

**BASIC STUDIES OF THE CROSS-LINKING
REACTION OF COLLAGEN WITH
OXAZOLIDINE AND PLANT POLYPHENOLS**

By

LIJIANG SONG

A thesis
submitted to
University of Leicester

In partial fulfilment of the requirements for the degree of
Doctor of Philosophy

British School of Leather Technology
University College Northampton

Northampton, UK, 2003



BASIC STUDIES OF THE CROSS-LINKING REACTION OF COLLAGEN WITH OXAZOLIDINE AND PLANT POLYPHENOLS

Lijiang Song

ABSTRACT

To elucidate the reaction between collagen, condensed tannins and oxazolidine, two prodelphinidin tannins have been studied: extracts from pecan nutshell pith and *myrica esculenta* bark. The tannin chemistry study showed pecan tannins are copolymers of catechin and gallo catechin with a ratio 1:6 and myrica tannins are prodelphinidins with 40% of the structural units gallated. The average molecular weight was measured by GPC and the number average molecular weight of tannins was found to be 2500-3000.

The molecular structure of oxazolidine, 1-aza-5-ethyl-1,3-dioxacyclo[3.3.0]octane, was confirmed by IR, GC-MS and ¹H-NMR spectra. From NMR, the ratio between *cis* and *trans* conformations is nearly 1:1. Complete hydrolysis of oxazolidine is slow under weakly acidic or neutral conditions.

The reaction of polyphenols with oxazolidine was modelled using simple phenols phloroglucinol, pyrogallol and resorcinol; the process was followed by chromatographic and NMR methods. The results show the reaction is second order: phloroglucinol has the highest reactivity. By using catechin and gallo catechin, it was shown that the cross-linking reaction of gallo catechins can happen between an A-ring and a B-ring, while for catechin, the reaction is only at the A-ring. Polyphenol polymer studies also support this result; more oxazolidine can be reacted with prodelphinidins.

The reaction of collagen with oxazolidine was studied; the reaction sites were determined using modified collagen. Few cross-linking reactions were found, which is supported by hydrothermal isometric tension (HIT) results. The reaction between polyphenol, collagen and oxazolidine was studied by HIT and it was found that the cross-linking is between collagen and tannins, which is responsible for the high stability of treated collagen. Based on this, organic combination tanning can be explained as an effect of covalent bonding, hydrogen bonding and hydrophobic bonding together, but the high shrinkage temperature is mainly due to the covalent bonding forming a complex matrix structure around the collagen fibres.

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ACKNOWLEDGEMENTS

I am deeply indebted to my supervisors, Professor Anthony D. Covington and Professor Edwin Haslam (Sheffield University), for their support, advice, enthusiasm and patience throughout this research project.

I would like to thank Professor G. Attenburrow, Dr. R.G. Paul, Dr. J. Ding, Professor T. Lilley (Sheffield University), Dr. R. W. Hemingway (USDA, USA) and Professor B. Shi (Sichuan University, China) for their kind helpful discussion and advice. I would like also to thank Dr. G. Griffith (Leicester University) for NMR help, Dr. A. Long for GC-MS and SEM help, Mr. P. Richardson, Mrs. P. Potter, Mrs. A. Michel, Mrs. L. Timson, Dr. M. Syed, Mr. R. Graves, Ms. S. Jeyapalina, Mr. T. Green of BSLT and BLC for their help, technical support and friendship during our time together.

I would like also to thank all the members of my family, especially to my wife - producing this thesis would have been impossible without her help.

To My Mother

ABBREVIATIONS

ΔH	Change in enthalpy
APT	Acetone pecan tannin fraction
BAW	n-butanol:acetic acid:water (14:1:5 v/v/v)
C	Catechin
Ca	Catechol
CBB	Coomassie Brilliant Blue
CHD	1,2-cyclohexanedione
D₂O	Deuterium oxide
D₆-acetone	Hexadeutero acetone
D₆-DMSO	Hexadeutero dimethyl sulphoxide
DSC	Differential scanning calorimetry
EA	Ethyl acetate
EC	Epi-catechin
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
EGC	Epi-gallocatechin
EGCG	Epi-gallocatechin-gallate
FDNB	1-fluoro-2,4-dinitrobenzene
For	Formaldehyde
GC	Gallocatechin
GC-MS	Gas chromatography-mass spectrometry
GPC	Gel permeation chromatography
GTA	Glutaraldehyde
HDC	Hexamethylenediisocyanate
HIT	Hydrothermal isometric tension
HP	Untanned hide powder
HPLC	High performance liquid chromatography
Hyp	Hydroxyproline
IR	Infrared spectroscopy
LC	Liquid chromatography
Me+P	Melamine resin+phosponium salt
MF	Melamine-formaldehyde

Mn	Number average molecular weight
MPT	Methanol pecan tannin fraction
MW	Molecular weight
Mw	Weight average molecular weight
myrica	<i>Myrica esculenta</i> bark extract
NMR	Nuclear magnetic resonance
Ox	Oxazolidine
PC	Procyanidin
Pc	Paper chromatography
PD	Prodelphinidin
Pd	Polydispersity
pecan	Pecan nutshell pith extract
PF	Profisetinidin
Ph	Phloroglucinol
PP	Plant polyphenol
PR	Prorobinetinidin
Py	Pyrogallol
Re	Resorcinol
rpm	Revolutions per minute
SDS-PAGE	Sodium dodecyl sulphate poly-acrylamide gel electrophoresis
SEM	Scanning electron microscopy
SLTC	Society of Leather Technologist and Chemists
T	Vegetable Tannins
Td	Denaturation temperature
THF	Tetrahydrofuran
THPS	Tetrakis hydroxymethyl phosphonium sulphate
TLC	Thin layer chromatography
TNBS	Trinitrobenzene sulphuric acid
TP	Tea polyphenol
Ts	Shrinkage temperature
UV-Vis	Ultraviolet visible light spectroscopy

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Chapter One

General Introduction

1.1 Background

Collagen is the most abundant structural protein in nature; collagen content can reach more than 50% of the dried weight of animal skins or hides or 80-90% of tendon and bone protein^{1-3,20,33,34}. The understanding and utilisation of collagen materials has a long history, probably predating civilisation, which has gradually formed an important industry - leather making⁴. Leather production is composed of a series of procedures that convert putrescible hide or skin into "leather", with chemical and physical stability, for such articles as clothes and shoes^{5,17}. It is worthy of note that nowadays collagen studies are not only the foundation of leather chemistry and engineering, but are of interest in gelatine, biomaterials, biology and related research areas^{1,6-15,19-23}. For example, because of the collagen materials' excellent biocompatibility, the booming tissue engineering technology has given research in collagen materials, such as artificial scaffolds, a new boost¹. Therefore, how to enhance the performance of collagen materials has potential commercial and industrial prospects, not limited to protein chemistry research.

As a natural high polymer, collagen has hydrothermal stability, which comes from inter-molecular and intra-molecular reactions, especially hydrogen bonding, hydrophobic bonding and covalent bonding^{3,8-13,16,35,36,81,83,264,271,272}. There is evidence that the hydrothermal stability of collagen is directly related to the hydroxyproline content, which serves an important function in forming hydrogen bonds^{4,32}. The denaturation temperature of mammalian skin is in the range 60 to 70°C, compared to 38 to 45°C for cold water fish and 50 to 56°C for warm water fish. The hydroxyproline contents in the collagen of these skins are about 13, 6 and 8% respectively. Because of the presence of many charged residues, the electrostatic interactions are also considered important for collagen's molecular organisation^{4,17}. In leather making, stability is determined in the form of the shrinkage temperature, T_s , which is the temperature at which the skin or leather starts to shrink in heated water medium^{5,18}. There is recognition that hydrothermal stability is a useful parameter to measure³² and, for some applications, it is a predictor of the performance of collagen materials. Thus, pursuing high thermal stability has become

one of the important criteria in developing new collagenic materials, including leathers.

The most effective and the commonly used strategies to increase hydrothermal stability are to introduce additional cross-links between the collagen polypeptide chains^{1,19,21-23}. The presence of many reactive sites on the collagen chains, such as carboxyl groups, amino groups, and hydroxyl groups, provide the possibility of natural or artificial cross-linking reactions. In the collagen ageing process, more stable natural cross-links are formed, which reflect the changing of collagen properties from young animals to old²¹⁻²³; for instance, the Ts of calfskin is 60-63°C, while the Ts of old cattle hide is 65-67°C. The tanning procedure, which converts raw skin to leather, actually is a process to introduce artificial cross-linking into collagen and stabilise the molecules. Tanning materials are the cross-linking reagents, which are bi-functional or multi-functional and can react with more than one collagen side group and form new cross-links²⁴. The conventional view is that the type and amount of inter-molecular cross-linking bonding of the chains determines the increase in the Ts¹⁷.

Leather production is one of the oldest known crafts. There are many tanning agents and tanning methods that have been developed in its long history, such as vegetable tannage, aldehyde tannage and chrome or aluminium tannage⁵. In order to achieve better tanning effect and leather properties, combination tannages also have been applied. For instance, vegetable-aluminium combination tannage, based on the synergistic effect between hydrolysable tannin and aluminium, produces leather with Ts higher than 100°C²⁵⁻²⁷. Different tanning agents or tanning methods give distinctive shrinkage temperatures, which mainly depend on the cross-linking circumstance in the collagen fibres structure, although some of the cross-linking reaction mechanisms are still not completely understood.

From the beginning of the last century, chrome tannage has become the dominating tanning method in the world's leather industry, in which more than 90% of production is chrome tanned^{5,17,28}. Vegetable tannage is mainly used to produce leather goods, sole leather and some upholstery leather⁹⁰. Aldehyde tannage is

mainly used in the post-tanning process of clothing or upper leather²⁶². Considering the pollution problem from the metal ions of traditional mineral tannage, especially chromium and aluminium, environmentally friendly tanning materials and methods have become urgently needed. At the same time, pure organic tanned leather may be preferable in some applications, such as car upholstery leather: for example, the car industry of Germany is presently demanding complete recyclability²⁹. Unfortunately, none of the traditional organic tannages, vegetable tannage or aldehyde tannage, can reach the requirements of the modern leather industry: among the properties required of organic tanned leather, high hydrothermal stability is the most difficult property to achieve³². The shrinkage temperature of vegetable tanned or aldehyde tanned leather is usually no more than 86°C (Fig 1.1).

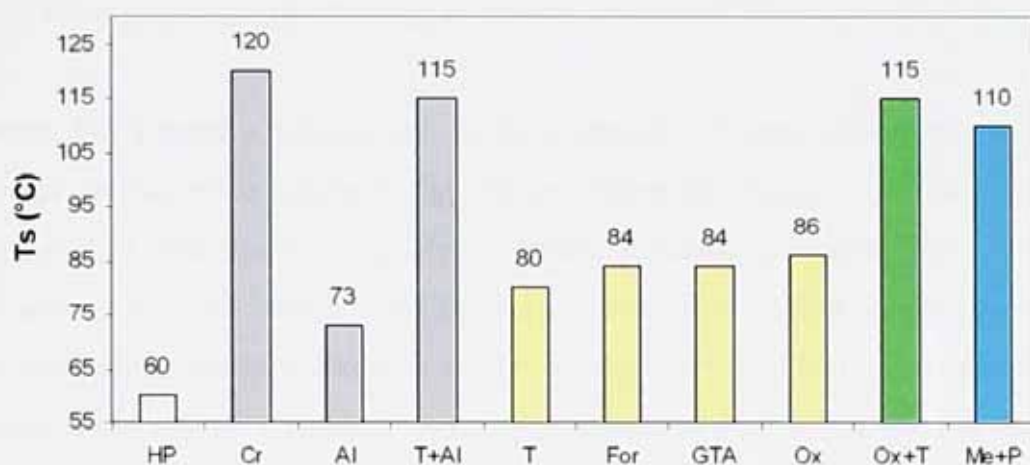


Fig 1.1 The shrinkage temperatures of different tanned leathers

(**HP**: untanned skin, **Cr**: chrome tannage, **Al**: aluminium tannage, **T+Al**: vegetable tannin and aluminium tannage, **T**: vegetable tannage, **For**: formaldehyde tannage, **GTA**: glutaraldehyde tannage, **Ox**: Oxazolidine tannage, **Ox+T**: oxazolidine and vegetable tannin combination tannage, **Me+P**: melamine resine and organic phosphonium salt tannage)

Based on the studies of Covington and Shi³⁰⁻³¹, it has been shown that condensed tannins and oxazolidine combination tannage can confer high hydrothermal stability; the shrinkage temperature can reach 115°C. This method employed environmentally friendly and renewable natural plant materials, vegetable tannins, and a reactive cross-linking reagent oxazolidine, making it suitable for the requirements of the future leather industry. This could be a substitute for chromium(III). It is not a traditional vegetable tannage or aldehydic tannage, so the mechanism of this

combination tannage is not established. It might include the reaction of collagen with oxazolidine, the reaction of polyphenols with oxazolidine and the reaction between all three components. In this work, the endeavour is to understand the origin of high hydrothermal stability. At the same time to address the general leather tanning mechanism, to obtain a comprehensive understanding of the cross-linking of collagen by pure organic chemicals at the molecular level.

1.2 Collagen structure and cross-linking

Based on the molecular structural characteristics, protein can be classified into two main groups: globular proteins, of which most are soluble in water, such as enzymes and insulin, and insoluble fibrous proteins, which serve to support the organs and to protect the organism, such as collagen, keratin and elastin^{4,17}.

Collagen has played a critical role in the evolution of large organisms, where it provides an insoluble scaffold for the provision of shape and form, for the attachment of macromolecules, glycoproteins, hydrated polymers and inorganic ions, and for cell attachment²⁻³. As the major constituent of skin, tendon, bone and other connective tissues, collagen is one of the most prevalent and widely distributed proteins in the animal kingdom, comprising about one third of the total proteins in the animal body². Based on the molecular composition and properties, collagen can be divided into different types. Types I, II, III and V are the collagens which are condensed into fibrils and thick fibres and fibre bundles, called fibril-forming collagens, but type IV does not form fibrils^{2,3,19}.

1.2.1 Molecular structure of collagen

Collagen has a fibrous structure and appearance, forming a variety of patterns in the various tissues of the animal body, especially in the skin and bone. A major component of collagen protein is the triple helical structural domain, composed of three polypeptide chains (α chains), which each have an extensive and characteristic "Glycine-X-Y" repeat sequence, where X and Y can be any amino acid but are frequently the imino acids proline and hydroxyproline respectively^{2-4,20,33,272}. Other

specific features of collagens are a high content of alanine and lysine, post-translational modifications that encompass hydroxylation of proline and lysine residues, various glycosylations and the formation of intermolecular cross-links through lysine and hydroxylysine residues. Chemically, collagen from all animal sources is distinguished by its unusually high content of glycine, proline and hydroxyproline, which together account for over 50% of the amino acid content².

Since Ramachandran and Kartha proposed the triple helix conformation, much progress has been made towards understanding the molecular structure of collagen^{2, 33}. The molecule they proposed, still widely accepted, consists of three left handed polypeptide chains, which are in turn wound into a right handed triple helix with approximately 3.3 residues per turn and a unit twist of about 108° ³⁴⁻³⁶. The structure is stabilised by two hydrogen bonds per triplet^{35, 36} (Fig 1.2): one formed between the **NH** group of a glycy residue and the **C=O** group of the residue in the second position of the triplet in the neighbouring chain and the second hydrogen bond is formed through the hydroxyl group of hydroxyproline in the third position of the triplet (**Gly-X-Y**).

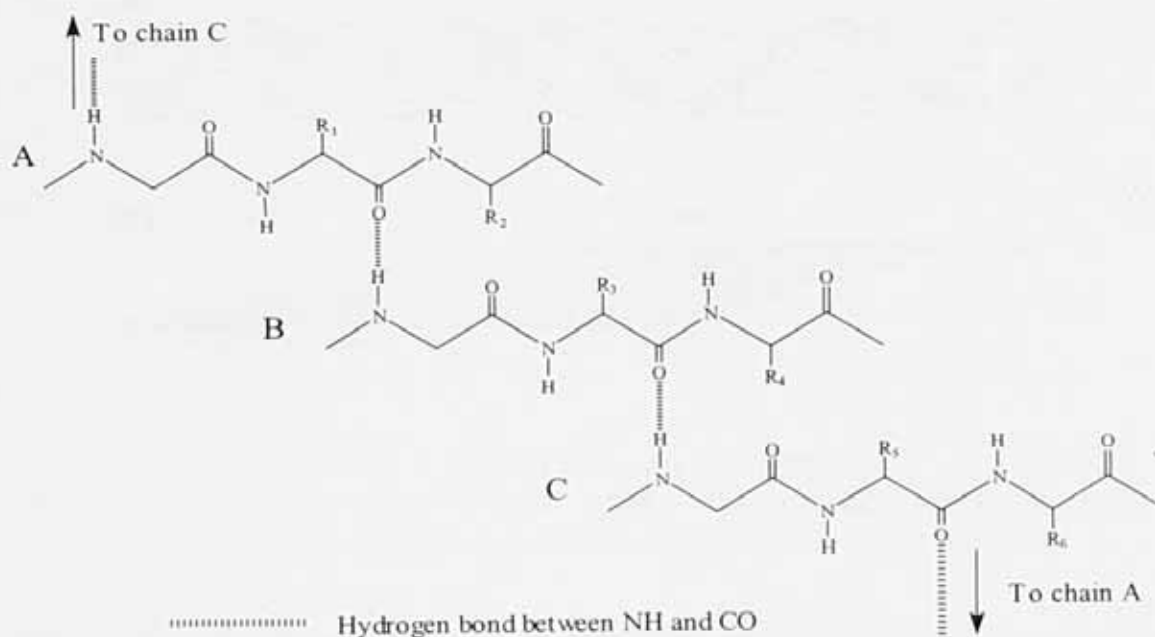


Fig 1.2 A schematic representation of the intramolecular hydrogen bonding between three tripeptide units of a collagen molecule, each tripeptide unit belongs to a different chain (A, B, C) and participates in two hydrogen bonds, R₁-R₆ represent side chains.

Collagen fibril assembly is an example of an entropy driven self-assembly process². Growth of the fibrils is largely directed by repeated clusters of hydrophobic and hydrophilic residues that divide the molecule into 4.4D units (where D = 67nm, the periodicity seen in collagen fibrils) and cause the monomers to associate, so that each one is staggered by one D unit. Under the electron microscope, collagen fibrils exhibit a characteristic banded appearance when negatively contrasted with heavy metal stains. Regions of gap and overlap zones are produced within the fibril: the overlap zone is about 0.4D and the gap zone is about 0.6D. There is some evidence to suggest that those portions of collagen molecules in the gap zone are more mobile than those that comprise the overlap zone^{1, 37, 38} (Fig 1.3).

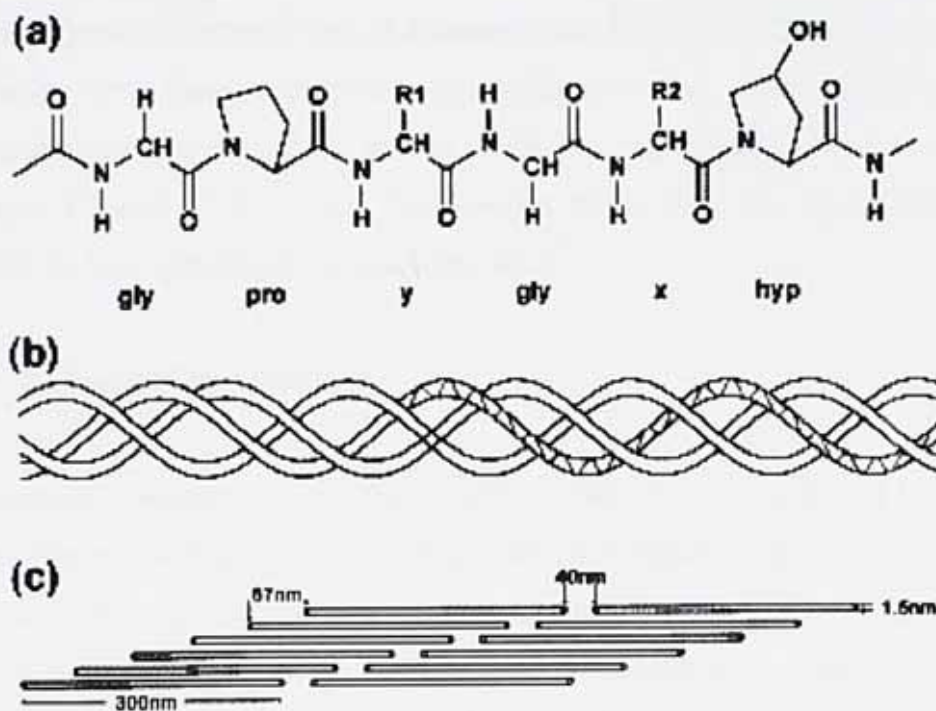


Fig 1.3 Type I Collagen structure

(a: Primary amino acid sequence, b: Secondary left handed helix and tertiary right handed triple-helix structure, c: Fourth staggered quaternary structure)

The structure of collagen can be described by four levels of hierarchy: the primary structure is the amino acid sequence (Fig 1.3, a); the secondary structure is the α chains forming left handed helices with a pitch of 0.87nm (Fig 1.3, b); the tertiary structure refers to the fundamental unit tropocollagen, which is three polypeptide chains intertwined to form a right-handed triple helix with a pitch of approximately 8.6nm and molecular weight about 300kD (Fig 1.3, b); in the quaternary structure

the triple-helical molecules are staggered longitudinally and bilaterally into fibrils with distinct periodicity (Fig 1.3, c). The collagen molecules aggregate through fibrillogenesis into microfibrils, consisting of four to eight collagen molecules, and further into fibrils. The collagen fibril is composed of rod-like triple helices arranged in a systematic manner.

To date, at least 25 types of collagen have been confirmed^{1,2,265}, which vary mainly in the length of the helix and the nature and the size of the non-helical portions. Type I collagen is the major collagen of hide of higher animals, containing two kinds of chains, two $\alpha 1(I)$ chains and one $\alpha 2(I)$. The tropocollagen molecule is about 290nm in length and about 1.5nm in diameter. The main body of the molecule consists of a triple helical domain (about 1000 amino acids long, 337 Gly-X-Y units from each contributing polypeptide chain), with short telopeptide sequences at the N-terminal (17 amino acids) and C-terminal (26 amino acids) for $\alpha 1(I)$ and 11 and 17 for $\alpha 2(I)$. Telopeptides differ from the main body of the molecule by their inability to form a triple helix².

1.2.2 Natural cross-linking

The systematic packing of the triple helices lends strength and resilience to the collagen fibres. Additional mechanical and chemical stability derives from intra- and inter-molecular chain cross-linking. The assembly of type I collagen fibrils is accompanied by the formation of inter- and intra-molecular covalent cross-links between α chains, the molecular chains linked in "head-to-tail" formation³⁹⁻⁴¹.

Natural cross-links are based on aldehyde formation and condensation, involving specific peptidyl lysine and hydroxylysine residues, catalysed by a single enzyme, lysyl oxidase, which oxidatively deaminates the amino group of certain lysyl and hydroxylysyl residues in the telopeptide regions of collagen molecules, to form reactive allysyl and hydroxyallysyl aldehydes respectively²⁶⁷⁻²⁷⁰. These aldehyde groups then react with the non-protonated free amino group of lysyl or hydroxylysyl residues to form the covalent bond cross-linking and can further react with a histidine residue to form stable tri-functional cross-linking^{39-40,267} (Fig 1.4).

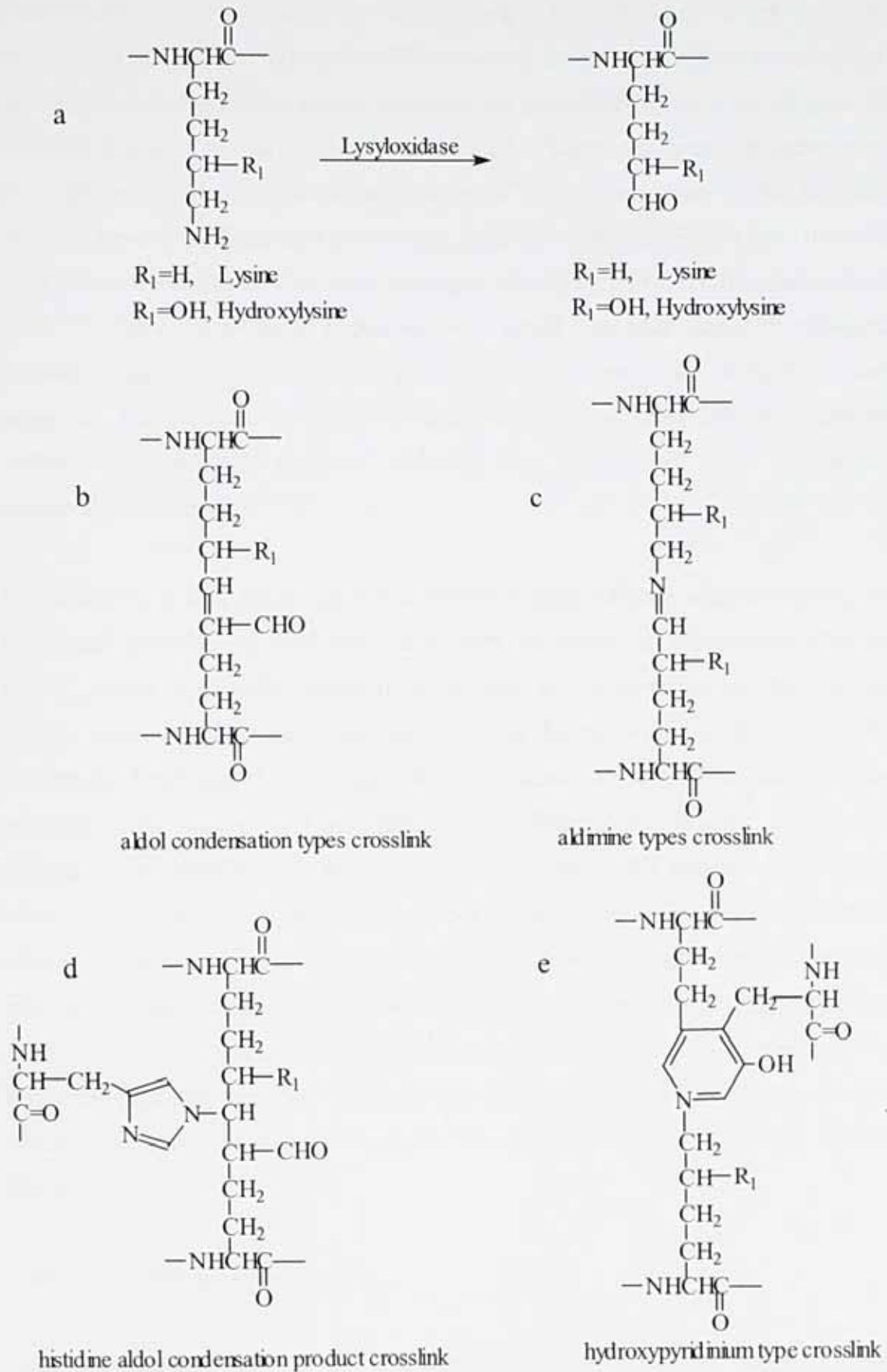


Fig 1.4 Natural cross-linking in type I collagen structure

The residues in type I collagen that are involved in this type of cross-link are $\alpha 1(I)$ Hyl-87, $\alpha 2(I)$ Hyl-87, $\alpha 1(I)$ Hyal-16C. Structural investigations have revealed that the features involved in cross-link formation are practically identical in all types of collagen. It is well known that type I and type III collagens are present together or as co-polymers in skin and other connective tissue⁴². Therefore, cross-linking between different types of collagen can occur under natural conditions. Henkel and Glanville have reported that this kind of cross-linking is present in human leiomyoma and calf aorta⁴²⁻⁴³. These cross-linking formations were found after treatment with cyanogen bromide, digesting with trypsin, chymotrypsin or bacterial collagenase and separating the products by HPLC. Therefore, complex cross-linking in natural collagen fibres could be proposed, although more proof needs to be obtained to support the conjecture.

The solubility in acid or salt solution of collagen from different sources depends on the natural cross-linking condition, the stability and density of cross-links. With the ageing process of animals, less and less collagen can be extracted into acid or salt solution from skin, because of the increasing of the cross-linking density and the conversion from non-stable cross-linking to stable bonds²⁶⁷. For example, the reducible cross-linking bond in calfskin can be disassociated when the extraction process is conducted with 0.5mol/l acetic acid²². That is, the aldimine bond of the Schiff base structure is hydrolysed and thus the collagen molecules become more soluble in this solution after losing the non-stable cross-linking structure¹. Once the non-stable bonds all change to stable cross-links in the collagen structure, the physical, biological and chemical properties all change significantly. This also is the fundamental principle of introducing additional artificial cross-linking into collagen structure, to make it more stable or provide unique properties for collagen-based biomaterials.

1.2.3 Artificial cross-linking

After pretreatment, such as the beamhouse processes in leather making, it has always been necessary to introduce extra artificial cross-linking to improve the biochemical resistance or hydrothermal stability¹⁷. Leather making is a process to

change the easily putrescible raw material to a non-putrescible material, which has good resistance to bacteria or fungi. High thermal stability is necessary in many applications, such as leather protective clothing, shoes or gloves. In collagen based tissue-engineering studies, artificial cross-linking is also important to control the degradation or mineralisation process.

Except for the natural cross-linking through the lysine or hydroxylysine groups, which mainly happens at both terminals or close to the terminals of collagen molecules, there are various active functional groups on the peptide side chains (Fig 1.5), such as the carboxyl groups of acidic amino acid residues, the amino groups and guanidyl groups of basic amino acid residues, which provide the possibility for further cross-linking reactions. For example, in leather tanning, the carboxyl groups have been shown to be the reaction sites for chromium(III) or aluminium(III)^{4,17}; the free amino groups have been shown to be the reaction sites for aldehydes^{17,44}. Collagen-based biomaterials are employed in various applications: here, glutaraldehyde (GTA), hexamethylenediisocyanate (HDC), polyepoxy compounds, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and acyl azide have all been widely used as stabilising (tanning) agents. Considering the biocompatibility and tissue regenerating capacity, the most promising results were obtained with EDC as the collagen crosslinking agent^{1,6}. In any case, the chemical treatment to be used depends on the application purpose.

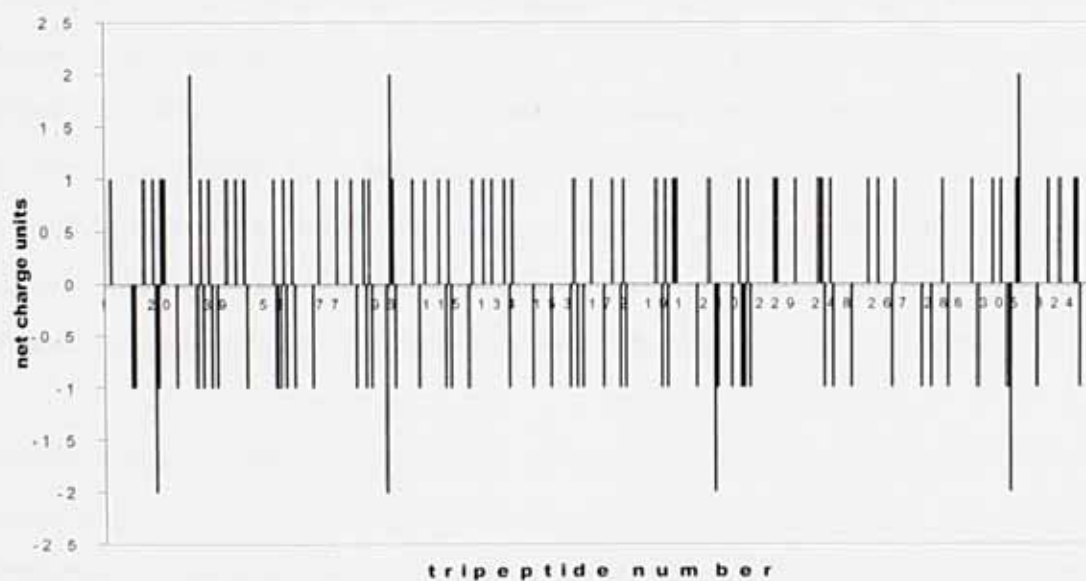


Fig 1.5 Distribution of carboxyl and amino or guanidyl groups on collagen chains

1.2.3.1 Mineral tanning

The most important mineral tanning material is basic chromium(III) sulphate, followed to a much lesser extent by aluminium(III), titanium(IV) and zirconium(IV) salts. The interactions of collagen with chrome have been extensively investigated since the end of the nineteenth century. The basic reaction of mineral tanning is the formation of complex bonds with the ionised carboxyl groups of aspartic and glutamic acid residues on collagen fibres^{5,28,45,46} (Fig 1.6).

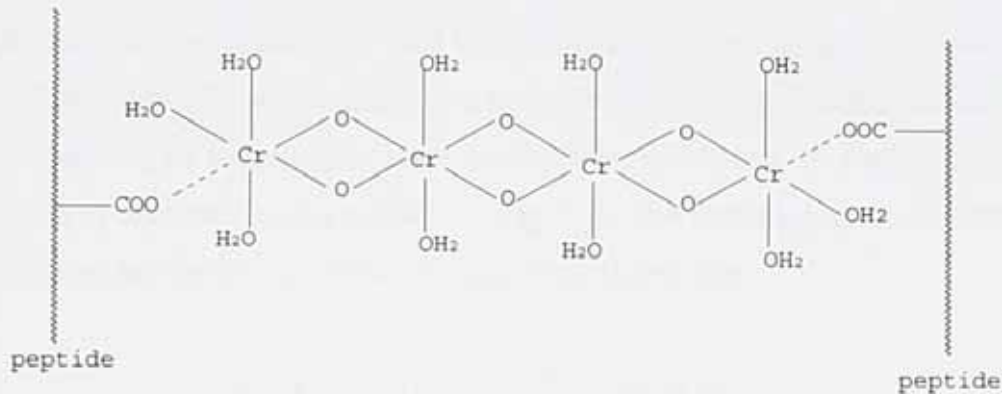


Fig1.6 The reaction model of chrome complex with carboxyl groups of collagen

Chromium tannage is typically accomplished by the addition of the pretreated raw materials into a 33% basic chromium sulphate solution (empirical formula $\text{Cr}(\text{OH})\text{SO}_4$). The effectiveness of tannage is dependent on the Cr(III) species present in the tanning bath²⁸. Based on the mechanism postulated by Gustavson, the carboxylate side chains of collagen interact with the bi- and tri-nuclear Cr(III) species in the tanning bath, when about 10% of the combined chromium is bonded by multipoint fixation, by the reaction between the Cr(III) and at least two collagen carboxyl side chains⁴⁵. Later investigations have proposed that only inter chain cross-linkings are responsible for the increased thermal stability of leather⁴⁶.

To date, more than 90% of the world's leather is tanned with chromium⁵, which is a consequence of the easy processing, the broad achievability and the excellent properties of chrome tanned leather. It should be noted that the environmental

hazard from the metal ions cannot be neglected and is an urgent problem to be solved.

Other metal tannages (aluminium(III), titanium(IV) and zirconium(IV)) should have similar reaction mechanisms to chromium. The use of aluminium in leathermaking is as old as leather making itself. The reaction of aluminium salt with collagen carboxyl groups is dominated by electrovalent bonding, thus much lower shrinkage temperature is obtained than with chrome⁵. The development of titanium and zirconium tannage is relatively recent⁵. Empirically, the chemistry of Ti(IV) is dominated by the titanyl ion TiO^{2+} and the species in the tanning agent are chains of $(\text{Ti-O})_n$. Zirconium salts are characterised by eight-coordination and high affinity for oxygen, resulting in a tetrameric core structure; the basic unit of structure is four Zr(IV) ions at the corners of a square^{5,17} (Fig 1.7). The tanning powers of titanium and zirconium are similar and both are better than aluminium.

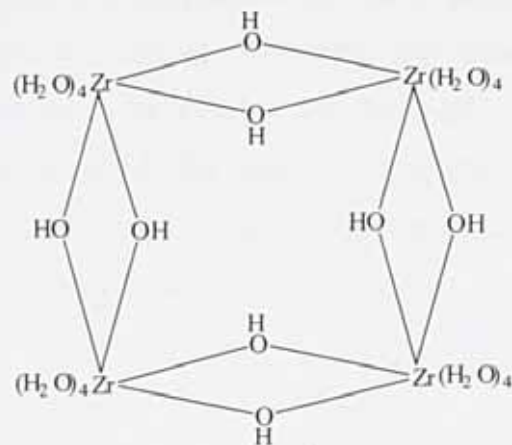


Fig 1.7 Basic molecular structure of Zr(IV) salt

1.2.3.2 Aldehyde tanning

The cross-linking reaction of collagen with aldehyde, especially formaldehyde, is not only applied in leather tanning but also in biomaterials and photographic film. Although the mechanism has been studied for a long time, it is still not completely clear^{5,47-53}. Most researchers accept that aldehydic compounds react with the free amino groups of lysine and form cross-links (Fig 1.8).



The N-hydroxymethyl group is highly reactive and cross-linking will occur at a second amino group:

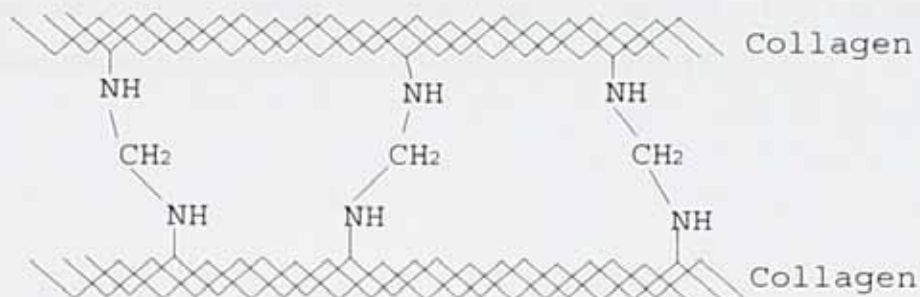


Fig 1.8 Model of cross-linking reaction of formaldehyde with collagen

As a multifunctional aldehyde, glutaraldehyde is polymerised in solution (Fig 1.9); the terminal hydroxyl groups of the polymer are active and capable of reacting with amino groups. Because of reasons of health and safety, formaldehyde and glutaraldehyde are unlikely to be suitable for the leather industry of the future. Another notable shortcoming of this kind of cross-link is that the achievable hydrothermal stability is not high; the shrinkage temperature of aldehyde tanned leather can only be raised to 80-85°C^{4,17,18}.

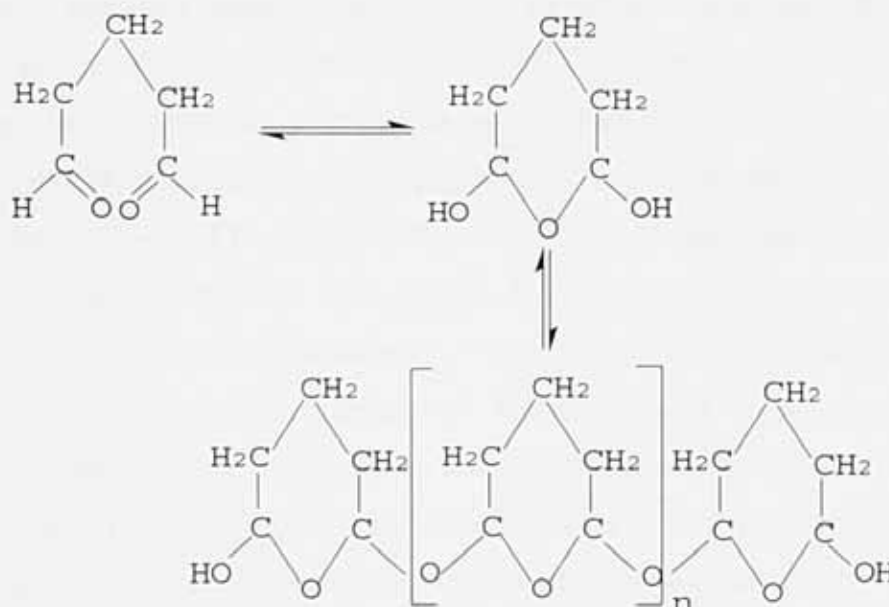


Fig 1.9 The reactivity of glutaraldehyde

An alternative to aldehyde is oxazolidine, proposed in the 1970's, although not widely used until more recently⁵⁴⁻⁵⁵. Oxazolidine has been shown to possess high reactivity and good tanning ability; leather tanned by oxazolidine II has similar properties to glutaraldehyde tanned leather. Under hydrolytic conditions, the rings open under hydrolysis to form an N-hydroxymethyl compound, which can react with one or more amino groups to produce effective cross-linking (Fig 1.10)⁵⁴.

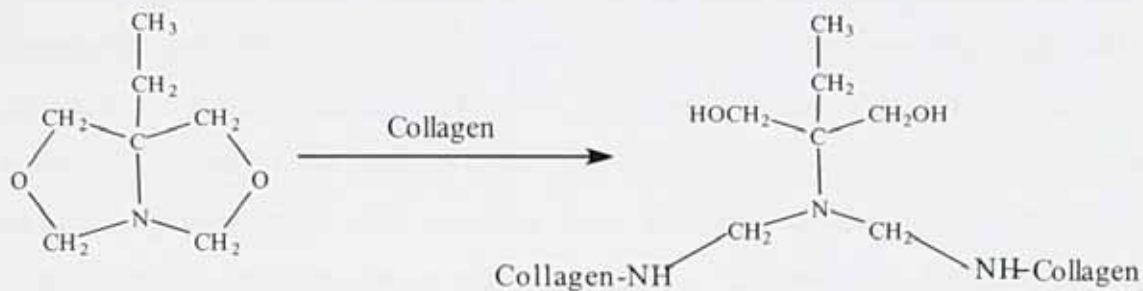


Fig 1.10 Cross-linking model of collagen with oxazolidine

1.3 Plant polyphenols

Plant polyphenols, also named vegetable tannins, are the secondary metabolism products of plants, present in high concentration and wide distribution: vegetable tannins are an abundant natural product. Following cellulose and lignin, they are the third most abundant natural materials in plants^{57,67,68}. The name “vegetable tannins” reflects the close relationship with leather tanning and they are the oldest tanning materials, probably used by prehistoric man^{56,58,59,63}. Vegetable tanning was the most important tanning method before the 19th century^{5,59}. Its tanning ability is owing to the strong complexing property with protein. It can effectively precipitate soluble protein from solution, such as in salivary protein, to produce the astringent feeling when tannin rich food or drink is consumed. This is the most important property of tannin⁵⁹⁻⁶². From its unique molecular structure and characteristics, tannin also has high chemically reactive ability, such as complexing with metal ions, reduction, nucleophilic reactions, electrophilic reactions etc. Because of these reactivities, plant polyphenols have been used and studied widely in many areas besides leather making, such as in the food and beverage industry, as special bio-chemicals, as effective anti-bacterials and anti-viral components in medicine. Plant polyphenols

are the active components in more than 70% of natural (herbal) medicines, fine chemicals, anti-UV formulations, wood preservatives and wood adhesives, anti-corrosives for metal, natural dyes, functional polymers, *et al.*^{63-68,71,78,79,102,105,142,143}

1.3.1 The molecular structure of plant polyphenols

Although vegetable tannins have been used for thousands of years, the molecular chemistry of these natural products was unknown until the 1950's because of their complexity and difficulty in isolation and purification^{57,63,71}. According to the early definition of Bate-Smith and White⁶⁹⁻⁷⁰, they are water soluble, possess relative molecular masses in the range 500 to 3000 Dalton and they can precipitate some alkaloids, gelatine and other proteins from solution, besides having the usual phenolic properties. The name 'plant polyphenol', suggested by Haslam⁷¹ in the 1980's, may provide a better way to summarise this kind of natural products' structural characteristics and mixture components, because plant polyphenols include not only the molecular weight range of 500 to 3000D, but other components, for example, the low molecular weight phenols, referred to as non-tannin^{66,71}. Because the term "vegetable tannin" has been used for many years, and most of time has the same meaning as "plant polyphenol", in this thesis the two terms will be used without distinction.

From the early work of Freudenberg in 1920's and 1930's, vegetable tannins can be classified into two groups based on the chemical nature and structural characteristics: hydrolysable tannins (Section 1.3.1.1) and condensed tannins (Section 1.3.1.2)^{57,63}.

1.3.1.1 Hydrolysable tannins

Usually, hydrolysable tannins have a carbohydrate (polyol glucose) core with pendant esterified gallic acid and/or hexahydroxydiphenolic acid derivatives or related acid. Depending on the polyphenolic acids that are obtained as products of hydrolysis, hydrolysable tannins can further be divided into gallotannins (**I**) and ellagitannins (**II**) (Fig 1.11). Gallotannins, such as Chinese gallotannin, Turkish gallotannin and sumac yield gallic acid and glucose on hydrolysis. The ellagitannins,

such as myrobalan nut and chestnut wood tannins, produce ellagic acid in addition to gallic acid and glucose on hydrolysis^{57,63,68,69,71-75}.

Table 1.1 Commercially used hydrolysable tannins⁶³

Tannins	Source	Tannin content (%)
Chinese tannins (tannic acid)	Galls, leaves of <i>Rhus semialata</i>	34-71
Turkish tannin	Galls on wood of <i>Quercus infectoria</i> (Aleppo galls)	25-45
Sumach	Leaves of <i>Rhus coriaria</i> , <i>R. typhina</i>	26-27
Myrobalans	Fruit of <i>Terminalia chebula</i>	30-50
Valonea	Acorn cups of <i>Quercus valonea</i>	15-36
Chestnut	Wood of <i>Castanea sativa</i>	10-12
Tara	Fruit pods of <i>Caesalpinia spinosa</i>	45-59

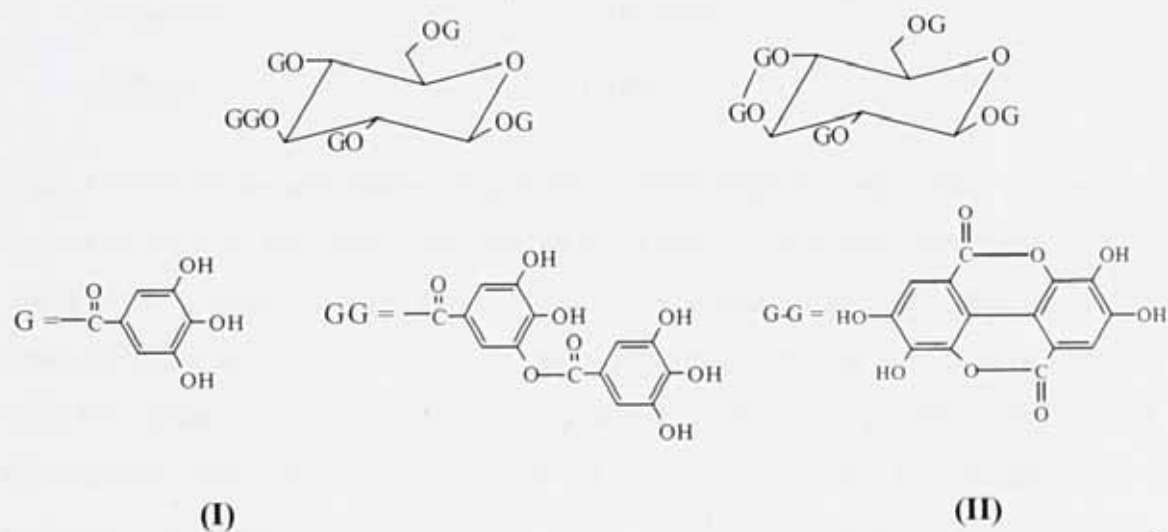
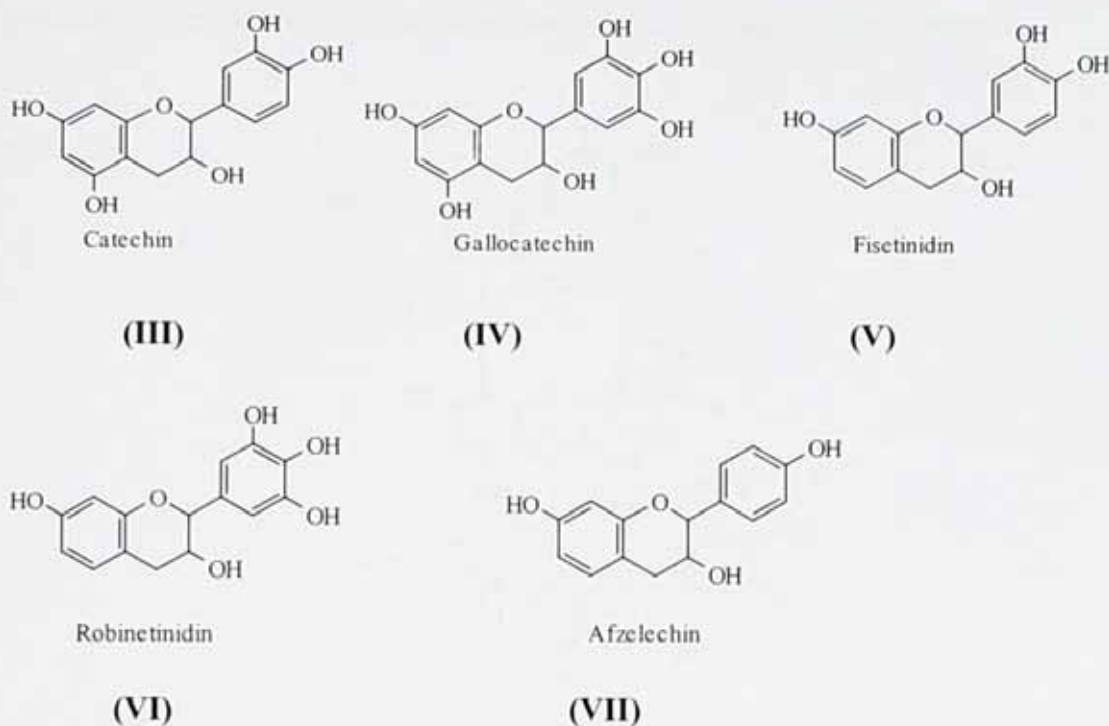


Fig 1.11 Molecular structures of hydrolysable tannins

1.3.1.2 Condensed tannins (proanthocyanidins)

Condensed tannins are now more correctly referred to as proanthocyanidins or polyflavanols^{71,76,77,84,91}. The fundamental structure unit in this group is the phenolic flavan-3-ol, present in catechin (III), gallocatechin (IV), fisetinidol (V) and robinetinidol (VI)^{76-80,84}. The flavan-3-ol units are linked principally through the C-4

to C-8 positions or C-4 to C-6 positions, the polymers are poly-dispersed and it has been shown that they may contain molecules with degrees of polymerisation ranging from 2 to 50 or even more, the chains are irregular, sometimes having some chain branches, as shown in Fig 1.12.



Each kind of condensed tannin often contains more than one type of configurational base unit, such as the condensed tannins from black wattle bark, which consists of about 75% prorobinetinidin units, about 20% profisetinidin and less than 5% prodelphinidin units¹¹²⁻¹¹⁵. The structures of proanthocyanidins polymers from more than 100 plants were studied by Porter and Foo in the 1980's⁷⁷, when they found procyanidin polymers occur widely, as do mixed procyanidin and prodelphinidin polymers, although the procyanidin units usually predominate in the mixed polymers. Prorobinetinidin and profisetinidin tannins are present in only a few plants.

Compared with the study of hydrolysable tannins, the properties of many condensed tannins are not very clear and many are in dispute. This is because the proanthocyanidins are more complex compositions and have higher reactivity than the hydrolysable tannins. Substantial progress in the chemistry of proanthocyanidins began to be made in the 1950's from the pioneering work of

Weinges and his collaborators^{63,71}: they were the first to isolate and fully characterise the four principal procyanidin dimers, B-1, B-2, B-3 and B-4, as their peracetates. Haslam and his group isolated and characterised their free phenolic forms in 1972⁷¹. The dimers, B-5, B-6, B-7 and B-8, linked by C-4 to C-6 bonds between the monomer units, have also been reported⁶⁶(Fig 1.13). The “B” class structure represents most of the proanthocyanidins’ molecular characteristics.

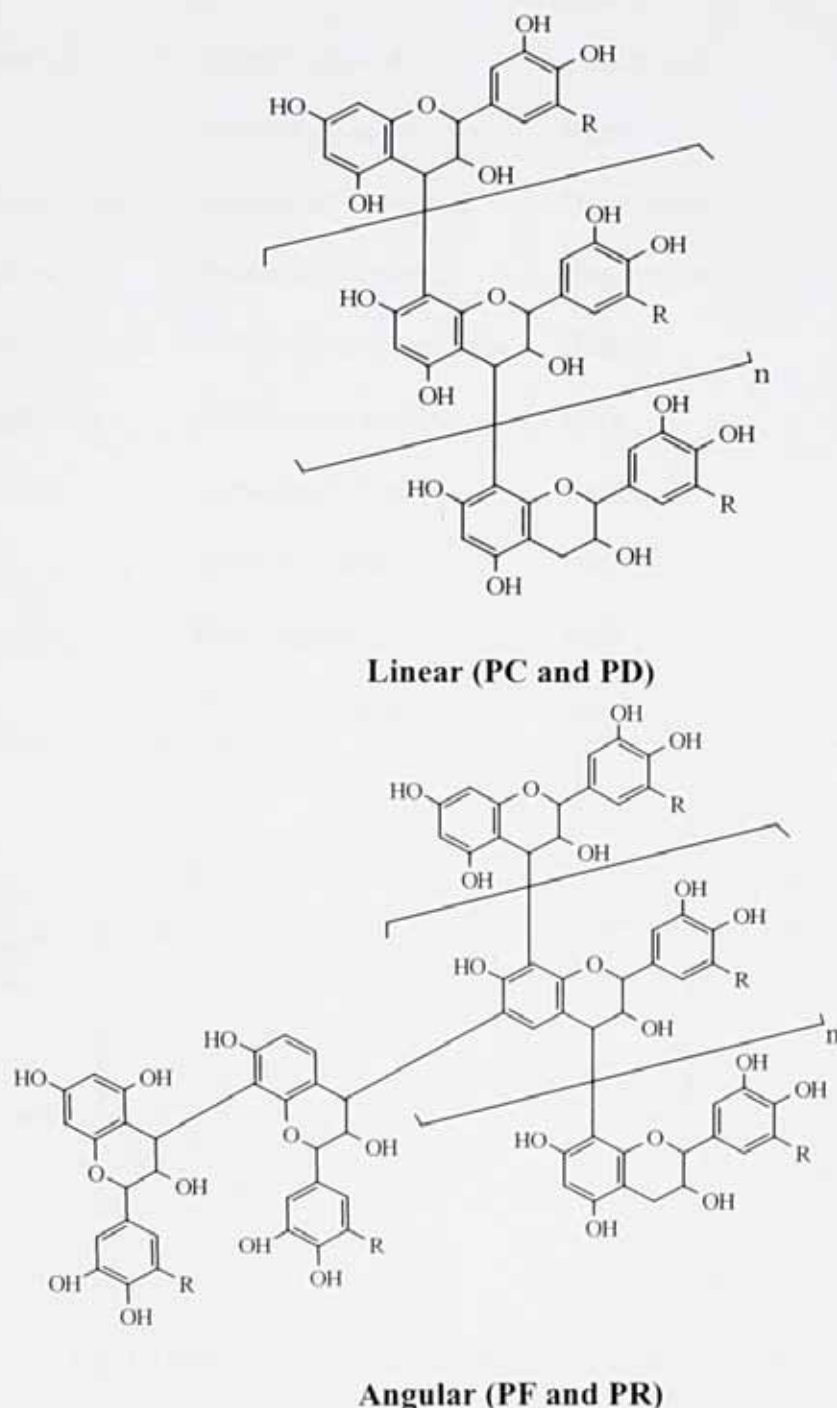


Fig 1.12 General structure for proanthocyanidin monomers and polymers
(PC: procyanidin, PD: prodelphinidin, PF: profisetinidin, PR: prorobinetinidin)

Table 1.2 Sources of condensed tannins⁶³

Family (Tannins)	Plant	Part of the plant	Tannin content (%)
Myrtaceae (Eucalyptus)	<i>Eucalyptus astringens</i>	Bark	40-50
	<i>Eucalyptus wandoo</i>	Bark&heartwood	12-15
Leguminosae (Mimosa)	<i>Acacia mollisima</i>	Bark	35-40
	<i>Acacia catechu</i>	Heartwood	15
	<i>Robinia pseudacacia</i>	Bark	7
Anacardiaceae (Quebracho)	<i>Schinopsis balansae</i>	Heartwood	20-25
	<i>Schinopsis lorentzii</i>	Heartwood	16-17
Rhizophoraceae (Mangrove)	<i>Rhizophora candelaria</i>	Bark	25-30
	<i>Rhizophora mangle</i>	Bark	20-30
Fagaceae (Oak)	<i>Castanea sativa</i>	Bark	8-14
	<i>Quercus robur</i>	Bark	12-16
Pinaceae	<i>Picea abies</i>	Bark	5-20
	<i>Pinus sylvestris</i>	Bark	16

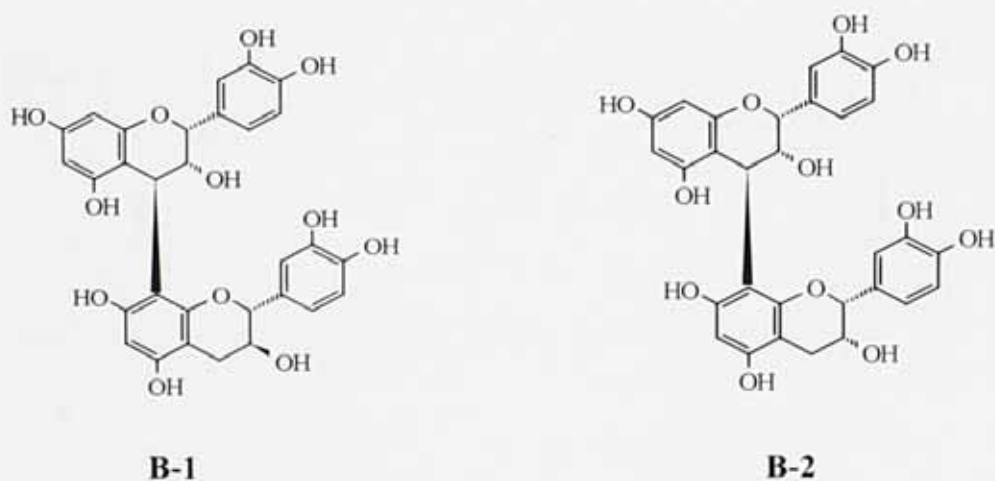
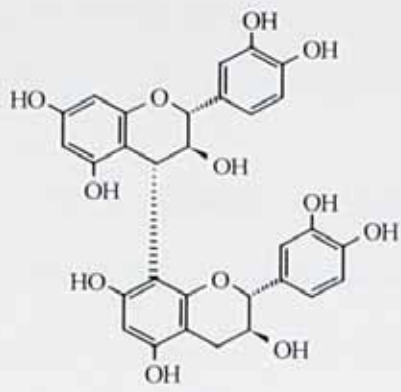
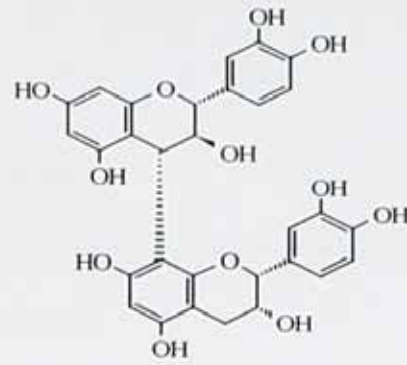


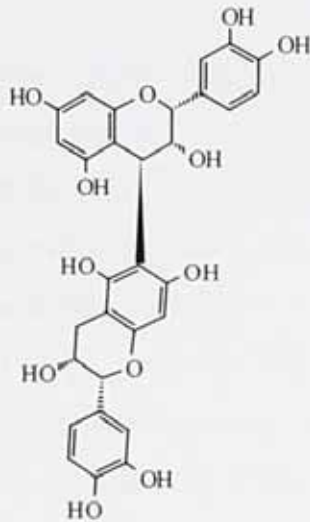
Fig 1.13 Procyanidins dimers B1, B2, B3, B4, B5, B6, B7 and B8



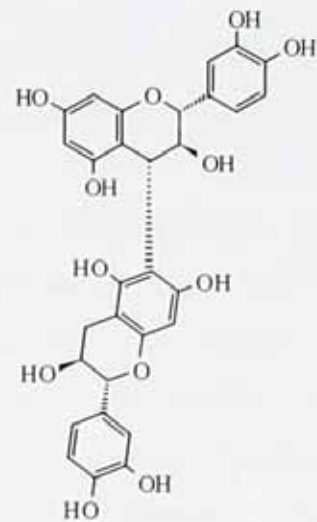
B-3



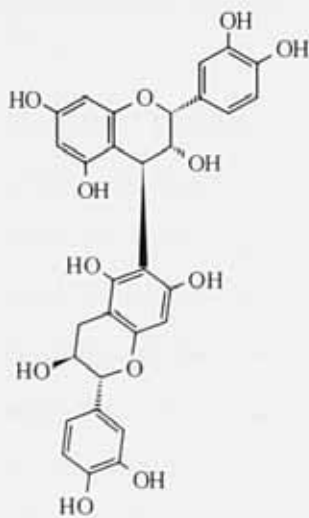
B-4



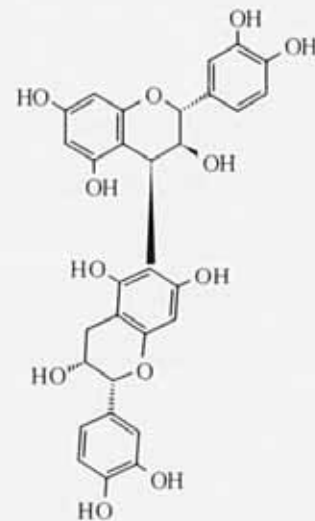
B-5



B-6



B-7



B-8

Fig 1.13, (Continued) Procyanidin dimers B1, B2, B3, B4, B5, B6, B7, B8

Because of the difficulty in obtaining pure proanthocyanidin compounds, it is usual to describe the tannin from four aspects^{57,76,78}.

1. *Average molecular weight and its distribution*
2. *The structure types of the monomer units and their ratio in the polymer*
3. *The conformation of C2 and C3 in the units*
4. *The conformation and configuration of inter-flavan linkages*

The variation in the molecular structure of condensed tannins, especially the different hydroxylation patterns at the A-ring and B-ring, create character differences when condensed tannins are compared, for instance, comparing the proanthocyanidins, which have a resorcinol A-ring as prorobinetinidin (PR) or profisetinidin (PF), with those which have the phloroglucinol A-ring, as procyanidin (PC) or prodelphinidin (PD). PR and PF polymers usually form "angular" chain structures with some branching (Fig 1.11), but the link bond in PC and PD is usually sited between C-4 and C-8, so a linear polymer structure is formed. This variation, the molecular weight and the conformation of C-2 and C-3 influence the chemical reactivity of proanthocyanidins, especially the cross-linking reaction with aldehydes, which will be studied in this work.

Compared with procyanidins, prodelphinidins are less known. The monomeric unit of prodelphinidins is (+)gallocatechin (**VIII**) and (-)epigallocatechin (**IX**), or partly replaced by (epi)gallocatechin-gallate (**X**)^{85-87,92,125-127,212-214}. Unlike procyanidins, prodelphinidins have a phloroglucinol A-ring and a pyrogallol B-ring, which gives them the highest chemical reactivity among all the vegetable tannins (Fig 1.14). The pyrogallol B-ring has been shown to be much more reactive than the catechol B-ring of procyanidins³⁰⁻³², with regard to complexation with metal ions or reaction with aldehyde⁶⁴⁻⁶⁵. The stereochemistry of prodelphinidins is quite similar to the procyanidins and similar dimers to the prodelphinidins B-1 to B-4 have been reported^{66,68,275}.

