show greater hydrothermal stabilisation when in retanning vegetable tanned samples, as compared to their hydrothermal stabilisation on untanned collagen (hide powder). This indicates that the transition metals may be more reactive towards the *o*-diphenol groups of the tannins rather than the carboxyl groups of collagen.

2.3.5. Synergistic hydrothermal stabilisation

In previous studies, the relative degree synergistic hydrothermal stabilisation in semi-metal samples has been compared in terms of the net increase of T_s beyond the summation of the individual the T_s values (Covington and Lampard 2004; Kallenberger and Hernandez 1984). The net gain of T_s of as a result of the synergistic effect of vegetable tannins and metals (synergy of hydrothermal stabilisation, denoted as (Y) can be calculated and compared as follows:-

$$Y=\Delta T_{sm} -(\Delta T_m+ \Delta T_{veg})$$

Where :-

Y is the synergy of hydrothermal stabilisation, defined as the net increase in T_s by semi-metal tanning beyond the summation increase in T_s by the individual tanning agents,

ΔT_{sm} is the total rise in the T_s of the semi-metal tanned samples relative to that of the untanned hide powder,

ΔT_m is the rise in the T_s due to metal tanning , as compared to that the untanned hide powder

ΔT_{veg} is the rise in T_s due to vegetable tanning, *i.e.* 12°C for myrabalan and 20°C for mimosa.

Except in the case of the semi-Cr(III) tanned samples, the increase in T_s in the semi-metal tanned samples is greater than summation of the increments of T_s caused by vegetable tanning and tannin-metal of hide powder. The Y values of the semi-metal tanned samples in this experiment are shown in Figure 2.4. Despite the high T_s value of the myrabalan-Cr(III) and mimosa-Cr(III) samples, the value of the synergy of semi-metal interactions (Y) is actually negative as shown in Figure 2.8. The ΔT_m value for the Cr(III) tanned collagen is 38 °C, which is significantly greater than the rise of T_s in the

semi-Cr(III) tanned samples, 31°C for myrabalan-Cr(III) and 21°C for mimosa-Cr(III) samples (shown in Figure 2.3). Hence it is assumed that the predominant interaction in both type of semi-Cr(III) tanned samples is the Cr(III)-collagen interaction.

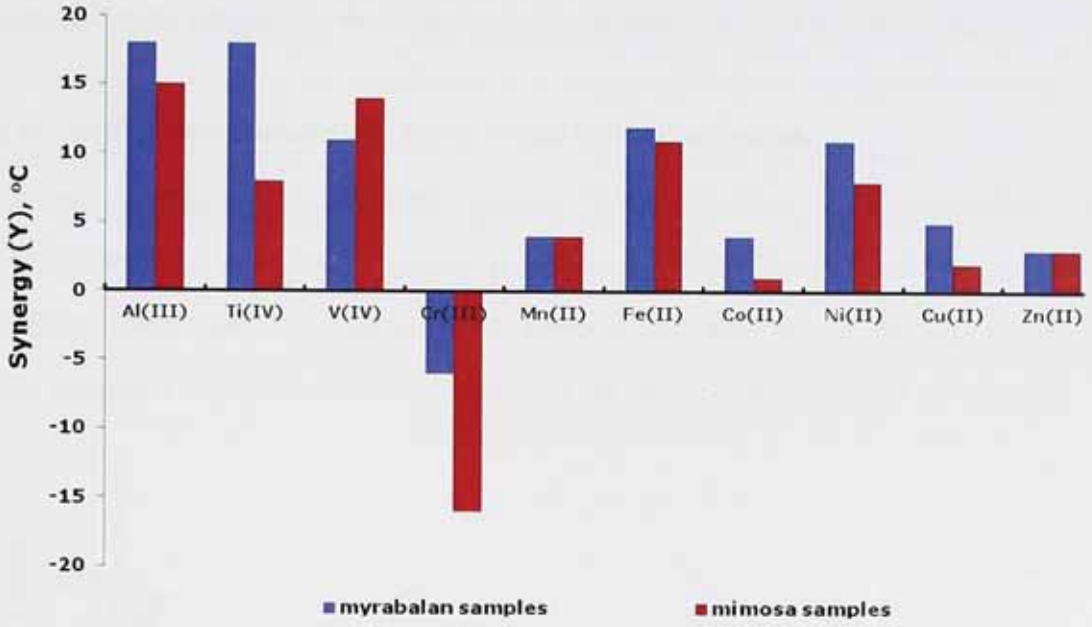


Figure 2.4. Synergy of hydrothermal stabilisation (Y) in semi-metal tanning of myrabalan and mimosa tanned hide powder using transition metals.

The oligomeric complexes of Cr(III) are invariably different from aqueous complexes of the transition metal ions. It is known that Cr(III) complexes form a stable covalent bond with carboxyl groups of collagen. This interaction may be much stronger than the Cr(III)-polyphenol interactions that may exist in the semi-Cr(III) tanned collagen. (Covington and Lampard 2004). Semi-Cr(III) tanning of collagen or retanning of vegetable tanned collagen with Cr(III) salt gives almost the same T_s as Cr(III)-tanned collagen. Hence, the case of Cr(III) retanning of vegetable tanned leather does not conform to the typical type of synergistic hydrothermal stabilisation observed in other semi-metal tanned samples.

The Y values are indicative of the degree of cross linking of the polyphenolic structures in the hide powder by tannin-metal complexation and formation of a rigid tanning matrix. The highest level of synergy of hydrothermal stabilisation (Y=18°C) was observed with the myrabalan-Al(III) and myrabalan-Ti(IV). The different types of the semi-Fe(II),

semi-Ni(II), semi-V(IV)-tanned samples and the mimosa-Ti(IV) show moderate level of synergy of hydrothermal stabilisation of collagen with Y values the range of 8-12°C. The relatively lower Y values (<5°C) of the semi-metal tanned samples of Zn(II), Cu(II), Co(II) and Mn(II) reflects their relatively weak interaction with polyphenols, as compared to the strength of the metal-polyphenol interaction of the other metals.

2.3.6. Total metal contents of semi-metal tanned samples

As shown in Figure 2.5, relatively greater metal uptake (>0.35 mmol/gram) was observed with the semi-metal tanned samples of Al(III), Ti(IV) and V(IV) as well as myrabalan-Cu(II) and myrabalan-Fe(II) samples. All samples of Mn(II), Co(II) and Zn(II) showed a relatively low metal uptake in the range of 0.04 to 0.17 mmol/g.

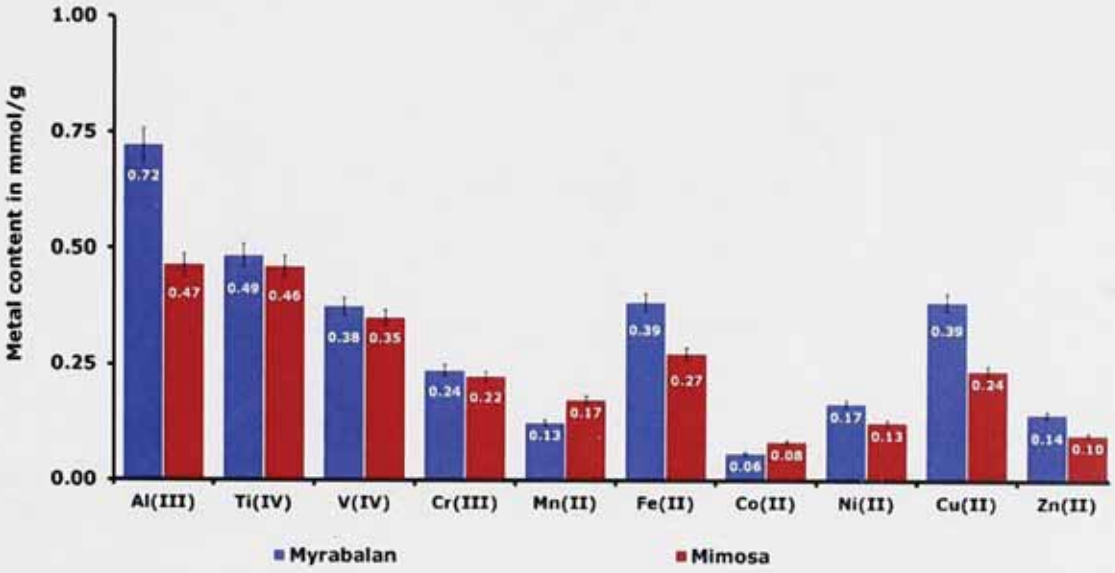


Figure 2.5 The Metal contents of semi-metal tanned hide powder samples in mmol per gram of dry weight.

As shown in Figure 2.5, the Ti(IV) and V(IV) containing samples also have relatively greater metal binding and degree of hydrothermal stabilisation with both types of vegetable tannins (Figure 2.2-3). In contrast, the weak tannin-metal binding interactions of Mn(II), Cu(II) and Zn(II) is also reflected in the relatively low metal uptake and the synergy values. With the exception of the myrabalan-Cu(II), metals that showed relatively greater metal binding also showed greater relatively greater hydrothermal stability.

The myrabalan-Cu(II) showed relatively lower value of synergy value (Y), while the metal binding is quite high (0.4 mmol/g), comparable to that of the myrabalan-V(IV). This may be related the type of complexation formed with the polyphenolic structure. Metal–polyphenol complex formation is pH dependent interaction (Madhan *et al.* 2006). Hydrolysable tannin compounds such as pentagallolyl-glucose can bind Al(III) and Fe(III) in solutions with ratios ranging from 2:1 (at pH 4) to 4.5:1 (at pH 6), similarly compounds of condensed tannins can bind metals in the ratios 1:1, 1:2 and 3:2 at pH 4 (Liu 2009, Slabbert 1992; McDonald, 1996). An example of metal binding at multiple sites of a hydrolysable tannin compound is shown in Figure 2.6.

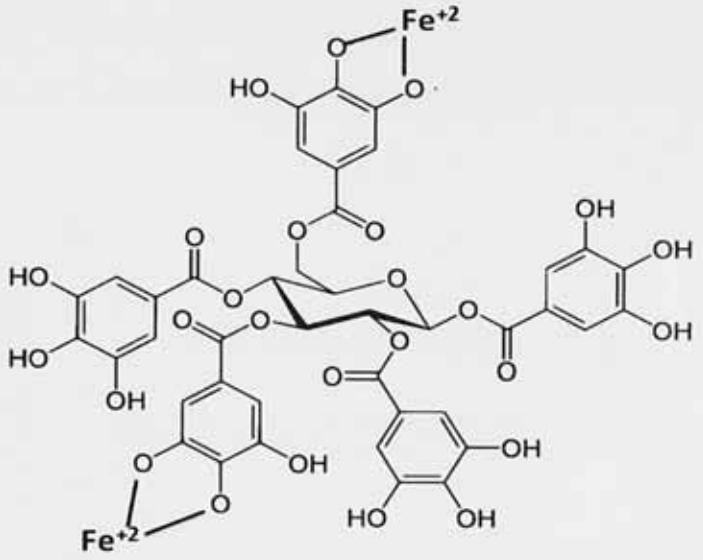


Figure 2.6 An example of multi-point binding of metal ions by tannin molecules: Fe^{+2} pentagallolyl-O-gallolyl glucose (2:1) complex (Liu *et al.* 2009)

In view of the mechanism of semi-metal tanning; the binding of metal ions at multiple chelating sites of tannins, without formation of crosslinks between polyphenols, may not bring about increase in T_s. The high metal content of the myrabalan-Cu(II) samples and its relatively low temperature of synergy indicates that most of the metal ions may be engaged in multi-point binding with the large tannin compounds that have multiple sites for complexation with metals. In this case, not all metal binding with tannin molecules may result in crosslink formation. On the other hand, it is also possible that the metal complexes might precipitate on the surface of fibres, without being chelated by the *o*-diphenol groups of tannins.

2.3.7. Permanence of tanning interactions

2.3.7.1. Metal tanned hide powders

All of the metal tanned samples maintained the initially attained T_s value after two months of storage at 21°C and 65% RH, except those of V(IV) and Fe(II). The average T_s of the metal tanned samples measured over 64 days is included in Appendix 2a. The results indicated that, with the exception of the Fe(II) and V(IV) tanned samples, all of the metal tanned sample maintained constant T_s. As shown in Figure 2.7, Fe(II) tanned samples showed slight increase in T_s by 3°C with one week of storage. The change in T_s was accompanied by a change in colour from white to orange, which is the characteristic colour of Fe(III) salts. This change is most likely due to the oxidation of Fe(II) ions by air, commonly observed in aqueous solutions (Greenwood and Earnshaw 1984; Stumm and Morgan 1996).

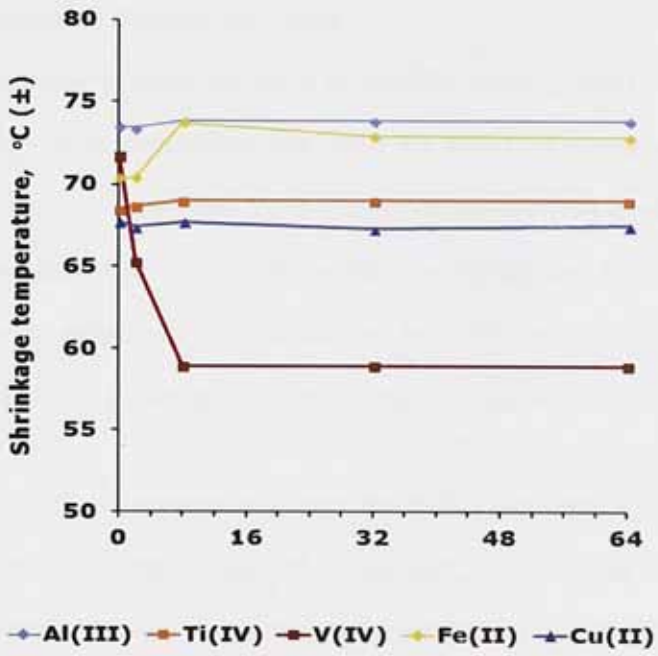


Figure 2.7 - Changes in the average shrinkage temperature (T_s) of different metal tanned hide powder samples

At the same time, the T_s of the V(IV)-tanned samples declined significantly from 72°C down to 58°C. The colour of the semi-V(IV) sample changed light blue to yellow. This change is suspected to be due to formation of vanadates by oxidation of V(IV) by air.

Aqueous complexes of vanadyl (VO^{2+}) ion are also known to undergo slow oxidation to V(V) species *i.e.* dioxovanadium ion (VO_2^+) and vanadates (Sharpe 1986).

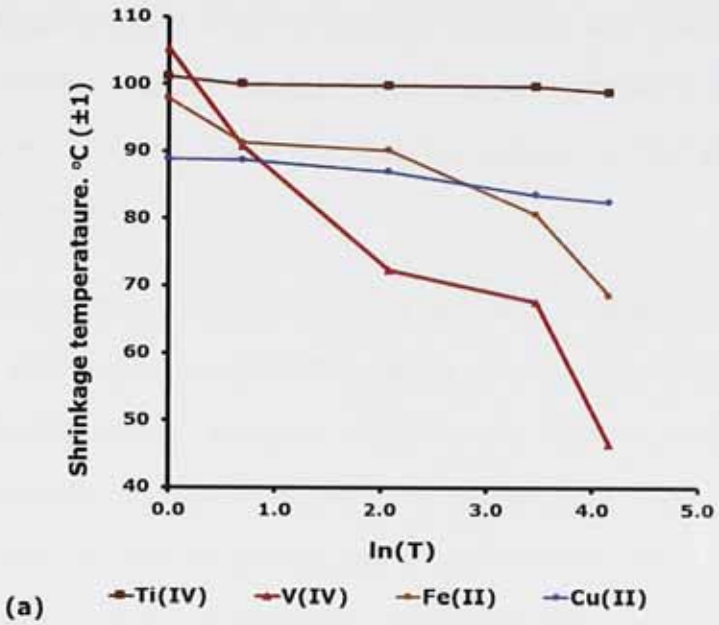
The changes observed in the Fe(II) and V(IV)-tanned samples were confirmed by preparing hide powder samples tanned with ammonium metavanadate [NH_4VO_3] and Fe(III) sulfate [Fe_2SO_4]. The ammonium metavanadate tanned sample has a bright yellow colour and T_s of 58-59°C, while the Fe(III) sulfate tanned sample was an orange-yellow coloured sample having T_s of 73 °C. Both of the samples did not show changes in hydrothermal stability after storage for 60 days at 21 °C and 65% RH. The fact that Fe(II) and V(IV) are oxidised *in-situ* in the metal tanned samples and the observed changes in pH indicated that oxidation is an important factor in determining the permanence of tanning interaction.

2.3.7.2. Semi-metal tanned samples

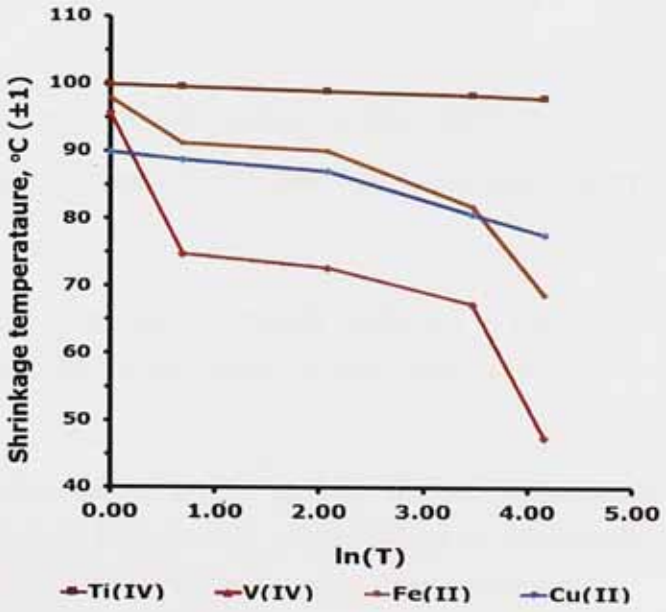
The T_s of the semi-metal tanned samples of Al(III), Cr(III), Mn(II), Co(II), Ni(II) and Zn(II) did not show large difference over the 64 days of storage (results shown in Appendix 2). At the same time, the T_s of the myrabalan-Ti(IV) and the mimosa-Ti(IV) samples a slight decreased T_s by 2-3 °C, while the myrabalan-Fe(II) and the mimosa-Fe(II) samples showed relatively greater decline by 23°C and 29°C, respectively. At the same time, the T_s of Cu(II) retanned samples were also lower by 6-12°C.

The largest decline in T_s were observed with the V(IV) retanned samples, the mimosa-V(IV) sample showed remarkable loss of T_s by 15°C in the first two days, while the myrabalan-V(IV) sample showed even more greater loss of T_s by 21°C. The decline of T_s in both of these samples progressed further and the decline in T_s of myrabalan-V(IV) and mimosa-V(IV) over the 64 days was 49°C and 60°C, respectively. The changes observed in these samples are peculiar in that the T_s dropped to 45-45°C, which is significantly lower than that of the untanned hide powder (60°C). As shown in Figure 2.8a&b, the decline of T_s of the samples is appears to be linear with respect to the logarithm of storage time (T in days)

These change occurred at ambient conditions of storage (*i.e.* 21°C and 65% RH) and in absence of additional chemical treatments. The decline of T_s below that of the vegetable tanned hide powder in the semi-Fe(II) and semi-V(IV) samples indicates that the initially established semi-metal tanning interaction has been disrupted and the original structure of tannins has also been altered.



(a)



(b)

Figure 2.8 –The shrinkage temperature (T_s) the **(a)** mimosa based and **(b)** myrabalan based semi-metal tanned hide powder samples, plotted against a logarithmic scale of days log (T) of storage at 21°C and 65% RH

The V(IV) retanned samples have shown significant decline during the drying time. After 64 days of storage, the T_s of the V(IV)-retanned samples became lower than that the untanned hide powder (59.5°C) indicated that the collagen in the samples has undergone denaturation.

The observed changes are assumed to be a V(IV) induced degradation process that has resulted in the decomposition of the polyphenol structure and breakdown of tanning interactions. In addition, the fact that the T_s has dropped below 60°C indicates that, not only the polyphenolic tanning matrix but also the collagen in the semi-V(IV) tanned samples has been altered.

Changes in the T_s of tanned collagen reflect the degree of stabilisation or destabilisation of the collagen matrix (Covington 2009). While tanning results in an increase in hydrothermal stability, certain chemical environments such as treatment of fibrous collagen with hydrogen bond breakers e.g. urea decrease the T_s by the breaking the hydrogen bonds that stabilise the protein (Usha and Ramasami 2004). The presence of excess acidity or formation of hydrolytic conditions also result in the structural denaturation of collagen that is also reflected in lowering of the Ts below 60°C (Chahine 2000; Larsen 1996).

The above described cases of semi-metal tanning that exhibited subsequent loss of hydrothermal stability (i.e. semi-V(IV), semi-Fe(II) and semi-Cu(II) tanned) represent the occurrence of :-

- *Initial stabilisation in the tanning matrix*: reflected in the initial increase in T_s as a result of tanning due to formation tannin-metal complexes in the polyphenolic matrix,
- *Progressive destabilisation in the tanning matrix:* reflected in the progressive decline in T_s, loss of the synergy of hydrothermal stabilisation by tannin-metal interaction and disruption of the polyphenolic tanning structure,
- *Denaturation of collagen*: reflected in the decline of T_s below 60°C, particularly observed in this experiment with the V(IV) containing samples.

Hence the semi-metal tanning interaction in these samples may be considered to be transient (unstable or metastable) tanning interactions. It is observed that the chemical reaction responsible for the decline of T_s may also caused changes in the structure and composition of the samples.

2.3.7.3. Change in the enthalpy of shrinkage

The shrinkage enthalpy (ΔH) values per dry weight of the semi-metal tanned hide powder samples were in the range of 11.0 to 12.4 J/g in the case of the myrabalan samples and 11.5-14.1 J/g with the mimosa samples (Appendix 2c). With the exception of semi-V(IV) samples, all ΔH the values remained relatively the consistent, showing variations by less than 1 J/G. However the ΔH of the semi-V(IV) samples was lowered to 6.5 J/g and 7.48 J/g after one month of storage (Table 2.3), at the same time the T_s was lowered considerably below that of untanned collagen. The significant lowering of the ΔH values after 32 days of storage (at 21°C and 65% RH) indicates that the collagen in the semi-V(IV) tanned samples has undergone structural changes *i.e.* the triple helical structure has been altered. Studies on the deterioration of vegetable tanned leather has shown that decline in the enthalpy of shrinkage may indicate the presence of denaturation of collagen (Chahine 2000). Treatment of collagen with 6M of urea $(NH_2)_2CO$, which is a known hydrogen bond breaker, causes denaturation that is reflected in the decline of both the T_s and the enthalpy of shrinkage (Usha and Ramasami 2004).

Table 2.3 – Changes in enthalpy (ΔH) and T_s of mimosa-V(IV)and myrabalan-V(IV) tanned samples

Time (days)	semi-V(IV) samples			
	mimosa-V(IV)		myrabalan-V(IV)	
	T_s, (±1) °C	ΔH,(±0.2) J/g	T_s, (±1) °C	ΔH, (±0.3) J/g
0	94.6	12.11	105.3	13.2
2	77.3	11.31	74.7	13.3
8	73.3	11.86	72.4	12.12
32	60.9	11.27	67.6	9.96
64	43.4	7.48	44.1	6.65

The decline in the value of the ΔH value of the semi-V(IV) after 32 days of storage at ambient condition indicates that the collagen in the sample has been denatured as a result of a reaction tannin-V(IV) complex. It was also observed that, a moist mimosa-V(IV)-tanned hide powder, after 64 days of storage at 21°C and 65% RH showed signs of gelatinisation in terms of physical appearance.

These may be related to oxidation of the metal ions by air, leading to reversal of tanning interactions in the same manner as in the V(IV) and Fe(II) tanned hide powder. In addition, the formation of V(V) by oxidation may have resulted in the decomposition of the tanning matrix. The denaturation of collagen as observed in the semi-V(IV) samples, indicates that there may be further reactions, originating from the tannin-metal redox interaction, that resulted in denaturation of the collagen. The progressive nature of the loss of T_s indicated that the mechanism of these changes may be related to a continued interaction of the metal containing samples with oxygen.

The V(V) and Fe(III) species that can be formed by oxidation of Fe(II) and V(IV) by air are known to have oxidative effect as observed with lower molecular weight *o*-diphenolic compounds (Kustin and Toppen 1974; Bark *et al.* 2012). Hence, the tannin-Fe(III) and tannin-V(V) interaction may involve redox reactions that contributed to the decomposition of the polyphenols, resulting in the observed decline of the T_s of the semi-metal tanned samples below the T_s of the vegetable tanned hide powder.

In relation to denaturation of collagen in the semi-V(IV) tanned samples; it is observed these metal ions do not cause lowering of the T_s of the hide powder significantly below 59.5 °C (Section 2.3.7.1), indicating that the collagen-V(V) interaction does not lead to denaturation. Therefore, it is assumed that the tannin-metal interactions in the semi-V(IV), semi-Fe(III) as well as semi-Cu(II) tanned samples may be responsible for the possible creation of a redox mechanism that caused denaturation of the collagen.

2.4. Summary

With the exception of Cr(III), tanning collagen samples with transition metal salts resulted in lower T_s (<73°C). Varying levels of rise in T_s were observed when vegetable tanned samples were retanned with metal salts (semi-metal tanning). The Semi-metal tanned samples of Al(III), Ti(IV), Cr(III) and V(IV) showed relatively high T_s exceeding 100°C. In these cases, the net increase of T_s resulting from retanning of the vegetable tanned samples with the metal salts (ΔT_s) was 20-31°C. On the other hand, the semi-metal tanned samples of Fe(II) and Ni(II) showed moderate level of hydrothermal stabilisation with the ΔT_s values being 15-19°C. The semi-metal tanned samples of Mn(II), Co(II),Cu(II) and Zn(II) showed a relatively lower ΔTs of 6-11°C. Increase in T_s during retanning of vegetable tanned collagen with metal ions occurs as a result crosslinks formed in the polyphenol tanning matrix by metal-tannin complexation.

The hydrothermal stability of the semi-metal tanned samples is determined mainly by the strength of tannin-metal interaction, rather than by the metal content. Metals that are susceptible to oxidation by air may cause changes in the tanning structure as observed with the changes in the T_s of the Fe(II), V(IV) and Cu(II) containing samples. The decline in T_s is attributed to decomposition of the tanning matrix caused by autoxidation of metal ions and tannin-metal redox interactions. The observed changes are considered to be cases of metal induced degradation of tanned collagen.

In the case of the semi-V(IV) samples, the T_s dropped below that the untanned collagen and the enthalpy of shrinkage transition became was lowered significantly after one month of storage at ambient condition, indicating the possible occurrence of denaturation. The progressive loss of hydrothermal stability in these samples reflects the metastable nature of the semi-metal tanning interactions and the presence of an oxidative mechanism resulted in denaturation of collagen.

The results described in this chapter were presented at the XXXII Conference of the International *Union of Leather Technologists and Chemists Societies* (IULTCS), May 28-31, 2013, Istanbul Turkey.

CHAPTER 3

STABILITY AND DEGRADATION IN SEMI-METAL TANNED LEATHERS

3.1. Introduction

The results described in Chapter 2 indicated that semi-V(IV), semi-Fe(II) and semi-Cu(III) exhibited decline in hydrothermal stability during storage at ambient conditions. The observed changes indicated that the metal-tannin interactions in the above mentioned samples were unstable. Furthermore the occurrence of collagen denaturation (particularly with the semi-V(IV) samples), showed the possibility of degradation of the leather due to the redox reactions triggered in by the metal-tannin complexes.

The autodegradation observed in the hide powder sampels was also expected to occur in leather samples. This chapter describes the experiments carried out to study changes in the properties of the semi-metal tanned leathers of V(IV), V(V), Fe(II), Fe(III), Ti(IV) and Al(III). The semi-metal tanned samples were studied in comparison with vegetable tanned leathers. The Fe(II) and V(IV) induced degradation of Cr(III) tanned leathers was also investigated. The features of metal induced degradation of leathers were characterised in terms of changes in the hydrothermal stability, tensile strength and fibre structure. In order to understand the changes occurring in the semi-metal tanning matrix, particularly in relation to degradation, analysis of pH-value and water-extractable metal content were carried out.

In the semi-metal tanned samples, redox reactions of metal ions may involve one-electron transfer with tannins. Accordingly, the possibility of formation for organic free-radicals in the leather samples was suspected. Therefore, electron paramagnetic resonance (EPR) spectroscopy was used to obtain qualitative information related to the oxidation states of iron and vanadium in the leathers. In addition, a quantitative EPR analysis on the relative abundance of free-radicals in different semi-metal tanned leathers was also done.

Based on the redox properties of V(IV) and Fe(II) and the results of the experiments carried out in this study, the features and possible mechanism of metal induced autodegradation of leather are summarised.

3.2. Materials and methods

Pickled sheep skins (pH 1.5) of UK origin were used for preparation of vegetable tanned and Cr(III) tanned leathers.

3.2.1. Chemicals

The following analytical grade chemicals were obtained from Fisher-Scientific Ltd. (UK): glacial acetic acid (99% w/w), anhydrous sodium acetate (99% w/w), nitric acid (70% w/w), perchloric acid (70% w/w), sulfuric acid (98% w/w) and standard solutions of metals (1000 ppm). The metal salts described in Chapter 1, Section 2.2.1 were also used in the experiments.

3.2.2. Tanning agents

The hydrolysable tanning powders, myrabalan and chestnut tanning extracts were obtained from (SilvaTeam Spa., Italy). The condensed tannins (Mimosa ME, Forestal Ltd., UK) and quebracho tannin (Indusol ATO, SilvaTeam Spa., Italy) were used for sample preparation. Basic Cr(III) sulfate (Chromosal B, Lanxess GmbH., Germany), having 25 % (w/w) Cr_2O_3 content and 33% basicity, was used for preparation of Cr(III) tanned samples.

3.2.3. Analytical instruments

Differential scanning calorimeter (DSC)

A differential scanning calorimeter (Mettler-Toledo 822e, Mettler-Toledo Ltd. Switzerland) was used in the experiments for measurement of the T_s of the samples.

Inductively coupled plasma – optical emission spectrometer (ICP-OES)

Measurement of metal contents in analyte solution was done using the ICP-OES Thermo-iCAP6000 (Thermo-scientific Ltd., Hemel Hempstead, UK)

Physical testing instrument

Instron 5567 (Instron Co. Ltd. USA) was used for measurement of the tensile strength and % elongation-at-break of the semi-metal tanned samples.

Scanning electron microscopy (SEM)

Assessment of the fibre structure in the leather samples was done using Hitachi 3000N (Hitachi Co. Ltd., Japan).

Electron paramagnetic resonance spectroscopy (EPR)

EPR analyses of samples was carried out at Manchester University, Chemistry department using a Bruker EMX Micro X-band EPR Spectrometer (Bruker Co. Ltd., USA), equipped with ET 4122 SHQ resonator.

Freeze drier

A CHRIST-LSC plus-16 freeze drier (Martin-Christ GmbH., Germany) was used in sample preparation for EPR and SEM analyses.

3.2.4. Sample preparations

3.2.4.1. Vegetable tanned leathers

Fourteen pickled sheep skins or twenty eight sides were tanned with quebracho, mimosa, chestnut and myrabalan tanning agents as described in Appendix 3. From each of the vegetable tanned leathers, two samples of equal size were cut out from the standard sampling location in accordance with the guideline of standard sampling position SLP 02 stated in the SLTC Official-methods of analysis (SLTC 2000).

3.2.4.2. Semi-metal tanned leathers

The semi-metal tanned samples, described in Table 3.1, were prepared by retanning the samples of different vegetable tanned leathers with the sulfate salts of Al(III), Ti(IV), V(IV), Fe(II), Fe(III) and ammonium metavanadate. Samples of the vegetable tanned leathers were kept as controls without retanning.

Table 3.1 Sample table of semi-metal tanned leathers

metal	vegetable tanned leathers			
	myrabalan	chestnut	mimosa	quebracho
Al(III)	myrabalan-Al(III)	chestnut-Al(III)	mimosa -Al(III)	quebracho - Al(III)
Ti(IV)	myrabalan-Ti(IV)	chestnut - Ti(IV)	mimosa -Ti(IV)	quebracho- Ti(IV)
V(IV)	myrabalan-V(IV)	chestnut-V(IV)	mimosa -V(IV)	quebracho-V(IV)
V(V)	myrabalan -V(V)	chestnut - V(V)	mimosa -V(V)	quebracho- V(V)
Fe(II)	myrabalan-Fe(II)	chestnut - Fe(II)	mimosa -Fe(II)	quebracho - Fe(II)
Fe(III)	myrabalan-Fe(III)	chestnut -Fe(III)	mimosa -e(III)	quebracho- Fe(III)

3.2.5. Cr(III) tanned leathers

Two picked sheep skins were tanned with basic chromium sulfate according to the process described in Appendix 5. Two pieces of Cr(III) tanned leathers were retanned separately with chestnut and mimosa tannins. The process of preparation of the Cr(III) tanned leather and retanning with the vegetable tanning agents are shown in Appendix 6. The vegetable retanned Cr(III) leathers samples are referred to as Cr(III)-chestnut samples and Cr(III)-mimosa samples.

3.2.6. Analysis of semi-metal tanned samples

After sample preparation, all of the samples were kept in a conditioned room at 21°C and 65%. Periodic analysis of the leather samples were carried, the tensile strength and % elongation-at-break of the samples were measured at different times of storage. The permanence of metal-tannin binding in the samples was examined by analysing the quantity of metal extractable by deionised water. The fibres structure of the leather samples was also examined using SEM.

3.2.7. Shrinkage temperature measurement

Shrinkage temperature measurements were carried out according to the method of Chahine (2000) as described in section 2.3.3.9 in chapter 2. Measurements of shrinkage temperature of each sample were carried out immediately after sample preparation, followed periodic measurements after 2, 4, 8, 32, 64, 128, 256 and 360 days of storage at 21°C and 65% RH.

3.2.7.1. Tensile strength and % elongation-at-break

The tensile strength at break (MPa) and % elongation-at-break of the leather samples stored at 21°C and 65% RH were measured according to the SLTC official methods of analysis SLP6, 'Measurement of tensile strength and % elongation-at-break' leather (SLTC 2000). The measurements tests were carried out initially (after 48 hours of drying at 21°C and 65% RH), followed by measurements after 1, 3, 6 and 12 months of storage at the same condition. All measurements were carried out using identical sized samples

that were cut out from the standard sampling locations using a standard cutter dies. The test length of the samples was 20 mm, the width of samples in all measurements was 4.0 mm and the thickness of samples varied between 1.20-1.40 mm. The values of the tensile strength at break (MPa) of the samples are calculated as the ratio of the maximum force recorded at the rupture of the samples to the initial cross-section area. The % elongation-at-break of the samples were calculated at percent ratio of the extension of the samples at the breaking point to the initial test length (20mm).

3.2.7.2. Determination of total metal content

The total metal content of the semi-metal tanned samples was determined according to the official methods of the International Union of Leather Technologists and Chemists, "Determination of Total Metal Content in Leather" IUC27/part 2 (IULTCS 2011). The procedure described in Chapter 1, Section 2.2.5.5 was used to determine the metal content of dry leather samples in mmol/g.

3.2.7.3. Determination of water-extractable metal content

To determine the water-extractable metal in the semi-metal tanned samples, 0.25g of each the leather samples were immersed in 5 mL of deionised water for 24 hours. After filtering out the leathers, the filtrates were digested with 5 mL of the ternary acid-mixture at 100°C for 2 hours, cooled and diluted to 50 mL in volumetric flasks. The concentration of metal in the analyte solution of aqueous extracts was determined from the spectrometric measurement. The relative quantity of extractable metal (%w/w) were calculated as the ratio of metal concentration of the analyte solution of aqueous extract to the concentration of the analyte solution of the digestion of leather samples.

$$\textbf{Extractable metal , \%(w/w)} = \frac{P_{ext}}{P_{tot}} \textbf{X100}$$

Where:-

- P_{ext} is the measured concentration of metal in mg/L of measured in the 50 mL analyte solution prepared from the digestion of aqueous extracts of leather samples,
- P_{tot} is the measured concentration of metal in mg/L of the analyte solutions prepared from digestion of leather samples.

3.2.7.4. Analysis of pH

The pH values of the samples were analysed according to the SLTC official method for "Determination of pH of leather", SLC-06 (SLTC 2000). Samples (weighing 1g) were cut to small pieces and immersed in 10 mL of deionised water for 24 hours and pH was measured.

3.2.7.5. Scanning electron microscopy

The fibre structure of the semi-metal tanned samples was observed using the SEM, Hitachi S3000N-SEM (Hitachi Co. Ltd., Japan). The porosity of the fibre structure and morphology of the fibres were examined visually after 6 months of storage at 21°C and 65% relative humidity.

3.2.8. Analysis of V(IV) and Fe(II) induced degradation

3.2.8.1. Mimosa tanned samples

Ten samples of mimosa tanned leather (each weighing 2 g) were cut into small pieces and rehydrated in sample tubes with 10 mL acetate buffer (pH 4.0). The first 5 samples were treated with different quantities of $VO(SO_4)$ salt equivalent to 2, 20, 40 and 80 mg of V(IV), which corresponds to 0.1, 1.0, 2.0, 4.0% (w/w) of V(IV) on the weight of the dry samples. The other five samples were treated in the same manner using V(V) ion using $NH_4(VO_3)$ salt. After addition of the salt, the samples were placed on a shaker for 15 minutes, the pH of the samples was adjusted to pH 4.0 and after 2 hours, the samples were filtered, dried and kept at 21°C and 65% RH. The T_s of the samples was measured immediately after sample preparation and after 2, 8, and 32 days of storage at 21°C and 65% RH.

3.2.8.2. Cr(III) tanned samples

Pieces of Cr(III)-tanned, Cr(III)-chestnut tanned and Cr(III)-mimosa tanned leathers (5g each) were fully rehydrated acetate buffer (pH 4.0) for 6 hours and treated with different concentrations of Fe(II) and V(IV) ions. The first three samples, from each type of leather, were transferred to separate bottles and treated with 250 mg of $VO(SO_4).5H_2O$ in 25 mL of acetate buffer (pH 4.0). The second set of the samples were

treated in the same manner but with 270 mg of $Fe_2(SO_4).7H_2O$ added to each sample as 250 mg of $Fe(SO_4).7H_2O$. In both cases the quantity of metal ion applied was 1% w/w relative to the weight of the leather samples.

The bottles containing in the V(IV) and Fe(II) treated samples were stirred on a rotary shaker for 2 hours, the treatment solution was discarded, the samples were dried and stored in an oven at 40°C. The hydrothermal stabilities of the samples were measured periodically and the changes in the fibre structure of the samples were observed using scanning electron microscope.