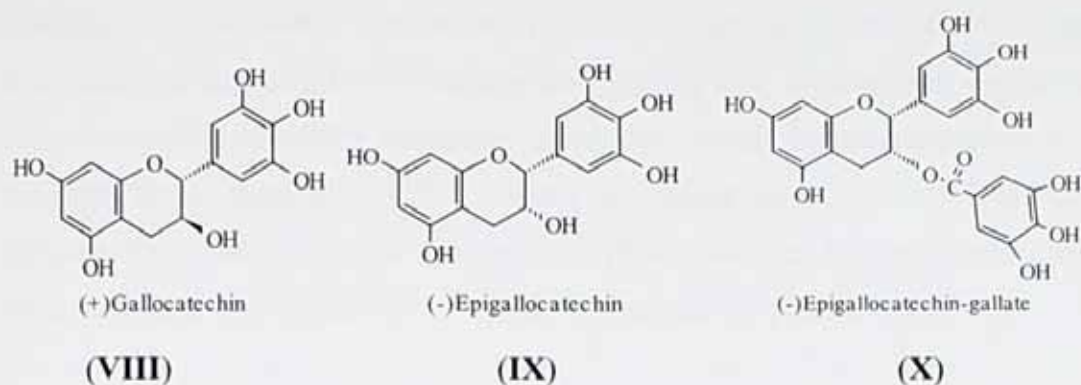


Fig 1.14 Prodelphinidin monomers

1.3.2 Reactivity of plant polyphenols

1.3.2.1 Complexation with protein

Precipitation of protein from solution is the most important property of vegetable tannins, applying to both the hydrolysable tannins and condensed tannins, and the combination of tannins with protein is known as the astringency of the plant polyphenols^{56,58,63,82,88-90,120-124,167,174,176,255}. The phenomenon of reversible polyphenol protein complexation has been known for centuries⁶³. There have been lots of studies about this reaction: it is now generally believed that the interaction between polyphenol and protein is a surface phenomenon with multi-site cross-linking, mainly through weak bonds. Haslam and co-workers have summarised the results^{66,82,255}. The principal mechanisms of polyphenol-protein binding are thought to be:

- (i) *Multi-point hydrogen bonding*
- (ii) *Hydrophobic bonding*

The importance of hydrogen bonding and hydrophobic bonding to complexation has been illustrated by Oh^{88,121}, who found that protein-proanthocyanidin complexes could be disassociated by addition of either polyvinylpyrrolidone (which disrupts hydrogen bonds) or the detergent Tween 80 (which disrupts hydrophobic interactions). Similarly, soluble protein-proanthocyanidin complexes were adsorbed on either Polyclar AT (through hydrogen bonding) or Phenyl-Sepharose Cl-4B

(through hydrophobic interactions). This suggests amphiphilic capacity (simultaneous capacity for hydrophilic and hydrophobic interactions) on the part of proanthocyanidins, which originates from the phenol hydroxyl groups and the benzene rings respectively^{82,89,90}. There are some side chains of the protein polypeptide chains, such as the residues phenylalanine, leucine, isoleucine and proline, which form hydrophobic regions on the peptide chains.

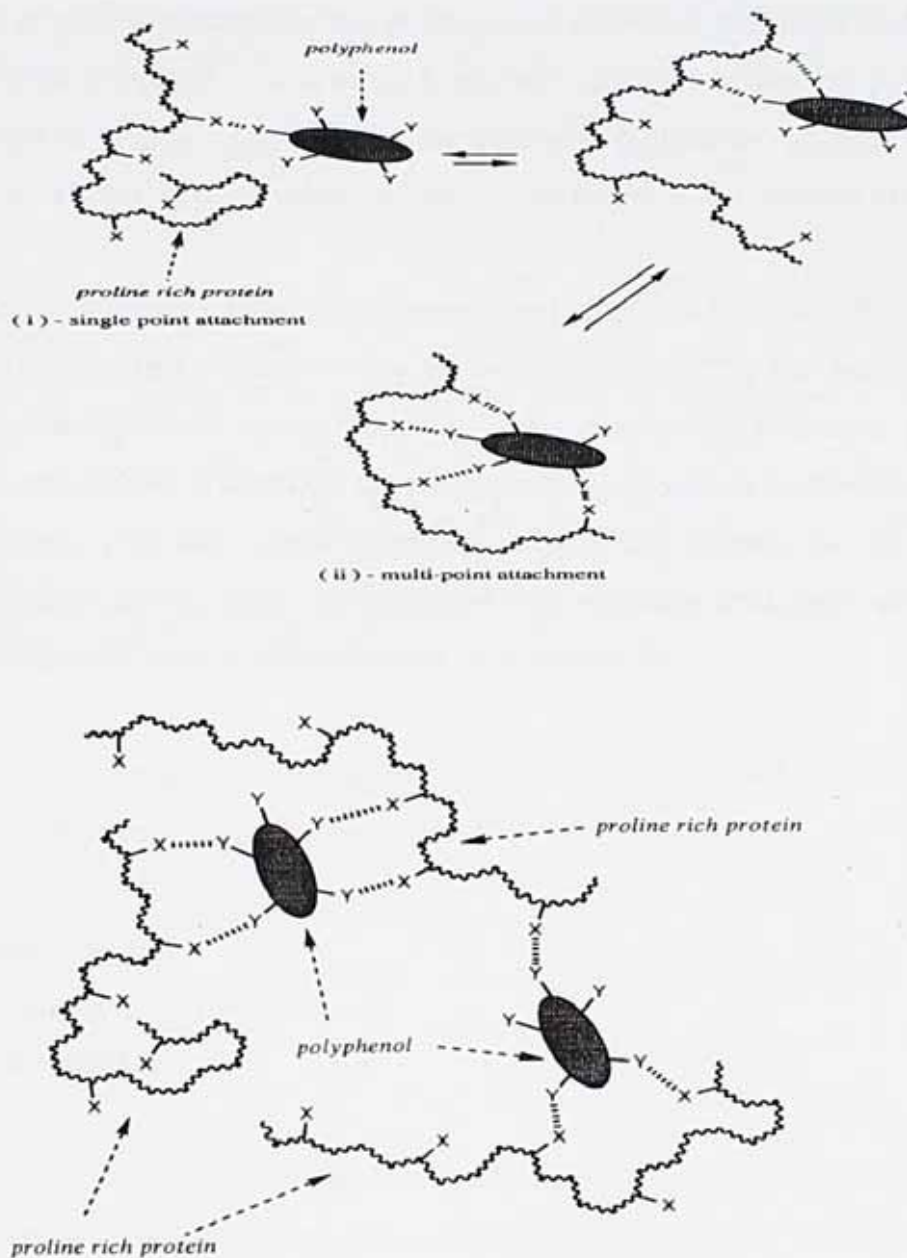


Fig 1.15 Interaction between plant polyphenols and proteins⁶⁶

The process of polyphenol combining with protein can be divided into two steps: first, the polyphenol molecules approach and enter the hydrophobic region of the protein, which is driven by the hydrophobic bonding; the second step is combination, strengthened by the consequent formation of hydrogen bonds between the hydroxyl groups of polyphenols and the polar groups of proteins, such as carboxyl groups, hydroxyl groups, amino groups and guanidyl groups (Fig 1.15). The hydroxyl groups of polyphenols function as proton donors and the carboxyl or hydroxyl groups of protein function as the proton acceptors. Although the hydrogen bond is weak compared to a covalent bond, the combination between polyphenols and protein is strong, especially for the proline-rich proteins, such as collagen, salivary protein and casein, which also have many hydrophobic residues or regions.

Irreversible combination between polyphenol and protein is thought to be due to the formation of covalent bonds between the two molecules^{5,30,65}. For example, auto-oxidation of polyphenols occurs, producing highly electrophilic structures, quinones, which are susceptible to attack by the nucleophilic groups, such as the amino group, **NH₂**, located on the side chains of protein polypeptides. Irreversible combination can form under the influence of some metal ions, enzymes, acid, base and oxygen, which enhance the auto-oxidation process of polyphenols.

There are three properties of polyphenols, which significantly affect their ability to associate with proteins in aqueous solution^{82,89}.

(1) Molecular size

(2) Conformational flexibility

(3) Water solubility

The molecular size of the polyphenol has to be suitable for the hydrophobic regions of the protein; the configuration and conformation of molecules also significantly affect the first step of the polyphenol-protein combination process. Once the polyphenol molecule loses its solubility in water, the ability to combine with protein reduces.

1.3.2.2 Chemical reactivity of condensed tannins

This work mainly concentrates on condensed tannins (proanthocyanidins) because they have high chemical reactivity on the A-ring and B-ring, such as electrophilic substitution, which hydrolysable tannins do not show. Most of the important commercial tannins, such as mimosa, quebracho, larch bark tannins, and gambier, belong to the condensed tannins.

1.3.2.2.1 Reaction of the proanthocyanidin A-ring

The common reactions at the A-ring of proanthocyanidins are examples of electrophilic aromatic substitution, when an electrophile displaces the hydrogen of the benzene ring. The hydroxylation pattern of A-ring controls both the reactivity and the orientation of substitution, partly by steric effects, but electronic factors have more influence. The phenolic hydroxyl group is a powerful electron donor, increasing the electron density in the ring and thereby making it a better nucleophile, as well as stabilising the carbocation intermediates that result from electrophilic attack on the positions *ortho* and *para* to the hydroxyl groups. For resorcinol derivatives, there is evidence that the favoured substitution site is C-6, thus avoiding substitution between the hydroxyl and alkoxy group^{64,65}.

For derivatives of phloroglucinol in the A-ring, numerous studies indicated that the substitution reaction occurs most readily at C-8^{64,65,106,131,137-141} (Figs 1.16, 1.17). The C-8 position is apparently sterically favoured, since the alkoxy oxygen is sterically fixed as part of the pyran ring and thus does not contribute any interference that would result from the rotation about the C-O bond. In contrast, C-6 has hydroxyl groups on each side, that may be free to rotate around the C-O bonds and thus partially block that position¹³¹. Basic conditions increase the reactivity of these phenolic ring systems. The oxyanions generated under basic conditions are significantly better electron donors than the corresponding hydroxyl groups. Under acid conditions, the reaction rate increases because of the formation of a more reactive electrophile.

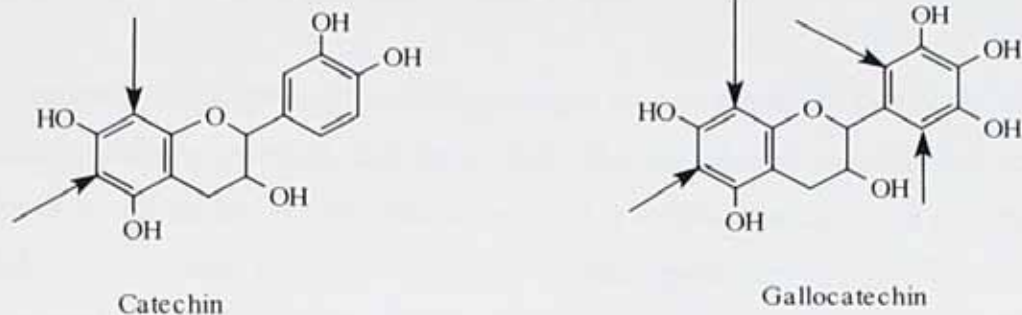


Fig 1.16 Reactive sites of catechin and gallocatechin with electrophilic agents

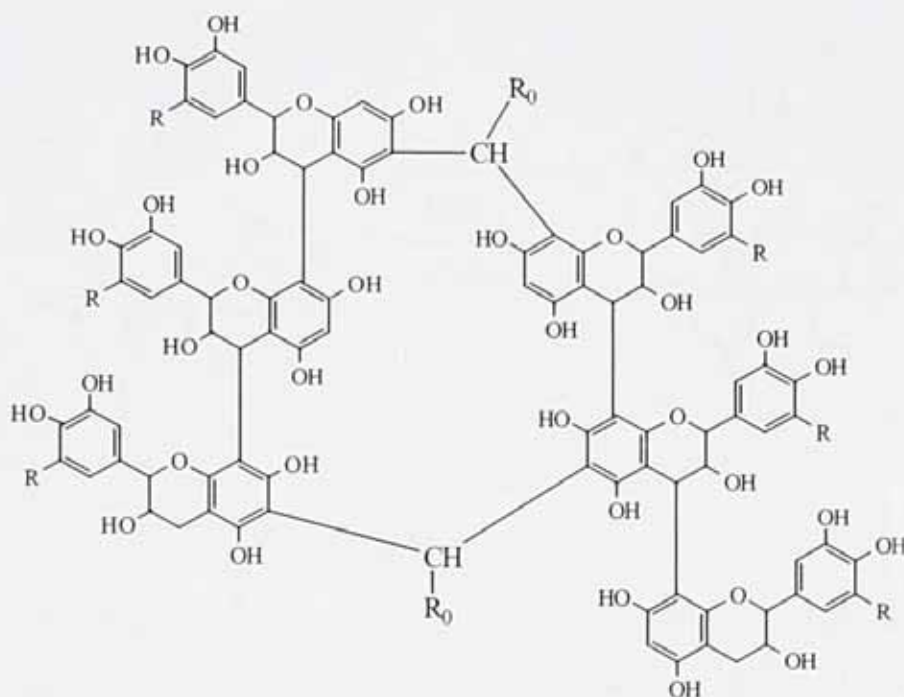


Fig 1.17 Reaction product of proanthocyanidins with aldehydes⁶⁶

The use of plant polyphenols as phenol replacement in phenol-formaldehyde resins naturally led to the investigation of the reaction of aldehyde with proanthocyanidins. Many researchers have studied the reaction with catechin/epicatechin as a model compound¹³⁷⁻¹⁴⁰. They have shown the reaction is on the A-ring: under a variety of conditions, formaldehyde can rapidly react with the phloroglucinolic A-ring system, giving 8-methylolcatechin, which can further condense to give the bis-(8-catechinyl) catechin dimers. The reaction of aldehyde with resorcinolic type phenols is more easily controlled, due to the decreased nucleophilicity of the resorcinol ring compared to the phloroglucinol ring.

1.3.2.2.2 Reaction of the proanthocyanidin B-ring

The chemistry of the proanthocyanidin B-ring is closely related to that of the parent phenols ie phenol, catechol, and pyrogallol. The polyphenol structure of tannins results in an instability property, the polyphenol is oxidised easily in the presence of sunlight, heat, oxidative agents, moisture, enzymes, metal ions^{64,65,129-131}. Vegetable tannins tend to undergo reddening in both direct and indirect sunlight. The effect is consistent with the mechanism as outlined below for a dimer flavanol, although no direct chemical proof has as yet been provided (Fig 1.18)¹³¹.

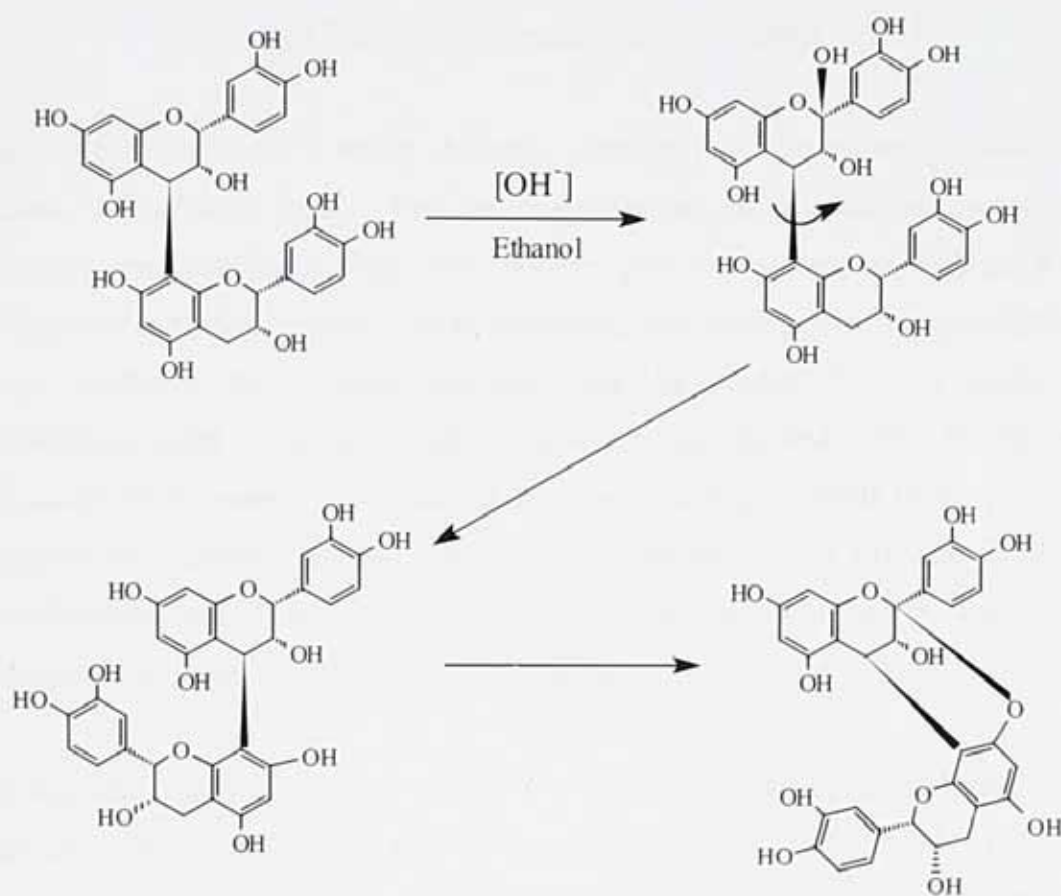


Fig 1.18 Oxidative reactions of procyanidin B2 under basic condition¹³¹

Many of the same reactions observed for the simple phenols can be reproduced in the proanthocyanidin B-ring¹³¹. In general, the degree of reactivity of the phenol and the complexity of its reactions increase with the addition of hydroxyl groups to the benzene nucleus, demonstrated when comparing gallo catechin tannin with other types of condensed tannin

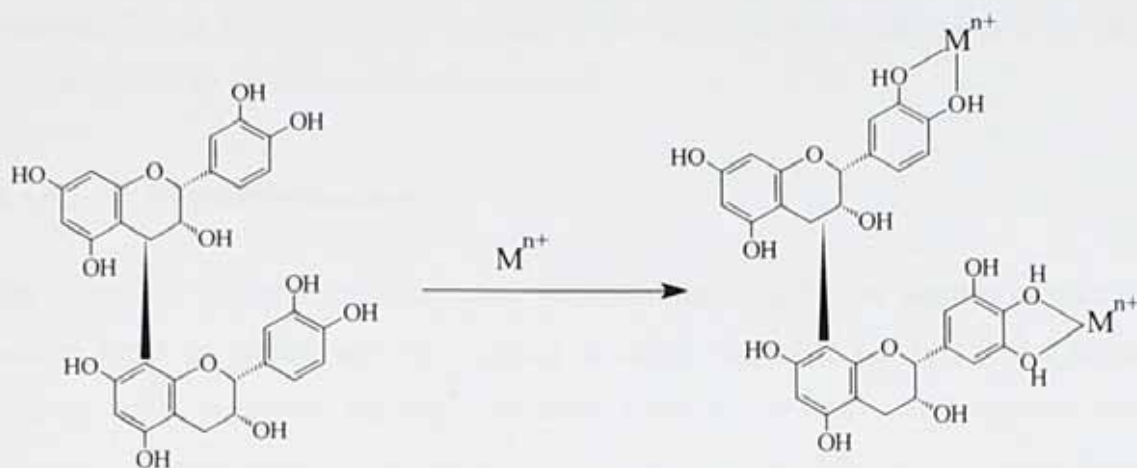


Fig 1.19 Polyphenol complexation with metal ions

Vegetable tannins have a strong ability to complex with metal ions because of the presence of hydroxyl groups. For condensed tannins, usually there are two or three hydroxyl groups on the B-ring. Two hydroxyl groups can complex with metal ions to form stable five-membered ring coordination. The presence of the third hydroxyl group facilitates the reaction and stabilises the products^{25,132}. A number of applications based on tannin chelation have been investigated¹³¹⁻¹³². For example, the use of polyflavonoid-containing barks to remove heavy metals from mining and industrial wastewaters has been studied comprehensively (Fig 1.19). The property of complexation with metal ions is the fundamental reaction of the combination tannage (semi-metal tannage) in leather making.

An arguable topic is whether or not the B-ring is reactive towards aldehydes. Normally, the catechol B-ring is quite unreactive towards most of kinds of aldehyde agents, but with one more hydroxyl group, the pyrogallol B-ring exhibits much more reactivity than the catechol B-ring. Pizzi has reported there is cross-linking bonding formed between the resorcinol A-ring and pyrogallol B-ring for proanthocyanidins when they are reacted with formaldehyde¹³⁷⁻¹⁴¹. Other researchers found that the hydroxyl pattern of the B-ring has an effect on the reactivity of the A-ring³⁰⁻³¹. The reaction of proanthocyanidin with aldehyde agent may be at the B-ring (C-2' and C-6') if the proanthocyanidin has a pyrogallol B-ring.

In this thesis, the reactivity of proanthocyanidins, the influence on the A-ring of different B-ring structures, cross-linking reaction sites and the ratio of different site reactions with oxazolidine will be presented.

1.3.3 Vegetable tannage

For thousands of years, man has used vegetable material rich in tannins to convert animal hides to leather and the method is called vegetable tanning^{5,56,58}. Leather making with vegetable tannage continues because, among many reasons, this process produces leather with strong natural feel, greater body and firmness. Most importantly, vegetable tannin is from the plant kingdom, it is a renewable resource. Consumers typically prefer more natural products, therefore, this is the main reason for vegetable tannin still being popular, even in the modern era of leather production.

1.3.3.1 Mechanism of vegetable tannage

Notwithstanding its long history, the molecular mechanisms, which underlie vegetable tannage are still not understood well. The mechanism of vegetable tannage is widely believed to involve the complexation of vegetable tannins with peptides, driven by hydrophobic effects and with multi-point hydrogen bonding to reinforce the initial interactions, which increase the hydrothermal stability of collagen. There is evidence that the peptide bond is the main proton acceptor in the formation of hydrogen bonds with the phenol hydroxyl groups of tannins^{57,58,63,89}.

The semi-colloidal property of tannin aqueous solution and skin collagen structure play important roles, which affect the penetration of tannin molecules in the skin cross-section. It is well known that vegetable tannins are poly-dispersed, the tannins also contain non-tannin components, such as simple phenols, acids, lipid, sugar and other species, which make the colloidal tannin solution stable. After penetration into the collagen fibres, the colloidal solution loses its stability and precipitates within the fibre structure, when the surface tension changes; this is the physical step in vegetable tannage. In practice, this physical deposition function of vegetable tannins cannot be neglected^{2,57}. Therefore, there is equilibrium between penetration and

combination of tannins in vegetable tannage. In the beamhouse processes of leather making, most of the non-collagenous components are removed from the pelt and the collagen structure is opened up and ready for the penetration of tannins: thereafter, the combination process can be enhanced by controlling the tanning conditions, such as temperature and pH.

Although the simple model for the combination of plant polyphenol with protein is widely accepted, the details of this process are still in debate, mainly concerned with the primary reaction sites on collagen. As discussed above, collagen has a D-periodic structure and the collagen fibril is composed of tropocollagen molecules arranged in a systematic manner. Each D-period comprises an overlap zone and a gap zone, Haslam has proposed that the gap zone is a probable site for vegetable tannins to bind^{56,58}. However, this suggestion needs more supporting experimental work.

1.3.3.2 Application of vegetable tannage

Vegetable tanned leathers are generally firm and full, so traditional vegetable tannage is used mainly for shoe soles and leather goods, such as saddle leather and industrial belting leather. Today, vegetable tannins are also widely used in upper leather re-tanning and combination tannage. However, vegetable tanned leather always has low shrinkage temperature: condensed tannin tanned leather always has a higher shrinkage temperature than hydrolysable tannins tanned leather (Table 1.3)⁵⁷.

Table 1.3 Shrinkage temperatures of vegetable tanned leather

	Vegetable tannins			
Condensed	Mimosa	Quebracho	Larch	Gambier
Ts (°C)	85	84	80	80
Hydrolysable	Valonea	Sumac	Chestnut	Myrobalans
Ts (°C)	75	75	78	72

Due to the long process (slow penetration), low shrinkage temperature and other unacceptable characteristics of vegetable tanned leather, new methods have been

developed, such as vegetable tannin-metal combination tannage, vegetable tannin-aldehyde combination tannage. High shrinkage temperature can be obtained from both of these new methods.

1.3.3.3 Vegetable tannin-metal combination tannage

In 1885, Procter described a semi-aluminium process, using basic aluminium salts for treating gambier-tanned leather for the production of patent leather, which could withstand higher temperature finishing treatment²⁸. More recently, Slabbert undertook a comprehensive study of mimosa-aluminium combination tannage²⁶. He postulated that the aluminium ions react with the ortho-dihydroxyl groups of the flavanoid pyrogallol B-ring in mimosa and suggested the mechanism involves the formation of aluminium cross-linking between two tannin molecules bound to collagen or one bound tannin molecule and collagen¹³³⁻¹³⁴(Fig 1.20). Further studies have shown the former is the more probable mechanism. With the increase in environmental pressure on chrome tanning, vegetable tannin-aluminium combination tannage has become one of the more promising substitutes for chrome.

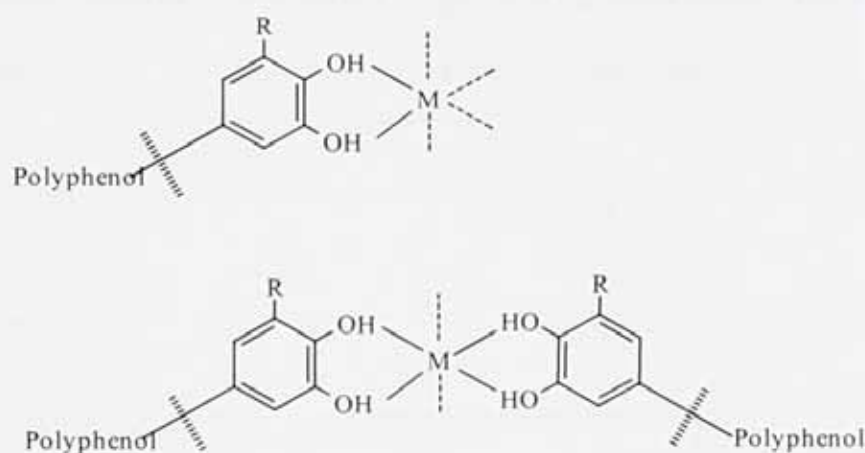


Fig 1.20 Structure of coordination of condensed tannins with metal ions

The leather tanned by aluminium-vegetable tannin method has a high shrinkage temperature (Table 1.4). Hydrolysable tannins or condensed tannins with a pyrogallol B-ring have been found to be more suitable for this method.

Table 1.4 Shrinkage temperature of vegetable-aluminium tanned leather (°C)

Tannins	Vegetable tanned	Veg-Al tanned
Tara	68	115
Myrobalan	68	120
Quebracho	78	88
Mimosa	78	98

1.3.3.4 Vegetable tannin-aldehyde combination tannage

The problem associated with semi-metal tannage is the presence of the metal ions, especially aluminium, which is regarded as a pollutant. Vegetable tannin-aldehyde combination tannage would avoid using metal ions and high shrinkage temperature can still be obtained^{35-37,135-137}. Condensed tannins are more suitable for this tannage, due to the highly nucleophilic properties of the flavanoid molecular structure, which has resorcinol or phloroglucinol as the A-ring^{64,65}.

Table 1.5 Shrinkage temperature (°C) of vegetable tanned leather (mimosa) retanned with different crosslinking reagents at elevated temperature⁵

Crosslinker	Reaction temperature (°C)		
	20	40	60
Control		84	
Phosphonium salt*	94	93	93
Glutaraldehyde	90	91	92
Oxazolidine	100	105	110

- Tetrakis-hydroxymethyl phosphonium sulphate

Aldehyde can react with the free amino group of collagen in a Mannich reaction and form cross-links at a second amino group or tannin molecule^{4,17}. The cross-linking can be classified as “tannin-aldehyde-tannin”, “tannin-aldehyde-collagen” and “collagen-aldehyde-collagen”. This means that there are at least three kinds of chemical functions responsible for the high stability: covalent bonding, hydrogen bonding and hydrophobic bonding. The first model has been widely believed to be

the main reaction in this process, because condensed tannin is more reactive than collagen amino groups. Therefore, the mechanisms of this reaction will depend on the tannin molecular characteristics, the reactivity of the aldehyde cross-linking agent (Table 1.5) and the reaction conditions, such as pH. So far, there is no direct experimental support for the mechanisms that have been suggested.

1.4 High stability organic tannage based on gallo catechin tannins

1.4.1 Environmental requirements for the global leather industry

The leather industry has had a bad reputation for environmental pollution, especially in the developing countries. Chromium(III) is a major heavy metal pollutant of aquatic bodies. Every year, a large amount of chrome is drained without treatment into the environment. Usually, chromium occurs in two oxidation states, Cr(III) and Cr(VI), and both forms are taken up by plants; Cr(III) is sparingly soluble and less toxic, while Cr(VI), being more soluble in water, is highly toxic to biota. Soluble Cr(VI) salts are absorbed more easily and cross cell membranes more readily than the trivalent chromium salts. The chromium pollution from the leather industry is mostly trivalent chrome, but the Cr(III) may be converted to Cr(VI) under some conditions (oxygen, UV, free radicals). Therefore, the potential danger from trivalent chromium cannot be neglected¹⁷. Aluminium cannot be the substitute for chrome, because it too has an environmental impact⁵.

For the future of leather production, tanning has to be environmentally friendly, preferably without involving any metal ions, so the leather should be able to be recycled completely. Bearing this in mind, leather chemists have to find a more suitable chrome-free or metal free tanning method, which at the same time confers the required commercial properties to leather.

1.4.2 Development of metal free tannage

In the search for an alternative tanning method to chrome, the most difficult property to achieve is the high hydrothermal stability, whilst retaining the other desirable

features of chrome tanning. Organic tannage probably holds the future for the leather tanning industry. To date, two kinds of organic tannage have been reported to produce high hydrothermal stability: one is based on melamine-formaldehyde resin, the other is condensed tannin-oxazolidine combination tannage.

1.4.2.1 Synthetic organic tannage

Synthetic organic tanning methods have been used in the leather industry for a long time, in the form of the widely used polyacrylates and syntans. Unfortunately, no syntan can confer high Ts to leather. That is, until the melamine-formaldehyde resin method was proposed by Covington⁵ (Fig 1.21). It was found that oligomers can be further reacted *in situ* on collagen with additional cross-linking agent, to raise the shrinkage temperature to previously unachievable high values ($T_s > 115^\circ\text{C}$). This can be done by controlling the resin particle size, then by cross-linking with organic phosphonium salt, in which the reactions are controlled by temperature rather than pH.

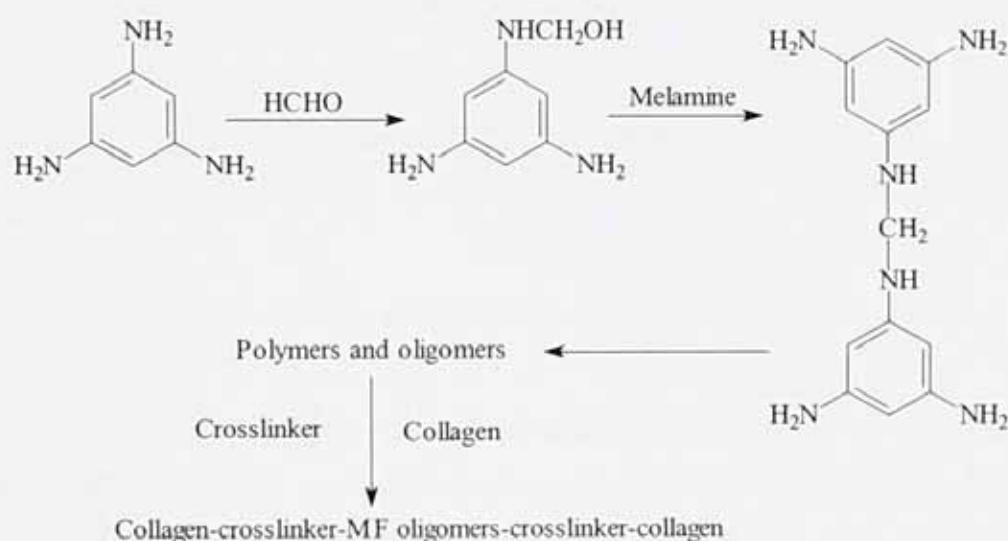


Fig 1.21 Melamine-formaldehyde (MF) oligomer tanning

1.4.2.2 Condensed tannin-oxazolidine combination tannage

Condensed tannin-oxazolidine combination tannage has been proposed only recently. It is analogous to organic synthetic tannage and the previously discussed

vegetable tannin-aldehyde tannage. In 1998, Covington and Shi reported that condensed tannin-oxazolidine combination tannage could confer high hydrothermal stability to leather³⁰⁻³¹; the shrinkage temperature could reach 115°C, which was previously unachievable based on vegetable tannins³¹⁻³². The process consists initially of penetration by the polyphenol at pH 5-6, followed by cross-linking with oxazolidine. They found that only condensed tannins exhibit a positive synergistic effect with oxazolidine. The best result was obtained from mimosa tannin and it was clearly shown that not all condensed tannins are successful in this process. It is interesting that the pyrogallol function of the B-ring apparently influences this reaction strongly, since the accompanying model study with epicatechin showed that the crosslinking with oxazolidine occurs only at the phloroglucinol A-ring (Fig 1.22).

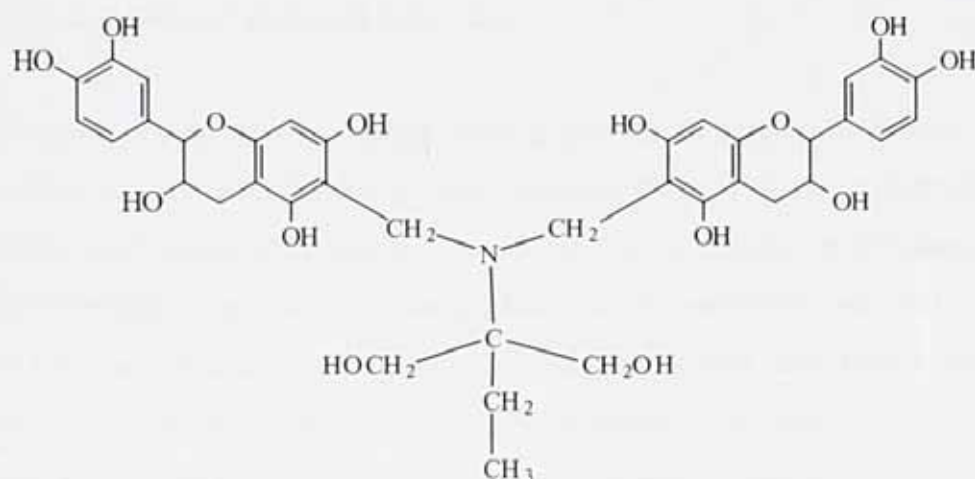


Fig 1.22 Cross-linking reaction between catechin and oxazolidine

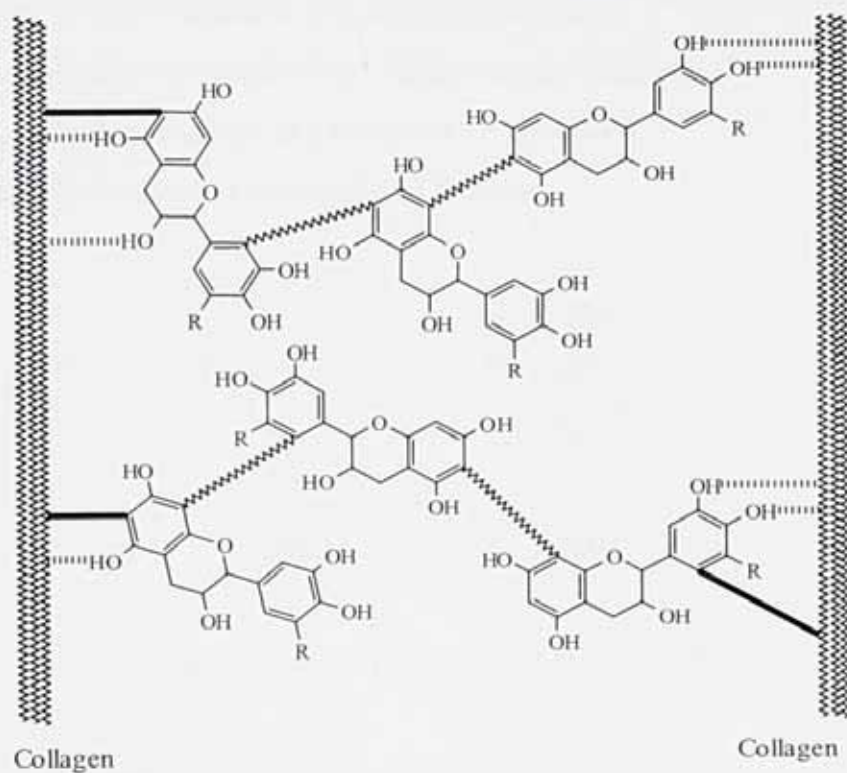
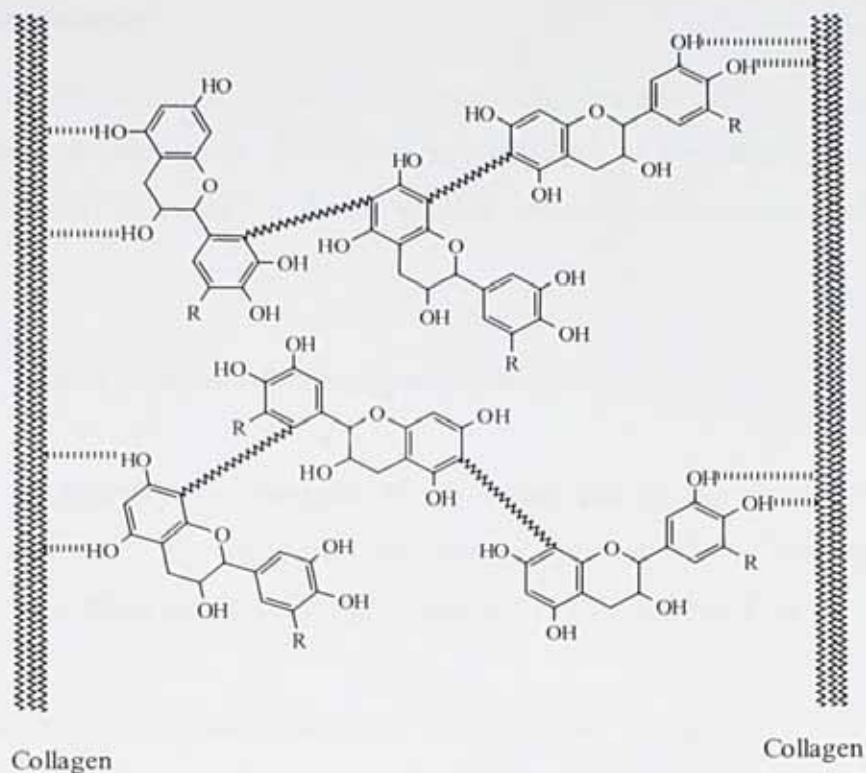
Comparing mimosa with quebracho, these tannins have similar molecular weight, similar conformation and configuration of the molecules, both have a resorcinol A-ring, but the only difference lies in the B-ring: mimosa has a pyrogallol B-ring in one of the major constituents, but quebracho has a catechol B-ring. It is well known that the phloroglucinol A-ring in condensed tannins is a stronger nucleophile than the resorcinol A-ring. Based on this, we can predict that the prodelphinidins (gallocatechin tannins) should have the highest reactivity towards oxazolidine, because they have a phloroglucinol A-ring and a pyrogallol B-ring.

1.4.3 Introduction to this project

Covington and Shi proposed that the cross-linking reaction happens between collagen molecules and high tannin polymers formed in the condensation process³¹. These condensation products combine with collagen fibres through multiple hydrophobic bonds and hydrogen bonds. This is similar to vegetable tannage; the difference is in the molecular weight. There is also argument about this, because the combination product should be labile to heat, if there are only hydrogen bonds between collagen and the tanning agent. Then where does the high stability come from? There is also the possibility of forming covalent bonds between tannins and collagen: the tannins would be immobilised on collagen fibres or fixed between fibres and so the high shrinkage temperature may come from the stable covalent combination rather than only hydrogen bonding. The elucidation of this problem is one of the objectives of this work (Fig 1.23).

As discussed above, the pyrogallol B-ring has an important influence on the crosslinking reaction, so, does the pyrogallol B-ring take part in the crosslinking and what is the main reason for mimosa being better than quebracho in the combination tannage? Therefore, high stability tannage based on the prodelphinidin gallo catechin tannins was studied in this work. Two prodelphinidin tannins have been used in this research: pecan nutshell tannin and *myrica esculenta* bark tannin, for which the botanical description and tannin chemistry are discussed in Chapter Two.

Oxazolidine was used through this work as the cross-linking agent; the molecular structure is given in Fig 1.9. Little is known about this compound, so its synthesis and hydrolysis were studied, as well as its reactivity with polyphenols and collagen. Das Gupta reported oxazolidine will hydrolyse and react with the amino groups of collagen to form cross-links to improve the shrinkage temperature⁵⁴⁻⁵⁵, but no experimental results were reported. Again, very little is known about the tanning properties of oxazolidine. All these effects will be discussed in following chapters.



- Hydrogen bond between collagen and tannins
- ~~~~~ Covalent bond between tannins
- Covalent bond between collagen and tannins

Fig 1.23 Cross-linking model for condensed tannin-oxazolidine combination tannage
(Upper: non-covalent bond model, Lower: covalent bond model)

1.5 Methodologies

Considering the complexity of both vegetable tannins and collagen, model compounds were employed, first in the investigation of the cross-linking reaction mechanism and followed by direct studies with macromolecular collagen and tannins.

1.5.1 Model compounds for polyphenols

Based on the hydroxylation patterns of the A-ring and B-ring, flavanol monomers can be modelled by mixtures of simple phenols¹³⁸⁻¹⁴¹: resorcinol (XI), phloroglucinol (XII), pyrogallol (XIII), catechol (XIV), see Fig 1.24.

- Resorcinol and phloroglucinol as models of the A-ring
- Pyrogallol and catechol as models of the B-ring
- Phloroglucinol+catechol as a model of catechin
- Phloroglucinol+pyrogallol as a model of gallocatechin
- Resorcinol+pyrogallol as a model of robinetinidol
- Resorcinol+catechol as a model of fisetinidol

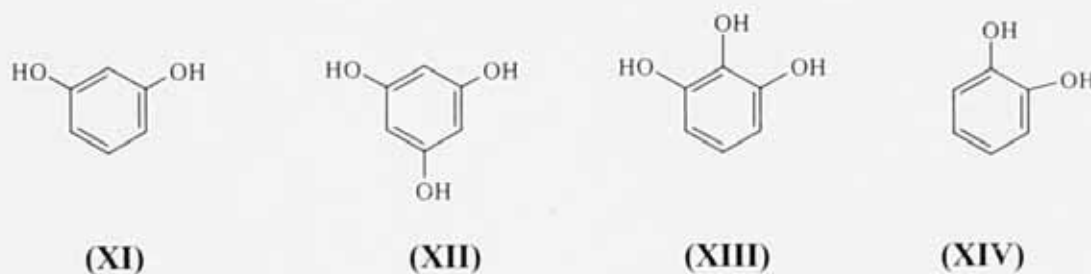


Fig 1.24 Model phenols used in this work

1.5.2 Model compounds for collagen

In the structure of collagen, the free amino groups, either from the N-termini or from lysine and hydroxylysine residues, are known to be cross-linking places for aldehyde or oxazolidine cross-linking; from the literature^{4,47}, some researchers also believe the guanidyl group from the arginine residues might take part in the cross-linking

reaction with aldehyde. Therefore, n-butane amine, glycine, lysine and arginine have been used here as models for collagen to study the cross-linking process. Acid soluble collagen, from young rat tail tendon was also used, as a more realistic model, avoiding penetration difficulties associated with skin of finite thickness.

1.6 Main aims

The main objective of this work was to understand high stability organic combination tanning, by addressing the following aspects:

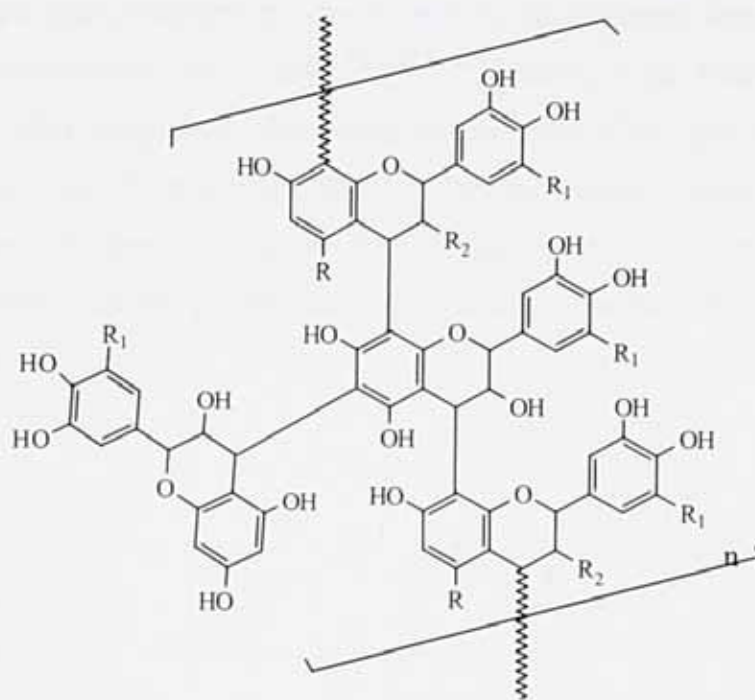
- 1 Preparation and characterisation of prodelphinidin condensed tannins**
- 2 Synthesis and hydrolysis of oxazolidine**
- 3 Mechanism of the reaction between polyphenols and oxazolidine**
- 4 Mechanism of the reaction between collagen and oxazolidine**
- 5 Mechanism of the reaction between polyphenols, collagen and oxazolidine**

Chapter Two

Preparation and characterisation of gallo catechin condensed tannins — prodelphinidins

2.1 Introduction

Since 1824, black wattle bark extract (mimosa tannin) has been sold as concentrated aqueous extract in Australia and since the end of the 19th century vegetable tannin powders have become common commercial chemical products for leather and other industries¹⁴². However, the understanding of the chemistry of vegetable tannins, especially condensed tannins, was vague until the 1950's. Significant progress was made between the 1960's and the 1990's by many research groups, such as Roux and Pizzi^{93-97,112-115,131,138-140,142} in South Africa, Haslam^{63,66,72,84-87,89,91,107,149} in UK, Okuda^{74,75,102,127,159} in Japan, Hemingway^{64,65,106,129,141,155,177} in USA and Porter and Foo^{76-78,92,116,126,145,151,175} in New Zealand. The chemistry of plant polyphenols and their main properties have been reviewed in Chapter 1. The rapid development of plant polyphenol chemistry has provided the foundation for many applications of these important natural products.



R	R ₁	R ₂		
OH	H	H	XV	Procyanidins (gambier, larch)
OH	OH	H	XVI	Prodelphinidins (pecan, myrica)
H	H	H	XVII	Profisetinidins (quebracho)
H	OH	H	XVIII	Prorobinetinidins (mimosa)

Fig 2.1 Proanthocyanidins polymers and some important monomer units

The most commonly used commercial condensed tannin extracts in the present leather industry are mimosa^{57,68,69,142,144,166} (prorobinetinidin), quebracho^{118,145,146} (profisetinidin), mangrove and gambier^{16,63,69,143,147} (procyanidin); none belongs to the prodelphinidin type tannin (gallocatechin tannins). As discussed in Chapter 1, the appearance of new prodelphinidin type condensed tannins will have the potential for application in tanning chemistry.

Many parameters influence the properties of vegetable tannins extracts, such as the state of the raw materials of plant (fresh or old bark, leaf, root, fruit, etc.), the extraction solvent system, temperature, pH condition, as well as the polyphenol chemical structure characteristics and its composition¹⁴⁷. Many methods have been used in the extraction studies of vegetable tannins^{86,87,96,107,108,117,147-154}. For phytochemistry studies, the most used solvents are 50-70% acetone aqueous solution, 50-100% methanol aqueous solution, ethanol and ethyl acetate. For the production of commercial tannin extracts, water with different contents of sodium sulphite (usually no more than 7-8%^{64,142,145,154,256}, based on the dried weight of raw materials) at high temperature has been widely used. The presence of sodium sulphite can increase the extraction yield, lighten the colour, enhance the solubility and protect the polyphenol molecules from oxidation by heat or sunlight. Sodium sulphite can react with the proanthocyanidin polymer and can partially degrade the structure (Fig 2.2).

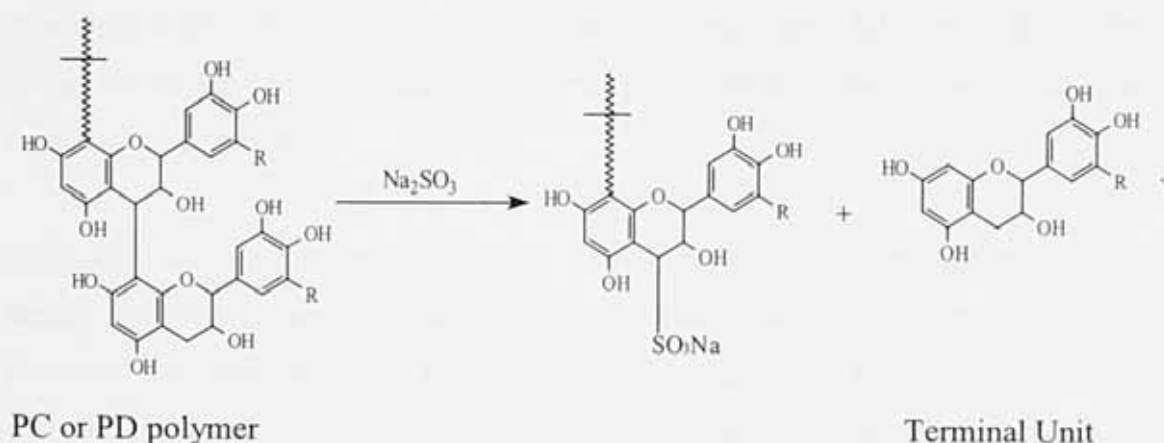


Fig 2.2 Degradation of proanthocyanidins by sodium sulphite

In the early days of polyphenol studies, 50% methanol was thought to be the best solvent to extract plant polyphenols, but Haslam and Porter have pointed out it may not be a good solvent for proanthocyanidin polymers^{63,66,70,147}. After applying the complex tannin solution to the top of a Sephadex LH-20 column, typically 50% methanol aqueous solution is used to wash out the non-tannins and low molecular weight tannins fraction from the proanthocyanidins extractions, then 50-70% acetone aqueous solution is used to wash out the purified proanthocyanidin polymers. 50% methanol still is used in the studies of tannin extraction, because it is believed that less oxidation happens with this method, compared to the 50% acetone solution system⁸⁴.

1% of methanolic hydrochloric acid has also been used to extract tannins¹⁴⁷. This solvent can increase the yield of proanthocyanidins from the grains of sorghum; this may due to de-polymerisation of insoluble proanthocyanidins in organic solvent, which is similar to the anthocyanidin reaction of proanthocyanidins, (see Section 2.3.2.2). This method has not been used widely, because the yield is usually much lower than with 50% acetone aqueous or ethanol.

In phytochemical studies, it is very important to avoid any chemical alteration in the extraction process. Proanthocyanidins can be oxidised easily at relatively high temperature or by exposure to oxygen and the presence of some metal ions will enhance the oxidation process^{64,65,84,128,131}. Therefore, the temperature for extraction is usually kept low (20-25°C) when organic solvent-water mixture is used. The water should be demineralised, in order to minimise the effect of auto-oxidation catalysis by metal ions.

Other than the main polyphenols in the tannin extracts, there are also many non-tannin components, such as sugar, polysaccharide (gum), lipid, salt, chlorophyll, terpenes, steroids, alkaloids and proteins, which have to be removed through dialysis, lead salt precipitation or organic solvent extractions in the tannin purification process. Sugar is removed usually through lead salt precipitation, the phenolic components will be precipitated out and the sugar can be removed after

centrifugation^{94-96,142}. The free polysaccharides can be removed by saturated alcohol solution (concentration>95%), to allow separation by centrifugation.

To elucidate the molecular structure of proanthocyanidins, chemical degradation under acidic condition, with or without the presence of nucleophilic agents (such as phloroglucinol or toluene- α -thiol)¹⁵⁵, and nuclear magnetic resonance (NMR) spectroscopy^{63,66,76-77,104,126,156} have been the most powerful methods, from which most of the molecular information can be obtained. The high polymers can be degraded to monomers, oligomers or derivatised products, and then separated and purified: thin layer chromatography (TLC), Sephadex G-25, G-50 or Sephadex LH-20 column chromatography^{66,101,103,109,168} and reverse-phase high performance liquid chromatography (RP-HPLC) with a C-18 column^{63,66,68,117,119,157,158,160,251} have proved to be the best methods for low molecular weight proanthocyanidins. Other analytical methods, such as circular dichroism (CD), infrared spectrometry (IR or FT-IR), also have been widely employed^{80,110,161-163}.

2.2 Raw materials

Two prodelphinidin tannins have been studied in this work. One raw material was pecan nutshell pith. Pecan trees are widely distributed in the United States and Central America. More than one million tonnes of pecan nutshell is produced every year as agricultural waste, in which the tannin content is higher than 40%^{79,106}. Another raw material was the bark of *Myrica esculenta*¹⁰⁸. This tree is widely distributed in Asia; the tree bark has been used in traditional medicine in China, India and other countries. The tanning properties of these two tannins are not well known yet.

This work concerns the extraction and analysis of tannins, the elucidation of the chemical structural characteristics and the tanning properties, compared with the commercial tannins mimosa and quebracho.

2.2.1 Pecan nutshell pith (*Carya illinoensis*)

The pecan tree is a member of the *Juglandaceae*, together with walnut, but it is more closely related to hickories than walnuts. The current binomial for the pecan is *Carya illinoensis*, although in the last 100 years it has been referred to as *Juglans pecan*, *Juglans illinoensis*, *Hicoria pecan*, *Carya pecan*¹⁶⁴. America Indians used pecans at least 8000 years ago in Texas. The term **pecan** comes from the Indian word “**pacane**”, meaning, “**nut so hard as to require a stone to crack**”. There are about 20 *Carya* species, a few of which contain edible nuts: they generally have low yields, thick, hard shells, and small kernels. Pecan is the most important native North American orchard species.

Pecan nut pith contains mixed procyanidin and prodelphinidin polymers in which the prodelphinidins predominate. Units with 2,3-*cis*-stereochemistry occur more than those with 2,3-*trans*-stereochemistry. Compared with more common procyanidin-based (3,5,7,3',4'-pentahydroxyflavan) vegetable tannin polymers, little is known about the chemistry of prodelphinidin-based (3,5,7,3',4',5'-hexahydroxyflavan) plant polyphenols (Fig 2.3)¹⁰⁶.

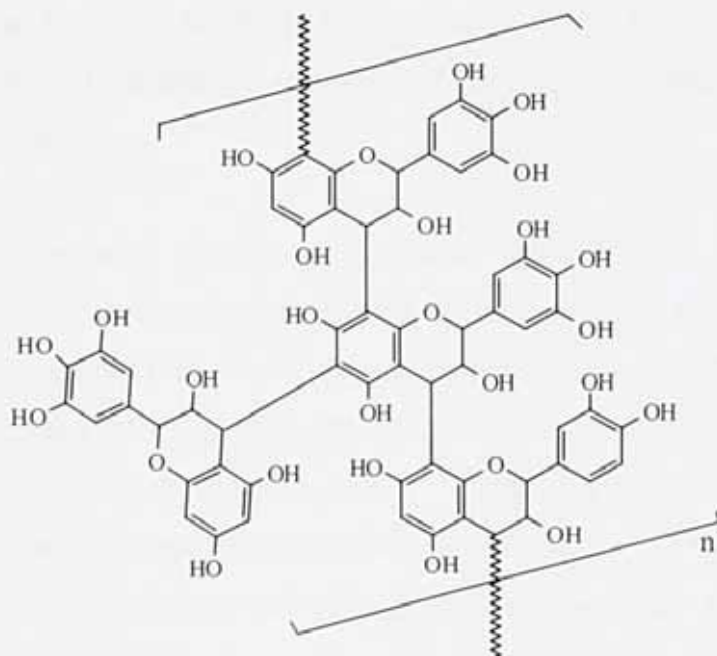


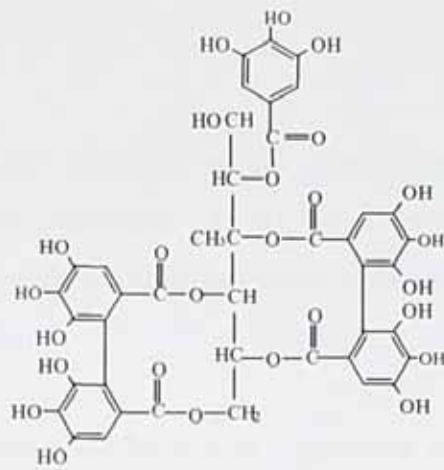
Fig 2.3 Proanthocyanidin (condensed tannin) from pecan nut pith¹⁰⁶

2.2.2 *Myrica esculenta* tree bark

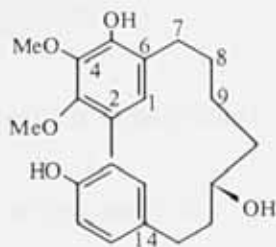
Myrica esculenta, as an important raw material for crude medicine, is widely distributed in the area of the Himalayan Mountains. It belongs to the family *Myricaceae*, also named hairy bayberry. The evergreen plant is a shrub or slender tree up to 40 feet high. The leaves are from 1 to 4 inches long, narrow, wedge-shaped, entire or with a few teeth, and have a fragrant odour when crushed. The flowers appear from March to May, according to locality, generally before the leaves are fully expanded. Male and female are borne on separate trees, the male flowers in cylindrical yellow clusters and the female flowers in green, shorter clusters. The fruit, which remains on the tree for several years, consists of clusters of round, single seeded, berrylike nuts covered with a whitish wax. The colour of bark is grey-brown. The bark is very hard with light yellow cross section; the powder has very strong astringent taste. The bark is mainly used for treatment of stomachache, headache and toothache; it is antipyretic, anti-spasmodic and a bronchial sedative.

Tannin extracts from *Myrica esculenta* bark have been used commercially in China since the early 1970's, with present production amounting to several thousand tonnes annually⁵⁷. Now *Myrica esculenta* bark tannins form the largest production in China, more than that from larch and Chinese valonea. But the tannin's properties have not been studied completely; in particular the reactive properties with protein and aldehyde are not clear.

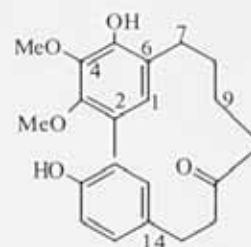
Sun conducted some work on this tannin in the 1980's^{68,108} and found the tannin content is about 20-30% based on the weight of dried bark. An interesting result is that the *Myrica esculenta* bark tannin is pure prodelphinidins, with about 40% of the C-3 hydroxyl groups esterified. The extracts also include ellagitannin, myricanol, myricanone, epigallocatechin-3-O-gallate, two prodelphinidin dimers epigallocatechin-(4 β -8)-epigallocatechin-3-O-gallate and 3-O-galloylepigallocatechin-(4 β -8)-epigallocatechin-3-O-gallate (Fig 2.4). The higher molecular weight fraction consists exclusively of prodelphinidin units with 2,3-cis-configurations of average molecular weight 5000D. The polyphenol polymer has epigallocatechin-3-O-gallate as the terminal unit. Very little is known about the non-tannins of this extract.



Ellagitannin



Myricanol



Myricanone

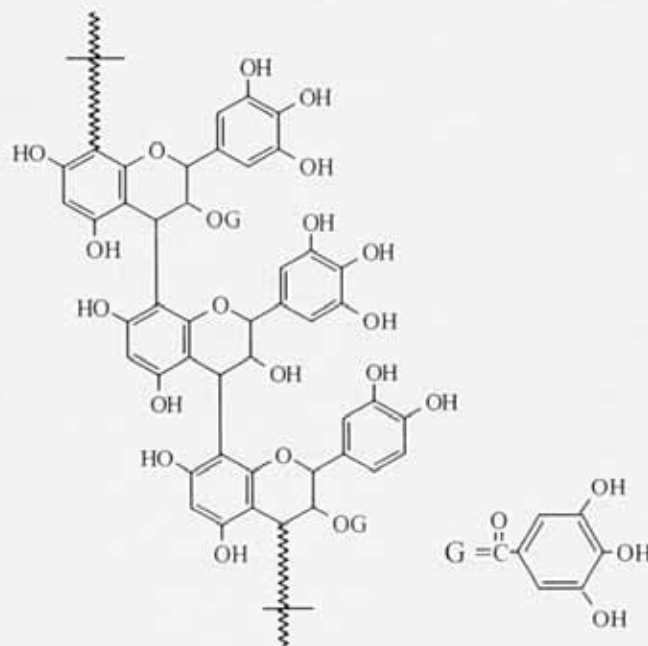


Fig 2.4 Proanthocyanidin and other components from *Myrica esculenta* bark

2.3 Experimental

2.3.1 Materials

All the chemicals used were analytical grade, obtained from Sigma-Aldrich (UK). Sephadex G-25, G-50 and Sephadex LH-20 were obtained from the Pharmacia Company (Sweden). A standard chromatography column C 40/26 (45x3mm) was used (UK, Sigma-Aldrich).

Air-dried pecan nutshell pith was received as a gift from Mr. Andy Sherrod (Royalty Pecan Company in America), one sample was fresh and a second sample was five years old. Air-dried fresh *Myrica esculenta* bark was received as gift from Wu-ming Vegetable Tannin Extracts Company of China; the bark was collected from 5-6 year old trees in autumn 2000.

2.3.2 Extraction experiment

2.3.2.1 Extraction methods

Air-dried pecan nutshell, and fresh dried *Myrica esculenta* bark were converted to fine powder (mesh 12-25) with an Apex grinder (NECO. Ltd, London).

Extraction with water

The powder (500g) was extracted with 1,500ml of water at different temperatures (25, 50, 85°C) in a 3 litre brown bottle^{86,87,108}. The process was carried out over 12 hours and then the debris was filtered off, the procedure was repeated a further four times and the extract solutions were combined. The total volume of aqueous extracts was concentrated by rotary vacuum evaporation at 35°C to about 500ml.

Extraction with 50% acetone aqueous solution (v/v) or ethanol

Considering the evaporation and efficiency of organic solvent, the two organic solvent systems were only applied at low temperature (25°C). A similar procedure to aqueous extraction was employed. The combined ethanol or acetone aqueous filtrates were concentrated under reduced pressure by rotary evaporation.

The concentrated aqueous extract solutions obtained from all the extraction methods were freeze-dried and kept at -4°C for further analysis.

Some of the concentrated water extracted tannin solution was used directly, to study physical properties and the normal tanning ability of the extracts. The solid content of the solution was determined by drying 20ml of solution in a vacuum oven (200mmHg) at 45-50°C. Aqueous acetone solution extracts were used for the most of the physical-chemical studies on the tannin molecular characteristics.

2.3.2.2 Analysis of crude extracts

2.3.2.2.1 Tannin/non-tannin analysis

The tannin content was measured with hide powder by the SLTC standard method (SLC 6).

2.3.2.2.2 Determination of sugar content

Tannin extracts were dissolved in 50 volumes of distilled water and 10% neutral lead acetate was added slowly with vigorous stirring^{94,95}. A clear white precipitate of lead tannate formed instantaneously; the lead salt precipitation was centrifuged at 2,000g for 20 minutes and washed four to five times with a little water to remove non-phenolic compounds and excess lead salt. The final product was suspended in distilled water and diluted oxalic or sulphuric acid was added to release the tannins fraction. A new lead salt precipitate was formed and separated by centrifugation. The solution was evaporated to dryness under reduced pressure, then redissolved in solvent (acetone and ethyl acetate) and the non-soluble part was filtered off. The procedure was repeated. The content of sugar in the extracts was calculated based on the dry weight of the extracts.

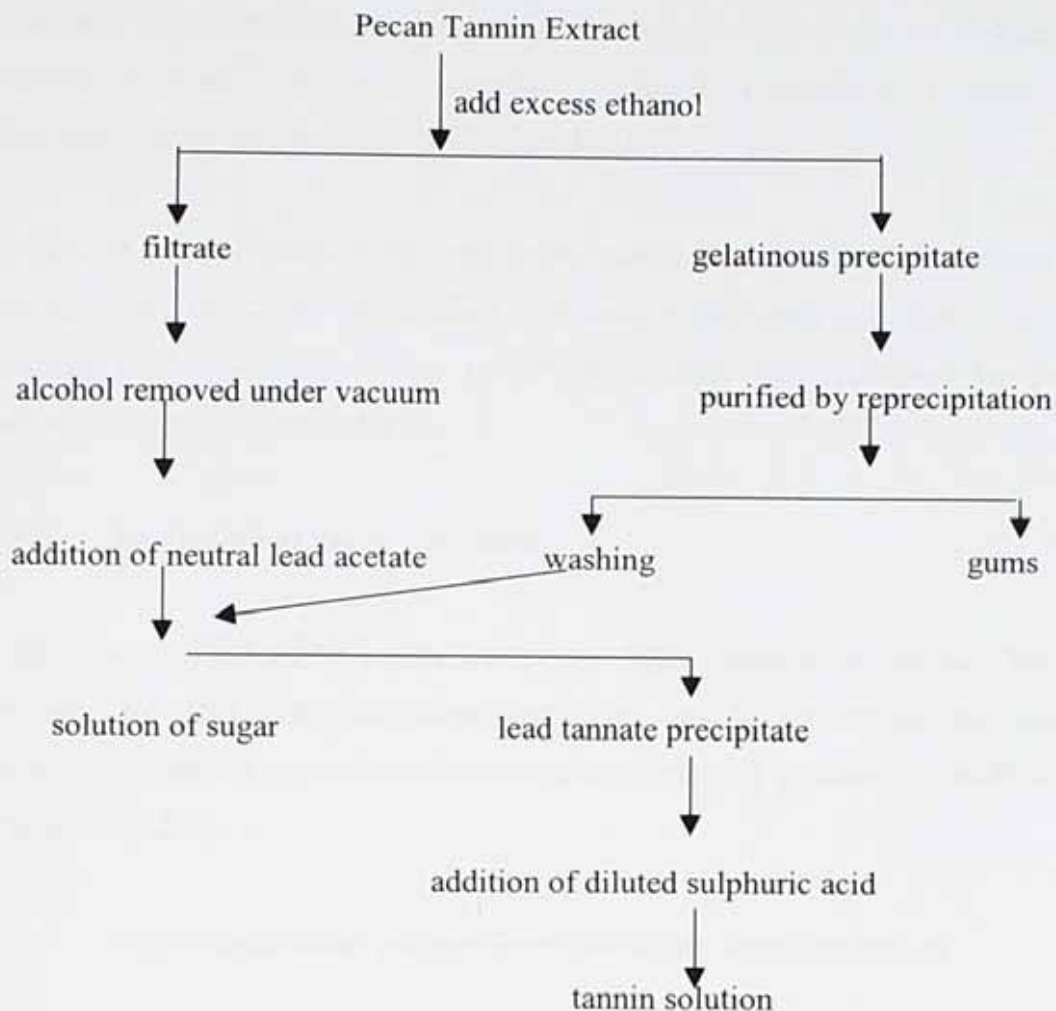


Fig 2.5 Procedure for measurement of sugar and gum content in extract

2.3.2.2.3 Determination of polysaccharides (gums)

1g of sugar free tannin material, dissolved in 20 volumes absolute ethanol, was centrifuged and the gelatinous residue was thoroughly washed five times with small amounts of ethanol until colourless; the washing solution was checked for polyphenol content by adding one drop of ferric chloride solution. After evaporating, gum free tannins were obtained⁹⁵.

2.3.2.2.4 Analysis of sugar and gums

The free sugar was examined by paper chromatography¹⁴⁷. The sugar solution was applied to Whatman No.1 paper using a capillary glass tube. The paper chromatogram was run with butan-2-ol-ethanol-water (5:1:4, v/v/v) and then silver

nitrate and naphthoresorcinol-phosphoric was used to develop the colour, as described by Syed¹⁴⁷. Reference samples, D-glucose, L-arabinose, L-fucose, D-xylose and D-galactose, were used as the standards.

The gum or the polysaccharides were hydrolysed with 1 mol/l sulphuric acid for eight hours at 100°C, then neutralised with barium carbonate and filtered through Whatman No.1 filter paper. The hydrolysis solution was examined by paper chromatography, as described above.

2.3.2.3 Extraction of pecan nut shucks

On the outside of the pecan nutshell, there is a layer called pecan shuck. This has been used to make activated carbon, but there are no reports on the tannins content¹⁰⁶. Extraction of pecan nut shuck was studied using the same methods as for pecan nutshell pith.

2.3.3 Physical-chemical properties of the tannin extract solution

Vegetable tannins are complex organic compounds, large molecules with wide range of molecular weights, which, without the presence of some strongly lyophilic groups in their structure, would be insoluble in water. The presence of a large number of hydroxyl groups in the molecular structure determines the hydrophilic and hydrophobic properties of tannin molecules. At the same time, the occurrence of non-tannins produces colloidal stability. The tanning property of vegetable tannin infusions is intimately linked with its semi colloidal nature. The presence of salt and acid in the extracts also has an important effect on the properties of vegetable tannin solutions.

Physical-chemical properties of natural tannins are of great significance for the proper understanding of vegetable tannin chemistry and the vegetable tanning process. In the early 1960's, Scaria¹⁶⁵ conducted a comprehensive study on the effects of the physical properties of tannin solutions on the mechanism of vegetable tanning and found the semi-colloidal property of tannin solution and the presence of

small molecules all play important roles¹⁶⁵. A study of some of the physical-chemical properties of these prodelphinidin condensed tannins may therefore furnish useful information in understanding the mechanism of the tanning reaction.

2.3.3.1 Viscosity of colloidal solution

One of the important properties of vegetable tannins solution, which is related to the particle size and molecular weight of tannins, is its viscosity. Viscosity may be considered as a indicator of different penetration powers of types of extract. Since the rate of kinetic diffusion varies inversely as the viscosity, low viscosity will facilitate the penetration of vegetable tannins into the pelt. The viscosity of tannin solutions were measured with a Ubbelodhe viscometer with inner diameter 0.16mm, using a water bath controlled at $25.0 \pm 0.2^\circ\text{C}$, solution conditions were pH 4.0-8.0 and concentration 10-200g/l¹⁵⁴.

2.3.3.2 Salt and acid content

Holmes¹⁴⁷ considered that the action of vegetable tanning liquor is firstly the action of ions from salts and acids to modify the structure of the untanned collagen pelt and secondly the combination of the vegetable tannin with the fibres. Here, the free salt and acids were measured by cationic exchange resin column using the SLTC standard method (SLC 4).

2.3.4 Chemistry of prodelphinidins

2.3.4.1 Separation and purification of plant polyphenols

After removing the acetone from the 50% acetone extraction, the aqueous tannin solution was extracted with equal volumes of chloroform twice and then twice with ether. Then the residue was extracted eight times with equal volumes of ethyl acetate, the gum of the pecan extracts suspended in the ethyl acetate fraction was removed by filtration and a low molecular weight polyphenol fraction A was obtained. The aqueous phase was concentrated and passed through a Sephadex LH-

20 column; the gum and low and medium molecular weight phenols were eluted out with 50% methanol and then the high molecular weight polyphenols, fraction B, were eluted with 50% acetone. Combination of fractions A and B produced the total tannins in the initial extracts^{63,66,86,87,106,108,117}.

Due to failure to separate gum from the pecan nut shell pith extracts with saturated ethanol solution, this process was carried out using Sephadex G-50 and LH-20 column chromatography. The extracts of *Myrica esculenta* bark were treated by the same procedure.

2.3.4.2 UV-Vis spectroscopy

The UV absorption of tannins solution, before and after purification by column chromatography, was measured in aqueous solution at concentration 30mg/l with a Philips UV S-280 spectrophotometer^{57,92,126}.

2.3.4.3 Infrared spectroscopy

Freeze-dried samples were examined as KBr discs with a Perkin-Elmer-781 infrared spectrophotometer; the base line was adjusted to 100% at 4000cm^{-1} with no sample in the optical path. The instrument settings were split 3; scan time 6 minutes; noise filter 1; abscissa expansion 0.5^{126,163}. The spectra were recorded over the wavelength range 4000 to 600cm^{-1} . Polystyrene film was used for calibration.

Procedure for preparation of KBr discs:

1mg of freeze-dried tannin sample and 200mg of KBr were mixed and ground to fine powder and placed in the closed IR sample tube together with two 3/16 inch balls and vibrated for 5 minutes, then treated under pressure (12tonnes/m^2) for 3 minutes. A transparent uniform salt plate was obtained with average thickness 0.3-0.5 mm.

2.3.4.4 Paper chromatography (Pc) and thin layer chromatography (TLC)

One and two dimensional paper chromatography and thin layer chromatography have been widely used as simple and powerful methods in the studies of plant phenolic materials. Most of the components have unique R_f values in one or two directions, therefore, Pc or TLC are useful methods for defining the complex mixtures^{67,86,87,94,100,109,160,168,169}. Before running Pc or TLC, the gum fraction was removed by passing through a short Sephadex LH-20 column (~3.5x5cm).

Developing systems for Pc and TLC:

6% acetic acid (A) and n-butanol: acetic acid: water 14:1:5 v/v/v (BAW) (B) have been used as the developing systems for one and two-dimensional chromatography since the 1970's. Although the molecular structure of polyphenols is usually determined by separating pure compounds, it is still possible to confirm some of the components from two-dimensional chromatography, if accurate reproduction of R_f values is obtained. Some of the proanthocyanidin monomers have also been confirmed by running control compounds^{63,66}.

2.3.4.5 Average molecular weight analysis with gel permeation chromatography

1g of sample was dissolved in freshly redistilled pyridine, then excess acetic anhydride was added and the mixtures were stood at room temperature for 48 hours. The reaction products mixture was poured into ice water and shaken well until a precipitate formed. The precipitate was filtered and washed with 10% acetic acid twice, then 10% sodium bicarbonate twice, and then was dissolved in chloroform. After drying over anhydrous sodium sulphate, the chloroform solutions were filtered, concentrated and poured into petroleum ether to form a precipitate. The precipitate was filtered off, washed with petroleum ether and dried. The modified samples were dissolved in tetrahydrofuran (THF) for GPC study^{57,93,149,170}.

Gel permeation chromatography was carried out using a column (Polymer 3 μ m Mixed E 300x7.5mm, Merck, Germany), one HPLC pump LC1120 (both from Polymer Laboratory, UK) and a data-collection unit PL-DCU. The sample was filtered through a PTFE (polytetrafluoroethylene) membrane (pore size 0.47 μ m) before injecting into the system. The flow rate was 1.0ml/min at pressure 4.4-4.5Mpa.

Molecular weight calibrations were performed with polystyrene standards (Polymer Laboratory, UK) and catechins (catechin, gallic acid and epigallocatechin-gallate) acetate derivatives.

2.3.4.6 Anthocyanidin reaction

Condensed tannins with a 5,7-dihydroxy A-ring are particularly susceptible to facile cleavage of the interflavonoid bond under acidic or basic conditions. The lability of the interflavonoid bond in these proanthocyanidins has provided some of the most widely used analytical methods for the quantitative and qualitative study of condensed tannins^{76,77,92,126}.

The molar ratio of catechin and gallic acid units in polymer (ratio of PC and PD) and the terminal unit were determined through the anthocyanidin reaction, as set out below. The iron reagent was a 2% (w/v) solution of $\text{NH}_4\text{Fe}(\text{SO}_4)_{12}\text{H}_2\text{O}$ in 2M hydrochloric acid. The reaction was carried out in 10ml capacity thick walled glass tubes with screw top.

1.0ml of methanol solution (0.2g/l) containing purified tannin polymer was mixed with 8.7ml of n-butanol/conc. HCl (95/5 v/v), followed by adding 0.3ml of ferric reagent solution (0.14% w/v) as catalyst, the tube was sealed and heated for 45 minutes at 95°C in a water bath. The final concentration of tannin was 0.02g/l and the concentration of Fe^{3+} was 0.045g/l. A deep red colour is produced in the solution. The reaction products were checked by paper chromatography, developed with the Forestal system (acetic acid:HCl:H₂O = 30:3:10 v/v/v)⁹². The acid degradation products were then separated by thin layer chromatography (cellulose

sheet, 20x20cm, 0.16mm thickness, Sigma, UK) and quantitatively studied by UV-vis spectrophotometry^{63,66,76-78,92,126}.

2.3.4.7 Acid degradation in the presence of nucleophiles, phloroglucinol and toluene-thiol

Reaction with phloroglucinol

Purified tannin (1g) and phloroglucinol (2g) were dissolved in dioxan-water (1:1 v/v 15ml) and the acidity adjusted to 0.5mole/l with concentrated hydrochloric acid. The solution was maintained at 20°C for 48 hours, then poured into 100ml water and then extracted with ethyl acetate (6x50ml). The extracts were dried and evaporated, then dissolved in ethanol and the excess phloroglucinol was removed through recrystallisation^{86, 92, 155}. Then the products were checked with two dimensional paper chromatography, using the A and B systems (see section 2.3.4.4).

Reaction with toluene-thiol

Purified tannin (1g) was suspended in 20ml of ethanol containing 6ml of toluene-thiol and 1ml of acetic acid, the mixture was heated to reflux under nitrogen for 50 hours¹⁵⁵. The solvent was removed by rotary vacuum evaporation and the residual oil was checked with two-dimensional paper chromatography, by using the A and B systems, to reveal the degradation products in the solution.

2.3.5 Tanning experiment

The astringency of prodelphinidins was studied by determining the shrinkage temperature of tanned collagen. Hide powder and pickled sheepskin were used to check the tanning properties of these two kinds of prodelphinidin tannins. The tanning method is given in Appendix I. Combination tannage with oxazolidine was conducted as previously described by Covington and Shi³⁰⁻³². The pH of pickled sheep skin was brought to 6.0 with sodium bicarbonate, then the skin was tanned with 20% vegetable tannins first, followed by cross-linking with 10% oxazolidine,

all based on the weight of pickled skin^{4,30,17,57,147}. The detailed method is given in Appendix I.

2.4 Results and discussion

2.4.1 Yields of tannin extraction

The main extraction results are shown in Table 2.1.

Table 2.1 Yield (% on dried weight of raw materials) and colour of extracts

	Water		85°C +5%NaHSO ₃	50% aqueous acetone	Ethanol
	25°C	85°C		25°C	25°C
Myrica	24	27	36	31	28
	Light brown	Brown	Brown	Light brown	Light brown
Fresh pecan	20	23	44	30	24
	Brown	Red brown	Brown	Brown	Brown
5 years old pecan	16	20	41	26	21
	Red brown)	Brown	Brown	Brown	Brown

From Table 2.1, there is no great difference between the two PD tannin extracts. From hot water extraction, the yields of pecan and myrica extract are similar to black wattle bark (40% yield), but higher than quebracho wood (25% yield). By extraction with 5% sodium bisulphite, the yield from pecan reached 41-44% and 36% for myrica, which is in good agreement with the literature^{106,108}. The sodium bisulfite extracts have lighter colour than from the other methods, which could be a potential benefit to the industry. Aqueous acetone extraction at room temperature gives the highest yield for all the materials. The 50% acetone extract solution has a red-brown colour and the colour changes to deep brown quickly, if the extract solution is exposed to sunlight. Extraction with ethanol produces lower yield than other methods: because the polysaccharide has low solubility in pure ethanol, the

polysaccharide remains in the raw material in this process. Further studies have shown that this method does not work when polysaccharide is combined with tannin molecules. Fresh pecan nutshell pith gives higher yield than older material. In time, the tannin may auto condense to high polymers or be oxidised and combined with other cell tissues, so it then becomes non-extractable. The freeze-dried ethyl acetate extracts of pecan and myrica both have a light brown colour.

2.4.2 Analysis of tannin extracts

The analytical results for the pecan and myrica extracts compared with mimosa (commercial black wattle bark extract) and quebracho are given in Table 2.2.

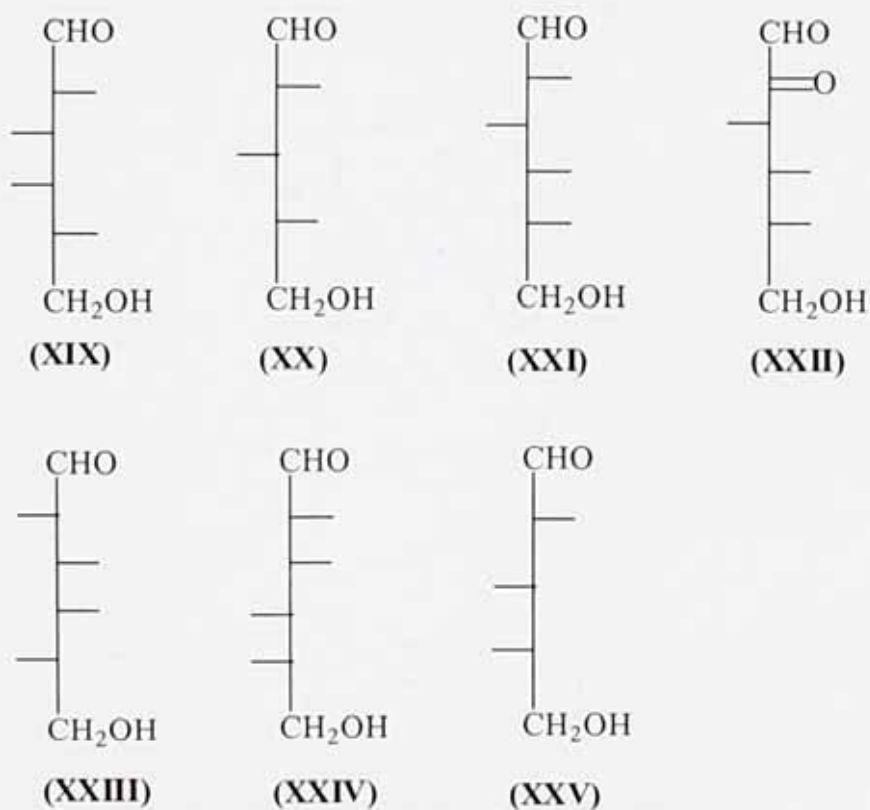
Both pecan extract and myrica extract have high tannin contents, which are a little lower than commercial mimosa or quebracho, the non tannin contents in the former two are higher than the latter two and insolubles are lower than quebracho. It is noteworthy that both of the PD tannin extracts have much higher sugar (especially gum) contents than mimosa and quebracho.

Table 2.2 Analysis of pecan and myrica extracts

	Pecan	Myrica	Mimosa	Quebracho	
Tannin (% on dry weight)	48	53	58	64	
Nontannin (% on dry weight)	41	35	31	16	
Sugar and gum (% on dry weight)	29	14	8	7	
Acid (mg/l, equivalent to acetic acid)	102	126	50	40	
Salt (mg/l, pH 5.5)	68	77	150	30	
Insolubles (% on dry weight)	6.0	5.4	2.6	12	
Moisture (% on dry weight)	4.1	6.1	8.4	8	
pH (30g/l)	4.5	4.2	4.8	4.6	
Lovibond tintometer readings					
Colour	Red	5.5	3.3	1.2	3.2
	Yellow	8.3	6.8	2.4	7.5

In the 1960's, Roux studied mimosa tannin and established most of the analytical methods⁹⁴⁻⁹⁷, that have been used in this work. Ethanol has been proved to be a powerful solvent to separate the gum from mimosa extracts, but it failed to separate the gum from pecan extracts. A likely reason is that the polysaccharides could be combined with tannin molecules, called glycosides, so changing the properties of the polysaccharide, making it soluble in ethanol (Fig 2.6). The sugar and gum contents of both extracts were measured with Sephadex LH-20 column before and after acid hydrolysis.

It has been found there is high sugar or gum content in pecan extract compared with myrica extract, mimosa and quebracho. In this work, from paper chromatography, the sugar in pecan extracts was found to be galactose (XIX) with a trace of xylose (XX), but in myrica extract it is glucose (XXI) and fructose (XXII). Further studies by hydrolysis of the gum with 1mole/l sulphuric acid demonstrated that fucose (XXIII), fructose and galactose are the main components in myrica extract gum. The gums of pecan extract and mimosa tannin extract have similar hydrolysed products: galactose, arabinose (XXIV) and rhamnose (XXV). The results are in agreement with reports in the literature^{106,108}.



The gum obtained from pecan nut pith extract was red-brown after washing ten times with ethanol. From this, it may be assumed the polysaccharide is combined with the tannin molecule with some kind of chemical bonding. In the free sugar analysis process, first there was some white precipitate produced, then after more lead acetate solution was added, a large amount of suspended substance was produced in the solution. It was difficult to centrifuge the precipitate, which appeared to be a kind of colloidal solution. This was not studied further.

Different extraction methods yield different compositions, which produce different analytical results (See Tables 2.3 and 2.4). Aqueous acetone extract produces higher tannin contents than extraction by water, which probably comes from the high solubility of proanthocyanidin polymers in organic solvent-aqueous media.

Table 2.3 Analysis of the tannin extracts from pecan shell (% , on dried weight)

Solvent		Tan	Non-Tan	Insoluble	Moisture
H ₂ O	25°C	53	41	2	3
	85°C	48	42	6	4
50% acetone		64	27	4	5

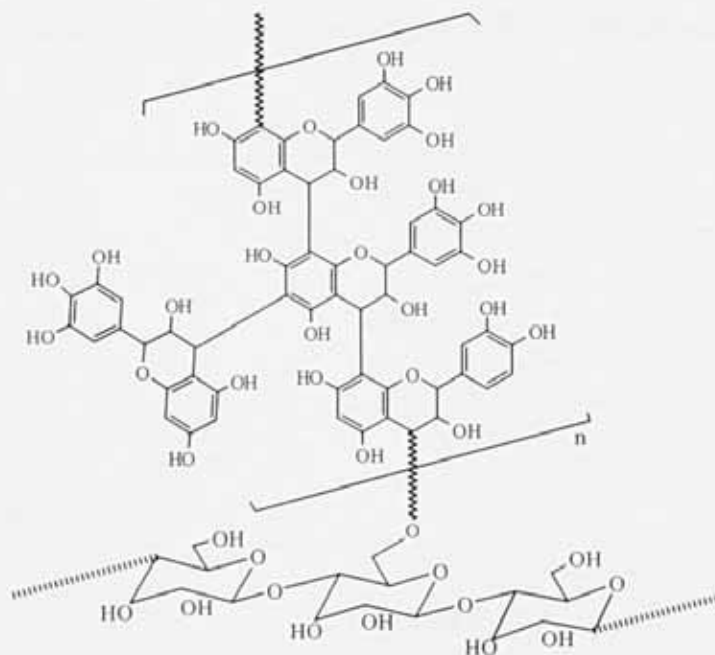


Fig 2.6 Possible structure of pecan tannin

Table 2.4 Analysis of the tannin extracts from myrica (% on dried weight)

Solvent		Tan	Non-tan	Insoluble	Moisture
H ₂ O	25°C	60	30	2	8
	85°C	53	35	6	6
50% acetone		76	13	5	6

2.4.3 Viscosity of tannins solution

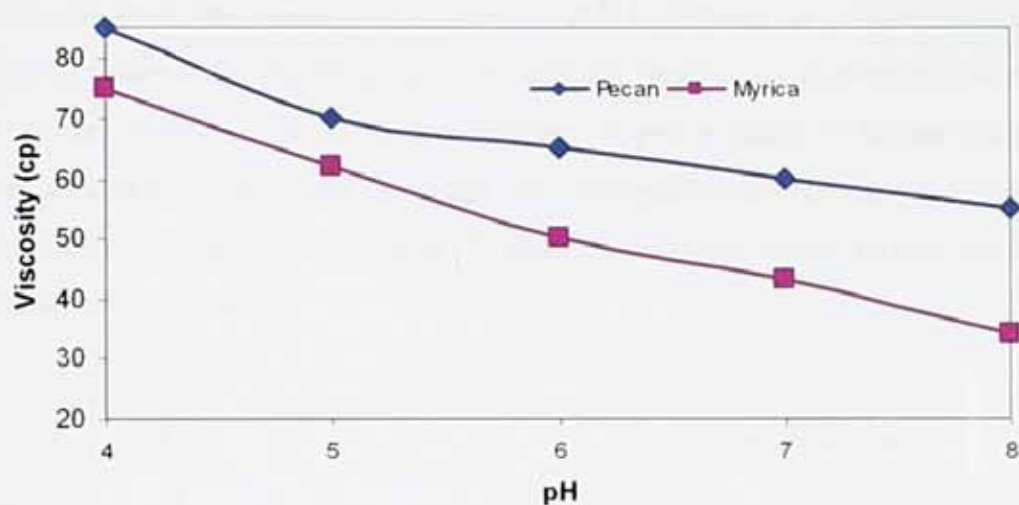


Fig 2.7 Relationship between viscosity and pH at concentration 30g/l

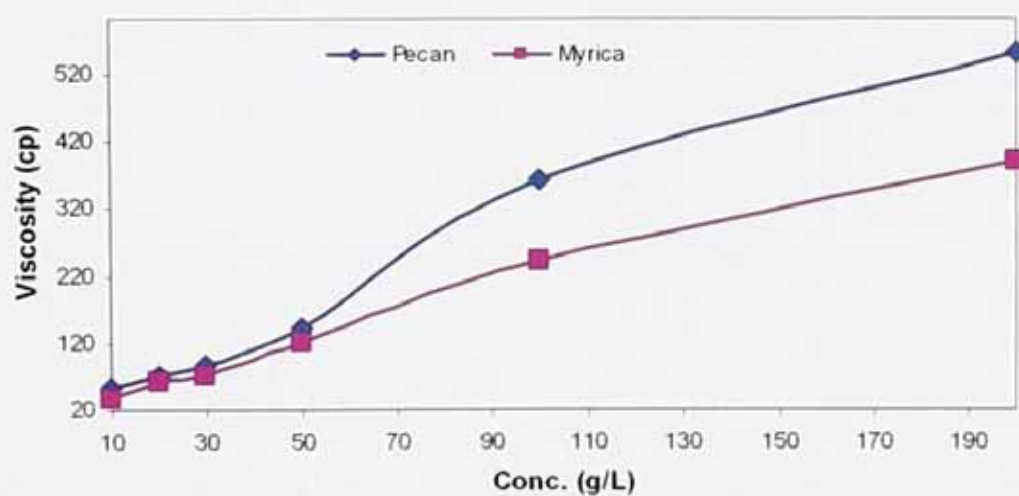


Fig 2.8 Relationship between viscosity and concentration at natural pH (pecan pH4.5, myrica pH 4.2)

The viscosity of pecan nutshell extract solution changes little with increasing pH value of the solution (Figs 2.7, 2.8). The reason may be the presence of the large amount of gum (polysaccharide), because there is little effect on carbohydrate chemistry when the pH of the solution is changed.

2.4.4 Tannins content of pecan shucks

There was no tannin found in the ethanol extraction of pecan shuck, based on the UV absorption curve (Fig 2.9). There are only two peaks at about 330nm and 310nm, no absorption at 270 to 280nm, where pecan tannins have strong absorption, but there is one shoulder or peak at 240 to 245nm in the shuck extract solution, which is similar to the tannin solution. From 200 to 220nm, all polyphenols show strong absorption from the benzene structure, but there is no absorption from the shuck extract solution. The extraction solution of pecan shuck is lighter coloured than pecan extract solution and the paper chromatography did not show any blue or green colour when sprayed with iron(III) chloride solution, which further confirms there is no tannin present.

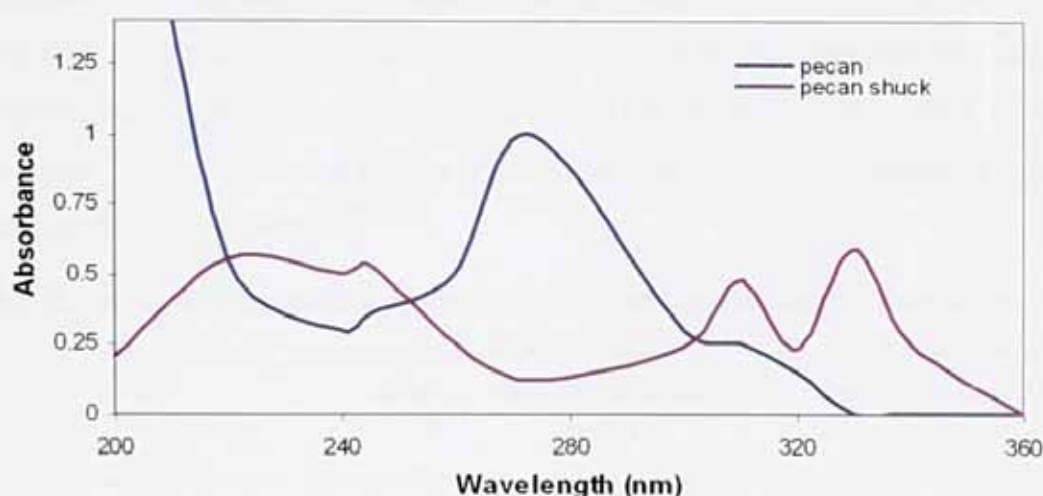


Fig 2.9 UV-vis absorption of pecan shuck and pecan extracts

2.4.5 Separation of tannin extracts

Usually, the separation of tannins extracts is achieved by precipitation, organic extraction and chromatography. The sugar can be removed by lead acetate precipitation and gum can be removed by saturated ethanol. But for pecan and

myrica extracts, which have high contents of non-tannins, these methods do not work well, because effective precipitation cannot be obtained. Good separation also cannot be achieved with organic extraction (ethyl acetate) alone, because there is some gum suspended in the solvent layer and the content in the solvent will be limited by polarity. When the 50% acetone extracts are separated on a Sephadex LH-20 column, the high molecular weight tannin compounds can be obtained, but the low and medium molecular weight polyphenols are washed out together with sugar and gum. Therefore, ethyl acetate extraction and column chromatography are better methods for high non-tannin types of extracts.

2.4.6 Characterisation of prodelphinidins

2.4.6.1 Average molecular weight and its distribution

Gel permeation chromatography (GPC) can give the number average molecular weight (Mn), the weight average molecular weight (Mw) and the polydispersity of the proanthocyanidins. The polydispersity ($Pd = Mw/Mn$) may be used as an indication of the breadth of the molecular weight range^{57,64,65,116,170}. The GPC results show both pecan and myrica tannins have higher molecular weights than mimosa and quebracho tannins. The molecular weight distributions are also wider than the latter two (Fig 2.10), but are similar to other procyanidin or prodelphinidin tannins, according to the literature^{57,77,78,147}.

Table 2.5 Average molecular weight of some condensed tannins polymers

	Mn	No of flavanol units	Mw	Pd
Pecan	3,000	10	7,530	2.5
Myrica	2,500	8	6,925	2.8
Quebracho	1,250	4	2,400	1.9
Mimosa	1,230	4	2,130	1.7
Gambier ⁵⁷	570	2	1,540	2.7
Larch bark ⁵⁷	2,800	9	8,390	3.0

The average molecular weight of pecan tannins is 2,800-3,000, but the molecular weight range is from 200 to 100,000, the Pd value is 2.5. The average molecular weight of myrica tannin is 2,500-2,700, the distribution is a little narrower than pecan pith tannins, from 200 to 50,000, with a Pd value at 2.8 (Table 2.5).

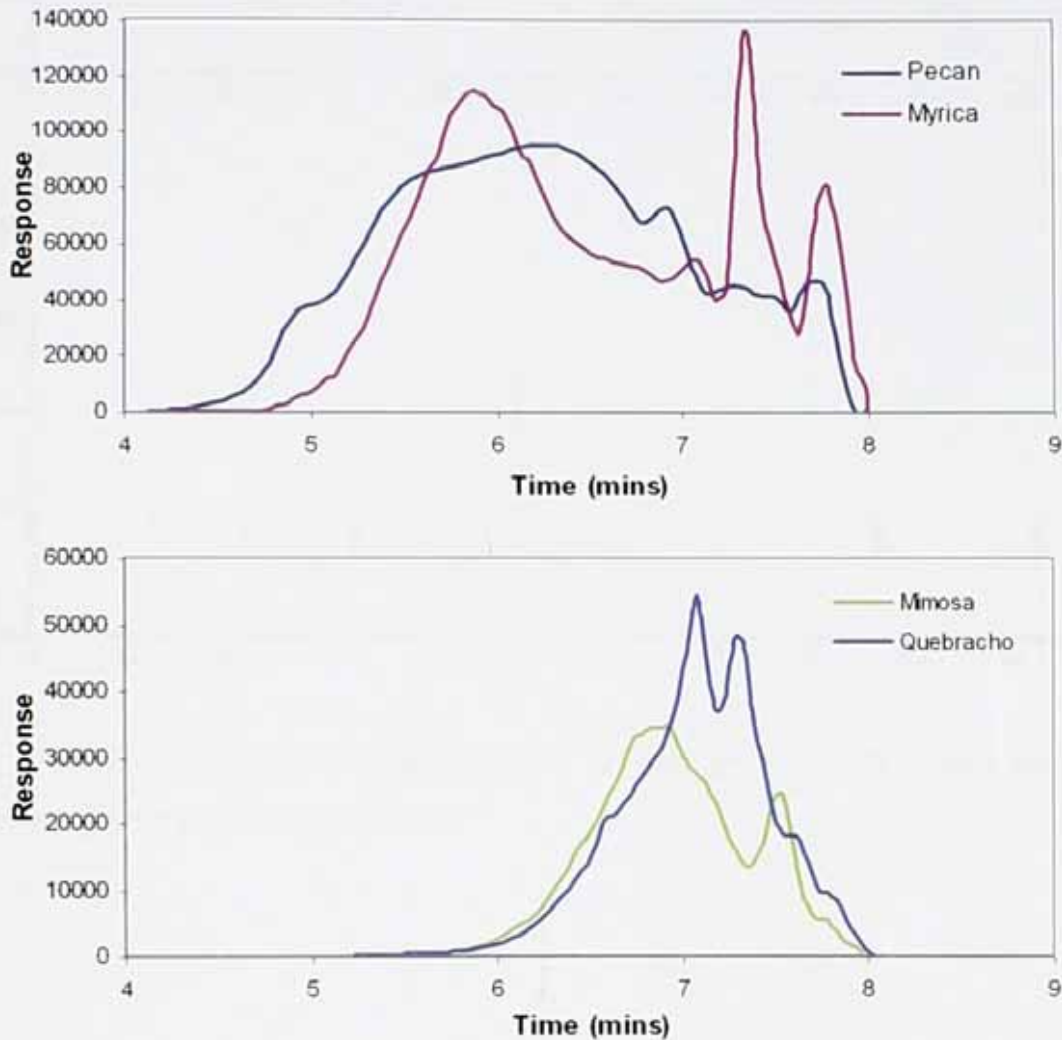


Fig 2.10 Gel permeation chromatograms of pecan (PT) and myrica (MT) tannins peracetate derivatives, compared with mimosa and quebracho

From Fig 2.10, there are much lower molecular weight components in myrica extract than pecan extract. Compared with the reference samples, the last peak in the myrica tannin GPC chromatogram is epigallocatechin-gallate (EGCG) and the second peak is from dimers of galliccatechins, which have been confirmed through HPLC and $^1\text{H-NMR}$ (Figs 2.11 and 2.12). There is a similar peak in the pecan tannin GPC chromatogram, which is galliccatechin or epigallocatechin, present only at low concentration.

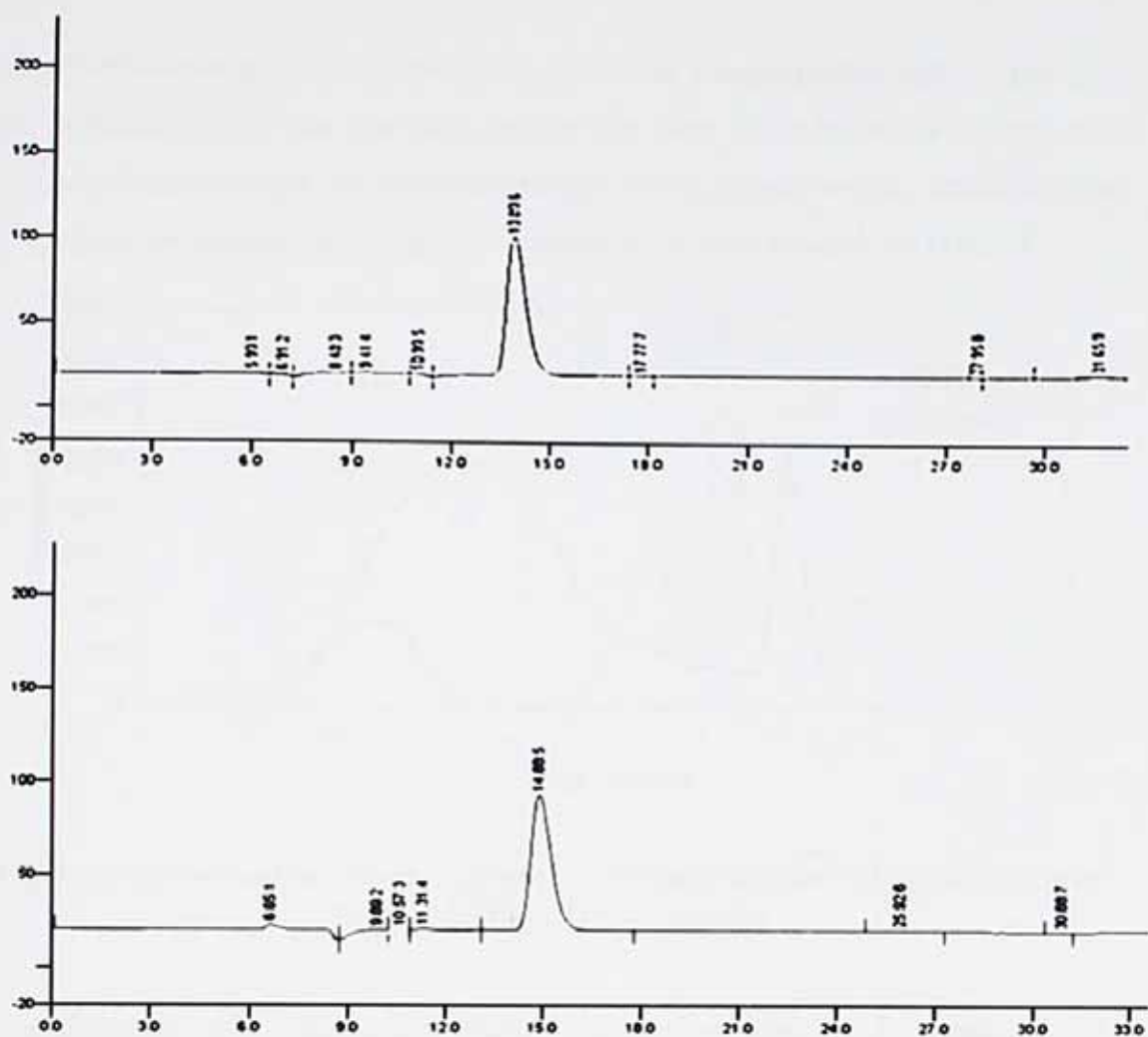


Fig 2.11 HPLC of epigallocatechin-gallate isolated from myrica extract (upper) comparing with pure EGCG (Lower)

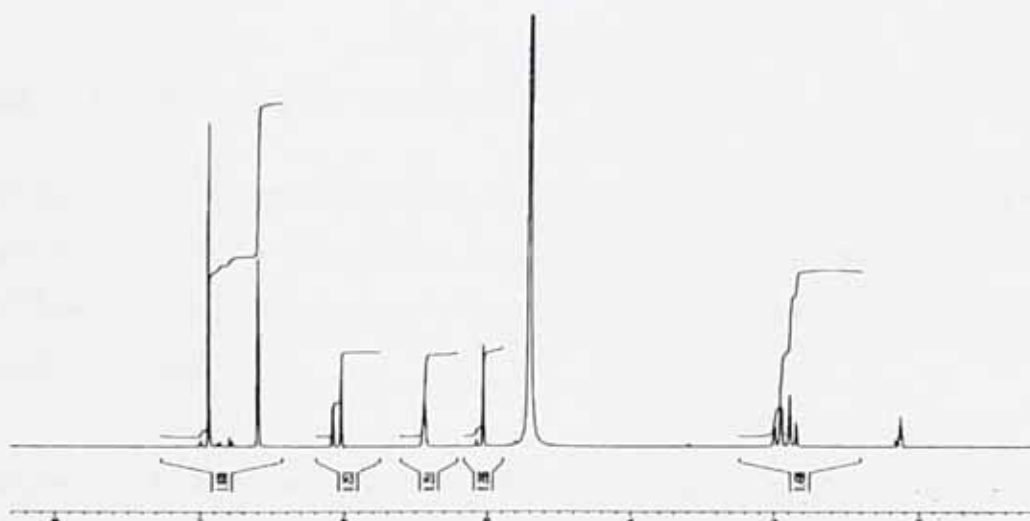


Fig 2.12 ^1H -NMR of epigallocatechin-gallate from myrica extract

The separation method affects the tannin molecular weight profile. After separation with Sephadex LH-20, the methanol fraction has three peaks in the GPC curve, one is high molecular weight and the other two are low molecular weight, indicating that the polysaccharides and low molecular phenols have been washed out (Fig 2.13).

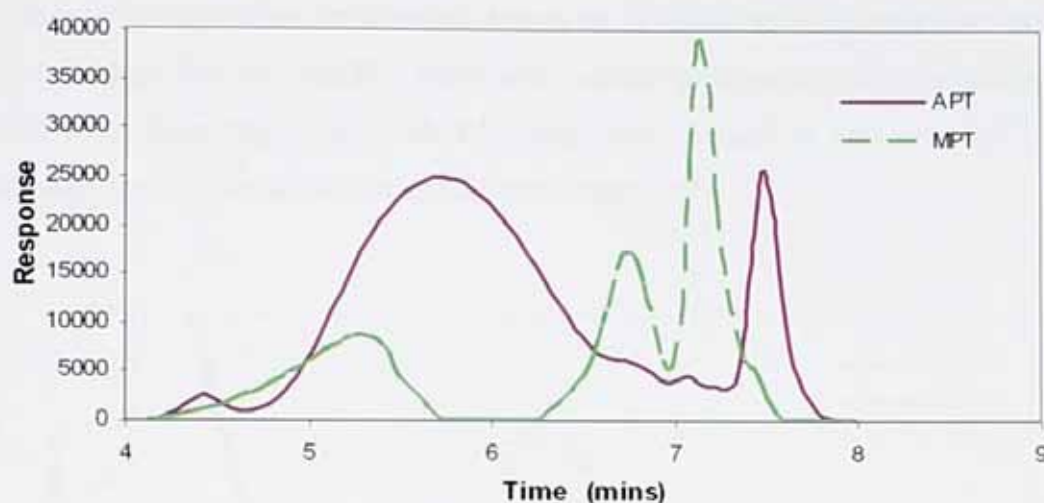


Fig 2.13 Gel permeation chromatograms of methanol fraction (MPT) and acetone fraction (APT) of pecan tannins