3.2.9. Electron paramagnetic resonance spectroscopy

The following brief outline of the basic principles of EPR spectroscopy is provided as described by Murphy (2009) and Duin (2013). Electron paramagnetic resonance spectroscopy is used to analyse chemical structures in substances containing free-radicals and coordination complexes with paramagnetic centres. Electrons are charged particles in spin and orbital motion and generate magnetic fields. An electron spins around its axis, with a spin angular quantum number (**S**) which can be parallel or co-linear with the axis of rotation, the spin quantum number may assume values of +½ or -½. Its spin magnetic momentum (μ_s) is expressed as:-

$$\mu_s = -g_e \cdot \mu_b \cdot S$$

Where:

- g_e is the Lande splitting factor (g-factor) of electron, a dimensionless constant having value of 2.0023
- μ_b is a fundamental constant for the magnetic dipole moment of electron (Bohr Magneton constant) $9.27400968 \times 10^{-24}$ J/T

The Zeeman Effect

When exposed to an external magnetic field (**B**), the spin magnetic momentum (μ_s) of an unpaired electron becomes directionally aligned or opposed to the magnetic momentum of the applied field. Subsequently, the electrons will be in different energy states due the interaction of the two magnetic fields. The resulting splitting of energy levels in the population of the unpaired electrons (degeneracy) is known as the Zeeman

Effect (shown in Figure 3.1a). The energy (**E**) of interaction between the external field and the election spin magnetic momentum is related to the applied magnetic field as:-

$$E = \mu_s.B = \pm(½).\ g_e\ .\ \mu_b.$$

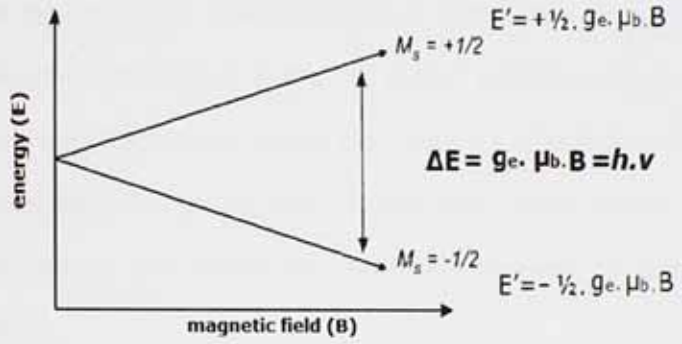
Hence, the electrons with M_s +½ or -½ will have an energy difference of :-

$$\Delta E = g_e.\ \mu_b.$$

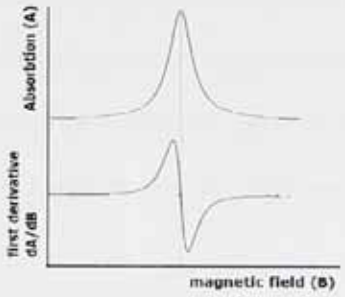
The unpaired electron may undergo transition between the upper and lower energy states by either absorbing or emitting a photon of energy of :-

$$\Delta E = h.v$$

Where **h** is the Planck's' constant (6.626 $\times 10^{-3}$ J/S) and **v** is the frequency of microwave radiation in Hertz (Hz).



(a)



(b)

Figure 3.1. A diagram showing (a) the Zeemann effect or splitting of energy states of an electron due to interaction with an external magnetic field and (b) profile of an EPR absorption spectra and the corresponding first-derivative.

The most commonly used EPR spectrometer is in the range of 9-10 GHz, known as the X-band. In EPR spectroscopy, the sample may be subjected to a constant microwave radiation and the spectra can be obtained by scanning the magnetic field. EPR data may be analysed as absorbance spectra or its first derivative as shown in Figure 3.1b.

Spin-relaxation and saturation

At thermal equilibrium and under the influence of the external applied magnetic field, the spin population is split between the two Zeeman levels according to the Maxwell – Boltzmann law:

$$\frac{N_1}{N_2} = e^{\Delta E/kT}$$

Where:-

k is the Boltzmann constant,

$\mathbf{N_1}$ and $\mathbf{N_2}$ the spin populations having $\mathbf{M_S}$ ($+½$ and $-½$ respectively)

T the absolute temperature in Kelvin

In a 300 mT magnetic field at the temperature of 298K, the distribution N_1/N_2 is 0.9986. Since the distribution between the two Zeeman levels is not equal, transitions from the M_s -½ to the M_s +½ states are possible. However, the observed signal may fade quickly because the transition would equalise the two energy states. To maintain the signal, there has to be energy loss in the system and a return back to the $N_2>N_1$ condition. The mechanism energy loss, known as spin relaxation, occurs through exponential decay of energy into vibrational and rotational energy (spin-lattice relaxation) and through exchange of energy between the two states (spin-spin relaxation).

When the spin-lattice relaxation time is too long, electrons do not have time to return to the ground state. The populations of the two levels tends to equal and the intensity of the signal decreases, being no longer proportional to the number of spins. This effect is known as Saturation. Since EPR analysis of samples has to be carried out at conditions of signal non-saturation, the incident microwave power is an important control parameter. Commonly, EPR signal saturation is prevened by tuning to a lower range of microwave power.

The g-factor and hyperfine splitting

EPR spectroscopy may also be used to obtain information regarding the environment of the paramagnetic centres or the free-radicals. Practically, the g-factor of an unpaired electron in a molecule or metal complex is not exactly equal to g_e. This is because the unpaired electron's net angular momentum also includes its orbital angular momentum. The g-value of a paramagnetic centre or radical is determined by the effects of the local magnetic fields produced by magnetic nuclei. Hence the effective magnetic field interacting with the unpaired electron and the effective g-factor may be expressed as:-

$$B_{eff} = B_0 (1-\sigma)$$

$$B_{eff} = B_0 \left(\frac{g_e}{g_{eff}} \right)$$

Where:-

- B_o is the applied magnetic field,
- B_{eff} is effective magnetic field,
- σ is the magnetic shield factor results in decreasing or increasing the effective field due to local environment
- g_e is g-factor of electron
- g_{eff} is the effective g-factor

In many organic radicals, the contribution of the orbital angular momentum is negligible, hence the g-factor is considered to be equal to $\mathbf{g_e}$. In contrast, unpaired spins in transition metal ions or complexes typically have larger values of angular orbital momentum and interact with a non-zero nuclear spin. The magnetic moment of the non-zero nuclear spin also interact with the spin angular momentum of the unpaired electron. As a result, the EPR spectrum is split into multiplet signals; this is known as hyperfine splitting. A nuclear magnetic quantum (I), which is multiple of ½, would result in 2I+1 splitting lines.

Quantitative EPR

The area under the absorption spectrum of an EPR signal may be integrated, similar to optical spectroscopy, as a direct measure for the concentration of unpaired electrons. Unlike electronic absorption spectroscopy, however, there is no 'extinction coefficient' in EPR spectrometry all S = ½ systems are assumed to absorb equally. Standards of known spin concentration (spin count per unit weight) are used for the estimation of spin concentration of samples.

3.2.9.1. Samples for EPR analysis

Hide powder samples

Preparation of V(IV) and V(V) treated hide powder samples was carried out as follows. Two hide powder samples (2g each) were rehydrated with 20 mL of acetate buffer (0.1M, pH 5.0) in separate flasks and treated with 101 mg of $VO(SO_4).5H_2O$ and 50 mg of $NH_3(VO_3)$ *i.e.* 20mg of metal relative to the on the dry weight of hide powder. The treated samples were dried in a desiccator over 72 hours.

Mimosa powder samples

Four samples of mimosa tanning powder (Forestal Co. Ltd., UK), weighing 1g each, were dissolved in 10 mL of deionised water, the first three of the samples were treated with $Al_2(SO_4)_3.16H_2O$, $VO(SO_4)$ and $Fe(SO_4).7H_2O$ in calculated quantities equivalent to 1% of metal ion (10 mg) on the weight of the tanning powder. In each case, the fourth sample (no metal salt) was kept as control. The samples were stirred for 30 minutes at room

temperature and then dried by evaporating the water under vacuum (0.1mbar) using a freeze drier over 24 hours at -20°C

Leather samples

The mimosa tanned leather and mimosa-based semi-metal tanned leathers of Al(III), Fe(II) and V(IV) were analysed with EPR spectroscopy. At the time of analysis, the samples have been kept at 21°C and 65% RH for more than 6 months. As described in Sections 3.3.2.1, 3 &4, while the T_s of the mimosa-Al(III) has remained the same, the other two have shown remarkable decline during storage. The main purpose of the EPR analysis of the samples was to ascertain the occurrence of redox transitions in the metal ions (particularly iron and vanadium species). In addition, comparative analysis of free-radical concentration was carried out (Section 3.2.9.3) in order to examine the relationship between the metal induced oxidative degradation in relation to formation of free-radicals in the leathers.

3.2.9.2. EPR spectral analysis

Leather samples were cut into 3.5 × 20 mm pieces were weighed and loaded into similar quartz tubes. Spectra were collected at ambient temperature (293 K) at X-band frequency (9.87 GHz) on a Bruker EMX Micro spectrometer. In each case, the average spectrum of 8-scans were obtained over the magnetic field range 0–700 mT with scan-time of 164.7 seconds.

3.2.9.3. Analysis of free-radical concentration in leather samples

For the quantitative analysis, spectra were acquired using modulation amplitude of 0.1 mT and microwave power of 10 mW. The receiver gain was adjusted to prevent signal saturation. The spin concentrations were quantified using a weak pitch standard (Bruker Co. Ltd. UK) with an estimated 1.2×10^{13} spins per cm containing carbonaceous radicals (Eaton *et al.* 2010).

A spectral measurement of the standard, diluted in potassium chloride (KCl), was recorded using the same parameters used for the samples and used for calibration. A

polynomial function of third-order was subtracted to remove baseline distortions in the spectrum. The signal (over 700 mT) was double integrated to determine the intensity. Likewise, a double integration of the signals of each leather sample performed in the same manner to estimate the spin concentration (spins per mg) in each sample.

3.3. Results and discussion

3.3.1. Colours of semi-metal tanned leathers

Retanning of the vegetable tanned leathers with the transition metal salts resulted in a distinct change of colour (shown in Table 3.2) that occurs due to metal-to-ligand charge transfer between the aromatic hydroxy ligands of tannin compounds and the metal ions (Pierponts and Lange 2007; McDonald, *et al.* 1996; Andjelković *et al.* 2006). It has been suggested that Fe(II) does not create stable complexes with the *o*-diphenol moieties of tannin, the distinct blue colour is assumed to be the result for formation of Fe(III)-tannin complexes (Slabbert, 1992). The semi-metal tanned samples of V(V) have a deep blue colour, the same as that of V(IV). It is known that the reaction between V(V) species and tannin compounds involves both redox transformation of the V(V) to V(IV) and blue complex formation with V(IV) ions (Nakajima *et al.* 2000; Ferguson and Kustin 1979).

Table 3.2. The colours of semi-metal tanned leather samples.

Type of sample	Vegetable tanned leathers	
	myrabalan and chestnut	mimosa and quebracho
No metal	yellow	light red-brown
semi-Al (III)	yellow	light red-brown
semi-Ti (IV)	orange	orange
semi-V(IV)	dark blue	dark blue
semi-V(V)	dark blue	dark blue
semi-Fe(II)	blue	blue
semi-Fe(III)	blue	blue

During the storage of samples at ambient conditions (21°C and 65% RH), the colour of the semi-V(IV) and semi-V(V) samples changed progressively from blue to green. At the same time, the semi-Fe(II) and semi-Fe(III) samples showed slight fading of the initial

deep blue colour. This may be related to the structural change in the chromophore groups of the tannin and formation of new complexes due to possible redox reaction taking place in the leather samples. The colour of the semi-Al(III) and semi-Ti(IV) did not show significant change.

3.3.2. Shrinkage temperatures (T_s) of semi-metal tanned leathers

The highest T_s, exceeding 110°C were observed with the chestnut and mimosa leathers retanned with Al(III), Fe(II) and V(IV) as shown in Figure 3.2. Nevertheless, the T_s of all of the samples were greater than 100°C, except for the semi-V(IV) samples and the myrabalan (V) sample.

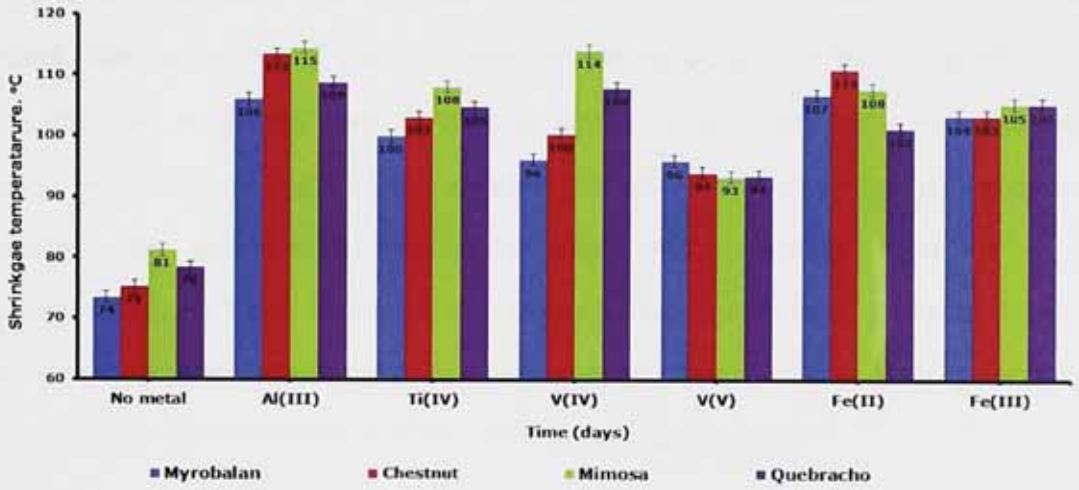


Figure 3.2. Shrinkage temperature of vegetable tanned leathers and a range of semi-metal tanned leathers prepared using sulfate salts of Al(III), Ti(IV), V(IV), V(V), Fe(II) and Fe(III).

Semi-metal tanning using hydrolysable tannins, that essentially have multiple gallolyl units, are generally expected to result in a greater increase of T_s in semi-metal tanning, as compared to most of the condensed tannins Sykes *et al.* (1980). Mimosa extract exhibits a semi-metal tanning effect similar to the hydrolysable tannins (Covington *et al.* 2005). Quebracho tannin, which is composed of profisetinidine and procyanidine tannins (*i.e.* no pyrogallol moieties) also showed similar effectiveness in terms of hydrothermal stabilisation through semi-metal tanning with different metals.

It is also noted that the semi-metal tanned samples of the hydrolysable tanning agent have slightly greater T_s value only in the case of the chestnut-Fe(II). With the other samples, the T_s of the mimosa based semi-metal tanned samples show greater T_s. The

semi-Fe(II) samples have slightly greater T_s than the semi-Fe(III). As suggested by Slabbert (1992), Fe(II) is readily oxidised in acidic medium to Fe(III), which exhibits stronger binding or complex formation with tannins. Hence the semi-Fe(II) tanning effects may be attributed to *in situ* formation of Fe(III) complexes in the polyphenolic matrix.

The T_s of the semi-V(V) samples are relatively lower as compared to the other samples. The V(V) used in preparation of the sample (metavanadate VO_3^-) is not expected to form stable complexes with polyphenols because its reaction with the *o*-diphenol groups at the tanning conditions (*i.e.* acidic media), is most likely to be a redox interaction rather than complexation. However, the increase in the T_s (or ΔT_s) for the semi-V(V) tanned samples, relative to that of the vegetable tanned controls, is in the range of 12-22°C, which indicates significant level of tanning action by V(V). According to Kustin *et al.* (1974), vanadium(V) species can oxidise the *o*-diphenol moieties of polyphenols. Therefore, the tannin-V(V) interaction most likely involves reduction of the metavanadate (VO_3^-) and formation of hydrated oxovanadium (VO^{2+}) species during the tanning process, the cationic V(IV) species can form complexes with tannins resulting in the increase of T_s as observed with the semi-V(IV) tanned leathers.

3.3.2.1. Vegetable tanned and semi-Al(III) tanned leathers

The T_s of the vegetable tanned leather (control samples) and semi-Al(III) tanned did not show significant difference over one year of storage at ambient conditions. The leather samples stored under ambient condition remained stable. The tanning interaction between polyphenols and collagen is not reversed spontaneously. Studies on deterioration of leather-made historical artefacts (Larsen 1996; Chahine 2000; Thomson 2006; Florian 2006) show that decline in hydrothermal stability and changes in physical properties in vegetable tanned leathers occurs only when the leathers are exposed to hydrolytic and oxidative conditions over a long period of time.

3.3.2.2. Semi-Ti(IV) tanned leathers

The semi-Ti(IV) leathers exhibited a slow decline in T_s, the average temperature of these samples was reduced from 106°C to 92°C over one year time (Figure 3.3 a&b). Since the T_s of the semi-Ti(IV) leathers was not reduced below that of the initial vegetable tanned leathers, the observed change is not considered to be a case of metal-induced degradation. This because the observed deline in T_s is indicative of only reversal of the metal-tannin interaction, not structural disruption in the polyphenolic tanning matrix. The tanning effect of the polyphenols in the leather prevail, regardless of the observed change in T_s.

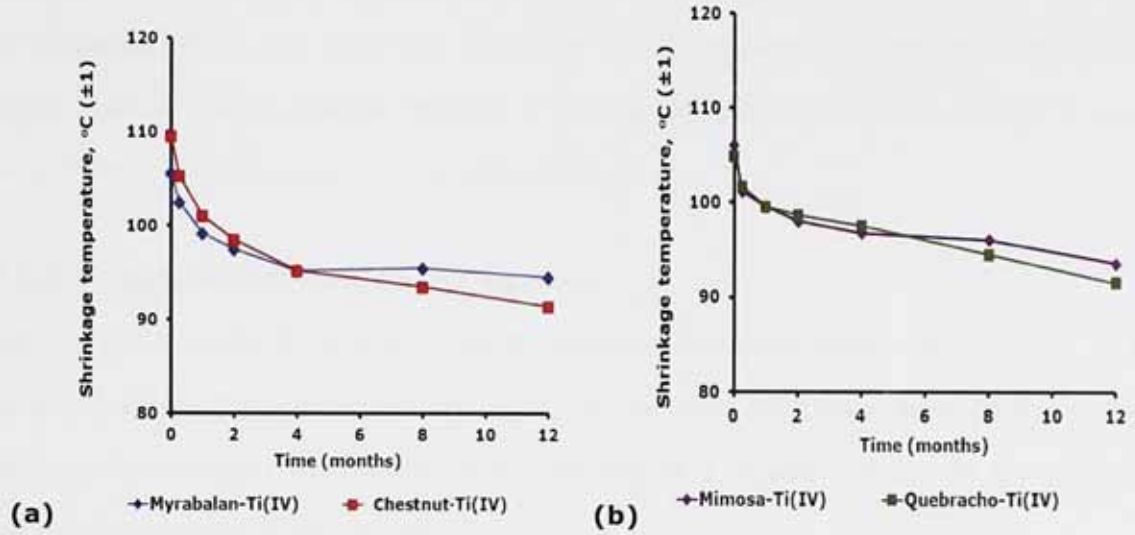


Figure 3.3. Results of periodic measurements of the T_s of semi-Ti(IV) tanned leathers over 12 months of storage at 21°C and 65% RH, showing samples prepared with (a) hydrolyable tannins and (b) condensed tannins.

The decrease in T_s by 14-15°C may represent the occurrence of certain structural changes in the Ti(IV)-tannin complex resulting in the reversal of the tanning interaction formed during the tanning process. A similar case of lowering of the T_s of semi-Ti(III) and semi-Ti(IV) leathers was observed earlier by Lampard (2000). Titanium(IV) or the titanyl ion has high charge density and is prone to hydrolysis in aqueous media (Persson 2010; Grzmil *et al.* 2008). The semi-Ti(IV) leather samples most likely would contain the polymeric ions (poly-titanyl ions, $[TiO^{+2}]_n$) forming a complex with the tannin molecules.

During retanning of the vegetable tanned leathers with Ti(IV), both the metal-tannin complex formation and the hydrolysis of Ti(IV) ion (formation of polytitanyl ion) may

occur concurrently as the pH is raised to 5.0. Subsequently, part of the Ti(IV) may have been deposited in the leather as polymeric metal ions species. During storage of the samples, the Ti(IV)–tannin complexation may be broken down due to further hydrolysis of titanium ions (i.e. further hydrolytic polymerisation into polytitanium ions).

Titanium(IV) is the most stable oxidation state of the metal, its electrochemical reduction may require acidic media *i.e.* pH<0.5 (Cservenyak *et al.* 1996). Hence, it is unlikely that it may be reduced by the polyphenols at the conditions retanning process *i.e.* pH 2-5. Analysis of the stability of low molecular weight polyphenols (described in chapter 4) showed that the metal doesn't cause change in the composition of the compounds. Hence, the observed decline in T_s may caused by redox interactions but rather related to to a possible reversal of tanning interactions, that occurred as a result of hydrolysis of the tannin-Ti(IV) complexation.

3.3.2.3. Semi-Fe(III) and Fe(II) leathers

Both of the semi-Fe(II) and semi-Fe(III) samples exhibited significant decline in T_s as shown in Figure 3.4 and Figure 3.5. The T_s of the semi-Fe(II) and semi-Fe(III) tanned samples was reduced by 10-15°C in 8 days (Figure 3.3a and 3.4a), with the mimosa-Fe(II) sample showing the largest decline.

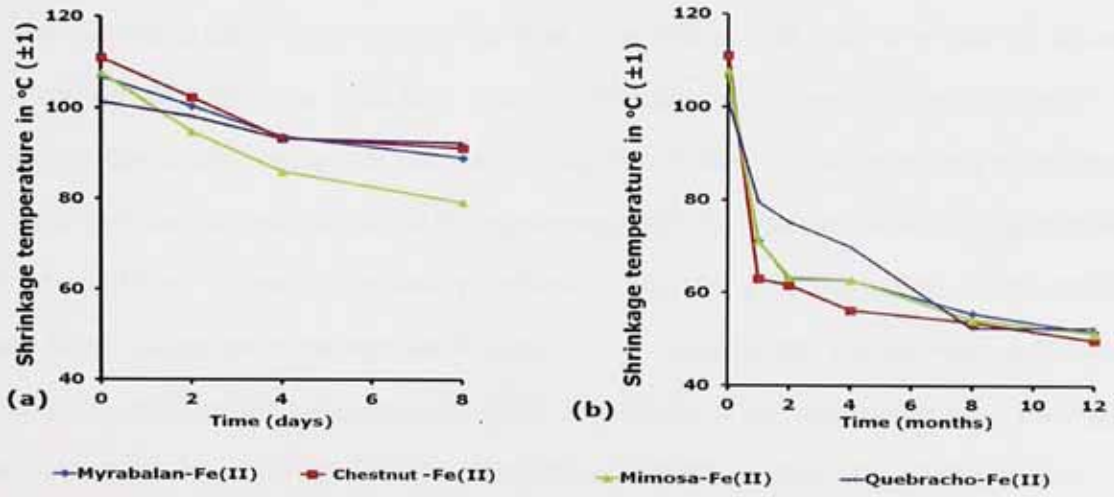


Figure 3.4 Results of periodic T_s measurements of the semi-Fe(II) leather samples (a) in the first 8 days after sample preparation and (b) over 12 months of storage at 21°C and 65% RH.

Further decrease was observed over the period of one year. The final T_s values of the semi-Fe(II), after one year of storage at ambient condition were between 50-53°C and that of the semi-Fe(III) samples were also reduced to 49-55°C. The similarity in the trend decline of T_s of the semi-Fe(II) and semi-Fe(III) samples may indicate the presence of a similar mechanism of destabilisation. The decline in T_s of the samples in both cases was also accompanied by physical deterioration (Section 3.3.3) and chemical decomposition as observed with strength measurement and analysis of water-extractable metal content (Section 3.3.6).

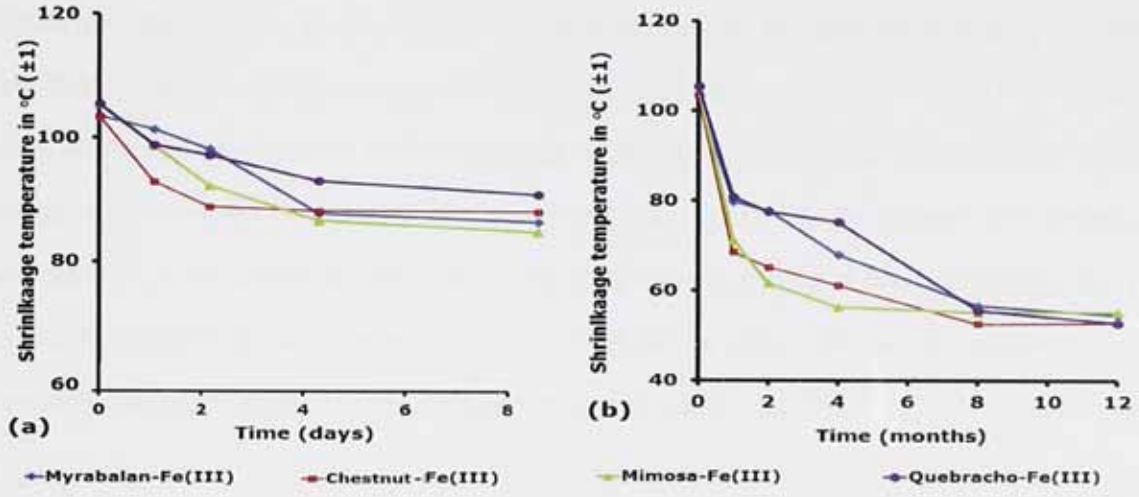


Figure 3.5 Results of periodic T_s measurements of the semi-Fe(III) leather samples (a) in the first 8 days after sample preparation (b) and over 12 months of storage at 21°C and 65% RH.

The observed decline in the T_s of the leathers is due to oxidative chemical decomposition of the vegetable tanning structure. The final T_s of the sample after one year of storage became significantly lower than that of untanned collagen, indicating denaturation of the collagen. Generally, the rate of decline in T_s does not seem to be dependent on the type of vegetable tanned leather. All of the semi-metal tanned leathers showed similar rate of decline and final T_s. Iron(II) is readily oxidised to Fe(III) by air in aqueous media and the rate of the oxidation increases particularly in the range of pH 3-8 (Morgan and Lahav 2007). The polyphenol complexes of Fe(III) are known to be more stable than the Fe(II) complexes (Slabbert 1992). On the other hand, Fe(III) is also an oxidising agent that may convert the *o*-diphenol groups to semi-quinone radicals and quinones (Perron *et al.* 2009). An investigation of the stability of polyphenols in the presence of Fe(II), Fe(III) and other metal ions is described in Chapter 4.

Treatment of pickled pelt with Fe(III) sulphate in the presence of sodium citrate (or acetate) gives a brown coloured leather having T_s of 80°C (Covington 2009). However, the presence of iron particularly in leathers containing polyphenolic tanning agents is associated with defects (iron stains), because of dark coloured spots (iron complexes) and the subsequent deterioration of the leathers (John 1997). As described in Chapter 1, the presence of trace quantities of iron has also been related to oxidative degradation of historical leathers (Haslam 1998; Stambolov 1996).

3.3.2.4. Semi-V(IV) and V(V) leathers

As shown in Figure 3.6a, the T_s of all the semi-V(IV) tanned samples were initially higher than 100°C, except for myrabalan-V(IV) sample (T_s 96°C). After 2 days (*i.e.* during drying of the samples) the T_s of the chestnut-V(IV) mimosa-V(IV) and quabracho-V(IV) samples were lowered by 10-13°C and the myrabalan-V(IV) sample showed that largest decrease in T_s in two days by 26°C. After the first 8 days, the T_s of the myrabalan-V(IV) and chestnut-V(IV) samples were lower than that of the vegetable tanned leathers (*i.e.* myrabalan tanned leather 74°C and chestnut tanned leather 75°C).

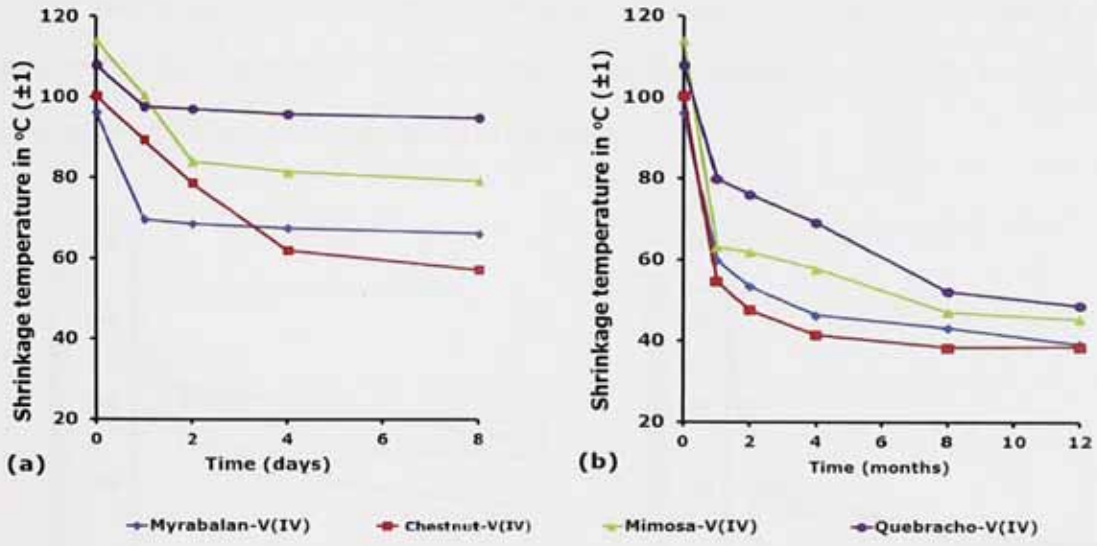


Figure 3.6 Results of periodic measurements of the T_s of semi-V(IV) leather samples **(a)** in the first 8 days after sample preparation **(b)** and over 12 months of storage at 21°C and 65% RH.

In the case of chestnut-V(IV) the T_s was even slightly lower than 60°C after 8 days of storage. This indicates the polyphenolic structure in the leather has been decomposed rapidly, after the initially observed semi-metal tanning interaction. The quebracho-V(IV)

sample after 8 days had T_s 97°C, showing relatively less decline as compared to the myrabalan-(IV) and chestnut-V(IV). At the same time the mimosa-V(IV) sample showed a larger decline from 114°C to 83°C, which is close to that of the mimosa tanned leather (84°C). The decline of T_s in the first eight days evidently may represent the reversal of the effect of semi-metal tanning interaction.

As shown in Figure 3.6b, the decline in T_s continued over the 12 months, and the final T_s of all of the samples were below that of the untanned collagen (60°C). As described in Chapter 2, Section 2.3.7.2, the of decline of T_s in the semi-V(IV) tanned hide powder samples reached to the level of denaturation of the collagen (T_s <59.5°C) in a month time. Nearly the same rate of decline of T_s was observed with the myrabalan-V(IV) and chestnut-V(IV) leathers as shown in Figure 3.6. The decline in T_s was comparably slower in the case of the mimosa-V(IV) and quebracho-V(IV) leathers, after 6 months that the T_s of the quebracho-V(IV) leather also decreased to less than 60°C. The initial Ts of the semi-V(V) leathers are relatively lower as compared the corresponding semi-V(IV) samples. However the decline of T_s decline (as shown in Figure 3.7a) are generally similar to the same decline in the semi-V(IV) samples. In all cases, the final Ts of the semi-V(IV) and semi-V(V) samples are all in the range of 38-49°C, indicating the structural alteration or denaturation of collagen.

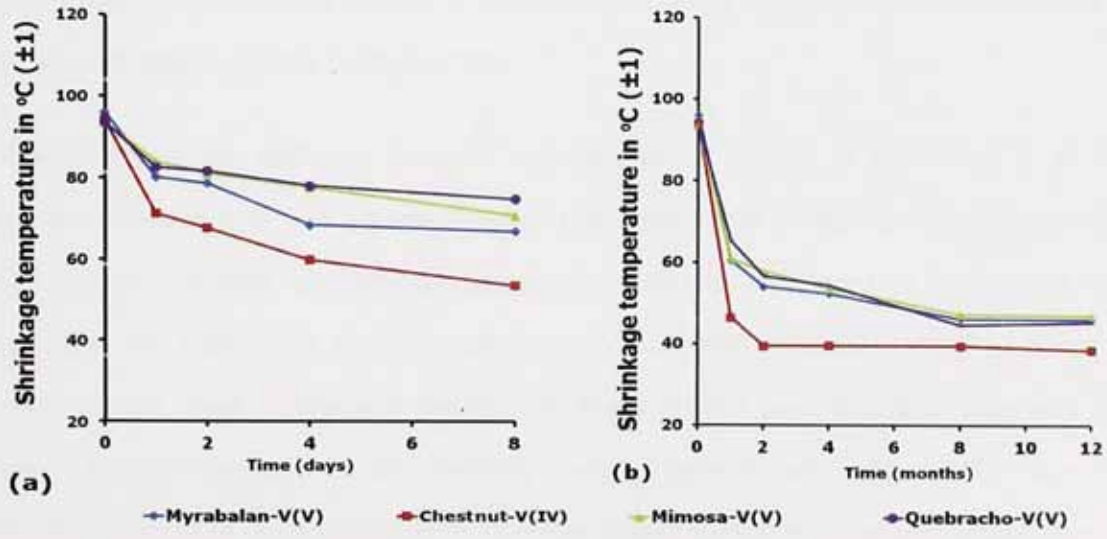


Figure 3.7 Results of periodic measurements of the T_s of semi-V(V) leather samples (a) in the first 8 days after sample preparation (b) and over 12 months of storage at 21°C and 65% RH.

3.3.3. Time dependence of decline in shrinkage temperature

The decline in T_s of the semi-metal tanned samples during drying (*i.e.* first 2 days of storage) was significant with the the Fe(II) and V(IV) retanned samples of myrabalan, chestnut and quebracho tanned leathers (Table 3.3), while the the titanium retanned leathers showed a relatively small change (2°C). The quebracho based semi-metal tanned samples of Fe(II) and Ti(IV) showed almost no difference in the first two days. But the quabracho-V(IV) samples more significant decline in by 8°C. The results indicate that the the semi-metal tanning interactions with the iron retanned and vanadium retanned samples are subjected to a rapid or spontaneous changes with the greater presence of moisture (i.e. during drying), the observed initial decline in T_s vary with the type of metal and the type of vegetable tannin.

Table 3.3 Decline in Ts of semi-metal tanned samples during drying over 2 days at 21°C and 65% RH

Metal	semi-metal tanned leather of:-			
	myrabalan	chestnut	mimosa	quebracho
Ti(IV)	-2	-2	-2	-1
Fe(II)	-6	-11	-13	-1
V(IV)	-27	-11	-14	-8

While the change in T_s over the the two days of storage apprears to be relatively rapid, The continued decline of T_s of the samples over one year is relatively slower particularly in the case of the the Fe(II) and V(IV) retanned samples. The plot of shrinkage temperature of the samples against a logarithmic scale of time or ln(t), i.e. 't' is time in day, showed linear pattern as Figure 3.8.

The slope the linear declining patterns apprears to be related to the initial T_s of the samples. Samples that had greater T_s after the second day of storage (e.g. myrabalan Fe(II) sample) showed relatively greater slope of decline, compared to others that had lower T_s at the same time (e.g. myrabalan-V(IV) sample). The semi-V(IV) samples of myrabalan and chestnut leathers already had lower T_s after two days of storage and the slope of decline apprears to be relatively lower. However, all of the V(IV) retanned samples showed the largest decline in T_s in the overall period of storage , as decribed in Section 3.3.2.4.

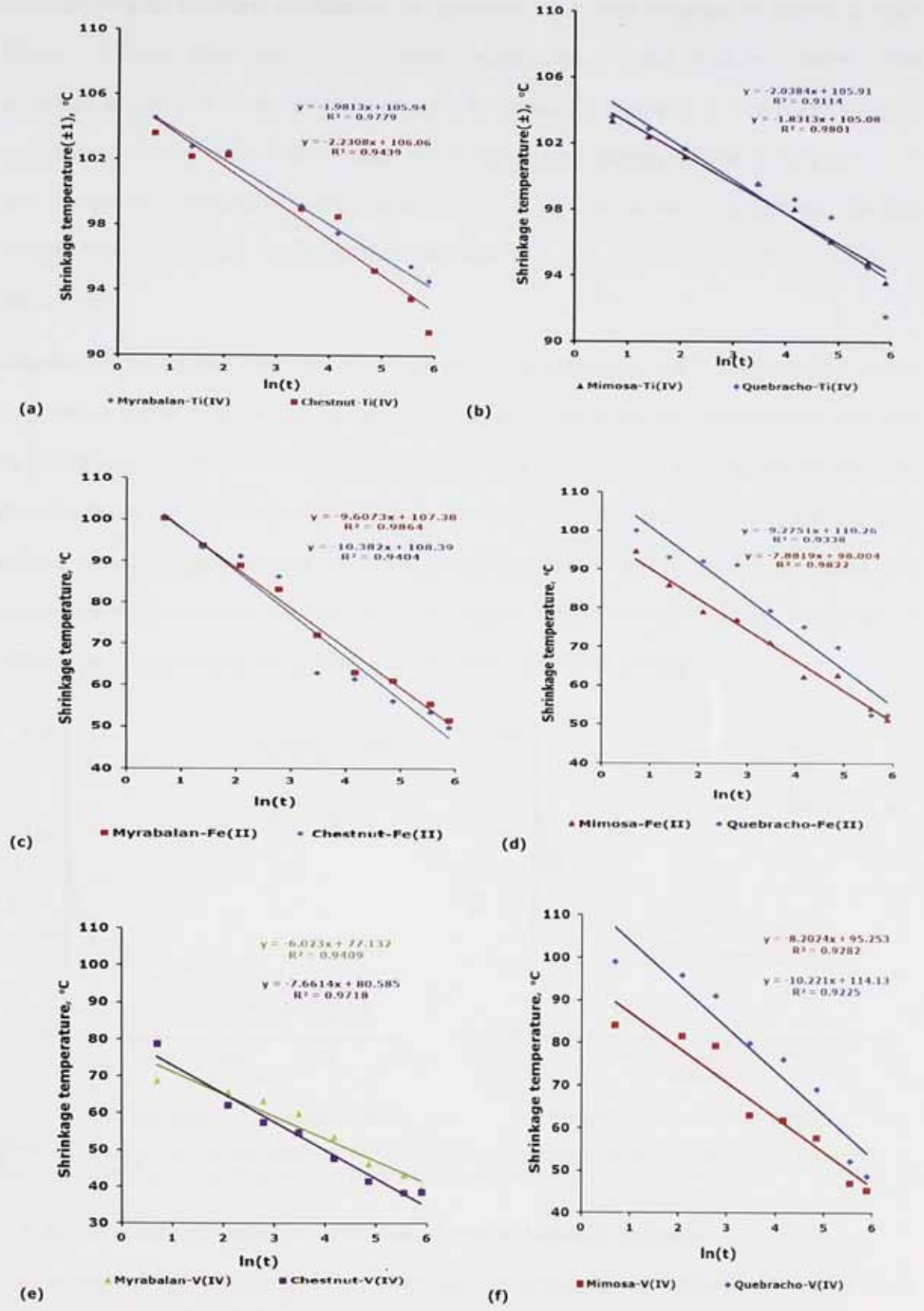


Figure 3.8 Graphs of the shrinkage temperature of samples against logarithmic time scale for the period of 2-360 days of storage: **(a, b)** semi-Ti(IV) leathers, **(c,d)** semi-Fe(II) leather and **(e,f)** semi-V(IV) leathers

The relationship between the decline of logarithm of T_s and duration of storge is non-linear, indicating that the V(IV) induced degradation process does not comply the condition psudo-first order kienetic model. As shown in Figure 3.9, the decline ln(Ts) occurs much faster in the first 64 days and a significantly slower decline of T_s is observed after 9 months. Changes of 3-4°C are observed in the T_s of the samples between the 256 ad 360 days of storage. Hence further decrease in T_s are assumed to occure in a much slower rate.

Assuming an extended duration of storage at same condition, the T_s may possibly reach a minimum value below which further decrease may not be occur. Theoretically, this can be viewed in relation to the occurrence of chemical denaturation to a significant level, so that the triple helical collagen structure in the leathers would be completely transformed into separate collagen strands or polypeptides of different length. In this condition, the measured hydrothermal stability may be closely similar to the melting transition of galatine which is around 37°C (Zhang *et al.* 2006; *Bigi et al.* 2004)

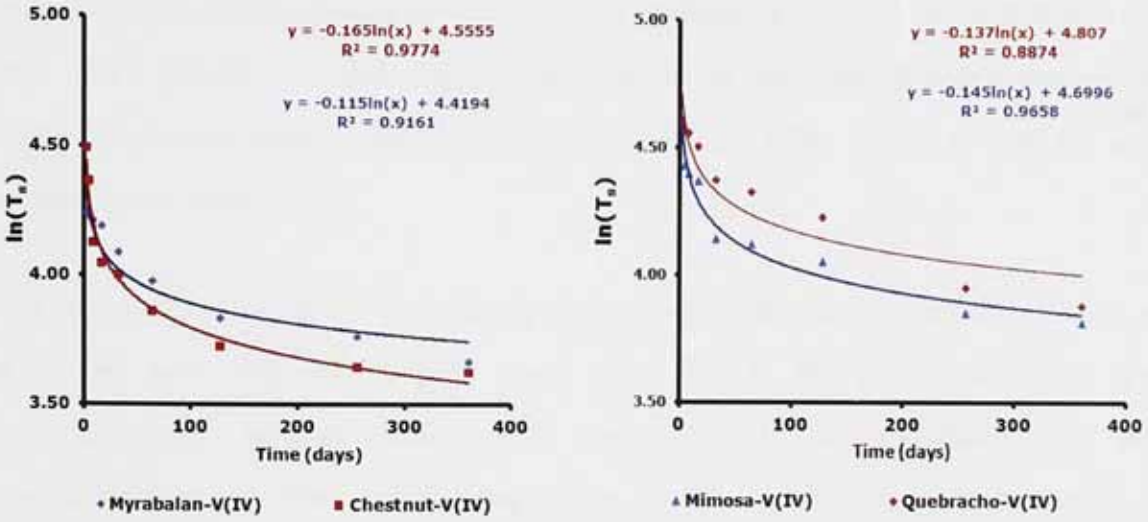


Figure 3.9 Graph of the logarithm of the shrinkage temperature values of semi-V(IV) tanned leather samples ln(T) against storage time (days), showing non-linear correlation

3.3.4. Physical degradation of the semi-metal tanned leathers

The records of tensile strength measurements (summarised in Appendix 7) shows that, the average of initial tensile strengths of the mimosa based semi-metal tanned leathers samples were between 10.0-12.0 Mpa. The mimosa based semi-metal tanned samples of Al(III) and Ti(IV) showed a relatively consistent tensile strength in the range of

11.0±0.3 MPa and 11.5±0.5 MPa throughout the one year of storage at 21°C and 65% RH.

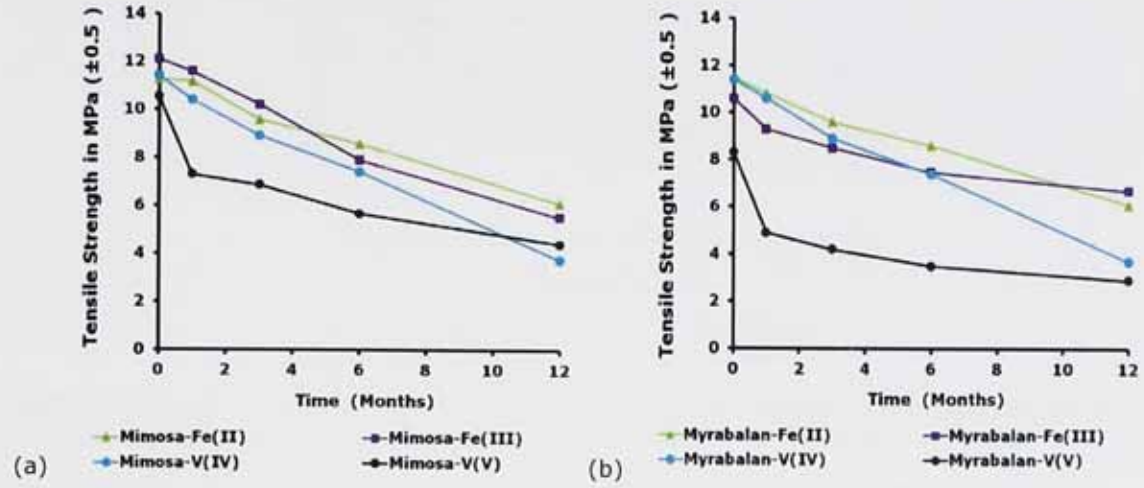


Figure 3.10 Results of periodic measurements of the tensile strength of (a) mimosa-based samples and (b) myrabalan based samples of semi-metal tanned leathers stored at 21°C and 65% RH for 12 months.

At the same time, the mimosa based semi-metal tanned samples of V(IV), V(V), Fe(II) and Fe(III) showed remarkable decline in tensile strength as shown in Figure 3.10a. The mimosa-Fe(II) sample showed loss of tensile by strength by 43.5% from 10.8 MPa to 6.1 MPa in 12 months. Similarly, the tensile strength of the mimosa based semi-metal tanned leathers of Fe(III), V(IV) and V(V) declined by 54.5%, 67.5% and 58.1% with respect to the initial values.

The myrabalan-V(IV) and myrabalan-V(V) samples showed tensile strength values of 8.25 MPa and 10.3 MPa, slightly lower than that of the myrabalan-Fe(III) and myrabalan-Fe(II) samples (Appendix 7, Figure 3.10b). The myrabalan-V(IV) and myrabalan-V(V) lost their tensile strength by 54.3% and 63.4% over the one year period; at the same time the myrabalan-Fe(II) and myrabalan-Fe(III) samples showed reduction of strength by 37.3% and 40.1% over one year of storage. In both cases, a linear decline in the tensile strength values of Fe(II), Fe(III) and V(IV) were observed. The initial tensile strength of the mimosa-V(V) and myrabalan-V(V) samples were relatively lower and sharper decline was also noted particularly in the two months of storage of these samples (Figure 3.10 a & b).

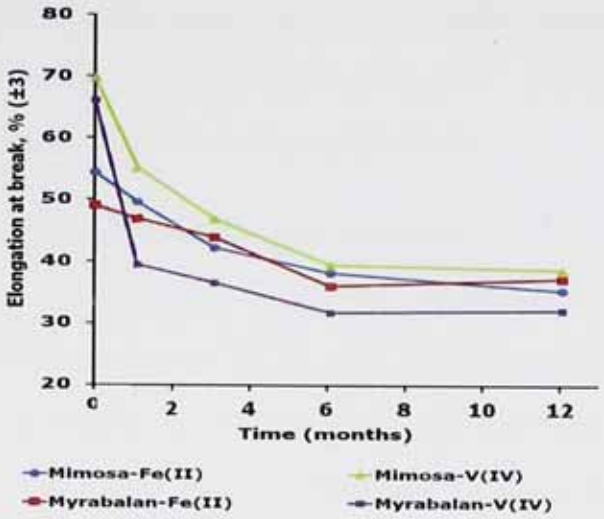


Figure 3.11 Results of periodic measurements of % elongation-at-break of semi-Fe(II) and semi-V(IV) leather samples stored at 21°C and 65% RH periodically measured for 12 months

The % elongation-at-break of the mimosa-V(IV) and myrabalan-V(IV) samples reduced significantly from an initial average of 67% to 39% after 6 months (Figure 3.11). The myrabalan-Fe(II) and mimosa-Fe(II) samples showed reduction of the % elongation-at-break values from 52% to 36% in the same time frame. The decline of both tensile strength and % elongation-at-break in the samples occurred largely after the three months of storage.

The significant physical deterioration, observed in terms loss of tensile strength and increased brittleness of fibre in the fibre structure *i.e.* lowering of % elongation-at-break may be related to two main aspects. The first is that the tannin-metal interactions with the iron and vanadium containing samples changes because of redox interactions. The initially formed metal-tanning complexation may be disrupted due redox reactions involving the metal ions; in addition, the polyphenolic tanning structure may also undergo complex structural changes, the effects being reflected in the physical properties. The relatively lower tensile strength of the V(V) retanned samples may be the result of the occurrence of a change in the polyphenolic tanning matrix possibly resulting from the oxidative effect of V(V) species on polyphenols (Kustin *et al.* 1974; Ferguson and Kustin 1979). Comparison of changes in the tensile strength of samples over the 12 months (Figure 3.10) and the changes in the T_s of samples (Figure 3.4-7) shows that both parameters showed quite similar pattern of decline. The occurrence of

simultaneous decline of T_s and the tensile strength vegetable tanned samples were also observed in artificially aged samples that undergone hydrolytic and oxidative degradation in sulfur dioxide chambers (Larsen *et al.* 1996).

3.3.5. The pH value of the semi-metal tanned leathers

As shown in Table 3.4, the pH value of the semi-Al(III) tanned leathers remained unchanged throughout the one year period. The initial pH of the leather samples indicates that the mimosa-Ti(IV) and mimosa-Fe(III) samples have relatively lower pH values as compared to the others samples. With both of the mimosa and myrabalan based semi-metal samples, the semi-Fe(II) samples showed a greater decline in pH after 3 months. This may be related to the formation of Fe(III) in the leather and hydrolysis of the metal ion during measurement. The mimosa-V(IV), mimosa-Fe(II) samples also became more acidic during storage, with lowering of pH by 0.7-0.8. Similar changes of pH were observed with the myrabalan based semi-metal tanned leathers (Appendix 7).

Table 3.4 The pH values of the mimosa based semi-metal tanned leathers, at different times of storage at 21°C and 65% RH.

Sample	Initial	3 months	6 months
mimosa	6.5	6.5	6.5
mimosa -Al(III)	6.1	6.1	6.1
mimosa -Ti(IV)	4.7	4.2	3.9
mimosa -V(IV)	5.2	4.5	4.5
mimosa -V(V)	5.4	4.7	4.6
mimosa -Fe(II)	5.2	4.3	3.9
mimosa -Fe(III)	4.8	4.1	3.8

The presence of relatively increased acidity of the samples may be related to the reversal of the semi-metal tanning interactions and chemical decomposition of the polyphenols. With the progress of degradation, increased proportion of metals became extractable by water. Lowering of the pH in most of the sample may be related to hydrolysis of the metal ions and solubilisation of degraded polyphenol components.

3.3.6. Metal contents of the semi-metal tanned leathers

The quantity of metal ions used during retanning was 1% w/w on the weight of the rehydrated vegetable tanned leather. Determination of the total metal content in the dry

samples indicates that the semi-Al(III) samples contain greater concentration of metal as compared to others. As shown in Figure 3.12, the mimosa and myrabalan leathers treated with the same metal, have similar metal contents. The semi-Ti(IV) samples show slightly higher metal content as compared to the semi-metal tanned samples of the transition metals. The percentage of the metal ions extracted when the leather samples are immersed in deionised water is shown in Figure 3.13. The analysis was carried out after 3 months of storage of the samples at 21°C and 65% RH.

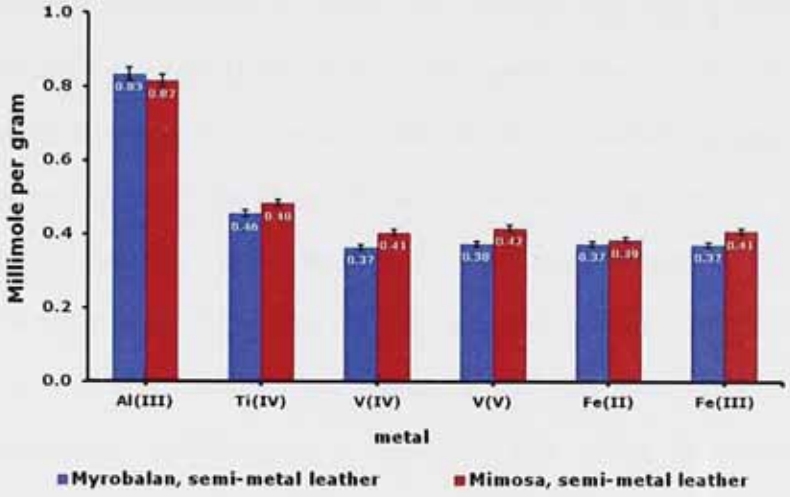


Figure 3.12 Metal contents in mMol/g of the dry semi-metal tanned leathers

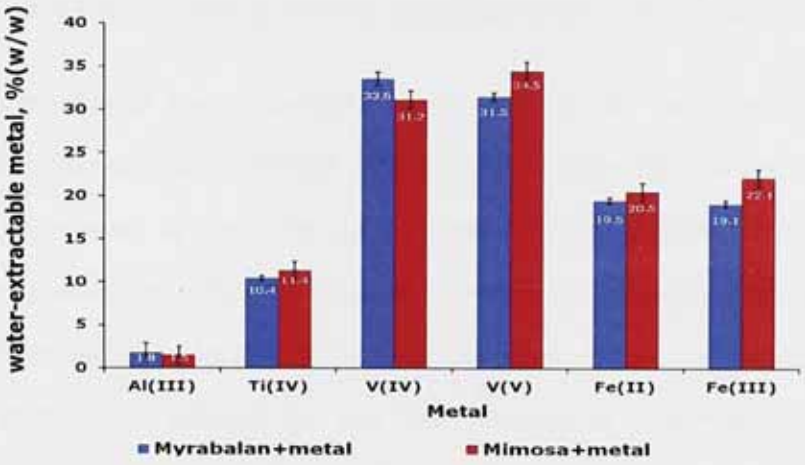


Figure 3.13 Relative quantity of water-extractable metal from the semi-metal tanned leathers expressed as %(w/w) of total metal content.

The result shows that metal ions in the semi-V(IV) and semi-V(V) samples have appreciably soluble in water, approximately one-third of the total metal content was extracted by water. The increased tendency of the meta ions to dissolve in water is indicative of the disruption of the metal-tanning complexation. The initial colour of the semi-V(IV) and semi-V(VI) leathers was dark-blue; however, the leathers and their

aqueous extracts (after 3 months) were dark-green. The cause of colour change is related to redox interactions resulting in the decomposition of tannins due to oxidation. In addition, the redox reactions may lead speciation of vanadium into the two oxidation states due to simultaneous occurrence of oxidation of V(IV) species by air and reduction of V(V) species by tannins.

Similarly, about one-fifth of the metal content of the semi-Fe(II) and semi-Fe(III) samples has become extractable by water. The extract was had a light blue colour, indicating solubilisation of Fe(III)-polyphenol complexes. The results indicated that the semi-metal tanning interaction in the iron and vanadium containing samples has been disrupted, allowing the metal to be solubilised. The fact that the iron and vanadium containing aqueous extracts from the leather have also indicated that certain low molecular weight fragments of the tannins (i.e. possible products of oxidation) may be solubilised along with the metals. This indicates that the structural changes in the tannin-metal crosslinks, occurring as a result of the redox reactions, may cause significant level of decompositon the large polyphenols (tannins), thereby the tanning matrix is eventually transformed to soluble molecular fragments of oxidised tannins.

The small percentage of the extractable metal content of the semi-Al(III) samples indicates the presence of loosely bound Al(III) complex deposited on the surface of the leather fibres. Whereas in the case of the semi-Ti(IV) samples, the presence of extractable metal indicates partial reversal of the semi-metal tanning interaction, which was also observed with the decline of T_s of the samples. Unlike the vanadium and iron leathers, the semi-Ti(IV) leathers did not show change in colour during storage *i.e.* remained orange, and the aqueous extract from the leather samples were colourless, indicating that there is no decomposition of the polyphenol structure into low molecular weight soluble species.

3.3.7. Vanadium content and the rate of degradation

The vanadium content of the mimosa tanned leather samples treated with different concentrations of V(IV) and V(V) were in the range of 0.11-1.42 %(w/w) relative to the

dry weight of the mimosa-V(IV) samples and 0.05-1.42 %with the mimosa-V(V) samples. As shown in Table 3.5-6, the T_s of the mimosa-V(IV) samples increased with the increase in the vanadium content. In the case of the mimosa-V(V) the increase in T_s (compared to the mimosa leather (*i.e.* T_s 84.5°C) is relatively low.

The T_s of all of the sampes of mimosa-V(IV) and mimosa-V(V) lowered to 57±3 °C in 60 days of storage at 21°C and 65% RH. The rate of decline in the T_s of the semi-V(IV) and semi-V(V) apprears to be less dependent of the quantity of the metals absorbed into the leather. The vanadium ions in the degradation process may act as triggers and catalysts of the an oxidative degradation process. Hence it may require the presence of a relatively small quantity (*i.e.* less than 1000 ppm) of vanadium to produce the same degradative effect as in the samples containing more than 1% w/w (or >10,000 ppm).

Table 3.5 Metal content of mimosa tanned leather samples treated with different concentrations of V(IV) and results of periodic T_s measurement carried out during storage of the samples at 21°C and 65% RH

vanadium applied on dry leather weight, % w/w	vanadium content in leather % w/w	Initial T_s	Time (days)			
			2	8	32	64
0.25% V(IV) as $VO(SO_4)$	0.05	89.0	87.2	82.2	69.3	54.7
1.0% V(IV) as $VO(SO_4)$	0.47	102.1	85.9	82.1	59.6	54.0
4.0% V(IV) as $VO(SO_4)$	1.23	112.5	86.5	75.8	71.0	59.7

Table 3.6 Metal content of mimosa tanned leathers treated with different concentrations of V(V) and results of periodic T_s measurements carried out during storage of the samples at 21°C and 65% RH

vanadium applied on dry leather weight, % w/w	Vanadium content in leather % w/w	Initial T_s	Time (days)			
			2	8	32	64
0.25% V(V) as $NH_4(VO_3)$	0.11	88.7	85.5	83.1	73.2	61.0
1.0% V(V) as $NH_4(VO_3)$	0.67	94.5	88.4	86.6	69.7	54.6
4.0% V(V) as $NH_4(VO_3)$	1.42	90.0	87.4	73.3	71.4	56.6

The results indicate that the presence of excess V(IV) or V(V), above certain thershold level, may have less effect on the rate of degradation in terms of speeding up the process. Hence the presence of V(IV) and V(V) in the leather may be considered as catalyst of oxidation of tannins.

3.3.8. Degradation of Cr(III) tanned leathers

The T_s of the Cr(III) tanned leather (111°C ±1) was not affected by treatment with 1% of V(IV) and Fe(II), the T_s remained the same. The V(V) and Fe(II) salts have no

additional hydrothermal stabilising effect on the Cr(III) tanned leather. In addition, no change in shrinkage temperaure of the samples was observed when V(IV) and Fe(II) sample were kept at ambient condition for 30 days. On the other hand, as shown in Figure 3.14 a&b, progressive decline in T_s was observed with the V(IV) and Fe(II) treated samples of Cr(III)-chestnut and Cr(III)-mimosa leathers that were stored for 30 days. During the storage, the colour of the V(IV) samples changed from blue to dark green, similar to the the case of the semi-V(V) leathers. The Cr(III)-mimosa and Cr(III)-chestnut tanned leathers that were treated with Fe(II) also showed similar decline in T_s from 115°C to 73°C in 30 days, as shown in Figure 3.14a. These changes are however are relatively lower as compared the corresponding V(IV) treated samples shown in Figure 3.14b.

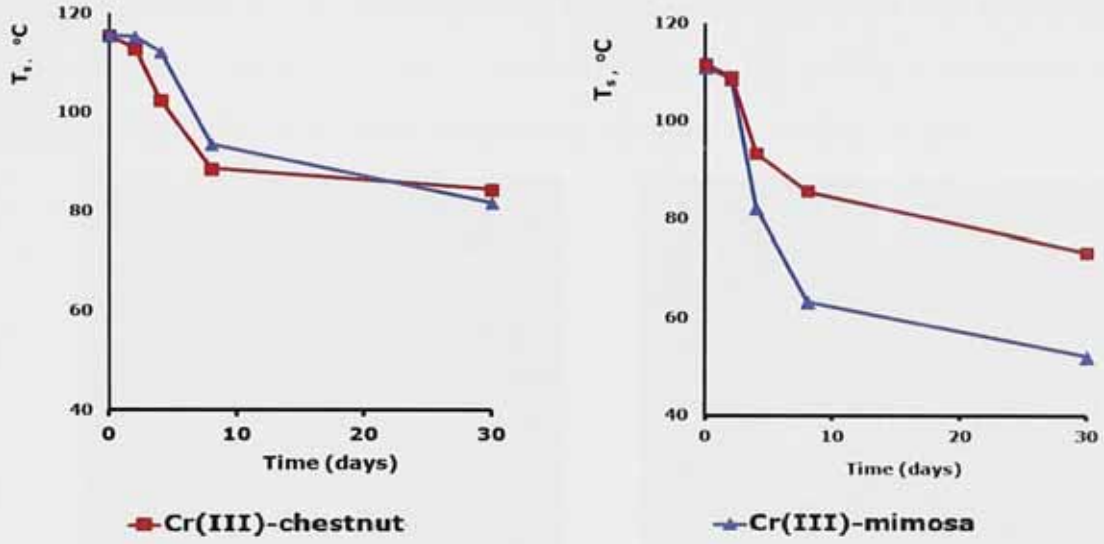


Figure 3.14 Shrinkage temperature Cr(III)-chestnut and Cr(III)-mimosa tanned leathers (a) treated with 1% of Fe(II) (b) treated with 1% of V(IV)

Based on these results, it is recognised that Fe(II) and V(IV) induced degradation, cause similar effects in vegetable tanned leathers and vegetable retanned Cr(III) leathers. More importantly, the results reveal that the loss of hydrothermal stability and physical degradation caused by the instablility in the Fe(II)-tannin or V(IV)-tannin interaction, is not affected by the presence of a Cr(III) tanning interactions in the samples. The T_s of the Cr(III)-chestnut sample, treated with V(IV), dropped significantly below 60°C. In this

case, it can be said that, the initial decline of T_s and the denaturation of collagen are not influenced by the presence of Cr(III) complexes in the tanning structture.

Tanning with Cr(III) salts imparts highest hydrothermal stability to leather (T_s >110°C) as a result of formation of stable coordinate-covalent links between Cr(III) and the collagen carboxyl groups (Riech 2007; Covington *et al.* 2008). However, with regards to occurrence of V(IV) and Fe(II) induced degradation, the presence of the Cr(III) tanning matrix does not prevent the degradation process.

3.3.9. The fibre structure in the degraded leathers

3.3.9.1. Semi-V(IV) tanned leather

The fibre structure of a degraded chestnut-V(IV) leather was visually compared with that of a undegraded sample of chestnut-Al(III) leather as reference. The SEM micrographs of the samples, shown in Figure 3.15 and Figure 3.16, indicates that there are observable differences in terms of porosity and apprearance of fibre bundles.

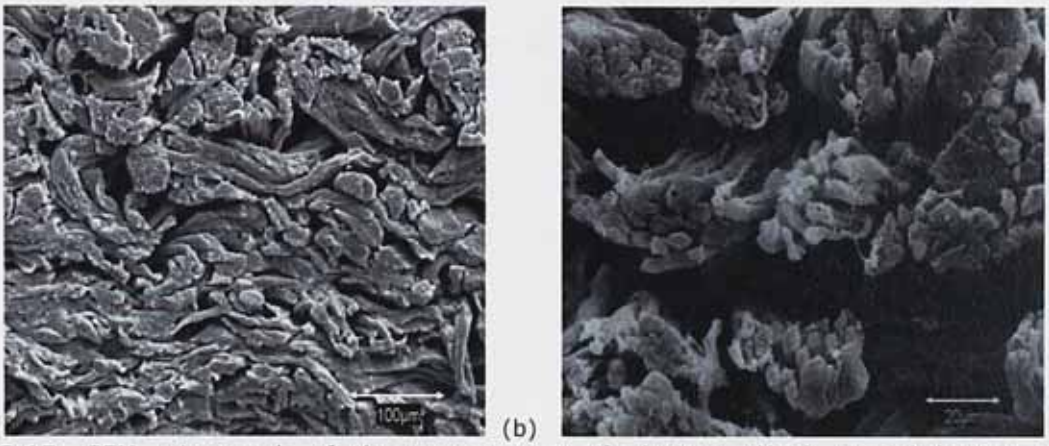


Figure 3.15. SEM micrograph of the cross-section of chestnut-Al(III) tanned leather at magnification of (a) X250 and (b) X1000.

The SEM micrograph of the chestnut-Al(III) sample shows distinct fibres and a relatively open structure as shown in Figure 3.15 a&b. In contrast, the inter-fibre space in the chestnut-V(IV) sample (Figure 3.16a-b) appears to be smaller and the structure looks relatively compact as compared to the chestnut-Al(III) sample. The fact that the T_s of the vanadium and iron containing semi-metal tanned samples was reduced below 60°C has indicated that the collagen in the samples has been denatured by the redox reactions that take place inside the leathers.

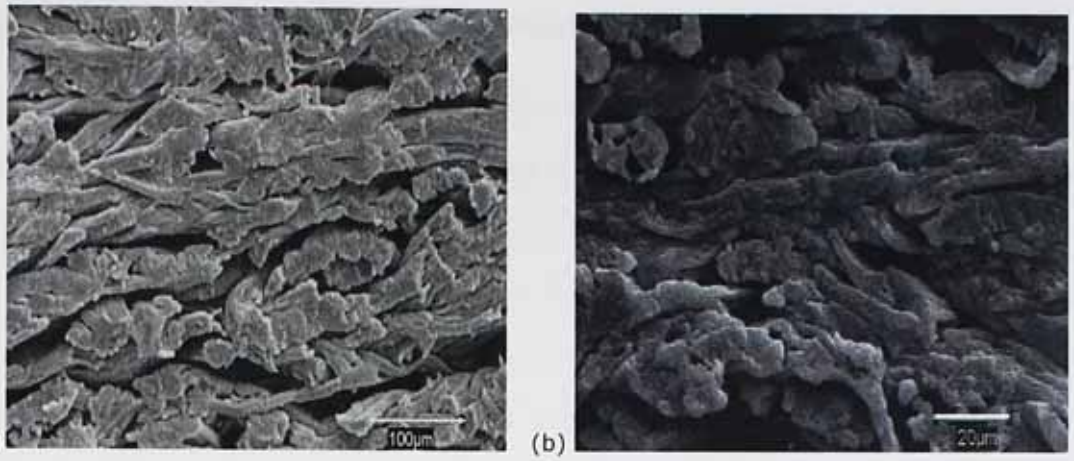


Figure 3.16. SEM micrograph of the cross-section of chestnut-V(V) tanned leather after storage for one year at 21°C and 65% RH at **(a)** magnification of X250 and **(b)** magnification of X1000.

In order to observe the effect of denaturation and ascertain the presence of partial gelatinisation, the semi-V(IV) samples were rehydrated with deionised water for 12 hours and subsequently freeze dried at -40°C for 24 hours to completely remove the water. After drying, the sample became brittle, the cross-sectional surface showed irregular structures that appreared like a 'melting' structure in which fibres are not visible distinctly (Figure 3.17a). The cross section of the sample showed what apprears to be fibre bundles fused together into irregular shapes (Figure 3.17b).

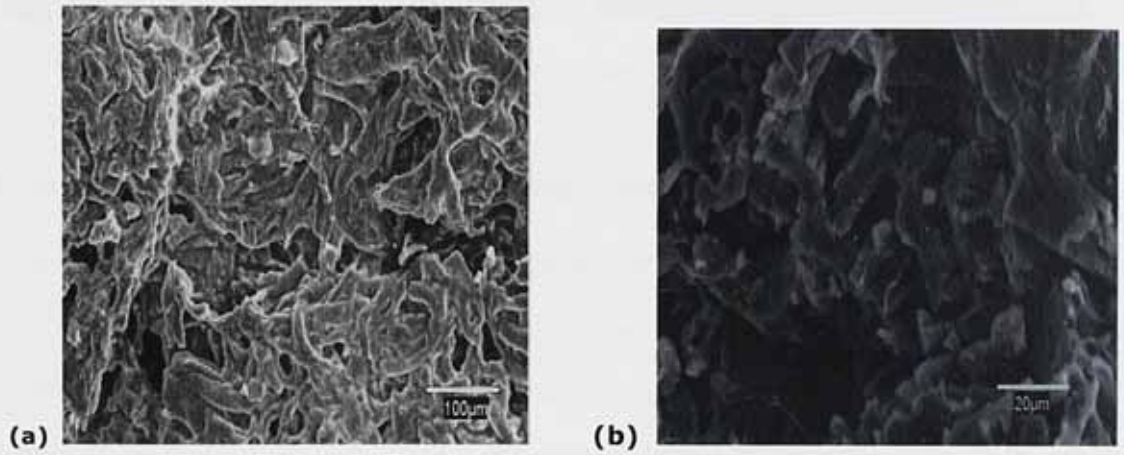


Figure 3.17 A SEM micrograph of chestnut-V(IV) sample, showing (a) gelatinised surface of the cross-section of the sample ,magnification X250 (b) internal section of the brittle sample, showing damaged fibre bundles fused together, magnification X1000.

The SEM observation of the samples indicates that degradtion in the semi-metal tanned leathers containing V(IV) and V(V) eventually results in disruption of the basic fibre structure and gelatinisation due occurrence of oxidative denaturation of the collagen .

3.3.9.2. Cr(III)-Chestnut and Cr(III)-Mimosa leathers

The Cr(III)-chestnut and Cr(III)-mimosa tanned leathers, treated with V(IV) and kept at ambient condition for 30 days, also showed a similar change in fibre structure as described in section 3.3.8.1. During storage at 21°C and 65% RH for 3 months, the leather became thiner and weak. Comparison of the normal sample of Cr(III)-chestnut leather and the V(IV) treated Cr(III)chestnut leather (Figure 3.18a-b) shows that the partially degraded leather has changed into a dense structure, where the fibre weave is not distinctly visible.

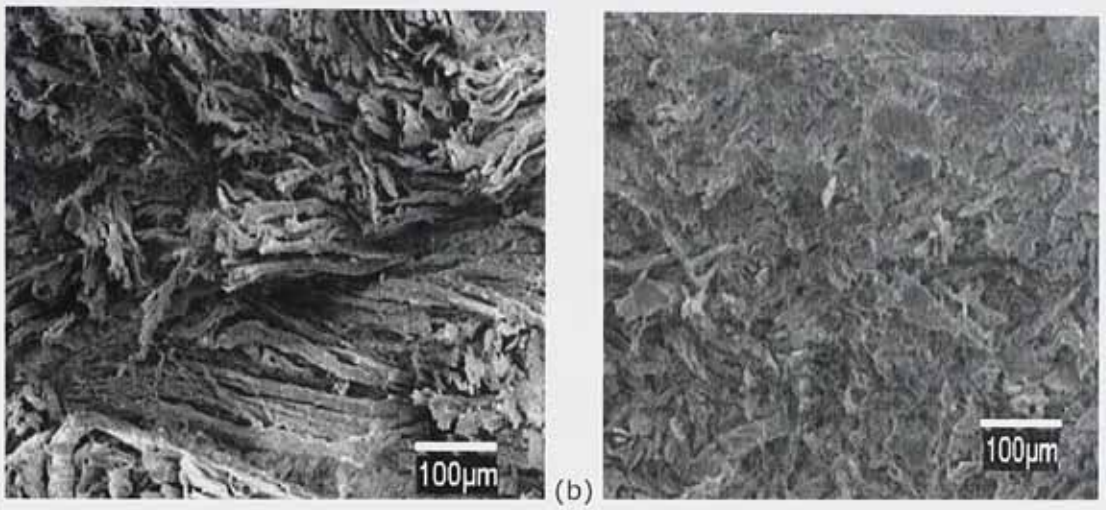


Figure 3.18. SEM micrograph of the cross-section of Cr(III)-Chestnut tanned leathers, magnification X250, after storage at 21°C and 65% RH; (a) control sample (b) sample treated with V(IV)

3.3.10. EPR spectroscopy of vanadium containing samples

The Vanadium(IV) ion carries an unpaired electron (d^1) in its electronic configuration *i.e.* $[Ar]3d^1$. The unpaired electron in oxovanadium ion (VO^{2+}) gives a characteristic eight-line hyperfine splitting pattern in its EPR spectrum. This originates from the interaction of the nuclear magnetic moment of the $\mathbf{V^{51}}$ nuclei (characterised by nuclear magnetic quantum I=7/2) with the magnetic moments of the unpaired electron (Murphy 2009).

As shown in Figure 3.19, the spectrum of the reference sample of $VO(SO_4)$ and the hide powder and mimosa leather treated with $VO(SO_4)$ showed hyperfine signals centred at 360 mT. It is observed that the width of the signal of the VO^{2+} treated mimosa tanned leather is narrow as compared to the others.

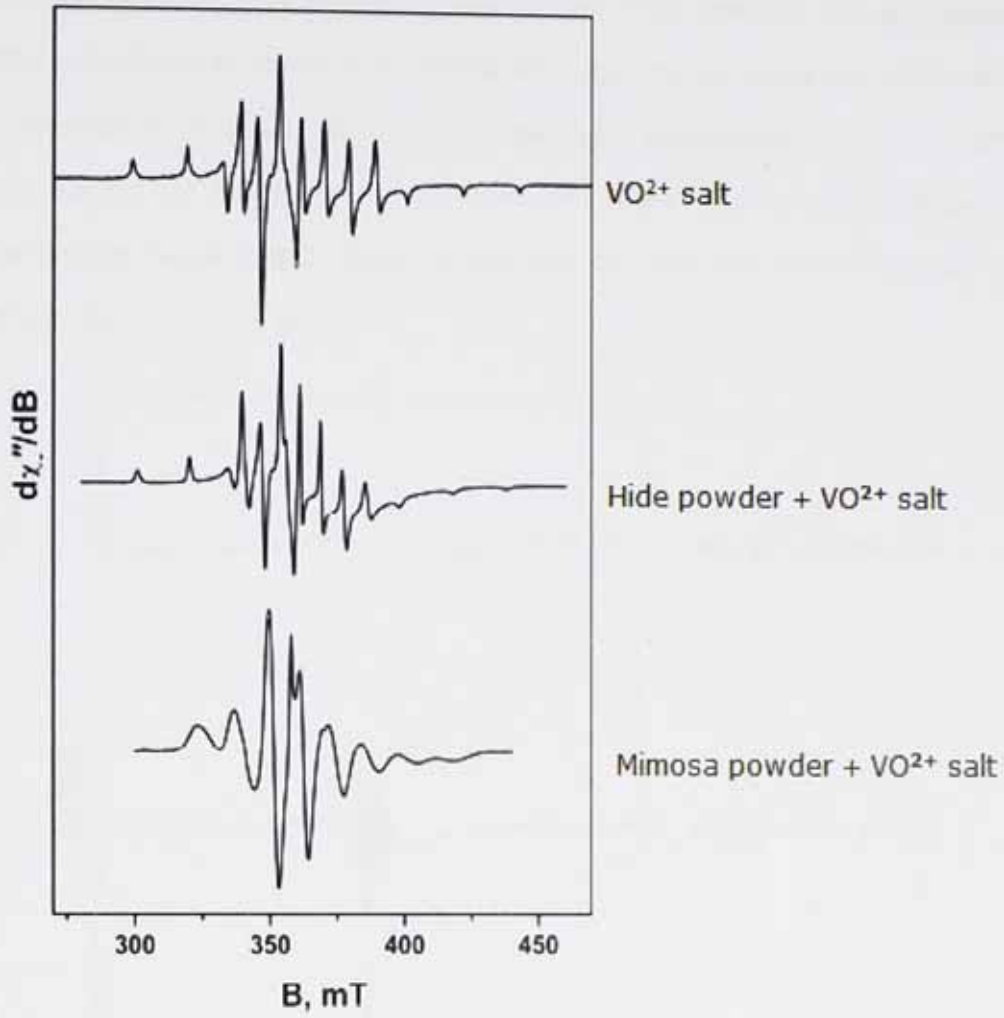


Figure 3.19: EPR spectra $VO(SO_4)$ salt and that of the hide powder and mimosa tannin powder samples treated with V(IV).

The oxovanadium ion (VO^{2+}) and its complexes have a distorted square pyramidal structure with one of the coordination sites being occupied by the V=O bond (Nagaraju *et al.* 2013; Greenwood and Earnshaw 1984). However, the oxo-ligand may also be displaced by a ligand leading to formation of a non-oxo V(IV) complex. As described by Rangel *et al.* (2006), formation of the six coordinated tris-catecholate V(IV) complex from oxo-vanadium in aqueous solution is characterised by narrowing of the width of the VO^{2+} hyperfine pattern. The hyperfine pattern of the mimosa-VO^{2+} sample, which contains complexes of the *o*-diphenolic complexes of VO^{2+}, is narrower compared to the width in the other two hyperfine spectra, indicating the possible presence of tris-catecholate type of structure of VO^{2+} complex *i.e.* complexed by three chelating ligands.

The EPR spectra of the reference sample of $NH_3(VO_3)$ and the sampes treated with $NH_3(VO_3)$, are shown in Figure 3.20. Unlike $VO(SO_4)$, the vanadate salt is EPR-silent due to the absence of unpaired electron in its electronic configuration *i.e.* V(V) with $[Ar]d^0$ (Murphy 2009). The spectra of the vanadate salt (Figure 3.20), merely shows a broad but non-distinct signal in the region of 150-600 mT that may have originated possibly from trace impurity in the salt.