From Hemingway¹⁰⁶, the 50% methanol fraction contains the polysaccharide or glycoside components. The hydrolysis experiment showed there is some dimer or trimer in the products. Analysis of myrica tannins shows similar results. The GPC chromatogram of the acetone fraction shows only mid-range molecular weight proanthocyanidin oligomers and polymers.

2.4.6.2 UV-Vis spectra

Ultraviolet-visible absorption spectra have been widely used in plant polyphenol studies from the middle of the 1950's. Harborne reviewed the progress made before 1963²⁶⁶ and summarised the relationship between spectra characteristics and phenol molecular structure.

Doub and Vandenbelt proposed that the short wavelength, high intensity absorption band of benzene at around 200nm is the primary band (1La), and the long wavelength, low intensity absorption band is the secondary band (1Lb)^{171, 173}. Frequently, there is another band at 180nm arising from the substituted benzene ring

structure, called the second primary band (1B). Substitutes on the benzene ring perturb the ring by resonance and inductive effects, which shift both the primary and secondary bands to longer wavelength, especially the secondary bands, which are not only shifted to red but also intensified¹⁷². For proanthocyanidin studies, different UV absorption curves are anticipated, due to the different hydroxylation patterns on A and B-rings. For PC and PD, both with a phloroglucinol A-ring, the difference should arise from the B-ring. Model compounds pyrogallol and catechol show maximum absorptions at 271nm and 278nm respectively.

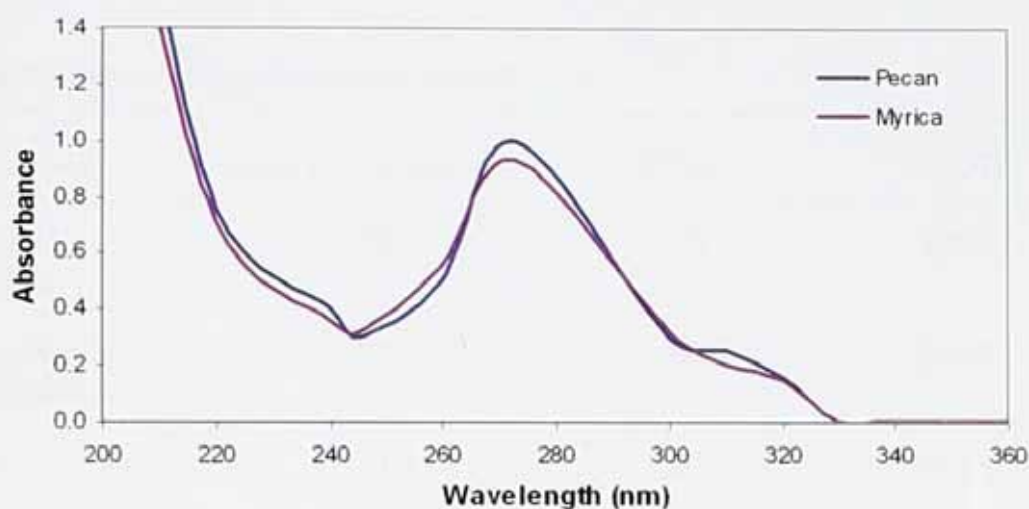


Fig 2.14 UV-Vis spectra of pecan and myrica tannin extracts

As Czochanska reported, the UV spectrum of proanthocyanidin polymer in water consists of a maximum absorption at 205nm for the 1B band (second primary band), a shoulder peak around 240nm for the 1La band (primary band) and the peak at 270-280nm is the 1Lb band (secondary band)⁷⁶. Procyanidins and prodelphinidins have the same phloroglucinol A-ring, the difference is only in the B-ring, which affects the 1Lb band. The 1Lb of catechol B-ring of procyanidin unit is about three times more intense than that of the prodelphinidin unit chromophore pyrogallol B-ring. If the ring A and B chromospheres behave as a set of uncoupled oscillators, they exhibit no conjugation, the observed λ_{max} for the 1Lb band should always lie between 270 and 280nm, its actual value depending on the ratio of PC and PD; a plot of $E_{1\%}^{1\text{cm}}$ against mole fraction of PC should be a straight line. From Czochanska's study, such a relationship is obeyed and yields values of $E_{1\%}^{1\text{cm}}$ of

130 and 62 for the λ_{\max} of the 1L_b band for pure PC and PD polymers, respectively⁷⁶.

From the $E^{1\%}_{1\text{cm}}$ value of 72 for pecan tannin (Table 2.6), the PC:PD ratio can be calculated and the value is 1:6. For pecan tannins, the highest absorbance of UV is near to 273nm, indicating that the main structures in this tannin are prodelphinidins with some procyanidins. For myrica tannin, the λ_{\max} peak is at 270nm, $E^{1\%}_{1\text{cm}}$ is 64, which is in good agreement with the tannins containing pure prodelphinidins (Fig 2.14).

Table 2.6 Ultraviolet spectroscopy results

Compounds	λ_{\max} of 1L_b band (nm)	$E^{1\%}_{1\text{cm}}$	PC:PD
Procyanidin (catechin)	280	130	100:0
Prodelfhinidin (gallo catechin)	270	62	0:100
Pecan	273	72	16:84
Myrica	270	64	100:0

2.4.6.3 Infrared spectroscopy



Fig 2.15 IR spectra of pecan pith extracts (upper line) and *Myrica esculenta* bark extracts (lower line)

From the work of Foo, gallocatechins differ from catechins in having an extra hydroxyl group in the B-ring and this distinction is seen in the 1540-1520 cm^{-1} region^{57,59,68,77,126,163}. The spectra of gallocatechin derivatives or prodelphinidin compounds exhibit distinct peaks at about 1520 and 1535 cm^{-1} . The major components of the pecan and myrica tannins were found to be prodelphinidins (Fig 2.15), from the double peaks at 1520-1535 cm^{-1} .

Table 2.7 Frequencies of the main IR spectral peaks (cm^{-1}) of pecan and myrica tannin.

Pecan			Myrica		
Extraction at 25°C	Ethanol extraction	50% acetone extraction	Extraction at 25°C	Ethanol extraction	50% acetone extraction
3500-3100	3450-3200	3450	3500-3200	3600-3000	3500-3000
2970	2975		2950		
2885			2890		
			1705	1710	1710
1620	1615	1625	1620	1620	1625
1525-1545	1540	1540	1525-1540	1530-1550	1530-1540
1455	1450	1455	1455	1450	1455
1330-1380	1350	1360	1355	1320-1360	1320-1360
1200	1200	1200	1210	1200-1220	1220
1110	1110	1120	1140	1120	1120
1050	1040	1040	1035	1040	1040
850	835	850	845	830	835
810			810	800	800
			775	770	770
740	730	735	730	735	740

The B-ring hydroxylation pattern is also reflected in the region 730-780 cm^{-1} . In the spectra of procyanidin polymers, there are pronounced bands at 770-780 cm^{-1} , but the spectra of prodelphinidins show bands at 730 cm^{-1} . Therefore, there will be absorption bands at 730 and 775 cm^{-1} if the polymer is mixed prodelphinidin and procyanidin. There is one obvious absorption band at 730 cm^{-1} in the spectrum of

pecan tannin. It confirms the polymer is mainly composed of prodelphinidin. The IR spectrum of myrica tannin exhibits two peaks at 730 and 775 cm^{-1} . This might suggest that myrica tannin is a mixture of procyanidin and prodelphinidin. But, the IR spectrum of epi-gallocatechin-gallate also has two peaks at similar frequencies, while there is only one peak at 730 cm^{-1} in the spectrum of epi-gallocatechin and one peak at 775 cm^{-1} in the IR spectrum of the gallotannin tannic acid. Based on these results, we can conclude that the peak at 775 cm^{-1} is from the gallate group (Fig 2.16, 2.17). This also confirms that gallocatechin-gallate is one of the monomer units in myrica tannin.

Information about the configuration of C-ring can be obtained from the region 795-800 cm^{-1} . The band from pecan tannin is distinct but small, indicating the polymers are made up of flavan units with approximately equal proportions of *cis* and *trans* configurations. The band from myrica tannin is about the same intensity as the peak at 730 cm^{-1} , which means flavanol units with the 2,3-*cis*-configuration. This result is in agreement with the literature¹⁰⁸, in which the ratio of 2,3-*cis* and 2,3-*trans* was found to be 9:1.



Fig 2.16 IR spectrum of epi-gallocatechin-gallate



Fig 2.17 IR spectrum of tannic acid

2.4.6.4 Paper chromatography and thin layer chromatography results

Two-dimensional paper and thin layer chromatography have shown pecan and myrica tannins are mainly prodelphinidins. The yield from ethyl acetate extraction of pecan pith tannins is low, only 0.6% of the weight of extracts. In myrica extracts, the yield from ethyl acetate extraction is much higher than from pecan extracts, 16-20% of the dried weight of the extract, which means there are lower and medium molecular weight components in myrica tannin. These data are in agreement with the gel permeation chromatography results. Scanned paper chromatograms can be found in Appendixes II, III and IV. The results of two-dimensional paper chromatography revealed there are epicatechin, epigallocatechin, three prodelphinidin dimers and one unidentified trimer in the ethyl acetate fraction of pecan tannin; the ethyl acetate fraction of myrica extract contains galocatechin, epigallocatechin, epigallocatechin gallate and some prodelphinidin dimers. No catechin or epicatechin was found in the myrica extract. From the literature, there is a low content of hydrolysable tannins – ellagitannins - presented in the myrica extract¹⁰⁸. But here, no ellagitannins were found from the paper chromatography results. Some gallic acid was found in the myrica extract, but this does not mean the

presence of ellagitannins, because gallic acid may originate from the epigallocatechin gallate.

Table 2.8 Some components of ethyl acetate fraction of pecan extract

Rf value ^a		Structure ^b
A	B	
0.53,	0.80	epicatechin
0.42,	0.61	epigallocatechin
0.45,	0.20	gallocatechin(C 4-6)epigallocatechin
0.44,	0.31	prodelphinidin dimer B-2
0.31,	0.29	prodelphinidin dimer B-4
0.20,	0.18	trimers (PC and PD)

a A: 6%acetic acid, B: BAW (14:1:5, v/v/v)

b Based on Rf value and literature

Table 2.9 Some components of ethyl acetate fraction of myrica extract

Rf value ^a		Structure ^b
A	B	
0.61,	0.54	epigallocatechin-epigallocatechin gallate
0.52,	0.76	gallocatechin
0.42,	0.74	epigallocatechin
0.48,	0.57	epigallocatechin gallate-galocatechin/ gallocatechin-epigallocatechin gallate
0.46,	0.41	epigallocatechin gallate-epigallocatechin gallate
0.56,	0.29	gallic acid
0.07,	0.47	unknown
0.08,	0.57	gallocatechin gallate
0.30,	0.14	oligomer
0.11,	0.72	epigallocatechin gallate

a A: 6%acetic acid, B: BAW (14:1:5, v/v/v)

b Based on Rf value and literature

2.4.6.5 Anthocyanidin reaction

Acid catalysed cleavage of the interflavanoid bond releases the upper unit as a carbocation, with the charge localised on the C-4 and the lower unit as a flavan-3-ol. The upper unit can then either be oxidised to give anthocyanidin, cyanidin or delphinidin (Fig 2.18) or in the presence of a nucleophile, undergo nucleophilic capture, which will be discussed in Section 4.6.6.

Although the anthocyanidin formation analytical method, as developed by Swain and Hillis in 1959, is not quantitative, it is still one of the most important methods for proanthocyanidins chemistry study to date⁹². The mechanism involved in the oxidation is complex and side reactions involving phlobaphene formation are thought to be involved. The method has been significantly improved by Porter, who discovered that the addition of ferric salts improved both the yield and reproducibility⁹². The results obtained from the anthocyanidin reaction of pecan and myrica tannins showed good reproducibility by employing the same method as described as Porter.

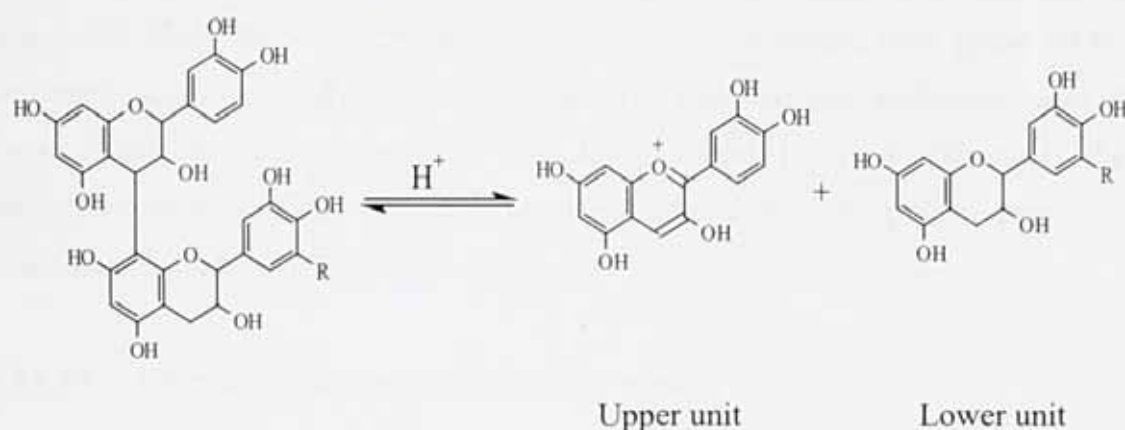


Fig 2.18 Anthocyanidin formation

2.4.6.5.1 Ratio of procyanidin units and prodelpinidin units

From the literature, the R_f values of cyanidin and delphinidin in the Forestal solvent system are 0.30 and 0.50 and the λ_{max} are 545nm and 555nm respectively. There are two pink spots in the paper chromatogram from acidic degradation products of

pecan tannin; the R_f values are 0.29 and 0.49 respectively. For myrica tannin, there is only one spot with R_f value 0.50, corresponding to delphinidin.

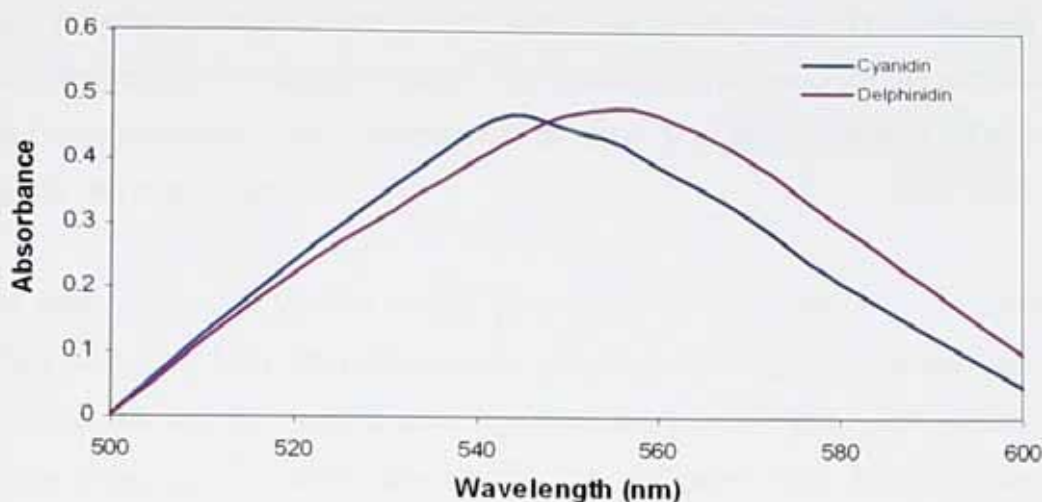


Fig 2.19 UV-Vis absorption of cyanidin and delphinidin

The degradation products of pecan and myrica tannins were separated by TLC and the cyanidin and delphinidin bands were cut out and eluted with ethanol (+ 0.01% HCl). The UV-Vis spectrum was recorded from 200nm to 650nm and the λ_{max} values were found to be 554nm and 555nm for cyanidin and delphinidin respectively (Fig 2.19). Then the concentrations of cyanidin and delphinidin were measured by the spectroscopic method at 545 and 555nm. The cyanidin and delphinidin ratio of pecan tannin degradation products was found to be 1:5.5-6.0. Therefore, the anthocyanidin is identified to contain prodelphinidin as the principal component in pecan tannin, with 14-16% procyanidin units.

2.4.6.5.2 Terminal units in pecan and myrica tannins

As mentioned above, the lower terminal unit of the proanthocyanidin polymers is released as monomeric flavan-3-ol. Therefore, the anthocyanidin reaction products of pecan and myrica tannins were also examined by two dimensional chromatography with systems A (6% acetic acid) and B (2-butanol:acetic acid:water 14:1:5 v/v/v). It was revealed that the terminal units for pecan tannin are (+)gallocatechin and (+)catechin, which were identified by comparing with standard authentic samples. The ratio of these two monomers was examined by comparing the intensity of the colour developed with ferric chloride. Also, by removing the

solvent from the degradation mixture and extracting with ethyl acetate, a light yellow-brown powder was obtained after evaporating to dryness and this was examined by TLC. The ratio of (+)gallocatechin to (+)catechin was 2:1, and the result is same as from the degradation reaction in the presence of nucleophiles. But, this is different to the literature data¹⁰⁶, in which McGraw reported the terminal unit was (+)gallocatechin and (+)catechin in the ratio 1:2 obtained from a toluene-thiol degradation experiment.

The terminal unit of myrica tannin polymers was found to be (+)gallocatechin gallate with very little epi-gallocatechin gallate, comparing with standard samples. Sun reported that the terminal unit in myrica tannin was only (-)epi-gallocatechin gallate, from the ¹³C-NMR spectrum¹⁰⁸. The difference may originate from the different methods employed.

2.4.6.6 Nucleophilic capture

2.4.6.6.1 Degradation with phloroglucinol

As discussed above, proanthocyanidins produce carbocation ions at C-4 under acidic conditions. In the presence of a nucleophile reagent, the carbocation will be captured and form derivatives with phloroglucinol and toluene-thiol (Fig 2.20). By examining the composition of pecan tannins extracts with paper chromatography before and after the degradation reaction with phloroglucinol, it was found that there are some oligomers, monomers and the derivatives epigallocatechin-phloroglucinol and epicatechin-phloroglucinol were produced (Table 2.10). The monomers were found to be (+)gallocatechin and (+)catechin in the ratio 2:1, which is in agreement with the anthocyanidin reaction result. After separation by Sephadex LH-20 column chromatography, the ratio of epigallocatechin-phloroglucinol and epicatechin-phloroglucinol was found to be 1:6.

In the degradation products of myrica tannin with nucleophilic agents, gallocatechin-gallate-phloroglucinol, epi-gallocatechin-gallate-phloroglucinol, and the monomer and dimers of gallocatechin tannins were obtained by separation on a Sephadex LH-20 column, eluting with ethanol. From paper chromatography, no

catechin or epi-catechin or its derivatives were found in the products of the degradative reaction. The terminal unit was found to be (+)gallocatechin gallate compared with the result of ethyl acetate fraction. Some phloroglucinolic derivatives were found in the degradation products (Table 2.11).

Table 2.10 Some components of pecan tannin degradation with phloroglucinol

Rf value ^a		Structure ^b
A	B	
0.71	0.78	phloroglucinol
0.66	0.52	4-(2,4,6-trihydroxyphenyl)flavan-3,3',4,4',5,7-hexaol
0.57	0.40	4-(2,4,6-trihydroxyphenyl)flavan-3,3',4,4',5,5',7-heptaol
0.48	0.52	(+)gallocatechin
0.51	0.69	(+)catechin
0.3-0.4	0.15-0.2	dimer and trimer

a A: 6%acetic acid, B: BAW (14:1:5, v/v/v)

b Based on Rf value and literature

Table 2.11 Some components of myrica tannin degradation with phloroglucinol

Rf value ^a		Structure ^b
A	B	
0.67	0.74	phloroglucinol
0.51	0.64	gallocatechin
0.37	0.54	epigallocatechin
0.56	0.45	EGC-EGCG, EGC-4-phloroglucinol
0.55	0.26	(monomer and dimer) phloroglucinolic derivatives
0.05	0.51	gallocatechin-gallate
0.26	0.17	trimer (PD)
0.45	0.24	trimer (PD)/ tetramer

a A: 6% acetic acid, B: 2-butanol: acetic acid: water = 14: 1:5 (v/v/v)

b Based on Rf value alone

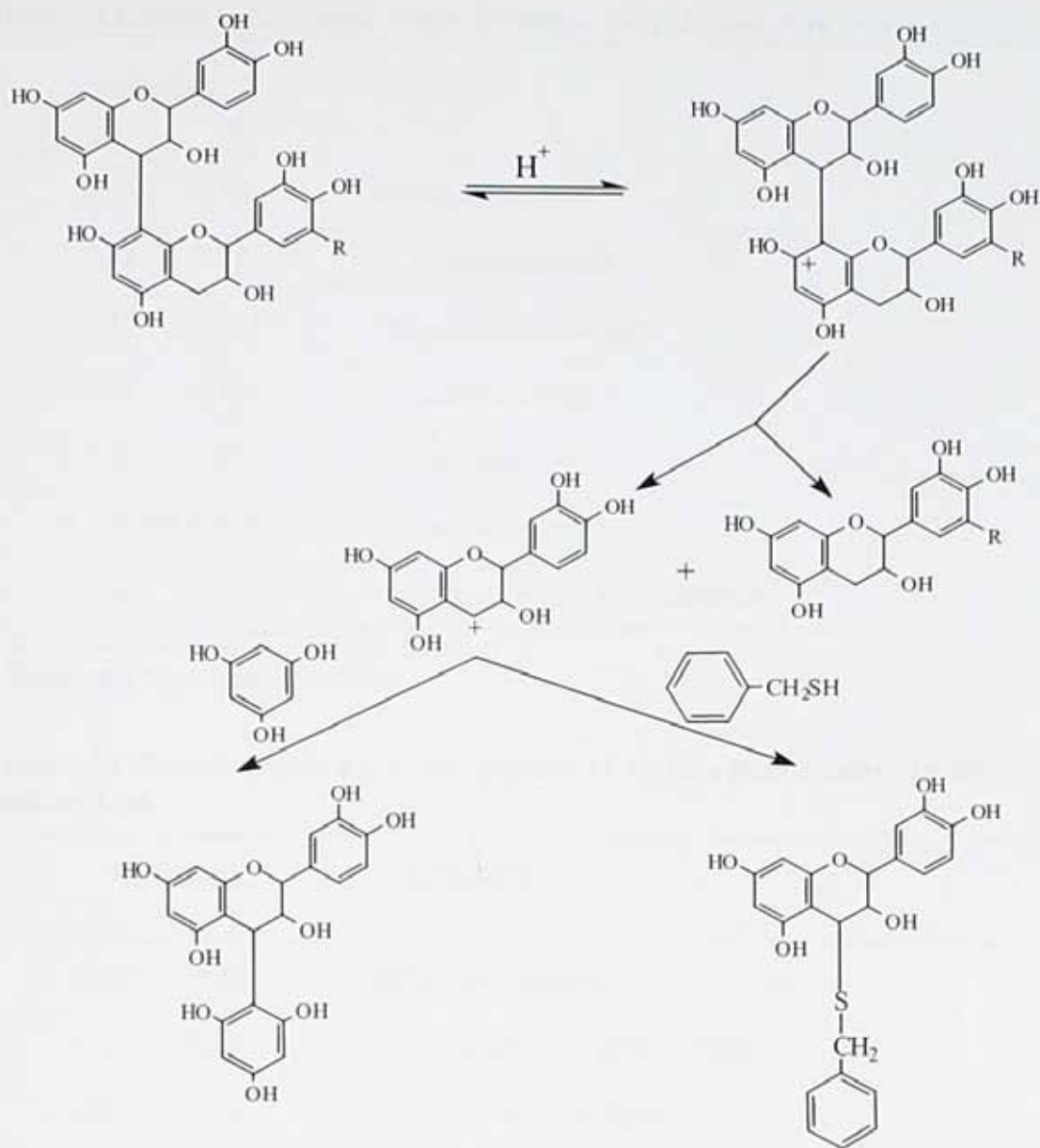


Fig 2.20 Degradation of proanthocyanidin with phloroglucinol and toluene-thiol

2.4.6.6.2 Degradation with toluene-thiol

The degradation reaction of proanthocyanidins under acidic condition in the presence of toluene-thiol has the same mechanism as phloroglucinol, but higher yield is obtained. Therefore it is necessary to examine the degradation products by comparing with the results obtained from the phloroglucinol experiments. The main results are shown in Tables 2.12 and 2.13.

Table 2.12 Some components of pecan tannin degradation with toluene-thiol

Rf value ^a		Structure ^b
A	B	
0.38	0.84	epicatechin-thiol
0.42	0.78	gallocatechin-thiol
0.35	0.72	epigallocatechin-thiol
0.43	0.52	(+)gallocatechin
0.46	0.67	(+)catechin
0.2-0.4	0.2-0.45	dimer and trimer
0.45	0.24	epigallocatechin-galocatechin

a A: 6%acetic acid, B: BAW (14:1:5, v/v/v)

b Based on Rf value and literature

Table 2.13 Some components of the product of myrica tannin degradation with toluene-thiol

Rf value ^a		Structure ^b
A	B	
0.38	0.86	gallocatechin-thiol
0.35	0.74	epi-galocatechin gallate-thiol
0.47	0.70	epi-galocatechin-thiol
0.37	0.56	epi-galocatechin
0.08	0.52	(+)gallocatechin gallate
0.2-0.4	0.2-0.45	dimer and trimer of prodelphinidins

a A: 6%acetic acid, B: BAW (14:1:5, v/v/v)

b Based on Rf value and literature

McGraw¹⁰⁶ and co-workers reported that during the thiolysis reaction of pecan tannin, significant quantities of phloroglucinol were produced as a side reaction product. They proposed the mechanism shown in Fig 2.21. Thiolysis of procyanidins and especially prodelphinidins was found to yield phloroglucinol. They suggested that this result makes it impossible to achieve “quantitative” information on the composition of mixed proanthocyanidins polymers and conduct molecular weight

studies. However, in our experiments, there was only very little phloroglucinol from the pecan tannin degradation products and no phloroglucinol was found in the paper chromatography examination of myrica tannin thiolysis products. Further research work is needed to elucidate this confusion.

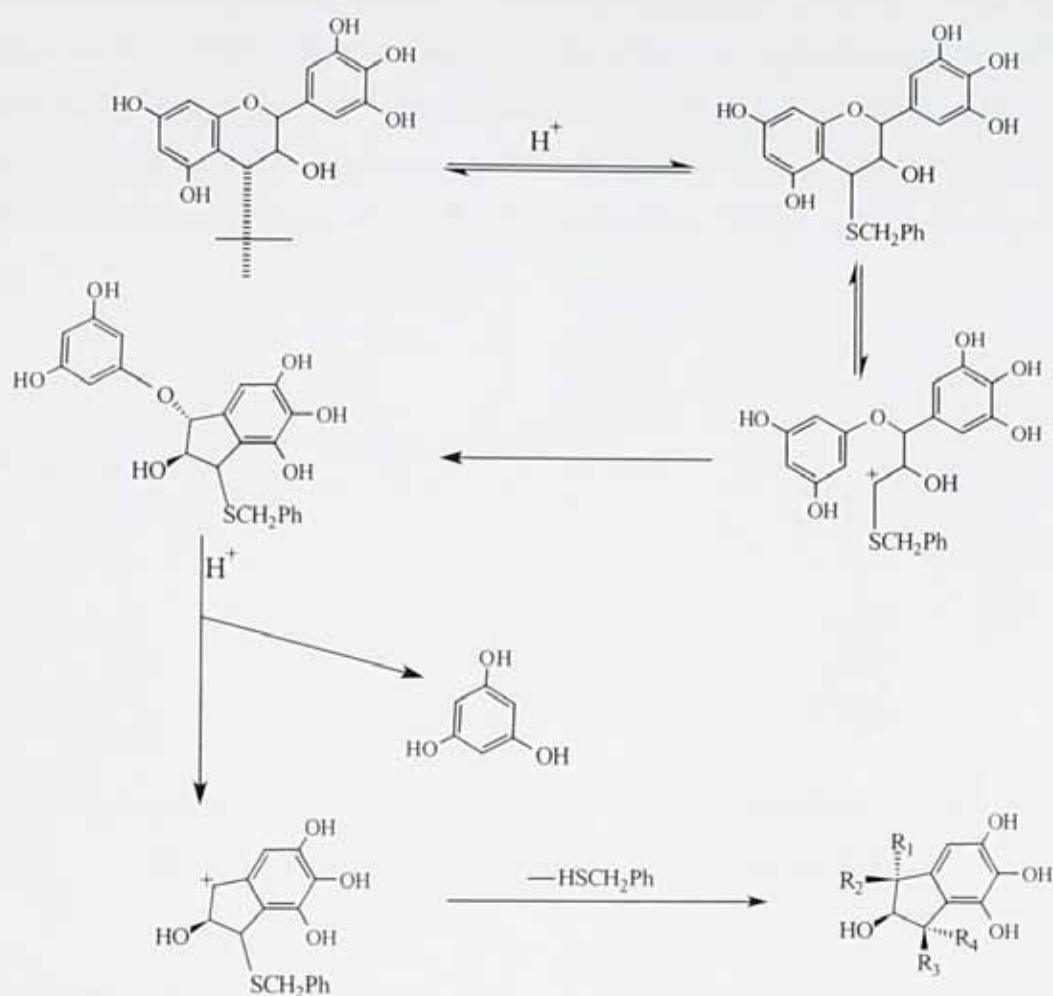


Fig 2.21 Proposed routes for the production of phloroglucinol during thiolysis of proanthocyanidins¹⁰⁶

The thiolysis products of pecan tannin were separated by TLC and it was found that the terminal units are (+)gallocatechin and (+)catechin in the ratio of 1.8:1, which is similar to the result obtained from the anthocyanidin reaction. The ratio of PC to PD was found to be 1:4.5. The difference between the results from the anthocyanidin reaction and the thiolysis degradation may be caused by the side reactions in both methods: it has been reported that the yields are significantly related to the reaction

conditions, such as concentration, purity of proanthocyanidin samples and temperature^{57,63,66,68,92,106}.

In the thiolysis product of myrica tannin, the terminal unit was found to be the same as from the anthocyanidin reaction, (+)gallocatechin gallate, with very little (-)epigallocatechin gallate. The ratios of 4-thioether of epigallocatechin gallate, gallocatechin-4-thioether and epigallocatechin-4-thioether were found to be 3:1:5 (Fig. 2.22), from which the amount of the galloyl groups is calculated to be about 40%; in other words, about 40% of the extender units possess a galloyl group at C-3.

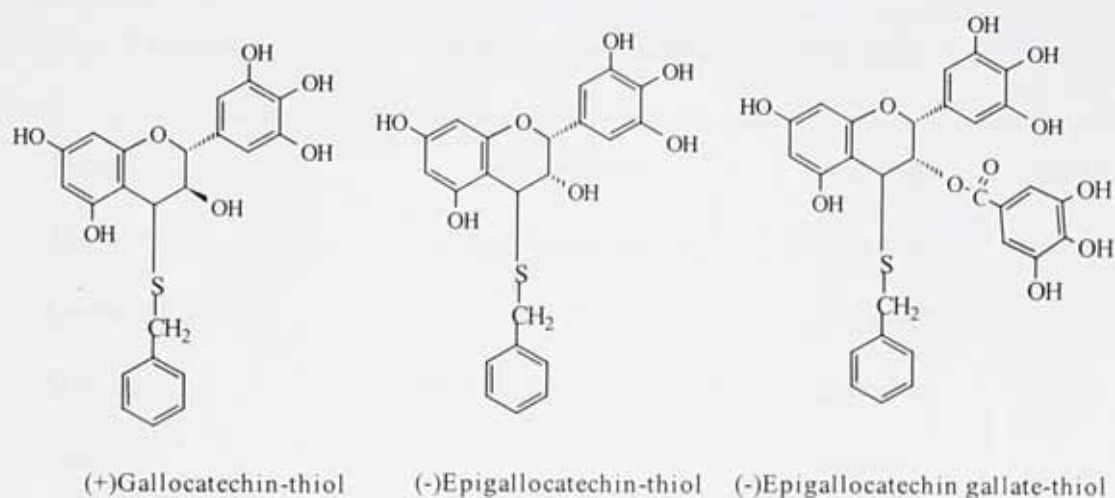


Fig 2.22 Prodelphinidin monomers, thio-adduct structures

2.5 Discussion

From the extraction experiment, the tannin content analysis indicated that the extraction yield and composition of extracts are directly related to the extraction method employed. Condensed tannins are present in plant tissues as extractable and non-extractable forms; they may exhibit different properties in different extraction solvents. 50% acetone cleaves the hydrogen bond between proanthocyanidins and carbohydrate; hot water with or without sodium sulphite can significantly increase the solubility of proanthocyanidin polymers; unlike sodium sulphite, acetone-water will not result in depolymerisation or opening of the pyran C-ring. Therefore, acetone-water extraction is of more interest for structural analysis of condensed

tannins. The chemical structural characteristics of pecan and myrica tannins are summarised in Table 2.14.

Myrica esculenta tannin constitutes a model for studies of gallocatechin reaction with protein or cross-linking agents. Pecan tannin presents the opportunity to study the cross-linking reaction of proanthocyanidins. Because in nature PC and PD are present together most of time, it is important to acquire some information about the mixture's reaction with cross-linking agents.

Table 2.14 Chemical structure characteristics of condensed tannin polymers

Tannins	Main component ^a	Mn (Dp ^b)	2,3-Trans:Cis
Pecan	PC: PD=1:6	3,000 (10)	50:50
Myrica	PD (40% gallated C-3)	2,500 (7-8)	90:10
Quebracho	PF	1,230 (4-5)	
Mimosa	PR: PF: PD = 7:2.5:0.5	1,250 (4-5)	
Gambier	PC	570 (2)	
Larch bark	PC	2,800 (9-10)	60:40

a PC: procyanidin; PD: Prodelphinidin; PF: Profisetinidin; PR Prorobinetinidin

b Dp: degree of polymerisation

2.6 Tanning properties of tannin extracts

Tanning studies showed pecan and myrica tannin have similar tanning properties to commercial condensed tannins, such as mimosa and quebracho. The shrinkage temperatures are 83 and 86°C respectively, compared to mimosa (84°C) and quebracho (81°C). The colour of leathers tanned with pecan and myrica is brown-red, darker than mimosa and quebracho, due to the pyrogallol B-ring in prodelphinidin tannins, which has been reported to be more easily oxidised, resulting in a deeper colour than profisetinidin and prorobinetinidin. Due to the higher average molecular weights, the penetration of pecan and myrica tannin through the leather cross-section is slower than mimosa (Fig 2.23 and Table 2.15).

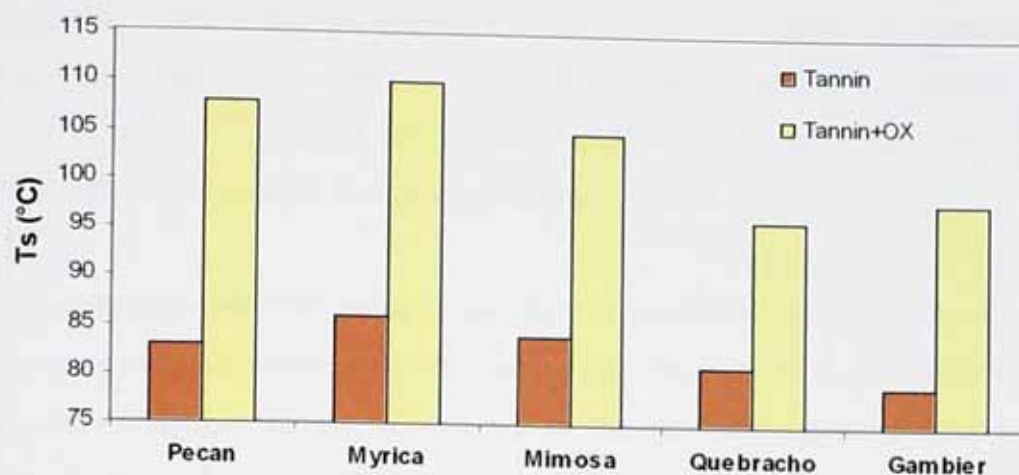


Fig 2.23 Shrinkage temperature of different tannin tanned leathers, retanned with oxazolidine

As anticipated, the shrinkage temperature of leather tanned by gallocatechin tannin with oxazolidine is the highest of the experimental tannins, reaching 111°C, compared to 105°C from mimosa and 96°C and 98°C for quebracho and gambier respectively.

Table 2.15 Tanning with different vegetable tannins

Tannins	pH	Time of penetration (h) ^a	Colour of leather	Ts (°C) with tannin alone	Ts (°C) of combination tan with oxazolidine
Pecan	6.0	6	Red-brown	83	108
Myrica	6.1	2	Brown	86	111
Mimosa	5.9	1	Light brown	84	105
Quebracho	6.0	1.5	Brown	81	96
Gambier	5.9	1.5	Red-brown	79	98

a Average thickness of cattle hide 2mm

From Table 2.15, the high Ts from combination tannage is determined mainly by the chemical structure of the condensed tannin, rather than its molecular weight. Gambier has a low average molecular weight, but the Ts still increases by 20°C after retanning with oxazolidine. The high shrinkage temperature of mimosa combination

tannage may come from the pyrogallol B-ring in the gallo catechin component of the polyphenol. Quebracho has similar average molecular weight and distribution to mimosa, but has a non-reactive catechol B-ring, which will not contribute to high stability leather¹⁰⁻¹³. So, it can be concluded that high shrinkage temperature is determined by the special feature of proanthocyanidins.

It is noteworthy that with pecan tannin there is a penetration problem in the tanning process, which may originate from gum or polysaccharides or glycoside with high molecular weight. All these will slow down the penetration process, even block the fibre structure. Another possible reason is the high average molecular weight of pecan tannin, with very few low molecular weight components. From the GPC results, the pecan tannin molecules contain on average 9-10 monomer units. For myrica tannin, the degree of polymerisation is 7-8 although the average molecular weight is similar because of the presence of gallate groups. Because of the presence of low and medium molecular weight components in myrica tannin, there is less of a penetration problem.

2.7 Summary

Two new gallo catechin-type (prodelphinidin) tanning materials have been analysed. The yield of extracts from pecan nut shell pith and *Myrica esculenta* bark is 34% and 30% respectively, in which the tannin contents are 56% and 54% respectively. Pecan pith tannins are copolymers of catechin and gallo catechin, in the ratio 1:6. The terminal units are (+)gallo catechin and (+)catechin (2:1). The molecular weight range is from 300 to 100,000; the number average molecular weight is 2,800. There is about 28% gum or sugar in pecan pith extracts, which makes it easily fermentable and causes penetration problems in the tanning process. *Myrica esculenta* tannins are partly gallated prodelphinidins (40%) with (+)gallo catechin-gallate as the terminal unit, the molecular weight range is 300-50,000; the number average molecular weight is 2,500. Both of the tannins exhibit good tanning ability, which produce shrinkage temperatures 83 and 86°C respectively. The combination tannage with oxazolidine gives Ts 108 and 111°C for pecan and myrica tannins respectively. *Myrica esculenta* bark extract is better than pecan pith extracts in penetration, tanning ability and lightness of colour of leather.

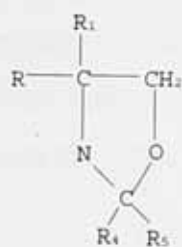
Chapter Three

Synthesis and Characterisation of Oxazolidine

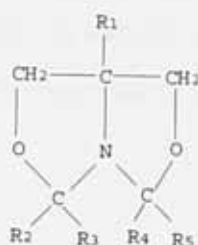
— 1-aza-5-ethyl-1,3-dioxabicyclo [3.3.0] octane

3.1 Introduction

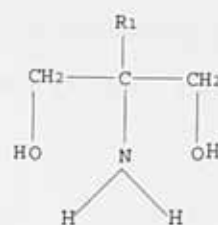
Oxazolidines are five-membered heterocyclic derivatives, with one nitrogen atom and one oxygen atom^{178-180,200} (XXVI), formed from the condensation reaction of β -amino alcohols with carbonyl chemicals (aldehyde or ketone). They are useful and important intermediates in organic synthesis and have wide potential application in many areas, mainly deriving from the unique five-membered ring structure¹⁸¹⁻¹⁸⁷. Completely hydrolysed oxazolidine exhibits free amino and hydroxyl functionality (XXVIII). In the structures of XXVI-XXVIII, R, R₁, R₂, R₃, R₄, R₅ mean aromatic or other substitutes, XXVII is the oxazolidine studied in this work (1-aza-5-ethyl-1,3-dioxacyclo[3.3.0]octane) where R₁ = -CH₂CH₃, R₂ = R₃ = R₄ = R₅ = H.



XXVI



XXVII



XXVIII

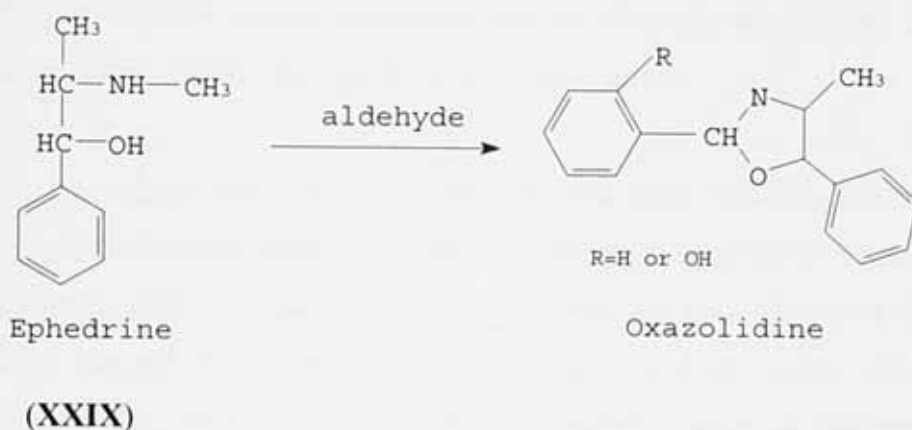
The oxazolidine molecular structure has been known for a long time. In 1901, Knorr and co-workers described the oxazolidine structure for first time¹⁷⁸⁻¹⁸⁰. Since then, most of the studies on oxazolidines have focused on organic synthesis and chemical properties¹⁹⁶⁻¹⁹⁹. Bergmann comprehensively reviewed the progress made before the 1950's¹⁷⁸. Between the 1970's and the 1990's; oxazolidines were studied as potential pro-drugs, for example, oxazolidine derived from ephedrine has shown considerable sympathomimetic activity in several animal models¹⁸¹⁻¹⁸⁴. At the same time, some oxazolidines have been studied in leather tanning chemistry and the paint industry as cross-linking reagents^{55,188-193,205}. However, the reaction mechanisms of oxazolidine are still not well understood.

Oxazolidine can be classified into two types, monocyclic (XXVI) and bicyclic (XXVII). Monocyclic oxazolidines have been widely studied, especially for pro-

drugs. Bicyclic oxazolidines have mainly been used in the paint industry and in the leather industry as cross-linking agents. Application of different types of oxazolidine depends on the functional mechanisms that derive from the molecular structural characteristics.

3.1.1 Oxazolidine as pro-drugs

Some drugs containing the β -amino alcohol moiety or carbonyl groups, may be converted to oxazolidines, to protect these functionalities and enhance the hydrophobic properties for delivery purpose^{181,183,184,187,195}. Most of the related work has concerned monocyclic oxazolidines, derived from (-)-ephedrine (XXIX) and benzaldehyde or salicylaldehyde as potential pro-drugs, which can improve both drug efficiency and safety. Fife, Bundgaard and Somani *et al.*^{184-185,194-196} studied the properties of these oxazolidines in detail, especially the hydrolysis process in aqueous media, and found the oxazolidine is hydrolysed *in vivo* and gradually releases the drug.



3.1.2 Oxazolidines used in paint industry

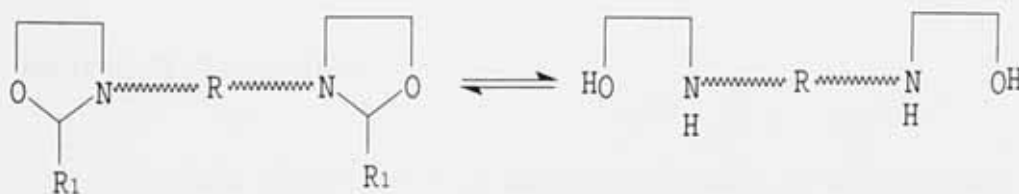


Fig. 3.1 Oxazolidine used in the paint industry

Oxazolidines have been used in the paint industry as cross-linking agents in polyurethane coatings¹⁸⁸. This kind of oxazolidine is synthesised from chemicals containing β -amino alcohol with a long chain aldehyde (eg. isobutyraldehyde). Monocyclic and bicyclic oxazolidines have been used in this context. Polyisocyanate will react with a ring-opened oxazolidine, which has lost the carbonyl reactants through hydrolysis, and thereby produce free hydroxyl and amino functional groups (Fig. 3.1). Carter studied the hydrolysis process of this kind of oxazolidine and found the reaction is fast: completely ring-opened oxazolidine could be obtained after 6-12 hours, depending on the water content¹⁸⁹.

3.1.3 Oxazolidines used in leather industry

Another important application of oxazolidine is the reaction with protein in leather tanning. These kinds of oxazolidines (**XXVII**) are mainly derived from 2-amino-2-ethyl-1,3-propanediol or 2-amino-2-hydroxymethyl-1,3-propanediol with formaldehyde^{178,188-189,191,197}. Usually, treatment with oxazolidine will give good thermal properties; the denaturation temperature of collagen is typically increased from 60 to 86°C. The tanning properties and the characteristics of the leather are similar to or better than traditional glutaraldehyde tannage.

Recently, Covington and Shi have reported that high stability leather can be obtained by combination tannage of proanthocyanidins with oxazolidine, 1-aza-5-ethyl-1,3-dioxacyclo[3.3.0]octane^{30,31,179}. They proposed that, after ring opening of oxazolidine through hydrolysis and forming an N-hydroxymethyl intermediate, oxazolidine would react with polyflavanols or amino groups of collagen. Recent studies also have shown that oxazolidine exhibits higher reactivity towards phenol and protein than formaldehyde^{32,261,262}. However the mechanism of oxazolidine reaction with protein or polyphenol still needs to be clarified and is addressed below.

3.2 Hydrolysis of oxazolidine

From the discussions above, the most important property of oxazolidine is the hydrolysis process, whether it used for synthesis or cross-linking. Different

oxazolidines may have their own mechanism in the hydrolysis reaction. It has been shown that different conformations of the ring have a pronounced effect on the process. Recently, Walker and coworkers have suggested the hydrolysis reaction of oxazolidine may include five steps (Fig. 3.2)¹⁸¹⁻¹⁸⁹. The rate-determining step is different under different conditions.

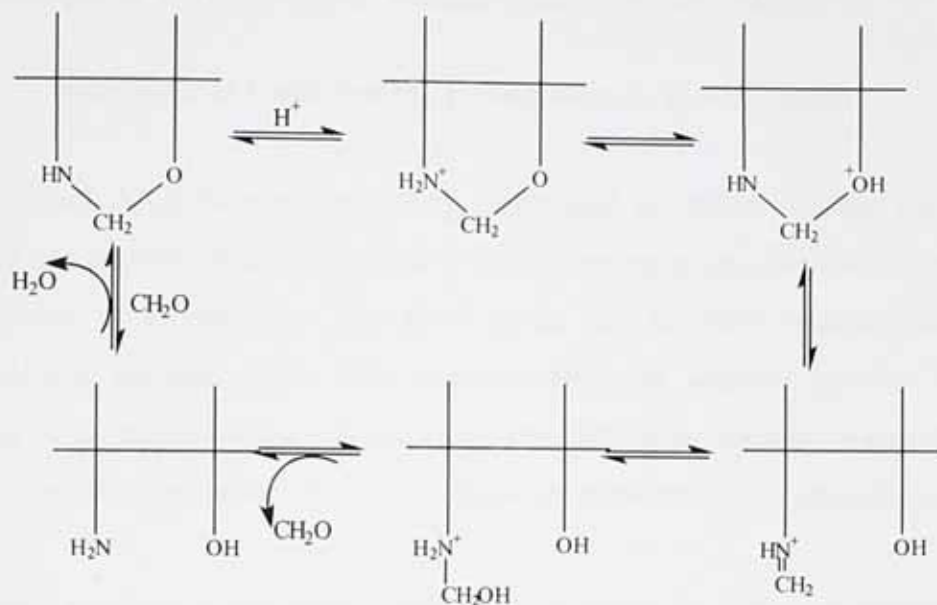


Fig. 3.2 The hydrolysis process of the oxazolidine five-membered ring under acidic conditions

In this work, we have been concerned with the synthesis and characterisation of oxazolidine 1-aza-5-ethyl-1,3-dioxacyclo[3.3.0]octane: the hydrolysis and cross-linking reaction with phenol or protein of this oxazolidine will be discussed in Chapters 4 and 5.

3.3 Experimental

3.3.1 Materials

All chemicals used were the highest purity available from Sigma, UK: 2-amino-2-ethyl-1,3-propanediol (98%), toluene, analytical grade (99%), formaldehyde, 37% aqueous solution, analytical grade, semicarbazide hydrochloride (99%).

3.3.2 Synthesis of 1-aza-5-ethyl-1, 3-dioxacyclo [3.3.0] octane

2-amino-2-ethyl-1,3-propanediol (23.8g, 0.2moles) in 200ml toluene (or without solvent) and formaldehyde (32.4ml of 37% aqueous solution, 0.4moles) were added and refluxed in a Dean-Stark trap for 2 hours with magnetic stirring, then water removal was initiated (both from the formaldehyde aqueous solution and the products of the condensation reaction) until stoichiometric water been removed, then toluene was removed under vacuum to yield the crude product of oxazolidine¹⁷⁸.

After low pressure (240mmHg) redistillation twice or more, colourless liquid oxazolidine was obtained. The process was monitored by paper chromatography with ethanol-water-acetic acid (70:28:2 v/v/v) until only one spot was observed from two dimensional testing, visualised in an iodine atmosphere.

Physical properties, such as density, refraction, boiling point, were measured as described in Organic Chemistry Laboratory Techniques²⁰⁴.

3.3.3 Infrared spectroscopy

Oxazolidine samples were examined by infrared spectroscopy before and after drying over anhydrous sodium sulphate. A Perkin-Elmer-781 infrared spectrophotometer was used; the spectrum was obtained from a thin oxazolidine liquid film formed between sodium chloride discs. The spectrum was recorded in the wavelength range $4000-600\text{cm}^{-1}$, calibration was as described in Chapter 2.

3.3.4 Gas chromatography-mass spectrometry

A gas chromatograph, Hewlett Packard 5890 Series II, was equipped with a BPX5 (SEG) column (30m long, 0.32mm inner diameter and film thickness 0.25 μ m) with auto-injector Hewlett Packard 7673. A Hewlett Packard 5971A mass selective detector ion trap mass spectrometer with upgraded short transfer line was operated with electron impact at 70eV. Mass spectra were recorded in the mass range 0-600.

10 μ l of each sample were injected at 280°C in the split mode with helium carrier gas at 5psi column head pressure. The oven conditions were initially at 45°C for 10mins, heated at the rate of 5°C/min up to 300°C, then held for 1 min.

3.3.5 ¹H-NMR spectroscopy

Proton NMR experiments were conducted with a Bruker AMX 400 spectrometer equipped with an Aspect X32 computer and a temperature probe. Spectra were obtained at 400MHz. All experiments were conducted in 5mm standard NMR tubes with 0.5ml D₆-acetone, D₆-DMSO or D₂O as the solvent.

3.3.6 Measurement of pK_b of oxazolidine

In 1953, Fritz established a relationship between the half-neutralisation potentials in acetonitrile of a number of organic bases and the dissociation constant values in water²⁰⁴. This method has been widely used in studying the basic strengths of organic bases in non-aqueous solutions. Some authors reported on the determination of the dissociation constants of substances which undergo change in structure upon the addition of acid or alkali^{203,204}. Oxazolidine derivatives are susceptible to hydrolysis; as a result, the pH of their half-neutralised solutions changes with time and introduces an error in the determination of pK_b values. This difficulty can be overcome by determining the pK_b value in different concentrations of methanol. Therefore, it is useful for understanding the hydrolysis properties of oxazolidines to compare the pK_b values obtained in methanol and water respectively.

The method used here is based on that of Soliman for the determination of apparent dissociation constants of unstable oxazolidines¹⁹⁵.

Demineralised, double distilled water that had been boiled for 20 minutes (to remove the dissolved carbon dioxide) was used to prepare all solutions. The apparent dissociation constant of oxazolidine was determined by measuring the pH of oxazolidine solution containing equivalent concentrations of organic base and its salt. These solutions were obtained by adding to a solution of oxazolidine the calculated amount of 0.1 mole/l hydrochloric acid required to bring the solution to half-neutralisation.

Oxazolidine was dissolved in 60%, 40%, 20% and 0% methanol (0.2mM), then the calculated amount of 0.1M hydrochloric acid was added to bring the solution to half neutralisation and the pH value monitored as a function of time. The pH values were plotted against time and, the resulting curve extrapolated back to zero time to give the apparent pK_b value at different methanol concentrations.

3.3.7 Kinetic studies of hydrolysis

All rate studies were performed in aqueous buffer solutions: formate, acetate and phosphate. The effect of inorganic salt on the hydrolysis process was also examined.

The rates of hydrolysis of oxazolidines were measured at given pH values (3-7) by trapping the formaldehyde formed with semicarbazide and following the increase in absorbance of the reaction product semicarbazone at wavelength 235nm. Semicarbazide hydrochloride was included in the buffer solution at a concentration of 5mM, to confirm it has no significant influence on the rate of the hydrolysis reaction of oxazolidine. The reactions were conducted over up to three days in a cuvette at 20.0±0.2°C.

The hydrolysis of oxazolidine was also investigated by ¹H-NMR. Oxazolidine solution in D₂O was prepared at a concentration of 0.01M in standard 5mm NMR tubes. The hydrolysis process was followed by checking the shift of related peaks

and the appearance of new peaks every 5 minutes. The reaction rate was calculated based on the formation of $-N-CH_2OH$ from the ring-opening process.

3.4 Results and discussion

3.4.1 Synthesis and physical properties of oxazolidine

The reaction process is described in Fig. 3.3. There are some coloured byproducts: Schiff base (4) is produced in the condensation reaction with the main products (3). The higher the temperature and the longer the time, the more Schiff base is produced, which indicates that there is equilibrium between the main products and the byproducts. In the purification process of oxazolidines by low pressure redistillation, more Schiff base is produced. After low pressure distillation, pure oxazolidine can be obtained. The colourless oxazolidine becomes light brown after a week or so, especially when exposed to direct light and heat.

Many solvents have been tried as the medium to synthesis oxazolidines, such as ether, chloroform, butyl ether, ethyl alcohol, benzene, toluene¹⁷⁸. Knorr and coworkers condensed the reactants in boiling ether in the presence of solid potassium carbonate¹⁷⁸⁻¹⁸⁰. Later work showed that the reaction could be carried out without any catalyst. From the literature, the best and most common method is azeotropic distillation¹⁷⁸, in which benzene is used as the water-entraining agent. Considering the toxicity of benzene, in this study, toluene was tried and high yield was obtained. It was found that high yield could also be obtained when no solvent is used in the process, just refluxing the mixture of one mole of 2-amino-2-ethyl-1, 3-propanediol to two moles of formaldehyde solution. Organic solvent free synthesis is more useful in the clean production of oxazolidine²⁰⁴. The yield is 94-97% when toluene is used as the reaction medium, close to the theoretical yield. Without any solvent medium, the yield is a little lower, 87-90%, but at the same time, there is more Schiff base produced.

The purity and molecular structure characteristics of the final oxazolidine product and the byproduct Schiff base were examined by IR, GC-MS and ¹H-NMR. Pure oxazolidine, 1-aza-5-ethyl-1,3-dioxacyclo[3.3.0]octane, is a colourless liquid with

slight odour. The boiling point for this oxazolidine is 133°C at 240mmHg. The density at 25°C is 1.076g/ml, which agrees with the literature data¹⁷⁸, and the refractive index n_D^{20} is 1.4610.

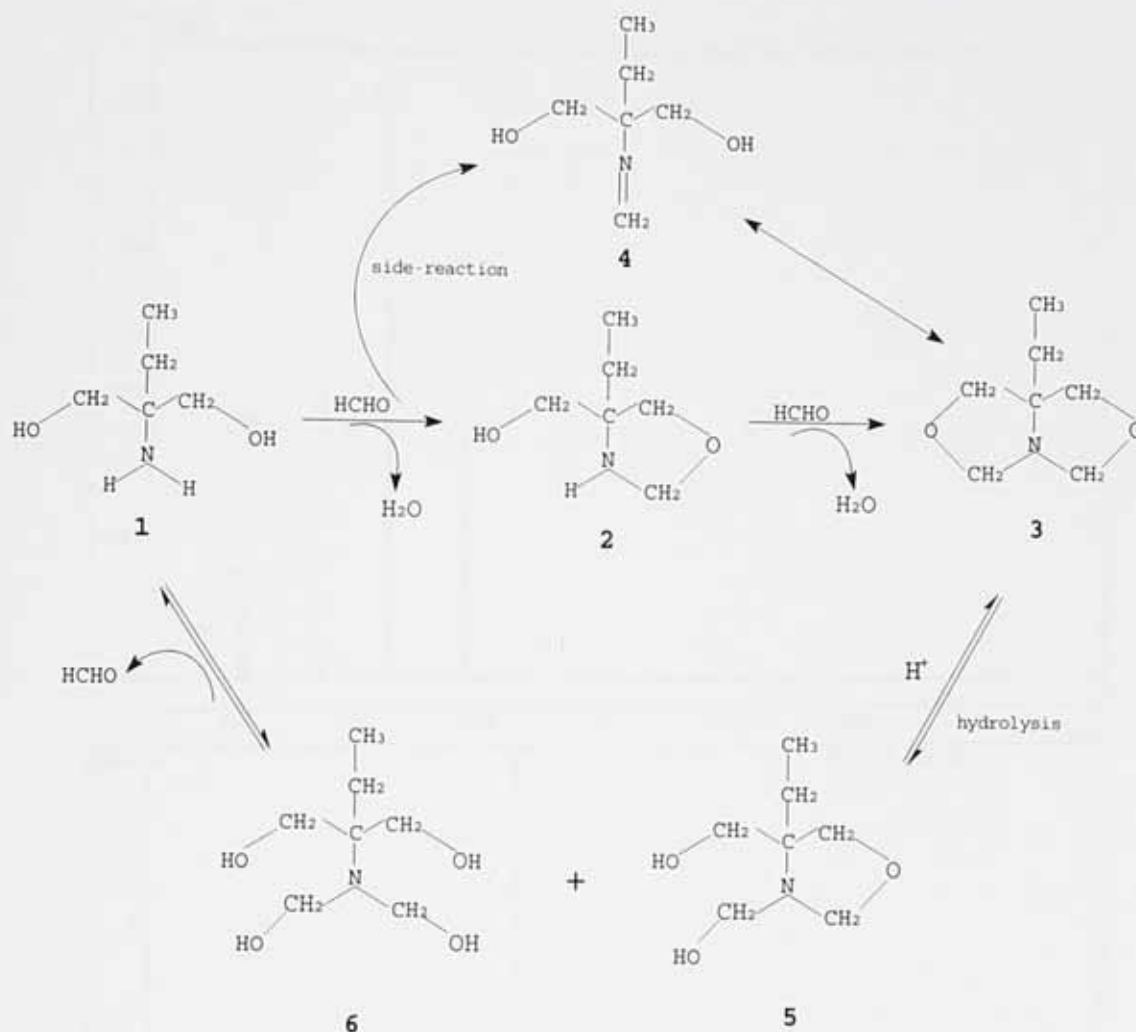


Fig 3.3 Synthesis and hydrolysis of 1-aza-5-ethyl-1,3-dioxacyclo [3.3.0]-octane from 2-amino-2-ethyl-1,3-propanediol and equal molar ratio of formaldehyde

3.4.2 Gas chromatography-mass spectra

From GC-MS (Fig 3.2), the product is pure after just redistillation and dehydration. The positive chemical ionisation mass spectrometry exhibited a mass of 144D (M+1) with base peak at 113, indicating its actual molar mass to be 143, which agrees with the calculated molecular weight.

Chromatogram Plot

File: c:\gc-m\data files\25may01\o.3mox.sms

Sample: o.3mox

Scan Range: 1 - 2857 Time Range: 0.00 - 40.98 min.

Operator: amanda long

Date: 5/25/01 6:47 PM

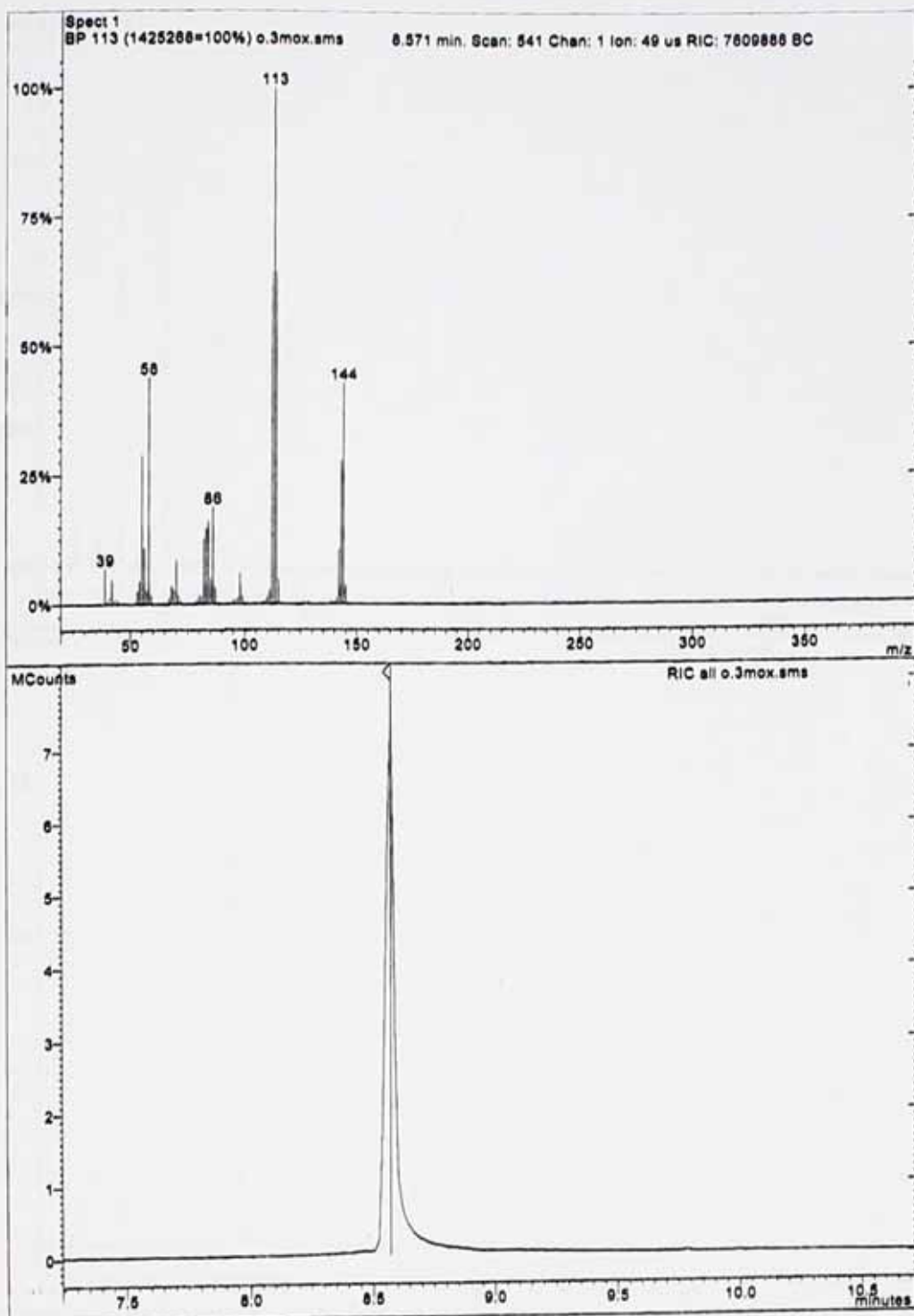


Fig. 3.4 GC-MS of oxazolidine 1-aza-5-ethyl-1,3-dioxacyclo[3.3.0]octane

Chromatogram Plot

File: c:\gc-ms\data files\02aug01\13.ms

Sample: 13

Scan Range: 1 - 2888 Time Range: 0.00 - 40.98 min.

Operator: Amanda Long

Date: 8/2/01 9:23 PM

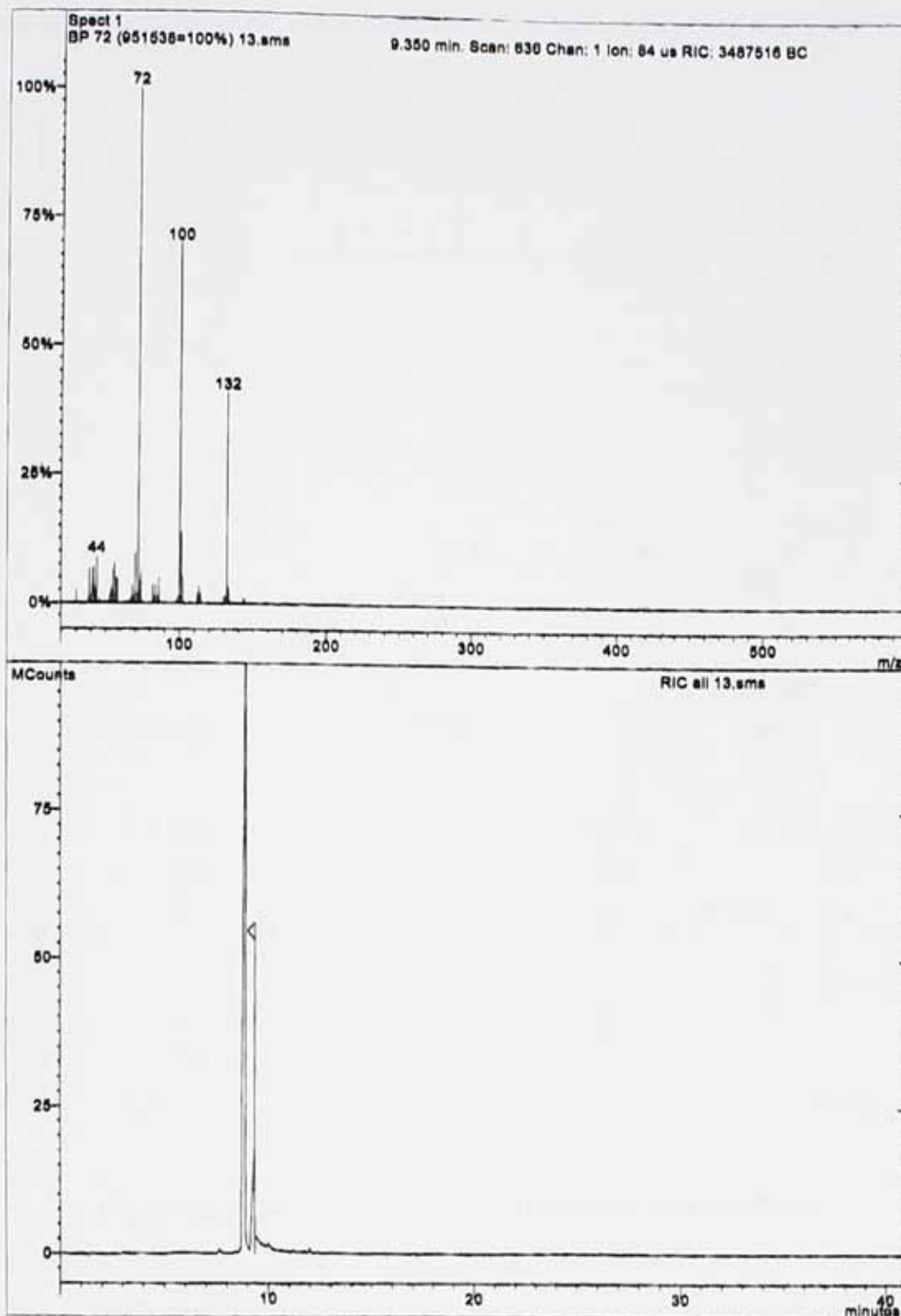