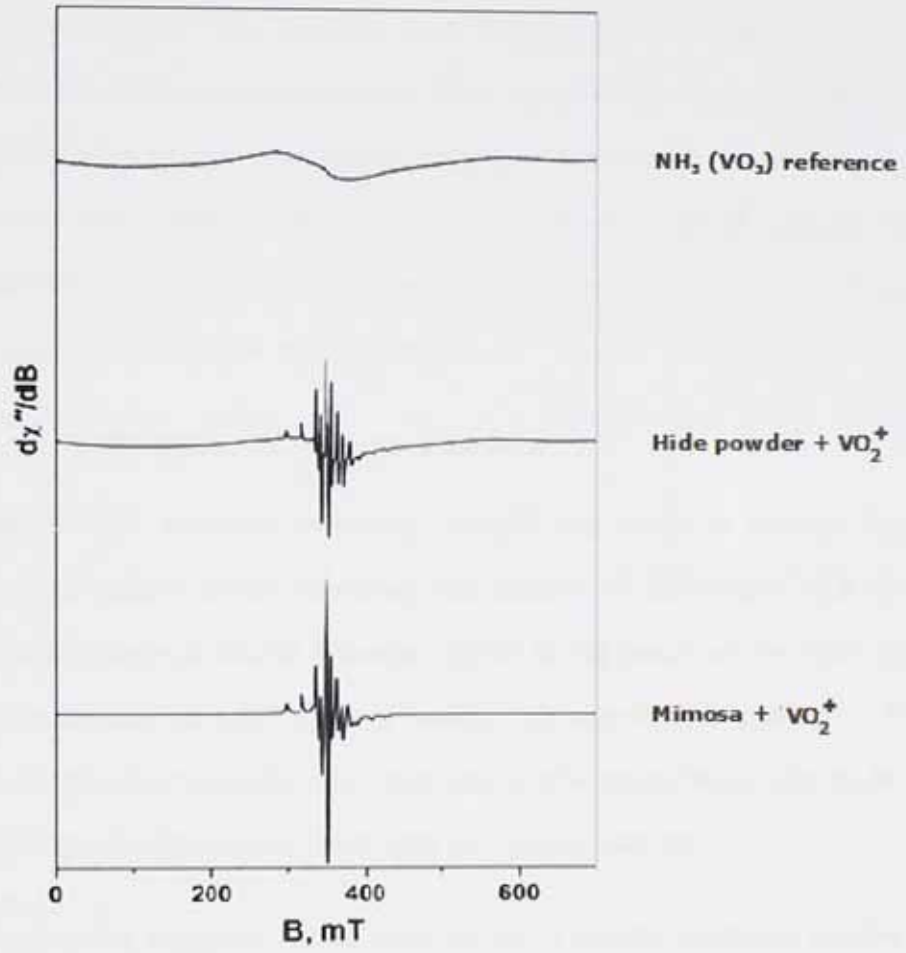


Figure 3.20 EPR spectra $NH_3(VO_3)$ salt and vanadate (VO_3^-) treated samples of hide powder and mimosa tannin powder

Both the hide powder well as the mimosa powder samples, treated with the vanadate salt, show the characteristic hyperfine spectral profile, similar to the samples treated with VO^{+2}. This suggests that the V(V) was reduced *in situ* by the hide powder and the mimosa tannin. Nakajima (2000) has confirmed that V(V) was reduced when it was absorbed from water by condensed tannin gel (exudate from persimmon fruit). Kustin *et al.* (1974) has also described that the interaction between catechol-derivatives

(polyphenols) and V(V) ion is a fast redox reaction, resulting in the formation of a blue coloured V(IV) complex (λ_{max} 760 nm). This is a clear evidence that V(V) ions are strong oxidising agents in their interaction with polyphenols. The result also indicates that V(IV) was formed in the hide powder sample as a result of a possible redox reaction with functional groups at the side chains of collagen.

As described in Chapter 1, no increase or decline of shrinkage temperature was observed on hide powder that was treated with $NH_3(VO_3)$. With respect to hydrothermal stabilisation, the V(V) salt is assumed to have no effective interaction with collagen. The spectra of the V(V) treated hide powder indicates the occurrence of reduction of V(V) in to,V(IV). Most likely, some of the side chain functional groups of collagen may be prone to oxidation by V(V). Nevertheless, effect of this redox reaction on the collagen molecule did not lead to denaturation of collagen.

3.3.11. EPR spectra of mimosa powders

The tannin powder samples, a control sample and metal containing samples, were analysed using quartz tubes. However, the spectra of the empty tube (Figure 3.21), showed the presence of Mn(II) impurity, signal at the centre of the spectrum (340-380 mT) that originated from EPR sample cavity. The signals of the organic radicals in the spectrum of the other samples also appeared in the same region and in all spectra the cavity signal (that of the empty tube) was subtracted from all.

As observed in the spectrum of the mimosa-Fe(II) powder samples, the low field signal (100-170 mT) corresponding to g=4.3 is attributed to Fe(III). This signal is also identified in Fe(III) containing mineral salts (Gopal *et al.* 2004; Allard *et al.* 2004). This characteristic signal was also used to confirm the presence of Fe(III) in archaeological leathers by Bardet *et al.* (2009). The formation of Fe(III) species in the mimosa-Fe(II) sample is due to oxidation of the Fe(II) by air.In all of the sample spectra, the characteristic signal of organic radicals (g=2.0023) is detected, appearing as sharp signals at 360 mT. In the case of the mimosa-V(IV), the organic radical signal overlaps with the broad V(IV) hyperfine signal which is characteristic of V(IV) complexes.

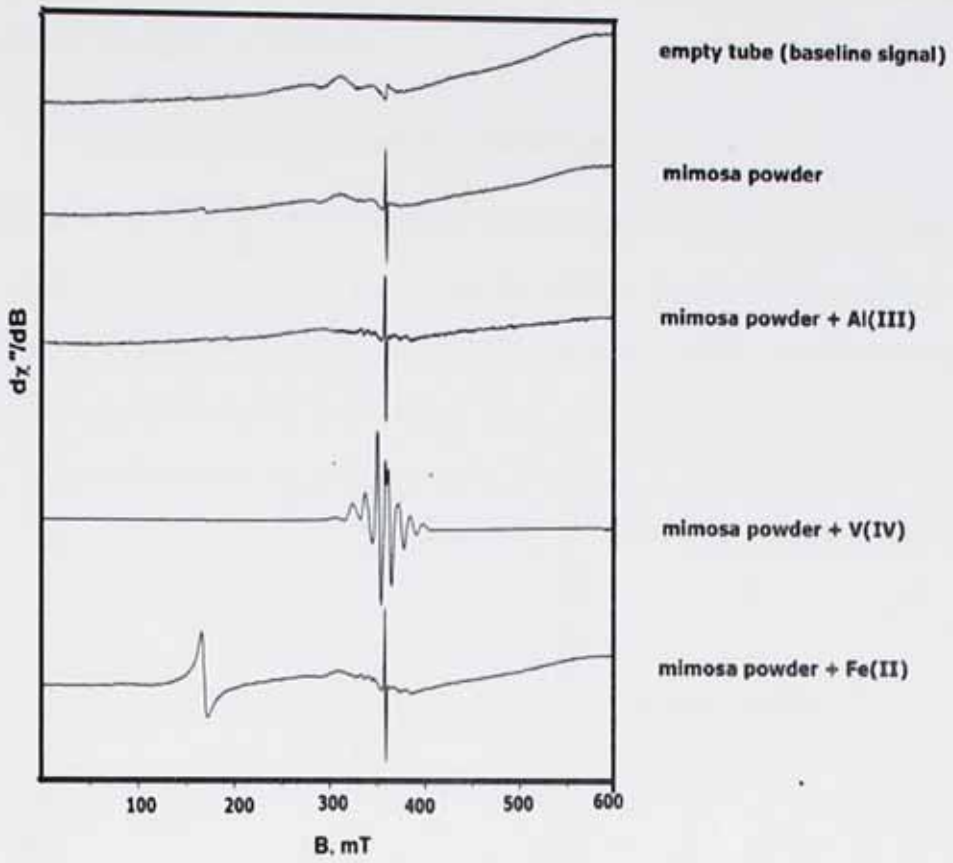


Figure 3.21 First derivative X-band EPR spectra of mimosa powder and mimosa powder samples containing Al(III), V(IV) and Fe(II)

The formation of free-radicals in the samples may be related to oxidation tannins reactions by air. Oxygen may abstract an electron from the *o*-diphenol groups producing semi-quinone radical intermediates and a subsequent structural rearrangement (Porter 1992; Lambert *et al.* 2010). On the other hand, since Fe(III) is capable of oxidising polyphenols (Hynes and Ó'Coinceanainn 2001), its presence in the vegetable tanned leathers, may enhance the oxidation of the *o*-diphenol groups into semi-quinone radical and quinone structures.

The formation of free-radicals in the semi-V(IV) leather may be enhanced by the oxidation of the *o*-diphenol moieties of tannins by V(V) species. In the EPR spectrum of the mimosa-V(IV) leather (Figure 3.21), the signal overlap of V(IV) does not allow clear analysis of the free-radical signal of the sample. It should be noted that, the formation of free-radicals in the tannin structures may be part of a spontaneous redox reactions

involving semi-quinone radical intermediates that are terminated by further oxidation resulting in the formation of quinones.

3.3.12. EPR spectra of mimosa tanned leathers

The spectra of the mimosa tanned leather and the mimosa based semi-metal tanned leathers (shown in Figure 3.22) are generally similar to the spectra of the mimosa powder samples (Figure 3.21). At the time of analysis, the leather samples were actually stored at 21°C and 65% RH for 6 months.

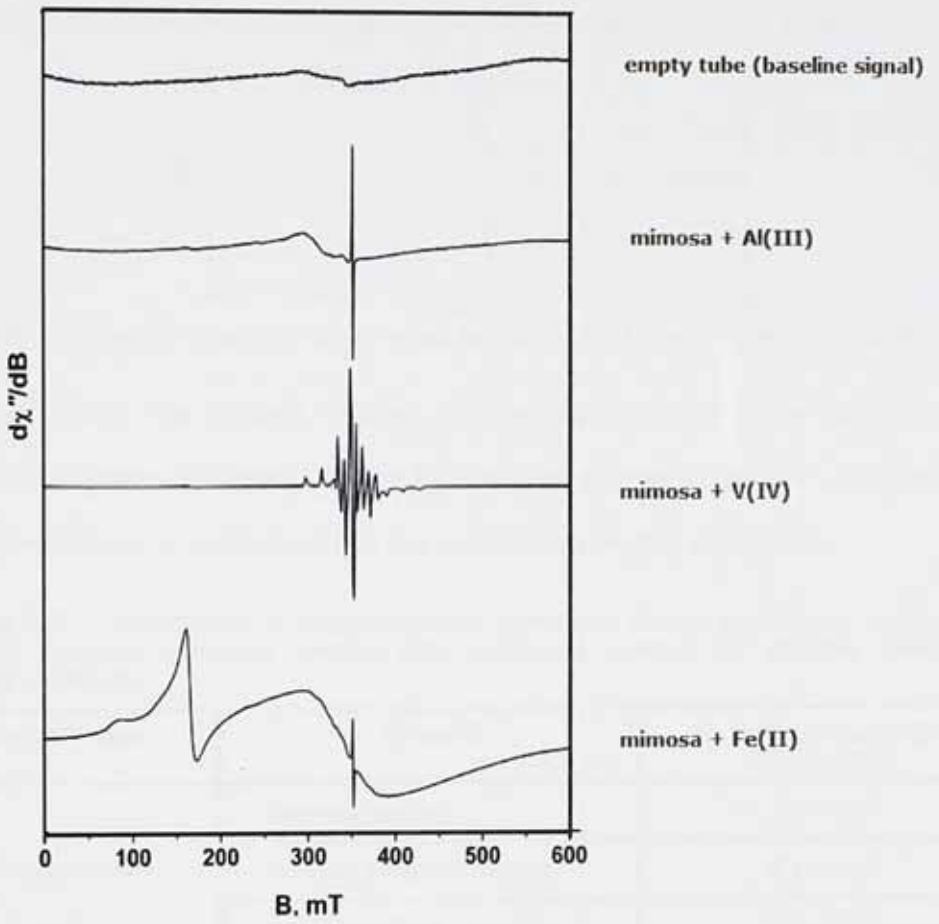


Figure 3.22 First derivative X-band EPR spectra of samples of mimosa tanned leather, mimosa-Al(III) tanned leather, mimosa-V(IV) tanned leather and mimosa-Fe(II) tanned leather stored at 21°C and 65% RH for 6 months.

The spectra of the mimosa-Fe(II) leather has the characteristic Fe(III) low-field peak at 170 mT but also showed a broad signal from 200-500mT. This signal is also observed in oligomeric Fe(III) species complexes with organic ligands. During storage of the mimosa-Fe(II) sample and the metal-tannin interaction might be disrupted allowing for

increased interaction between metal complexes, hence leading formation of oligomeric Fe(III) species.

3.3.13. Free-radical concentrations

The spectrum of the weak pitch standard, occupying the entire 2 cm height of the cavity, has estimated spin signal of 2.4 × 10^{13} spins (*i.e.* calculated as 2cm x 1.2X10^{13} spins/cm based on the specification of the manufacturer Bruker Co. Ltd.) USA). The spectra of the standard (Figure 3.23) was collected under identical conditions used in the analysis of the sample, a sharp resonance signal of the pitch standard (at g = 2.007)

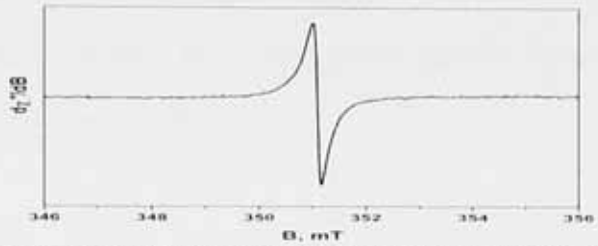


Figure 3.23. X-band EPR spectrum of the weak pitch standard recorded at 293 K and 9.861 GHz.

The quantities of free organic radicals determined using the standard, focusing at g=2.0023, are shown in Table 3.7. The free-radical concentration in the mimosa powder samples is relatively low compared to the that contain Fe(II) and Al(III).

Table 3.7. Concentration of organic radicals in mimosa tannin powder and semi-metal tanned leathers: reference samples (not containing metals) and samples containing Al(III) and Fe(II)

Sample type	Sample	Radical concentration (spins/mg)
Tannin powders	Mimosa powder	2.5 × 10^{13}
	Mimosa powder +Al (III)	8.9 × 10^{13}
	Mimosa powder + Fe(II)	7.2 × 10^{13}
Leather samples	Mimosa tanned	13.8 × 10^{13}
	Mimosa + Al(III) tanned	154.6 × 10^{13}
	Mimosa + Fe(II) tanned	18.8 × 10^{13}

The Al(III) containing powder samples concentration of free radicals greater than that of the Fe(III) containing sample by 24% and nearly 3 times that of the mimosa powder. On the other hand, with the leather samples, the Al(III) treated leather show markedly the highest free-radical concentration. The Fe(II) treated leather shows only slightly greater

concentration as compared to the control sample. The implication of the greater spin count in the Al(III)-mimosa sample is that the metal may either enhance or stabilise the formation free radicals..

On account of its redox properties, not having multiple oxidation states, the Al(III) in the leather does not engage in redox reactions through one-electron transfer. On the other hand, free-radicals may be formed in the polyphenolic matrix due to oxidation of tannins by air. The *o*-diphenol moieties are susceptible to oxidation (Tanaka *et al.* 2009). It is known that oxidation in the presence of light in vegetable tanned leathers results in change of colour or reddening (Covington 2009). The reaction may involve oxidation of the *o*-diphenol groups of tannins to semi-quinone radicals and quinone structures as shown in Figure 3.24 (Lambert *et al.* 2010).

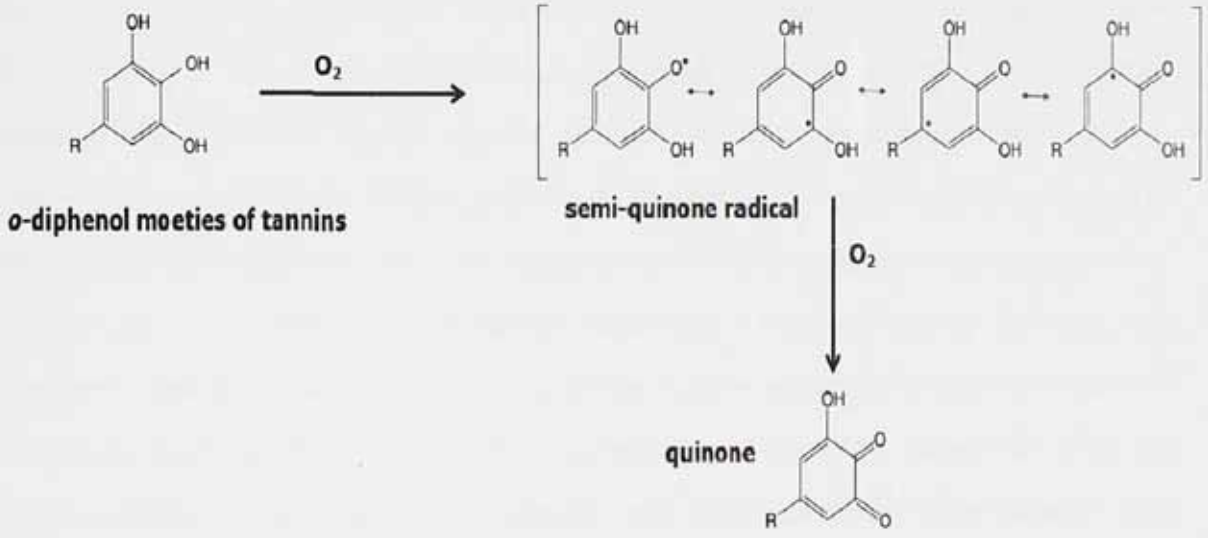


Figure 3.24 Oxidation of the *o*-diphenol moieties of tannins into quinone structures by oxygen, through formation of resonance stabilised semi-quinone structures (Lambert *et al.* 2010).

The quinone structures are reactive and may form coupling products by reacting with an unoxidised catechin (Bark *et al.* 2011; Hathaway *et al.* 1958). This coupling reaction is also observed with enzymatic oxidation of catechin at pH 6-7 using polyphenol oxidase (Tanaka *et al.* 2009). The oxidation of the *o*-diphenol moieties in the mimosa tanned leather (and other similar leathers) may also lead to coupling reactions between adjacent monomeric units in the tanning matrix. The free-radical concentration related to

the mimosa powder and the mimosa tanned leather sample may be related to the presence of the semi-quinone radicals as short-lived intermediates of the oxidation reactions.

In the mimosa-Fe(II) tanned leather, the oxidation of the *o*-diphenol moieties of tannins may occur in the same manner as described in Figure 3.24. However, the reaction is could be fast because iron ions (Fe^{2+}/Fe^{3+}) to catalyse univalent redox reactions (Perron *et al.* 2010). The Fe(II) species are readily oxidised in air to form Fe(III) ions, which are capable of oxidising the *o*-diphenol groups, thereby enhancing the formation of the semi-quinone and quinone structures The slightly higher concentration of free-radicals in the Fe(II) containing samples (Table 3.6) as compared to the control sample may be related to an increased rate of oxidation reaction.

The relatively strong interaction of Al(III) ions with the *o*-diphenol effectively prevents the oxidation of the *o*-diphenol groups to quinones. Nevertheless, the third hydroxyl group of the *o*-diphenol ligands may be oxidised, forming a resonance stabilised free-radical (Figure 3.25). Since further oxidation is prevented by complexation, the B-ring of the flavonoid structure may carry the unpaired electron delocalised in the aromatic ring. The Al^{3+} ion in the Al(III)-mimosa leather appears to have the effect of stabilising the resonance stabilised free-radical, by preventing further oxidation of the semi-quinone radical to quinone. As a result, the free-radical concentration, measured with the mimosa-Al(III) tanned leather is remarkably high compared to the other samples *e.g.* more than 8 times that of the mimosa-Fe(II) tanned leather sample.

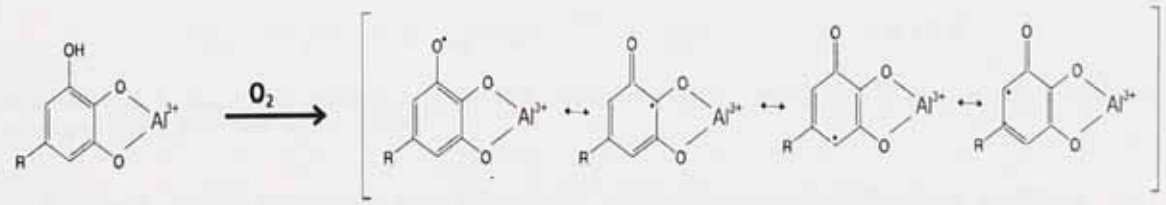


Figure 3.25. The possible formation of resonance stabilised free-radicals by oxidation of Al(III) complexed *o*-diphenol moieties.

Semi-alum leathers are known for their resistance to ageing as observed from artificial ageing tests (Larsen 1996). The semi-Al(III) tanning process is a confirmed standard for

making bookbinding leathers *i.e.* standard number BS 7451:1991 (BSI 2011). This property may be related to the strength of tannin-Al(III) interaction and inhibition of the of oxidation of tannins in vegetable tanned leathers.

3.3.14. General aspects of metal induced autodegradation of leather

With regards to the decline in the hydrothermal stability and the physical degradation that resulted from treatment of vegetable tanned (or retanned) samples with iron and vanadium salts, the implications of the results described in this chapter are summarised as follows.

3.3.13.1. The stabilities of semi-metal tanning interactions

The initially observed hydrothermal stabilisation by semi-metal tanning was only a transient form of stability imparted by tannin-metal complex formation. Storage of the samples at ambient conditions has resulted in loss of the hydrothermal stability. The changes observed in semi-V(IV) and semi-Fe(II) leathers may be related to oxidation of tannins by metal ions. The autoxidation of the Fe^{2+} and VO^{2+} leads to formation of the Fe^{+3} and VO_2^{+}. This has been shown with the detection of the characteristic Fe(III) and V(V). EPR signal features in the EPR spectra of semi-Fe(II) and semi-V(IV) leathers, respectively. Due to their relatively greater reduction potential (shown in Equation 3.1), Fe(III) and V(V) are oxidative ions, capable of oxidising the *o*-diphenol moieties of tannins (Perrron *et al.*2010; Hynes and Ó'Coinceanainn 2009; Kustin *et al.* 1974).

$$VO_2^{+} + 2H^{+} + e^{-} \rightleftharpoons VO^{2+} + H_2O \qquad \mathbf{E^{\circ} = 1.00\ V}$$

$$Fe^{3+} + e^{-} \rightleftharpoons Fe^{2+} \qquad \mathbf{E^{\circ} = 0.77\ V}$$

$$TiO^{2+} + 2H^{+} + e^{-} \rightleftharpoons Ti^{3+} + H_2O \qquad \mathbf{E^{\circ} = 0.10\ V}$$

Equation 3.1. Standard reduction potentials of aqueous V(V), Fe(III) and Ti(IV) species in acidic media at 25°C (Shriver *et al.* 1979)

In contrast, Ti(IV) ions have low affinity for electrons and do not act as oxidising agent in its interaction with tannins. Hence, the decline of T_s in semi-Ti(IV) leathers is considered to be a case of a reversal of the semi-metal tanning interaction, possibly caused by structural changes and hydrolysis, rather than oxidation of tannins. In

contrast, the metal-tanning complexation interactions in semi-Fe(II) and semi-V(IV) leathers may be stable only until oxidation of the metal ions by atmospheric oxygen.

3.3.13.2. Redox reactions in semi-metal tanned leathers

The interaction of the metal with the polyphenolic tanning matrix may create a series of reactions that not only cause reversal of the semi-metal tanning interaction but also causes decomposition of the polyphenolic structure in the leather. It was observed that part of the polyphenol and the metal from these samples became extractable with water, indicating that the large molecular structures have decomposed into low molecular weight soluble components.

The oxidation of the *o*-diphenol groups may involve formation of the free-radical intermediates (semi-quinone radicals) and quinone groups. On the other hand, oxidised polyphenols are capable of undergoing oxidative coupling, leading to formation of a more cross-linked structure.

The oxidation of the *o*-diphenol groups (and the subsequent reactions oxidative coupling) does not explain the degradation of the polyphenol matrix in the semi-metal tanned leathers. In fact, the opposite effect *i.e.* cross-linking of the tannin molecules, could be expected. However, the most important redox reaction with respect to the degradation of the polyphenol and collagen structure in the leather may be the formation of the superoxide anion ($O_2^{\cdot -}$) and its derivatives *i.e.* hydroxyl radicals, hydrogen peroxide and hydroxy radicals. The oxidation of metals such as Fe(II) and V(IV) may be generalised as shown in Equation 3.2.

$$M^{n+} + O_2 \longrightarrow M^{+(n+1)} + O_2^{\cdot -}$$

Equation 3.2. Oxidation of metal ions by atmospheric oxygen

The Fe(II) and V(IV) ions that are chelated by the *o*-diphenol groups may be subjected to the above oxidation reaction. The consequences of oxidation o-diphenol complexed metal ions in the semi-metal tanned leather would be primarily the reversal of the semi-metal tanning interaction and formation of superoxide anion. Secondly, the higher oxidation states of the metals *i.e.* Fe(III) and V(V) species may be reduced by o-

diphenol groups. Hence, a redox cycling of the metal ions, with oxygen being the oxidant and polyphenols being the reductant, may be established. The presence of superoxide anion may lead to the formation of hydroxyl radicals, which are powerful oxidants that are capable of decomposing large organic molecules (Barbusiński 2001). The basic aspects related to the possible mechanism of the formation of hydroxyl radicals and their effect in the stability of leather are described in Chapter 4 and Chapter 5.

3.3.13.3. Denaturation of the collagen

The collagen in these samples was denatured as observed with the lowering the T_s values below that of the untanned collagen and loss of physical strength of the leathers; Chahine (2000) has observed similar changes in the T_s of artificially aged leather samples and deteriorated historical leathers. The effect of environmental conditions on the collagen degradation, in historical leathers and parchments, is mostly assessed in terms of the lowering of the T_s values below the 60°C (Badea *et al.* 2011). The occurrence of gelatinisation in semi-V(IV) tanned leathers, that have undergone degradative changes during storage at 21°C and 65% RH, has been physically confirmed with the SEM observation of the fibre structure in the degraded semi-V(IV) leather samples. The observed gelatinisation may be caused by the formation of oxygen-derived free-radicals. Collagen is susceptible to oxidative damage by hydroxyl radicals through cleavage of the peptide chain (Uchida *et al.* 1990; Kato *et al.* 1992) and reactions taking place in the side chain (Komsa-Penkova *et al.* 2000).

3.4. Summary

During semi-metal tanning, slightly greater T_s values were observed with the mimosa and chestnut based samples. With the exception of the non-transition metal Al(III), all of the semi-metal tanned samples showed characteristic colour changes during retanning with metal ions. The identical colour of the semi-V(IV) and semi-V(V) leathers *i.e.* dark blue, indicated that the tannin-metal complex in both cases may be the same. Similarly, semi-Fe(II) and semi-Fe(III) showed the same blue colour. Based on the redox properties of the metal ions, it is assumed that the formation of tannin-metal complexes

in the case of semi-V(IV) ions may involve reduction of the metal ion. While in the case of Fe(II),the complexation may occur, along with oxidation of the metal to Fe(III).

The vegetable tanned samples and the semi-Al(III) tanned samples remained consistently stable in terms of physical properties and Ts during storage at 21°C and 65% RH for 12 months. At the same time, the semi-Ti(IV) exhibited a relatively slow decrease Ts, but no significant change in colour and physical properties was observed. The reason for the observed change may be attributed to occurrence of reversal of the semi-metal tanning interaction due to hydrolysis.

In contrast, semi-Fe(II), semi(III), semi-V(IV) and semi-V(V) samples showed greater decline in T_s as well as reduction in tensile strength and % elongation-at-break. These changes indicate the occurrence of a progressive metal-induced degradation process; in which, the initially established tannin-metal complex interactions were disrupted (reversal of semi-metal tanning interaction), the polyphenol structure undergone decomposition and eventually the collagen in the samples was subjected to denaturation.The degradation process originating from the tannin-Fe(II) and tannin-V(IV) interactions may also occur in the presence of a Cr(III) tanning structure, as observed with autodegrdation of Cr(III)-mimosa and Cr(III)-chestnut tanned leathers. The results showed that the observed cases of metal induced degradation are dependent on V(IV)-tannin or Fe(II)-tannin interactions.

On the other hand, treatment leather tanned with Cr(III) salt (not retanned) with V(IV) and Fe(II) did not result in degradative changes, inducating that In addition, Cr(III) tanning does not create chemical stabilisation against the V(IV) or Fe(II) induced degration process. It is also observed that V(IV) and V(V) induced degradation of vegetable tanned leather samples occurs in a catalytic manner, the rate of decline of T_s was found to be less dependent of metal concentration. The presence of a minimum threshold level of metal ion *i.e* < 0.1% w/w in the leather was sufficient to cause decline in T_s to that the untanned collagen (60°C) and further below.

Using EPR analysis of leather samples, it was confirmed that oligomeric Fe(III) species are formed during storage of the semi-Fe(II) samples due to oxidation. Similarly, evidence for the formation of V(IV) species in the semi-V(V) tanned leathers was obtained. The results suggested the possibility of creation of a redox cycling process in which the lower oxidation states of the metals i.e Fe(II) and V(IV) may be oxidised by the atmospheric oxygen, while the higher oxidation states *i.e.* Fe(III) and V(V) may be reduced by the *o*-diphenol moieties of polyphenols.

The evidence that the most stable semi-metal tanned sample (semi-Al(III) tanned leather) has shown the largest concentration of free-radicals suggests that the chemical process of metal-induced degradation of leather is not determined by the presence of organic radicals but rather by the presence of catalytic metal ions that also result in the foration of reactive oxygen radicals (i.e. superoxide anion and hydroxy radicals). The progressive decomposition of the tanning structure and the denaturation of collagen may be related to the formation of superoxide anion and other similar oxidative species.

The degradation of the iron and vanadium containing leathers occurred at ambient conditions of storage (21°C and 65% RH) due to internal redox reactions in the samples that also involved atmospheric oxygen. In this context, the changes are recognised as oxidative autodegradation of leathers, catalysed by the transition metals.

CHAPTER 4

INTERACTIONS OF POLYPHENOLS WITH IRON AND VANADIUM IONS IN SOLUTION

4.1. Introduction

As described in Chapter 1, hydrolysable tannins are polyphenolic molecules, while condensed tannins are polymeric flavonoids. In the structures of both types of tannins there are multiple *o*-diphenol moieties These functional groups are the most important with respect to the interaction of tannins with metal ions (Slabbert 1992). In the investigation of tannin-metal complexes, low molecular weight polyphenols (*e.g.* gallic acid, methyl gallate, catechol and (+)-catechin) have been used as models of the *o*-diphenolic moieties of tannins to analyse the features of complexation reactions and the stabilities of different types of metal-tannin complexes in aqueous solutions (Sykes *et al.* 1980; Madhan *et al.* 2006). The stoichiometry of complexation of polyphenols with metals is influenced by pH (Liu *et al.* 2011; Madhan *et al.* 2006; McDonald *et al.* 1996).

In addition to complex formation, the *o*-diphenol moieties in tannin structures may also be oxidised by metal ions through a one electron transfer process (Bark *et al.* 2011; Yasuda *et al.* 2012). The purpose of the experimental work described in this chapter was to explain the observed changes in the semi-metal tanned leathers (Chapter 2 & 3) in terms of the features of the stabilities of selected metal-polyphenol complexes in aqueous solution.

In the experiments, the changes brought about by oxidation of the polyphenol compounds were examined at different pH. The stability of the polyphenol compounds in the presence and absence of transition metal ions *i.e* Ti(IV), Fe(II) and Fe(III) was studied. In addition, free-radical scavenging assay was used to study the relative effect of the metal ions in oxidising the *o*-diphenol moieties of polyphenols.

4.2. Materials and methods

4.2.1. Chemicals

Analytical grade metal salts titanium(IV) sulfate [$Ti(SO_4)_2$ 15%w/v solution], vanadium(IV) sulfate [$VO(SO_4).5H_2O$], iron(II) sulfate heptahydrate [$Fe(SO_4).7H_2O$], anhydrous ammonium metavanadate [$NH_4(VO_3)$] and anhydrous iron(III) sulfate [$Fe_2(SO_4)_3$] were sourced from Fisher Scientific Ltd., UK. HPLC purified samples of (+)-catechin, methyl gallate and 2,2 diphynyl picryl-1-hydrazyl (DPPH) were purchased from Sigma-Aldrich Ltd, UK.

4.2.2. Analytical instruments and apparatus

UV-Vis spectrophotometer

A Shimadzu UV-2501PC double beam spectrophotometer (Shimadzu Co. Ltd., Japan) was used. Measurements were performed using quartz cuvettes (1.5 mL, path length 10 mm) in the range of 200-800nm, at 1 nm resolution and at scan rate of 600 nm/minute.

Aeration pump and nitrogen gas

A linear aeration pump (Dymax 2, Charles Austen Ltd. UK) having a fixed pump rate of 2 L/minute was used for aeration of sample solutions. Nitrogen gas with was also used for purging of sample solution at the flow rate of 250 cm^3/min.

Rotary evaporator

A Bucci rotary vacuum-evaporator (Büchi Labortechnik AG, Germany) was used to evaporate water from sample solutions under vacuum.

4.2.3. Analysis of aerated solutions of polyphenol at different pH

Acetate buffers (pH 4.5, 6.0), phosphate buffer (pH 7.5) and carbonate buffer (pH 9.2), each having concentration of 0.01M were prepared. The buffer solutions were used for preparation of 0.1 mM solutions of methyl gallate and (+)-catechin. The solutions were aerated for 10 minutes by bubbling air and kept for 6 hours at room temperature. Then UV-Vis absorbance spectra of the solutions scanned in the range of 200-800 nm using the UV-Vis spectrophotometer.

4.2.4. Analysis of metal containing polyphenol solutions

Metal containing solutions of (+)-catechin and methyl gallate were prepared in a deoxygenated acetate buffer solution (pH 5.0) as described in Section 4.2.4.1-2. A half portions of each of the solutions was aerated for 10 minutes every day for four days. The other half portion of each solution was kept oxygen free by purging with nitrogen gas. The progressive changes in absorption spectra of the sample solutions were monitored every 24 hours over a period of 4 days. The changes in the absorption spectra of aerated versus deoxygenated samples were compared.

4.2.4.1. (+)-Catechin and methyl gallate solutions

Acetate buffer solution (0.01 M, pH 5.0) was degassed under vacuum for 30 minutes to remove oxygen and then purged with nitrogen gas for 10 minutes. Solutions of polyphenol compounds (3 mM) were prepared by dissolving 110 mg methyl gallate and 176 mg (+)-catechin in 200 mL of acetate buffer (0.01 M, pH 5.0).

4.2.4.2. Metal containing (+)-catechin and methyl gallate solutions

Solutions of 0.1M of Ti(IV), Fe(II) and V(IV) were prepared by dissolving calculated quantities of the sulfate salts (Section 4.2.1) in acetate buffer (pH 5.0). To prepare the metal containing polyphenol solution, 50 µL of the metal solutions were added to separate samples tubes containing 50 mL of 3 mM solution of (+)-catechin and methyl gallate solutions. The mole ratio of metal ions to polyphenol was 1:30 (*i.e.* 0.1 mM of metal ions and 3 mM of polyphenol). Metal-free sample solutions of methyl gallate and (+)-catechin were used as controls. The effect of the presence a relatively small proportion of the different metal ions in the stability of the polyphenol compounds was studied by analysing the UV-Vis spectra of the solutions aerated and deoxygenated samples.

4.2.4.3. UV-Vis spectrophotometric analysis

The initial absorption spectra of the control solutions and the metal containing solutions of (+)-catechin and methyl gallate were scanned in the wavelength range range 200-800 nm with the spectrophotometer using the quartz cuvettes (1.5 mL, path length 10mm).

Then each of the control samples and metal containing solutions were divided into two equal portions and treated as follows

(i) deoxygenated samples : 25mL portions of each sample solution were purged with nitrogen in separate sample tubes and kept closed at room temperature,

(ii) aerated samples: 25 mL of the samples were oxygenated by bubbling air into the sample tubes for 10 minutes and kept at room temperature,

Spectral measurements were carried out every 24 hours over four days. Aeration and purging of the sample solutions with nitrogen gas were done each UV-Vis measurement and the changes in the spectral patterns were analysed.

4.2.5. Thin layer chromatographic analysis

After completion of the experiment described in Section 4.2.5.3, the water from the solutions was removed using a rotary vacuum-evaporator at 35°C. The dry residue of the samples were dissolved in methanol (4 mg/mL) and spotted on to an aluminium backed thin layer chromatogram plate (Florescent- silica gel, 254nm) and developed with chloroform: ethyl acetate: formic acid at the ratio of 5:4:1. The chromatograms were viewed in UV-chamber at 254nm and developed by spraying 2% Fe(III) chloride solution prepared in 50% methanol.

4.2.6. Free-radical scavenging assay

Polyphenol compounds found in various fruits and plant extracts from plants have been widely investigated to characterise their potential as free-radical scavengers or antioxidants (Dudonne *et al.* 2009; Villano *et al.* 2007; Jovanovic *et al.* 1994). Polyphenol compounds exhibit antioxidant activity by terminating free-radical reactions in biological system mainly through the hydrogen atom transfer (HAT) mechanism (Brand-Williams *et al.* 1995). In the HAT reactions, the *o*-diphenol groups are converted to semi-quinone radicals and quinone products as shown in Figure 4.1a.

The most common method for evaluating and comparing the antioxidant efficiency of polyphenol compounds is by a free-radical scavenging assay method in which the stable free radical 2,2 diphenyl picryl-1-hydrazyl radical (DPPH) is used (referred to as DPPH

assay). In this assay method, methanolic solution of DPPH, which has an intense purple colour with λ_{max} at 517nm. The colour of DPPH changes into yellow when the DPPH radical is completely converted to a non-radical product by hydrogen atom transfer (HAT) reaction with hydroxyls of polyphenols (Dudonne et al. 2009) as shown in Figure 4.1b. The reduction in absorbance at the 517 nm resulting mainly from HAT reactions is used to determine the quantity of the DPPH radical scavenged by the HAT reaction.

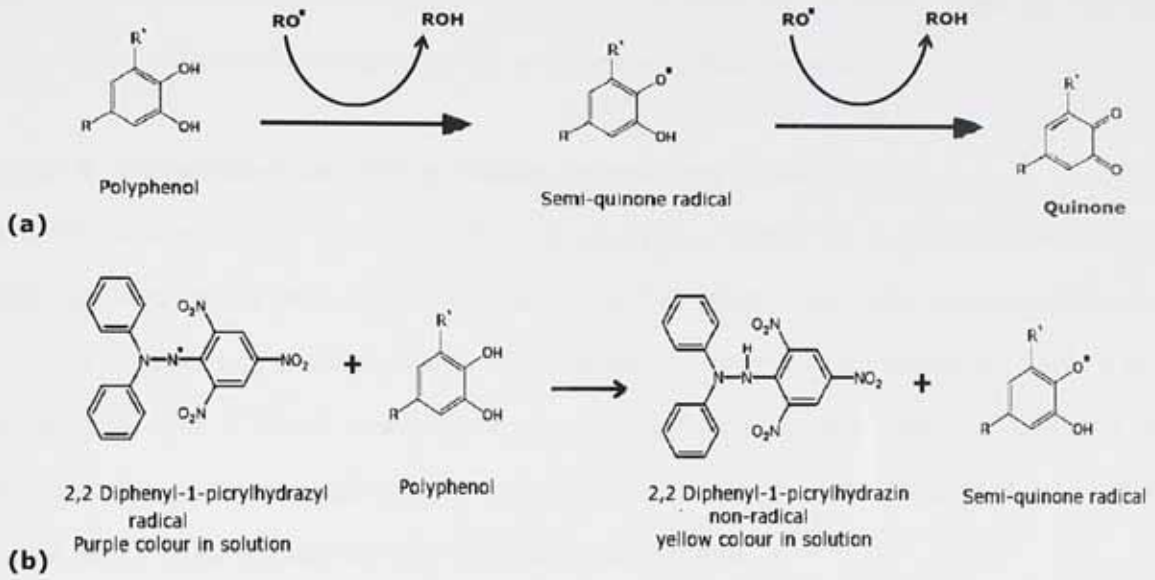


Figure 4.1: Hydrogen atom transfer reactions:(a) anti-oxidant reaction of polyphenols with biological free-radicals denoted as RO• (e.g. lipid peroxy radicals), and (b) radical scavenging reaction of polyphenols with DPPH radical (Ionita 2005).

In flavonoid compounds, the aromatic hydroxy group in the C-ring of the structure may also take part in scavenging DPPH radical (Osman 2011). Oxidation of polyphenols results in the conversion of the *o*-diphenol groups of the polyphenols to quinone groups. Unlike the *o*-diphenol groups, quinone groups do not react with DPPH due to the absence of a labile (transferable) hydrogen atom. Hence, oxidation of the *o*-diphenol moieties of polyphenols is reflected in the loss of free-radical scavenging capacity. In this experiment the extent of metal induced oxidation of methyl gallate is examined in terms of the change in the free-radical scavenging activities of a metal containing solutions. The procedures are described in Sections 4.2.7.1-2.

4.2.7.1. Metal containing solutions of methyl gallate

A solution of methyl gallate (140 mL, 1mM) was prepared in 50% methanolic acetate buffer (0.01 M, pH 5.0) solution and divided into 20 mL portions, 20 µL of the 0.1M solutions of Al(III), Ti(IV), Fe(II), Fe(III), V(IV) and V(V) were added into the first six solutions. A 20 mL portion was used as a control, *i.e.* no metal solution added. The mole ratio of methyl gallate to metal ion in the test samples was 10:1 in all cases. The sample solutions were kept in closed tubes for 24 hours at room temperature. Then the relative free-radical scavenging capacity of the solutions was determined.

4.2.7.2. Determination of free-radical scavenging capacity

A 1 mM solution of 2,2 diphynyl-picryl-1-hydrazyl was prepared in 50%(V/V) methanol that was adjusted to pH 5.0 using HCl (0.01 M). The solution was appropriately diluted in in 50% methanol to prepare five gradient solutions with concentrations of 0.04, 0.08, 0.12, 0.16 and 0.20mM. Measurements of absorbance of the gradient solutions at 517nm were performed using glass cuvetts (3.5 mL, 10 mm path length). A calibration curve with a linear correlation (R^2=0.9968) was obtained.

Determination of the free-radical scavenging activity of the methyl gallate solutions was carried out as follows. Initially, 3400 µL of freshly prepared solution DPPH (0.03 mM) was transferred in to the cuvettes, then 100 µL the methyl gallate solutions were added into each. A blank control sample was prepared by adding 100 µL of 50% methanolic acetate buffer (pH 5.0). The sample cuvettes were kept for 30 minutes and the concentration of DPPH remaining after incubation was determined spectrophotometrically using the calibration curve. In each case, triplets measurements of each sample were carried out. The free-radical scavenging activity of the solutions were calculated as follows.

$$\text{Free radical scavenging in \%} = \frac{C - C_M}{C} \times 100$$

Where:

- **C** is the concentration of the DPPH solution alone after 30 minutes of incubation,
- C_M is the concentration of DPPH after incubation with the methyl gallate solutions i.e. a control and metal containing samples.

4.3. Results and discussion

4.3.1. Autoxidation of (+)-catechin and methyl gallate

An aqueous solution of (+)-catechin in acidic media is colourless and shows a characteristic band of absorption in the UV-region with λ_{max} at 276 nm (Bark *et al.* 2011). In this experiment, the (+)-catechin solutions at pH 4.5 and pH 6.0 have produced the characteristic spectra (Figure 4.2a). However, the samples prepared at pH 7.5 and 9.2 showed an additional absorption with a broader wavelength range 370nm to 550 nm having peak absorbance at 430nm (shown in Figure 4.2b)

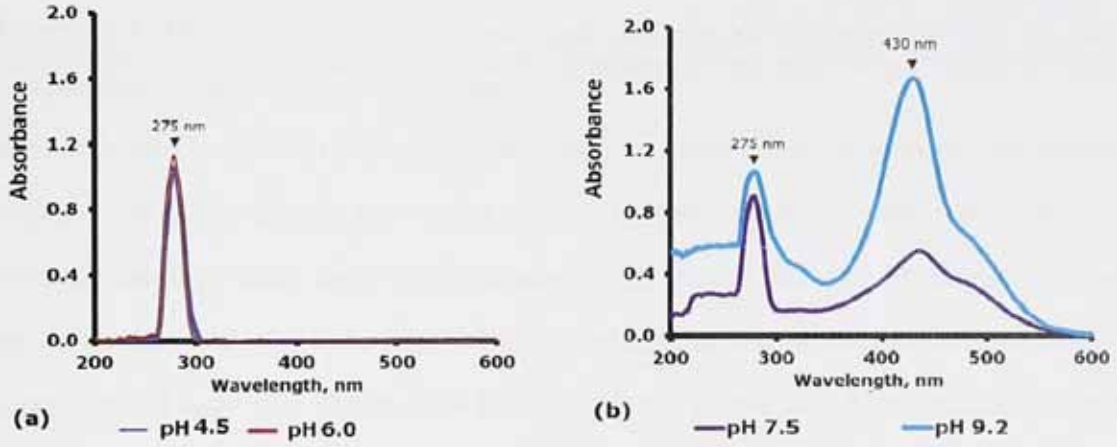


Figure 4.2 UV-Vis spectra of (+)-catechin solutions at (a) pH 4.5 and 6.0 in acetate buffer (b) at pH 7.5 in phosphate buffer and at pH 9.2 in carbonate buffer.

The UV-Vis spectra of (+)-catechin in alkaline media has been investigated by (Bark *et al.* 2011) and a similar pattern of changes were reported. According to Porter (1992), (+)-catechin and other flavonoid compounds undergo autoxidation in alkaline pH, resulting in the formation of a semi-quinone radical and the quinone methide intermediate as shown in Figure 4.3. The reaction between molecular oxygen and the o-diphenol groups is enhanced by ionisation of the hydroxyl groups *i.e.* formation of phenolate groups (Bark *et al.* 2011). Rearrangement of the quinone methide intermediate leads to formation of an epimerisation product, namely (-) epicatechin and another intermolecular rearrangement product (+) catechinic acid (Ferreira *et al.* 1992). The emergence of the new band of absorption of the (+)-catechin spectra in alkaline (Figure 4.2b) media may

be related to deprotonation of the *o*-diphenol and subsequent intermolecular rearrangement reactions as shown in Figure 4.3.

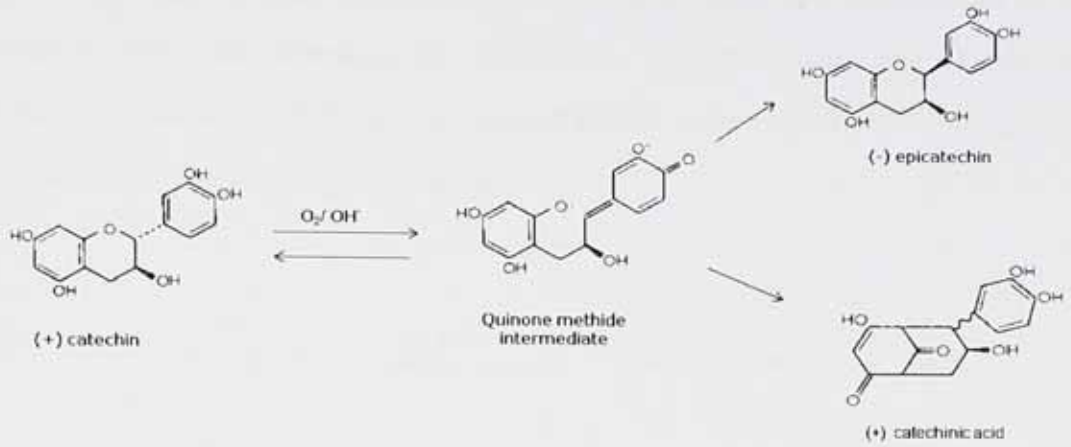


Figure 4.3. Epimerisation of (+)-catechin and formation of (+)-catechinic acid by oxidative rearrangement through a quinone methide intermediate formed by opening of the pyran ring at C-2 of the flavon-3-ol structure (Porter,1992)

Similar to the case of (+)-catechin, the methyl gallate solutions with pH 6.0 and below showed the characteristic absorption spectra of the compound with a peak at 271nm, with narrow absorption band in the between 300 nm and 250 nm (Figure 4.4a). However, at pH 7.5 and 9.2, the absorption bands of methyl gallate at 271 nm became broad; no other distinct peak was observed in both cases, but rather broad bands of absorbance in the region of 500nm and 300nm were obtained (Figure 4.4b).

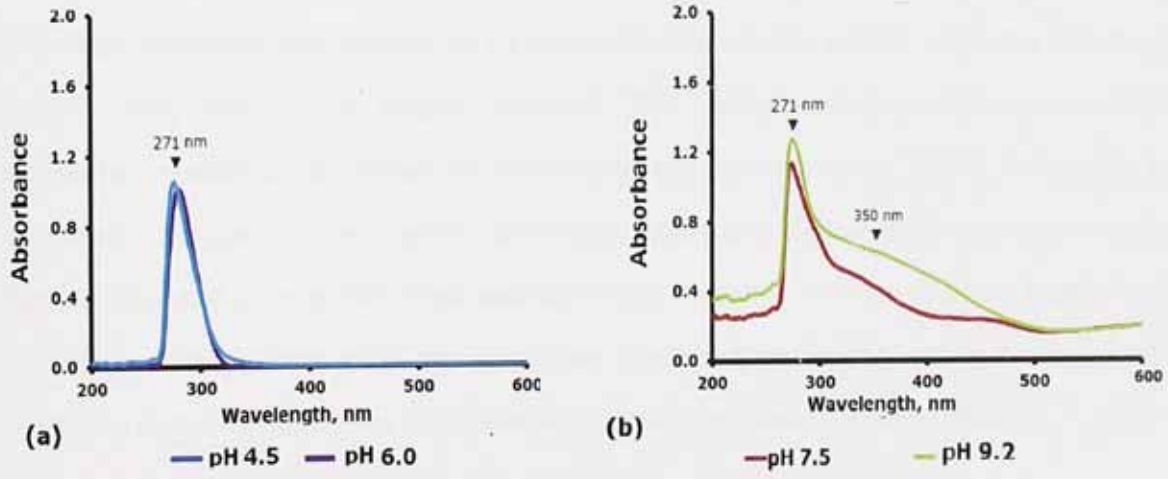


Figure 4.4 UV-Vis spectra of methyl gallate solutions at (a) pH 4.5 and 6.0 in acetate buffer (b) at pH 7.5 in phosphate buffer and pH 9.2 in carbonate buffer

These spectral changes are the result of alkali catalysed oxidative reactions and intermolecular coupling reactions. According to Thulayathan (1989), gallic acid (Figure

4.7a, structure 1) is oxidised to the respective quinone (Figure 4.5a, structure 2) in alkaline media. Then the quinone compound undergoes coupling reactions to form di-gallic acid structure (Figure 4.5b, structure 3). The di-gallic acid intermediate exists in equilibrium with the intermolecular-estrification product ellagic acid (Figure 4.5b, structure 4). However di-gallic acid is oxidised further to form di-quinone product (Figure 4.5b structure 5) and hydrogen peroxide.

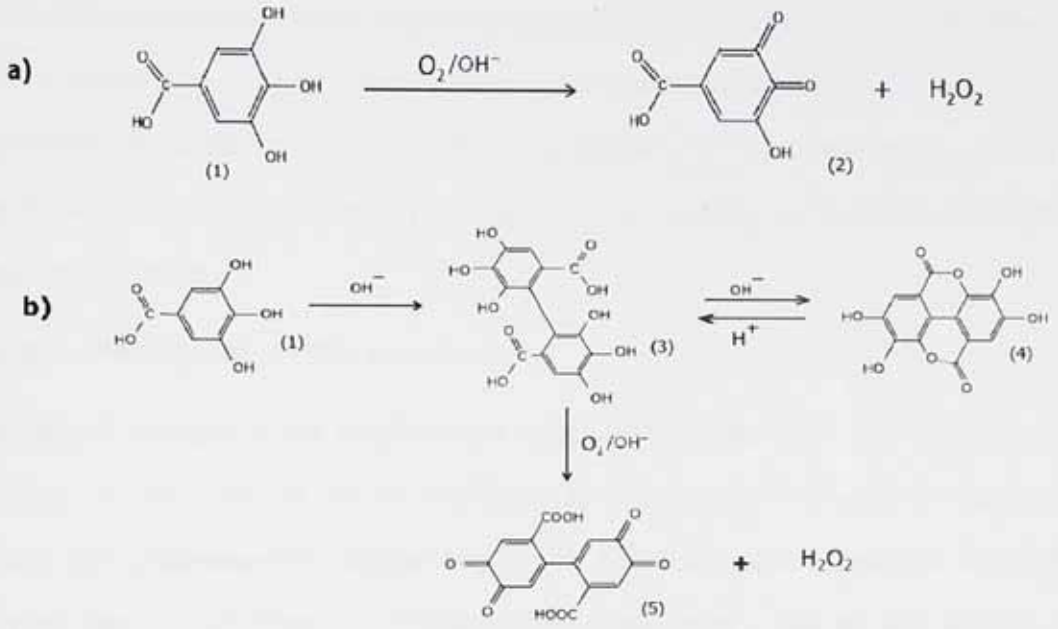


Figure 4.5 Reaction of autoxidation and dimerisation of gallic acid in aerobic environment in alkaline media showing autoxidation of (a) gallic acid and (b) di-gallic acid (Thulayathan 1989)

The alkali catalysed autoxidation of (+)-catechin and methyl gallate indicates that large tannins, may also exhibit similar reactions. The products of autoxidation are larger structures created by intermolecular rearrangement (Ferriera *et al.* 1992). In addition to structural changes in the organic compound, the autoxidation processes involve the formation of the superoxide anion and hydrogen peroxide. Mochizuki *et al.* (2002) and Tulyathan *et al.* (1989) have demonstrated the quantitative conversion of oxygen to hydrogen peroxide during the autoxidation of condensed tannin compounds.

4.3.2. Effect of metals on the stability polyphenols in acidic media.

In order to study the effect of oxygen, aerated and deoxygenated (nitrogen purged) sample solutions were used in the experiment, the spectra of the methyl gallate and (+)-

catechin solutions, in the presence and absence of metal ions, were monitored over 4 days. The results are described in sections 4.3.2.1-3

4.3.2.1. Polyphenol-titanium(IV) solution

The absorption spectra of the methyl gallate and (+)-catechin solutions (0.1 mM) containing with Ti(IV) in the ratio 30:1 (polyphenol: metal) showed no change in UV-Vis spectra over the five days regardless of the presence or absence of aeration. Ti(IV) is stable to autoxidation in acidic media (Lee 1998) and reduction of Ti(IV) can be achieved only in highly acidic media (pH<1) using electrochemical methods; the resulting Ti(III) spontaneously oxidises back to Ti(IV) in the presence of air (Kiekens *et al.* 1981). The results shows that the interaction of Ti(IV) with the polyphenol compounds is limited to complex formation.

4.3.2.2. Polyphenol-V(IV) solution

Progressive changes in the spectra were observed over the four days of aeration of the samples. In the case of the (+)-catechin solution (Figure 4.6a), the absorbance at 275nm was progressively reduced and a new absorption band emerged with the peak centred around 370-380nm. Whereas, the absorbance peak of the methyl gallate solution at 271nm declined and showed a hypsochromic shift to 240nm (Figure 4.6b).

When V(IV) was added to the methyl gallate and (+)-catechin solutions, the colourless solution turned to a blue colour, which indicated the formations of V(IV) complex with the *o*-diphenol groups. However, the blue colour of the V(IV) containing samples turned to green in four days. The change in colour of the solution could be due to changes in the polyphenol structure and formation of new vanadium complexes as a result of oxidation. At the same time, the samples that were purged with nitrogen gas and showed no change of colour and the initial spectra also remained unchanged over the four days. The results indicate that the V(IV) may cause oxidation of the polyphenols in aerobic conditions only.

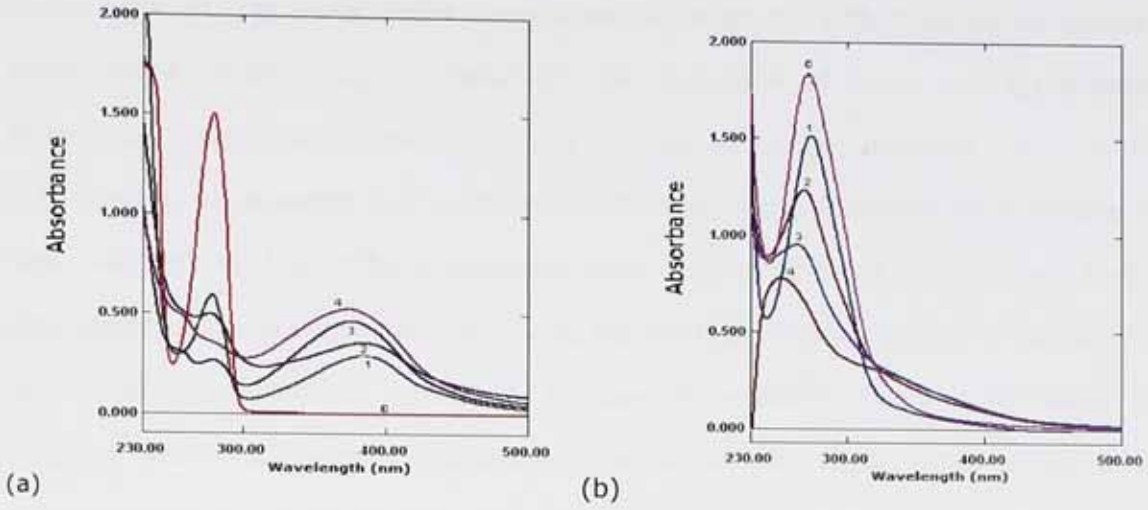


Figure 4.6 Spectra of polyphenol solutions containing V(IV) in the ratio 30:1 scanned over 4 days of aeration of (a) (+)-catechin solution (b) methyl gallate solution

Both methyl gallate and (+)-catechin are relatively stable to oxidation in acidic media with respect to autoxidation (Bark *et al.* 2011; Friedmann *et al.* 2006; Zhu *et al.* 1997). Hence the changes in the spectra (Figure 4.6), that indicate change in the composition of the solution, are evidently the results of the oxidation of V(IV) and subsequent reactions with the polyphenols. Vanadium compounds occur in four different oxidation states (II, III, IV and V, but the most common oxidation states are IV and V. In aqueous solution, the two oxidation states exist as oxovanadium [VO^{2+}] and dioxo-vanadium [VO_2^{1+}] in acidic media (pH <4). With increase in pH the dioxovanadium(IV) ions hydrolyse to form metavanadate (VO_3^-), orthovanadate (VO_4^{3-}) and other oligomeric oxy-anions such as decavanadate [$V_{10}O_{28}^{6-}$] (Lee 1998; Sharpe 1986).

Aqueous V(IV) is known to oxidises in the presence of air to V(V) species (Greenwood *et al.* 1984), the resulting V(V) species exhibit the property of a strong oxidising agent, particularly in presence of reducing agents like SO_2 as well as polyphenols (Sharpe 1986; Ramasarma 2003; Lee 1998). The redox interaction of V(V) with the catechol derivatives, which results in the formation of V(IV) and quinone, is known to be spontaneous because of the electron rich *o*-diphenol functional groups (Ferguson *et al.* 1979; Racheva *et al.* 2008).

Kustin *et al.* (1974) investigated the reaction of vanadates with catechol derivatives in acidic media under anaerobic condition, the formation of V(IV) was quantitatively determined spectrophotometric analysis of ion-exchange fractions at 750nm . Furthermore, by scanning in the region of 300-500nm the formation of *o*-quinone was confirmed with its characteristic absorption peak at 380nm; it was also observed that the absorbance of the quinone decayed during the reaction, indicating that formation of the quinone structure could be a precursor for coupling reactions. This may be related to a coupling reaction between unoxidised polyphenols and quinones, which is also is also observed when (+)-catechin and similar compounds are enzymaticaly oxidised in acidic media (Bailey *et al.* 1993; Matsuo *et al.* 2008)

The observed changes in the spectra (Figure 4.6) of the aerated solution may be related to oxidation of the V(IV) to V(V) and subsequent oxidation of the *o*-diphenol ligands by V(V), leading to the formation of quinone products and V(IV). The emergence of a new absorption band centred at 380nm may also be related to a similar formation of quinone species in the solution as a result of oxidation of the *o*-diphenols of the B-ring of (+)-catechin. The changes in the spectra in the (+)-catechin and methyl gallate solution may is related to oxidation of the metal complex due to aeration. Based on the literature on V(IV)-polyphenol interaction and the results in this experiment, it is proposed that the reactions described in Figure 4.7 may occur in the process of V(IV) catalysed oxidative autoxidation of polyphenols.

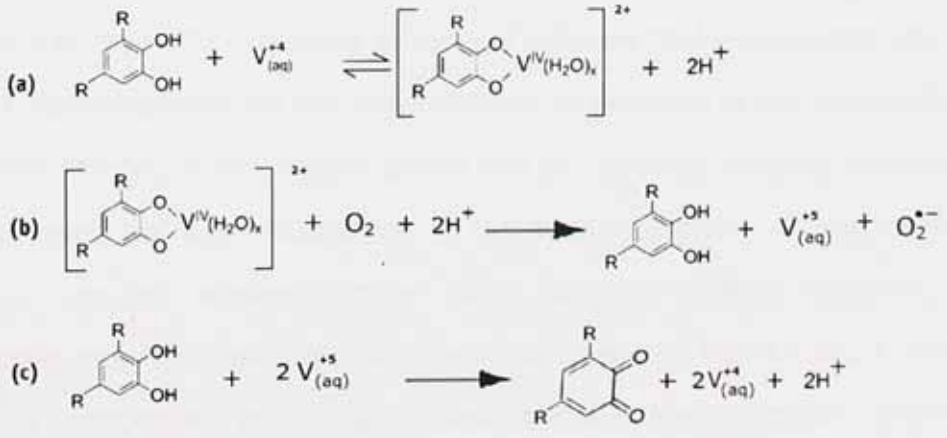


Figure 4.7 Interactions of V(IV) with *o*-diphenol groups of polyphenols in acidic media under aerobic conditions.

The reactions shown in Figure 4.8 involve:-

- Complex formation reaction between the V(IV) and the *o*-diphenol groups of polyphenols in acidic media, shown in Figure 4.7a (Yasarawan *et al.* 2013; Kustin *et al.* 1974; Racheva *et al.* 2002).
- Oxidation of the V(IV) complex by oxygen, resulting in the breaking of the coordinate covalent bonds of the complex and the formation of the superoxide radical, shown in Figure 4.7b (Valko *et al.* 2006; Kustin *et al.* 1974; Ferguson *et al.* 1979).
- Oxidation of the polyphenol by V(V) resulting in the formation of a quinone compound and V(IV), shown in Figure 4.7c (Nakajoma *et al.* 2002, Kustin *et al.* 1974; Ferguson *et al.* 1979; Butler *et al.* 1992).

Based on the above suggested mechanism, the concentration of the V(V) ions may be kept replenished by oxidation of V(IV) while the concentration of the V(IV) is also maintained as the V(V) is reduced by the *o*-diphenol groups. The mechanism is in effect a cyclic redox process (i.e. with respect to V(IV)/V(V) changes). At the same time, the oxidative effect of V(V) results in the depletion of the concentrations of methyl gallate and (+)-catechin as reflected with progressive changes in the spectra of the aerated solutions.

4.3.2.3. Polyphenol-Fe(II) solution

The spectra of the Fe(II) containing polyphenol solutions that were purged with nitrogen remained unchanged over the four days, showing no reduction in the peak absorbance at 271nm and 276 nm for the methyl gallate and (+)-catechin samples, respectively. On the other hand, the peak absorbance of the aerated samples of both of the Fe(II) containing samples showed decline after aeration,indicating lowering of the concentration of polyphenols. With the (+)-catechin sample (Figure 4.8a), a new band of absorbance emerged with peak at 400nm and its absorbance successively increased over the four days.

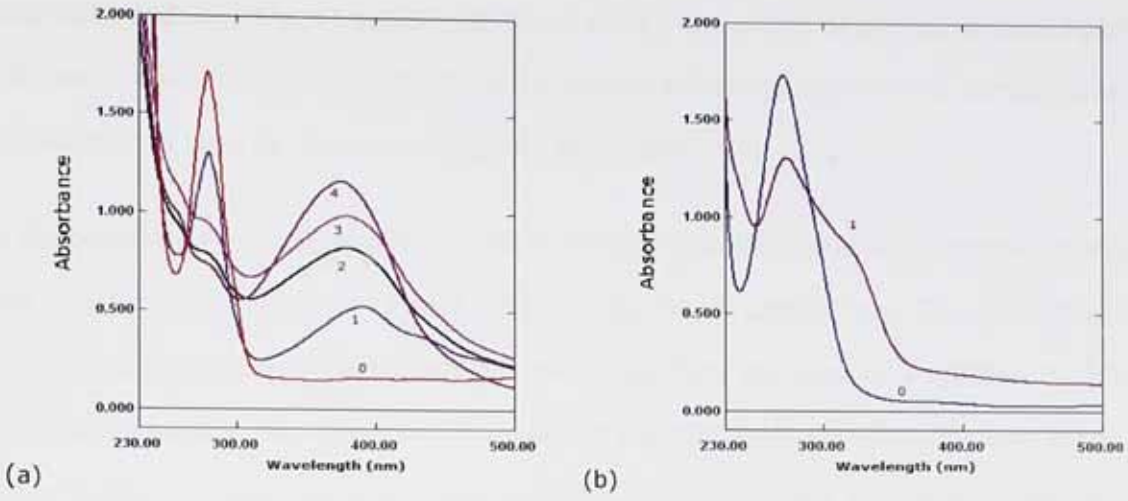


Figure 4.8 Overlaid spectra of polyphenol solutions containing Fe(II) in the ratio 30:1 scanned over 4 days of aeration **(a)** (+)-catechin solution **(b)** methyl gallate solution

In the case of the Fe(II) containing methyl gallate solution (Figure 4.8b) , after the first day of aeration, the spectra of the sample showed a decrease in absorbance at 271 nm and formation of a shoulder peak between 360 nm and 270 nm. Further analysis of the Fe(II) containing methyl gallate solution was not possible due to the formation of a precipitate in the sample, that may be related to the The precipitate in the Fe(II) containing methyl gallate solution may be related to the formation of water insoluble Fe(III) complexes. On the other hand, the oxidation of the *o*-diphenol groups of methyl gallate might have resulted in oxidative coupling reactions, similar to the case of autoxidation of gallic acid described by Thulayathan (1989) and Nikolic *et al. (*2011).

The observed changes in the spectra of the Fe(II) containing samples may be because of oxidation of Fe(II) to Fe(III) and the subsequent redox reactions of Fe(III) with the *o*-diphenol groups of the compounds. Similar to V(IV), aqueous Fe(II) also oxidises in the aerobic conditions, it is known that autoxidation of Fe(II) in solution is influenced by the pH which determines the proportion of the different species of the metal, namely Fe^{2+}, $Fe(OH)^{1+}$ and $Fe(OH)_2$ (Morgan *et al.* 2007). Hydrolysed ferrous species are more readily oxidised than the hydrolysed complexes in the increasing order of Fe^{2+}< $Fe(OH)^{1+}$< $Fe(OH)_2^{o}$ (Stumm *et al.* 1996; Santana-Casiano 2006). According to Morgan *et al.* (2007), the presence of anions in the aqueous solution may slow down the rate of oxidation of Fe(II) because of complex formation. Since Fe(II) does not form stable

complexes with *o*-diphenol groups (Slabbert 1992), the effect of the polyphenol ligands in slowing down the autoxidation of Fe(II) is likely to be less effective as compared with anionic ligands such as chloride, carbonate and sulfate

As described by Perron *et al.* (2010), both complex formation and redox transformations may occur concurrently when Fe(II) binds to polyphenol compounds. The initial reaction may be the formation of Fe(II) complex of *o*-diphenolic compounds as shown in Figure 4.9a. Subsequently, an intermolecular electron transfer from Fe(II) to the oxy-ligand of the *o*-diphenol group may occur (Figure 4.9b), resulting in the formation of an Fe(III) complex with a free-radical bearing *o*-diphenolic ligand. At the same time, the *o*-diphenolic complex of Fe(II) complex may also be oxidised to produce the corresponding complex of Fe(III) and superoxide anion (Figure 4.9c).

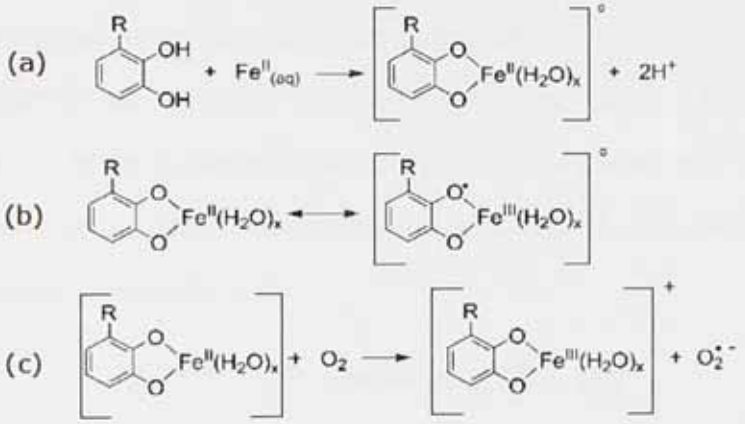


Figure 4.9 Interaction of polyphenols with Fe(II) in under aerobic condition in acidic media (Perron *et al.* 2010)

Aqueous Fe(II) oxidises relatively slowly in aerobic condition, Fe(II)-polyphenols complex formation may speed up the rate of oxidation of the metal, mainly because the oxidation product (Fe(III)-polyphenol complex) is more stable than the Fe(II)-polyphenol complex (Kawabata *et al.* 1996, Perron *et al.* 2010). On the other hand, aqueous Fe(III) oxidises the *o*-diphenol groups of polyphenols into quinone structures. Bark *et al.* (2012) demonstrated that deaerated methanolic solution of (+)-catechin is oxidised by Fe(III) in acidic media. A study by Hynes and Ó'Coinceanainn (2001) and Hider *et al.* (2001) have shown that Fe(III) forms 1:1 complexes with (+)-catechin and gallic acid derivatives in acidic media (Figure 4.10a). However, the concentration of the complexes in all cases

decays due to an internal redox reactions (within the complex) that subsequently leads to dissociation in to Fe(II) and phenolate ion (Figure 4.10b).

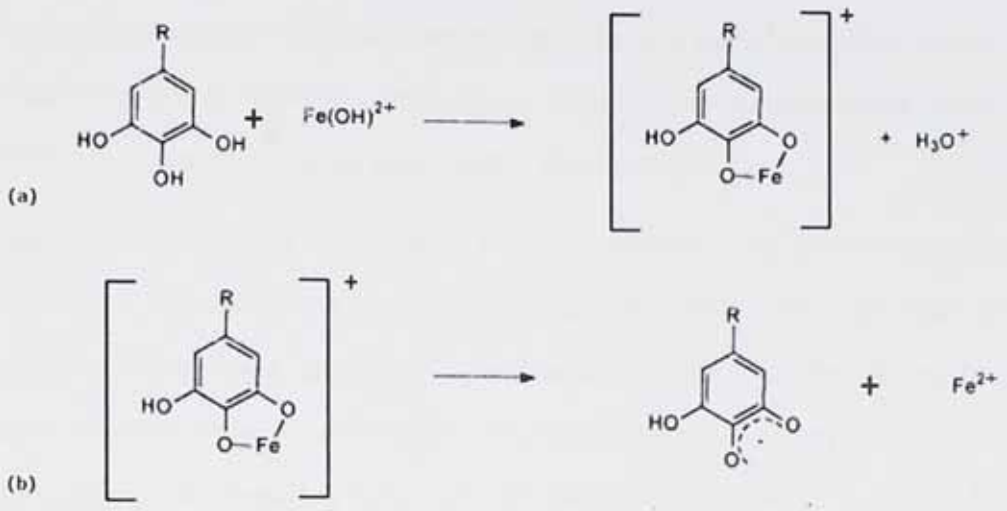


Figure 4.10 Reduction of Fe(III) by o-diphenolic groups in acid media (Hynes and Ó'Coinceanainn 2001)

4.3.3. Metal catalysed autoxidation of polyphenols

An important aspect of the reactions described in Sections 4.3.2.2-3 (Figure 4.7 & Figure 4.9) is the formation of the superoxide radical ($O_2^{\bullet}$). In the presence of water superoxide anions are protonated to form hydroperoxyl radical ($HO_2^{\bullet}$), the two species exist in equilibrium as shown in Equation 4.1.

$$O_2^{\bullet -} + H_2O \rightleftharpoons HO_2^{\bullet} + OH^-$$

Equation 4.1. The equilibrium of superoxideanion and hydroperoxylradical in aqueous media (Stumm *et al.* 1996)

The superoxide anion and hydroperoxyl radical are oxidants; in the presence of polyphenols, they these radicals form hydrogen peroxide by abstracting hydrogen from the hydroxy groups of polyphenols. In the presence of metal ions such as Fe(III), Fe(II), V(IV) and Cu(II), hydrogen peroxide is decomposed to form hydroxyl radical ($HO^{\bullet}$) through the Fenton reaction (Valko *et al.* 2006; Barbusiński *et al.* (2009). Therefore, the role of the redox-active metal ions in the oxidative transformation of the compounds may occur in reactions that may involve:-

- oxidation of the *o*-diphenol groups by the most oxidised form of the transition metals ions *i.e.* Fe(III),V(V)
- formation of oxygen derived free radicals, as the lower oxidation states of the metal ions are oxidised by atmospheric oxygen, the reaction being enhanced by oxidation of polyphenols (as described in Section 4.3.2.3).

In biological systems, metal ions such as Fe^{2+}, Cu^{+} and VO^{2+} act as pro-oxidants due to the their role in catalysing the formation of hydroxyl radicals from hydrogen peroxide (Carmichael 1989, Halliwell *et al.* 1990). On the other hand, polyphenols are commonly are known for their anti-oxidant roles as they inhibit formation of hydroxy radicals from hydrogen peroxide by chelating metal ions. In addition, polyphenols involve in hydrogen atom transfer (HAT) that converts other organic radicals (*e.g.* lipid-peroxy radicals) into non-radical species (Apak *et al.* 2013, Niki *et al.* 2005). However, Nakayama *et al.* (2002) suggested that polyphenols may also create a pro-oxidant mechanism, particularly in the presence of $Fe^{3+}$and Cu^{2+}. This is related to the formation of hydrogen peroxide from the superoxide anion generated by metal catalysed autoxidation of polyphenols, as described in Sections 4.3.2.2-3. Despite the initial formation of metal-polyphenol complexes; in aerobic conditions, the effect of the redox active transition metal ions, such as Fe(II) and V(IV), is to act as catalyst in speeding up autoxidation of polyphenols in acidic media (Haslam 1998; Bark *et al.* 2012; Kustin *et al.*1974) .

4.3.4. Thin layer chromatography of metal containing polyphenol solutions

The TLC of the metal containing solutions of methyl gallate and (+)-catechin shows that the deoxygenated samples remained unaffected by the presence of metals. In addition there was no difference in the TLC profiles of the samples as a result of storage at room temperature in nitrogen-purged sample tubes (shown Figure 4.11a). In the chromatogram of the deoxygenated samples (Figure 4.11 a&b), the coloured complexes of the metal-polyphenol complex, did not migrate upwards in the solvent system chloroform: ethyl-acetate:formic acid (50:40:10). The retardation factor (R_f) values and description of the spots in the chromatogram are given in Table 4.1. In all of the

nitrogen purged samples, the methyl gallate spots appear at the R_f value of 0.78 as marked by the reference sample spotted alongside the metal containing samples.

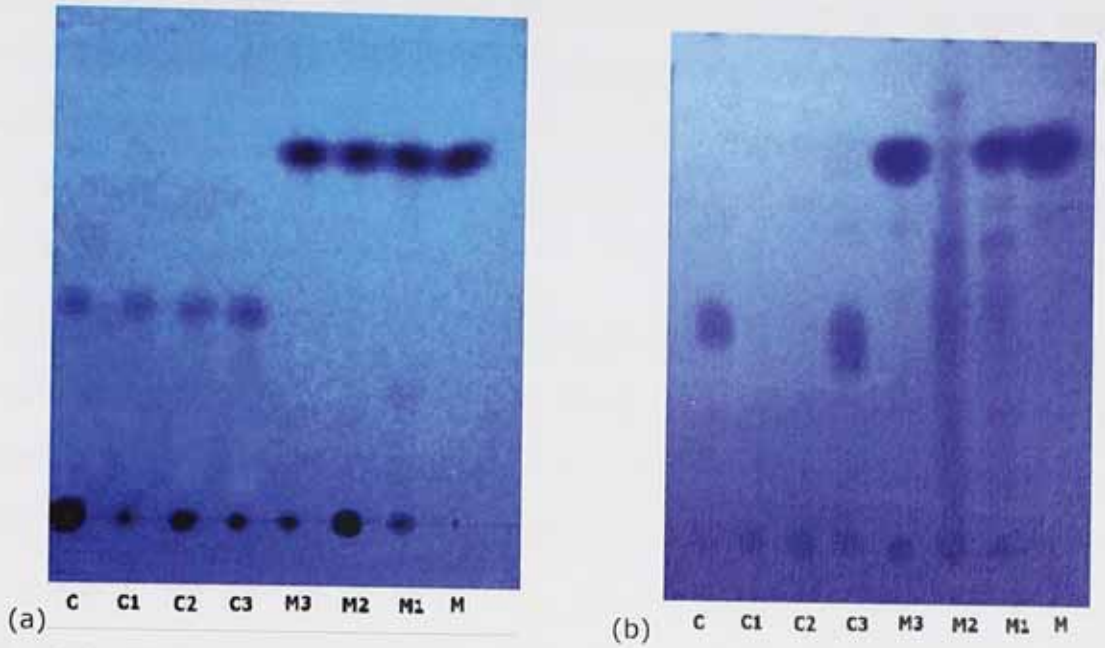


Figure 4.11 Thin layer chromatograms of V(IV), Fe(II) and Ti(IV) containing (+)-catechin and methyl gallate solutions (a) samples kept in deoxygenated condition (b) aerated samples. Details of the TLC are described in Table 4.1

Table 4.1 Description of the thin layer chromatogram of V(IV), Fe(II) and Ti(IV) containing solutions of (+)-catechin and methyl gallate (aerated samples)

Label/Sample	R_f values	Description
C -(+)-catechin	0.44	(+)-catechin s, reference sample
C1-(+)-catechin + V(IV)	0	(+)-catechin- V(IV) complex (green coloured) and polar oxidation products, No (+)-catechin spot
C2-(+)-catechin + Fe(II)	0	(+)-catechin-Fe(II) complex (blue coloured,) and polar oxidation products, No (+)-catechin spot
C3-(+)-catechin+ Ti(IV)	0	(+)-catechin-Fe(II) complex (orange coloured)
	0.44	(+)-catechin
M-methyl gallate	0.78	methyl gallate, reference spot
M1-methyl gallate +V(IV)	0	methyl gallate-V(IV) complex (green coloured)
	<0.78	methyl gallate spot
	0.78	Tailing spots (oxidation products)
M2-methyl gallate+ Fe(II)	0	Blue, methyl gallate-Fe(II)
	0.78	methyl gallate
	0.78-0.85	Oxidation product
	<0.78	Tailing spots (oxidation products)
M3-methyl gallate +Ti(IV)	0	Methy gallate - Ti(IV) complex (orange coloured)
	0.78	methyl gallate

Similarly the (+)-catechin spots showed R_f value of 0.44. No other extra spots are detected, hence the sample solutions are pure and their composition is unaltered in the anaerobic environment. The chromatogram of the aerated sample solutions (Figure 4.11b) shows that the (+)-catechin spots containing Fe(II) and V(IV) have completely disappeared, the spots of both of the aerated metal containing (+)-catechin solutions were immobile, which indicates the products of the metal catalysed reactions might be have lower affinity for the mobile phase.