Fig. 3.5 GC-MS of the byproduct Schiff base

From positive chemical ionisation mass spectrometry, the molecular weight of the byproduct Schiff base was shown to be 132 (M+1) with a base peak at 72, which indicates the actual molecular weight to be 131 (Fig 3.5). The possible fragmentation pathway for oxazolidine and Schiff base is proposed in Fig 3.6.

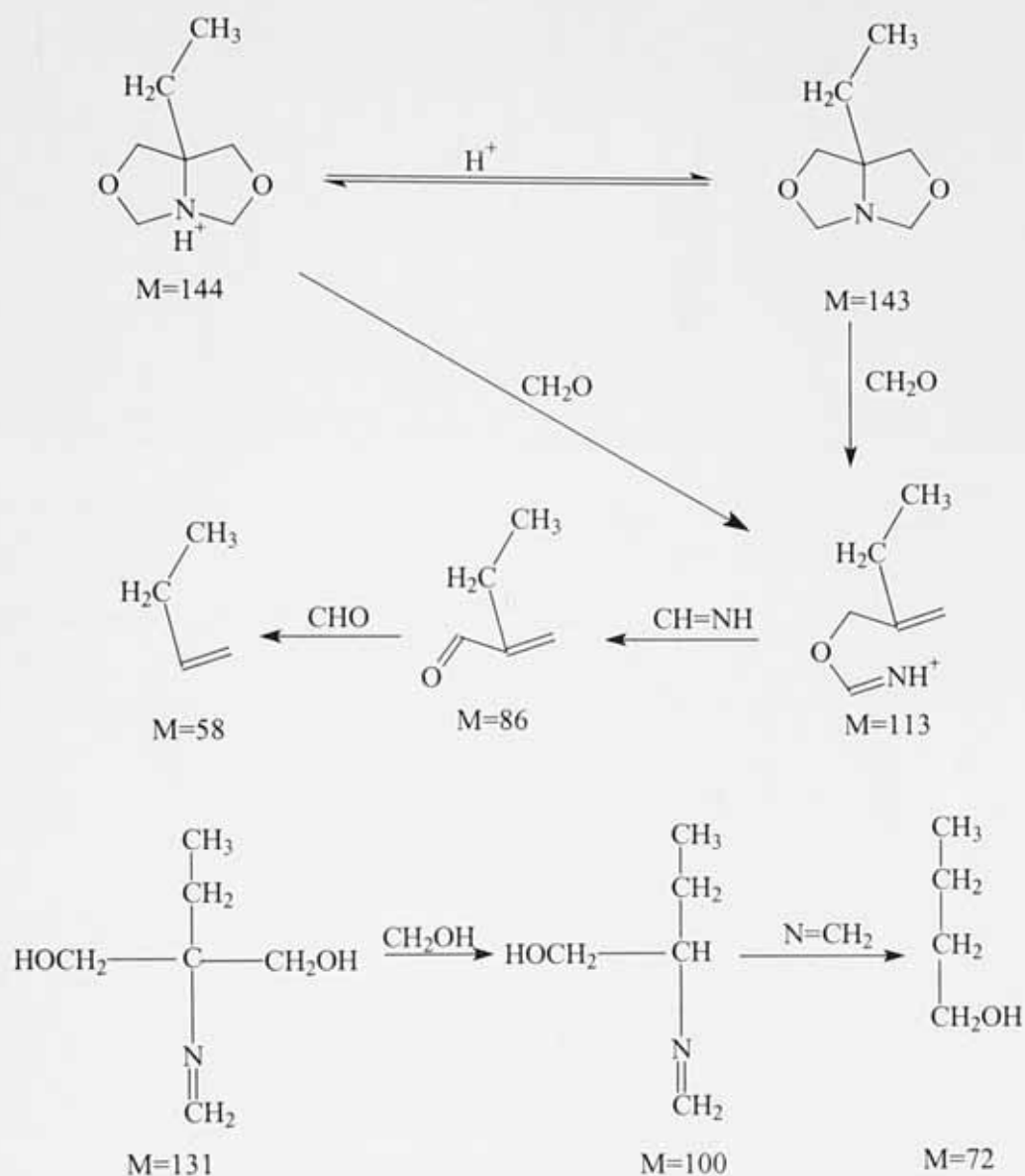


Fig 3.6 Fragmentation pathway for oxazolidine and Schiff base

3.4.3 Infrared spectra

Figs 3.7 and 3.8 show the IR spectra of the crude oxazolidine product, purified oxazolidine and Schiff base respectively.

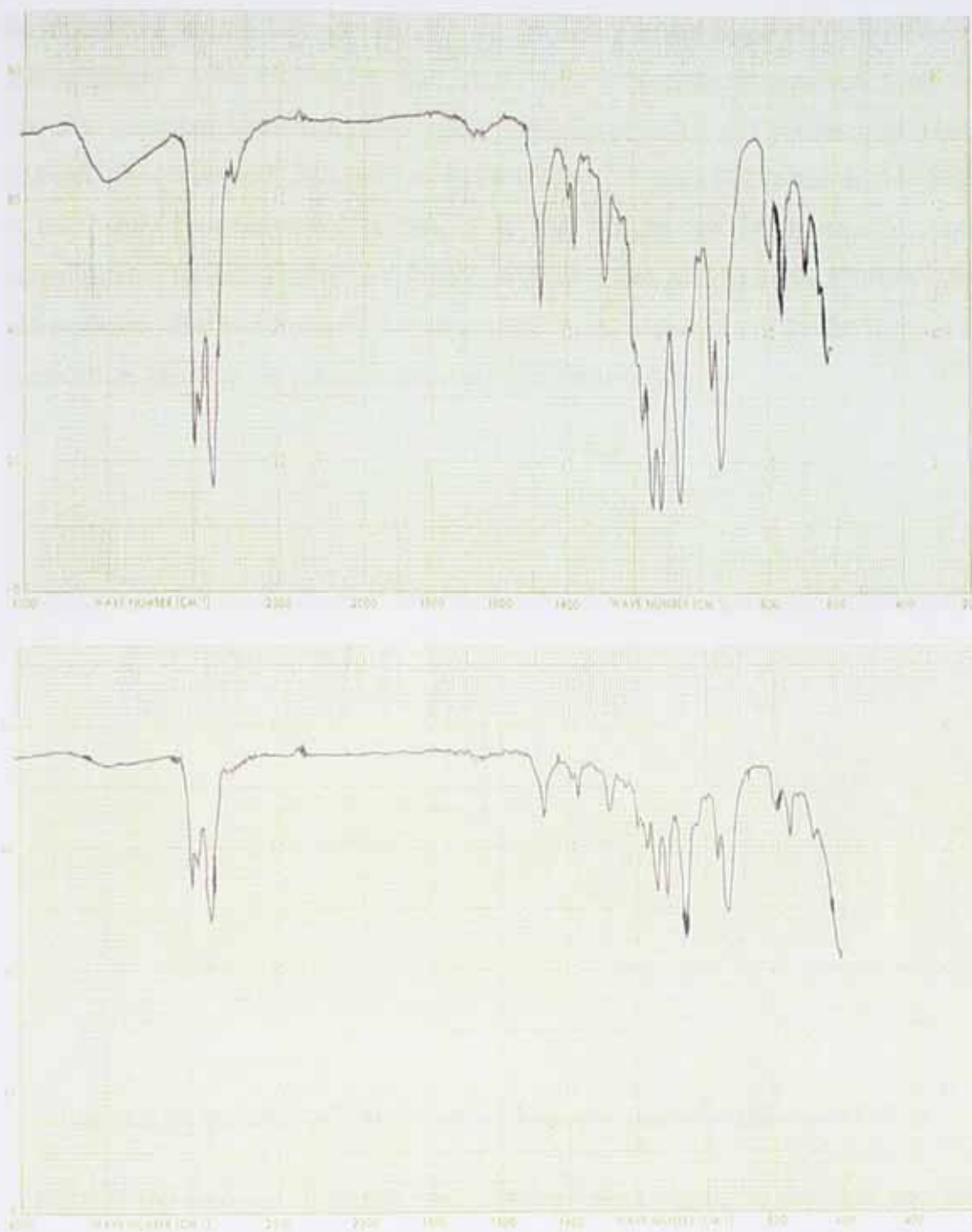


Fig. 3.7 IR spectra of oxazolidine before (upper) and after (lower) dehydration

From Fig 3.5, the peaks from the amino group and the hydroxyl groups ($3100\text{-}3500\text{cm}^{-1}$, O-H stretch and N-H stretch)²⁰² of 2-amino-2-ethyl-1,3-propanediol become weak after condensation with formaldehyde. After purification of the crude oxazolidine, these peaks disappear completely, indicating there is no free amino or hydroxyl group left. There is no hydrogen bonding between oxazolidine molecules, from the disappearance of the peaks between $3100\text{-}3500\text{cm}^{-1}$. IR also confirms that

the mixture of oxazolidine and Schiff base can be separated based on their different boiling points. After distillation, the Schiff base is completely removed from the mixture. A strong absorbance associated with an ether (C-O-C) stretch at 1100-1200 cm^{-1} was observed. The peak at 1650-1700 cm^{-1} is assigned to the double bond of the Schiff base structure (C=N), **4** in Fig 3.3. In the IR spectra of crude oxazolidine or the residues after distillation, there is one peak at 1650-1700 cm^{-1} , but it disappears after purification. However, this peak becomes big in the residue of oxazolidine, because the concentration of Schiff base is high.

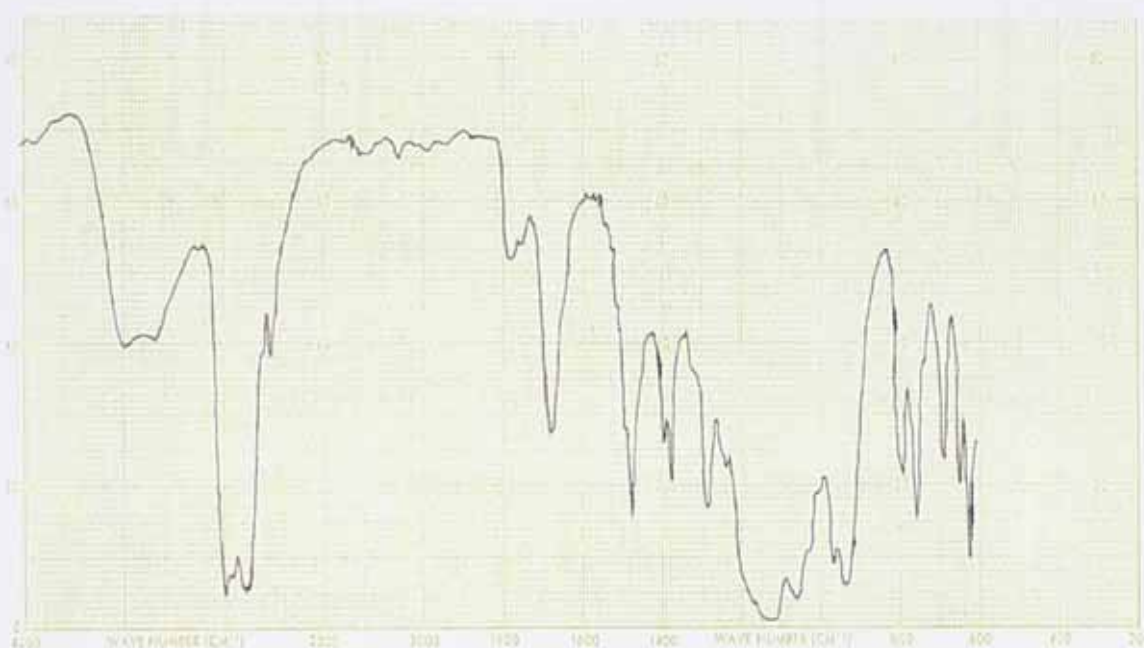


Fig 3.8 IR spectrum of Schiff base residue of oxazolidine after distillation

In Fig 3.8, the peaks at 3300 cm^{-1} and 3500 cm^{-1} are assigned to the N-H and O-H stretch respectively, which are present in the Schiff base molecular structure. The peak at 1100 cm^{-1} also becomes smaller, which indicates the loss of the ring structure in the Schiff base.

3.4.4 $^1\text{H-NMR}$ spectra

The molecular structure of 1-aza-5-ethyl-1,3-dioxacyclo [3.3.0] octane is confirmed by the $^1\text{H-NMR}$ spectrum (Fig 3.9). From the integration of peaks, there are four hydrogens at 4.3-4.5ppm, and another four at 3.7-3.9ppm; the peaks at 0.9ppm

(triplet, three hydrogens) and 1.7ppm (quartet, two hydrogens) result from the ethyl group at C-5.

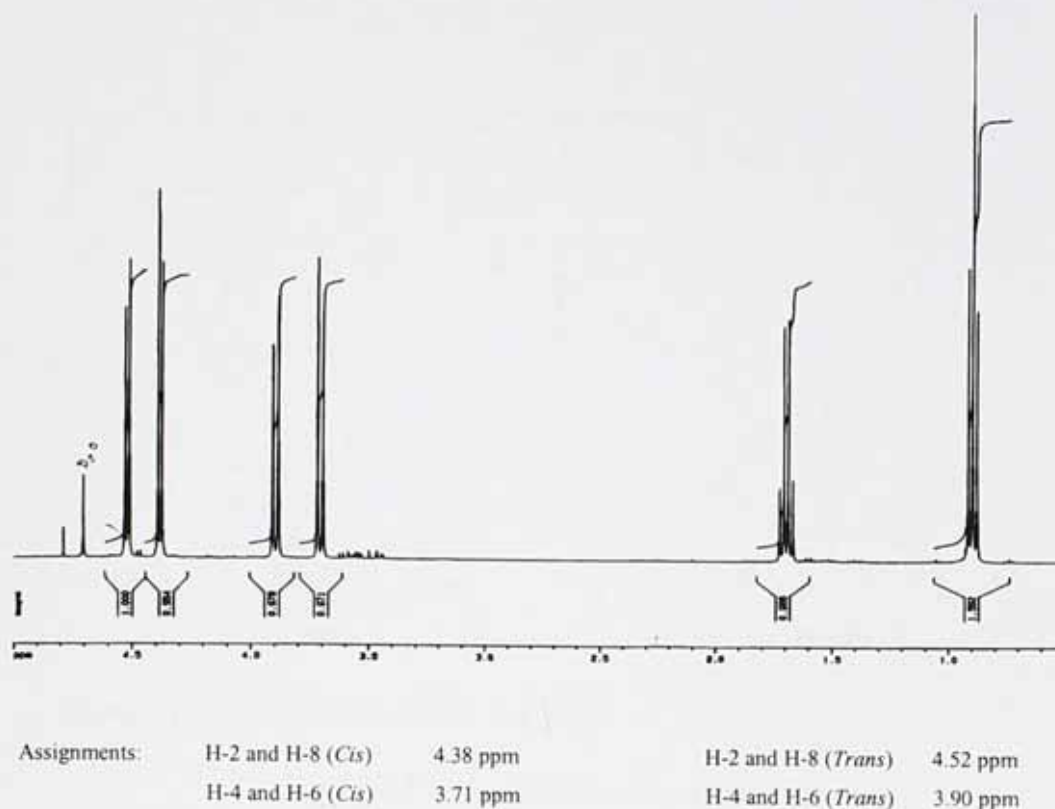


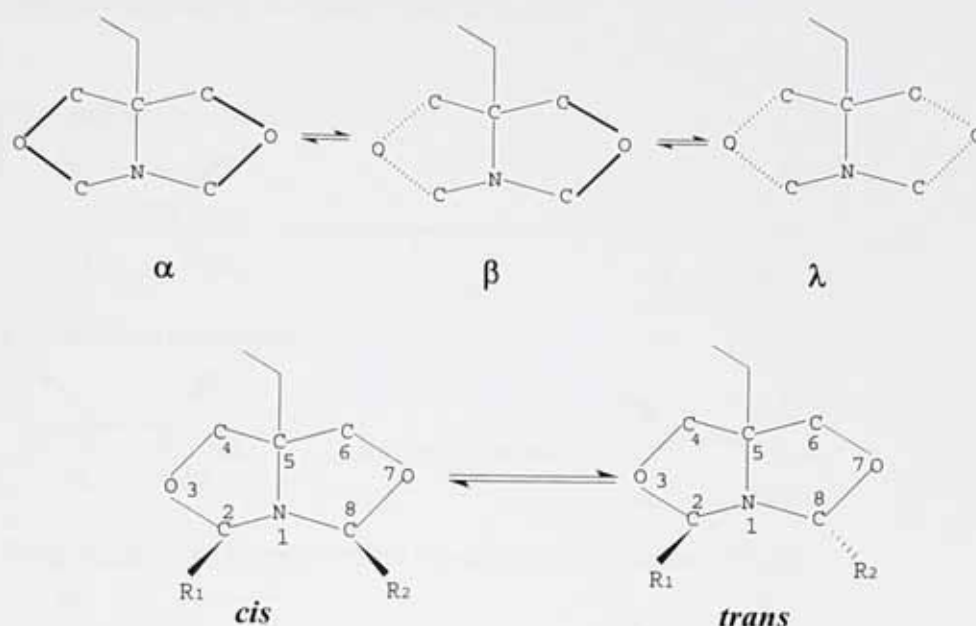
Fig 3.9 ^1H -NMR spectrum of oxazolidine in D_2O

3.4.4.1 Stereochemistry

Many reports in the literature have shown that different stereochemistry of substituents bonded to the oxazolidine ring often has a pronounced effect on the hydrolysis rates^{187,197,198,199}. Therefore, it is important to check the stereochemistry of oxazolidines. From the work of Darabantu and co-workers, the stereochemistry of oxazolidine can be studied by high-resolution ^1H -NMR^{197,198,199}. The diastereoselectivity ratios can be calculated from the integration of different peaks (Fig 3.9).

Oxazolidines have different stereochemistries and the most stable conformation has been found by Darabantu to be β (Fig 3.10)¹⁹⁹. Plane N1-C5-CH_2 - is a symmetry plane for the conformers α and λ , but a chirality plane for β . The β conformation

has two conformations, *trans* and *cis*: from the $^1\text{H-NMR}$ spectrum (Fig.3.9), the ratio between *cis* and *trans* is nearly 1:1, and remains unchanged in the hydrolysis process.



$R_1=R_2=H$ for oxazolidine II in this work

Fig 3.10 Conformations of oxazolidine

3.4.4.2 Hydrolysis observed through NMR

When the NMR experiment is carried out with oxazolidine in D_2O solution, some small peaks at 3.40-3.65ppm appear in the spectra, assigned to be from the hydrolysis products of oxazolidine ($-\text{CH}_2\text{OH}$). These peaks change with time, but slowly. From Fig 3.3, hydroxymethyl or N-hydroxymethyl groups are produced after the ring-opening process. Only about 10% of the oxazolidine is hydrolysed to the ring-opened position after 24 hours at 20°C , based on the integration of these new peaks. One point is clear, there are no peaks from formaldehyde or its derivatives (HCHO , HOCH_2OH or the polymers of formaldehyde)^{47,49,201,202}, which means there is no free formaldehyde produced in the hydrolysis process. These results are in agreement with the results from the following hydrolysis reaction studies. Much research work has shown that most of the oxazolidines can be hydrolysed completely to give the original reactants, but that appears to be significantly influenced by the substitutes at C-2 or C-8, which originate from the

carbonyl moiety^{178,185}. Therefore, from the spectra ¹H-NMR, the hydrolysis of 1-aza-5-ethyl-1, 3-dioxacyclo [3.3.0]octane in D₂O is slow under these experimental conditions or the rate determining step is slow and only little oxazolidine is hydrolysed completely to formaldehyde, if at all.

Step 1 Protonation of nitrogen



Step 2 Protonation of oxygen



Step 3 Ring-opening of O-protonated oxazolidine to cationic imine



Step 4 Hydration of cationic imine to protonated carbinolamine



Step 5 Decomposition of protonated carbinolamine



Step 6 Re-protonation of nitrogen



Fig 3.11 Hydrolysis process proposed by Walker¹⁸¹

Unfortunately, there is no way to check which ring will be opened first or whether both rings open at the same rate to produce species **5** and **6** of Fig 3.3, which are present in the solution at same time, nor can the effect of the different conformations be measured. By studying the process with computational methods¹⁸¹, Walker and co-workers deduced the hydrolysis reaction of oxazolidine is as described in Figure 3.11 and the rate determining step of the hydrolysis reaction of oxazolidine is the decomposition of the protonated carbinolamine intermediate.

3.5 Hydrolysis of oxazolidine

3.5.1 Measurement of pK_b of oxazolidine

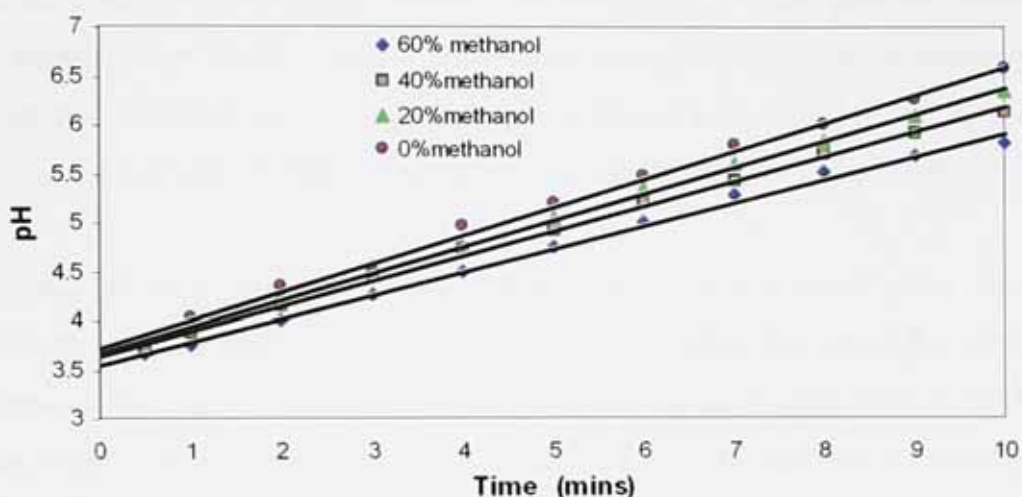


Fig 3.12 Plots of pH values of half neutralised oxazolidine solution against time

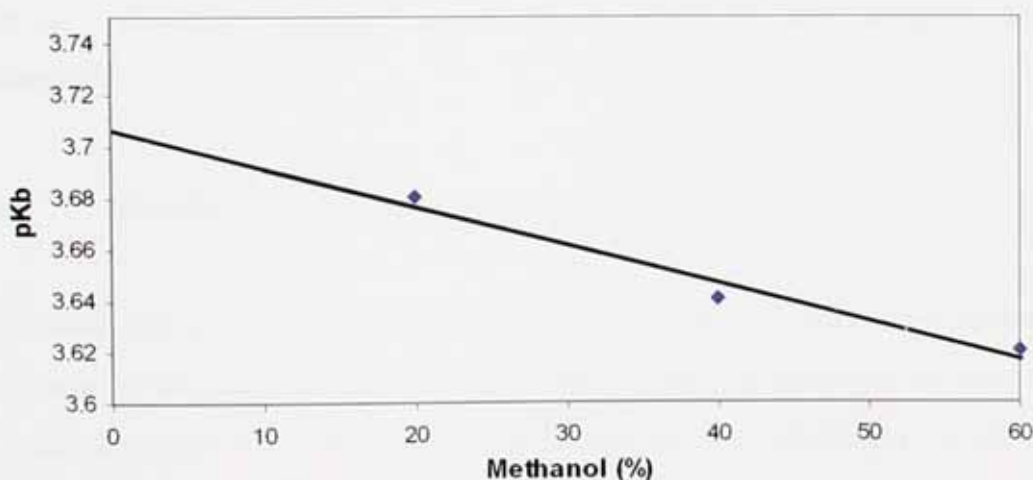


Fig. 3.13 The effect of methanol concentration on pK_b value of oxazolidine

The pH values of half neutralized oxazolidine increased slowly from 3.30-3.70 to 6.80-7.00. The process takes more than 3 hours to finish for all the methanol concentration conditions (Fig 3.12, data only shown for the first 10 minutes). This indicates that the hydrolysis reaction of oxazolidine is slow after a quick equilibrium is obtained. By extrapolation, at time zero, the pH value is the acidic pKa value or pKb for the organic base (Fig 3.12). The pKb value of oxazolidine is then determined by plotting pKb values versus methanol concentration: the value extrapolated back to 0% methanol is the pKb value for oxazolidine in water (Fig 3.13). So, the pKb value of this oxazolidine is about 3.7. The same result can be obtained by directly carrying out this process in water, which indicates that the hydrolysis of this oxazolidine in water did not produce free amine. The pKb value is similar to the pKb of the tertiary amine group of various derivatives of dimethylammonium, ranging from 3.0 to 4.8^{203,204}, such as triethylamine indicating that the nitrogen is chemically similar. The hydrolysis process does not produce a free amino group. The dissociation constant of oxazolidine in different compositions of aqueous methanol solution is a little lower than it is in water, which may affect the reactivity of oxazolidine in organic solvent, discussed in detail in Chapter 4.

The final pH of the half-neutralised oxazolidine solution is much lower than the expected value of the amine, the pH value of 2-ethyl-2-amine-1,3-propan-diol solution is 10.0. This may indicate that the hydrolysis is not complete even after 3 hours, which has been proposed in the literature^{181,187}. The reaction is controlled by one of the steps 1 to 4 in Fig 3.11. So, we can assume the first four steps of hydrolysis to open the heterocyclic ring are relatively fast, compared with the next step to produce the original alcohol amino chemicals and carbonyl reactant (aldehyde).

3.5.2 Hydrolysis in different buffer solutions

First order rate constants were determined from ultraviolet absorption measurement at 235nm by plotting $\log(A_{\infty}-A_t)$ against time. A_{∞} and A_t represent the absorbance of semicarbazone at the reaction end point and time t respectively. In all cases, stable end points (A_{∞}) were observed after 72 hours, giving different reaction rate

constants under different conditions (Table 3.1). It is possible to use semicarbazide to follow the hydrolysis of oxazolidine, because the reaction of formaldehyde with semicarbazide is much faster than the hydrolysis reaction of oxazolidine.

Table 3.1 Hydrolysis reaction rates under different conditions

Compounds	Buffer pH value and buffer concentration	k (10^{-3} s^{-1})	
Oxazolidine	7.2 (phosphate, 0.1M)	0.13±0.01	
	6.2	acetate buffer conc. 0.2M	0.33±0.03
		acetate buffer conc. 0.1M	0.22±0.02
		acetate buffer conc. 0.05M	0.18±0.03
	5.2 (acetate, 0.1M)	0.34±0.03	
	4.2 (acetate, 0.1M)	0.74±0.05	
	3.2 (formate, 0.1M)	0.91±0.08	
Formaldehyde	6.2 (acetate, 0.1M)	12.6±0.7	

From UV spectroscopy studies, the hydrolysis of oxazolidine is slow under weak acidic and neutral conditions. The rate constant ranges from 1.3 to $9.1 \times 10^{-4} \text{ s}^{-1}$, which is much slower than the rate of formaldehyde reaction with semicarbazide. The reaction is much faster when the pH is lower than 3.2. There is no formaldehyde produced when the pH is higher than 7.2, which is similar to the result from pK_b measurement.

The concentration of buffer solution affects the hydrolysis reaction¹⁸⁵. This is in agreement with the literature^{185,195,196}; the hydrolysis reaction becomes faster with increasing buffer concentration, which is probably due to the catalytic effect of the presence of other ions. However the effect is small compared with other kinds of oxazolidine¹⁷⁸.

3.6 Discussion and summary

1-aza-5-ethyl-1,3-dioxacyclo[3.3.0]octane was synthesised by different methods and the molecular structure was confirmed by IR, GC-MS and $^1\text{H-NMR}$ spectra. From NMR, the ratio between *cis* and *trans* conformations is nearly 1:1.

From $^1\text{H-NMR}$ and the results obtained from the semicarbazide studies, complete hydrolysis of 1-aza-5-ethyl-1,3-dioxacyclo[3.3.0]octane is slow under the experimental conditions of weakly acidic or neutral pH. In the incomplete hydrolysis process, the N-hydroxymethyl intermediate is present as the most abundant product and this is the main reactive component when oxazolidine is used as a cross-linking agent for protein or phenol. No free formaldehyde is produced in the hydrolysis process, making this chemical safer than other formaldehyde substitutes.

Chapter Four

Cross-linking reaction of polyphenols with oxazolidine

Section 4.1 Simple phenols study

4.1.1 Introduction

The condensation reaction of phenols with aldehydes, especially formaldehyde, has been the foundation of polymer chemistry and polymer materials science^{206,225,226}. The reaction has been studied extensively and comprehensively in the resin and adhesive areas, which are the main applications of phenol-aldehyde resins. As the first commercial synthetic resin, phenolic resin was developed as early as 1908^{206,209}. In the last century, phenolic-formaldehyde condensation products were exploited to meet the requirements of industry markets, such as paints, adhesives, plastics and functional resins^{206,210,215,218,221-224,225-229,231,232}. As well as the main application, the phenol-aldehyde reaction also has been exploited in polyphenol-aldehyde combination tannage studies in the leather industry^{17,230}.

The reaction of phenol with aldehydes is a typical electrophilic substitution process and can be catalysed by acid, base or some metal ions^{203,204,207-209,211,225}. The reaction of phenols with formaldehyde has been shown to be a second order reaction^{216,217,220}. Formaldehyde is the most reactive, most common and cheapest aldehyde employed in the condensation reaction. But, there are increasingly stringent restrictions to emission in developed countries, due to its toxicity and its known carcinogenicity²¹⁹. In fact, not only phenolic resins, but also amino-plastic resins (urea-formaldehyde, melamine-formaldehyde, phenol-melamine-formaldehyde) face the same problem, because of the presence of formaldehyde. Therefore, the pursuit of a formaldehyde substitute cross-linking agent, or "F-zero" material, is an important research direction in green chemistry and environmentally friendly technology.

Oxazolidine is probably one of the better choices to replace formaldehyde. Recently, Covington and Shi have suggested a new kind of new organic tannage, using oxazolidine and plant polyphenols^{6,30-32}. The main reaction is believed to happen between polyphenols and oxazolidine, which is similar to the traditional vegetable – aldehyde combination tannage in cross-linking style, but probably has a different

mechanism. Compared with formaldehyde, oxazolidine has higher reactivity and will produce a longer bridge chain in cross-linking. From Chapter 3, the first step of ring opening is fast and no formaldehyde has been observed, except under strong acidic conditions. Many studies have also shown there is no formaldehyde emission when oxazolidine is used as a cross-linking agent in the adhesive or coating industries^{185,189,192,194,198}. However, at present the mechanism and kinetics of this reaction are far from well understood yet. This is because the complexity of this process makes it difficult to characterise the intermediates or final products. In this work, such difficulties were presented, as oxazolidine showed higher reactivity than formaldehyde with phenols.

As a new cross-linking agent for phenolic compounds, the reaction process of oxazolidine is different to formaldehyde. For formaldehyde, the first step of cross-linking process is methylation, producing a hydroxymethyl group $-\text{CH}_2\text{OH}$ on a benzene ring, which will attack another phenol's reactive site and form a condensation product^{219,225,226}. The reaction is believed to be controlled by the methylation step (Fig 4.1).

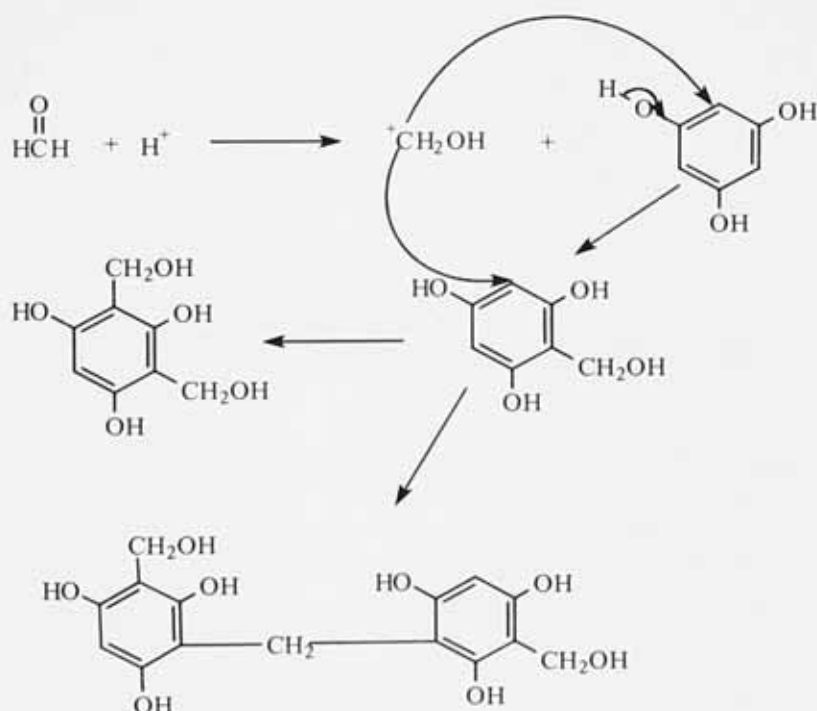


Fig 4.1 Reaction of formaldehyde with phloroglucinol

For oxazolidine, the mechanism should include two steps: first is the ring opening of oxazolidine; second is the substitution at nucleophilic sites of phenol and loss of a water molecule. The ring-opened oxazolidine has two $-NCH_2OH$ groups that have high reactivity with polyphenols. For oxazolidine, the first substitution and further condensation are nearly same, all from the attack of NCH_2OH on phenols (Fig 4.2).

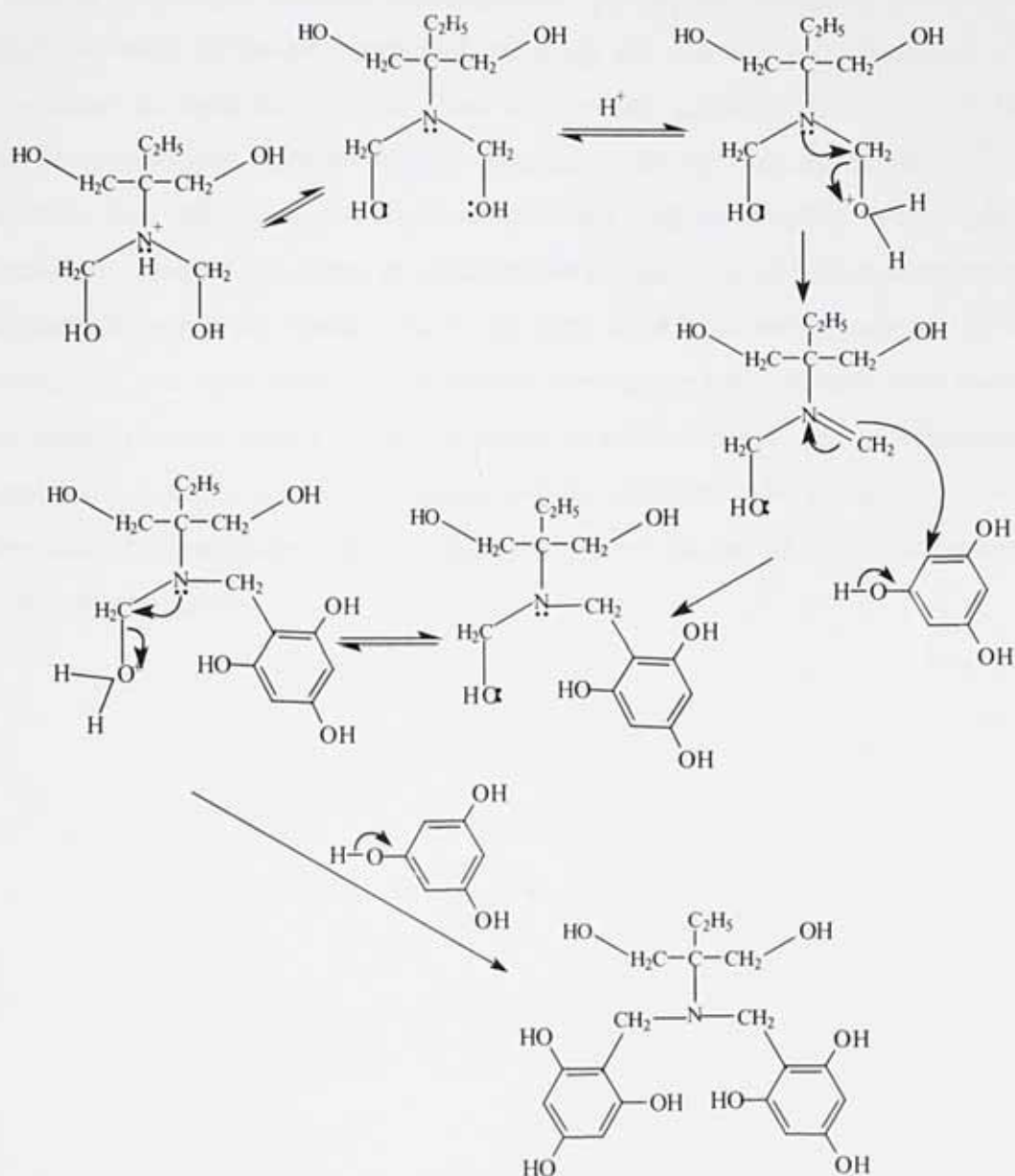


Fig 4.2 Reaction of oxazolidine with phloroglucinol

This work only concerns condensed tannins (proanthocyanidins), discussed in Chapters 1 and 2. They are flavonoid polymers with different hydroxylation patterns of A-ring and B-ring (See Chapter 1, Fig 1.12), which lead to different nucleophilic properties in the benzene ring. Due to the complexity and high reactivity of proanthocyanidins, pure compounds have always been difficult to obtain. Therefore, model compounds have been employed in this study.

In Section 4.1, simple phenols, phloroglucinol, pyrogallol, resorcinol, catechol and phenol are used as model compounds to study the reaction of polyphenols with oxazolidine in aqueous solution. Zinc acetate and calcium chloride have been reported as effective catalysts for the reaction of phenol with formaldehyde²¹⁶⁻²¹⁷; therefore, their effects on the reaction of phenol with oxazolidine were explored. Aluminium sulphate and borax are well known for their complexation reaction with polyphenols, especially borax, which can form soluble complex compounds with polyphenol⁵⁷, so, their effects on the phenol-oxazolidine reaction have been studied. The simple phenols results should be useful to explain the reaction mechanism of polyphenols or its monomers (catechins or gallocatechins) with oxazolidine. At the same time, this constitutes a fundamental study about the use of cross-linking agents in the leather industry.

4.1.2 Experimental

All the chemicals employed in this work, phloroglucinol, resorcinol, pyrogallol, catechol and phenol, zinc acetate, calcium chloride, aluminium sulphate, borax, sodium sulphate and sodium chloride, were analytical grade, obtained from Sigma (UK). Oxazolidine was synthesised as described in Chapter 3.

4.1.2.1 Preparation of stock solution

A known weight of phloroglucinol, resorcinol, pyrogallol, catechol, phenol was dissolved in water or acetate buffer solution (at designed pH) or 10% aqueous acetone or methanol solution to give a concentration of 0.1mol/l, nitrogen was bubbled into solution for 10 minutes to prevent autooxidation, then the phenol solution was used within 30 minutes. All the phenols could be dissolved in water at this concentration.

Oxazolidine was diluted to 1mol/l with distilled water or acetate buffer solution, adjusted to the required pH value with 1.0mol/l HCl, then nitrogen was bubbled in the solution for 10 minutes. Oxazolidine solution was prepared fresh for each experiment.

Zinc acetate or calcium chloride were dissolved in water to give a concentration of 0.2mol/l, borax was at 0.05mol/l. Sodium chloride and sodium sulphate were prepared at a concentration of 0.5mol/l.

4.1.2.2 Reaction procedure

All the reactions were conducted in aqueous solution or 10% aqueous acetone solution: organic solvent can be used to slow down the reaction and so better $^1\text{H-NMR}$ results can be obtained¹⁴¹. Various pH conditions (3.5, 5.0, 6.5, 8.0), temperatures (20, 40, 60°C) and molar ratios (phenol:ox = 2:1, 1:1, 1:2) were employed in this study.

The reaction processes were followed by paper chromatography (Pc), gel permeation chromatography (GPC, for molecular weight distribution), UV-Vis spectroscopy, gas chromatography-mass spectrometry (GC-MS) and $^1\text{H-NMR}$ spectrometry. From Pc, the disappearance of the original phenols and formation of new products could be observed directly. The effect of molar ratio of phenol to oxazolidine and the completion time of the reaction were determined by Pc and GPC methods. The determination of reaction sites and kinetic studies were mainly carried out by following the disappearance of signals in $^1\text{H-NMR}$ spectra, calculated from spectra integration or from the UV-Vis absorbance change.

Determining the turbidity of solution has been used in colloid chemistry and polymer reactions for a long time, sometimes referred to as the Tindall phenomenon. Due to the changing of the composition of a solution, such as polymerisation or combination between molecules through weak bonds, the optical properties of the solution will change. Light cannot pass through a solution when the particle size becomes significant (diameter over 500nm). Although the appearance of turbidity is a rather indefinite factor, the information can still be useful. The turbidity of reaction in water was recorded at 650nm at different times to reflect the molecular particle size and reaction progress.

Consumption of oxazolidine is a key question in this study and it was measured by the amount of oxazolidine left in solution when reaction had finished, using GC-MS or nitrogen determination methods.

One dimensional paper chromatograms were developed in solvent system A (6% acetic acid/water, v/v); two- dimensional paper chromatograms were developed in A for the first direction, followed by B (BAW (14:1:5, butan-2-ol:acetic acid:water, v/v/v)) in the second direction, and the filter paper was allowed to dry between the two developing systems. Whatman No. 1 paper was used throughout these experiments. The final results were examined first by UV light, followed by soaking in 1% ferric chloride aqueous solution for 30 seconds and washing with 2M HCl to remove excess ferric chloride or by iodine atmosphere to develop colour.

4.1.2.3 Effect of electrolytes

In this work, zinc acetate (0.2mol/l), borax (0.05mol/l), calcium chloride (0.2mol/l), and aluminium sulphate (0.1mol/l) were used. The concentration of ions in the phenol solutions was kept at 0.01mol/l or 0.02mol/l, then an equal molar amount of oxazolidine to phenol was added to the solution and the pH was adjusted to 6.0. The reaction progress was checked by paper chromatography and the turbidity meter (to check the percentage of light passing through at 650nm) at different times (1, 2, 3, 4, 5, 10, 15, 20, 30, 60 minutes).

4.1.2.4 UV-Vis spectroscopy

0.1ml of stock phenol solution was put into a UV cell, followed by pH 6.0 sodium acetate buffer solution and oxazolidine to give phenol and oxazolidine concentrations 0.0003mol/l and 0.03mol/l respectively. After shaking for 10 seconds, the absorbance was recorded at minute intervals until no more change was observed.

4.1.2.5 ¹H-NMR spectroscopy

Phenol was dissolved in D₂O or 10% D₆-acetone or D₆-DMSO in D₂O and the pH was adjusted to 6.0 with DCl solution. 0.5 ml of solution was used and then oxazolidine was added to the NMR tubes at a molar ratio of 1:1 phenol:oxazolidine, but for phloroglucinol the ratio was 2:3. The spectrum was recorded every 2 or 5 minutes, depending on the reaction rate.

A Bruker AMX 400 NMR spectrometer was used for all the NMR experiments, as described in Chapter 3.

4.1.2.6 Gas chromatography-mass spectroscopy

The cross-linked product mixture was studied by GC-MS. A known weight of phloroglucinol, resorcinol, pyrogallol or catechol was dissolved in methanol to make

the concentration 0.01mol/l. 1 to 10 times of the molar offer of oxazolidine was added and the mixture was held for 24 hours at 25°C before running the GC-MS. The method was as described in Chapter 3.

4.1.2.7 Molecular weight distribution of reaction products

The reaction product was freeze-dried (equipment and drying conditions as described in Chapter 2 for polyphenol extracts) and dissolved in freshly redistilled pyridine. Excess acetic anhydride was added and the mixture was kept in the dark for 48 hours at 25°C. The solution was poured into ice water to obtain a precipitate, which was washed with equal volumes of 10% acetic acid and 10% sodium carbonate twice each, then the precipitate was dissolved in THF for GPC analysis as described in Chapter 2. Polystyrene samples were used for calibration.

4.1.2.8 Kinetic studies

The mechanism of the reaction of phenols with aldehydes has been widely studied: Pizzi, Porter and co-workers concluded the reaction is second order.^{222,223,227}

$$V = k [\text{phenol}][\text{aldehyde}]$$

Where:

V is the reaction rate

k is the reaction rate constant

[phenol], [oxazolidine] are the concentrations of phenol and oxazolidine reactive sites respectively

For the reaction of phenols with oxazolidine, the reaction rate can be expressed in the same way.

4.1.3 Results and discussion

4.1.3.1 Reaction of phloroglucinol with oxazolidine

4.1.3.1.1 $^1\text{H-NMR}$ spectra

The $^1\text{H-NMR}$ spectra show that the peak for H-2, H-4, H-6 at δ 5.99 ppm disappears with time (Figs 4.3, 4.4). No chemical shifts were observed in the reaction process, which indicates the three reaction sites are consumed at nearly the same rate, so no intermediates were produced. The reaction proceeded slowly when it was conducted in 10% acetone solution, but there was also no chemical shift observed in the $^1\text{H-NMR}$ spectrum. All three available sites can react with oxazolidine, but it is not likely all of them could combine with another phloroglucinol molecule, because of the steric hindrance effect, which will be discussed in Section 4.1.3.1.4.2.

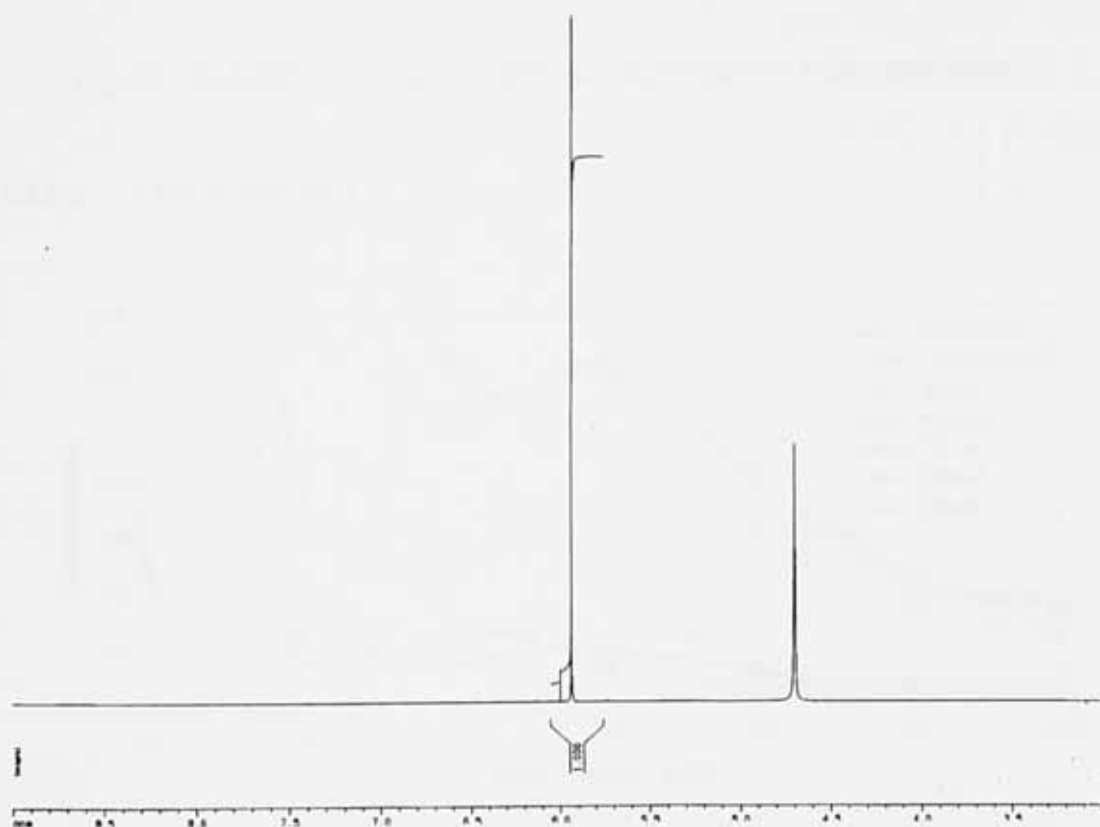


Fig 4.3 $^1\text{H-NMR}$ spectrum of phloroglucinol

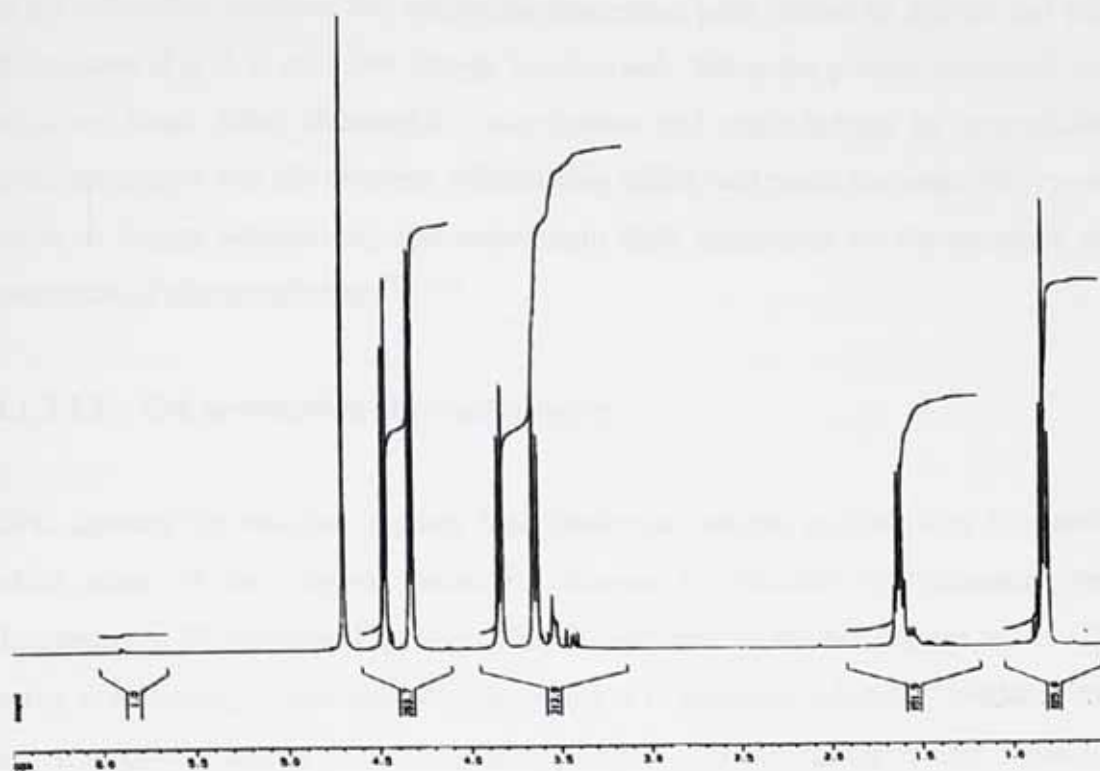


Fig 4.4 $^1\text{H-NMR}$ spectrum of phloroglucinol reacted with oxazolidine

4.1.3.1.2 UV-Vis spectrum

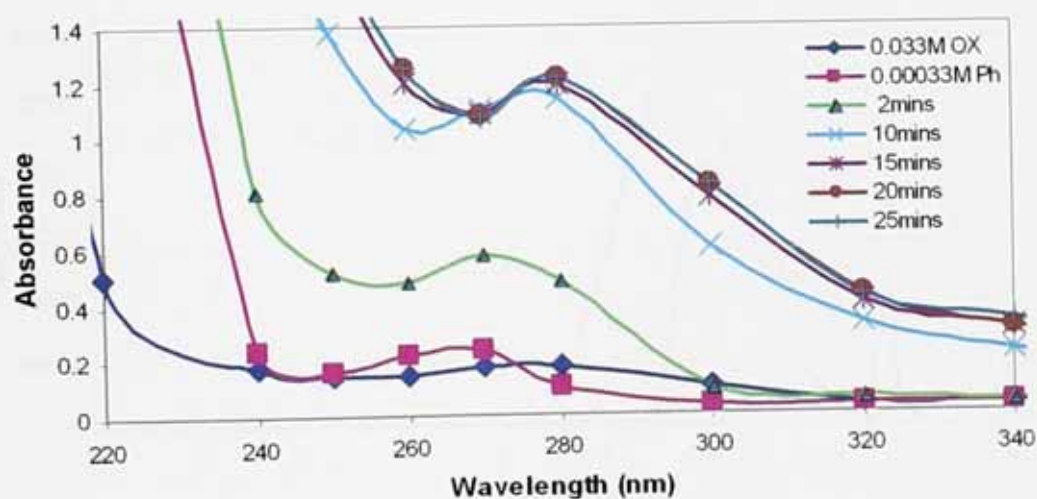


Fig 4.5 UV absorbance of phloroglucinol (Ph) reacted with oxazolidine (OX)

There is big shift in the UV peak of phloroglucinol after reaction with oxazolidine. The phloroglucinol has maximum absorption at 267nm in aqueous solution, while

oxazolidine does not show any evident absorption. After reaction with oxazolidine in pH 6.0 buffer solution, the maximum absorption peak moves to 283nm and after 20 minutes (Fig 4.5) no more change is observed. When the phenol molecular size becomes larger after electrophilic substitution and cross-linking by oxazolidine, steric hindrance and the electron withdrawing effect will make the absorption peaks move to longer wavelength, the wavelength shift depending on the numbers and properties of the substitutions¹⁷¹⁻¹⁷³.

4.1.3.1.3 Gel permeation chromatography

GPC showed the reaction creates high molecular weight polymers in 5 minutes, while some of the original monomer remains in solution; the monomer peak disappears in 20 minutes (Fig 4.6). After 20 minutes, no further change in the GPC curve is observed, which indicates the reaction is complete when the reactants ratio is 1:1, at pH 6.5 and at room temperature (20°C). The formation of high molecular weight polymer indicates that reactive intermediates produced from the initial process of this reaction further react with phloroglucinol or other intermediates to form polycondensation products. The formaldehyde reaction produced high polymers after 15 minutes.

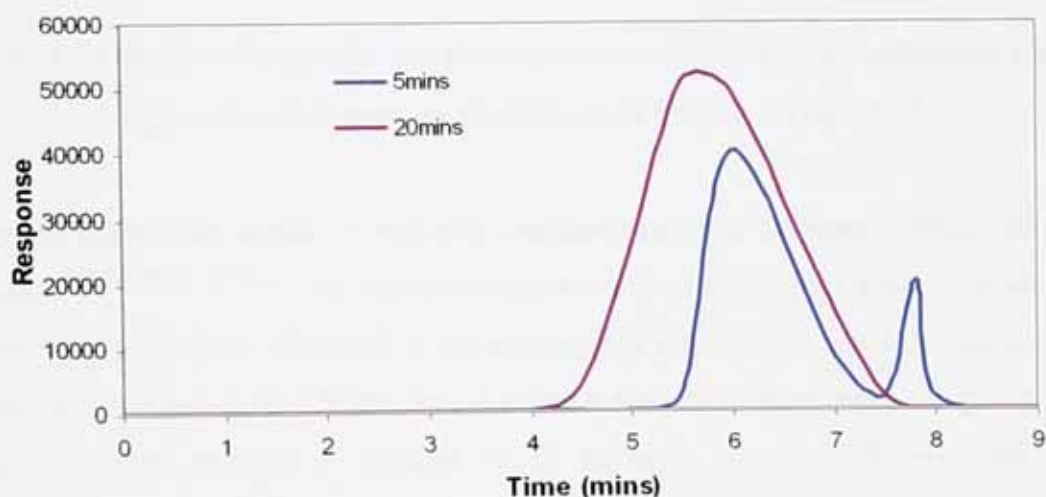


Fig 4.6 Gel permeation chromatograms of the reaction product of phloroglucinol with oxazolidine after 5 and 20 minutes

4.1.3.1.4 Influences of the reaction of phloroglucinol

4.1.3.1.4.1 Effects of pH

Under all the pH conditions for the phloroglucinol reaction, precipitates or turbidity are obtained immediately after addition of oxazolidine; the solution becomes cloudy followed by precipitation. In the case of formaldehyde, the solution at the beginning is clear, but becomes cloudy in 15-60 minutes, depending on the pH. For example, when the ratio of phloroglucinol to oxazolidine is 1:1, the reaction is finished in 20 minutes at pH 6.5 and 20°C; this is much faster than the formaldehyde reaction, which takes 60 minutes.

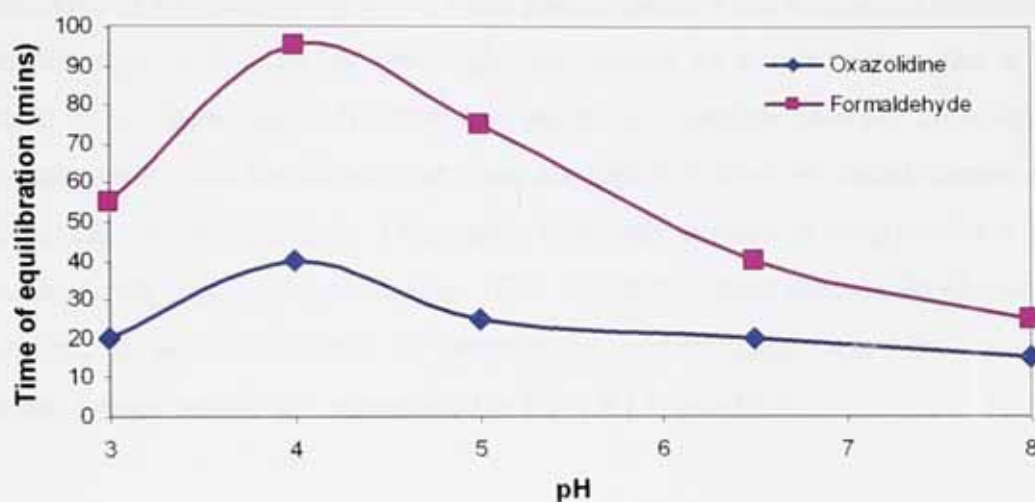


Fig. 4.7 Completion times for the phloroglucinol reactions with oxazolidine and formaldehyde (molar ratio of Ph:Ox or Ph:F is 1:1) at 20°C

It is well known that acidic or basic pH conditions catalyse the phenol-formaldehyde reaction^{209-210,216,217,222-223}; the lowest reaction rate is usually at pH 4.0-4.5. A similar phenomenon has been observed in the oxazolidine reactions: it takes 20 minutes to finish the reaction at pH 6.5, the time is extended to 40 minutes when the pH is 4.5, at pH 3.5 the reaction is finished in 25 minutes. No big difference for the oxazolidine reaction rate has been found when the pH is higher than 5.5. Both acidic and basic pH exhibit catalytic function in the oxazolidine reaction (Fig 4.7). Considering the differences between formaldehyde and oxazolidine: first, the reaction of oxazolidine can be conducted at pH 3.5 to 4.5, where formaldehyde shows low reactivity; second, the effect of pH is smaller for oxazolidine than

formaldehyde. That means oxazolidine can be used over a wider pH range, especially in neutral and weakly acidic conditions.

4.1.3.1.4.2 Effect of molar ratio (phenol:oxazolidine)

There are three reactive sites on phloroglucinol and there are two functional groups in ring-opened oxazolidine. Theoretically, the complete reaction ratio of phenol with oxazolidine should be 2:3: one mole of phloroglucinol can react with 1.5 moles oxazolidine if all the reactive sites on the phenol and the oxazolidine are cross-linked; but one phloroglucinol molecule can react with three oxazolidines if there is no cross-linking. From paper chromatography, the minimum reaction molar ratio of phloroglucinol to oxazolidine is 1:1, when phloroglucinol can be reacted completely. When the ratio is 2:1, all the phloroglucinol cannot be reacted even after a long reaction time. These results indicate that not all the reactive sites are cross-linked. Chromatography also has shown that there are high polymers produced immediately after mixing the two reactants. Oligomers were only present in solution for a short time, then only high polymer remains. This indicates, under the employed reaction conditions, it will be difficult to observe the intermediates. Some of the paper chromatography results are represented in Fig 4.8 (Appendix V).

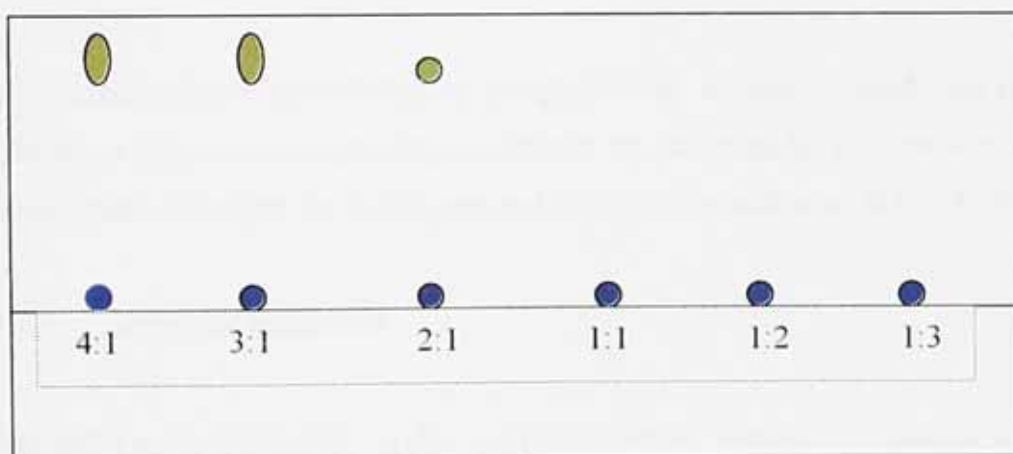


Fig 4.8 Representation of one dimensional paper chromatography of the reaction of phloroglucinol with oxazolidine (molar ratio from 4:1 to 1:3)

More oxazolidine can be consumed when excess oxazolidine is present in the reaction system. It was found that 2.05 moles of oxazolidine can be combined per mole of phloroglucinol when the ratio is 1:5. The value reduces to 1.60 moles per mole of phloroglucinol when the ratio is 1:3. No more oxazolidine is consumed when the ratio is higher than 1:5 (Fig 4.9). This is further proof that few intermediates are present in the final products, even when oxazolidine is in excess over phloroglucinol.

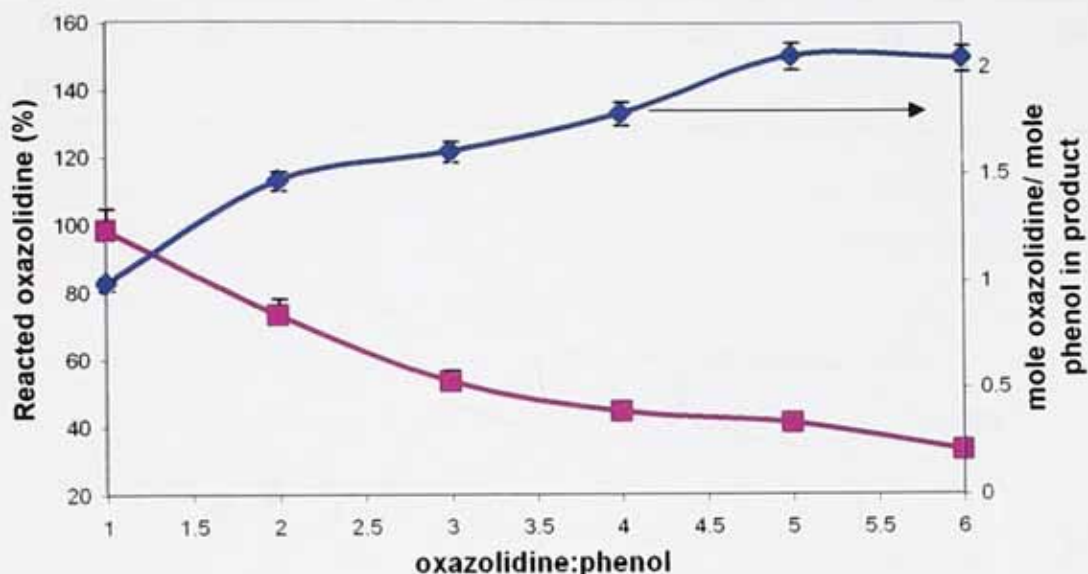


Fig 4.9 Consumption of oxazolidine by phloroglucinol (pH 4.5, 20°C) and stoichiometry of the product (n=3)

From the consumption of oxazolidine by phloroglucinol, we can conclude that most of the reactions sites on phloroglucinol molecules are occupied by the reaction. The product polymers should be three-dimensional structures as indicated in Fig 4.10.

4.1.3.1.4.3 Effect of metal ions

Freeman and Lewis found that oxides or hydroxides of alkaline or alkaline earth metals and some other metal salts, such as magnesium, calcium, zinc and aluminium, can be used to accelerate the condensation reaction of phenol with formaldehyde^{225,226,231,232}. Pizzi explained the mechanism of the reaction as similar to that of the formation of metallic acetylacetonate complexes^{138,232}; this involves

the formation of chelate rings between metal ions and phenols. The rate of metal exchange in solution and the instability of the complex formed determine the accelerating or inhibiting effect of the metal in the reaction.

Table 4.1 Effects of salts on the reaction of phloroglucinol with oxazolidine (molar ratio 1:1) at concentration 0.02M, pH 6.5 and 20°C, completion time of reaction.

	Ph	Ph+CaCl ₂	Ph+ZnCl ₂	Ph+NaCl	Ph+Na ₂ SO ₄	Ph+Borax
Time (mins)	20	14	17	20	19	40

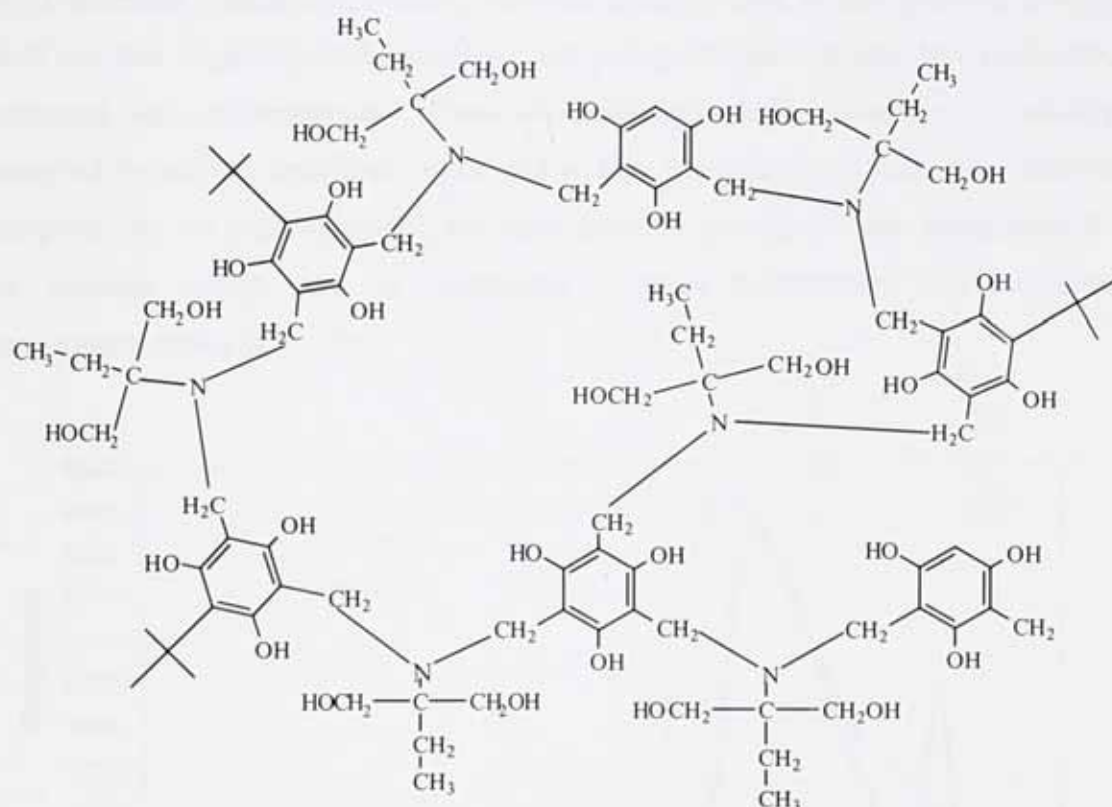


Fig 4.10 Indicative reaction product of phloroglucinol with oxazolidine

The most effective catalyst for phloroglucinol condensation with oxazolidine is calcium chloride, followed by zinc acetate: the complete reaction times are 10 and 14 minutes respectively, while the uncatalysed reaction time is 20 minutes. Not all

the salts catalyse this reaction: borax reduces the reaction rate, so that the completion time is prolonged to 40 minutes.

Sodium chloride and sodium sulphate were used to check the effect of neutral salt on the phloroglucinol-oxazolidine reaction process. Sodium salt has no effect on accelerating the condensation reaction. There will be some salting out effect because high molecular weight polymers will be produced in the reaction, resulting in more rapid precipitation.

4.1.3.1.4.4 Effect of temperature

Temperature has an important influence in phenol-formaldehyde resin production or wood adhesive production. Among different phenols used in this process, phenol itself has low reactivity and resorcinol and pyrogallol also exhibit low reactivity, compared with phloroglucinol. Phenol-formaldehyde resin production is usually catalysed by sodium hydroxide and cured at high temperature to make the reaction complete. As for phloroglucinol, the most reactive phenol towards formaldehyde, the reaction usually can be completed at room temperature and increased temperature shows little effect.

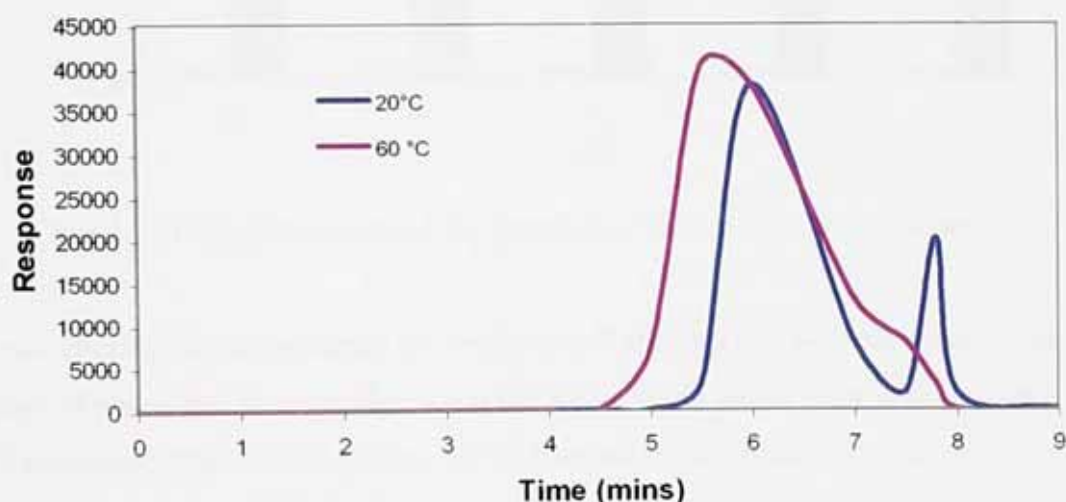


Fig 4.11 Gel permeation chromatograms of phloroglucinol-oxazolidine reaction product after 5 minutes at 20 or 60 °C

The same result is obtained from the oxazolidine reaction with phloroglucinol. From GPC, high polymers are produced more quickly when the temperature is increased to 40-60°C, which indicates the higher temperature enhances the polycondensation reaction (Fig 4.11), but the difference is not great due to the high reactivity of phloroglucinol with oxazolidine.

4.1.3.2 Reaction of resorcinol with oxazolidine

The reaction of resorcinol is much slower than phloroglucinol. When the molar ratio of resorcinol to oxazolidine is 1:2, it takes nearly 360 minutes to finish the reaction at room temperature and pH 5.0. Increasing the temperature to 40°C shortens this time to 130 minutes (Fig 4.12, 4.17). When the ratio is 1:1, the resorcinol is not reacted completely even after 10 days.

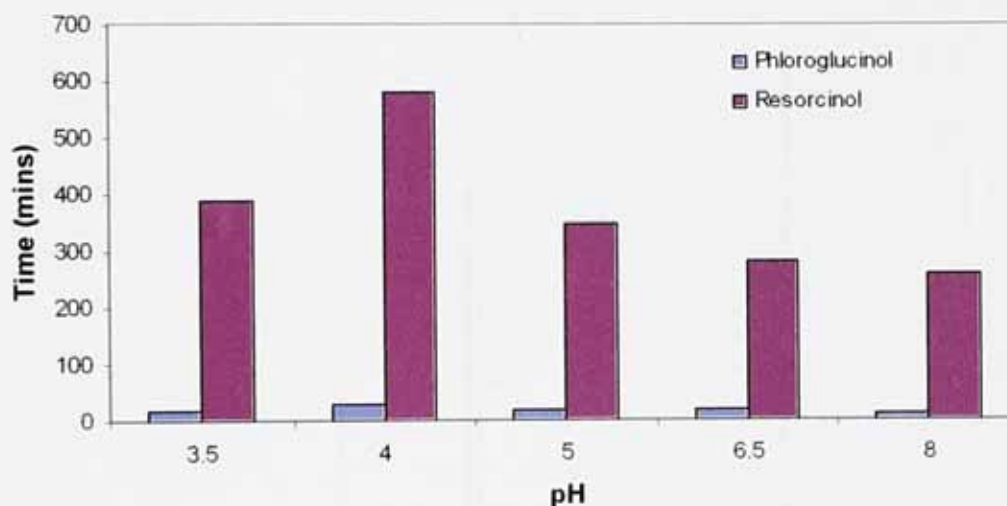


Fig 4.12 Completion time of the reaction of phenol with oxazolidine (1:2)

Less oxazolidine is consumed by resorcinol (Table 4.2). 0.94 mole oxazolidine per mole of resorcinol is consumed when the oxazolidine molar offer is three times that of resorcinol, the value increases to 1.34 moles oxazolidine per mole of resorcinol when the ratio is 1:5. From the consumption of oxazolidine by resorcinol, the most likely product is a linear structure with branching. There is evidence that resorcinol can consume no more than 0.9 moles formaldehyde per mole resorcinol, even at pH 7.5, and it may take several hundred hours to finish the reaction, unless the

temperature is increased. This shows oxazolidine is much more reactive than formaldehyde towards resorcinol under similar conditions.

Table 4.2 Oxazolidine reaction with phenols at pH 8 and 60°C.

Phenol	Ratio phenol:ox	Oxazolidine reacted (%)	Mole Ox/mole phenol in product
Phloroglucinol	1:5	44	2.16
	1:3	68	2.03
Resorcinol	1:5	25	1.27
	1:3	31	0.93
Pyrogallol	1:5	16	0.82
	1:3	24	0.73

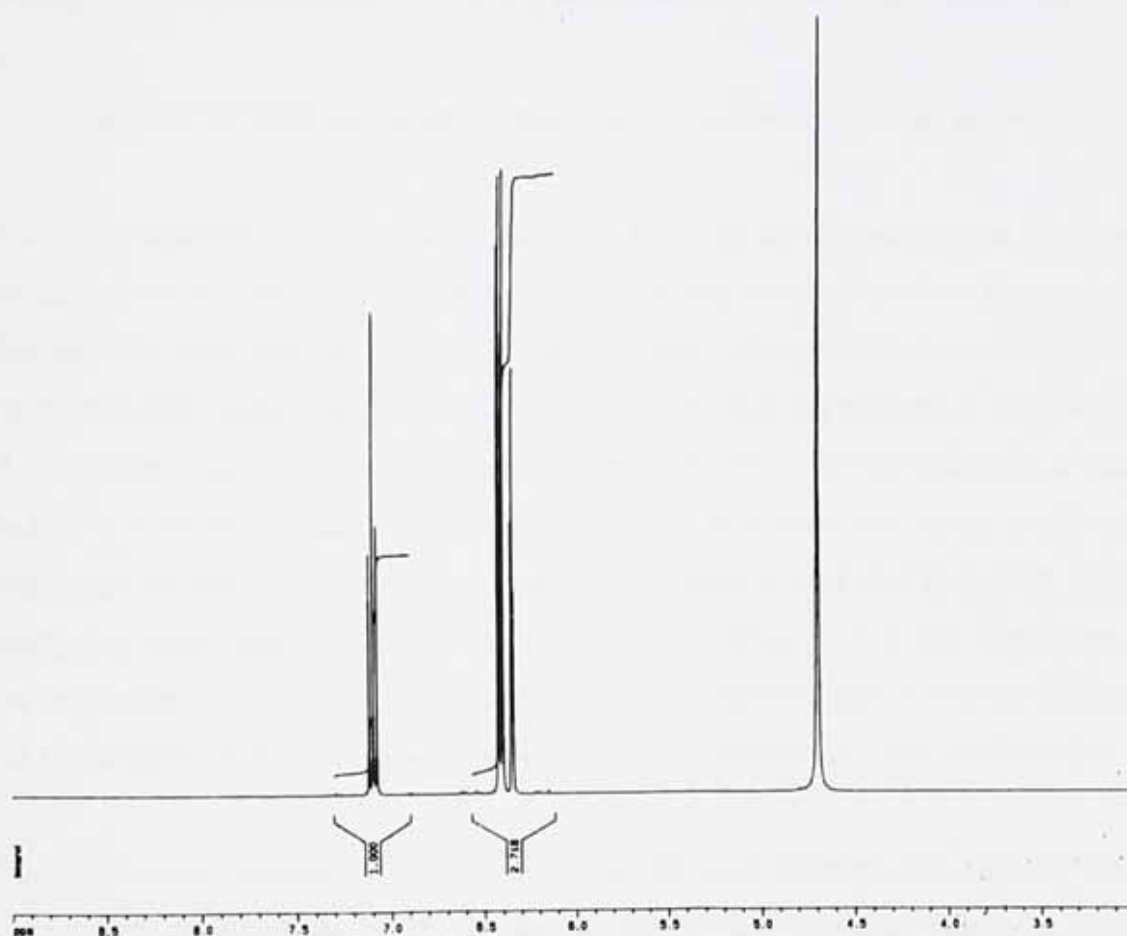


Fig 4.13 ¹H-NMR spectrum of resorcinol

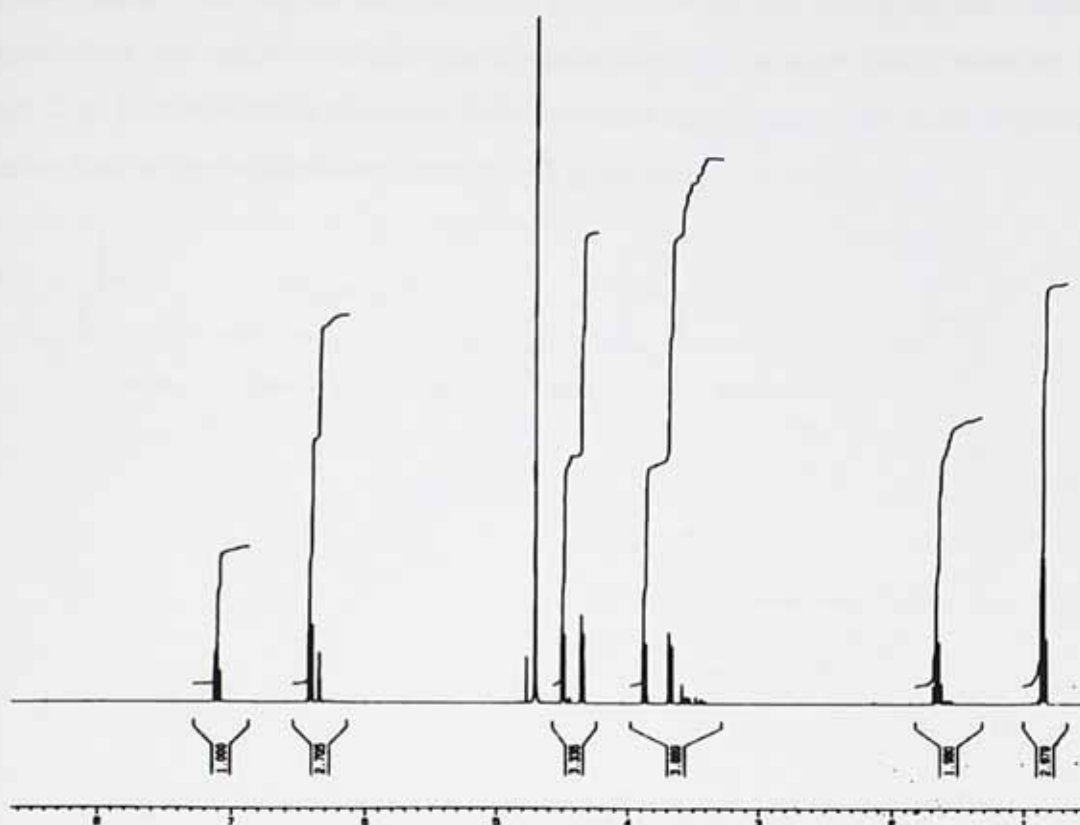


Fig 4.14 ^1H -NMR spectrum of resorcinol + oxazolidine reaction product

From the molecular structural characteristics, there are three possible reactive sites on resorcinol, H-2, H-4 and H-6, but it is unlikely that H-2 will react or form cross-linking with other sites because of the steric effect. After studying the reaction of resorcinol with formaldehyde, Pizzi^{138,209} pointed out that the proportion of H-4 and H-6 linkages relative to H-2 linkages is of the order 10:1 and the reactivity of H-4 and H-6 is about 5 times that of H-2. H-4 and H-6 have the same reactivity, confirmed by our ^1H -NMR results, in which the peak of H-4 and H-6 (6.53 ppm) disappears more quickly than H-2 during the reaction (Figs 4.13, 4.14). Considering the molecular size of oxazolidine, a steric effect is expected and it will be difficult for H-2 to contribute to the cross-linking process, in competition with H-4 and H-6.

Due to the high reactivity of oxazolidine, the H-2 peak does become smaller with time, which indicates the NCH_2 - group can also occupy this site. This is in good agreement with the calculated oxazolidine consumption. It is not clear whether the

cross-linking will happen between C-2 and C 4-6 or not, but from the different reactivities, we might conclude that cross-linking will be more likely between C-4 and C-6. This will make the cross-linked product more linear, with some branching, rather than a three-dimensional network (Fig 4.15).

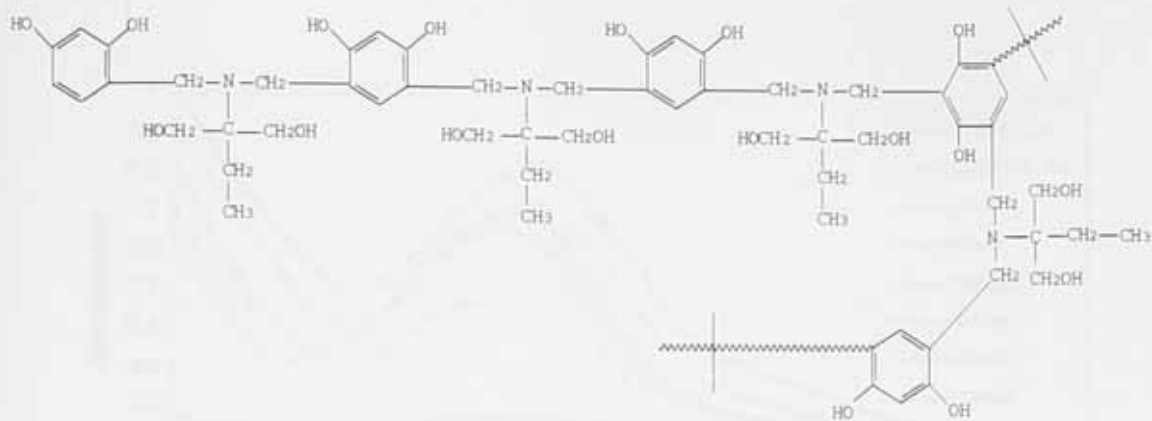


Fig 4.15 Indicative reaction product of resorcinol with oxazolidine

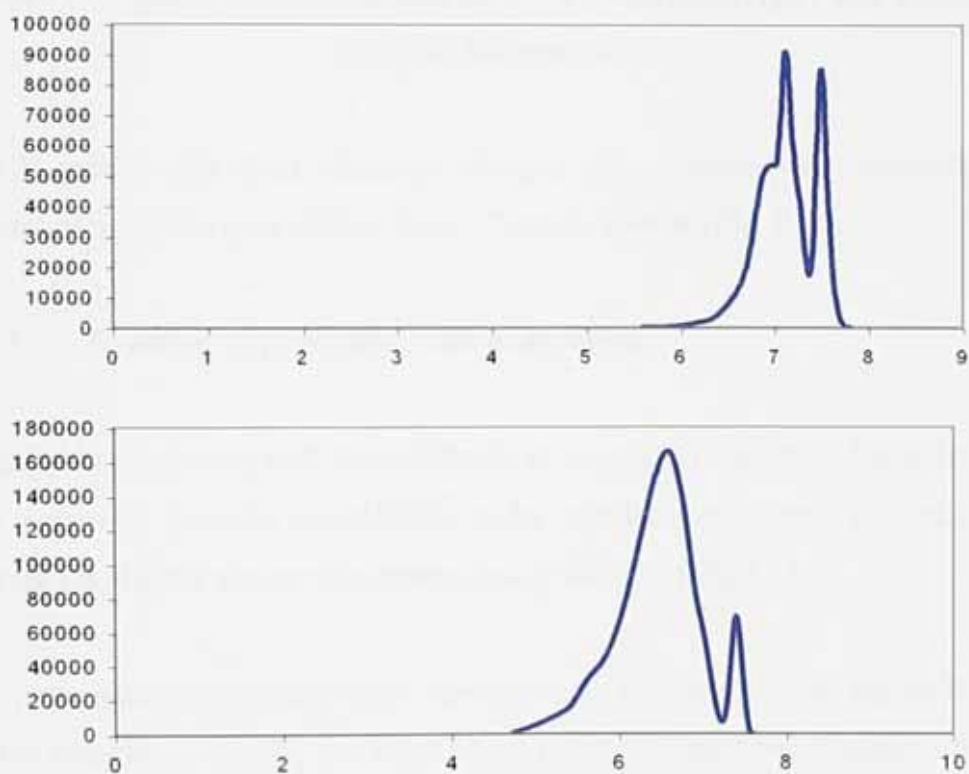


Fig 4.16 Gel permeation chromatograms of resorcinol reaction product with oxazolidine after 20 minutes (upper) and 300 minutes (lower).

Calcium and zinc salts show similar catalytic effects for phloroglucinol or resorcinol with oxazolidine. GPC results show the reaction is slow compared with phloroglucinol: most of the resorcinol is polymerised to high polymers after 6 hours at room temperature, although some high polymers are produced after just 10-20 minutes, when most of the resorcinol is still in solution (Fig 4.16).

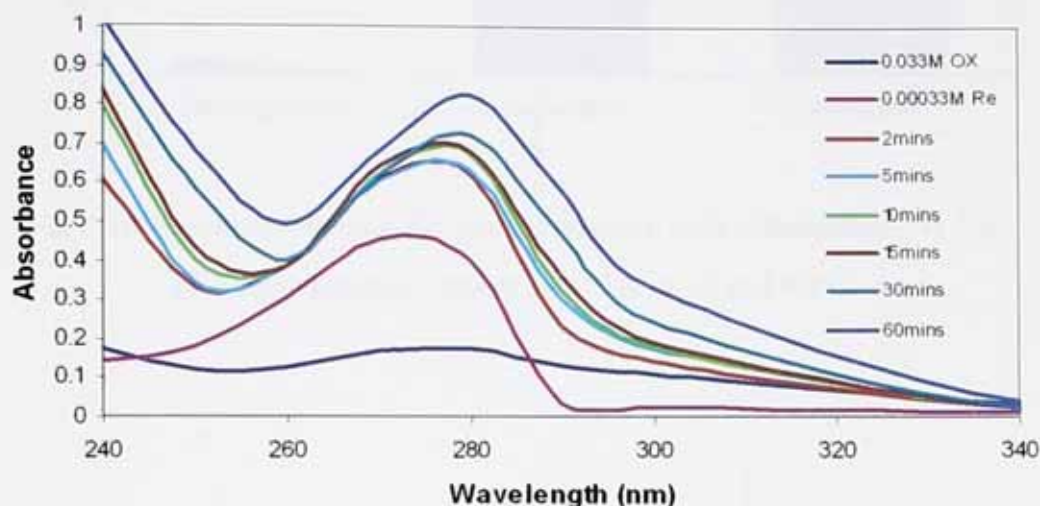


Fig 4.17 UV spectrum of the reaction products of resorcinol (Re) with oxazolidine (OX) at different times

The UV spectra also show chemical changes after reaction with oxazolidine; the maximum absorption peak moves from 273nm to 284nm (Fig 4.17).

4.1.3.3 Reaction of pyrogallol with oxazolidine

Pyrogallol hardly reacts with formaldehyde at neutral pH and 20°C, but it does show some reactivity towards oxazolidine under similar conditions. The reaction of pyrogallol is slightly slower than resorcinol at 40-60°C (Fig 4.18).

From paper chromatography, some new products are created with higher R_f values than the original pyrogallol, but most of the pyrogallol molecules remain unreacted. After 3 hours at pH 8.0 and 60°C, most of the pyrogallol is cross-linked into high polymers: 0.82 moles oxazolidine are consumed by each mole of pyrogallol when the molar ratio of pyrogallol with oxazolidine is 1:5 (Table 4.2).

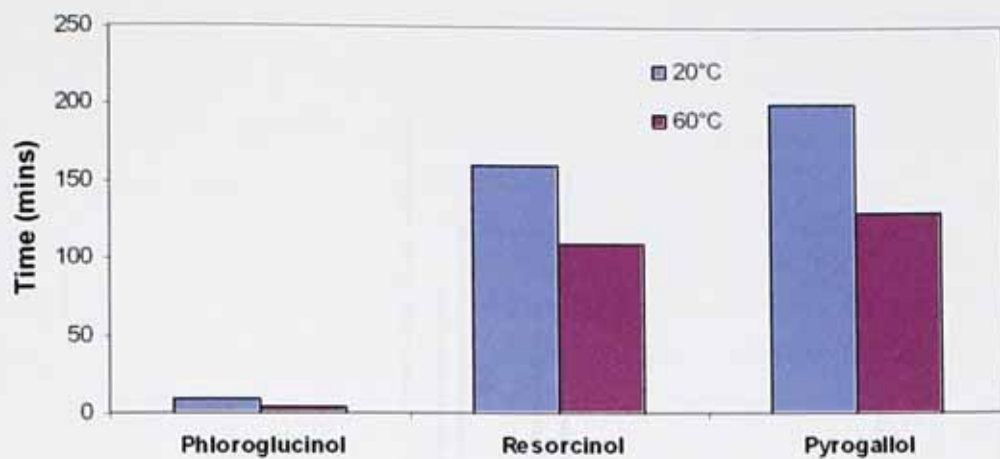


Fig 4.18 Equilibration time for phenol reaction with oxazolidine pH 5.5, phenol:oxazolidine molar ratio 1:2 at 40 and 60°C.

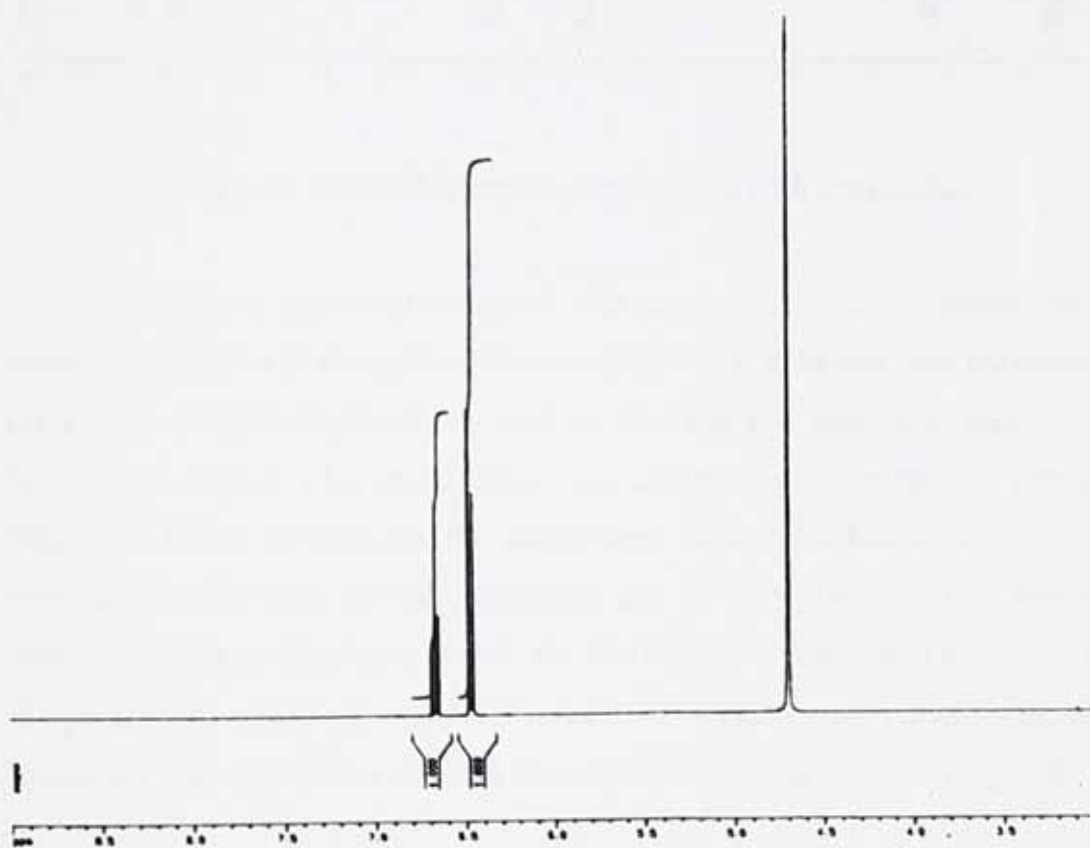


Fig 4.19 ¹H-NMR spectrum of pyrogallol

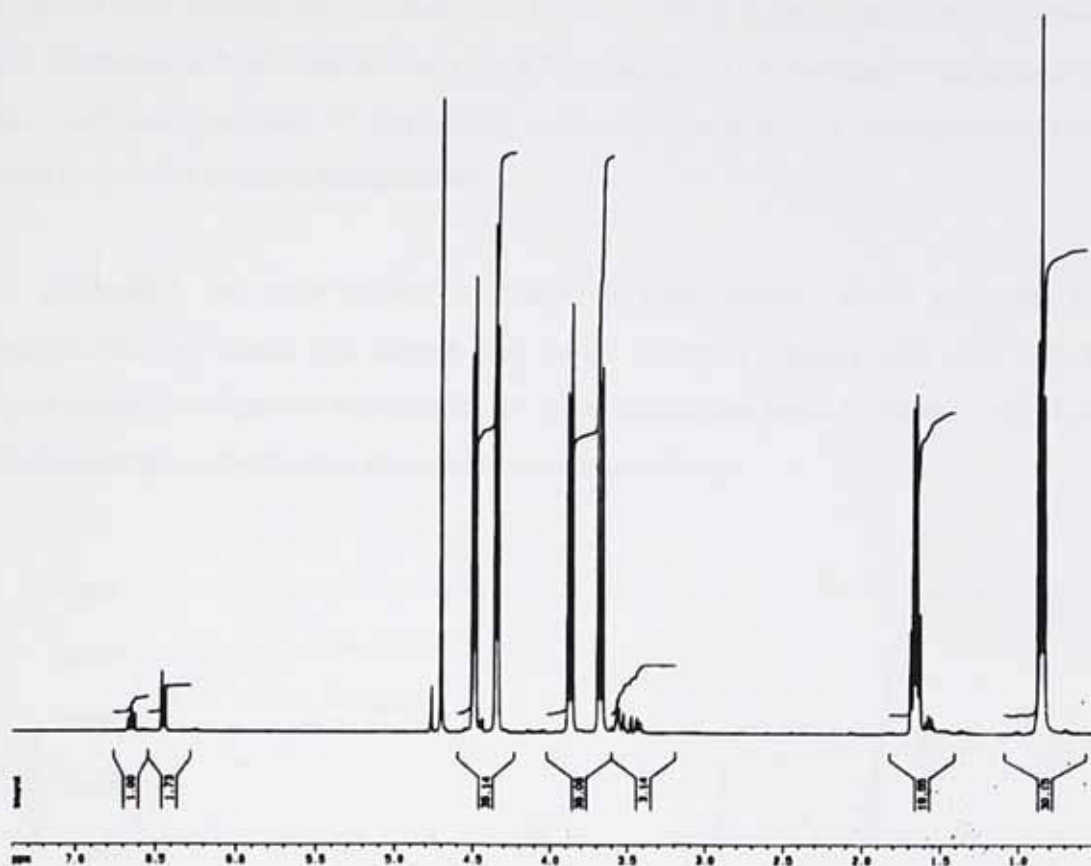


Fig 4.20 $^1\text{H-NMR}$ spectrum of pyrogallol with oxazolidine

Less oxazolidine is consumed compared with resorcinol, probably because there are fewer reaction sites in pyrogallol. When oxazolidine is in excess over pyrogallol, at pH 8.0 and 60°C , the pyrogallol is used up. This has also been confirmed by GPC. In UV spectroscopy, a big shift ($>20\text{nm}$) was observed in the reaction of pyrogallol with oxazolidine. It indicates the substituents have a marked influence on the molecular structures of pyrogallol products and strongly affect the UV absorption curve. $^1\text{H-NMR}$ studies have shown the H-4 and H-6 peaks at δ 6.48-6.49 ppm disappear at the same rate, while H-5 at δ 6.65-6.70 ppm remains unchanged, which indicates a linear product is obtained from the condensation reaction (Fig 4.20).

Compared with phloroglucinol and resorcinol, pyrogallol produces fewer high polymers at room temperature after 6 hours. For phloroglucinol, high polymers are produced immediately after mixing the reactants; for resorcinol, polymers are

produced after mixing the reactants for 15-30 minutes; for pyrogallol, precipitation only happened at high temperature (Fig 4.21). Increased temperature is important for resorcinol and especially for pyrogallol, while the reaction with phloroglucinol may be carried out at ambient temperature.