According to Azam *et al.* (2004), TLC analysis oxidised (+) catechin developed using solvent mixture of toluene:ethyl acetate (1:8), also showed immobile spots. The immobility of the oxidation products may be related to formation of polymeric structures. Bailey *et al.* (1993) showed that oxidation of (+)catechin and other flavan-3-ol compounds in the presence of metal salts such as potassium hexacyanoferrate(III) in acid media results in the formation of high molecular weight coloured compounds that are insoluble in water. Dimerisation of (+)-catechin and similar polyphenols occurs with initial oxidation of the o-diphenol groups of the B-ring, followed by coupling reaction between the quinone groups of an oxidised polyphenol and the B-ring of the initial flavan-3-ol molecule (Guyot *et al.* 1996).

The chromatogram of the metal containing methyl gallate samples show the presence of the original compound in the aerated samples, however additional spots of oxidation products also emerged. The Fe(II) containing sample showed overlapping or tailing spots with most them having lower R_f than that of methyl gallate. The V(IV) containing methyl gallate spot showed similar appearance of the spots of oxidation products with R_f values than that of methyl gallate. The tri-hydroxy groups of gallic acid and gallates are prone to oxidation with the formation of quinone and semi-quinone (Tulyathan *et al.* 1989), the oxidations of the *o*-diphenol groups of methyl gallate may be similar to that of gallic acid, except for difference that from the presence of the methylation, that prevents reversible intra-molecular ester link formation in di-gallic to form ellagic acid. In acidicmedia, the oxidation of the trihydroxy groups of the gallic acid derivative (methyl

gallate) may take place through direct oxidation of the chelating *o*-diphenols by the metal ions *i.e.* Fe(III) and V(V). In addition, the oxygen derived free-radicals ($O_2^{\bullet}$, $HO^{\bullet}$) and hydrogen peroxide (H_2O_2), generated as a result of autoxidation of the metal ions in acidic media, create a more oxidative environment.

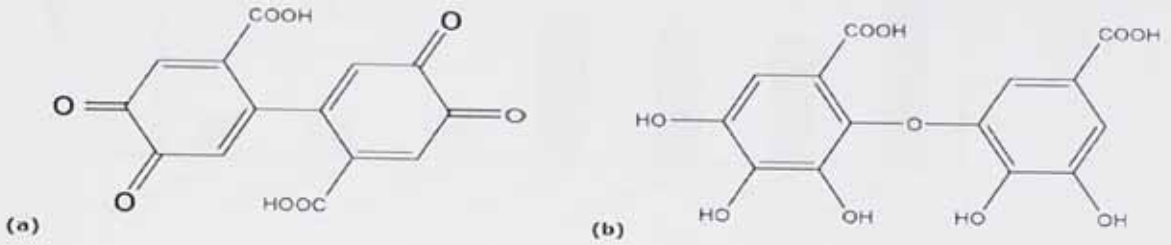


Figure 4.12. Proposed structures of oxidation products of gallic acid (a) C-C dimer oxidation product (b) C-O dimer oxidation product (Nikolić *et al.* 2011)

Using thin layer chromatography, Tulayathan *et al.* (1989) has demonstrated that the oxidation products derived of gallic acid and the ellagic acid (product of gallic acid coupling) are similar and it has been suggested that further oxidation of quinoid oxidation products may lead to molecular rearrangements. In addition to the C-C dimer products (Section 4.3.1). Nikolic *et al.* (2011) has also described that C-O dimeric oxidation products (Figure 4.12) may also be formed. Hence, the multiple spots observed in the chromatogram of the Fe(II) and V(IV) containing solutions of methyl gallate could be the different forms of the oxidation products of the tri-hydroxyl units.

4.3.5. Determination of free-radical scavenging activity

As shown in Figure 4.13, the methyl gallate solution caused the greatest reduction in DPPH concentration, scavenging 95% of the free-radical. During the 30 minutes of incubation, the initial DPPH concentration (0.3 mM) was consumed by hydrogen atom transfer reaction and reduced to 0.067 mM. The pyrogallol groups of polyphenols such as gallocatechins have greater reactivty with respect to free-redical scavenging reactions as compared to compounds having only di-hydroxyl units (Rice-Evans *et al.* 1997; Villano *et al.* 2007).

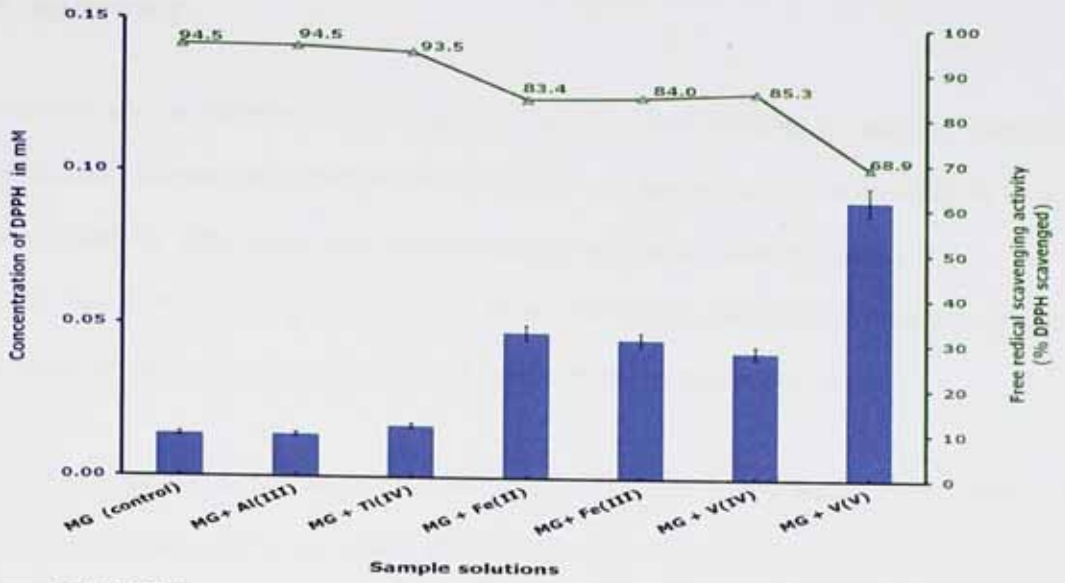


Figure 4.13 DPPH concentration after 30 minutes of incubation (primary axis) and the relataive free-radical scavenging activity (secondary axis) methyl gallate solutions; control samples and metal containing solutions.

The presence of metals in the methyl gallate solutions, at the mole ratio 1:10 (metal: methyl gallate) resulted in significant changes in the free-radical scavenging action of the polyphenol ($P<0.05$ one-way ANOVA, Appendix 9). The Al(III) and Ti(IV) containing methyl gallate solutions also showed nearly the same free-radical scavenging activity as that of the methyl gallate solution. This indicates that the presence of the metal ions and the reversible formation complex with the trihydroxy group of methyl gallate does nct influence the hydrogen atom transfer reaction of the compound with the DPPH radical. Whereas, the Fe(II), Fe(III) and V(IV) containing solutions showed free-radical scanvenging activity that are relatively lower by 8-11%, compared to the corresponding value of the methyl gallate reference solution and the Al(III) or Ti(IV) containing methyl gallatesolutions.

The free-radical scavenging activity of the V(V) containing methyl gallate solution (69%) was found to be the lowest, nearly one third of the DPPH radical remained unreacted. The main reason for the decline in the DPPH radical scavenging activity of the Fe(II), Fe(III), V(IV) and V(V) is related to the occurrence of redox interaction between the metal ions and the polyphenol as described in the earlier sections. The conversion of the o-dihydroxy groups into quinone groups lowers the possibility of formation of hydrogen atom transfer reaction with the DPPH radical (Brand-Williams *et al.* 1995).

4.4. Summary

Evaluation of the stability of (+)-catechin and methyl gallate, in aerobic conditions at different pH, showed that *o*-diphenol moieties are susceptible to autoxidation in alkaline media; however they may have relative stability in acidic media below pH 6.0. The metal ions of Fe(II), Fe(III), V(IV) and V(V) form complexes with the *o*-diphenol groups and also undergo redox interactions in acid media in the presence of oxygen.

Autoxidation of the polyphenols in acidic media may be catalysed by the transition metal ions. The catalysis of the oxidation of (+)-catechin and methyl gallate by Fe(II) and V(IV) ions may involve the *in situ* oxidation of the metal ions in the metal-polyphenol complex as well as reduction of Fe(III) and V(V) species by *o*-diphenol groups. Vanadium(V) appears to have relatively greater effect in oxidising the polyphenols as compared to that of Fe(III), as observed with the greater decline of the free-radical scavenging activity of the V(V) containing methyl gallate solution. In addition, oxidation of Fe(II) and V(IV) in aqueous media results in the formation of superoxide radical ($O_2^{\bullet}$) and other oxygen derived radicals. The presence of oxygen radicals speeds up the oxidation of the *o*-diphenol groups and leads to the formation of hydrogen peroxide.

The oxidation of tannins as well as low molecular weight polyphenols in aerobic conditions are catalysed by redox reactions involving redox cycling of Fe^{2+}/Fe^{3+} or V^{4+}/V^{5+}driven by autoxidation of lower oxidation state metal ions and reduction of the higher oxidation states by the *o*-diphenol groups of polyphenols. These redox reactions are also likely to occur with the tannin-V(IV) and tannin-Fe(II) interaction in semi-metal tanned leathers and subsequently result in the autodegradation of the leathers at ambient conditions (described in Chapter 3).

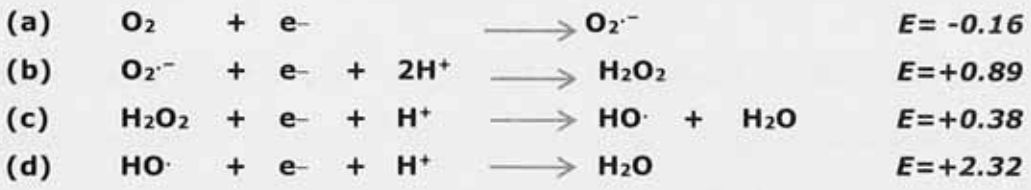
CHAPTER 5

EFFECT OF HYDROXY RADICALS ON THE STABILITY OF LEATHER

5.1. Introduction

The effect of Fe(II) and V(IV) in triggering and catalysing oxidative degradation in the semi-metal tanned samples (described in Chapter 2 and 3) were explained in terms of the redox reactions between the metal ions and tannins (Chapter 4). Results of experiments indicated that the interactions responsible for the Fe(II) and V(IV) catalysed oxidation of polyphenols may involve *in-situ* oxidation of the metals in the tannin complexes of Fe(II) and V(IV) by molecular oxygen (O_2) and the reduction of Fe(III) and V(V) species by the *o*-diphenol moieties of tannins.

The involvement of molecular oxygen in the redox interactions results in the formation of the superoxide anion that subsequently forms hydrogen peroxide (H_2O_2) as shown in Equation 5.1a-b. Hydrogen peroxide is relatively a weak oxidant as can be observed from its reduction potential. However, it can be reduced by certain metal ions and generate hydroxyl radicals (Equation 5.1c). Hydroxyl radicals are powerful electrophilic agents (oxidants) as reflected by their greater reduction potential (Equation 5.1). In contrast, the reduction of molecular oxygen is slow (i.e. negative value of standard potential)



Equation 5.1 Successive steps of one electron reduction of oxygen (O_2) in aqueous media in the presence metal ions or molecules with labile electrons (Stumm and Morgan 1996) and the corresponding standard reduction potential (Wood 1998).

In the formation of oxygen derived free radicals and hydrogen peroxide through the above mentioned redox reactions, the formation of superoxide anion (Equation 5.1a) is the slowest reaction and hence a rate limiting step (Perron *et al.* 2010). The superoxide

anion and hydroxyl radical are stronger oxidants than molecular oxygen and more reactive in terms of reacting with organic molecules (Kashima 1999; Mochizuki *et. al* 2002; Wood 1998).

H_2O_2 can be spontaneously reduced to hydroxyl radical and hydroxide anion by metal ions such as Fe(II), Fe(III), V(IV), Cu(II) in acidic media. The reaction of metal catalysed decomposition of H_2O_2 to form hydroxyl radicals is known as the Fenton reaction (Barbusinski *et al.* 2009).

5.1.1. The chemistry of Fenton reaction

The Fenton reaction generates the powerful oxidative free radicals (hydroxyl radicals) in acidic media. It was discovered more than a century ago by a British chemist Henry J. H. Fenton, who observed the complete oxidation of tartaric acid by H_2O_2 in the presence of Fe(II) ions (Kehrer 2000).

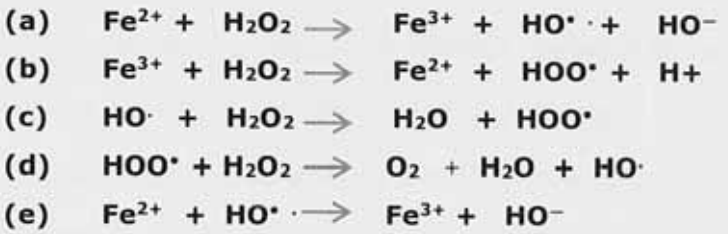
The Fenton reaction has also been applied for oxidation organic compounds (Barbusinski *et al.* 2001) in waste treatment activities such as enhancement the biodegradability of organic compounds (Chamarro *et al.* 2001; Kitis *et al.* 1999), effluent treatment and detoxification (Pulgarin *et al.* 1994; Oller *et al.* 2011), degradation and disposal of pesticides (Barbusinski and Filipek 2001; Felsot *et al.* 2003) and treatment of leachate from composting sites (Trujillo *et al.* 2006).

The occurrence of the Fenton reaction and its mechanisms has also been investigated in research related to free-radical mediated oxidative damage of biological molecules and the pro-oxidant role of metal ions *in vivo* (Henle and Linn 1997; Halliwell and Gutteridge 1990; Valko *et al.* 2006). The mechanism of metal catalysed generation of hydroxyl radicals *via* the Fenton or Fenton-like reactions has been a subject of controversy. While there is general agreement on the catalytic formation of oxidative species by the reaction, there are differing views on the type of oxidative species formed and the pathway of the reaction; a free-radical mechanism (Section 5.1.2) and a non-radical

mechanism (Section 5.1.3) have been suggested by various researchers (Barbusiński 2009)

5.1.2. The free-radical mechanism of the Fenton reaction

According to the description of the Fenton reaction by Haber *et al.* (1934), formation of free-radical species by iron catalysed decomposition of H_2O_2 is a chain reaction initiated by a spontaneous oxidation of Fe(II) ions by H_2O_2 (Equation 5.2a). Another initiation of the reaction may also be the reduction of Fe(III), which is a slow reaction that generates hydroperoxyl radicals ($HOO^{\bullet}$) as shown in Equation 5.2b. Reduction of H_2O_2 with hydroxyl radicals ($HO^{\bullet}$) and hydroperoxyl radicals ($HOO^{\bullet}$) shown in Equation 5.2 b&c are the propagation steps. At the end of the reaction, the hydroxyl radicals are terminated by oxidation of the Fe(II) with hydroxyl radical.



(a) $Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + HO^{\bullet} + HO^-$

(b) $Fe^{3+} + H_2O_2 \longrightarrow Fe^{2+} + HOO^{\bullet} + H+$

(c) $HO^{\bullet} + H_2O_2 \longrightarrow H_2O + HOO^{\bullet}$

(d) $HOO^{\bullet} + H_2O_2 \longrightarrow O_2 + H_2O + HO^{\bullet}$

(e) $Fe^{2+} + HO^{\bullet} \longrightarrow Fe^{3+} + HO^-$

Equation 5.2 Mechanism of Fenton reaction, iron catalysed decomposition of H_2O_2, originally proposed by (Haber and Weiss 1934)

The radical generation mechanism of Haber and Weiss (1934) was later revised by Barb *et al.* (1951), who suggested that the oxygen evolution occurs as a result of reduction of Fe(III) by hydroperoxyl radical ($HOO^{\bullet}$) as shown in Equation 5.3a, rather than the reduction of H_2O_2 by hydroperoxyl radical (Equation 5.2c). In addition, oxidation of Fe(II) by hydroperoxyl radical (Equation 5.3b) was also suggested.

(a) $Fe^{3+} + HOO^{\bullet} \longrightarrow Fe^{2+} + H^+ + O_2$

(b) $Fe^{2+} + HOO^{\bullet} \longrightarrow Fe^{3+} + HOO^-$

Equation 5.3 Reactions of the hydroperoxyl radical with Fe^{2+} and Fe^{3+} proposed by Barb *et al.* (1951) as modification of the Fenton chain reaction originally suggested by Haber and Weiss (1934)

The Fenton reaction may also take place with several transition ions such as Cu^{+}, Cr^{3+}, Co^{2+}, Ti^{3+} and Ni^{2+}, V^{4+} and V^{2+} (Barbusinski *et al.*2009) and with some inner-transition metal ions such as the cerium (Ce^{3+}) ion (Heckert *et al.* 2003). A general mechanism of metal catalysed free-radical generating mechanism is shown in Equation 5.4.

$$M^{n+} + H_2O_2 \longrightarrow M^{(n+1)} + HO^- + HO^{\bullet}$$

Equation 5.4 General formula of the initiation reaction of Fenton and Fenton-like reaction reactions exhibited by Fe(II) and other cations. (Walling *et al.* 1975; Barbusinsky 2009).

According to Strlič *et al.* (2003), the catalytic efficiency of some of the transition metal ion in Fenton-like reactions at neutral pH is in the order of Cu(II) > Cr(III) > Co(II) > Fe(III) > Mn(II) > Ni(II).

5.1.3. The non-radical mechanism of the Fenton reaction

In contrast to the free-radical mechanism, an alternative non-radical mechanism (Equation 5.5) has also been proposed in which a metastable Fe^{+4} oxycation species, known as ferryl ion $(Fe=O)^{2+}$ can be formed as an intermediate (Bray et al. 1932; Kramer 1999). It has been demonstrated that the ferryl ion is known to be a powerful oxidant It is extremely electrophilic, to the extent that it can abstract electrons from poor electron from poor electron donor such as aliphatic C–H bonds, the C–H bond orbital acting as electron donor in a charge transfer type of interaction (Louwerse and Baerends 2007).

$$\textbf{(a)} \quad Fe^{2+} + H_2O_2 \longrightarrow FeO^{2+} + H_2O$$

$$\textbf{(b)} \quad FeO^{2+} + H_2O_2 \longrightarrow Fe^{2+} + H_2O + O_2$$

Equation 5.5 Non-radical mechanism of the Fenton reaction through (a) formation of a ferryl cation intermediate $(FeO)^{2+}$ and (b) subsequent oxidation of H_2O_2 (Kremer 1999)

It is assumed that ferryl ion may be stable only near the neutral pH, at lower pH it may become protonated into $(Fe\text{-}OH)^{+3}$ and dissociate releasing the hydroxyl radical ($OH^{\bullet}$) and Fe(III) ion (Gozzo *et al.* 2001; Rush *et al.* 1998). There is evidence indicating the validity of both the radical the non-radical pathways (Barbusinsky 2009). Reviews of the Fenton reaction mechanism (Prousek 2007; Liochev and Fridovich 2002) suggested that

the free-radical and non-radical pathways may occur at different conditions of pH and type of ligands present in the system.

Currently, the free-radical pathway of the Fenton reaction is the most accepted mechanism, commonly invoked to explain the pro-oxidant mechanisms of metal complexes in biochemical systems (Valko *et al.* 2007; Halliwell *et al.* 1990; Moriwaki *et al.* 2008; Henle *et al.* 1997; Carmichael 1989)

5.1.4. Free-radical mediated oxidation of leather

Based on the features of oxidation of polyphenols in aqueous solution in the presence of V(IV) and Fe(II) and the degradation of the semi-V(IV) and semi-Fe(II) leathers, it was postulated that the occurrence of oxidative degradation might be related to the formation of hydroxyl radicals that caused non-specific oxidation, including damage to the collagen of the leather samples. The potential formation of H_2O_2 and iron catalysed formation of hydroxyl radical has also been suggested by Phillips (1954) and Haslam (1998). Literatures on the degradation of aged vegetable tanned leathers have also related the presence of certain types of metals (*e.g.* iron and copper) to the enhancement of the oxidative deterioration of leathers (Thomson 2006; Florian 1987 and Thomson 2006).

In Chapter 4, the catalytic effect of Fe(II) and V(IV) in oxidation of polyphenols was demonstrated and the possibility for the formation of H_2O_2 in acid media was also indicated. It is believed that the formation of H_2O_2 may occur in the semi-metal tanned samples as a result of tannin-metal redox interaction occurring in aerobic conditions. In the presence of the Fe(II) and V(IV), hydroxyl ions may also be formed through the Fenton mechanism (Equation 5.5). In the experiments described in this chapter, the effects of H_2O_2 and Fenton-generated hydroxyl radicals on the hydrothermal stability and structural integrity of leather were studied.

5.2. Materials and methods

5.2.1. Chemicals

Analytical grade hydrogen peroxide (30% w/w, H_2O_2, specific gravity 1.10), iron(II) sulfate [$FeSO_4.7H_2O$], glacial acetic acid (99% w/w, CH_3COOH) and sodium acetate (CH_3COONa) obtained from Fisher-Scientific Ltd (UK) were used in the experiment.

5.2.2. Samples

The following samples were used in the experiments described in this chapter.

Untanned hide powder: acetone dried pelt prepared as described in Section 2.2.3 of Chapter 2 for the hide power preparation,

Mimosa tanned leather : leather sample prepared by tanning pickled sheep skins with a condensed tannin powder (Mimosa ME, Forestal Ltd., UK) as described in Appendix 3,

Chromium(III) tanned leather : leather sample prepared by tanning pickled sheep skins with Chromosal B (chromium(III) sulfate, 33% basicity, 25% Cr_2O_3, Lanxess GmbH., Germany) as described in Appendix 5,

Cr(III)-mimosa tanned leather: leather sample prepared by retanning chromium(III) tanned leather using Mimosa ME as described in Appendix 6.

5.2.3. Analytical instruments

Differential scanning calorimeter (DSC): *Mettler-Toledo 822e DSC* (Mettler-Toledo Gmbh. Switzerland) was used to measure the shrinkage transition of samples.

Scanning electron microscope (SEM): *Hitachi 3000N SEM* (Hitachi Co. Ltd., Japan) was used to assess the fibre structure of dry samples.

pH meter : *Multi-Seven pH* meter (Mettler-Toledo Gmbh., Switzerland) calibrated with standard buffer solutions was used.

5.2.4. Procedures of sample treatment and analysis

Samples described in Section 5.2.2, each weighing 5g were placed in separate beakers and conditioned to pH 6.0 over 24 hours using 20 mL of 1.0 M acetate buffer. One sample of each type was kept as control in the acetate buffer without further treatment. The remaining three samples of each type were treated as follows.

5.2.4.1. Treatment with hydrogen peroxide

Into the each of the beakers containing the rehydrated samples, 10 mL of a 2% w/v solution H_2O_2 was added. The concentration of H_2O_2 in the treatment solution was 0.2 M. The pH of all sample solutions after addition of the H_2O_2 was in the range of 5.6-6.0. The samples were kept in the solution for 48 hours at ambient temperature and then washed excess deionised water.

5.2.4.2. Treatment with Fenton reaction

Two separate treatments of the samples (Section 5.2.2) were carried out in the same manner but with different concentration of H_2O_2 used. The Fenton treatment with relatively lower concentration of H_2O_2 is designated as Fenton-A treatment and the other treatment carried out with greater concentration of H_2O_2 is designated as Fenton-B treatment.

Fenton-A : samples were immersed in separate flasks containing 20 mL of 0.05M $Fe(SO_4)$ prepared in the acetate buffer and stirred on a rotary shaker for 2 hours to allow absorption of Fe(II) ions into the samples. Then, 10 mL of 2% (w/v) H_2O_2 solution was added to each of them. The samples were kept covered for 48 hours at room temperature. Finally, the samples were rinsed with excess deionised water and dried at room temperature.

Fenton-B: another set of the four samples were treated in the same manner as described in Fenton-A treatment, the only difference was addition of 10 mL of 4% H_2O_2 instead of 10 mL 2% w/w H_2O_2 solution.

The concentration of Fe^{2+} in the treatment solutions was 0.03 M in both cases. However, the concentrations of H_2O_2 in the Fenton-A and Fenton-B treatment solutions were 0.2M and 0.4M. Hence the mole ratio (H_2O_2 to Fe^{2+}) in the first and second Fenton reactions was 6:1 and 12:1, respectively.

5.2.4.3. Measurement of shrinkage temperature (T_s)

Measurement of T_s of the samples was carried out using DSC in standard aluminium pans (40 µL) by scanning in the range of 0–140°C at the heating rate of 5°C/minute. Triplet measurements were carried out and the averages and standard deviation of on-set temperature values were calculated

5.2.4.4. Scanning electron microscopy

Sections of the samples were cut perpendicular to the grain surface and coated with gold using an Emitec K550 vacuum sputter coater (Quorum technologies Ltd., UK). The fibre structures in the samples were examined at different magnifications.

5.3. Results and discussion

5.3.1. Effect of treatment with hydrogen peroxide

As shown in Table 5.1, the T_s of the untanned collagen was slightly reduced by 5°C after treatment with H_2O_2. While the that of the Cr(III) tanned and Cr(III)-mimosa tanned leathers were reduced to a large extent by 59°C and 30°C, respectively. The change indicates that the covalent link between Cr(III) and collagen is broken and oxidative reactions have disrupted the tanning structure of the Cr(III) tanned leather, which is composed of interlinked oligomeric Cr(III) complexes (Covington 2008). According to Bakore *et al.* (2011), dimer and trimer Cr(III) complexes may be oxidised by H_2O_2 at pH>5, the rate the reaction increases with pH.

In alkaline media, H_2O_2 is utilised in an oxidative detanning process Cr(III) tanned shaving waste Cot *et al.* (1991). Since the Cr(III)-collagen covalent bond is broken as a

result of oxidation into Cr(VI), the tanning effect of Cr(III) complexes in the H_2O_2 treated products is considered to be disrupted or non-existent.

Table 5.1. Average T_s of control samples of untanned collagen and leather samples and samples treated with 0.2M H_2O_2 alone and Fenton A (0.03MFe^{2+}/ 0.2M H_2O_2) and Fenton B (0.03MFe^{2+}/ 0.5M H_2O_2).

Sample treatment	untanned hide powder	mimosa tanned leather	Cr(III)-mimosa tanned leather	Cr(III) tanned leather
No treatment (control)	60.1±1	81±1	111.6±1	110.9±1
0.2 M H_2O_2 solution	54.5±1	80.2±1	82.9±4	52.1±2
Fenton-A : 0.03 M Fe(II) + 0.2 M H_2O_2	36.5±3	38.6±2	37.9±2	42.7±1
Fenton-B : 0.03 M Fe(II) + 0.4 M H_2O_2	-	-	-	-

In addition, investigations on the pro-oxidant effect of chromium species in biological system indicate that a Fenton-like reaction may also occur with Cr(III) and H_2O_2 system, generating hydroxy radicals as well as intermediate species of Cr(IV) and Cr(V), as shown in Equation 5.6a-d (Tsou and Yang 1996; Strlič, *et al.* 2003). The reaction occurs through successive steps in which other Cr(IV) species are involved in further oxidation with to Cr(V) with H_2O_2 (Equation 5.6 b&c) as well as a disproportionation reaction (Equation 5.6d).

(a) $Cr^{3+} + H_2O_2 \longrightarrow Cr^{4+} + OH^- + HO^\bullet$

(b) $Cr^{4+} + H_2O_2 \longrightarrow Cr^{5+} + OH^- + HO^\bullet$

(c) $Cr^{5+} + H_2O_2 \longrightarrow Cr^{6+} + OH^- + HO^\bullet$

(d) $2\,Cr^{4+} \longrightarrow Cr^{5+} + Cr^{3+}$

Equation 5.6. Oxidation of Cr(III) to Cr(III) in neutral and alkaline solutions, involving formation of hydroxyl radicals (Bokare *et al.* 2011)

Shi *et al.* (1998) showed that Cr(III) may be reduced to Cr(II) by superoxide anion to form H_2O_2, thereby creating a Fenton-like reaction in which both Cr(II) and Cr(III) may decompose the H_2O_2 to hydroxyl radical. The Fenton-like reactions exhibited by the chromium ions, with oxidation states ranging from +2 to +6, is evidently more complex than that of iron, which involves oxidation states +2, +3 and possibly +4 (*via* the non-

radical Fenton mechanism). The decline in the T_s of the peroxide treated Cr(III) tanned leather might also be related to occurrence of Fenton-like reaction in the leather (Figure 5.6). The T_s of the Cr(III) tanned sample dropped to 89°C (Table 5.1). However, it is known that complete detanning of Cr(III) tanned leather may also be achieved with greater concentrations of H_2O_2 applied in alkaline media i.e. pH >8.0 (Cot *et al.* 1991).

On the other hand, the mimosa tanned sample did not show decline of T_s, indicating that the polyphenolic tanning matrix doesn't react with H_2O_2, which actually has lower reduction potential as compared to hydroxyl and superoxide radicals (Equation 5.1). The decrease in the T_s of the Fenton-A treated sample of Cr(III)-mimosa tanned leather is relatively lower than that of the Cr(III) tanned leather. It appears that, the polyphenolic matrix in the Cr(III)-mimosa leather has not been affected significantly. The decline in T_s in this sample may be related mainly to the oxidation of Cr(III) by H_2O_2.

5.3.2. The effects of Fenton reaction

The T_s of all samples after treatment with the Fenton-A system resulted in a remarkable decrease of T_s, most of the sample showed T_s values of 40°C and below. Whereas, in the case of the samples treated with the Fenton-B, it was not possible to obtain distinct thermal transitions in the DSC measurements. The collagen in the Fenton-A treated samples is considered to be partially denatured. The degree of structural disruption and denaturation by oxidation appears to occur to a greater extent in the Fenton-B treated samples. It appears that the triple helical structure of the collagen in the Fenton-B samples may already been structurally disrupted by the chemical treatment, hence no endothermic transition of denaturation could be detected during DSC analysis.

With the samples treated using Fenton-A, the decrease in the T_s of all of the samples appears to occur to a narrow range of 36-42°C, regardless of the large difference in the T_s of the initial samples, as shown in Table 5.1. The extent of lowering of the hydrothermal stability observed with the Fenton treated samples indicates that both the

tanning structures and the collagen matrix are damaged by the oxidative action. Unlike the case of the treatment H_2O_2 alone, treatment with the Fenton-A and Fenton-B systems have caused oxidative damage in all of the samples, including the mimosa tanned sample.

5.3.3. The fibre structure of the Fenton treated samples

The structure of fibre network and morphological appearance in the oxidised samples showed significant difference as compared to the corresponding untreated control samples. The SEM micrograph of the control samples of mimosa tanned leathers (Figure 5.1 a&b) show an intact structure with distinctly visible fibre weave and fibre-bundles. In contrast, the samples after treatment with Fenton-A (Figure 5.1 c&d) have a structure that is relatively loose and composed of irregularly shaped fibres fused together.

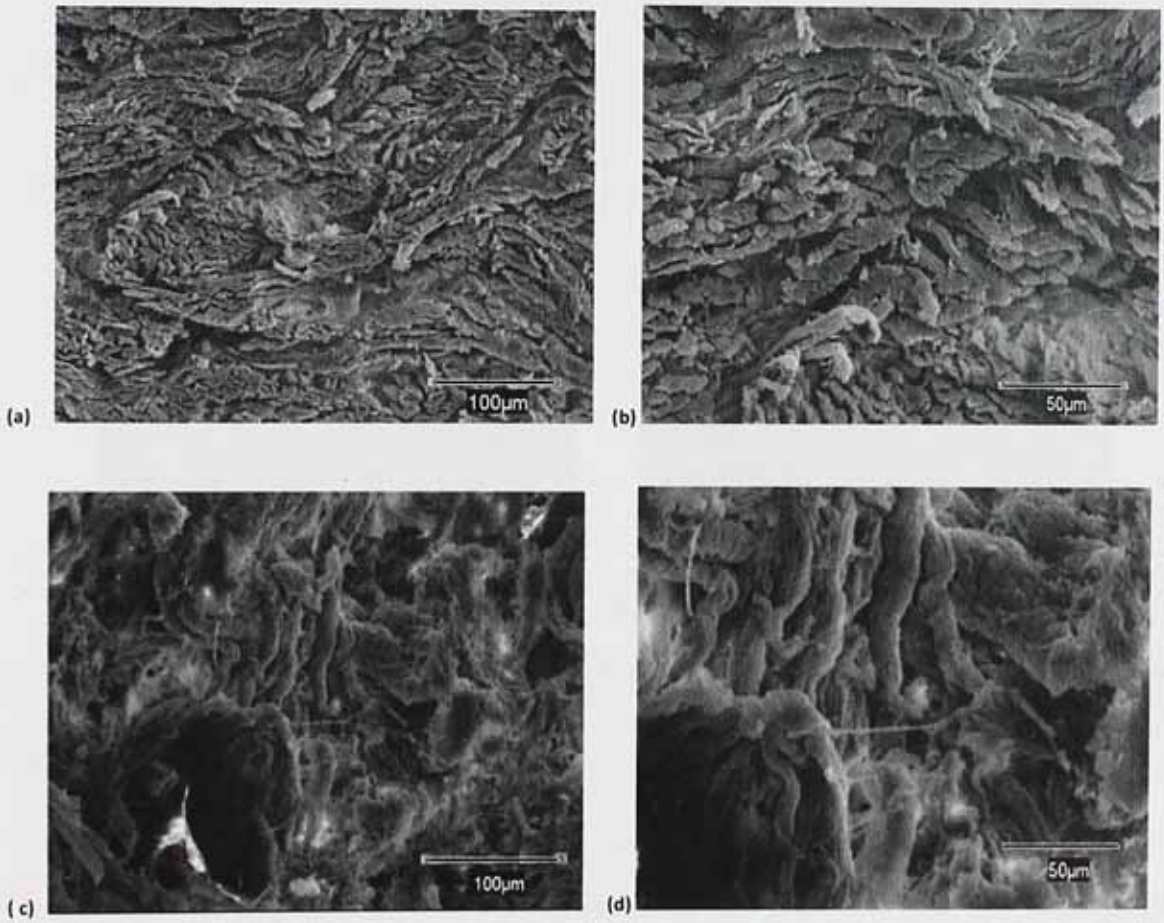


Figure 5.1. SEM micrograph of the cross-sectional view of mimosa tanned leather at (a) magnification X250, (b) magnification X600; cross-sectional view of mimosa tanned leather treated with Fenton reaction using 0.03M Fe^{2+} and 0.2 M H_2O_2 at (c) magnification of X250 and (d) magnification X600.

The Cr(III)-mimosa tanned samples treated with Fenton-A system also showed change in the fibre structure, similar to the case of the mimosa tanned. As shown in Figure 5.2, the normal fibre structure of the leather (Figure 5.2 a&b) was converted to an amorphous structure of fibres having distorted shapes in the Fenton treated Cr(III)-mimosa leather (Figure 5.2 c&d). The physical degradation and the decline of T_s of the Fenton treated Cr(III)-mimosa and Mimosa-tanned leathers appear to occur similarly, regardless of the difference in the composition of the leather in terms of the presence or absence of Cr(III) tanning.

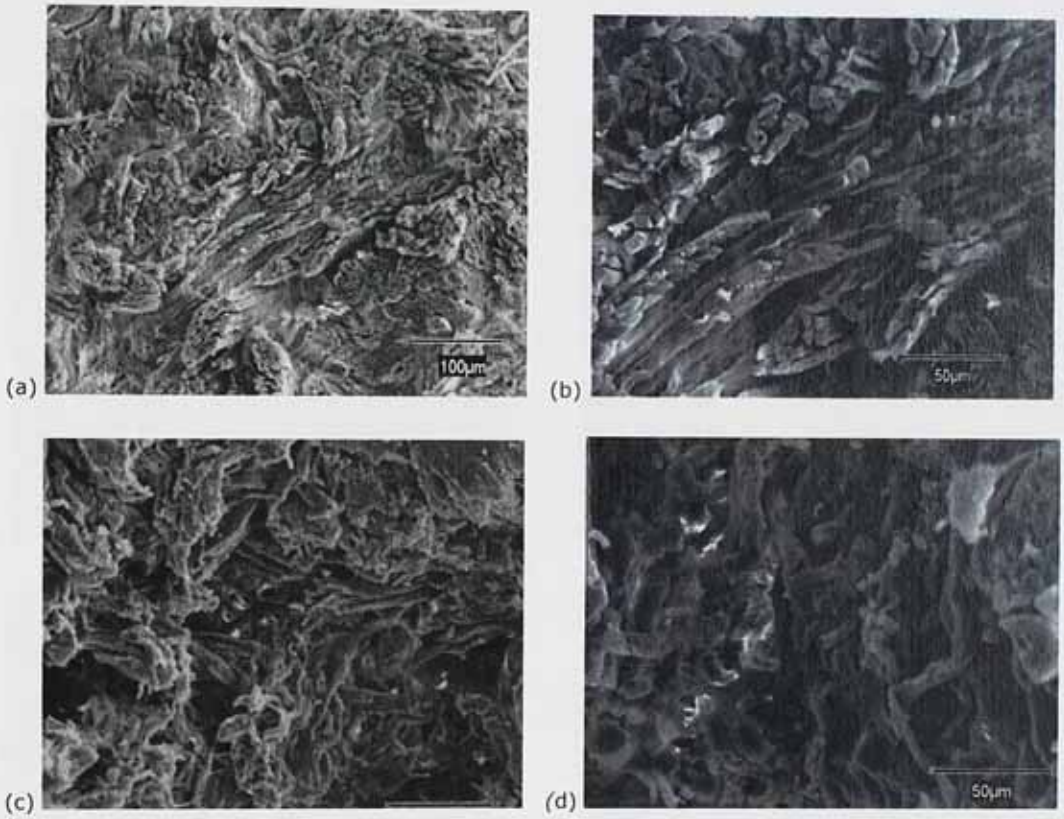


Figure 5.2. SEM micrograph of the cross-sectional view of Cr(III)-mimosa tanned leather at (a) magnification X250, (b) magnification X600 and the corresponding micrographs of Cr(III)-mimosa tanned leather treated with Fenton reaction using 0.03M Fe^{2+} and 0.2 M H_2O_2 at (c) magnification of X250 and (d) magnification of X600.

The Cr(III) tanned leather also showed a similar type of physical degradation as a result of treatment with the Fenton reaction, regardless of the high hydrothermal stability of the leather. The Fenton-A treated sample (Figure 5.3 c&d) showed a different appearance as compared to the controls (Figure 5.3 a&b). Broken fibres with irregular

morphology were observed (Figure 5.3 c&d) and the cross-section became a compact structure.

The physical degradation of the leathers by the Fenton reaction indicates that the presence of hydroxyl radicals in leather causes oxidative degradation in which, not only tanning structures may be decomposed, but the fibrous collagen matrix may also be denatured. The results indicate that leather is susceptible to a rapid oxidative damage under ambient conditions with application of dilute Fenton reagent (Fe^{2+}/H_2O_2). The degree of oxidative damage may be proportional to the quantity of oxidant used and extent of oxidative damage could extend to complete structural denaturation as well as decomposition of the whole material.

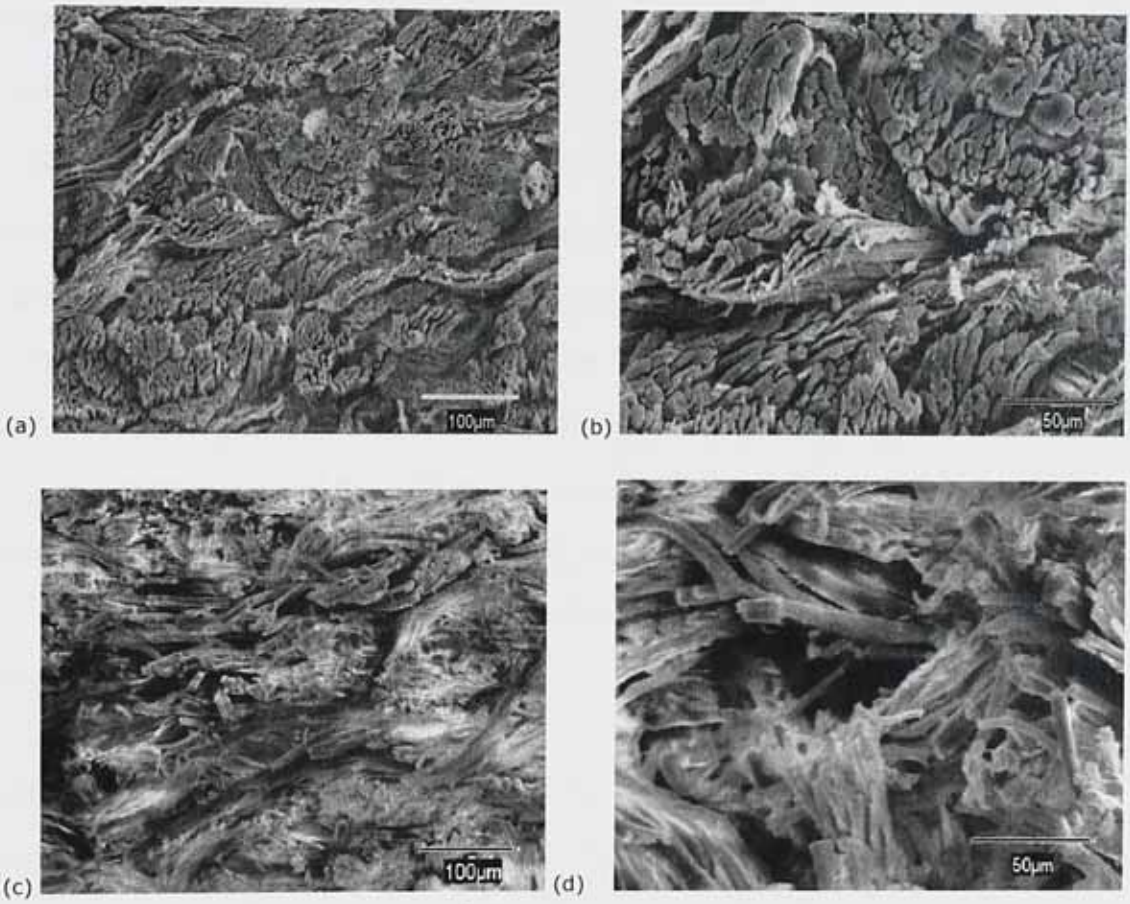


Figure 5.3. SEM micrograph of cross-sectional view of Cr(III)-tanned leather at (a) magnification X250, (b) magnification X600 and the corresponding micrographs of Cr(III)-mimosa tanned leather treated with Fenton reaction using 0.03M Fe^{2+} and 0.2 M H_2O_2 (c) at magnification of X250 and (d) magnification of X600

5.3.4. Free-radical mediated degradation of leather

The mechanism for oxidative degradation of tannins by metals in semi-metal tanned has already been described earlier as a chemical process. The potential for the formation of free-radicals was also described in Chapter 4, based on evidence from research on metal-tannin interaction and the pro-oxidant mechanisms of metal ions, *i.e.* Fe(II), V(IV).

The results shown in Sections 5.3.2 indicate the formation of hydroxyl radicals in vegetable tanned leather causes a deleterious effect on the polyphenolic tanning matrix as the causes denaturation of the collagen. Thus, the decline in T_s and degradation observed on the Fe(II) and V(IV) containing leather samples can be attributed to the formation of H_2O_2 and hydroxyl radicals as a result of the metal catalysed oxidation of tannins (Section 5.1) and Fenton reaction (Equation 5.4)

In relation to the deterioration of age old historical leather artefacts, it has been assumed that the presence of certain metal ions in vegetable tanned leathers, originating from the tanning process may enhance oxidative breakdown of the collagen matrix in the leathers (Kite 2006, Florian 1987). The formation of H_2O_2 during accelerated-ageing of vegetable tanned leathers in sulphur dioxide chambers is assumed to be an important part of the oxidative degradation of leather in acid media (Stambolov 1969). The catalytic effect of metal ions in decomposing H_2O_2 and enhancing oxidative degradation historical leathers (vegetable tanned) was proposed in earlier studies by Phillips (1954).

Haslam(1998) proposed that H_2O_2 may also be formed due to the autoxidation of polyphenols and the principal mechanism for the degradation of vegetable tanned leather is may be related to the catalytic effect of trace quantities of iron ions present in the leather. Assuming the presence of trace amounts of Fe(II) or Fe(III) ions in leather, the redox interactions (Figure 5.4) that involves reduction of Fe(III) by aromatic

hydroxyl or phenolate ions (Figure 5.4a) and oxidation of Fe(II) by air with subsequent formation of superoxide radical (Figure 5.4b) were suggested.

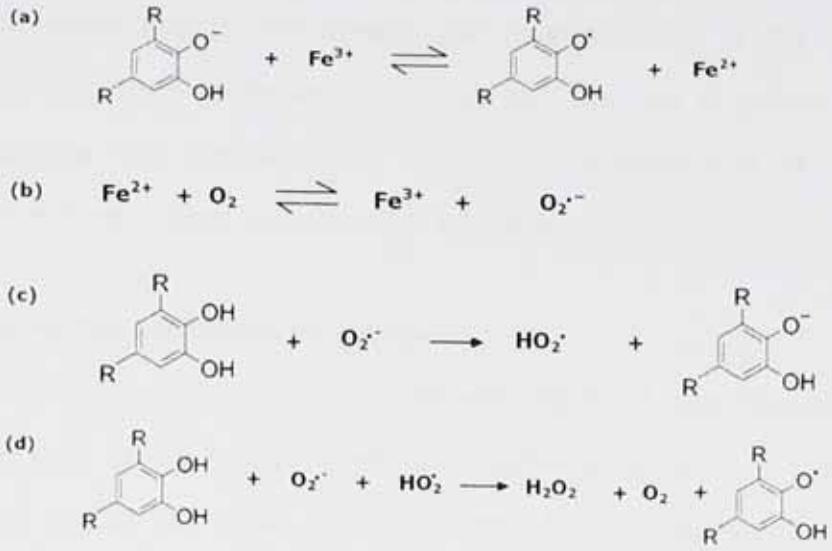


Figure 5.4 Proposed mechanism of iron-catalysed degradation of vegetable tanned leather (Haslam 1998) involving (a) oxidation of polyphenols by Fe^{3+}, (b) autoxidation of Fe^{2+}, (c) formation of hydroperoxyl radical (HO_2·) and (d) formation of H_2O_2.

The superoxide radical formed in the leather may abstract a hydrogen ion from the phenolic hydroxyl groups (Figure 5.4c) or react with the hydroperoxyl radical to form H_2O_2 (Figure 5.4d). The presence of H_2O_2 leathers would give rise to the catalytic formation of hydroxyl radicals (OH•) and subsequent oxidative breakdown of the collagen. The suggested sequence of reactions represents a mechanism in which molecular oxygen may be continually converted in to oxygen derived radicals and H_2O_2, through the catalytic effect of assume trace quantities of iron.

The experiments described in Chapter 4 on the stability of phenolic compounds in the presence of Fe(II) and V(IV) ions, indicate that the autoxidation of phenolic compounds may occur in acid media, in which the o-diphenol groups are largely not ionised. Therefore, the presence of phenolate ions is not a requirement for the reaction of polyphenols with Fe^{3+}. On the other hand, it is known that at conditions where phenolate ions are formed in large proportions (*i.e.* alkaline conditions), autoxidation of polyphenols occurs spontaneously resulting in oxidative rearrangement reactions

(Ferriera *et al.* 1992). The reactions in proposed mechanism of Haslam (1998) may not completely represent the redox interaction responsible for degradation of vegetable tanned leather. But it highlights the important role of metal ions and oxygen derived radicals in the leather degradation process. The general concept of this mechanism, in relation to the possibility of formation of H_2O_2 and the role of metals in catalysing oxidative reactions was considered in explaining the occurrence of metal-induced degradation in the semi-metal tanned leathers (Chapter 3)

5.3.5. Oxidative fragmentation of collagen

The collagen in the leather samples treated with Fenton-A and Fenton-B systems is evidently damaged by the oxidative effect of hydroxyl radicals. An investigation by Komsa-Penkova (2000) has shown that the treatment of collagen with hydroxyl radicals has shown that the quantity of free amino groups in the oxidised product was significantly lower than acid-hydrolysed collagen. Accordingly, it is assumed that the highly electrophilic hydroxyl radicals are most likely to attack side chain amino groups mainly lysine, argenine and Histidine.

Uchida *et al.* (1990) and Kato *et al.* (1992) have also investigated the oxidative breakdown of collagen by hydroxyl radicals. It was discovered that the peptide links at the proline residues (prolyl peptides) may be the initial sites for the fragmentation of the collagen polypeptide. Nevertheless, considering that hydroxyl radicals are powerful oxidants, the oxidative fragmentation of collagen is also assumed in other location of the collagen chain. As demonstrated by Mukhopadhyay *et al.* (1994), oxidatively fragmented collagen is most likely to be susceptible to proteolytic attack by enzymes that are normally incapable of hydrolysing intact collagen.

5.4. Conclusion

The formation of oxygen derived free radicals plays a crucial role in metal induced degradation of leathers. Vegetable tanned leather are not susceptible to oxidation by H_2O_2, while the T_s of Cr(III) tanned leathers is lowered by nearly 59°C due to the oxidative action of a 2% w/v solution of H_2O_2 *via* a Fenton-like reaction with Cr(III) ion. Collagen also showed a slight decrease in T_s from 60°c to 54°C. All leather samples and the hide powder sample were degraded by Fenton reaction in 2% of H_2O_2.

The T_s of hide powder and leather samples treated with the Fenton reaction *i.e.* 0.2M of H_2O_2 and 0.03M Fe^{2+}dropped to less than 40°C, indicating the occurrence of oxidative denaturation of collagen by hydroxyl radicals. The effect of hydroxyl radical mediated oxidation of hide powder and leather samples, observed with SEM, showed deformation of the fibres and formation of amorphous structures. Application of increased quantity of H_2O_2 in a separate Fenton treatment of samples i.e. 0.4 M H_2O_2 caused greater oxidative decomposition of the samples. In this case, the fact that no thermal transition was detected might indicate that the collagen in the samples has undergone complete structural disruption. The results described in this chapter also provide further evidence regarding the role of hydroxyl radicals in the autoxidative degradation of the semi-Fe(II) and semi-V(IV) tanned samples (described in Chapter 2 and 3).

In addition, the observed changes in the leathers treated with Fenton reactions represent a case of an efficient means of degrading leather. The Fenton reaction is widely applied industrially and in waste management operations. Based on the results obtained in this research, it is suggested that oxidative pre-treatment of leather waste using Fenton reagent may be useful in developing efficient methods for recycling of leather waste through biodegradation methods. This may be viewed in terms of the efficiency of the reaction in bringing about chemical breakdown of the macro-molecular structures in leather, thereby enhancing its susceptibility to enzymatic and/or microbial degradation.

CHAPTER 6

ENZYMATIC HYDROLYSIS OF OXIDATIVELY DEGRADED LEATHERS

6.1. Introduction

Resistance to putrefaction and enzymatic degradation are the fundamental property of leather and defining criteria for tanning (Reich 2007). Tanning agents prevent proteolytic degradation of leather by through steric hindrance, blocking of the active sites of the enzymes and causing changes in the secondary structure of the enzymes (Krishnamoorthy *et al.* 2008;Gayatri *et al.* 2000). It is known that removal of tanning agents from leather by detanning treatments and thermal denaturation of the collagen in leather may enhance the susceptibility of leather to degradation by enzymes (Dhayalan *et al.* 2005; Yagoub 2006).

As demonstrated in Chapter 3, the occurrence of metal induced autodegradation has led a decrease in the shrinkage temperature (T_s) of the leathers below that of the untanned collagen. The collagen in the degraded leather samples is considered to chemically denatured by oxidative reactions. As demonstrated in Chapter 5, treatment of leathers with dilute solution of Fenton reagent (H_2O_2/Fe^{2+}) has resulted in oxidative degradation in which the collagen in the leather was denatured. Metal-tannin redox reactions and hydroxyl radicals in leather causes significant changes in the structure of the tanning matrix and the collagen. Therefore, an increased susceptibility of partially degraded leathers to proteolytic degradation is also expected to be a possible outcome.

This chapter includes descriptions of the experiments carried out to evaluate the action of bacterial enzymes on the oxidatively degraded leather samples. The relevance of the results obtained in the experiments are also discussed in the context of application of oxidative treatments to enhance the biodegradation of leather waste.

6.2. Materials and methods

6.2.1. Chemicals and enzymes

The buffer salt tris-hydroxymethylaminomethane-HCl (Tris-HCl), chloramine-T reagent, hydroxyproline standard (L-4-hydroxyproline >99.9% w/w) and p-Dimethyl-amino-benzaldehyde were purchased from Sigma-Aldrich Ltd (UK). Calcium chloride [99.9% w/w $CaCl_2$] , propan-2-ole, concentrated hydrochloric acid (36.5% w/w) concentrated perchloric acid (70% w/w), citric acid monohydrate, anhydrous sodium acetate and trisodium citrate, iron(II) sulfate heptahydrate [$Fe(SO_4).7H_2O$], chromium(III) chloride [$CrCl_6$. $6H_2O$], vanadium sulfate [$VO(SO_4).5H_2O$] and hydrochloric acid (36% w/v) were obtained from Fisher Scientific Ltd.(UK). The bacterial enzymes used in the experiments (Table 6.1) were obtained from Sigma-Aldrich Ltd., (UK).

Table 6.1 - General description of bacterial enzymes, collagenase and subtilisin, based on product information from Sigma-Aldrich Ltd. (UK) and general information from Barrett *et al.* (1998).

Enzyme name	collagenase	subtilisin
Type and main enzyme component	Metalloprotinase; collagenase Type I	Serine endopeptidase; subtilisin A
Source (microorganism/culture)	*Clostridium Histolyticum*	*Bacillus Licheniformis*
Enzyme Commission number	EC 3.4.24.3	EC 3.4.21.62
Substrate specificity	Digestion of native collagen in the triple helical region	Hydrolyses peptide amides., broad specificity with preference to uncharged residues
Optimum pH	7.5	8.0
Temperature	37 °C	45-50°C
Form of enzyme product	Salt-free Lyophilised powder	200 mg/mL solution in borate buffer (pH 8.0), specific gravity 1.25 g/ml.

6.2.2. Untanned collagen and leather samples

The main samples for the investigation of enzymatic degradability were the leather samples that have undergone autodegradation during storage at 21°C and 65% RH for more than 6 months. The first group of samples are the semi-V(IV), semi-Fe(II) leathers. In addition, the Cr(III)-mimosa and Cr(III)-chestnut leathers that showed autodegradation at the ambient condition of storage after treatment with V(IV) and

Fe(II) salts were also used in the experiment (Table 6.2). The second group of degraded samples were the leathers that were oxidised by the Fenton reaction as described in Chapter 5, *i.e.* Fenton-A treatment method with 0.2 M H_2O_2 and 0.03 M Fe^{2+}.

In addition, reference samples of untanned collagen and control samples of leathers (not degraded or not exhibiting lowering of T_s) were used for comparative analysis. A description of the samples and their T_s at the time of the enzyme degradability analysis is given in Table. 6.2

Table 6.2 - Description of samples used in the analysis of enzymatic degradability of leather using collagenase and subtilisin enzymes.

Type and name of Samples	T_s (°C)	Description
a) Hide powder	59.5	Untanned collagen
b) Leather samples		leather samples prepared as described in Appendix 3 and Appendix 5
• Cr(III) tanned	110.5	
• mimosa tanned	84.4	
• chestnut tanned	76.2	
c) Partially degraded leather samples		semi-metal tanned leathers that have undergone autodegradation at ambient condition for six months, Cr(III)-mimosa and Cr(III)-chestnut tanned leathers treated with V(IV) and Fe(II) and undergone during storage at ambient condition for 6 months
• mimosa-V(IV)	40.1	
• chestnut-V(IV)	38.5	
• mimosa-Fe(II)	52.5	
• chestnut-Fe(II)	56.2	
• Cr(III)-mimosa +Fe(II)	84.3	
• Cr(III)-chestnut +Fe(II)	87.9	
• Cr(III)-mimosa +V(IV)	43.4	
• Cr(III)-chestnut +V(V)	42.1	
d) Fenton oxidised samples		Leather samples treated with Fenton reaction (0.03M Fe^{2+} and 0.2M H_2O_2) for 48 hours as described in Chapter 5, Section 5.2.4.2 as Fenton-A treatment
• Hide powder	36.5	
• Chrome tanned	42.7	
• mimosa tanned	38.6	
• Cr(III)-mimosa tanned	37.9	

6.2.3. Determination of the degree of enzymatic hydrolysis

Enzymatic hydrolysis of the samples by collagenase and subtilisin enzymes was measured in terms of the hydroxyproline concentration in the digestion solutions after 48 hours of incubation. The samples (Table 6.2) were dried in a desiccator for 48 hours. A set of five samples (100mg) of each sample type were treated as follows.

Acid digestion: - Samples from each type were digested in sample tubes with 5 mL of 6M HCl at 100°C for 6 hours and prepared as analyte solutions of 50 mL volume.

Enzymatic digestion: - Two samples were separately incubated collagenase and subtilisin enzymes for 48 hours as described in Section 6.3.2.1. After incubation, all sample tubes were cooled, centrifuged at 3000 rpm for 30 minutes at 4°C and filtered. The filtrates were digested at 100°C for 6 hours after addition of 5 mL of 6M HCl solution. The resulting solutions were prepared as analyte solutions by adjusting to 50 mL in using deionised water.

Incubation in buffer solutions: The remaining two samples that were incubated for 48 hours in the buffer solutions in absence of enzyme (control samples) and the filtrates (5 mL) from each sample were digested with 5M of HCl for 6 hours and prepared as analyte solutions by adjusting to 50 mL.

The concentration of hydroxyproline in each of the analyte solution was determined. The experiment procedures were carried out twice and the average of hydroxyproline concentrations were calculated. The degree of enzymatic hydrolysis for each sample was estimated by calculating the percentage of hydroxyproline concentration in the analyte solutions of the enzyme digestions with respect to that obtained by acid digestion *i.e.* the maximum hydroxyproline concentration from the sample obtained by chemical digestion.

$$\text{Enzymatic degradation (\%)} = \left(\frac{\text{hydroxyproline (mg/mL) in the analyte of enzymatic-digestion}}{\text{hydroxyproline (mg/mL) in the analyte of acid-digestion}} \right) \times 100$$

6.2.3.1. Enzymatic digestion of samples

Samples were treated with collagenase and subtilisin enzymes as follows.

- **Digestion with collagenase**: 100µL of 10 mg/mL methanolic solution of collagenase was added in to sample tubes containing the 100 mg samples immersed in 5 mL of 0.1M tris-HCl buffer (pH 7.5) containing 40 mM of $CaCl_2$. Incubation was done at 37°C in a water bath for 48 hours. In each case, a control sample was also incubated concurrently in the absence of the enzyme.

- **Digestion with subtilisin**: 50 µL of subtilisin enzyme (200 mg/mL) was added to sample tubes containing 5 mL of tris-HCl buffer (pH 8.0) containing 40 mM $CaCl_2$ and the 100mg samples. Incubation was done at 50°C for 48 hours. In each case, a control sample was also incubated concurrently in the absence of the enzyme.

6.2.3.2. Determination of hydroxyproline content

The concentrations of hydroxyproline in the analyte solutions (50 mL) were determined using the method of Bergman *et al.* (1963). The method is based on the oxidation of free hydroxyproline and the subsequent spectrophotometric determination of the chromophore generated by the condensation of the oxidation product with para-diaminobenzaldehyde. The chloramin T reagent (oxidising agent) was prepared by dissolving 1.4g of N-chloro-4-methylbenzenesulfonamide in 100 mL of citrate-acetate buffer (pH 6.0) containing 50% (v/v) of propan-2-ol. Ehrlich's reagent was prepared by dissolving 24 g of dimethylaminobenzaldehyde with 36 mL of perchloric acid and adding 200 mL of propan-2-ol.

A blank and five standard hydroxyproline sample solutions of 1.0, 2.5, 5.0, 7.5 and 10.0 mg/L were prepared, 0.55 mL portions of each of the standard solutions were mixed with 1.27 mL of 67% v/v propane-2-ole and 0.88 mL of chloramin T reagent in sample tubes and left for 5 minutes. Then Ehlrich's reagent (2.3 mL) was added followed by heating for 20 minutes at 70°C. After cooling to room temperature, the absorbance of each sample was measured at 555 nm using a spectrophotometer (Shimadzu 2400PC, Shimadzu Ltd. Japan) using glass cuvettes (3.5 mL volume, 10mm path-length).

The standard curve of absorbance versus concentration of hydroxyproline concentration with a linear correlation coefficient (R^2) of 0.9984 was obtained. The analyte solutions were diluted 50 times with deionised water and treated in the same manner as the standard hydroxyproline standard solutions. From the calibration curve, the hydroxyproline concentrations of the analyte solutions were obtained

6.2.4. Analysis of collagenase activity in presence of metal ions

To investigate the inhibition effect of metal ions on the enzymatic activity of collagenase, enzymatic digestions of untanned collagen samples (hide powder) were carried out in the absence and presence of V(IV), Cr(III) and Fe(II) ions. Four groups of six samples of dry hide powder samples (100 mg) were weighed in to digestion tubes. Buffer solutions (pH 7.5) with the following compositions were used for the incubations of the four groups of samples.

- 5.5 mL tris-HCl buffer (pH 7.5) + 200 µL of 1M $CaCl_2$
- 5.0 mL tris-HCl buffer (pH 7.5) + 200 µL of 1M $CaCl_2$ + 500 µL 0.5M $Fe(SO_4)$
- 5.0 mL tris-HCl buffer (pH 7.5) + 200 µL of 1M $CaCl_2$ + 500 µL 0.5M $CrCl_3$
- 5.0 mL tris-HCl buffer (pH 7.5) + 200 µL of 1M $CaCl_2$ + 500 µL 0.5M $VO(SO_4)$

In each case, the buffer immersed hide powder samples were allowed to rehydrate for 2 hours at 37°C in the buffer solutions containing $CaCl_2$. In each case, incubation at 37°C was started with addition of 100 µL of 10 mg/mL collagenase solution to all of the samples at the same time. Incubation of the individual samples was stopped every 30 minute by instant freezing, *i.e.* dipping the sample tubes in liquid nitrogen; six samples with incubation times of 30, 60, 90, 120, 150 and 180 minutes were produced.

The frozen samples were thawed at 5°C, centrifuged at 3000 rpm for 30 minutes and then filtered. The filtrates were digested with 5 mL of 6M HCl, analyte solutions were prepared by adjusting the volume of the digest solutions to 50 mL. Determination of hydroxyproline content of the filtrates was carried out as described in Section 6.2.3.2. The extent of collagen degradation at different incubation times, in the presence and absence of the different metal ions were compared.

6.3. Results and Discussion

6.3.1. Action of enzymes on leather samples and hide powder

The untanned collagen samples were digested by both the enzymes, complete solubilisation of 100 mg sample of hide powder with collagenase was observed in 6 hours time, while subtilisin took more than 12 hours to completely solubilise the collagen hide powder. The vegetable tanned and Cr(III) tanned leather samples showed no enzymatic degradation after incubation with both of the enzymes. No hydroxyproline was detected in the filtrates of the enzyme incubations as well as the respective controls. This is an expected result considering that the tanning structure is still intact and hence leather is not enzymatically degradable.

A form of subtilisin enzyme, commercialised as *Alcalase 2.5L,* (Novozymes Co. Ltd, Denmark) has been used to enzymatic hydrolysis of Cr(III) containing leathers waste at temperatures reaching 50°C (Mu *et al.* 2003; Kupec *et al.* 2002). The enzymatic action is effective only after an extended alkaline treatment of the leather waste, often in the presence of magnesium oxide or lime. This treatment partially removes the Cr(III) for the leather waste making the collagen more vulnerable to enzymatic digestion. In this experiment, subtilisin was applied in a weak alkaline buffer medium in which there was no condition for sufficient detanning of the leather. Hence the Cr(III) tanned samples are not degrade at all by the alkaline protease. However, subtilisin effectively degraded the untanned collagen sample (hide powder) at pH 8 and temperature of 50°C. Subtilisin-enzymes are known to have collagenolytic action (Okamoto *et al.* 2001; Kurata *et al.* 2010)and are thermophilic due to their stability and increased activity at higher temperatures reaching 50°C.

6.3.2. Effect of enzymes on oxidatively degraded leathers

Based on the observation that the semi-V(IV) tanned samples (autodegraded for more than 6 months) showed gelatinization, it was suspected that the protein in the autodegraded semi-metal tanned samples may possibly be dissolved without the action of enzymes. The control samples digested with buffer solutions (without enzyme) did not

solubilise the collagen i.e. no hydroxyproline was detected in the analyte solutions. This may indicate that there are no sufficiently degraded peptides to be solubilised.

It appears that denaturation of collagen in the degraded samples, e.g. semi-V(IV) tanned samples, and the possible formation of peptide segments do not result solubilisation of amino acids or peptides in the aqueous media. Interactions between oxidised polyphenols and functional groups of the protein may prevent solubilisation of the peptide fragments created. one of such interactions could be the formation of quinone mediated crosslinks (Heidemann 1993; Haslam 1998). Quinones, derived from the oxidation of polyphenols are generally known to be highly reactive and undergo polymerisation reaction (Hathway 1958). In oxidatively degraded vegetable tanned leathers, quinones may form covalent links with the amino groups of peptide fragments as shown in Figure 6.1. The effect of quinone crosslink formation could be that the peptide segments formed by oxidative denaturation would be insoluble in water.

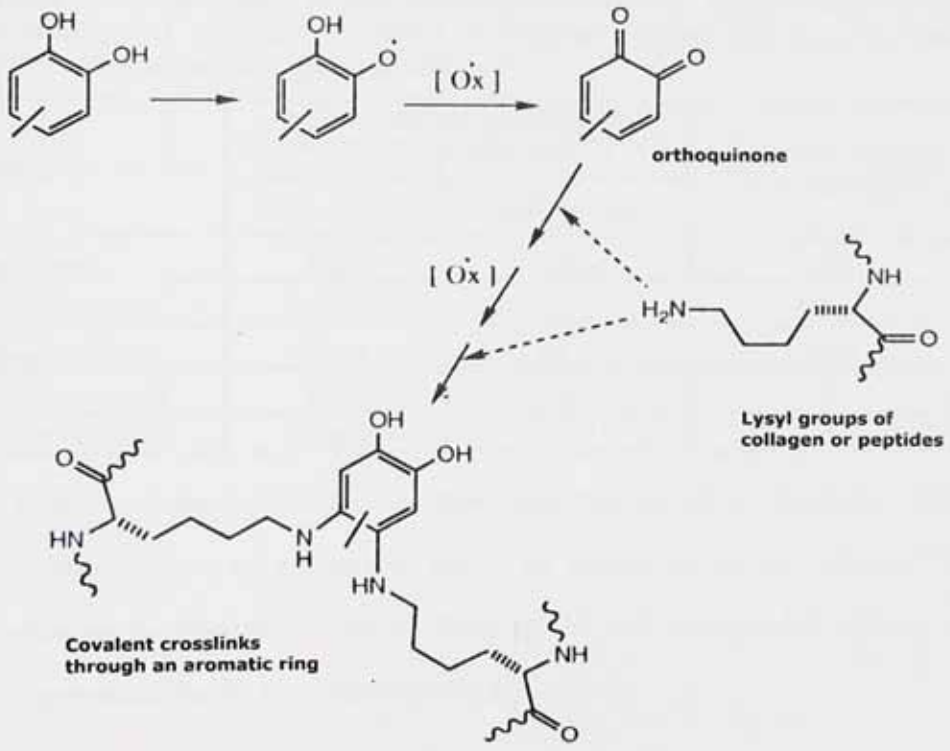


Figure 6.1 Mechanism of formation of quinone-crosslinks between lysine groups of collagen (peptides) in degraded vegetable tanned leather (Haslam 1998).

Incubation of some the semi-metal tanned leathers and the Fenton-oxidised leather samples in the presence of collagenase and subtilisin showed varying levels of enzymatic digestion as observed with quantitative determination of the hydroxyproline liberated.

6.3.2.1. Semi-metal tanned leathers

Analysis of hydroxyproline content in the control incubation, in the absence of enzyme, showed no hydroxyproline content in all of the cases. The collagenase enzyme completely degraded the hide powder sample completely as expected. The mimosa-V(IV) and chestnut-V(IV) leather samples were also partially hydrolysed by collagenase, showing 58-61 µg/L of hydroxyproline in the analyte solutions (Table 6.3), which corresponds to a degradation of 45-48% of the total hydroxyproline content. No hydroxyproline was detected in the analyte solutions from collagenase treatment of mimosa-Fe(II) and chestnut-Fe(II) samples, indicating that the samples did not respond to the action of the enzyme.

Table 6.3 Concentration of hydroxyproline in analyte solutions (50 mL) of acid-hydrolysis and enzymatic digestions of untanned collagen and autodegraded semi-metal tanned leathers using collagenase.

Sample	Hydroxyproline concentration (µg/ml) in analyte solutions (50 mL)		Degree of enzymatic hydrolysis (%)
	acid hydrolysis	collagenase	
collagen	286.0	278.8	98.4
mimosa-V(IV)	130.4	58.5	44.9
chestnut-V(IV)	125.5	61.0	48.6
mimosa-Fe(II)	132.2	0	0
chestnut-Fe(II)	128.2	0	0

About 80% of the untanned collagen sample was hydrolysed by subtilisin (Table 6.4), showing a slightly reduced enzymatic action as compared to collagenase. However, subtilisin showed hydrolysis of protein from all of the semi-metal tanned samples, including the mimosa-Fe(II) and chestnut-Fe(II) leathers.

The T_s of the mimosa-Fe(II) and chestnut-Fe(II) samples were 52-57°C (Table 6.1), indicating that the -metal tanning matrix that initially created greater hydrothermal stability of 95-110°C has been disrupted by metal induced oxidation. However, the

collagen in the semi-Fe(II) samples having T_s of 52-56°C may have relatively less degree of degradation compared to the semi-V(IV) samples which has T_s <40°C. On the other hand, the absence of enzymatic degradation with the semi-Fe(II) tanned samples may also be related to the greater degree of inhibition of collagenase by the Fe(II) ions as compared the V(IV) ions (as described in Section 6.3.3).

Table 6.4 - Concentration of hydroxyproline in analyte solutions (50 mL) of acid-hydrolysis and enzymatic digestions of untanned collagen and autodegraded semi-metal tanned leathers using subtilisin.

Sample	Hydroxyproline concentration (µg/ml) in analyte solutions (50 mL)		Degree of enzymatic hydrolysis (%)
	acid hydrolysis	enzymatic hydrolysis with subtilisin	
collagen	286.0	229.5	80.2
mimosa-V(IV)	130.4	57.4	44.0
chestnut-V(IV)	125.5	49.3	39.3
mimosa-Fe(II)	132.2	43.1	32.6
chestnut-Fe(II)	128.2	38.9	30.3

The difference in the response of the semi-V(IV) and semi-Fe(II) samples to the action of collagenase could be related to the degree of susceptibility of the sample to enzymatic action and the degree of inhibition of the enzyme by the metal ions in the sample. Based on comparison of T_s, it can be observed that the semi-V(IV) leathers have greater degree of physico-chemical degradation as compared to the semi-Fe(II) leathers. Consequently, the protein in the semi-V(IV) samples may be more readily hydrolysed by enzymes.

Subtilisin enzymes are known for their greater proteolytic activity in alkaline media and their stability at temperatures exceeding 50°C (Toyokawa *et al.* 2010, Barrett *et al.* 1998). At such conditions the untanned collagen samples as well as the other samples may undergo thermal denaturation over the long hours of incubation at the slightly alkaline medium, thereby enhancing the hydrolysis of peptide bonds by the enzyme.

Similarly, enzymatic treatments of the mimosa-V(IV) and chestnut-V(IV) samples with collagenase was carried out at 37°C, this temperature is nearly the same as the

observed onset temperature of shrinkage for the semi-V(IV) leathers (*i.e.* 38-39°C). Hence, thermal denaturation of the collagen in the partially degraded samples and the subsequent unravelling of the remaining triple helical segments should have resulted in complete solubilisation of the collagen in the leathers. Since no hydroxyproline was detected in the control incubations (without enzyme) the hydroxyproline found in the enzyme digestion solutions of the semi-V(IV) leather leathers is totally attributed to the actions of enzymes.

6.3.2.2. Cr(III) tanned leathers

The Cr(III)-mimosa and Cr(III)-chestnut tanned leathers treated with V(IV) had T_s of 42-43°C after 6 months of autodegradation at 21°C and 65% RH, These samples showed enzymatic degradation by subtilisin as shown in Table 6.5. None of the Cr(III) containing samples showed degradation by collagenase. The absence of collagenolytic action in this case may be related to the inhibition of the enzyme by Fe(II) and Cr(III) complexes (See section 6.3.3).

Table 6.5 Enzymatic degradation of Cr(III)-mimosa and Cr(III)-chestnut tanned leathers that have undergone autodegradation for after treatment with vanadium(IV)

Sample	Hydroxyproline concentration (µg/ml)		Degree of enzymatic hydrolysis (%)
	Acid hydrolysis	enzymatic hydrolysis with subtilisin	
Cr(III)-mimosa -V(IV)	132.3	29.81	22.5
Cr(III)-chestnut-V(IV)	141.5	27.32	19.3

On the other hand, the Fe(II) treated Cr(III)-mimosa and Cr(III)-chestnut leathers, having T_s of 84-87°C showed no sign of susceptibility to enzymatic action. The T_s of these samples are above 80°C, indicating that the Fe(II) induced degradation has not resulted in a complete decomposition of tanning structure. Hence, the collagen may be still protected from enzymatic action by Cr(III) complexes as well as the polyphenolic tanning matrix. The results in indicated that enzymatic digestion of the leathers occurs only when the tanning matrix has been disrupted and after the collagen molecule has also undergone significant structural changes as a result of the oxidative degradation.

6.3.2.3. Fenton oxidised samples

As demonstrated in Chapter 5, oxidation of leather and untanned collagen through Fenton reaction resulted in large decline of T_s and deformation of fibre structure in the samples. The oxidation of leathers by the Fenton reaction appears to be a simple procedure that brings about a rapid decomposition of the tanning matrix and denaturation of the collagen in the leather. The results of enzymatic treatments of the Fenton oxidised samples (Table 6.6 and Table 6.7) also showed that the protein component of the samples is susceptible to solubilisation by the action of the enzymes.

Table 6.6 - Concentrations of hydroxyproline in analyte solutions (50 mL) of acid-hydrolysis and enzymatic digestions of samples oxidised with the Fenton reaction (0.2M H_2O_2/0.03M Fe^{2+}) using collagenase; the degree of enzymatic hydrolysis (%)

Sample	Hydroxyproline concentration (µg/ml) in analyte solutions (50 mL)		Degree of enzymatic hydrolysis (%)
	acid hydrolysis	collagenase	
collagen	212.5	85.0	40.2
Cr(III)-Leather	146.0	0	0
Cr(III)-mimosa leather	112.0	0	0
Cr(III)-chestnut leather	105.0	0	0
mimosa	191.0	55.0	29.6
chestnut	202.0	85.0	42.8

Table 6.7 Concentrations of hydroxyproline in analyte solutions (50 mL) of acid-hydrolysis and enzymatic digestions of Fenton-oxidised samples (0.2M H_2O_2/0.03 M Fe^{2+}) using subtilisin; degree of enzymatic hydrolysis (%)

Sample	Hydroxyproline concentration (µg/ml) in analyte solutions (50 mL)		Degree of enzymatic hydrolysis (%)
	acid hydrolysis	subtilisin	
collagen	212.5	147.0	69.3
Cr(III) tanned leather	146.0	48.5	33.3
Cr(III)-mimosa leather	112.0	34.5	30.7
Cr(III)-chestnut leather	105.0	41.5	39.8
Mimosa tanned leather	191.0	57.0	29.8
Chestnut leather	202.0	71.0	35.1

As shown in, collagenase hydrolysed 30-43% the protein component of the oxidised vegetable tanned leathers, about 40 % of the Fenton oxidised collagen was also hydrolysed by subtilisin. In contrast, all of the Fenton oxidised Cr(III) containing samples, showed no sign degradation activity with collagenase (Table 6.6). In contrast,

all of the Fenton treated leathers were partially digested with subtilisin enzyme, showing solubilisation of the protein in the samples, including the Cr(III) containing samples, by by 49-71% (Table 6.7).