For pyrogallol, the most effective catalyst is zinc acetate, which increases the reaction rate 5-6 times: one reason may be the complex formed with zinc ions, to create stable coordination chemicals. All other metal ions seem to have no effect on the condensation reaction of pyrogallol with oxazolidine.

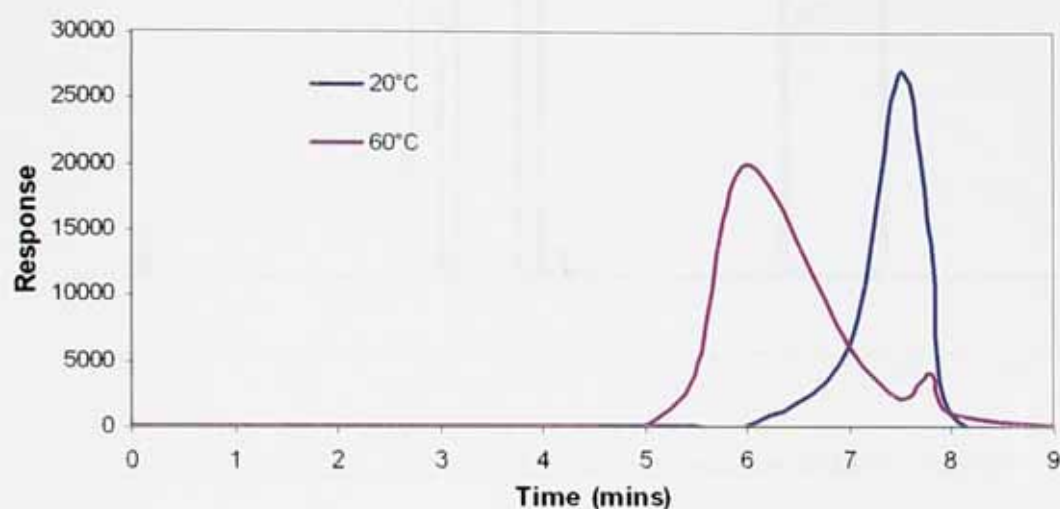


Fig 4.21 Gel permeation chromatograms of pyrogallol + oxazolidine reaction products at reactant ratio 1:3 and 20 or 60°C

4.1.3.4 Reaction of catechol and phenol

Phenol reacts with oxazolidine in a similar way to formaldehyde; there is little change in the reaction at room temperature, even at pH 8.0. The reaction at pH 8.0 can be conducted at 40-60°C for oxazolidine, but for formaldehyde 80°C is required.

No significant reaction was observed for catechol and oxazolidine under similar conditions. The *ortho*- and *para*- sites of one hydroxyl group are another hydroxyl group's *meta*- sites, which makes the electrophilic aromatic substitution reaction difficult^{203,204}. ¹H-NMR spectra showed no change after 6 hours after addition of oxazolidine at ambient temperature and pH from 4.5 to 8.0 (Fig 4.22). GPC also

gave similar results; most of the catechol remained unchanged even after 24 hours at room temperature, except for some oxidation. The reaction can only be conducted under conditions of high temperature or high pH.

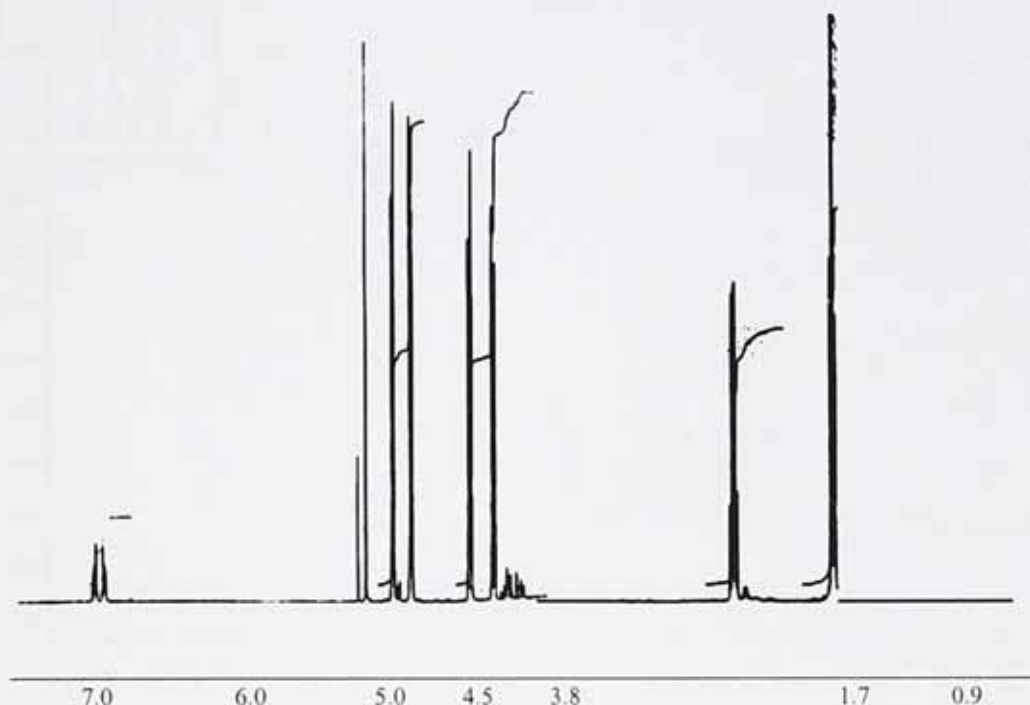


Fig 4.22 ^1H -NMR spectrum for catechol reaction with oxazolidine

4.1.3.5 Discussion

In the reaction mechanism studies of phenols with oxazolidine, it has been difficult to separate intermediate products to explain the reaction pathway, due to the high reactivity and the characteristics of the process. From paper chromatography, it was observed that the intermediates were present only for a very short time, then were converted to high polymers. Therefore, to slow it down, the reaction was carried out in methanol and the reaction process was followed by GC-MS (Figs. 4.23-4.25). From the retention time and molecular weight analysis, it is possible to propose the pathway of this reaction (Fig 4.26).

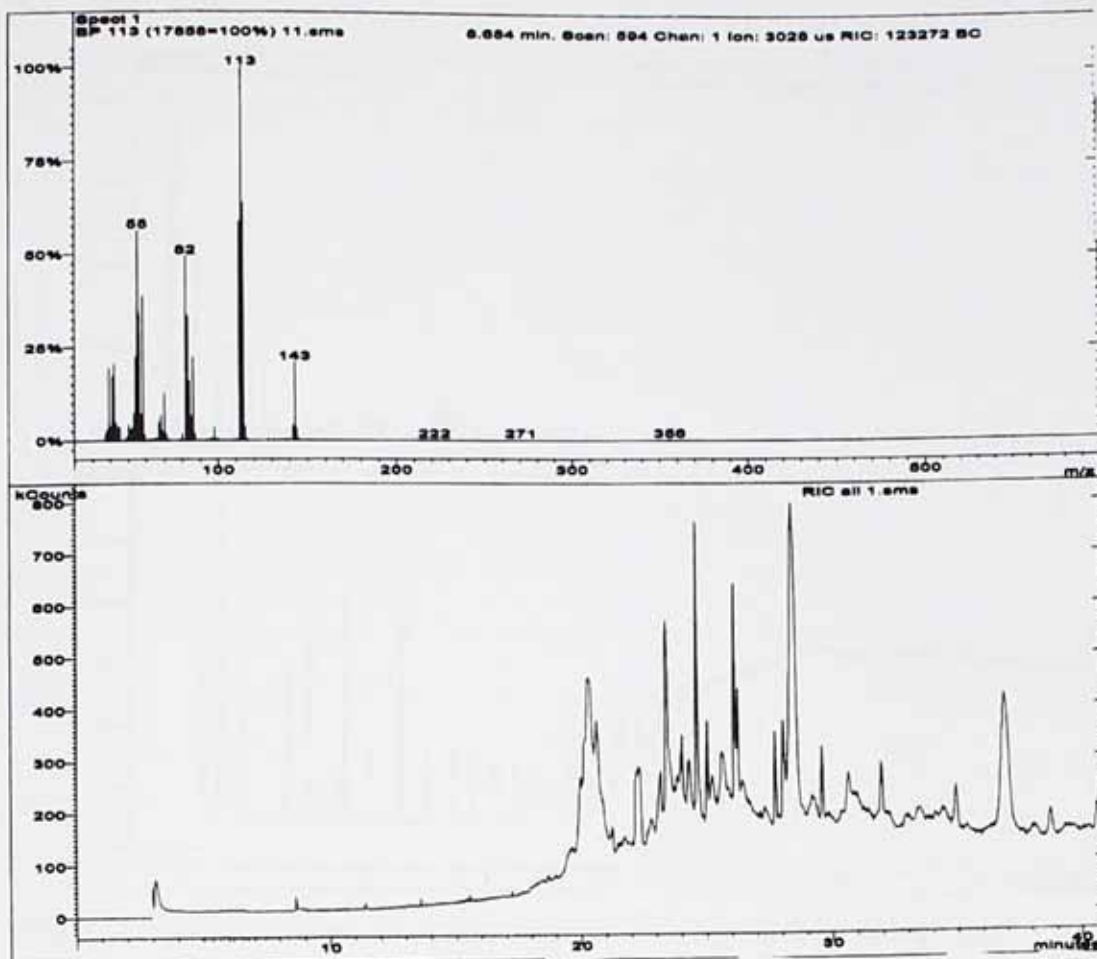


Fig 4.23 Gas chromatogram of phloroglucinol with oxazolidine (molar ratio 1:1) in methanol

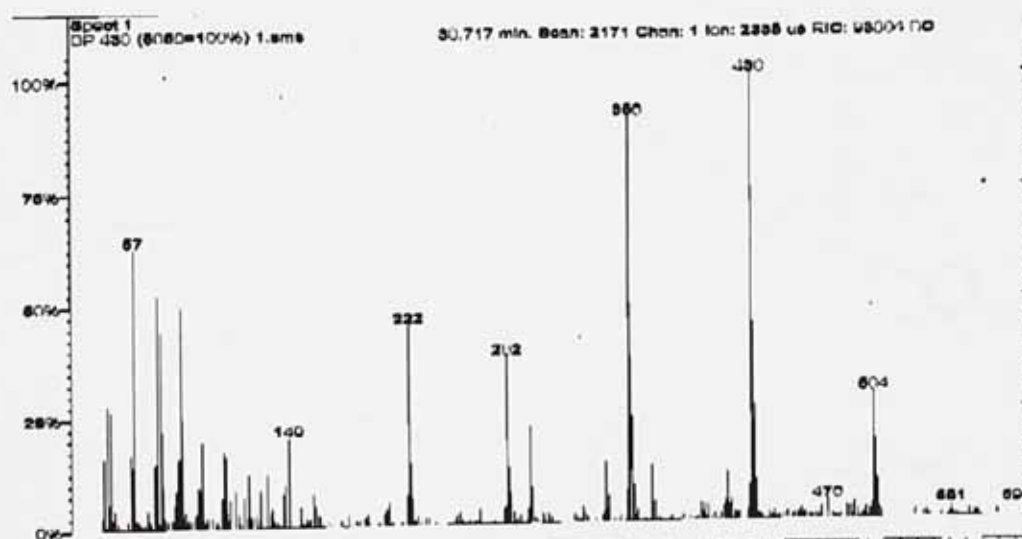


Fig 4.24 Mass spectrum of phloroglucinol with oxazolidine at molar ratio 1:1 in methanol

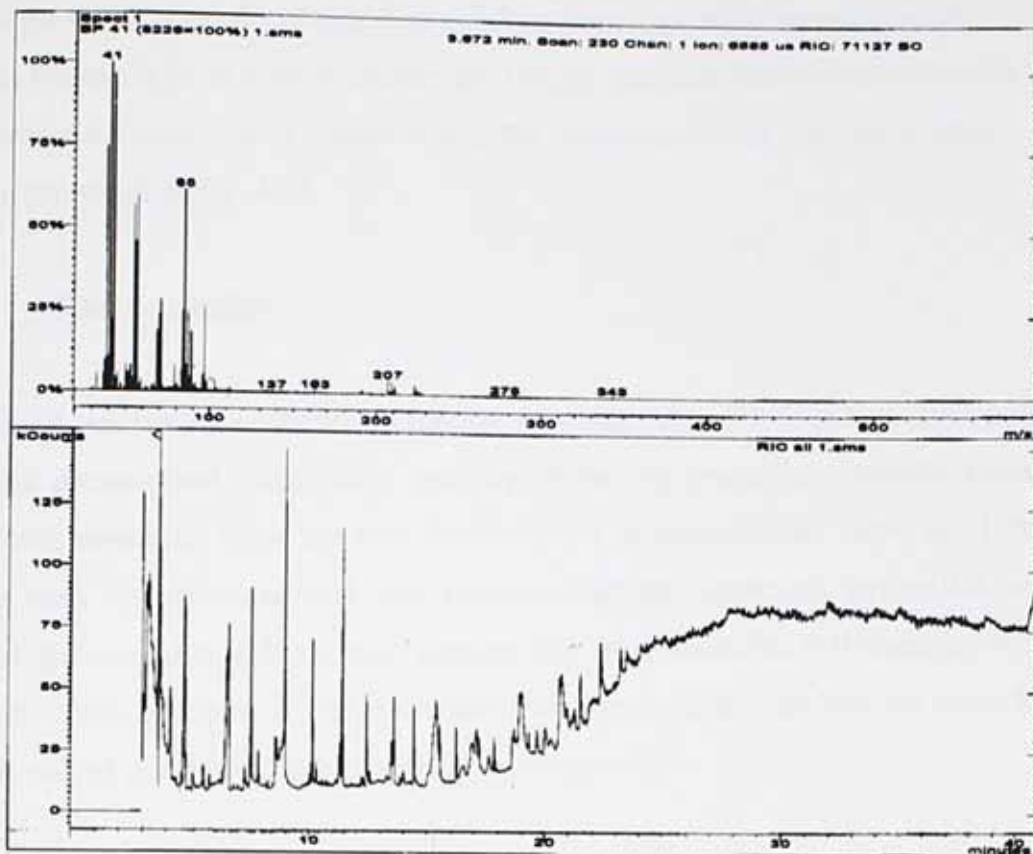


Fig 4.25 Gas chromatogram of resorcinol with oxazolidine at molar ratio 1:2 in methanol

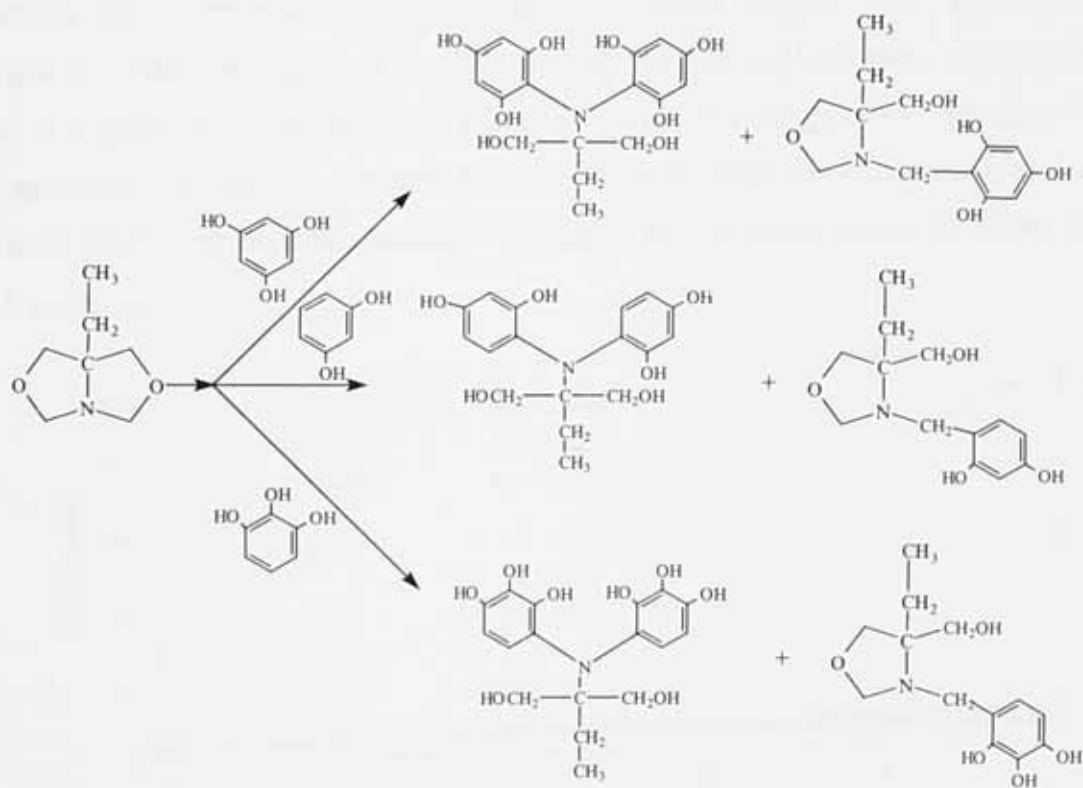


Fig 4.26 Some of the intermediates in the reaction of phenols with oxazolidine

Some of the GC-MS results of phloroglucinol or resorcinol with oxazolidine are shown in Figures 4.23 to 4.25, it can be seen that the reaction products are complex. By analysis each peak's mass spectroscopy, the possible reaction reaction process has been proposed in Fig. 4.26.

4.1.3.6 Kinetic studies

The ratio of reactants was chosen so that the concentration of the reactive sites, both on model phenols and oxazolidine, was equimolar, by assuming complete cross-linking and assuming there are two reactive sites in oxazolidine. There are three reactive sites on phloroglucinol, two reactive sites on resorcinol, pyrogallol and catechol, then in cross-linking, each reactive site of oxazolidine will combine with one reactive site on phenol. The concentration of oxazolidine was kept the same for each phenol, to provide a consistent basis for comparison.

In this work, the kinetics of the reaction of phenols with oxazolidine was measured by $^1\text{H-NMR}$: it was found that the reaction is second order in the initial stage, by plotting the reciprocal of concentration of phenol reactive site against time (Fig.4.27). After the high polymer is produced, the reaction becomes more complex and it is difficult to determine the reaction order. After the phenol's reaction with oxazolidine, the reactive benzene hydrogen is substituted by oxazolidine and the related NMR peak becomes smaller with time. The results are shown in Tables 4.3-4.6 (Appendix VI).

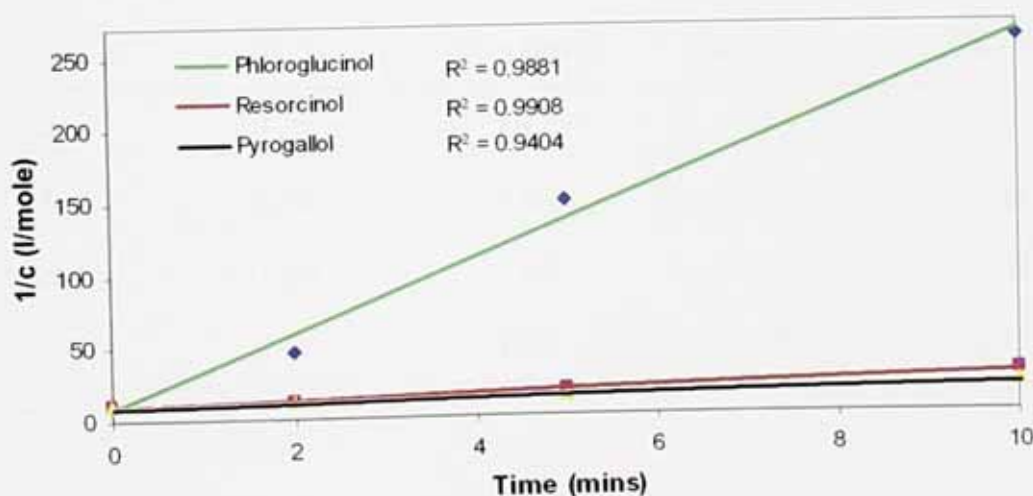


Fig 4.27 Reaction of phenols with oxazolidine

Table 4.3 Integrals of ¹H-NMR peaks of phloroglucinol and oxazolidine in D₂O

Time (min)	5.9ppm (Ph)	4.3-4.5ppm (H-2, 8 Ox)	1.7ppm (CH ₂ Ox)	0.9ppm (CH ₃ Ox)
0	1.00	-----	-----	-----
2	1.00	15.1	8.3	12.4
5	1.00	32.1	17.7	26.9
10	1.00	63.6	35.1	53.8
15	1.00	264	152	226

Table 4.4 Integrals of ¹H-NMR peaks of resorcinol and oxazolidine in D₂O

Time (min)	7.05ppm (H-5)	6.40ppm (H-4 and 6)	6.32ppm (H-2)	1.65ppm (CH ₂ Ox)
0	1.00	1.88	0.91	-----
5	1.00	1.76	0.90	1.97
10	1.00	1.68	0.89	1.98
25	1.00	1.55	0.82	1.99

Table 4.5 Integrals of ¹H-NMR peaks of pyrogallol and oxazolidine in D₂O

Time (min)	6.65ppm (H-5)	5.95ppm (H-4 and 6)	4.3-4.5ppm (H-2, 8 Ox)	1.65ppm (CH ₂ Ox)
0	1.00	1.87	-----	-----
5	1.00	1.83	12.69	7.25
15	1.00	1.80	14.26	8.09
25	1.00	1.78	15.21	8.77

Table 4.6 Reaction rates (mole ratio phenol to aldehyde agent 1:1, pH 6.0, 20°C)

Compounds	k (M ⁻¹ s ⁻¹)	
	Oxazolidine	Formaldehyde
Phloroglucinol	0.433	0.234
Resorcinol	0.024	0.008
Pyrogallol	0.018	—*
Catechol	<0.001	<0.001

* No literature data available

From Table 4.6, formaldehyde and oxazolidine exhibit the same reactivity sequence for the four phenols. Phloroglucinol has the highest reaction rate; the reaction rate with oxazolidine is 19 and 24 times that of resorcinol and pyrogallol respectively. Catechol does not show any reactivity towards either formaldehyde or oxazolidine.

4.1.4 Summary

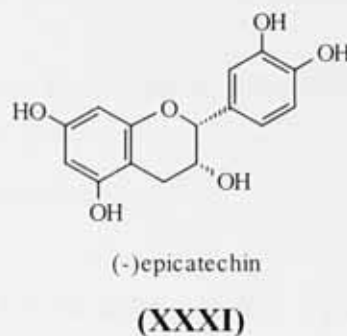
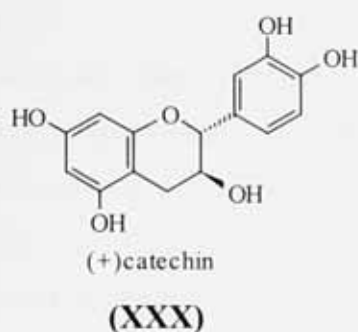
Compared with formaldehyde, oxazolidine shows similar cross-linking characteristics, but is more reactive towards phenols, which indicates a different mechanism. Unlike the formaldehyde reaction, temperature is a more important parameter in oxazolidine cross-linking than pH, especially for the resorcinol and pyrogallol reaction. For pyrogallol, formaldehyde is unreactive, but with oxazolidine the reaction proceeds at elevated temperature. This indicates the pyrogallol B-ring of proanthocyanidins, such as prorobinetinidin and prodelphinidin, can react with oxazolidine under similar conditions. Oxazolidine shows reactive properties over a wider pH range, from 5.0 to 9.0. At pH 6.5, the reaction rate of oxazolidine with phloroglucinol is 2-3 times that of formaldehyde. As with formaldehyde, oxazolidine also shows lowest reactivity at pH 4.0-4.5, but the reaction still proceeds. Due to the different mechanism mentioned above, at medium pH the first step of the formaldehyde reaction is difficult, which slows down the whole reaction system. There is only one step for oxazolidine to combine with the nucleophilic site

on phenols and it is not significantly affected by pH. This makes oxazolidine more suitable for the reaction with phenols. Because of the higher reactivity and the polymerised nature of formaldehyde, the consumption of oxazolidine per mole of phenol is higher than formaldehyde. For phloroglucinol, at pH 6.5 and 20°C, the consumption of oxazolidine is 1.4 times that of formaldehyde, which ensures more complete cross-linking.

Part 4.2 Catechins and gallocatechins study

4.2.1 Introduction

Catechins and gallocatechins are the most important monomer units for proanthocyanidins^{63,66}, having a phloroglucinol pattern A-ring with one hydroxyl group engaged in the heterocyclic bond: (+)catechin (**XXX**, **C**), (-)epicatechin (**XXXI**, **EC**) have a phloroglucinol pattern A-ring and a catechol B-ring; (+)gallocatechin (**XIII**, **GC**) and (-)epigallocatechin (**IX**, **EGC**) have a pyrogallol B-ring.



In the 1950's, Hillis and Urbach studied the reaction of catechin with formaldehyde^{216,217}, but the kinetics of this reaction were not elucidated until the 1980's by Kiatgrajai¹³⁷ and Porter²²². The reaction was found to be second order.

In Section 4.1, the cross-linking reaction between simple phenols and oxazolidine was investigated and the results showed that oxazolidine has similar electrophilic properties, but higher activity than formaldehyde. Among the simple phenols, there is a reactivity sequence towards aldehydic agents:

phloroglucinol >> resorcinol > pyrogallol > phenol > catechol.

Composed of different simple phenols in their structures, hydroxyflavan-3-ols have unique characteristics. Different hydroxyl substitution of the A-ring and the B-ring

endows the flavans with different nucleophilic properties, especially for the A-ring. For a phloroglucinol pattern A-ring, such as in prodelphinidin, there is evidence that the favoured electrophilic substitution site is C-8, while for a resorcinol pattern A-ring, such as in profisetinidin, the more reactive site is C-6.

Covington and Shi have studied the reaction chemistry between polyphenol and oxazolidine^{5, 30-31}: they pointed out that a pyrogallol B-ring appears to aid the cross-linking reaction substantially. The model compound study showed a catechol B-ring does not take part in the reaction, which is in accordance with the known reactions of simple phenols. In order to elucidate the reaction, we need to find the answers to the following questions:

- Concerning the reaction sites on catechin and gallocatechin, is the B-ring reactive or not?
- What are the effects of the heterocyclic C-ring, the conformation of the C-3 hydroxyl group and the presence of a gallate group on the reaction?
- How may the reaction mechanism and kinetics be defined?

The application of separate models for the A-ring and the B-ring makes it possible to find the relative reactivity of each part of the flavonol monomer molecule. Considering that the basic structure consists predominantly of a phloroglucinol or resorcinol A-ring linked to either a pyrogallol or catechol B-ring, the strong nucleophilic A-ring and weak nucleophilic B-ring are present at the same time and in the ratio of 1:1. It is of interest to know how the cross-linking reaction with oxazolidine proceeds in this mixed system.

In this study, mixtures of different phenols were used as models for the compounds, in which the two phenols are represented as the A-ring and B-ring respectively.

(phloroglucinol + catechol) as the model of **catechins**;

(phloroglucinol + pyrogallol) as the model of **gallocatechins**;

(resorcinol + catechol) as the model of **fisetinidols**;

(resorcinol + pyrogallol) as the model of **robinetinidols**.

There is more steric hindrance and fewer reactive sites in proanthocyanidin monomers: the C-4a is occupied by one alkyl group which results in one reactive site less in the A-ring; the presence of 1'-alkyl group and the C-ring result in steric hindrance on the A-ring and B-ring. The model system makes it possible to check the two rings separately. After studying the reaction of catechin or gallic catechin with oxazolidine directly, the influence of different conformations of the A-, B- and C-rings on this reaction will be considered, then a better understanding about the reaction of these proanthocyanidin monomers with cross-linking reagents may be obtained.

4.2.2 Experimental

4.2.2.1 Materials

Analytical grade phloroglucinol, pyrogallol, resorcinol and catechol were obtained from Sigma (UK) and used as received. Catechin (C) and epicatechin (EC) were obtained from Sigma (UK) and examined by paper chromatography and reverse phase-high performance liquid chromatography (RP-HPLC) to confirm the purity (>95%) of these products and they were used without further purification. Gallic catechin (GC) and epigallocatechin (EGC) from green tea extract were separated by a Sephadex LH-20 column, eluted with ethanol. The eluents for GC and EGC were combined and freeze-dried, light yellow powders were obtained and the purity (>95%) was checked with paper chromatography and HPLC. Epigallocatechin-gallate was received from Prof. E. Haslam (University of Sheffield, UK) as a gift. The purity (>95%) was checked by paper chromatography and HPLC before use. Oxazolidine was prepared as previously discussed in Chapter 3; the purity was checked by paper chromatography, ¹H-NMR and GC-MS, as described in Chapter 3.

4.2.2.2 Preparation of stock solution

0.1mol/l solution of phenol and 0.05mol/l of flavone monomer were made by dissolving a known weight of phenol in 10% acetone or hot water and kept in a

brown bottle out of direct light. Oxazolidine was diluted to 1mol/l with sodium acetate buffer solution (pH 6.0) before adding to the phenol solution.

4.2.2.3 Reaction procedure

Reactions of model compounds were made either in 10% aqueous acetone solutions or water at 0.1mol/l concentration, at initial pH 4.0, 5.5 and 6.5 and ambient temperature ($20.0 \pm 1.0^\circ\text{C}$). Then oxazolidine was added at molar ratio of 2:1, 1:1 or 1:2 oxazolidine:total phenol. The literature and preliminary experiments showed these conditions were suitable for kinetic studies. The presence of some organic solvent in the system slows down the reaction rate and stabilises the condensation reaction, as reported by Roux *et al.*²²⁴.

4.2.2.4 Effects of electrolytes

Based on the studies in Section 4.1, zinc acetate, barium acetate were studied as described in Section 4.1.

4.2.2.5 UV-Vis spectroscopy

The monomers were dissolved in 10% aqueous acetone at a concentration of 0.05mol/l. The same method was employed as described in Section 4.1.

4.2.2.6 ¹H-NMR spectroscopy

The same method was employed as described in Section 4.1, except to use a lower phenol concentration, 0.05mol/l instead of 0.1mol/l.

4.2.2.7 Kinetic studies

According to the di-functional property of ring-opened oxazolidine and the two reactive sites on the catechin or gallicocatechin A-ring, the kinetic studies were carried out with both reactants at the same concentration, 0.05mol/l. The reaction

order was determined by plotting the reciprocal of catechin concentration versus time. The reaction rate constant was calculated from the $^1\text{H-NMR}$ integral data of corresponding peaks.

4.2.3 Results and discussion

4.2.3.1 Reaction of phenol mixture with oxazolidine

4.2.3.1.1 Chromatography investigation

The reaction was followed by paper chromatography, to examine the amount of each phenol left in the solution (Fig 4.28). Indicative chromatograms are shown in Fig 4.28.

In the model for gallocatechins, the reaction of phloroglucinol and pyrogallol mixture with oxazolidine demonstrated that there are some competitive effects and a synergistic effect present at the same time in the system. Both phenols react by cross-linking at room temperature and pH 7.0. Some high polymer precipitation appeared soon after the addition of oxazolidine, free phloroglucinol (R_f 0.65) disappeared in 10 minutes and pyrogallol (R_f 0.73) disappeared in 20 minutes when some intermediates were formed and then the reaction equilibrated after 50 minutes. In the simple pyrogallol system, the reaction was not equilibrated even after 400 minutes (Table 4.7). This indicated that pyrogallol reacts with oxazolidine much faster in the mixture system than by itself; similarly, the reaction of phloroglucinol slows down.

Table 4.7 Equilibration time for phenols react with oxazolidine (mins)

	Phloroglucinol	Pyrogallol	Resorcinol	Catechol
Phloroglucinol	10	50	>600	>600
Pyrogallol	50	400	320	>600
Resorcinol	>600	320	280	>600
Catechol	>600	>600	>600	>600

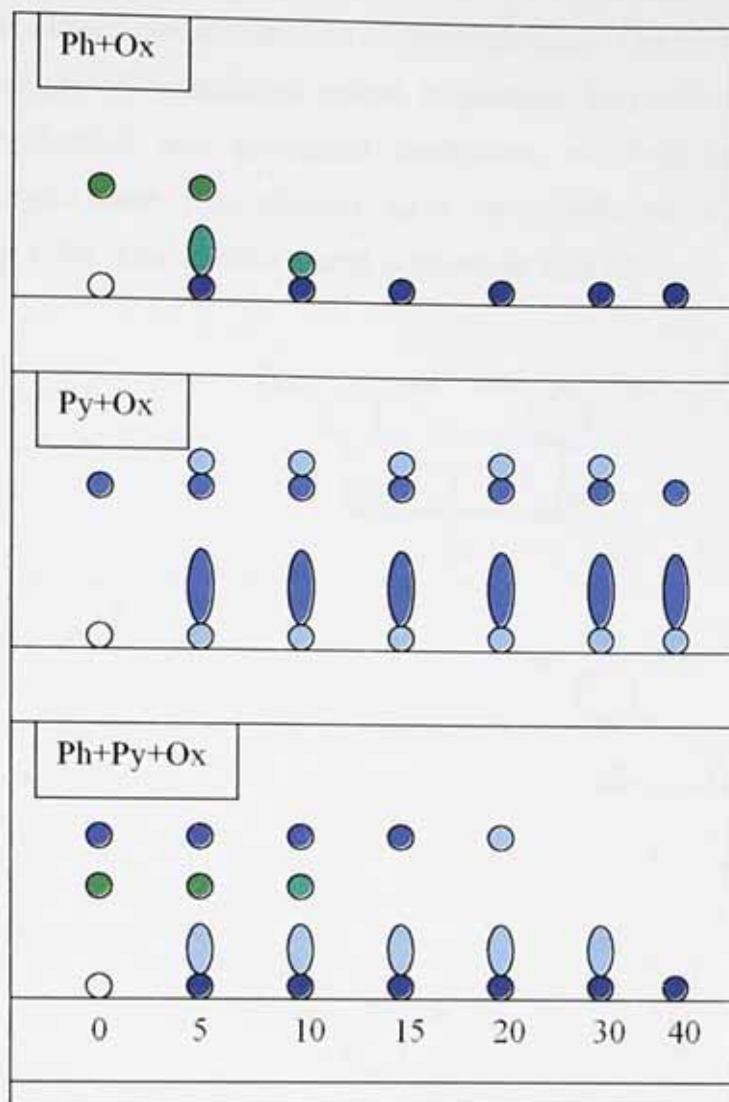


Fig 4.28 Representation of paper chromatograms of the mixtures of phloroglucinol and pyrogallol reacting with oxazolidine at ratios Ph+Py:Ox = (1+1):2, at 0, 5, 10, 15, 20, 30, 40 minutes

In the mixture of phloroglucinol and pyrogallol, phloroglucinol reacted first, which can be explained by its higher reactivity than pyrogallol. Unlike the solo phenol reactions, there are many intermediates produced (R_f value between 0.10 and 0.65), which indicates pyrogallol had taken part in a condensation reaction with phloroglucinol. When pyrogallol alone reacts with oxazolidine, there are some intermediates (R_f 0.85) produced, but none were observed in this mixture system. This phenomenon indicates that the presence of phloroglucinol makes the reaction of pyrogallol fast, by reacting with its intermediates. The mechanism suggests that the intermediates from pyrogallol with oxazolidine would be further exhausted by

phloroglucinol or its intermediates; this will enhance the reaction of pyrogallol and at the same time slow down the reaction of phloroglucinol. The high reactivity and longer bridge chain of oxazolidine makes it possible to produce cross-linking between phloroglucinol and pyrogallol molecules, to form co-condensation products, although these two phenols have very different reactivities with oxazolidine (Fig 4.29). The reaction model is shown in table 4.8.

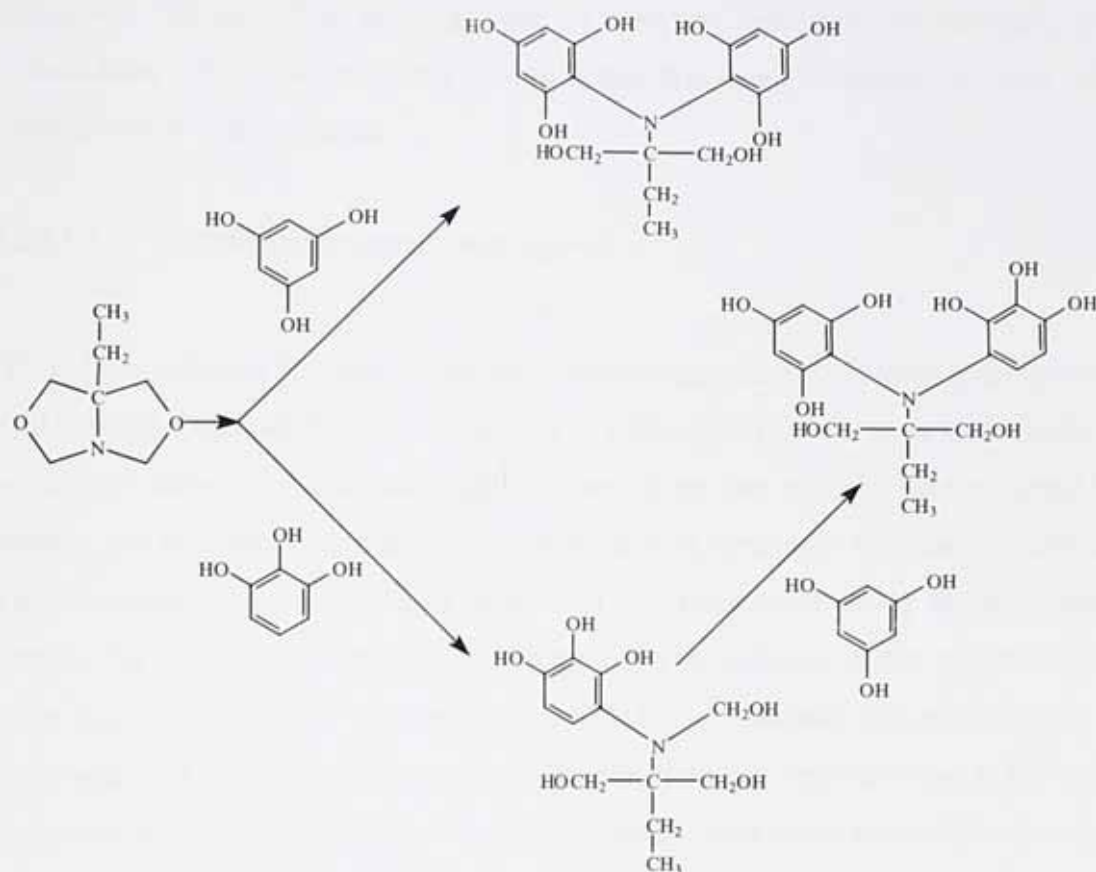


Fig 4.29 Reaction of phloroglucinol and pyrogallol with oxazolidine

As a model for robinetinidol, the mixture of resorcinol and pyrogallol has greater possibility for cross-linking by oxazolidine, because of the small difference between their reactivities. Chromatography has shown cross-linking occurred between resorcinol and pyrogallol at pH<7.0 and room temperature, because both disappear from the solution at nearly the same rate, although the process is much slower than phloroglucinol with pyrogallol.

From Fig. 4.28, there is a synergistic function between the two phenols in the phloroglucinol and pyrogallol mixture, which is more apparent when the reaction is conducted at increased temperature or pH. This phenomenon also indicates that in the reaction process, the pyrogallol-oxazolidine intermediate will react with the more reactive phloroglucinol or resorcinol.

The mixture of resorcinol or phloroglucinol with catechol did not show any synergistic effects. This may be because of the low reactivity of catechol with oxazolidine, only phloroglucinol or resorcinol has been observed to react with oxazolidine in these systems.

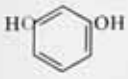
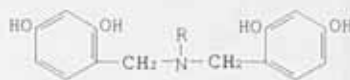
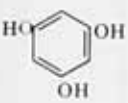
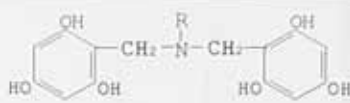
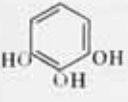
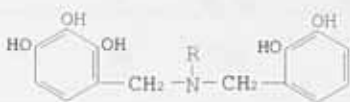
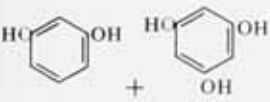
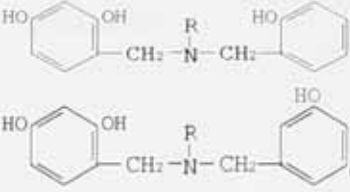
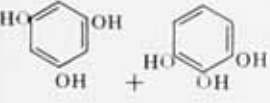
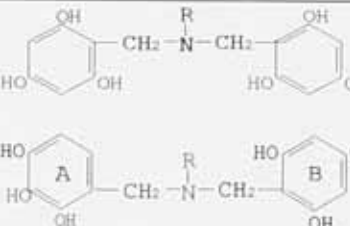
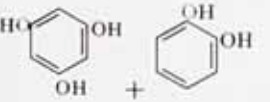
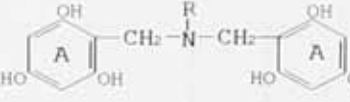
4.2.3.1.2 ¹H-NMR spectrometry investigation

¹H-NMR confirmed the chromatography results; importantly, relative reaction rates could be obtained and the data are shown in Table 4.8. The reactions were found to be second order in the initial stage, derived from the straight line obtained by plotting the reciprocal of phenol concentration with time over the first 30 minutes. The calculation was as described in Section 4.1. Compared to the simple phenols system, the reaction rate constant of pyrogallol in the mixture system was measured to be higher, $0.056\text{M}^{-1}\text{s}^{-1}$ compared to $0.036\text{M}^{-1}\text{s}^{-1}$. Similarly the reaction rate of phloroglucinol appeared slightly slower, the reaction rate constant was $0.393\text{M}^{-1}\text{s}^{-1}$ compared to $0.433\text{M}^{-1}\text{s}^{-1}$ (Table 4.8). Similar results were obtained for the mixture of resorcinol and pyrogallol with oxazolidine: the reaction rate constant of pyrogallol was higher, $0.050\text{M}^{-1}\text{s}^{-1}$ compared to $0.036\text{M}^{-1}\text{s}^{-1}$, but for resorcinol the rate was the same. The kinetic studies demonstrate that the presence of phenols more reactive than pyrogallol will catalyze the reaction of pyrogallol with oxazolidine.

In this way, it is concluded that cross-linking occurs between phloroglucinol (or resorcinol) and pyrogallol. These competitive and synergistic effects in the system of phenol mixtures reacting with oxazolidine suggest the possible reaction mode of flavonols with the same cross-linker, which was confirmed directly by the following studies.

Table 4.8 Reaction kinetic studies of phenols react with cross-linking reagent

(pH 7.0, 20°C, phenol:oxazolidine = 1:1)

Phenols	Reaction rate constant $k/M^{-1}s^{-1}$	Cross-linking mode
	0.048	
	0.433	
	0.036	
	Re: 0.046 Py: 0.050	
	Ph: 0.393 Py: 0.056	
	Ph: 0.396 Re: <0.001	

R: the side chain of oxazolidine [-C(CH₂OH)₂CH₂CH₃]

4.2.3.2 Reaction of catechin with oxazolidine

When catechin or epicatechin, which have a phloroglucinol A-ring and catechol B-ring, react with oxazolidine (molar ratio 1:2) at pH 7.0 and 20°C, the reaction is much slower than phloroglucinol, completed in 50 minutes while phloroglucinol takes 20 minutes.

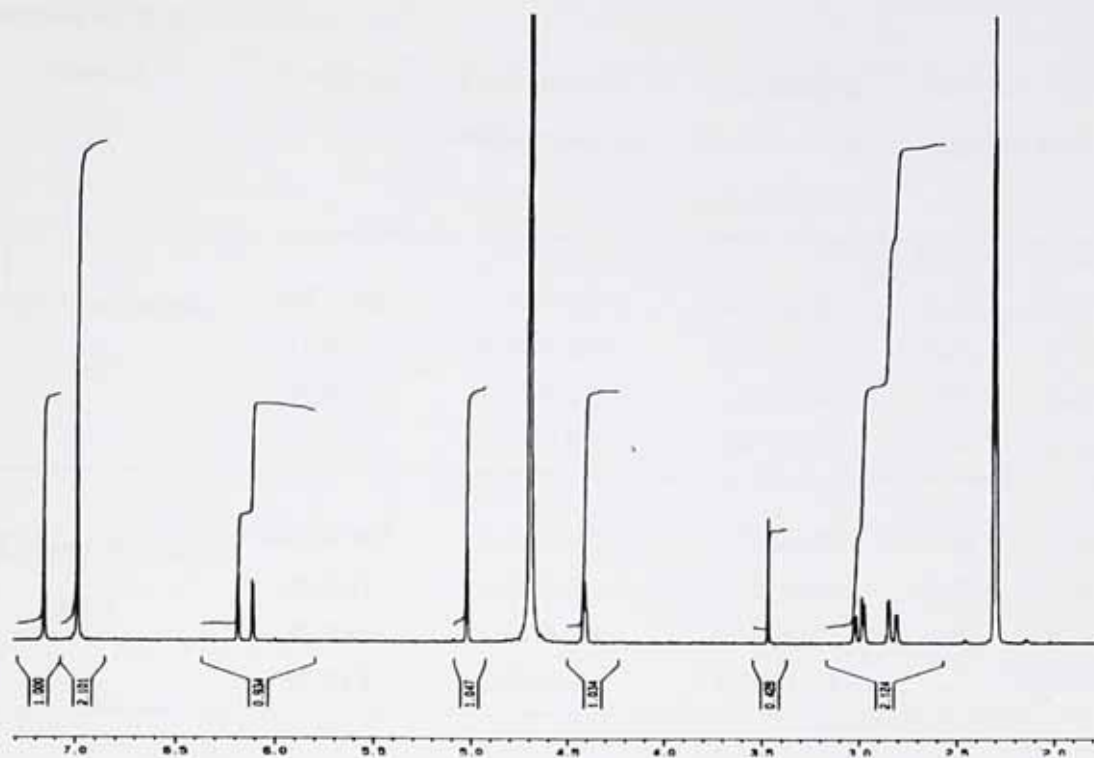


Fig 4.30 $^1\text{H-NMR}$ spectrum of epicatechin

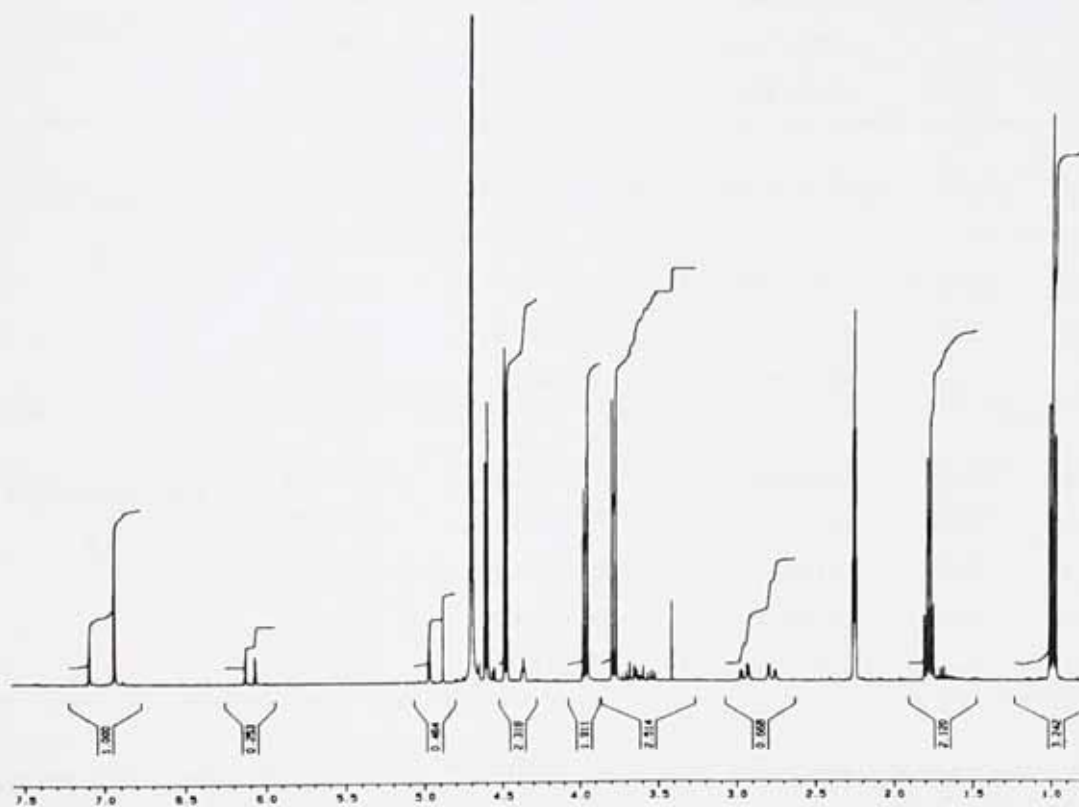


Fig 4.31 $^1\text{H-NMR}$ spectrum of epicatechin reaction product with oxazolidine

Table 4.9 ¹H-NMR spectra of gallocatechins and catechins before and after reaction with oxazolidine

Phenols	Positions	Peak integral	Peak integral	Integral ratio	
		before reaction and δ (ppm)	after reaction and δ (ppm)	against H-2 Before	against H-2 After
(+)Galocatechin GC	H-2',6'(B)	1.00 (6.60)	0.82 (6.60)	2.32	1.87
	H-8(A)	0.35 (6.06)	0.19 (6.06)	0.81	0.43
	H-6(A)	0.35 (6.02)	0.19 (6.02)	0.81	0.43
	H-2(C)	0.43 (4.92)	0.44 (4.92)	-----	-----
(-)Epigallocatechin EGC	H-2',6'(B)	1.00 (6.60)	0.79 (6.60)	2.50	1.97
	H-8(A)	0.33 (6.06)	0.18 (6.06)	0.83	0.45
	H-6(A)	0.33 (6.02)	0.18 (6.02)	0.83	0.45
	H-2(C)	0.40 (4.92)	0.40 (4.92)	-----	-----
(-)Epigallocatechin gallate EGCG	H-2',6'(B)	0.47 (6.58)	0.39 (6.60)	1.88	1.56
	H-8(A)	0.11 (6.07)	0.06 (6.06)	0.44	0.24
	H-6(A)	0.14 (6.02)	0.08 (6.02)	0.56	0.32
	H-2(C)	0.25 (5.42)	0.25 (4.92)	-----	-----
	H-2'',6'' (D)	0.47 (6.93)	0.48 (6.94)	1.88	1.92
(+)Catechin C	H-2',6'(B)	1.00 (6.77-6.90)	1.00 (6.77-6.90)	3.03	2.96
	H-5'(B)				
	H-8(A)	0.13 (6.04)	0.03 (6.06)	0.39	0.09
	H-6(A)	0.11 (5.95)	0.02 (6.02)	0.33	0.06
	H-3(C)	0.33 (4.16)	0.34 (4.16)	-----	-----
(-)Epicatechin EC	H-2',6'(B)	2.10 (6.98)	0.69 (6.98)	2.00	1.97
	H-5'(B)	1.00 (7.15)	0.33 (7.15)	0.95	0.95
	H-8(A)	0.47 (6.18)	0.12 (6.18)	0.45	0.34
	H-6(A)	0.47 (6.10)	0.12 (6.10)	0.45	0.34
	H-2(C)	1.05 (5.00)	0.35 (4.92)	-----	-----

Figures 4.30 and 4.31 shows the ¹H-NMR spectra of epicatechin before and after reaction with oxazolidine at a molar ratio of 1:2 (same ratio as the phenol mixtures experiment, the total moles of A-ring and B-ring to oxazolidine = 1:1). Only the peaks for the A-ring become smaller in the reaction process, but the peaks for B-ring

hydrogens and the heterocyclic C-ring (C-2, C-3 and C-4) do not change in the process. This shows the B-ring and C-ring do not take part in the cross-linking reaction with oxazolidine. The data from the H-NMR spectra have been summarised in Table 4.9, which shows the ratio between the integrals of A-ring and B-ring sites in the reaction. Little loss of B-ring peaks would be expected from oxidation of the catechol group. These results are in good agreement with previous results from the mixed phloroglucinol and catechol system and are similar to the catechin-formaldehyde reaction.

There are two reactive sites in catechin, available for cross-linking. ¹H-NMR has shown the reaction sites are C-6 and C-8 and the measured ratio of integrals for C-6 and C-8 remained at 1:1, with no change observed in the reaction; this indicates there is no noticeable electrophilic activity difference towards oxazolidine between C-6 and C-8 in the catechin structure.

As Hemingway has described, considering the molecular structure of catechins, there is a strong tendency to form hydrogen bonds between 3-hydroxyl and the pyran ether oxygen¹⁴¹. In (+)catechin, such bonding rotates the catechol B-ring into an axial position and crowds the spacing around the C-8 position. A similar hydrogen bond in the (-)epicatechin isomer rotates the B-ring into an equatorial plane so that there is no crowding of the C-8 position of the A-ring. For this reason, some difference between (+)catechin and (-)epicatechin was anticipated in the reaction with oxazolidine. A comparison of the condensation rates of (+)catechin and (-)epicatechin with oxazolidine has shown that (-)epicatechin is slightly more reactive than (+)catechin¹⁴¹. The following kinetic studies have confirmed this.

4.2.3.3 Reaction of gallocatechin with oxazolidine

Galocatechins have a pyrogallol B-ring, which is much more reactive than the catechol B-ring of catechin. The previous studies have shown some synergistic effect in the reaction with oxazolidine when phloroglucinol and pyrogallol are both present.

Chromatography has shown that the reactivity of gallocatechins to oxazolidine lies between catechins and phloroglucinol. When the molar ratio of gallocatechin to oxazolidine is 1:2, the gallocatechin molecules are reacted in 40 minutes at pH 7.0 and 20°C.

In the A-ring of the gallocatechin molecular structure, the reactive sites are one less than phloroglucinol, because one hydroxyl group is engaged in the heterocyclic ring; the presence of a steric hindrance by the C-ring will also further reduce the reactivity of the A-ring. But in contrast, the electron donating substitute at C-1' will increase the reactivity of the B-ring pyrogallol, although steric hindrance will also affect the B-ring somewhat. Then, based on the studies of the mixture of phloroglucinol and pyrogallol with oxazolidine, it may be estimated there is potential for cross-linking between the reactivity reduced A-ring of one flavanol and the barely changed B-ring of the other. This hypothesis is given support mainly by NMR studies (Figures 4.32 and 4.33).

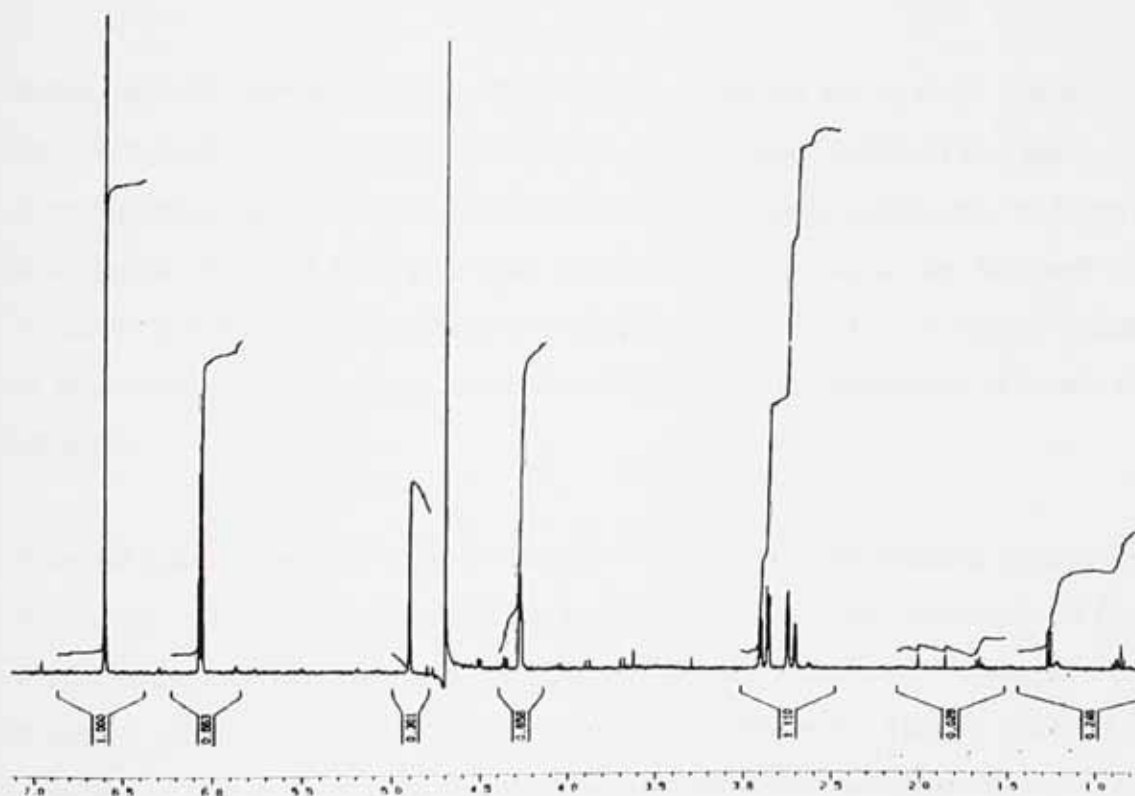


Fig 4.32 ¹H-NMR spectrum of epigallocatechin

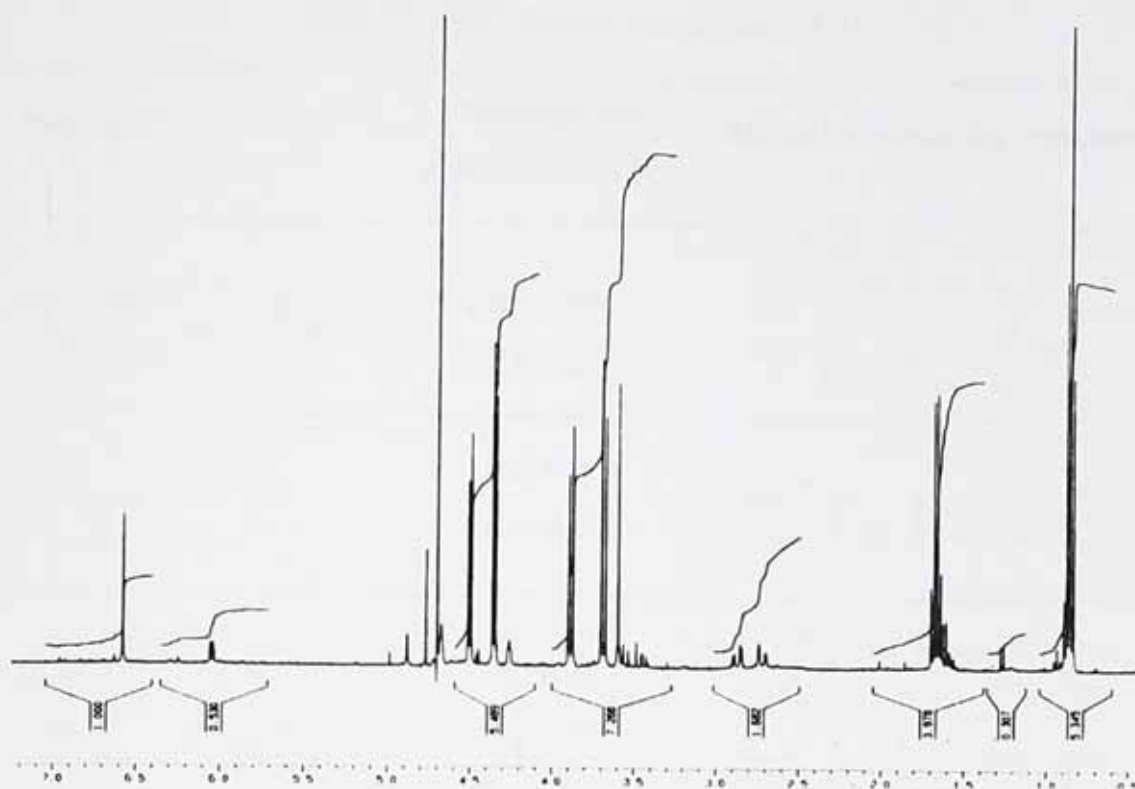


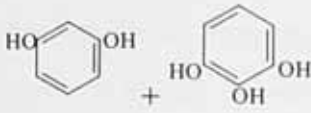
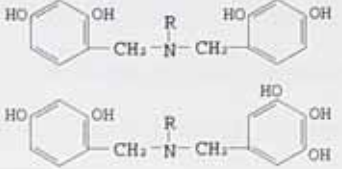
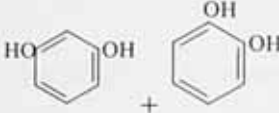
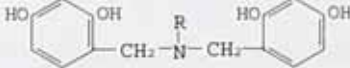
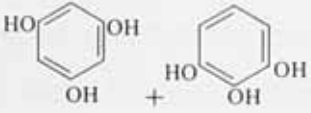
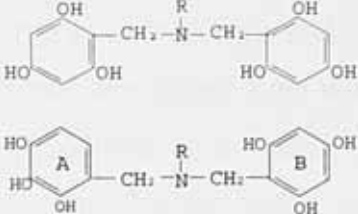
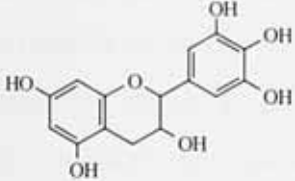
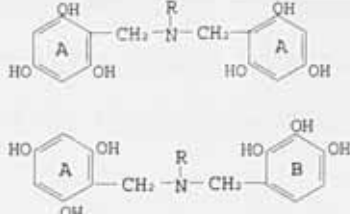
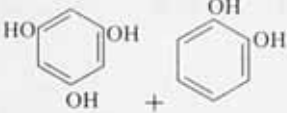
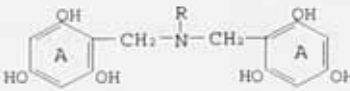
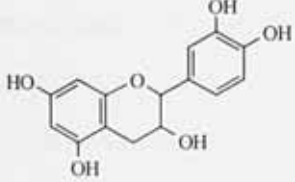
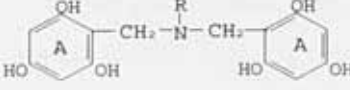
Fig 4.33 $^1\text{H-NMR}$ spectrum of epigallocatechin reaction product with oxazolidine

In the $^1\text{H-NMR}$ spectra, the peaks from the A-ring and the B-ring both reduced with time compared to the unchanging C-ring (Fig 4.31), which indicates that the B-ring does engage in the reaction. The peaks of the A-ring change rapidly after addition of oxazolidine; peaks at 6.12 ppm and 5.89 ppm, correspond to the H-8 and H-6 respectively and the measured ratio of the integrals remain at 1:1 in the initial stage of this reaction, which means these two sites react with oxazolidine at nearly the same rate.

After 30 minutes reaction, there is more H-8 signal left in the reaction system than H-6, which indicates that more H-6 has reacted in the first stage, which results from the different reactivities of these two sites, as the steric effect of the B-ring and C-3 hydroxyl group influence H-8 more than H-6 (Table 4.9). This is different to catechins, where these two reactive sites have the same nucleophilic character.

Table 4.10 Reaction kinetic studies of phenols react with cross-linking reagent

(pH 7.0, 20°C, Phenol:oxazolidine = 1:1)

Phenols	Reaction rate constant k ($M^{-1}s^{-1}$)	Model cross-linking structure
	Re: 0.046 Py: 0.050	
	Re: 0.048 Ca < 0.001	
	Ph: 0.393 Py: 0.056	
	A-ring: 0.217 B-ring: 0.058	
	Ph: 0.396 Re: <0.001	
	A-ring: 0.203 B-ring: <0.001	

- "R" the residue of ring-opened oxazolidine [$CH_3CH_2(HOCH_2)_2C-$]
- Ph: phloroglucinol, Re: resorcinol, Py: pyrogallol, Ca: catechol, C: catechin, GC: gallic acid
- There is also B-ring to B-ring reaction in the case of gallic acid

With the disappearance of A-ring peaks, the peak at 7.12ppm for the B-ring also becomes smaller, but the rate is faster than free pyrogallol reacting with oxazolidine.

This result is based on assuming the ethyl group of oxazolidine does not take part in this reaction. There is hardly any change in the B-ring peaks after the disappearance of the A-ring peaks. In other words, the pyrogallol B-ring only reacts slowly with oxazolidine without the presence of the phloroglucinol A-ring.

In fact, there is no synergistic effect when only the pyrogallol B-ring reactive sites are left. From these points, we could conclude that at least some of the cross-linking reaction occurs between the A-ring and B-ring, but little cross-linking between B-ring and B-ring. This reaction mode is shown in Table 4.10.

The difference between epigallocatechin-gallate and epigallocatechin lies in the C-3 gallate group. The conformation of the third benzene ring (D-ring, the gallate group) in this structure may show some influence on the A-ring, after forming a hydrogen bond with the alkoxy of the heterocyclic ring and it is also anticipated there will be a steric hindrance effect on the B-ring. There is no big difference in the reactivities of galocatechin and its gallate. This result proves the notion, that the gallate group does not take part in the cross-linking process, but there would be no significant influence on either the A-ring or B-ring. The presence of the electron withdrawing carboxyl group at C-1'' makes the C-2'' and C-6'' of the D-ring non-reactive to electrophilic aromatic substitution.

4.2.3.4 Kinetic studies of catechin and galocatechin with oxazolidine

It was expected that the cross-linking reaction of catechins or galocatechins would be second order in the first stage, based on the studies of simple phenol reaction with oxazolidine (See section 4.1). This assumption was shown to be valid under the conditions used in this work (Fig 4.34, Table 4.11-14, also see Appendix VI).

The results showed that epicatechin ($k=0.21\text{M}^{-1}\text{s}^{-1}$) is slightly more reactive than (+)catechin, reaction rate constant $k=0.18\text{M}^{-1}\text{s}^{-1}$. The same reaction is much slower in 10% D₆-acetone solution, when the reaction rate constant k is $0.020\text{M}^{-1}\text{s}^{-1}$ for epicatechin and $0.017\text{M}^{-1}\text{s}^{-1}$ for catechin.

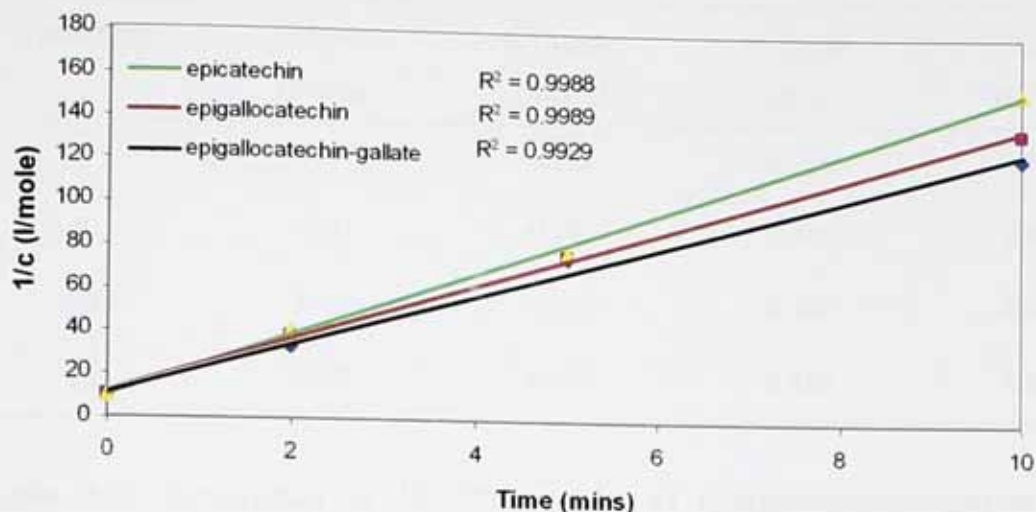


Fig 4.34 Second order rate plot for epicatechin, epigallocatechin and epigallocatechingallate with oxazolidine, 20°C, ratio 1:1, aqueous solution, pH 7.0

Table 4.11 Integration of ¹H-NMR peaks of catechin with oxazolidine in D₂O

Time/min	7.00ppm (B-ring)	6.05ppm (A-ring)	4.7ppm (H-2)	4.2ppm (H-3)	2.6-3.0ppm (H-4)
0	1.000	0.456	0.431	0.324	0.664
5	1.000	0.279	0.430	0.322	0.665
10	1.000	0.268	0.433	0.320	0.659
20	1.000	0.213	0.434	0.327	0.661

Table 4.12 Integration of ¹H-NMR peaks of epicatechin with oxazolidine in D₂O

Time/min	7.1-6.9ppm (B-ring)	6.15ppm (A-ring)	5.0ppm (H-2)	4.3ppm (H-3)	2.8-3.0ppm (H-4)
0	1.000	0.461	0.349	0.324	0.664
5	1.000	0.275	0.348	0.326	0.665
10	1.000	0.266	0.343	0.331	0.659
20	1.000	0.253	0.344	0.327	0.668

Table 4.13 Integration of ¹H-NMR peaks of EGC with oxazolidine in D₂O

Time/min	6.8ppm (B-ring)	6.15ppm (A-ring)	5.0ppm (H-2)	3.1ppm (H-4 Ox)
0	0.937	0.903	0.351	1.002
8	0.871	0.247	0.347	0.951
15	0.868	0.213	0.340	0.978
25	0.849	0.169	0.339	0.969

Table 4.14 Integration of ¹H-NMR peaks of epigallocatechin-gallate with oxazolidine in 10% D₆-acetone in D₂O

Time/min	7.2ppm (D ring)	6.8ppm (B-ring)	6.15ppm (A-ring)	3.1ppm (H-4 Ox)
0	1.00	0.923	0.898	1.003
8	1.00	0.861	0.231	0.940
15	1.00	0.875	0.201	0.964
25	1.00	0.860	0.150	0.960

Galocatechin has nearly the same reaction rate constant as epigallocatechin, $k=0.21\text{M}^{-1}\text{s}^{-1}$, but slightly lower than epigallocatechin-gallate with $k=0.24\text{M}^{-1}\text{s}^{-1}$.

From the kinetic results, the phloroglucinol A-ring always shows higher reactivity compared to the B-ring, the reaction rate constant is $0.24\text{M}^{-1}\text{s}^{-1}$, but this is only half of the rate of free phloroglucinol, $0.40\text{M}^{-1}\text{s}^{-1}$. There are two possible reasons for the reduced of reactivity in the A-ring: first, there is one reactive site less in the A-ring than free phloroglucinol, which is the C-4a site occupied by an alkyl substituent; second, there is the presence of the heterocyclic C-ring, in which one hydroxyl group also has been reacted to form an alkoxyl structure.

No reaction was observed in the catechol B-ring under these experiment conditions. This is in good agreement with the model compounds studies. The cross-linking

reaction only happened in the A-ring for catechin or epicatechin, but there is no experimental data available to decide whether the linking bridge is C8-C8, C8-C6 or C6-C6. The ¹H-NMR only showed the two sites have nearly the same reactivity towards oxazolidine. Regarding the gallo catechins or epigallocatechin-gallate derivatives, the A-ring has similar reactivity to catechins, but the B-ring has much higher reactivity than free pyrogallol, which makes it possible to form cross-links between the A-rings and the B-rings. The reduced reactivity and greater steric hindrance effect in the A-ring also makes cross-linking between A-ring and B-ring more possible. In the model study of phenol mixture, the ratio of the reactivities of phloroglucinol and pyrogallol is 12:1, but this ratio in gallo catechin is reduced to 4:1, which shows the influence of the flavanol structure on the reaction properties. Gallo catechin will have a different cross-linking reaction to catechins, in which more oxazolidine can be consumed; the following study has confirmed this view.

4.2.3.5 Consumption of oxazolidine

Table 4.15 Consumption of oxazolidine at pH 7.0

Flavonols Monomers	Ratio phenol:oxazolidine	Oxazolidine reacted (%)	Moles oxazolidine per mole phenol
Catechin	1:2	61.5	1.23
Epicatechin	1:2	63.0	1.26
Galocatechin	1:2	76.5	1.53
Epigallocatechin	1:2	79.5	1.59
Epigallocatechin gallate	1:2	74.0	1.48

When catechin is in excess (2:1), oxazolidine will be exhausted, some high polymers are produced but some of the original catechin remains in the solution. When the ratio is 1:1, catechin can be exhausted completely. Some polymer can be seen by paper chromatography when oxazolidine is excess (1:2), the reaction is faster and all the catechin is reacted and only polymeric phenol is present in the final solution.

These results indicate the optimum molar ratio of catechin with oxazolidine is close to 1:1.

By checking the consumption of oxazolidine, gallo catechins (GC, EGC and EGCG) can react with more oxazolidine than catechins under same conditions (Table 4.15). This phenomenon can be explained by the pyrogallol B-ring taking part in the cross-linking reaction to consume more oxazolidine, a higher density of cross-linking can be formed, so the optimum molar ratio of gallo catechin with oxazolidine is close to 1:1.2. From Table 4.15, not all the sites on the pyrogallol B-ring of gallo catechin are reacted, so there is less oxazolidine consumption than anticipated, which indicates the consumption of oxazolidine may also be determined by other effects, such as steric hindrance.

There is no significant difference between different conformations of the same molecule, so 1.23 and 1.26 moles oxazolidine are consumed by catechin and epicatechin respectively.

4.2.3.6 Effect of pH and metal ions

Table 4.16 Effects of zinc(II) acetate on the reaction of catechins and gallo catechins

Compounds	Rate constant without Zn ²⁺	Rate constant with Zn ²⁺
	(M ⁻¹ s ⁻¹)	(M ⁻¹ s ⁻¹)
Catechin	0.18	0.29
Epicatechin	0.21	0.33
Epigallo catechin	0.22	0.41
Epigallo catechin-gallate	0.24	0.46

Studies on the reaction of catechin or gallo catechin with formaldehyde have shown that pH is one of most important influences. Catechin has the lowest reactivity with formaldehyde at pH 4.5. Similar results for the reaction with oxazolidine for catechin or gallo catechin were obtained. Chromatography indicated the oxazolidine reaction at pH 4.5 is slower than at pH 7.0, but the difference is smaller than the

formaldehyde reaction. This phenomenon was also observed for EC, GC and EGC in the reaction with oxazolidine.

Zinc(II) acetate has been found to be the most effective catalyst for proanthocyanidins monomers reacting with oxazolidine. 0.02mol/l can increase the reaction rate significantly (Table 4.16). Gallocatechin and epigallocatechin have shown larger effects with zinc acetate, which may arise from the stronger complexation between the pyrogallol B-ring and the metal ions. Many researchers have shown the pyrogallol B-ring can form more stable complexes than the catechol-B-ring⁵⁷, where the presence of the third hydroxyl group in the pyrogallol B-ring stabilises the complexation.

4.2.4 Discussion and summary

The reaction of oxazolidine with proanthocyanidin monomers including catechin, epicatechin, gallocatechin, epigallocatechin and gallate derivatives, were modelled by simple phenol mixtures.

(1) The sequence of reactivity is:



The pyrogallol B-ring of gallocatechins has higher reactivity than free pyrogallol.

(2) There is a synergistic effect between the A-ring and the pyrogallol B-ring. The cross-linking reaction mainly happens on the A-ring. The pyrogallol B-ring of gallocatechins can take part in the reaction, but the catechol B-ring of catechins will not react with oxazolidine under similar conditions.

(3) The lowest reaction rates were obtained at pH 4.0-4.5. Increased temperature will enhance the reaction. Zinc(II) acetate is the best catalyst for this process, especially for the pyrogallol B-ring type flavanol.

(4) Prodelphinidins (gallocatechin) have the highest reactivity for oxazolidine and the highest oxazolidine consumption per unit in the natural flavonols. If one more phloroglucinol group is introduced into the flavonol molecules, it increases the reactivity further. But the gallate group of EGCG has shown little influence on the reactivity.

More work is needed to confirm cross-link formation at C6-C6, C8-C8 or C6-C8 for the phloroglucinol A-ring and similar reactions at the A and B rings for gallo catechins.

These results have indicated that among all different types of condensed tannins, poly-prodelphinidins tannins (gallo catechin tannins) have the highest reactivity towards the cross-linker oxazolidine. As for poly-prorobinetinidins (resorcinol A-ring and pyrogallol B-ring) and poly-profisetinidins (resorcinol A-ring and pyrogallol B-ring), the results here indicate that the former is more reactive than the latter.

Part 4.3 Proanthocyanidins polymer studies

4.3.1 Introduction

Considered as high molecular weight natural polymers, the condensed tannins exhibit many properties of individual flavan-3-ol units. Condensed tannins also show distinct nucleophilic substitution activity, although it may be modified by the steric effect from the high polymers and loss of reactive sites^{211,222}. As discussed in Chapter 1, the linkage bonds between condensed tannin monomer units are formed through either C4-8 or C4-6, which means there is one reactive site occupied in each extension unit.

Based on the results of Sections 4.1 and 4.2, monomeric gallo catechin (prodelphinidin) has the highest reactivity and oxazolidine consumption of all the proanthocyanidins monomer units, in which cross-linking reactions happen between A-ring and A-ring or A-ring and pyrogallol B-ring. Here, different types of condensed tannins, especially prodelphinidin tannins have been directly used to study the reaction with oxazolidine, to examine the following features:

- The difference between monomeric and polymer proanthocyanidins
- The relative reactivity of different proanthocyanidin polymers
- The kinetic characteristics of the polymerisation reaction
- The difference between oxazolidine and formaldehyde for polymerisation

The prodelphinidin tannins used in these studies were pecan tannin and myrica tannin, discussed in Chapter 2. The former is a copolymer of epicatechin and epigallocatechin (at ratio 1:5-6); the latter is pure prodelphinidin polymer with about 40% C-3 hydroxyl group gallated²¹¹⁻²¹⁴. These tannins are compared here with mimosa (prorobinetinidin), quebracho (profisetinidin) and gambier (procyanidin) tannins.

The complexity of proanthocyanidin polymers makes the condensation reaction with oxazolidine too complicated to be followed by NMR or UV methods. Therefore, the main methods in this work are similar to the traditional methods of tannin-formaldehyde adhesive studies. The gel time can reflect the reactivity of different polymers, the consumption of oxazolidine will shed some light on the reaction process and cross-linking modes. The kinetic studies were undertaken by measuring the change of oxazolidine concentration in the reaction.

4.3.2 Experimental

The commercial tannins mimosa, quebracho and gambier were received from Hodgson Chemicals PLC, UK. All tannins were treated as follows. The average molecular weight distribution and average molecular weight of these purified tannins were determined by GPC. The composition and structural characteristics of each polymer were also examined by using chromatography, IR and UV spectroscopy

A known weight of tannin was dissolved in 20 volumes of water, extracted with equal volumes of chloroform and ether. After the solvent was removed from the aqueous solution, then two times its weight of hide powder was added and the mixture was kept in water bath at $30.0 \pm 0.5^\circ\text{C}$ for 4 hours. The tannins fraction was absorbed on hide powder, the non-tannin fraction remained in the solution⁹⁴. The mixture was filtered through Whatman No.1 paper and the absorbed tannins were released from the hide powder by washing with 50% acetone.

4.3.2.1 Gel time study

Gel time is a parameter used in wood adhesive production to characterise the reaction with formaldehyde²⁰⁸⁻²⁰⁹. Here the method was used to distinguish the reactions of different proanthocyanidins towards oxazolidine.

Purified tannin polymers were dissolved in pH 6.5 acetate buffer at a concentration of 30g/l, which is equivalent to 0.1mol/l for monomer units, assuming the average monomer molecular weight is 300D. The solution was adjusted with 3mole/l sodium

hydroxide solution to pH 6.5. 10ml of each tannin solution were transferred to 30ml bottles with stoppers, kept in a water bath at $25.0 \pm 1.0^\circ\text{C}$, 1ml of 1mol/l oxazolidine (solution was adjusted to pH 6.5) was added to the tannin solution, the bottle was sealed and the gel time was recorded.

4.3.2.2 Effects of metal ions

As described in Chapter 1, proanthocyanidins, especially those with a pyrogallol functional group, show strong complexation ability towards some metal ions^{64,65,224}. Therefore it is necessary to evaluate the effects of metal ions on the reaction with oxazolidine. Based on the results of Sections 4.1 and 4.2, only the effect of zinc(II) ion was studied, as described in Section 4.1.

4.3.2.3 Consumption of oxazolidine

Tannins were dissolved in different sodium acetate buffer solutions (pH 3.5, 5.0, 6.5,) and phosphate buffer for pH 8.0, made to 30g/l, so the concentration of monomer units was 0.1mol/l, and then equal volumes of 0.3mol/l oxazolidine solution at different pH values were added, then kept at different temperatures in water baths (at 20, 40, 60 and 80°C) for 12 hours. Tannins or condensation products were removed by reducing the pH to 2.5-3.0 when tannins are precipitated. After centrifuging for 30 minutes at 3,000g and washing with distilled water three times, the upper liquid layer and the washing water were collected in a volumetric flask and made up to 50ml. Then the oxazolidine content was measured by GC-MS as described in Chapter 3.

4.3.2.4 Kinetic studies

Kinetic studies were carried out using a similar method as above: the free oxazolidine concentration in the reaction medium at a given time was measured by checking the oxazolidine level after centrifugation at 3,500g for 5 minutes, using GC-MS.

4.3.2.5 Molecular weight distribution of reaction products

The tannin solution, before and after reaction with oxazolidine, was freeze-dried. The number average molecular weight of purified tannins was determined as described in Chapter 2. But the tannin-oxazolidine reaction products do not dissolve well in pyridine, therefore, the molecular weight of the polymerised product was not studied.

4.3.3 Results and discussion

4.3.3.1 Structural characteristics, reactivity and pre-treatment of condensed tannins

The tannin materials in this work were purified; the results are shown in Table 4.17.

Table 4.17 Characteristics of purified condensed tannins

Tannins	Main component ^a	Mn ^b	Dp ^c	Anticipated reactive sites	Configuration
Pecan	PC: PD=1:6	3200	11	C-6 & B-ring	Linear
Myrica	PD (40% gallated C-3)	2700	9	C-6 & B-ring	Linear
Quebracho	PF	1430	5	C-8	Angular
Mimosa	PR: PF: PD = 7: 2.5: 0.5	1500	5	C-8 & B-ring	Angular
Gambier	PC	970	3	C-6	Linear

a PC: procyanidin; PD: prodelphinidin; PF: profisetinidin; PR prorobinetinidin

b Mn: number average molecular weight, determined by GPC

c Dp: average degree of polymerisation

After removing most of the non-tannins, the number average molecular weight of pecan and myrica tannins rises about 10%, from 3,000 and 2,500; similarly for quebracho, mimosa and gambier, the number average molecular weights were 1200, 1230 and 570 respectively before purification^{93,149,170}.

4.3.3.2 Comparison of tannin activity

The reaction of proanthocyanidins with aldehydic agents normally involves formation of cross-links between molecules, to give an increase in molecular weight and finally to form an insoluble product or gel. Four main factors affect the gel time for proanthocyanidins with cross-linking agents: concentration of polymer, ratio of polymer to oxazolidine, pH and temperature.

4.3.3.2.1 Effect of molecular structural characteristics

Oxazolidine has higher reactivity than formaldehyde, so the polymers are gelled by oxazolidine in a shorter time than with formaldehyde (Table 4.18). Chromatography has shown that both formaldehyde and oxazolidine form high polymers in the initial stage of the reaction with polyphenols. The reaction is fast. In the case of oxazolidine, mimosa polyphenol becomes a high polymer completely in 45 minutes at pH 6.5 and 20 °C; the same process takes 90 minutes with formaldehyde.

Table 4.18 Gel time of different proanthocyanidins polymers

(tannin concentration 3% w/v, ratio of monomer unit:oxazolidine =1:1, pH 6.5, 20°C)

Proanthocyanidins	Time (mins)	
	Oxazolidine	Formaldehyde
Pecan	6	10
Myrica	5	10
Mimosa	45	90
Quebracho	75	140
Gambier	35	60

Prodelphinidin tannins (pecan and myrica) exhibit shorter gel times than prorobinetinidin (mimosa) or profisetinidin (quebracho) tannin. This indicates the phloroglucinol A-ring is one of the determining factors. The main difference between myrica tannins and mimosa tannins, is the A-ring, they both possess pyrogallol B-rings.

There is little difference between pecan and myrica tannins: pecan tannin has higher average molecular weight, 3000D, than myrica tannin, 2500D, but 15-16% of the pecan tannin is epicatechin units, while myrica tannin is pure prodelphinidin. The presence of epicatechin units in pecan tannin will make the B-ring less reactive compared with myrica tannin, because the catechol B-ring cannot react with oxazolidine under these experimental conditions. The higher molecular weight of pecan tannin compared to myrica tannins, makes little difference to the gel time.

The reaction of procyanidin tannin (gambier) is slow compared to prodelphinidin: one reason is the low average molecular weight of gambier tannin, so it will take a longer time to form high polymers, another reason is the lower reactivity of the catechol B-ring compared the pyrogallol B-ring of prodelphinidin.

The difference between mimosa and quebracho tannins arises from the different B-rings, because these tannins have similar molecular weight distributions and molecular structure, also 25% of the monomer units in mimosa tannins are profisetinidin, which is same as the quebracho structure. But 70% of the structural units in mimosa are prodelphinidin with a pyrogallol B-ring, which makes it more reactive than quebracho tannins.

4.3.3.2.2 Effect of pH

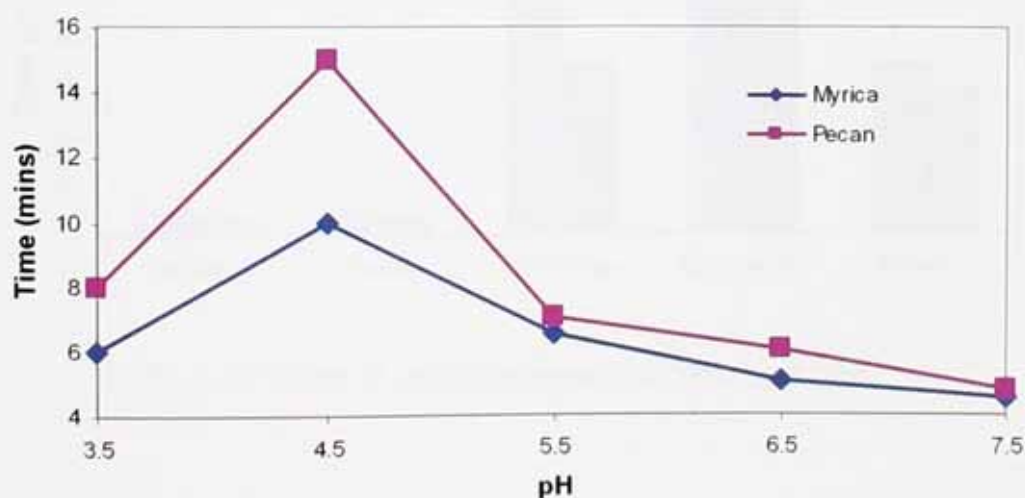


Fig 4.36 Gel times of myrica and pecan tannins at different pH values

The reaction of polyphenols with aldehydic agents may be base or acid catalysed and pH is a determining factor in the reaction with formaldehyde. But for oxazolidine, there is less effect due to pH, the lowest reaction rate was found at pH 4.5, the reaction is only slightly faster at pH 3.5 and there is little increase in the reaction rate above pH 7.5 (Fig 4.36).

4.3.3.2.3 Effects of zinc(II) acetate

In Sections 4.1 and 4.2, zinc(II) acetate was found to be an effective catalyst for the reaction of phenols with oxazolidine. The addition of zinc acetate to the proanthocyanidin polymers increases the speed of the gelling process and the consumption of oxazolidine: for prodelpinidins, such as pecan tannin and myrica tannins (Fig 4.37), zinc acetate was more effective than for the procyanidin gambier tannin. Zinc(II) acetate also shows catalytic function for mimosa, which indicates the effect of the pyrogallol B-ring: the presence of trihydroxyl group in the B-ring stabilises the coordination products and increases the catalytic effect of metal ions. The presence of zinc(II) acetate makes the pyrogallol B-ring more reactive and makes a distinct difference between a catechol B-ring and a pyrogallol B-ring.

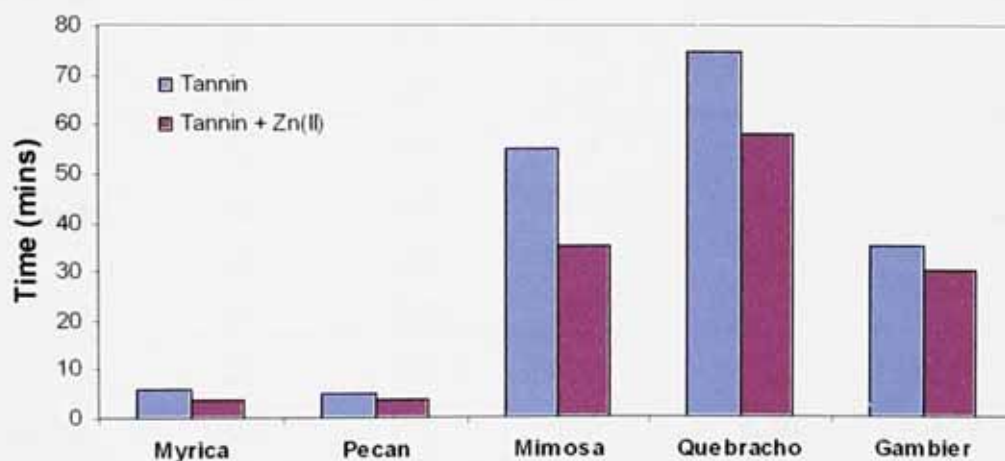


Fig 4.37 Effect of zinc(II) acetate (0.02M) on gel time

4.3.3.3 Consumption of oxazolidine

Hillis and Urbach studied the reaction of catechin and polyphenols with formaldehyde by determining the consumption of formaldehyde²¹⁶⁻²¹⁷. In this work, the consumption of oxazolidine by the five condensed tannins was studied by a similar method (Table 4.19).

Table 4.19 Consumption of oxazolidine by condensed tannins at pH 6.5^a

Tannins	Temperature (°C)	Ox (%)	Observed X ^b	X predicted ^c	
				0.5 model Crosslinked	1.0 model Non-crosslinked
Pecan	20	18.3	0.55		
	40	23.7	0.71	0.65	1.30
	60	27.7	0.73		
	80	21.8	0.65		
Myrica	20	19.0	0.57		
	40	23.3	0.70	0.70	1.40
	60	25.3	0.76		
	80	22.3	0.67		
Mimosa	20	10.0	0.30		
	40	17.0	0.51	0.73	1.45
	60	20.0	0.60		
	80	16.7	0.50		
Quebracho	20	9.0	0.27		
	40	16.7	0.50	0.60	1.20
	60	18.3	0.55		
	80	20.0	0.60		
Gambier	20	9.0	0.27		
	40	15.0	0.45	0.66	1.33
	60	15.0	0.45		
	80	13.7	0.41		

a Tannin solution 30 g/l in different buffer solution, assuming the average monomer unit molecular weight is 300 D, then the concentration of monomer unit 0.1 mol/l, 1ml of 3 mol/l oxazolidine was added to 10 ml tannin solution, monomer unit: oxazolidine = 1:3

b X = moles of oxazolidine consumed per monomer unit

c Predicted X, assuming 0.5 or 1.0 mole oxazolidine consumed per reactive site on the monomer molecule, 0.5 model means all reactive sites have been cross-linked

The calculation of the reaction stoichiometry was mainly based on the theory proposed by Porter²⁰². There are two possibilities in the reaction of tannins with oxazolidine: one is mono-point combination, when only one of the functional groups of oxazolidine reacts with phenols, the second is cross-linked combination, when each oxazolidine can combine with two reactive sites. The results in Table 4.19 indicate that the reaction mode is closer to the cross-linked model, in other words, both of the reactive sites on oxazolidine combine with proanthocyanidin monomer. This indicates that cross-linking forms between monomer units.

4.3.3.3.1 Effect of molecular structure of proanthocyanidins

Previous studies have shown that pecan and myrica tannins are both linear prodelphinidin polymers (Chapter 2). Compared with the branched structures of mimosa and quebracho tannins, these two prodelphinidin tannins can combine with more oxazolidine per monomer unit, because there is less steric hindrance and more reactive sites are available in the cross-linking process. From Table 4.18, it is clear that prodelphinidins do react with more oxazolidine than procyanidins, prorobinetinidins or profisetinidins.

Porter studied the reaction of procyanidin polymer, oligomer and monomer with formaldehyde by measuring the consumption of formaldehyde²²², assuming the reaction only happened at A-ring: effective substantial cross-linking could be produced when polymerisation is lower than 6-7 units, above that the reaction is mainly mono-point. The failure of cross-linking was thought to be due to the short bridge length of formaldehyde²²². The average polymer chain lengths of pecan, myrica, mimosa, quebracho and gambier are 10, 7, 5, 5, and 3 flavanoid units respectively: each unit has one reaction site at the A-ring, with the exception of the upper terminal units which have two. For pecan, myrica and mimosa tannins, the B-ring should also be considered. In this work, the reactivity of the gallocatechin A-ring is four times that of the B-ring, so the pyrogallol B-ring sites may be treated as one quarter of a reaction site. The quebracho and gambier tannins reactive sites were calculated as described by Porter, assuming the catechol B-ring is non-reactive. From Table 4.17, X was defined as the amount of oxazolidine reacted with each

monomer unit. From the observed X values, all the tannin reactions were in good agreement with the 0.5 mole model, which indicated effective cross-linking is formed, even for high polymers of pecan tannins. The X values of pecan and myrica tannins are slightly higher than the predicted value from the 0.5 model, meaning some of the cross-linking failed to form when the distance between two available reactive sites is too long. But for the lower molecular weights of mimosa and quebracho, the observed X is a little lower than the predicted value. This may be caused by the low reactivity of the resorcinol A-ring. After completion of the reaction, the data imply that all the reactive sites have cross-links. Porter has reported that each unit of *Chaenomeles speciosa* polymers (where polymerisation is as high as 10) can consume 0.96 moles of formaldehyde, which is close to the 1 mole model prediction of 1.08, as this indicates cross-linking formation can occur in this high polymer. Compared with formaldehyde, oxazolidine has shown good ability in cross-linking pecan tannins, where polymerisation is also 10.

4.3.3.3.2 Effect of temperature

The reaction of polymers is more dependent on temperature compared with the simple phenols and flavanol monomers. The oxazolidine consumption by polymers is much lower at room temperature, 25°C; it cannot reach the same level as for monomers, even at 60°C and a long time (720 hours). This indicates the significant effect of the molecular weight or the chain length of phenols on the reaction: the higher molecular weight, the more steric effects there are on the cross-linking reaction. The reaction has to be forced by heat to complete and the relatively long bridge chain of ring-opened oxazolidine makes further cross-linking possible for separated reaction sites, but the short chain of formaldehyde cannot reach to form bridges. But, if the temperature is higher than 80°C, the consumption of oxazolidine is reduced. The influence of temperature on the consumption oxazolidine is shown in Fig 4.38.

All the tannins showed a reduction in oxazolidine consumption at high temperature, 80°C. The highest values are always observed at 40-60°C. At high temperature, the

reaction becomes fast and less oxazolidine is needed because the gel forms quickly, making some of the reactive sites unavailable.

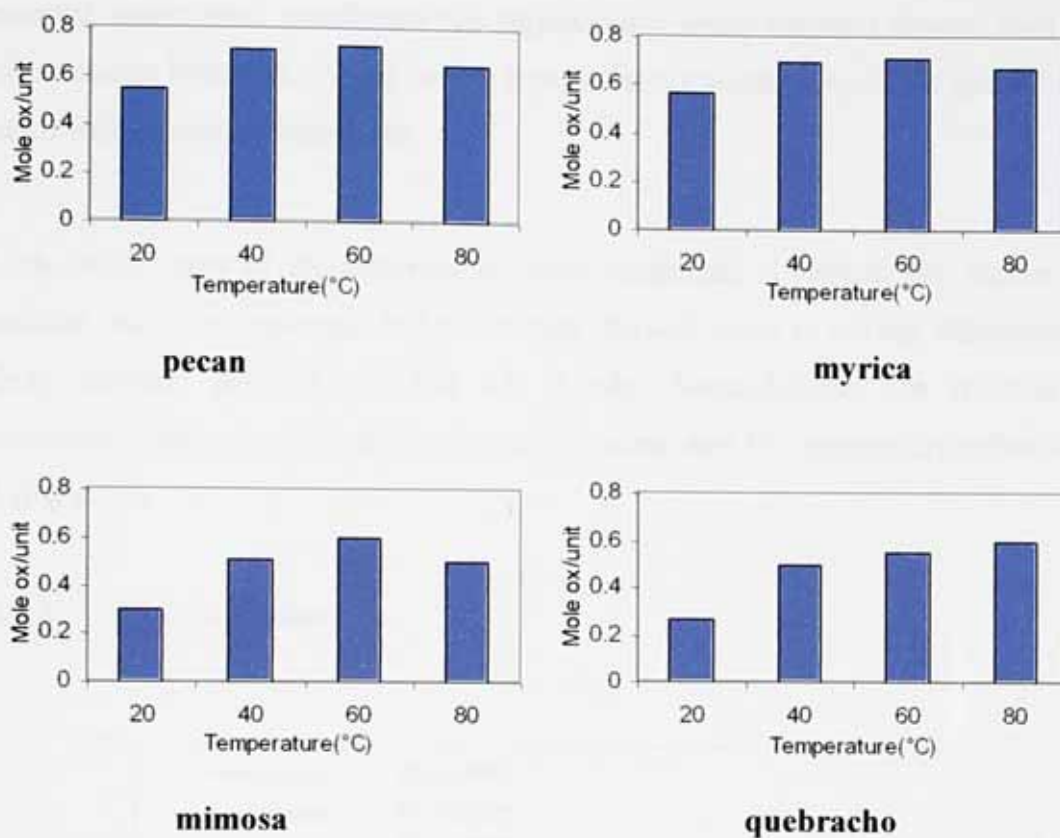


Fig 4.38 Consumption of oxazolidine by condensed tannins

4.3.3.3.3 Effect of pH

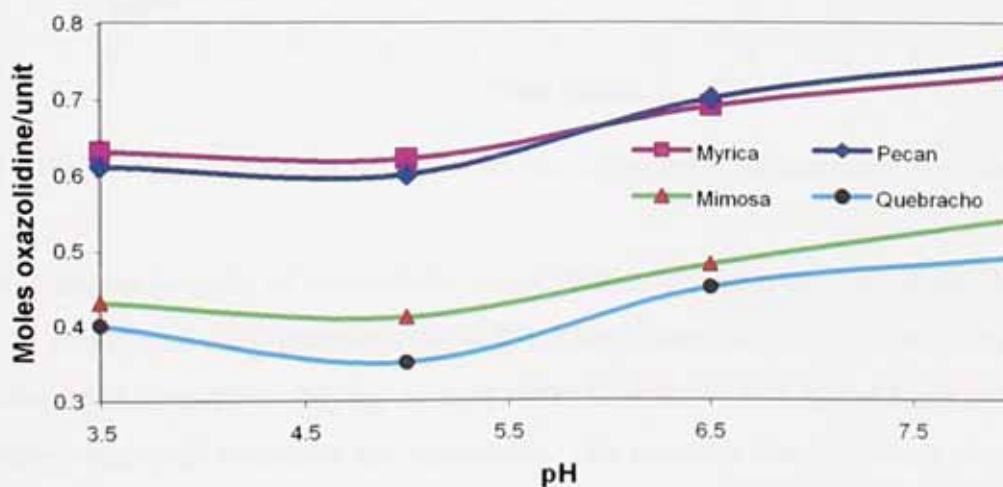


Fig 4.39 Effect of pH on the consumption of oxazolidine

As Pizzi and MacGraw^{223,231,232} have pointed out, pH has always been a key when studying the reaction of phenols with aldehyde cross-linking agents. Basic conditions increase the reactivity of the phenol ring system. The oxyanions generated under basic conditions are significantly better electron donors than the corresponding hydroxyl groups, which means the nucleophilicity of the phenol ring system will increase substantially.

In this work, most of the experiments were conducted at neutral pH, higher pH condition was also explored, but the results showed there is no big difference in effects between pH 6.5 and pH 8.0. Unlike formaldehyde, the reaction of oxazolidine with proanthocyanidins is mainly controlled by temperature rather than pH (Fig 4.39).

4.3.3.4 Kinetic studies

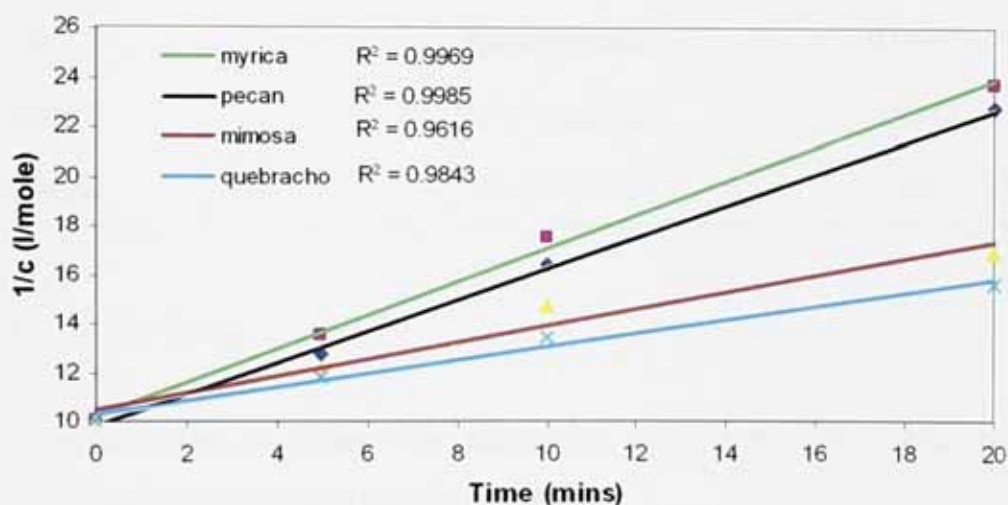


Fig 4.40 Kinetic plots of the reactions of four condensed tannins with oxazolidine

Plots of the reciprocals of oxazolidine concentration versus time were linear for the first 10 minutes of each reaction (Fig 4.40). These observations may be interpreted in terms of the reaction obeying second order kinetics in the initial stage of these processes when the reactants are equimolar. The reaction then becomes slow; the reaction of proanthocyanidin with oxazolidine also becomes very complex after 30 minutes under these conditions. The reaction rate constants are shown in Table 4.19.

As expected, k values for these proanthocyanidin polymers are much lower than for the related monomers, which might be explained by the fact that they possess fewer reaction sites per monomer unit than monomers and also by the steric hindrance effect.

Porter has reported the reaction rate constant of procyanidin polymers with formaldehyde is $0.0036\text{M}^{-1}\text{s}^{-1}$ at pH 8.0 and 30°C and for the dimers of procyanidins it is $0.095\text{M}^{-1}\text{s}^{-1}$ under the same conditions. Proanthocyanidin polymers ($D_p = 10$) pecan tannins have a rate constant $0.0443\text{M}^{-1}\text{s}^{-1}$ at pH 6.5 and $0.0591\text{M}^{-1}\text{s}^{-1}$ at pH 8.0 at 30°C , showing the reactivity of oxazolidine is nearly twice that of formaldehyde reactivity under similar conditions.

Table 4.18 Rate constants for proanthocyanidin polymer reaction with oxazolidine, at pH 6.5, ratio 1:1, $20.0\pm 0.2^\circ\text{C}$

Tannins	Flavonoid type	k ($\text{M}^{-1}\text{s}^{-1}$)
Pecan	Prodelphinidin	0.0443
Myrica	Prodelphinidin	0.0480
Mimosa	Prorobinetinidin	0.0072
Quebracho	Profisetinidin	0.0067

4.3.4 Summary

The current studies confirmed that oxazolidine is a better cross-linking agent for proanthocyanidins polymers than formaldehyde.

- Effective cross-linking can be obtained even when the degree of polymerisation is higher than 7-10, but formaldehyde cross-linking fails at proanthocyanidin chain length of 6-7. More oxazolidine is consumed than formaldehyde, which produces higher cross-linking density in the condensation products.
- The kinetic studies have also shown oxazolidine has higher reactivity than formaldehyde, the cross-linking reaction can be carried out at neutral pH and

room temperature; the reaction is second order at the first stage of the process, the optimum condition and pH for the reaction are 60°C and pH 6.0-7.0.

- The cross-linking reaction is probably of two types, proanthocyanidin A-ring to A-ring and A-ring to pyrogallol B-ring; the cross-linking between B-ring and B-ring is unlikely, due to the lack of reactivity of B-ring. Gallocatechin tannins have the highest reactivity among all types of condensed tannins, but the high molecular weight is a disadvantage.

Chapter 5

Reaction of collagen with oxazolidine

5.1 Introduction

There have always been arguments about the mechanism of collagen cross-linking reactions, including aldehyde, where and how the cross-linking reactions occur on the collagen molecules. Traditionally, it was believed that the reaction happens between the free amino groups of lysine residues or the terminal amino groups^{1,5,17,32}. High pH condition enhances the process, because the protonated amino group is believed to be unavailable for reaction with aldehyde; for the same reason, the guanidyl groups of arginine residues were believed to be non-reactive to formaldehyde at neutral pH. While many researchers have questioned this view^{47,49}, Gold, Smith, Albert, Peters and Taylor *et al.* proved by ¹³C-NMR that the cross-linking reaction of gelatine with formaldehyde happens between lysine and arginine side groups^{47,49-51}. Polylysine and polyarginine have been used as models to examine cross-linking with formaldehyde: there is no cross-linking formed between lysine and lysine or arginine and arginine and the only cross-linking observed is between lysine and arginine, but the reason is unknown⁵¹. There is evidence that the cross-linking reaction of collagen with formaldehyde does not link the neighbouring amino groups: the reaction is between one amino group and another non-amino group, such as a guanidyl group of arginine⁴⁹⁻⁵⁰. In recent diabetes mechanism studies, the guanidyl groups of arginine residues have been found to be more reactive towards dicarbonyl compounds, such as glyoxal or methylglyoxal^{53,81,83}, which also suggests the cross-linking reaction in collagen is not limited to the amino groups.

From the measurement of formaldehyde fixed on collagen, it has been shown that the reaction is mono-point combination, rather than multi-point cross-linking²³⁰, although more work is still needed to confirm this, because formaldehyde may be present as oligomers in concentrated aqueous solution rather than monomers. It is also believed there is monomer formation in dilute formaldehyde aqueous solution, but a similar result was obtained from low concentration of formaldehyde. Now, a query arises here, is there any cross-linking or not? No matter which is the right answer, the mechanisms of reaction of collagen by aldehyde compounds and their effect on collagen stability are far from well understood.

In this work, we have studied a relatively new cross-linking agent – oxazolidine, 5-ethyl-1,3-dioxacyclo[3.3.0]octane: it was synthesised from 2-amino-2-ethyl-1,3-dipropanol and formaldehyde (Fig 3.1, as discussed in detail in Chapter 3). It has shown good cross-linking ability, the shrinkage temperature of oxazolidine tanned leather increases from 60°C (pickled skin) to 86°C, but the reaction mechanism is unknown at present. In this part of work, the following questions concerning for oxazolidine as a cross-linking agent is addressed: first, where is the reaction site and second, how many cross-linking bonds are formed in this process?

5.2 Experimental

5.2.1 Collagen materials

In research on collagen cross-linking, the collagen material adopted is usually varied according to the application background. In leather chemistry studies, the most convenient raw material is animal skin or hide powder and untreated white hide powder can be regarded as a relatively pure collagen¹⁷. As for photographic film or drug delivery purpose, the most used material is gelatine^{50,51}, which is heat denatured collagen, the chemical composition can be considered to be the same, with the same amino acid sequence and peptide chains, but the conformation of the collagen fibre structure has been modified or even partially destroyed. In biomaterials studies, the most used collagen materials are water or acid soluble collagen, usually obtained from young animal skin or tendons, when the triple helix structure remains intact. Acid soluble collagen can still form collagen fibres under physiological conditions and display typical collagen fibre structure characteristics^{14,278}. The difference between normal skin collagen and acid soluble collagen is that there are less cross-linking bonds in acid soluble collagen and this is why it can dissolve in water¹. In this work, white hide powder and skin have been employed; gelatine was not used because the collagen has been denatured and the conformation or configuration of collagen molecules is no longer intact. There are a few advantages to using skin or hide powder, for example it is convenient to determine the absorption of cross-linking agents or to study the physical properties of cross-linked products. While the effect of the physical structure of skin on the

reaction of collagen with cross-linking agents is unclear, there is always a surface binding-penetration problem. Acid soluble collagen can be a useful substitute for skin to solve this problem. Therefore, in this chapter, white hide powder has been the main collagen material model for reacting with oxazolidine, but acid soluble collagen also was used in mechanistic studies of the reaction.

5.2.1.1 Preparation of white hide powder

Hide powder was made from calf skin using the official method (SLC 10). Briefly, calf skin was pretreated with a series of processes, which including soaking, unhairing, liming, deliming, bating and pickling. After these steps, most of the non-collagenous components had been removed, so relatively pure collagen was obtained, supported by hydroxyproline content determination. The hide was dehydrated four times with dried acetone, then dried under vacuum at room temperature and ground to prepare white hide powder.

5.2.1.2 Preparation of acid soluble collagen

Rat tail tendons were carefully drawn out from tails of six months old rats and then soaked in 0.5mol/l acetic acid overnight at room temperature (17-19°C). The collagen solution was filtered with glass wool to remove undissolved tissues, and then the grease or fat components were removed by extracting with chloroform twice to give a clear solution. After the organic solvent was removed through rotary vacuum evaporation, the pH was slowly increased to 7.4 with concentrated sodium hydroxide solution, 3mol/l. The solution was left overnight at 30°C, when most of the collagen precipitated out and the supernatant solution was discarded. The precipitate was dialysed against distilled water to remove acid and other small molecules or salt, changing the water four times at 12 hours intervals. Semi-permeable membrane tubes (diameter 2.5 cm) with molecular weight 10,000D cut-off were used in the process. Finally, the collagen was freeze-dried and kept at -4°C for further analysis.

5.2.1.3 Measurement of collagen purity

(1) Hydroxyproline content

Hydroxyproline (Hyp) is a post-translational product of proline hydroxylation catalyzed by an enzyme prolyhydroxylase. The occurrence of this imino acid is thought to be confined almost exclusively to the connective tissue collagen^{2,35}. Because of its restricted and unique distribution in connective tissue collagen, a useful indicator for collagen purity analysis is to measure the hydroxyproline content. In 1980, Huszar described a simple procedure for determining Hyp in order to monitor collagen and its fragments²⁷⁰.

I. Digestion for hydroxyproline determination

The procedure is based on acid hydrolysis of the tissue homogenate and subsequent determination of free hydroxyproline in hydrolysates. Chloramine-T was used to oxidise the free hydroxyproline to a pyrrole. The addition of Ehrlich's reagent results in the formation of a chromophore that can be measured at 555nm. Pure hydroxyproline solution was used to make the calibration curve.

0.200-0.400 grams of freeze-dried collagen was weighed into a digestion tube, 10ml of 50% hydrochloric acid were added and the tube was immediately closed with a lid containing a PTFE insert. Then the hydrolysis process was conducted for 16 hours at 100°C. The hydrolysate was cooled down to room temperature and transferred to a 100ml volumetric flask, which contained 40ml distilled water. The tube was washed with distilled water, these washings were transferred to the volumetric flask, then the volume was brought to the mark with distilled water.

II. Measurement of hydroxyproline

Materials and methods:

Chemicals: Chloramine-T, p-dimethylaminobenzaldehyde, L-hydroxyproline were citric acid, perchloric acid, n-propanol, sodium acetate, trisodium citrate were analytical grade, purchased from Sigma Chemical Co.

Preparation of reagent

Hydroxyproline stock

A solution containing 100mg/l of hydroxyproline was prepared in distilled water.

Chloramine T

1.4g chloramine T was dissolved in 20 ml of distilled water, then made up to 100 ml with buffer solution. The buffer solution was prepared as below:

sodium acetate anhydrous 34.4g
trisodium citrate 37.5g
citric acid 5.5g
400ml propan-2-ol

The salts were taken up in 300-400ml distilled water, propan-2-ol was then added, the volume was then made up to 1 litre with distilled water (solution stable for 1-2 months at room temperature.)

Ehrlich's reagent

dimethylaminobenzaldehyde 24 g
60% perchloric acid 36 ml
propan-2-ol 200 ml

The solid was dissolved in 60% perchloric acid and propan-2-ol was added immediately prior to use.

The calibration curve was created with diluted standard solutions at 2.5, 5.0, 10, 15, 20mg/l with distilled water, using distilled water as blank. 0.55ml of sample solution was put into a 5ml tube, 1.27ml of 2:1 propan-2-ol:water was added, 0.88ml Chloramine T was added, mixed and the mixture was left for 5 minutes at room temperature, then Ehrlich's reagent 2.3ml was added, mixed and kept in a 70°C oven for 10 minutes, then the absorbance at 555 nm was measured using a Philips PU8700 UV-Vis spectrophotometer. The hydroxyproline content of the collagen digestion solution was measured by the same method. For hydroxyproline analysis of hide powder, a similar method was employed.

(2) Total nitrogen

The nitrogen content was determined by the standard Kjeldahl method, SLC6.

(3) SDS-PAGE

The purity of isolated soluble collagen was analysed by sodium dodecyl sulphate poly-acrylamide gel electrophoresis (SDS-PAGE) Acid soluble collagen was denatured by boiling in water for 10 minutes under reducing conditions (5% v/v 2-mercaptoethanol). The denatured collagen solution was loaded on a 12% w/v gel and visualised by staining with Coomassie Brilliant Blue (CBB) R-250 solution (0.1% w/v)⁴³.

5.2.2 Reaction of amino acid with oxazolidine

Two basic amino acids (lysine and arginine) and two neutral amino acids (glycine and alanine) were studied. The amino acids were dissolved in acetate buffer at 0.1mol/l and pH 6.0. Oxazolidine was diluted in acetate buffer to 5mol/l at pH 6.0. 5ml of amino acid solution was transferred to a 25ml flask, then oxazolidine solution was added and the volume was adjusted to 25ml with buffer solution. The reaction was carried out for two hours in water bath at $60.0 \pm 0.2^\circ\text{C}$. The amount of oxazolidine capable of combining with each amino acid was measured by adding 0.25, 0.5, 1, 2, 4, 6, 8, 10 and 12 times of the molar equivalent of the oxazolidine to the amino acid solution.

The reaction was monitored by measuring the free amino acid in the system by the ninhydrin method and TLC, oxazolidine was measured by GC-MS.

5.2.2.1 Ninhydrin reaction

2ml of amino acid-oxazolidine reaction mixture was transferred into a 10ml tube and 5ml of 2% ninhydrin acetone solution was added, the tube was sealed and put into a water bath at 90°C for 30 minutes until a purple-red colour was obtained²⁸⁰. Then the liquid was cooled on ice and the absorbance at 555nm was measured by a Philips PU8700 UV-Vis spectrophotometer.

5.2.2.2 Thin layer chromatography

5-10 μ l of reaction mixture was transferred onto chromatography paper (Whatman No.1). After drying in air, the paper chromatogram was developed by BAW (n-butanol:acetic acid:water 4:1:2, v/v/v). The result was determined by spraying ninhydrin acetone solution on the paper and drying in an oven at 100°C for 10 minutes until the purple colour was fully developed, then the colour intensity of each point was compared with standards, using a chroma meter with a xenon lamp, Minolta (Osaka, Japan).

5.2.3 Reaction of collagen with oxazolidine

5.2.3.1 Reaction of intact collagen with oxazolidine

The cross-linking reaction was conducted in different buffer solutions (sodium acetate for pH 3.0, 4.0, 5.0 and 6.0; potassium phosphate for pH 9.0) at 20, 40 and 60°C. One gram of collagen material (white hide powder or acid soluble collagen) was suspended in 20ml of buffer solution for 12 hours before adding oxazolidine. The offer of oxazolidine was 2, 4, 6, 8, 10, 12 and 20% based on the dry weight of collagen or hide powder. The residue of oxazolidine in solution was measured after removing the collagen solid by centrifugation and filtration; hide powder or soluble collagen solution without oxazolidine were used as controls. The residual oxazolidine was measured by GC-MS.

5.2.3.2 Reaction of modified collagen with oxazolidine

The reaction sites on collagen with oxazolidine can be confirmed by using modified collagens; two modifications were used in this work.

(1) Deamination

Deaminated collagen was prepared as described by Hu¹⁴. Nitrous acid solution with a final pH of 3.6 was freshly prepared by mixing equal volumes of aqueous 1mol/l acetic acid and 1mol/l sodium nitrite. The collagen was suspended in the solution for

48 hours at 24°C. After neutralisation to pH 6.8, the collagen was obtained through centrifugation at 3,500g for 20 minutes, then freeze-dried.

(2) Blocking of arginine residues

1,2-Cyclohexanedione (CHD) was used to block the guanidine groups of arginine residues as described by Hu¹⁴. Collagen was suspended in 0.5mol/l CHD phosphate buffer solution and then the pH value was adjusted to 12.0. The solution was kept for 20 days at 4°C. The pH of the collagen solution was then reduced to 6.8 and centrifuged at 3,500g for 20 minutes, then freeze-dried.

5.2.3.3 Reaction characterisation

(1) Microscopy

Scanning electron microscopy (SEM): Freeze-dried acid soluble collagen film, before and after cross-linking with oxazolidine, was mounted on stubs and covered with an ultra thin layer of gold in a Polaron E5100 SEM coating system. Samples were studied with a JEOL JSM-6310 SEM (Oxford) microscope at an accelerating voltage of 10 kV.

(2) Differential Scanning Calorimetry (DSC) analysis

The denaturation temperature (Td) of hide powder, before or after cross-linking with oxazolidine, was determined using a Perkin-Elmer DSC-7. 10-15mg of wet sample (moisture content around 85%) was sealed in standard aluminium DSC pans. The denaturation was performed by heating at a rate of 5°C/min from 35°C to 120°C. The sample pans were then dried overnight in an oven at 100°C, and then the dried weight of collagen was recorded. The Td was recorded as onset temperature and denaturation enthalpy change was calculated based on the dried weight of collagen material.

(3) Enzyme degradation of collagen

Cross-linked or untreated soluble collagen samples, 0.1g dry weight, were suspended in 15ml of 0.1M phosphate sodium buffer solution (pH 7.6) for 4 hours at $37.0 \pm 0.2^\circ\text{C}$, then 1% of trypsin (1g/l solution, buffer pH 7.6) was added and the mixture kept for 22 hours at the same temperature. The reaction was stopped by cooling on ice to 0°C , followed by centrifugation for 30 minutes at 3,000g. The precipitate was washed twice with cold water and then freeze-dried. The dried samples were hydrolysed with 50% hydrochloric acid for 16 hours at 100°C for hydroxyproline analysis and then the proportion of degraded collagen was calculated from the hydroxyproline content.

(4) Determination of free amino groups and arginine groups by 1-fluoro-2,4-dinitro-benzene

Amino group: After cross-linking, 0.1g of dried hide powder was suspended in 10ml of 10% sodium bicarbonate (pH 8.5), then 1ml of 10% 1-fluoro-2,4-dinitrobenzene (FDNB) in ethanol was added. The solution was shaken for 2 hours at 20°C in the dark, then filtered and washed with water, ethanol and ether respectively to remove the excess FDNB. The dried hide powder was hydrolysed with 5mol/l HCl for 16 hours at 110°C . 1ml of hydrolysate was diluted to 5ml, extracted with ether five times to remove the DNP- α -amino acid and the DNP-lysine was measured by UV/Vis spectroscopy at 300nm and $364\text{nm}^{250,254}$.

Guanidyl group: 0.1g of cross-linked hide powder was hydrolysed with 5mol/l HCl for 16 hours at 110°C . 1ml of hydrolysate was dried under vacuum at 60°C and then dissolved in 2ml of 10% sodium bicarbonate, pH was adjusted to 9.0 and then 2ml of 10% FDNB was added and reacted for 3 hours in the dark. The solution was extracted with ether five times to remove FDNB and then concentrated HCl was added to give 1.0 mol/l. The solution was then extracted with ether five times to remove DNP- α -amino acid, when only DNP-arginine and DNP-histidine were left in solution, measured by UV/Vis spectroscopy at 375nm for DNP-arginine, using a sample without collagen hydrolysate as blank^{250,254,257}.

5.3 Results and discussion

5.3.1 Analysis of collagen materials

The hydroxyproline and total nitrogen content of acid soluble collagen or hide powder is shown in Table 5.1. Compared with reported results, this showed that the acid soluble collagen is close to 100% purity and the hide powder has about 86% collagen substance.

Table 5.1 Analysis results of collagen materials (% on dry weight)

	White hide powder		Acid soluble collagen	
	Experimental	Literature ^{34,6}	Experimental	Literature ^{34,6}
Hydroxyproline	13.8	14.0	12.1	12.1
Total nitrogen	17.9	18.0	15.4	18.0

The purity of the soluble collagen was also confirmed by SDS-PAGE, which indicated the isolated soluble collagen is free of other protein: there were two clear bands for $\alpha_1(I)$ and $\alpha_2(I)$ respectively and some high molecular weight bands for β dimer and λ trimer chains (Fig 5.1).

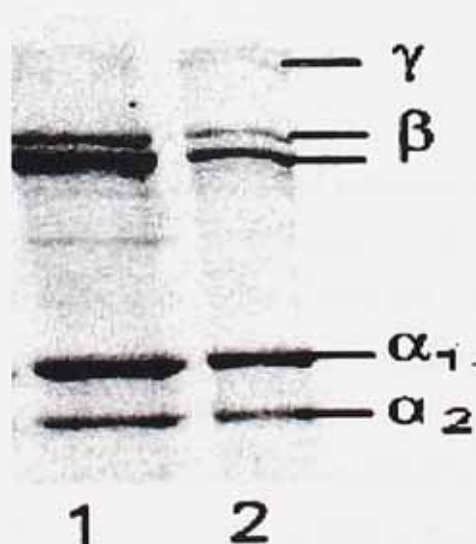


Fig 5.1 SDS-PAGE of acid soluble collagen

5.3.2 Reaction of amino acid with oxazolidine

The amino acid composition in the hide powder or soluble collagen is fundamental for its chemical reactivity, Table 5.2 is a typical amino acid analysis for type I collagen: the two main basic amino acid and their distribution are the main reaction sites for aldehyde cross-linking. For one α chain of type I collagen, there are 34-36 lysine and hydroxylysine, 52-55 arginine.

Table 5.2 Main amino acid composition in type I collagen (α 1(I) chain)²⁶³

Amino acid	Amount per 1000 residues	Property
Lysine	25	Basic
Hydroxylysine	8	Basic
Arginine	52	Basic
Histidine	3	Basic
Glycine	335	Neutral
Alanine	120	Neutral
Proline	122	Neutral
Hydroxyproline	94	Neutral
Glutamic acid	76	Acidic
Aspartic acid	46	Acidic
Asparagine		Neutral
Glutamine	45	Neutral