It was also observed that the hydroxyproline concentration obtained from acid hydrolysis of the Fenton oxidised collagen sample (212.5 µg/mL) was lower than that of the same analyte solution obtained by acid hydrolysis of the control sample of untanned collagen (286.0 µg/ml). This is because the oxidation collagen may involve reduction in the number of hydroxyproline residues. It is known that oxidation of hydroxyproline containing peptides results in the formation of pyrrol ring (Heidemann 1980). Earlier methods of hydroxyproline analysis indicate that oxidation of hydrolysed collagen can be done using hydroxyl radicals generated by Cu^{2+}/H_2O_2 (Neuman and Logan 1950).

6.3.3. Inhibition of ccollagenase by metal ions

Comparison of the rate of hydroxyproline released by enzymatic digestion of untanned collagen (hide powder) in the presence and absence of metal ions (Figure 6.5), indicates that V(IV), Fe(II) and Cr(III) cause lowering in concentration of hydroxyproline liberated as compared to the control sample (not containing metal).

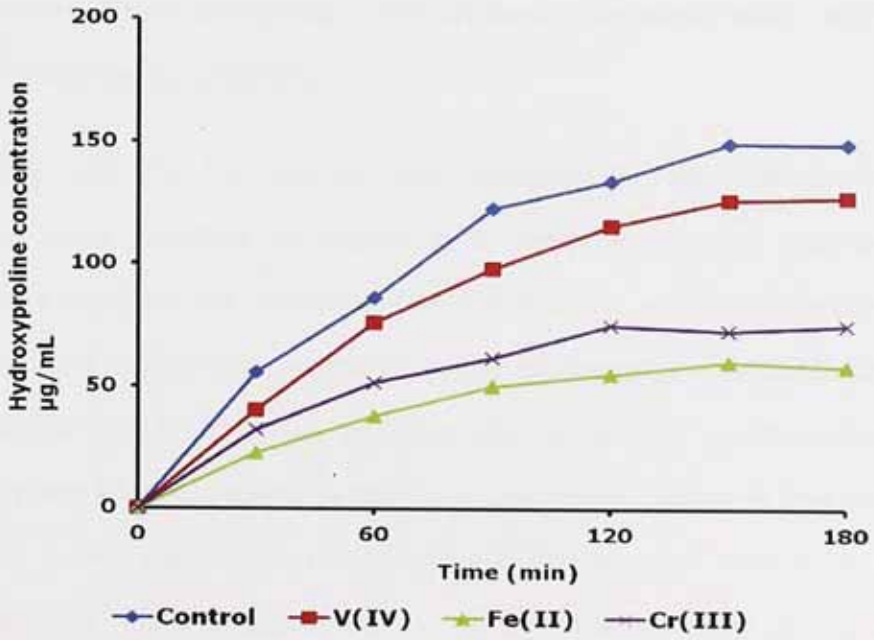


Figure 6.2 Concentrations of hydroxyproline (µg/mL) liberated by digestion of hide powder (100mg) using collagenase enzyme in the presence and absence of metal ions.

After 30 minutes of incubation, the control sample showed 55 µg/mL of hydroxyproline in the 50 mL analyte solutions; whereas the corresponding values for the Fe(II), Cr(III) and V(IV) containing samples were 22, 36 and 40 µg/mL, respectively. Compared to the control sample, the initial rate of the enzyme reactions in the Fe(II), Cr(III), and V(IV) containing samples were inhibited by 60%, 35% and 28% respectively. A similar pattern of differences in the enzyme activity was observed with the metal containing samples incubated for longer durations (i.e. 60-180 minutes)

This result clearly indicates that the presence of transition metals ions causes inhibition of collagenase activity in the a decreasing order of Fe(II)>Cr(III)>V(IV). According to Gayatri *et al.* (2000), inhibition of collagenase by Cr(III) complexes may occur by blocking the active sites and causing changes on the secondary structure of the enzyme. Collagenase enzymes contain Zn^{2+} ions in their structure at the active sites (Bond *et al.* 1984). According to Karakiulakis *et al.* (1991), the inhibitory effect of Fe(II) may be due to displacement of the Zn^{2+} ions in the enzyme structure by the other cations. The absence of collagen degradation during digestion of the Fenton-oxidised Cr(III) containing samples with collagenase (Section 6.3.2.2-3) could be related to inhibition of the enzyme by Cr(III), rather than resistance of the partially degraded substrate to the enzymatic action. In both cases, the Cr(III) containing samples showed relatively greater degree of degradation by subtilisin.

It is observed that the thermophilic and bread-specific bacterial alkaline protease (subtilisin) is more effective in degrading oxidatively degraded leathers. Bacterial enzymes with a subtilisin-like properties (*i.e.* thermophilic and bread-specificity) may be the most important degradative agents in biodegradation of protein containing waste. In fact, the subtilisin and other similar enzymes are produced by a wide range of bacteria that also survive in soil, including *Bacillus Lichniformis*, which is the source of the enzyme used in this experiment (see enzyme descriptions in Table 6.1), the Bacillus species, commonly found in soil, are also one the different types of micro-organisms responsible for the biodegradation in composting and anaerobic digestion.

6.4. Summary

The results in this chapter indicate that metal-induced degradation of vegetable tanned leathers and direct oxidative degradation of leather through Fenton reaction result in an increased susceptibility of all types of leathers to enzymatic degradation. However, the collagenase activity may also be inhibited by metal ions, particularly by Fe(II) and Cr(III).

The overall results of the enzymatic degradation tests indicate that the thermophilic and broad-specificity enzymes such as subtilisin may be effective in hydrolysing oxidatively degraded leather as they can remain stable and active at higher temperatures. Oxidative degradation of leather causes decomposition of tanning structures and denaturation the collagen. Subsequently, leather that have undergone oxidative degradation are significantly degradable by enzymes.

In terms of the application of this finding in waste management, the implication of the results obtained in this research is that, oxidative pre-treatment may be applied to make leather waste biodegradable; this may open new possibilities for recycling of leather waste through aerobic digestion (composting) and anaerobic digestion methods.

CHAPTER 7

CONCLUSIONS AND FUTURE WORK

7. Conclusions and Future Work

The results obtained in this research on the stability of semi-metal tanned leathers and metal catalysed oxidative degradation are hereby summarised in Sections 7.1-3 and the potential application of Fenton reaction in the recycling of leather waste are also described in Section 7.4 along with important aspects on future research work in this area (Section 7.4)

7.1. Reversal of tanning interaction and autodegradation of leather

Cross-linking of polyphenolic structures in vegetable tanned leathers by formation of chelate complexes with metals, reinforces tanning structure. As a result, semi-metal tanned leathers, prepared by retanning vegetable tanned leathers with metal, generally have show increased hydrothermal stability. The results described in Chapter 2 (Section 2.3.4) showed that vegetable tanned samples retanned with Al(III) salt show the highest shrinkage temperature. Similarly, the semi-metal tanned samples prepared using Ti(IV), V(IV) and Cr(III) salts showed shrinkage temperature greater than 100°C. The increased hydrothermal stabilisation in the semi-Al(III), semi-Ti(IV) and semi-V(IV) samples is attributed to a synergistic effect of the metal-tannin, tannin-collagen and metal-collagen interactions. Whereas, in the case of semi-Cr(III) tanned leathers, the relatively greater T_s (100-103°C) is considered to be mainly due to the Cr(III)-collagen interaction

Periodic DSC analysis of semi-metal tanned hide powder samples showed that while most of the semi-metal tanned samples of the first-row transition metals (i.e. sulfate salts) showed consistent T_s during storage, the V(IV) and Fe(II) retanned samples exhibited progressive lowering T_s. Based on comparative analysis of the observed changes in the T_s of metal-tanned and semi-metal tanned samples, it is concluded that in T_s in the semi-V(IV) and semi-Fe(II) samples is caused by redox reactions involving the tannins and the metal ions in the samples (Chapter 2, Section 2.3.7.1-2). It is observed that autoxidation of metal ions (e.g. Fe^{2+}/Fe^{3+} and VO^{2+}/VO_2^{+}) and subsequent metal-tannin redox reaction may lead to decomposition polyphenolic tannin structures and

subsequently cause collagen denaturation as observed with the results of periodic analysis of enthalpy and temperature of shrinkage of the semi-V(IV) tanned hide powder samples (Chapter 1, Sections 2.3.7.2-3).

As described in Chapter 3 (Section 3.3.2.2), The semi-Ti(IV) leathers, produced using different vegetable tanned leathers, exhibited a slow decline of hydrothermal stability over 12 months year to that of the vegetable tanned leathers. The observed changes are considered to be results of a reversal of the Ti(IV)-tannin interaction occurring due to hydrolysis of Ti(IV) ions.

On the other hand, progressive lowering of T_s as well as occurrence of physical degradation and chemical denaturation of collagen were observed in the semi-metal tanned leather samples produced using iron (Fe^{2+}, Fe^{3+}) and vanadium (VO^{2+} and VO_2^{+}) as described in Sections 3.3.2.2-3. These changes were characterised as metal-induced autodegradation occurring at ambient conditions. It was shown that V(IV) and Fe(II) induced degradations may also occur in the presence of Cr(III) tanning structure *i.e.* Cr(III)-mimosa and Cr(III)-chestnut tanned leathers (Section 3.3.7). Based on the fact that hide powder and Cr(III)-tanned samples, treated with V(IV) or Fe(II) salts did not show degradation, it is considered that the degradation mechanism is essentially related to metal-tannin interactions, rather than metal-collagen interactions. In addition to DSC analysis, the process of autodegration in the different semi-metal tanned leathers was also characterised by periodic analysis of tensile strength, extractable metal content, pH value of the leathers as well as SEM analysis of fibre structure (Sections 3.3)

The results of comparison of changes in T_s of mimosa tanned leather samples treated with different concentrations of V(IV) and V(V) revealed that V(IV) induced degradation may occurs in the presence of a relatively small quantity (<1000 ppm) of vanadium (Chapter 3, Section 3.3.6). It is concluded that the role of V(IV) in the autodegradation

of the leather is related to triggering of redox reactions and catalysis of the oxidation of tannins in the tanning matrix.

7.2. Mechanism of metal induced degradation

The loss of hydrothermal stability and autodegradation observed in the semi-Fe(II) and semi-V(IV) tanned leathers are considered to be the results of redox interactions. The occurrence of Fe(II) and V(IV) catalysed oxidative decomposition of polyphenols was explained based on the results the experiments described in (Chapter 4, Sections 4.3.2-5) and literature metal-tannin redox reactions. The formation of oxygen derived free radicals is considered to be an important aspect in relation to catalysis of the oxidative decomposition of tannins in the presence of iron (Fe^{2+}/Fe^{3+}), vanadium (V^{4+}/V^{5+}) is considered to be an important aspect, particularly in relation to the cause of the observed denaturation of collagen. The mechanism proposed to explain metal-induced degradation show that the reactions responsible for metal induced degradation of leather includes autoxidation of Fe(II) and V(IV) by air to the higher oxidation states with subsequent formation of superoxide anion and reduction of the higher oxidation states of the metal ions by polyphenols.

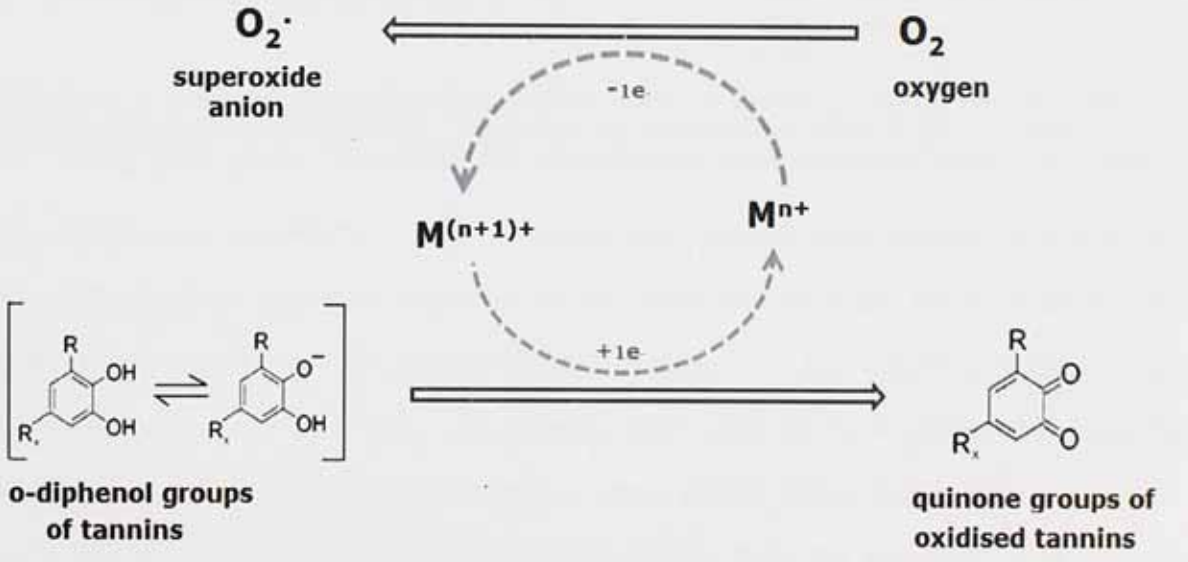


Figure 7.1. Schematic representation of the redox cycling of metal ions involving oxidation of tannins to quinoid structures and reduction of oxygen to superoxide anion; M^{n+} represent metal ions such as Fe(II) and V(IV) ions that can undergo autoxidation in aerobic conditions to higher the oxidations states $M^{(n+1)+}$

The concurrence of these two reactions (Figure 6.1) leads to creation of a cyclic redox mechanism in which the polyphenols are continually oxidised *i.e.* the *o*-diphenol groups of the tannins are converted to quinone groups. The degradation of the metal containing vegetable tanned leathers is enhanced further due to the formation of hydrogen peroxide through a subsequent catalytic generation of hydroxyl radicals (the Fenton mechanism) as shown in Figure 7.2. Hydroxyl radicals are powerful oxidants that can progressively oxidise the large polyphenolic structure of tannins as well as the collagen in the leather.

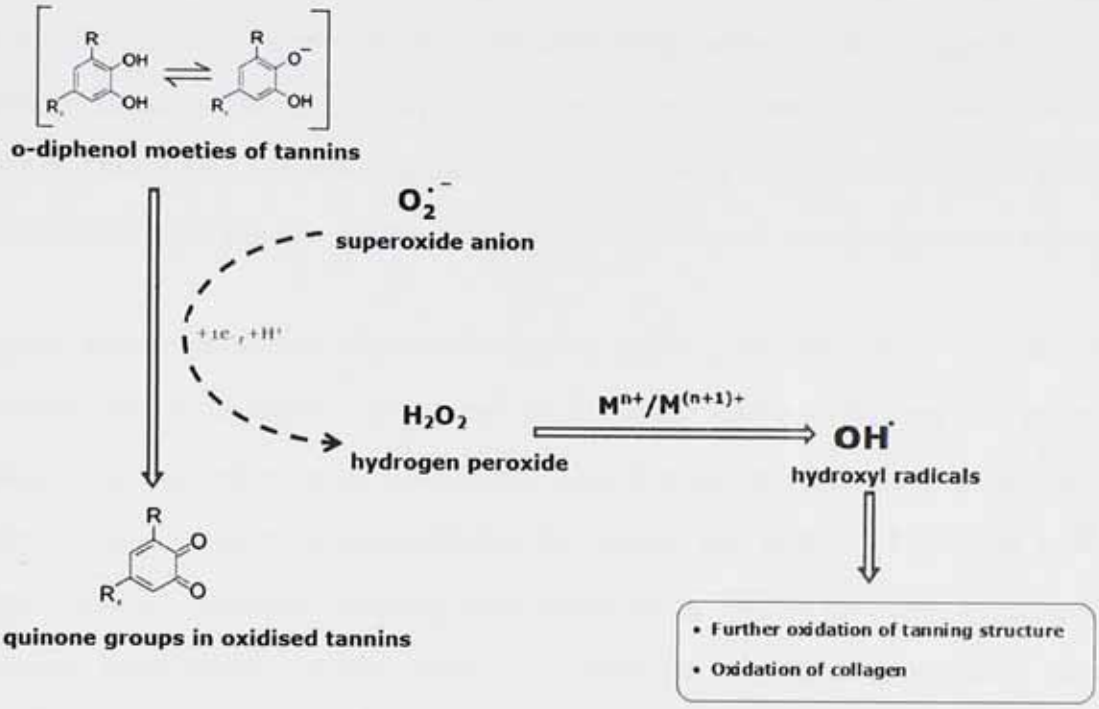


Figure 7.2. Schematic representation of redox reactions including oxidation of the o-diphenol moieties of tannins by superoxide anions leading to formation of hydrogen peroxide and the formation of hydroxyl radicals through the metal catalysed decomposition of hydrogen peroxide.

The observation that some transition metals may catalyse deterioration of leather has been described by literature of leather conservation (Haines 2006; Thomson 2006). The mechanism proposed in this research (shown in Figure 7.1 and 7.2) may also be applied to explain the role of metal ions (mainly Fe^{2+} and Cu^{2+}) in catalysing oxidative deterioration of vegetable tanned leathers. Investigation of the degradation mechanism of Fe(II) and V(IV) has led to the understanding that the principal cause for the observed physico-chemical degradation and collagen denaturation is the formation of hydroxyl radicals in leather through metal catalysed redox processes. As described in

Chapter 5 (Sections 5.3.2-4), exposure of leathers to hydroxyl radical generated by Fenton reaction resulted in a rapid oxidative degradation of the tanning structure as well as the collagen in the leather.

7.3. Enzymatic degradability of oxidatively degraded leather

As described in Chapter 6, oxidative degradation of leather causes chemical decomposition of the tanning matrix and chemical denaturation of the collagen in the leathers, thereby subsequently resulting in an increased level of enzymatic degradability. Thermophilic alkaline protease enzymes that have broad substrate specificity (e.g. subtilisin) substrate specificity may be more effective in digesting the partially degraded leathers. Oxidatively degraded leathers may also be digested by collagenase enzyme; but susceptibility of the enzyme to inhibition by metal ions is also an important aspect.

Previous studies related to recycling of leather waste (Dhayalan 2005; Yagoub 2006), indicated that susceptibility of leather to microbial degradation may be enhanced through chemical treatments (detanning) and thermal denaturation. Based on the findings in this research, it is considered that partial degradation of different types of leathers through oxidative methods, particularly using Fenton reactions, may result in increased susceptibility of the resulting material to microbial degradation. Hence, oxidative pre-treatment of leather waste has a potential application in developing recycling methods such as composting and anaerobic digestion of leather waste.

7.4. Potential application of oxidative degradation of leather

The stability of leather against biodegradation has been the major hindrance with respect to attempts made in the past to recycle leather waste into fertilizer (through composting) as well as into biogas (through anaerobic digestion). The possibility for enhancement of the biodegradability of different types of leather waste through detanning-treatments, thermal treatments and alkaline hydrolysis was recognised (Ferriera *et. al.* 2013; Lima *et al.* 2010).

As described in Chapter 1 (Section 1.9.3), Covington (2009), suggested that the mechanism of vanadium(IV) induced degradation of vegetable tanned leather may be emulated to develop tanning methods for producing usable leather that also incorporates within it a system of slow degradation. However, the results described in Chapter 3 indicated that the presence of vanadium may cause a rapid degradation even at relatively small quantities (<1000ppm). The spontaneous nature of V(IV) induced degradation would negatively affect the usability of the leather. Hence, producing autodegradable leather using metal ions such as vanadium is most likely to be impractical. On the other hand, the results from this research on the mechanism of metal catalysed oxidative degradation of leather revealed that the degradation process occurs through decomposition of tanning structures and denaturation of collagen, eventually leading to susceptibility of the leather to enzymatic degradation. As stated in Section 7.2, metal induced degradation of leathers (containing iron and vanadium ions) occurs through a mechanism that involves hydroxyl radical mediated oxidation.

A direct application of hydroxyl radical mediated oxidative degradation, by means of Fenton reaction (solution of H_2O_2, in presence of Fe^{2+}) causes a rapid degradation of different types of leathers in less than a day (Chapter 5). The concentration the Fenton reagent required for oxidative treatment (lab scale) is relatively dilute *i.e.* 0.2M H_2O_2, 0.07M Fe^{2+}. This shows that Fenton reaction may be an efficient oxidation method that may be applied for pre-treatment of leather waste to enhance its biodegradation, particularly in the context of recycling through composting or anaerobic digestion. The efficiency of the reaction implies that at larger scale biodegradation enhancing pre-treatment operations may be cost-effective.

7.5. Future work

The Fenton reaction has already been for detoxification of organic waste, treatment of pesticides and leachate from composting plants. Treatment of leather waste Fenton oxidation process may be developed as efficient method for pre-treatment of leather waste, prior to biodegradation of leather waste. Alternatively, composting of leather

waste may carried out in the form of an accelerate-compost process in which an optimised application Fenton reagent (Fe^{2+}/H_2O_2) may be used enhance decomposition of organic matter and enhance biodegradation. The effectiveness of Fenton reaction in compost acceleration thorough enhancement of decomposition of macromolecules (such as lignin) has been demonstrated by Balis *et al.* (2002).

The application of Fenton oxidation for treatment of non-Cr(III) tanned leathers may be developed into an effective recycling of leather waste to fertiliser. On the other hand, the presence of Cr(III) in most types of leathers would also entail the need to extract the metal through an effective method. In Europe, the allowable limits of Cr(III) for compost fertilisers are 70-200 mg/kg of dry matter, while in North America the Cr(III) limit is 1200 mg/kg (Hogg *et al.* 2002; Brinton 2000). In addition, oxidative treatment of Cr(III) containing leather waste through Fenton reaction may involve formation of Cr(VI), which is a carcinogen. Based on recognition of the potential of the Fenton reaction as a means of enhancing biodegradability of leather waste and considering the environmental issues related to Cr(VI), the following areas of further research are indicated:-

- Oxidative treatment of leather waste using Fenton reaction may have to be optimised considering parameters such as particle size, pH profile of the reaction, oxidant/substrate ratio, and further study in this area is relevant.

- The extraction of Cr(III) may be considered as part of the oxidative treatment of leather waste; in this regard, the detanning using α-hydroxyl carboxylates (Malek et al. 2009) such as tartrate salts, may be investigated further.

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APPENDICES

Appendix 1

A general outline of operations in the leather manufacturing

Process	Operation	Main effects of the operation on the pelt	Description of operation and chemicals used
Beam house process	Soaking	○ Removal of surface dirt preservation salt ○ Uniform rehydration	○ Washing ○ Treatment of pelts at pH 7-9 in presence of bactericide and surfactants.
	Liming	○ Removal of hair ○ alkaline hydrolysis of non-collagen proteins and proteoglycans ○ Saponification of fats ○ Increased separation of fibres (opening up of fibre structure)	○ Treatment with sodium sulfide (Na_2S) and other unhairing chemicals in presence of lime (CaO) ○ Treatment of pelts in alkaline media (pH>12.5) in saturated solution of lime (CaO)
	Mechanical operations	○ Removal of subcutaneous tissue ○ Thickness adjustment	○ Mechanical operations (fleshing and splitting)
	Deliming	○ Lowering the pH to 8.0-8.5 ○ Removal of Ca^{2+} ions	○ Treatment with ammonium salts or weak salts
	Bating	○ Further removal of partially degraded non-collagenous impurities	○ Treatment using pancreatic or bacterial enzymes
	Pickling	○ Adjustment of the pH of pelts to prepare for subsequent tanning operations	○ Acidification in the presence of common salt (NaCl)
Tanning process	Tanning	○ Permanent stabilisation of collagen against putrefaction ○ Increase in hydrothermal stability and change in physical characteristics	○ Treatment of pelt with a selected tanning chemical
Post tanning process	Retanning	○ Modification of physical and chemical properties (e.g. fullness, stiffness, resistance to perspiration,)	○ Additional tanning operations to using a one or more tanning agents
	Dyeing	○ Altering the colour imparted by tannage as desired in the final product	○ Dyeing mainly with anionic, cationic and metal-complex dyes
	Fatliquoring	○ Prevention of fibre cohesion upon drying ○ Enhancing softness	○ Treatment with oil-in-water emulsions using neutral oils and modified oils (sulfated and sulfited oils)
Finishing process	Finishing	○ Formation of a protective layer, modification of surface texture and colour ○ Enhancement of aesthetic quality	○ Application of finish films by spraying and roller-coating.

Appendix 2

Shrinkage temperature and enthalpy of hide powder samples

(a) Metal tanned hide powder samples

Shrinkage temperature (T_s,°C) of hide powder samples (2g) tanned with metal salts (equivalent to 100 mg of metal ion) and stored at 21°C and 65% RH for 64 days

	Time (days)				
Metal	0	2	8	32	64
Al(III)	73.5	73.4	73.8	73.8	73.8
Ti(IV)	68.5	68.7	69.0	69.0	69.0
V(IV)	71.7	65.3	58.9	58.9	58.9
V(V)	60.1	60.1	59.2	59.5	58.9
Cr(III)	94.0	94.0	95.0	94.8	94.9
Mn(II)	62.5	62.5	61.4	61.4	61.4
Fe(II)	70.4	70.4	73.7	72.9	72.8
Co(II)	69.6	69.9	70.2	70.3	70.3
Ni(II)	64.7	64.9	64.0	64.3	64.1
Cu(II)	67.7	67.4	67.7	67.3	67.5
Zn(II)	61.3	61.3	61.6	60.7	61.1

(b) Semi-metal tanned hide powder samples

Shrinkage temperatures (T_s) of hide powder samples tanned with 10 % (w/w) of myrababan powder mimosa powder and retanned with different metal salts. Measurements of T_s were carried out at different times over 64 days of storage at 21°C and 65% relative humidity

Myrabalan based semi-metal tanned hide powder samples					
	Time (days)				
metal	0	2	8	32	64
Al(III)	103.4	103.2	103.5	103.9	103.9
Ti(IV)	99.8	99.5	98.8	98.2	97.8
V(IV)	95.7	74.7	72.6	67.3	47.4
Cr(III)	103.0	104.1	105.2	104.2	104.1
Mn(II)	76.8	72.1	72.2	74.2	74.1
Fe(II)	97.9	91.2	90.0	81.7	68.7
Co(II)	78.0	76.6	77.9	77.3	77.6
Ni(II)	78.7	77.2	76.0	76.3	76.1
Cu(II)	89.9	88.7	87.0	80.6	77.6
Zn(II)	75.7	76.5	77.5	76.5	77.1

Mimosa based semi-metal tanned hide powder samples					
	Time (days)				
metal	0	2	8	32	64
Al(III)	108.6	109.3	108.5	108.3	108.7
Ti(IV)	101.1	100.0	99.8	99.7	98.9
V(IV)	105.3	90.8	72.4	67.6	46.5
Cr(III)	104.1	105.4	101.5	104.5	104.2
Mn(II)	86.6	86.3	85.7	85.0	84.5
Fe(II)	97.9	91.3	90.2	80.7	68.6
Co(II)	85.8	84.7	86.1	84.3	84.1
Ni(II)	84.0	84.9	83.0	82.7	84.0
Cu(II)	88.9	88.7	87.0	83.6	82.5
Zn(II)	84.9	85.0	86.4	84.2	84.0

Appendix 2 (continued)

Enthalpy of the shrinkage transition in Joules per gram of the semi-metal tanned hide powder samples, measured at different times of storage at 21°C and 65% RH

Myrabalan bases semi-metal tanned hide powder samples

metal	Time (days)				
	0	2	8	32	64
Al(III)	11.9	11.2	11.9	12.2	11.4
Ti(IV)	11.4	12.3	11.6	12.4	13.5
V(IV)	13.2	13.3	12.1	9.8	6.7
Cr(III)	11.0	11.6	12.4	12.5	13.1
Mn(II)	12.4	11.3	11.6	11.4	12.4
Fe(II)	12.4	11.5	11.6	12.5	12.1
Co(II)	12.4	12.7	13.6	12.2	13.2
Ni(II)	12.4	11.0	11.6	11.4	11.7
Cu(II)	11.4	12.2	12.8	13.4	11.5
Zn(II)	12.4	13.1	12.0	11.7	11.9

Mimosa based semi-metal tanned hide powder samples

metal	Time (days)				
	0	2	8	32	64
Al(III)	12.6	11.6	12.9	11.1	12.5
Ti(IV)	12.0	13.7	12.2	12.7	14.8
V(IV)	12.1	11.3	11.9	11.3	7.5
Cr(III)	13.6	13.4	11.7	10.9	12.9
Mn(II)	14.1	12.2	11.7	11.4	11.8
Fe(II)	12.5	12.7	11.8	13.7	12.2
Co(II)	13.6	12.5	13.7	12.8	12.7
Ni(II)	12.5	10.8	10.6	11.7	11.8
Cu(II)	11.5	12.6	12.8	13.5	12.3
Zn(II)	13.6	13.4	11.7	10.9	12.9

Appendix 3

Preparation of vegetable tanned sheep skins

Raw material: Degreased pickled sheep skins

Vessel: Pilot scale trial drum, DOSE drum, 60cm diameter (Dose Gmbh, Germany)

Process	Inputs		Parameters			
	Material and Chemicals	Qty. (%)[a]	Run Time (min)	Temp. °C (±1)	pH (±0.1)	Specific gravity
Weighing						
De-pickling	Water	150		20		
	Salt	7.5	5			6°Be
pH adjustment	Load pickled skins.		30		2.3	
	Sodium formate	0.5			3.2	
Pre-tanning	Derugan 3080 [b]	3%	60	25		
	Sodium formate	1.0%	45		4.2	
	Sodium bicarbonate	0.75 %	45		6.0	
	Sodium hydrogen sulfite	0.1%	20			
Degreasing	Pastozol BZ conc. [c]	1%	90	35		
	Wash (2X),water	150		35		
	Water	150		25		6°Be
	Sodium sulfate	8%	10			
Tanning	Veg-tan powder [d]	3%	30			
	Veg-tan powder	3%	60			
	Veg-tan powder	4%	120			
Check pH	Formic acid	0.5%		30	4.0	
	Water	100	5			
Antifungal	Preventol WB [e]	0.1%	5			
Horse up overnight, Samm, Shave, Toggle dry						

(a) All weights are calculated as percents of the weight of the pickled skins

(b) Derugan 3080 : Glutaraldehyde based pre-tanning agent (from Trumpler Chemicals Gmbh),

(c) Pastozole BZ : Non-ionic degreasing agent (surfactant), Trumpler Gmbh

(d) Veg-tan powder: four different type of samples were prepared using myrobalan, Quebracho (Indusol ATO) and chestnut tanning powders (from SilvaTeam Spa.); Mimosa ME tanning powder from Mimosa-chemicals SA Ltd

(e) Preventol WB : Anti-fungal chemical from Lanxess Ltd. UK

Appendix 4

Preparation of semi-metal tanned sheep skin samples

Raw material: Vegetable tanned crust leather sample pieces (20cmX20cm)

Vessel: Lab-scale drums (30 cm diameter)

Process	Inputs		Parameters		
	Material and Chemicals	Qty. (%) [a]	Run Time (min)	Temp. °C	pH (±0.1)
Re-wetting	Water	1 lt		35	
	Corilene W85 [b]	5 g			
	Oxalic acid	1 g	20		
Wash,drain and weigh					
Re-tanning	Water	100%	20	25	
	Acetic acid	0.5%	20		3.2-3.5
	Metal ion [c]	1%	120		
Basification to pH4.0	Sodium Bicarbonate [d]	0.25%	30		5.0
Run drum			60	35	
Drain					
Rinse,	Water	100%			
Drying in air					

(a) All quantities mentioned in % were calculated based on the weight of the wet vegetable tanned leathers.

(b) Corilene W385 (from Stahl Chemicals B.V, Netherlands) = anionic surfactant (wetting agent) generally used for uniform rehydration dry leather

(c) Metal ion:- added in the 1% of the metal (on the weight of the wet-leather)

(d) Basification: addition of 0.25% sodium bicarbonate and running 20 minute, carried out repeatedly until the pH reached 5.0±0.1

Appendix 5

Preparation of Cr(III) tanned sheep skins

Raw material: Degreased pickled sheep skins

Vessel: Pilot scale trial drum, DOSE drum, 60cm diameter (Dose Gmbh, Germany)

Process	Inputs		Parameters			
	Material and Chemicals	Qty.[a]	Time (min)		Temp. °C (±1)	pH (±1)
			Run	Rest		
Weighing						
pH adjustment	Water	200			25	
	Sodium chloride	5.%	10			
	Load pickled skins		30			2.3
	Sodium bicarbonate	1.5%	45			4.5
Degreasing	Pastozol BZ	1.0	60		30	
	Wash, Drain					
	Water	100	5			6.5
	Salt	5.0	5			
	Formic acid	0.75	20			2
	Sulfuric acid	1.0	20	O/N		2.7
Tanning	Chromosal B [b]	6%	180		30	
	Preventol WB	0.1%				
	Sod. bicarbonate	0.5	30			4.0
Drain, Horse up 24 hours						
Sammying (remove excess water),						
Shaving : thickness 1.5mm						

(a) All weights calculated as percent of the pickled weight

(b) Chromosal B (Lanxess Ltd. UK)- Basic Chromium(III) Sulfate powder (25%Cr_2O_3, 33% basicity)

Appendix 6

Preparation of Cr(III)-Mimosa and Cr(III)-Chestnut leathers

Raw material: Chromium(III) tanned skins, sammed and shaved to 1.5 mm

Vessel: Pilot scale trial drum, DOSE drum, 60cm diameter (Dose Gmbh, Germany)

Process	Inputs		Parameters		
	Material and Chemicals	**Qty.[a]**	**Time (min)**	**Temp. °C (±1)**	**pH (±1)**
Rehydration	Chromium(III) tanned leather				
	Water	100%			
	Corilene W385				
Neutralisation /pH adjustment/	Sodium acetate	0.50%			
	Sodium bicarbonate	0.75%	60	35	5.4
Tanning	Veg-tanning powder	5%	120	35	
pH adjustment	Formic acid	0.5%	30		3.8-4.0
Horse up 24hours, Wash, Sammying (remove excess water)					
Dry and store at 21°C and 65% relative humidity					

(a) All weights calculated as percent of the wet Cr(III) tanned leather after shaving

Appendix 7

Tensile strength and Elongation-at-break of semi-metal tanned leathers

(a) Tensile strength (MPa) of mimosa based semi-metal tanned leathers stored at 21°C and 65% RH for a year

Metal	Time (months)				
	0	1	3	6	12
Al(III)	11.4	11.5	11.3	11.0	11.2
Ti(IV)	11.1	10.8	11.0	10.8	10.6
Fe(II)	10.8	11.2	9.6	8.6	6.1
Fe(III)	12.1	11.6	10.2	7.9	5.5
V(IV)	11.4	10.4	8.9	7.4	3.7
V(V)	10.5	7.3	6.9	5.7	4.4

(b) Elongation at break (%) of mimosa based semi-metal tanned leathers stored at 21°C and 65% RH for a year

Metal	Time (months)				
	0	1	3	6	12
Al(III)	45.8±2.6	46.6±3.1	42.5±3.0	48.7±3.5	45.0±2.2
Ti(IV)	69.9±5.1	68.4±4.8	69.5±6.0	70.2±5.2	64.9+4.6
Fe(II)	54.4±5.1	49.6±3.3	38.1±3.6	39.2±4.8	35.3+3.4
Fe(III)	60.3+5.4	55.8+5.1	45.3±2.8	38.6±3.2	33.1+2.8
V(IV)	69.8+5.5	55.2±4.8	46.9±4.6	39.4±4.0	38.7+2.6
V(V)	53.3+2.8	50.0+2.5	44.9+4.2	42.9+5.5	30.2+4.8

(c) Tensile strength (MPa) of Myrabalan based semi-metal tanned leathers stored at 21°C and 65% RH for a year

Metal	Time (months)				
	0	1	3	6	12
Al(III)	11.0	10.7	10.2	10.7	10.4
Ti(IV)	9.9	10.3	10.2	10.2	10.4
Fe(II)	10.6	9.3	8.8	7.5	6.7
Fe(III)	9.9	8.7	7.9	7.1	5.9
V(IV)	6.3	4.9	4.2	3.5	2.9
V(V)	8.4	5.5	5.0	3.5	3.1

(d) Elongation at break (%) of Myrabalan based semi-metal tanned leathers stored at 21°C and 65% RH for a year

Metal	Time (months)				
	Initial	1	3	6	12
Al(III)	47.0±3.6	48.6±7.1	48.1±5.1	47.6±5.2	49.2+2.1
Ti(IV)	60.1±4.1	55.1±6.8	61.2±6.3	58.3+5.9	57.3±3.9
Fe(II)	44.9±5.0	46.9±5.2	48.1±5.4	36.0+5.2	37.2±2.5
Fe(III)	47.3±6.3	46.5±4.8	34.9±4.8	32.5+4.1	30.2±4.7
V(IV)	66.1±6.2	39.4±4.2	36.5±3.5	31.7+4.4	32.1±2.0
V(V)	56.0±4.0	59.0+5.7	45.0±5.1	32.1+5.6	28.1±4.2

Appendix 8

pH of myrabalan-based semi-metal tanned leathers

Table 3.5 The pH values (±0.1) of the Myrabalan based semi-metal tanned leathers

Sample	initial	after 3 months	after 6 months
Myrabalan	5.5	5.5	5.5
Myrabalan -Al(III)	5.2	5.2	5.1
Myrabalan -Ti(IV)	3.9	3.8	3.8
Myrabalan -V(IV)	4.4	3.7	3.7
Myrabalan -V(V)	4.5	4.2	4.1
Myrabalan -Fe(II)	4.8	4.2	3.4
Myrabalan -Fe(III)	4.4	3.7	3.6

Appendix 9

Summary of ANOVA - Free radical scavenging assay

Absorbance data

S/N	Sample	Absorbance data (2 trials, triplet measurements)						Mean	SD	Error, %
1	DPPH blank	2.347	2.351	2.358	2.408	2.348	2.347	2.360	0.024	1.017
2	Methyl gallate	0.108	0.109	0.110	0.109	0.109	0.108	0.109	0.001	0.569
3	Methyl gallate- Al(III)	0.109	0.108	0.109	0.109	0.109	0.109	0.109	0.000	0.324
4	Methyl gallate- Ti(IV)	0.365	0.357	0.364	0.367	0.365	0.367	0.364	0.004	1.100
5	Methyl gallate- Fe(II)	0.371	0.357	0.356	0.362	0.357	0.361	0.363	0.012	1.204
6	Methyl gallate- Fe(III)	0.353	0.359	0.352	0.355	0.347	0.352	0.353	0.004	1.142
7	Methyl gallate-V(IV)	0.333	0.332	0.331	0.330	0.331	0.333	0.332	0.001	0.404
8	Methyl gallate-V(V)	0.716	0.716	0.716	0.719	0.714	0.722	0.717	0.003	0.388

N.B.:- Standard deviation is denoted as SD and the Error % is estimated as relative standard deviation calculated as percentage of the SD to the mean value

Descriptive Statistics

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	25.520	13	1.963	100.566	.000
Within Groups	1.366	70	.020		
Total	26.886	83			

Oneway ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	25.520	13	1.963	100.566	.000
Within Groups	1.366	70	.020		
Total	26.886	83			

Hypothesis test summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Absorbance is the same across categories of Sample.	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis

Asymptotic significances are displayed. The significance level is .05.