As discussed in Chapter 3, oxazolidine hydrolysis produces two N-hydroxymethyl groups, which act as strong electrophilic agents to react with nucleophilic groups in collagen. There are two main basic amino acids in collagen, lysine and arginine (Fig 5.2). Disregarding the α -amino group, there is an ϵ -amino group in lysine and a guanidyl group in arginine. They have different pKa values, so different reactivity towards oxazolidine is anticipated. Based on traditional theory, the α -amino group is always more reactive than the ϵ -amino group and the guanidyl group, due to the high pKa values, so ionised amino or guanidyl groups are thought not to be reactive

to aldehyde. Considering their abundance in collagen, only lysine and arginine have been used as models for side chains of collagen. The N-terminal α -amino groups were modelled by glycine and alanine.

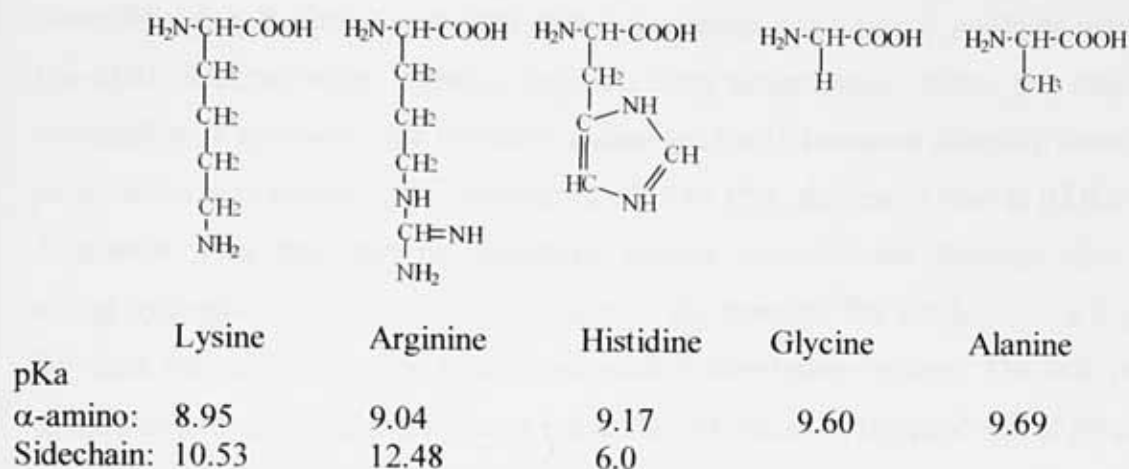


Fig 5.2 Structures and pKa values of basic and neutral amino acids²⁶³

No precipitation was observed in the reaction of amino acids with oxazolidine at neutral pH. The colourless solution was checked by TLC. Fig 5.3 is an indicative chromatogram of lysine-oxazolidine reaction products formed at different reactant ratios.

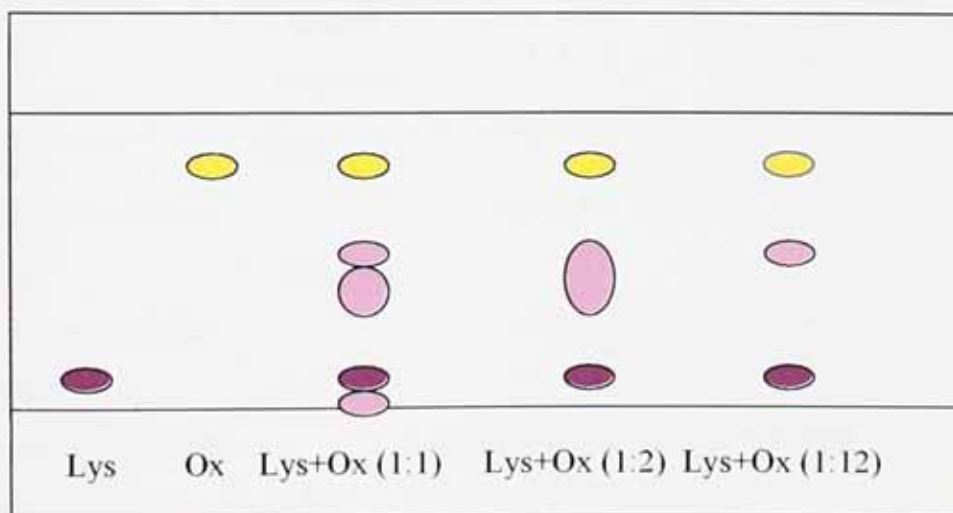


Fig. 5.3 Representation of paper chromatography of lysine reaction with oxazolidine (pH6.0, 60°C)

When the amount of oxazolidine is low, the products are a complex system, which can be seen from the chromatogram. Under the developing condition, four major spots at Rf 0.1, 0.2, 0.3-0.5 and 0.6 appeared when the ratio is 1:1; the spots at 0.2 and 0.6 are unreacted lysine and oxazolidine. The Rf 0.1 spot represents the complicated cross-linked products mixture, since high molecular weight results in a lower Rf value. It can be postulated that the complex cross-linked products may be Lys-Ox-Lys copolymers, because they are both bifunctional. When the ratio of oxazolidine is increased, the products system gradually becomes simpler; when the oxazolidine is in excess, oxazolidine:lysine 8:1 or 12:1, the major spot at Rf 0.3-0.5 dominates. This spot may be monopoint reacted intermediates, because after the strong hydrophilic amino group is blocked in the reaction, the product has a higher Rf value than original lysine in the hydrophobic developing system. The behaviour of the other amino acids is similar to lysine: the Rf values of the products of glycine, alanine and arginine are displayed in Table 5.3.

From Table 5.3, the amount of oxazolidine consumed varies according to the amino acid. This can be further quantitatively expressed by measuring how much amino group and oxazolidine take part in the reaction.

Table 5.3 Rf values of amino acid-oxazolidine product (molar ratios 1:1, 1:2 and 1:12)

	Before reaction	1:1	1:2	1:12
Glycine	0.28			
Glycine+oxazolidine		0.50,0.57	0.50	0.50
Alanine	0.36			
Alanine+oxazolidine		0.48,0.57	0.48	0.48
Arginine	0.17			
Arginine+oxazolidine		0.01,0.40,0.49,0.57	0.40,0.48	0.48
Lysine	0.16			
Lysine+oxazolidine		0.01,0.33,0.50	0.33,0.50	0.50
Oxazolidine	0.57			

For glycine, nearly 80% of the amino groups reacted when 50% oxazolidine was reacted; for alanine, 75% reacted under same conditions, which indicates the reactivity of α -amino groups is similar in all of the amino acids. For basic amino acids, when the oxazolidine offer is 50%, 40-45% of the α -amino group on lysine and arginine are reacted (Fig. 5.4), which indicates that the ϵ -amino group and the guanidyl group react with oxazolidine at similar rates.

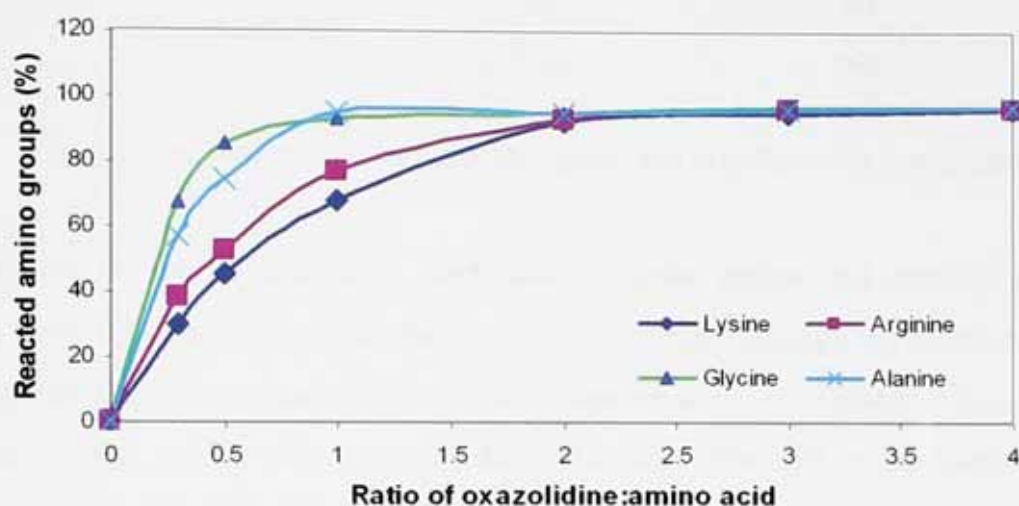


Fig 5.4 Amino acids reacted with oxazolidine measured by ninhydrin

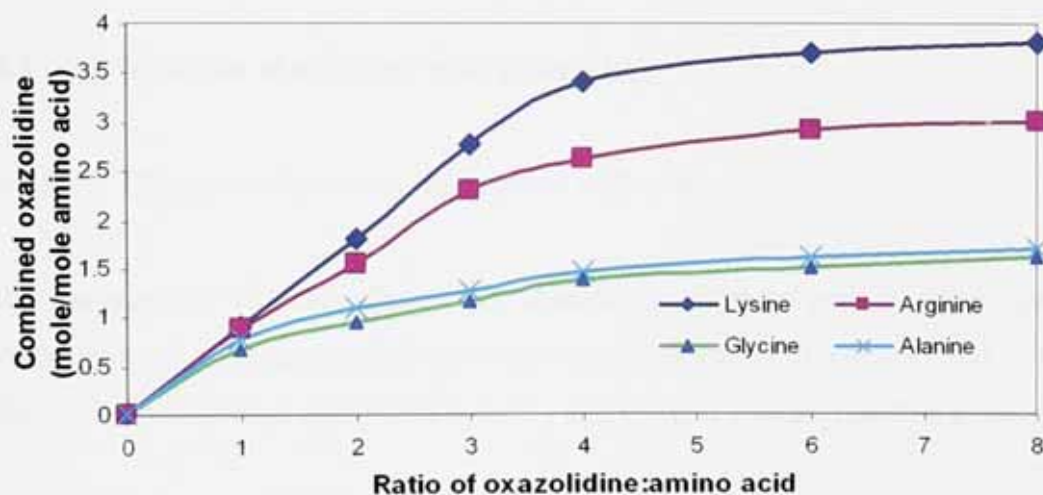


Fig. 5.5 Combination of oxazolidine on amino acids

From the consumption of oxazolidine (Fig. 5.5), 1 mole of lysine and arginine can combine with 3.8 and 3.0 moles of oxazolidine respectively, when the ratio of oxazolidine to amino acid is 8:1. Under the same conditions, the α -amino group of glycine and alanine can combine with 1.6-1.7 moles of oxazolidine, which means

the ϵ -amino group can react with 2 moles of oxazolidine, if the α -amino groups can be considered as similar for all the amino acids. The guanidyl group can react with 1.3 moles of oxazolidine, when the reactant ratio is higher than 8:1. The model for reaction between amino acid and oxazolidine can be described as set out in Fig 5.6.

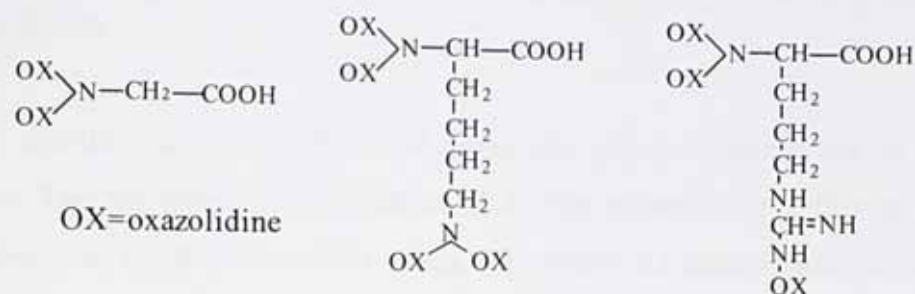


Fig. 5.6 Reaction products of glycine, lysine and arginine with oxazolidine

Based on the amino acid study, electrophilic ϵ -amino groups and guanidyl groups can react with oxazolidine just like the α -amino group, although the reaction pH is far below their pKa values. The ϵ -amino groups are even more reactive than the α -amino groups and the difference may be due to the steric effects in the conformation of amino acids. The long sidechain of lysine or arginine on the collagen peptide chains may be more easily reached by oxazolidine.

5.3.3 Reaction of collagen with oxazolidine

5.3.3.1 Characterisation of the reacted collagen

After the reaction with oxazolidine, the character and the properties of collagen are greatly changed, which can be observed directly under the microscopy, and it is also reflected in the increase of thermal stability and enzyme bio-degradation stability.

Freeze-drying of collagen solution results in a network structure, in which different structural characteristics are determined by the solution condition (pH value or salt) and the microenvironment of collagen fibres^{256,259,260}. In collagen based biomaterials research, it has been typical to observe the change in the appearance of the freeze-dried collagen material before and after modification. After cross-linking, the porous surface of collagen is changed, a completely collapsed fibrous structure was

observed (result see Appendix VIII), with the fibres all stuck together, which is probably due to the increase in hydrophobic function after losing the polar groups¹⁵. When the strong polar groups, such as amino and guanidyl groups, are deactivated through cross-linking reaction with oxazolidine, this will result in a more hydrophobic environment around the collagen fibres and change the interactions between chains.

Thermal stability is one of the most important physical properties of collagen materials. The denaturation temperature (Td) can reflect the stability of collagen under controlled conditions and Td increase is usually a critical feature of tanning or cross-linking agent reaction. Similarly, enzyme degradation of the collagen material is also a parameter of its stability. Trypsin breaks the amide bond after the basic amino acids, such as lysine and arginine, modification or cross-linking on these amino acid residues will prevent this process happening²⁵⁹. As it can be seen from Table 5.4, oxazolidine treated collagen showed similar enhancement of thermal stability and enzyme degradation stability to formaldehyde and glutaraldehyde.

Table 5.4 Stability of collagen tanned by different methods

Tannage	Reagent offer (%)	Td (°C)	Trypsin degradation (%)
None	0	60	9.5±0.3
Oxazolidine	4	86	3.3±0.2
Formaldehyde	8	85	3.2±0.2
Glutaraldehyde	10	82	3.6±0.2

Compared with formaldehyde and glutaraldehyde, oxazolidine is more effective in blocking the basic groups on collagen, resulting in low collagen loss in the enzyme degradation process, so less oxazolidine is needed to achieve the same effect obtained from formaldehyde and glutaraldehyde. Both oxazolidine and glutaraldehyde are difunctional cross-linking agent, but there is an apparent difference between them, which indicates they may have different reaction mechanisms.

5.3.3.2 Optimum conditions for the reaction

5.3.3.2.1 Effect of the offer of oxazolidine

From Fig 5.7, the thermal stability of collagen increases with increasing oxazolidine offer from 0 to 4%, but the Td decreases with increasing offer above 4%. When the offer of oxazolidine was 4-5%, the highest Td, 86°C was obtained. When the offer was 10-20%, the Td reduced to 80°C. This may be due to different reactions modes, cross-linking or monopoint combination. When the oxazolidine offer is in excess, monopoint combination is more likely to be formed.

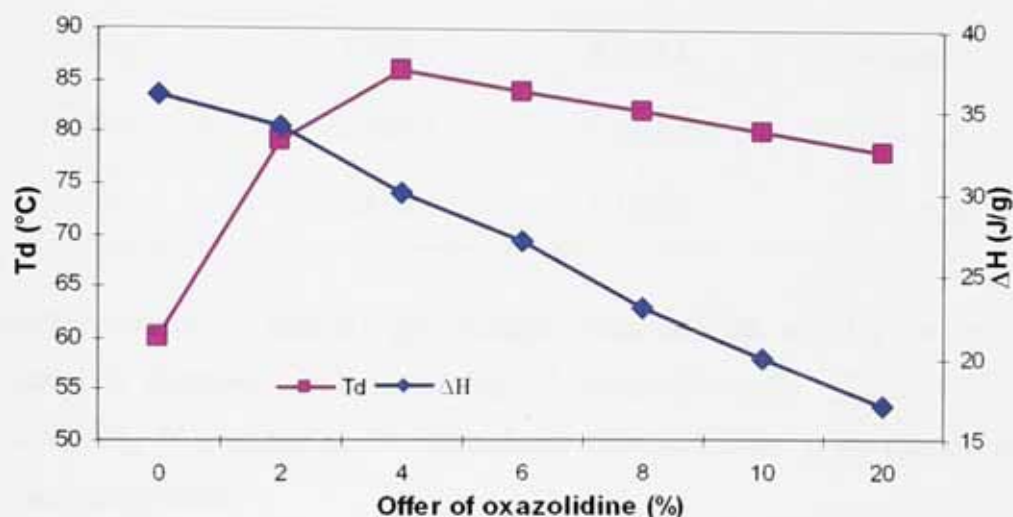


Fig 5.7 Denaturation temperature (Td) and the enthalpy of denaturation (ΔH) of oxazolidine reacted collagen

From Fig. 5.7, the enthalpy change in the denaturation process reduces after reaction with oxazolidine. For intact collagen, ΔH is 36J/g, but with increasing oxazolidine offer from 0 to 20%, the ΔH value reduced to 14J/g. The enthalpy change is determined by the type of bonds broken in the denaturation process; the increased stability may be associated with the presence of bonds that break exothermally¹¹⁻¹³. Hydrophobic bond rupture is an exothermic process, and such bonding should be formed after the reaction of collagen with oxazolidine, as the presence of increased non-polar interactions is consistent with the observed thermal effects (Fig. 5.8). This

indicates that the reacted collagen is stabilised by additional hydrophobic bonds after the polar groups are modified by oxazolidine²⁴¹. This is similar to results obtained from other aldehydes^{252,253}.

Table 5.5 Degradation of cross-linked or non cross-linked collagen (% w/w on dried weight of collagen) by trypsin (Data obtained as average of three measurements)

Cross-linker (%)	Oxazolidine	Formaldehyde	Glutaraldehyde
0	9.5±0.3	9.5±0.3	9.5±0.3
2	4.3±0.2	6.5±0.4	7.5±0.4
4	3.3±0.2	5.3±0.3	5.5±0.5
6	3.2±0.1	4.0±0.4	4.4±0.3
8	2.7±0.3	3.2±0.2	3.6±0.2
10	2.8±0.1	3.1±0.4	3.3±0.2

As another aspect of stability of collagen material, the stability to enzyme degradation is improved with increasing of oxazolidine offer (Table 5.5). The highest stability is obtained at 4% oxazolidine offer and there is no change when more oxazolidine used.

5.3.3.2.2 Effect of pH

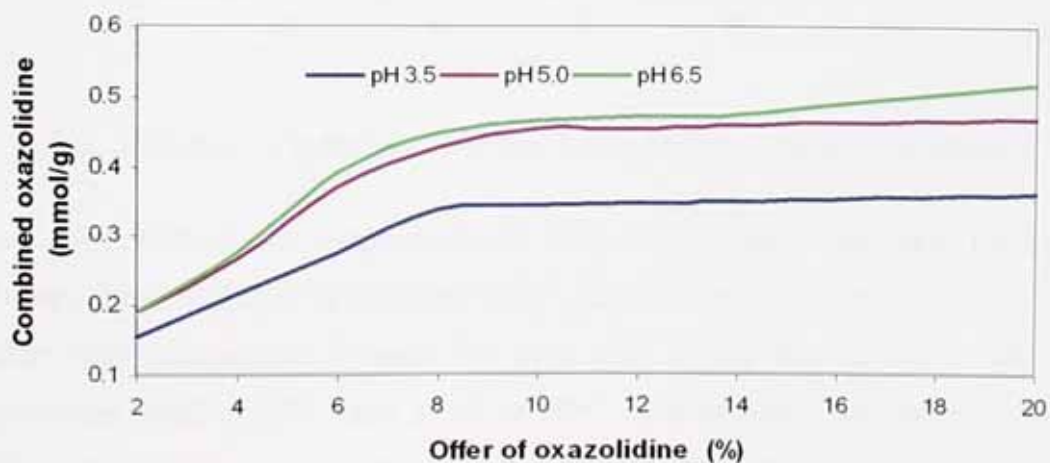


Fig 5.8 Effect of pH on the fixation of oxazolidine on collagen

As known in formaldehyde or glutaraldehyde tannage, basic pH condition enhances the reaction with collagen. The suggested pH for formaldehyde tannage is pH 8, as the free amino and guanidyl groups on collagen have high pKa values, as shown in Fig 5.2. But for oxazolidine, the amino acid experiment has proved that the reaction can be carried out even at pH 6.0. This has been confirmed from the collagen studies (Fig 5.8). Even at pH 3.5, oxazolidine can still combine at 0.3-0.4mmole per gram of collagen. With the increasing of pH, more oxazolidine can be fixed up to pH 6.0. After that point, no difference was observed.

5.3.3.2.3 Effect of temperature

In the study of combination tannage with condensed tannins and oxazolidine, Covington found that temperature is an important effect in the cross-linking process³⁰⁻³². After increasing the temperature from 20 to 60°C for 2 hours, the shrinkage temperature of leather is increased significantly.

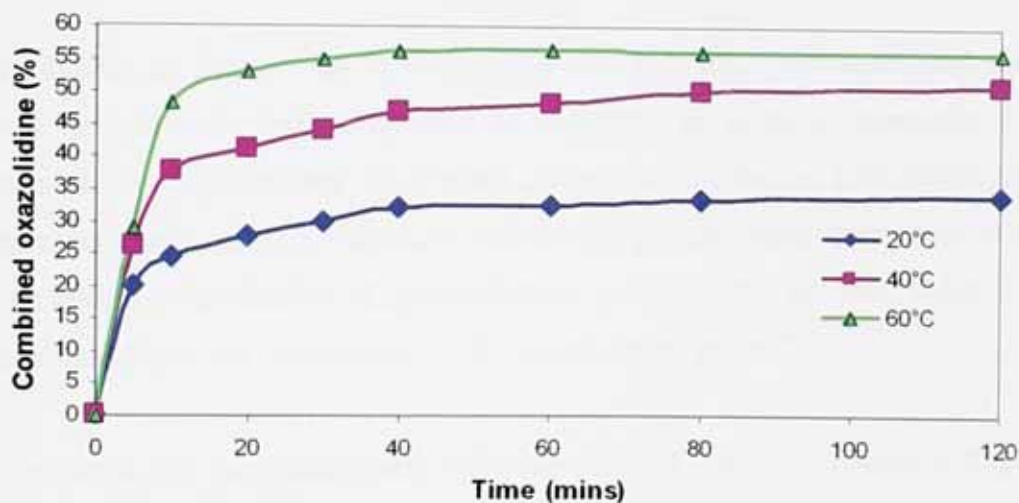


Fig. 5.9 Effect of temperature on the fixation of oxazolidine on collagen

From Fig 5.9, the higher temperature, the more oxazolidine is combined. The effect of temperature is similar to the effect of pH. Equilibrium can be quickly reached no matter what temperature is used, but even after a long time, there is still less oxazolidine fixed at 20°C than at 40 or 60°C. This indicates that there are some amino groups in the peptide chains are less reactive than others, perhaps because of the steric effect within the collagen fibre structure. This has also been shown in the

modification reaction of collagen, where not all the functional groups could be modified. Further studies have shown that no additional oxazolidine can be fixed above 60°C.

5.3.3.2.4 Effect of time

Considering the physical structure of collagen, the collagen reaction has two stages: first the reactants need to penetrate into the fibre structure and then fixation will take place. From Fig 5.9, it can be seen that the reaction does not reach equilibrium after 2 hours at 20°C. When the reaction temperature is increased to 40°C, the reaction is much faster, it takes 2 hours to complete the reaction, but it takes only 1 hour to reach the equilibrium point at 60°C. Due to the denaturation temperature of intact collagen at 60°C, it may be risky to use this temperature as the starting point. In practice, this process can be carried out at 20°C for 2 hours, followed by 60°C for another hour, when the highest denaturation temperature can be obtained.

5.3.4 Reaction mechanism

Oxazolidine is present as the monomer in solution, but formaldehyde and glutaraldehyde both form oligomers or polymers in solution especially at high concentration. Damink and co-workers found that pentamer glutaraldehyde, not glutaraldehyde monomer, combines with amino groups rather than cross-linking¹⁵. When a high concentration of glutaraldehyde is used in the process, about 3 moles of glutaraldehyde are fixed per mole of reacted amino group⁴⁴.

In contrast to the free amino acids, when the reaction happens between collagen and oxazolidine, it mainly takes place at the basic amino acid residues, but not α -amino groups, because they are blocked by the peptide bonds except for the N-terminal α -amino groups. Besides that, the distribution and amount of available reactive sites and the conformation of reactive groups on peptide sidechains have to be considered as well.

From Fig. 5.9, maximum oxazolidine fixation on collagen is about 5.6%. By assuming the molecular weight of one chain in the triple helix is 100,000, there are 34 side chain amino groups from lysine and 53 guanidyl groups from arginine. If only these reactive sites are counted, there are 87 sites available in each chain and 261 sites in a triple helix. 5.6% oxazolidine is equivalent to 117 moles per triple helix, so it can combine with 234 moles of reactive sites, if each oxazolidine reacts with two sites: if the histidine is also to be considered, then it indicates not all the reactive sites are reacted: if less than 80% of the reactive sites have been occupied, it is likely that monopoint combination is still present in the reaction process.

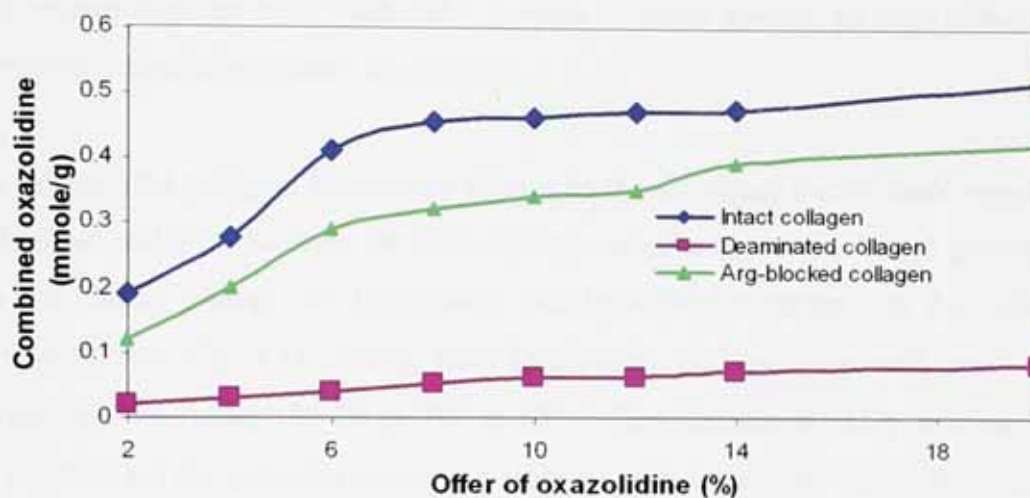


Fig 5.10 Effect of side chain modifications on the reaction of collagen with oxazolidine

Hu and co-workers¹⁴ have shown that deamination with nitrous acid and blocking of arginine with CHD is effective, by checking the amino acid composition. In this way, more than 85% of the amino groups and nearly 100% of the guanidyl groups are deactivated¹⁴. This has been confirmed by the FDNB experiments.

From the amount of oxazolidine fixed by deaminated collagen, it can be concluded that only a limited number of guanidyl groups can react with oxazolidine at neutral pH. This has been confirmed from the guanidyl-blocked collagen study, Fig 5.10, which showed higher oxazolidine fixation than in deaminated collagen. Fixation of oxazolidine on deaminated collagen is reduced by 75-80%. The results indicate that about 20% of all guanidyl groups take part in the cross-linking process at neutral

pH. Intact collagen can combine with 45-50 moles of oxazolidine per mole. After deamination, about 85% of the lysine amino groups are changed to hydroxyl groups and this reduces the combination of oxazolidine to 5-10 moles per mole collagen. This residual effect should be from the presence of guanidyl groups. Therefore, lysine amino groups fix 75-80% of the oxazolidine. If cross-linking is between lysine and arginine side chain groups, this observation will mean only few cross-links are formed in this process. If cross-linking can be formed between lysine and lysine or arginine and arginine, it is still limited. Considering 80% of the basic groups in collagen, this means there are about 60 sites available for reaction with oxazolidine, 45-50 moles of oxazolidine are combined on each chain, which means each oxazolidine can react with 1.2-1.3 moles of basic groups, so only 15% of the combined oxazolidine creates cross-linking.

The deaminated collagen showed no change in the shrinkage temperature compared with intact collagen, because the amino groups are changed to hydroxyl groups and this will hardly change the hydrophilic and hydrophobic balance in the collagen fibre structure. Fig 5.11 shows that deaminated collagen can still react with oxazolidine, increasing the Td by 7°C to 67°C. This increase is likely to come from the reaction on the guanidyl groups or other reactive sites. The Td of the guanidyl group modified collagen was not measured, because the collagen was already partly denatured by the modification.

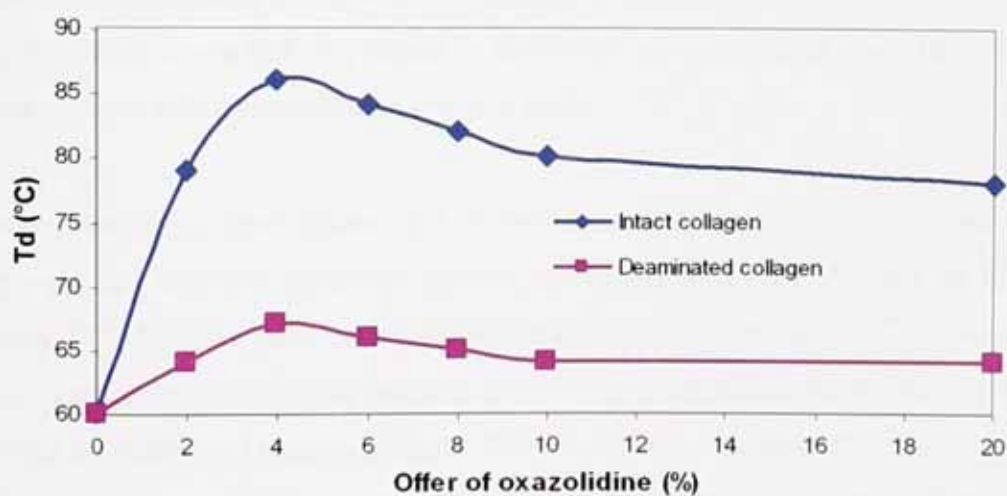


Fig. 5.11 Denaturation temperature of collagen after reaction with oxazolidine

Table 5.6 The effect of oxazolidine reaction on the basic groups in collagen and the associated denaturation temperature

Oxazolidine (%)	Free guanidyl groups per 1000 residues	Free amino groups per 1000 residues	Td (°C)
0	52±5	36±3	60
2	48±7	18±2	79
4	45±4	16±4	86
6	40±5	14±2	84
8	36±6	7±3	82
10	32±4	5±2	80

Free basic group analyses are displayed in Table 5.6. The amino group decreased from 34 to 6-7 per 1000 residues after reaction with 8% oxazolidine. This further indicates not all the amino groups can react with oxazolidine: only 80-85% of the amino groups reacted even with an oxazolidine offer of 20%. The guanidyl groups exhibit little change, compared with the amino groups. When the oxazolidine offer is 4%, 13.5% of guanidyl groups are reacted; this is similar to the result obtained from the cross-linking reaction of deaminated collagen. However, the FDNB method for guanidyl groups needs more work to confirm its reliability, because the stability of the reacted guanidyl group with oxazolidine in the hydrolysis process will affect the result. If the cross-linked product is destroyed by strong acid, then the result from this method will be higher than the true value.

Many researches have shown that cross-linking only happens between lysine amino groups and arginine guanidyl groups when formaldehyde is used as the cross-linker⁴⁷⁻⁴⁹, but few have addressed the reaction of glutaraldehyde⁴⁴. Oxazolidine is likely to undergo a similar reaction process to glutaraldehyde, so the cross-linking could be different to formaldehyde. The results here indicate that the lysine amino groups are responsible for reaction with oxazolidine, but most of them undergo monopoint combination rather than cross-linking.

5.4 Summary

Oxazolidine could be used as a new cross-linking agent in the leather or gelatine industries. 3-5% oxazolidine can be fixed on collagen, equivalent to 30-40 moles per mole collagen. High temperature or high pH enhances the reaction. More than 80% of the oxazolidine combines with lysine amino groups. Arginine guanidyl group fixes less than 20% of the oxazolidine and may take part in the cross-linking reaction. The high stability of collagen after reacting with aldehyde may arise from increasing hydrophobic function in the collagen structure or the disturbance of the supermolecular water layer^{32,277}, rather than cross-linking.

Chapter 6

Reaction between collagen, polyphenols and oxazolidine

Section 6.1

Mechanistic study of combination tannage of condensed tannins with oxazolidine

6.1.1 Introduction

Based on the studies of the reactions of polyphenol with oxazolidine (Chapter 4) and collagen with oxazolidine (Chapter 5), the objective of this chapter is to understand the reaction occurring between all three components: polyphenol, collagen and oxazolidine.

In the search for an alternative tanning system for chrome tannage so far, the condensed tannin-oxazolidine combination tannage might be regarded as one of the most promising choices, because it is based on renewable natural resources and environmental friendly materials, while high thermal stability can still be achievable^{5, 30-32}. However, the mechanism of this process has not yet been explored. In Chapters 4 and 5, it was demonstrated that gallocatechin tannins (prodelphinidins) have the highest cross-linking activity with oxazolidine, because the reaction takes place at both the flavanol A ring and the pyrogallol type B-ring. In the three component system, the collagen would usually be treated with polyphenol first; this reaction is believed mainly to occur through hydrogen and hydrophobic bonding^{5, 56-59}. One question arising here is, where are the condensed tannins fixed on the collagen and how will this affect the subsequent reaction with oxazolidine?

There are three types of interaction to be considered in the three components system:

- polyphenol-oxazolidine-polyphenol
- collagen-oxazolidine-collagen
- collagen-oxazolidine-polyphenol

The first two have already been discussed in detail in Chapters 4 and 5, but the third type of cross-linking reaction is the most complicated and has not been demonstrated at any level. Therefore, this study is intended to determine the cross-linking type and the ratio between different cross-links, to explain which type of cross-linking dominates and which kind of cross-linking is responsible for the high hydrothermal stability of combination tanned leather.

In the present work, the combination patterns of tannins and oxazolidine on collagen fibres have been studied by series methods, such as determining the uptake of oxazolidine and the affinity of condensed tannins at various conditions. Simple phenol and amino acids were used as models for polyphenol and collagen to explore the reaction model in the three component system. Glycine, alanine, lysine and arginine were used in this process. Glycine and alanine are two of the most important and abundant of amino acids in collagen, the amino groups on these amino acids can be used as the model for terminal amino groups of collagen chains. Lysine and arginine were the possible reactive sites on collagen for oxazolidine, so the presence of the ϵ -amino groups or guanidyl groups could be used as the model for the reactive sites on collagen sidechains.

This Chapter seeks to answer the following questions: where does the high stability tannage effect come from? If a multiply bound tanning system is created within the collagen matrix, then how do the three components interact?

6.1.2 Experimental

6.1.2.1 Model studies with amino acid and simple phenols

The cross-linking reactions of simple phenols, phloroglucinol, resorcinol and pyrogallol, with oxazolidine were carried out at pH 6.0 in acetate buffer, as described in Chapter 4, Section 4.1, but in the presence of different amino acids. The phenol and amino acid in the reaction system were set at an initial molar ratio of 1:1. Both phenol and amino acid initial concentration were 0.1mol/l. Equimolar offer of oxazolidine was added to 10ml of phenol-amino acid mixture solution, so the initial molar ratio of phenol:oxazolidine:amino acid was 1:1:1. The mixture was kept for 4 hours at $20.0\pm 0.5^{\circ}\text{C}$ in a water bath.

The reaction process was followed by paper chromatography and by measuring the precipitate formed in the reaction process, which was analysed by the nitrogen content and its dried weight. The precipitate was collected after the reaction mixture, centrifuged for 30 minutes at 3500g and then dried in a 100°C oven for 48 hours. The weight was recorded. The total nitrogen content in the precipitate was also measured by using a standard method (Kjeldahl). The un-reacted amino acid was analysed by the ninhydrin (triketohydrindene hydrate) method, in which α -amino acids yield a purple product that absorbs maximally at 555nm^{253} . A calibration curve was prepared for each amino acid. The method used was as described in Chapter 5. Based on the measurement of free amino acid and total nitrogen content in the precipitate, the ratio of amino acid to oxazolidine could be calculated.

6.1.2.2 Combination tannage of hide powder

Five condensed vegetable tannins have been used in this part of work, myrica and pecan as described in Chapter 2 are both prodelphinidin tannins, mimosa is mainly prorobinetinidin tannin¹⁴², quebracho is a pure profisetinidin tannin¹⁴⁵, Neem bark tannin was used as a representative procyanidin tannin¹⁴⁷.

1 gram of hide powder was soaked in 20ml of pH 6.0 sodium acetate buffer solution in a 50ml conical flask, the flask was shaken for 8 hours at $20\pm 1^{\circ}\text{C}$, and then 20% or

200% vegetable tannin (or 5% simple phenol) was added and reacted for 16 hours at the same temperature. The mixture was filtered to remove uncombined tannin. The tanned hide powder was soaked in the same buffer solution again, 10% oxazolidine was added and the mixture was kept for 30 minutes at 20°C, then the temperature was increased to 60°C for 4 hours. After completion of the tanning process, the mixture was filtered and washed with 10ml of buffer solution twice. All the liquid were collected into a volumetric flask, made to 500ml with water and used to measure the residue of oxazolidine. The oxazolidine in the solution was determined by GC-MS. Hide powder treated with tannins alone was used as control.

6.1.2.3 Determination of tannin fixation rate on leather

Vegetable tanned or combination tanned hide powder was soaked in 50ml of 50% acetone or pH 12.0 sodium carbonate solution for 30 minutes and then washed with 300ml of the same solution for 20 minutes at 20°C. All liquid was collected and the amount of tannins washed out from hide powder was analysed by the gravimetric method and the hide powder was analysed by DSC.

6.1.2.4 Determination of oxazolidine combination rate on leather

The oxazolidine content in the reaction system was measured by the GC-MS method, as described in Chapter 3. The liquid was filtered through 0.46µm PTFE membrane before injection into the GC. The peak was identified from the retention time and molecular weight; the amount of oxazolidine was calculated based on integration of the peak area.

6.1.2.5 Differential scanning calorimetry

The tanned hide powder with vegetable tannins, phenols or oxazolidine was adjusted to neutral pH and the solvent (acetone) was removed under vacuum. The DSC method was used as described in Chapter 5.

6.1.3 Results and discussion

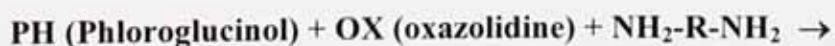
6.1.3.1 Reaction of simple phenol in the presence of amino acid

In the presence of glycine and alanine, the reaction of phloroglucinol, resorcinol and pyrogallol with oxazolidine all finished as clear solution, no precipitation was obtained in these processes. The relative precipitation result are listed in Table 6.1

Table 6.1 Relative yield of precipitate formed between phenol and oxazolidine in 10ml solution (phenol:oxazolidine:amino acid =1:1:1, pH 6.0, 20°C) (g)

	Phloroglucinol	Resorcinol	Pyrogallol
No Amino acid	0.21	0.12	0.09
Glycine	0	0	0
Alanine	0	0	0
Lysine	0.37	0.28	0.26
Arginine	0.35	0.23	0.21

In contrast to glycine and alanine, lysine and arginine show a strong effect on the phenol-oxazolidine reaction. The reaction progresses quickly, a large amount of precipitate forming immediately after adding the oxazolidine. For resorcinol and pyrogallol, the presence of amino acid increases the reaction rate significantly and more precipitate is formed than with phenol alone. Based on the calculation of the precipitate weight, it clearly shows that lysine and arginine do take part in the cross-linking process. Due to the dual functionality on these amino acids, only a kind of bridge unit is formed, unlike glycine and alanine, so the condensation reaction will carry on even after reacting with one of the amino acids. Therefore high polymers are formed in this process, as showed in the following equation:



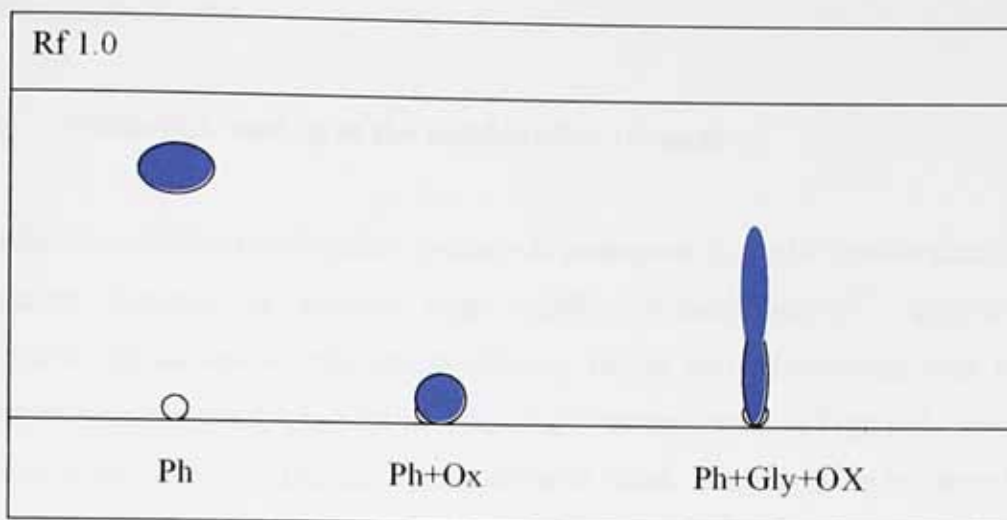


Fig 6.1 Representation of paper chromatography of phloroglucinol and glycine reaction with oxazolidine (pH 6.0, 20.0°C)

From Fig 6.1, the indicative chromatogram shows the reaction progresses slower without the amino acid, but there are lots of condensation products, polymers are formed (the Rf value showed as 0.0, Fig 6.1), which indicates the condensation products are soluble in water, but strongly combined on cellulose. This could be explained as follows:



The final condensation product has one or more hydrophilic carboxyl groups and this makes the polymer soluble in water. When amino acid takes part in the chain cross-linking reaction, it will act as a terminal agent. Therefore, it should also reduce the average molecular weight of the condensation product.

From the model studies, the amino groups and the guanidyl groups do take part in the cross-linking reaction of phenols with oxazolidine. Comparing phloroglucinol and resorcinol, pyrogallol, the less reactive phenols is more likely to be affected by the presence of amino acid in the reaction with oxazolidine. The condensed tannins or catechin and gallic acid have reactivity between phloroglucinol and pyrogallol, but the polymers have a much bigger molecular size, so there should be more

likelihood for the cross-linking reaction happening between collagen and polyflavonoid polymers.

6.1.3.2 Cross-link models in the combination tannage

Vegetable-oxazolidine combination tannage is analogous to hydrolysable tannin-Al combination tannage to achieve high stability synergistically⁵⁷. Similar to hydrolysable tannin and aluminium, condensed tannin and oxazolidine both have their distinct protein combining abilities and they can condense as high polymer; the difference is the former is formed by coordinated bonds and the latter is formed by C-C covalent bonds. Attempting to explain the mechanism of the vegetable-Al combination tannages there are several theories, of which most popular is "polymerisation" of the tannins by aluminium to form collagen-tannin-Al-tannin-collagen interactions¹³²⁻¹³⁴. Whether this theory can also be used to explain vegetable-oxazolidine combination tannage needs further discussion.

Vegetable tannage gives leather Ts 80°C^{56, 58}. The accepted theory is that, due to the multi-hydrogen bonding, tannin molecules are aggregated in a semi-colloid cluster form, rather than a molecular form, uniformly covering the collagen fibres and filling in the spaces between fibres⁵⁷. On the other hand, oxazolidine attacks amino groups or guanidyl groups on collagen by *N*-hydroxymethyl groups to form covalent bonds with collagen; consequently the Ts of oxazolidine tanned leather can typically be raised to 85°C^{5, 54}. Therefore, the vegetable-oxazolidine combination tanned leather is a complicated system, in which crosslinks between polyphenol-collagen, polyphenol-oxazolidine, and oxazolidine-collagen coexist.

From previous studies³¹⁻³², it was found that high molecular weight phenols-oxazolidine condensation compounds are formed under the retannage conditions and polyphenol is more reactive than protein in the competition reaction with the same crosslinking agent. In the case of vegetable tanned collagen, since the normal aldehyde reaction sites, the amino groups and the peptide links, are effectively masked by polyphenolic tanning agent, then oxazolidine in the retannage may only take part in cross-linking with polyphenol. In this way, a multiply bound tanning

matrix is created within the collagen matrix and large phenol condensation products could be the reason for high stability. However, the observations from our series of experiments are against this hypothesis.

6.1.3.3 Uptake of oxazolidine in tanning system

Table 6.2 Uptake of oxazolidine by vegetable tanned hide powder (% on dry weight of hide powder)^a

Tannins	Tannin uptake	OX_{tannin}^b	OX_{collagen}^b	OX^b	$OX - OX_{\text{tannin}} - OX_{\text{collagen}}$
Pecan	15.1	4.7	4.6	8.5	-0.8
Myrica	15.3	4.3	4.6	8.2	-0.7
Mimosa	12.8	3.8	4.6	7.5	-0.9
Quebracho	13.4	3.7	4.6	7.6	-0.7
Neem	14.7	3.9	4.6	7.5	-1.0
Control	—	—	4.6	—	—

^a 1 gram hide powder tanned by 20% tannins at pH 6.0, then the cross-linking reaction was carried out at pH 6.0 and 60°C for 4 hours, the offer of oxazolidine was 10%

^b OX_{tannin} , OX_{collagen} and OX represent the oxazolidine reacted by tannins, hide powder and tanned hide powder respectively

There is still a possibility for oxazolidine to take part in cross-linking reactions with collagen in vegetable tanned collagen; this can be postulated from the uptake of oxazolidine. Table 6.2 shows the maximum uptake of oxazolidine by vegetable tanned hide powder. Compared to the oxazolidine uptake by vegetable tannin (OX_{tannin}) or by hide powder (OX_{collagen}) under same conditions, it can be seen OX is close to ($OX_{\text{tannin}} + OX_{\text{collagen}}$) but with 5-10% deduction. If tannin actually had masked the vegetable tanned collagen^{248,249}, the uptake of oxazolidine by the tanned hide powder would be reduced to close to the amount by reacted tannin itself (OX_{tannin}). The results show the total uptake of oxazolidine was not significantly

affected by the reactions between tannins and collagen. In other words, most of the reactive sites are still available on collagen or condensed tannin molecules after the vegetable tanning process. This is aided by the increased temperature, which will help to conquer the high steric hindrance in the vegetable tannins-collagen system, or the "barrier effect" both from collagen and tannin molecules. The result is also in agreement with the literature, in which it was showed that vegetable tannage has little effect on the ability of the collagen to combine with acid or formaldehyde; in other words, the basic groups on collagen are hardly modified by vegetable tannins¹³⁶. The relatively small cross-linking molecules can manage to reach the reactive sites on collagen or on tannins molecules.

From the results above, different tannins give vegetable tanned collagen different reactivity to oxazolidine: gallo catechin (prodelphinidin) tannins such as pecan and myrica have higher uptake by collagen, hence the tanned collagen can absorb more oxazolidine. These data determine the maximum dosage for the two tanning materials in practice.

6.1.3.4 Combination of tannins in the tanning system

6.1.3.4.1 Intact collagen

In the history of vegetable tannage mechanism studies, many methods have been developed to measure the combined tannins. Lyotropic agents such as 50% acetone, 6M urea or strong Na_2CO_3 solution have been used to break down hydrogen bonds and release the tannin from tanned leather or hide powder¹⁶⁶. In vegetable tanned leather, most tannin is combined by weak bonds or adsorption on collagen. Up to 95% of the tannins can be washed out from leather by 50% acetone, leaving 5% irreversibly combined with collagen¹⁶⁶. From Table 6.3, less tannin can be washed out after the cross-linking process, showing 20-30% is irreversibly bonded to collagen. There are three possible reasons for this phenomenon: first, some of the tannin molecules are fixed on collagen by covalent bonding; second, the high polymer product of condensation with oxazolidine is held between the collagen fibres by physical interaction; third, stronger hydrophobic interaction are formed

after the blocking of the polar basic groups of collagen and they are resistant to the hydrogen bond breakers. The tannins or the condensation product with oxazolidine could be washed out by high pH sodium carbonate solution or 50% acetone if the cross-linking only happened between the tannins molecules, in other words, if there was no covalent cross-linking between collagen and tannin molecules. This means about 30g tannin is fixed in 100g collagen by oxazolidine through covalent bonds and the amount depends on the type of condensed tannin.

Table 6.3 Affinity of condensed tannins on collagen (% on dry weight of hide powder)

Tannin	Intact collagen				Deaminated collagen			
	T_1^a	T_2^b	Tox_1^c	Tox_2^d	T_1^a	T_2^b	Tox_1^c	Tox_2^d
Pecans	60	3	32	26	38	3	10	8
Myrica	63	4	32	27	40	4	11	7
Mimosa	55	4	26	23	34	2	9	6
Queb.	52	3	24	18	32	3	7	7
Neem	58	5	32	21	33	3	10	6

^a T_1 , tannin bound by hide powder, 200% tannin offer,

^b T_2 , irreversibly bound tannin, vegetable tanned hide powder washed with 50% acetone

^c Tox_1 , fixed tannin after combination tannage, tanned hide powder washed with 50% acetone

^d Tox_2 , fixed tannin after combination tannage, tanned hide powder washed with pH12 Na_2CO_3 solution

6.1.3.4.2 Deaminated collagen

How the tannin is fixed on the collagen can be further rationalised by washing experiments on deaminated collagen. From the literature, about 85% of the amino groups and some of the guanidyl groups are removed in deaminated hide powder²⁴. Studies have shown that removing or modifying of the amino groups in hide powder does not affect the shrinkage temperature of vegetable tanned leather although the

combination of tannins is reduced by about 25% (Table 6.3) and it prevents aldehyde tannage^{135,136,250,254}.

From the washing experiments, most of the combined tannins can be removed before or after oxazolidine function in the case of deaminated hide powder, which implies the amino groups of collagen play the key role in the combination tannage. Without the presence of side chain amino groups of collagen, tannin combination via oxazolidine are reduced (Fig 6.2).

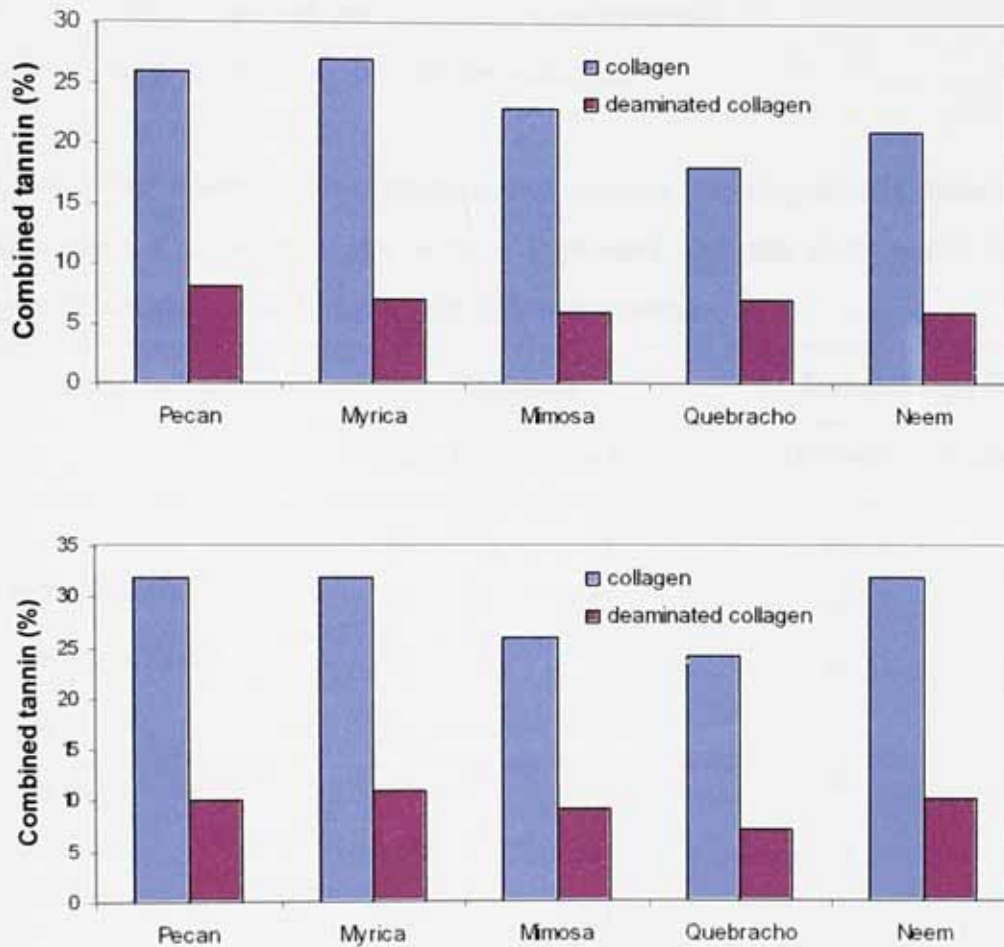


Fig 6.2 Covalent bonded tannins on collagen, measured by acetone washing (upper) and pH 12 sodium carbonate solution washing (lower)

This could be explained by losing reactive amino groups for the cross-linking reaction. After losing of most of the amino groups of collagen, only 6-10% tannin was combined through oxazolidine cross-linking after washing with pH 12 Na_2CO_3 solution, which means 6-10% of combined tannins in the combination tannage is

fixed through the cross-linking agent oxazolidine at other reactive groups, such as the guanidyl groups.

6.1.3.5 Differential Scanning Calorimetry

From the analysis above, cross-linking patterns coexist:

- type I: collagen-oxazolidine-polyphenol,
- typer II: collagen-oxazolidine-polyphenol-oxazolidine-collagen,
- type III: collagen-oxazolidine-collagen,
- type IV: polyphenol-oxazolidine-polyphenol,
- type V: collagen-polyphenol-collagen.

Table 6.4 The effects of deamination and acetone washing on the denaturation temperature (°C) of tannage with 5% phenol and tea polyphenol or 20% vegetable tannin, cross-linking with 8% oxazolidine

Tanning method	Collagen		Deaminated collagen	
	Unwashed	Acetone washed	Unwashed	Acetone washed
None	60	59	60	59
OX (oxazolidine)	83	83	66	66
Ph (phloroglucinol)	60	60	60	60
Ph+OX	95	93	66	65
TP (tea polyphenol)	68	60	60	60
TP+OX	101	96	73	64
Pecan	83	67	83	65
Pecan+OX	112	104	92	75
Myrica	85	68	84	65
Myrica+OX	113	105	92	77
Mimosa	82	67	80	65
Mimosa+OX	110	104	90	76
Quebracho	80	66	79	65
Quebracho+OX	98	93	88	65

In type I and type II reactions, the polyphenol molecules are connected to collagen through covalent bonds; while in type III and type IV reactions, the combination of polyphenols is mainly through hydrogen bonding and hydrophobic bonding. All these create a network woven around collagen, but which one is the mode for high stability of combination tanned leather? The answer can be found from DSC analysis of tanned hide powder and the results are shown in Table 6.4.

The washing process has no influence on intact collagen, the denaturation temperature remains unchanged. A similar result is obtained from the oxazolidine tanning process; the covalent cross-linking function is not changed by washing with 50% acetone or sodium carbonate solution.

If we compare the polyphenols used in this experiment, there are some common phenomena:

- (1) For the vegetable tanned collagen, washing with 50% acetone strips most of the polyphenols, to reduce the T_s nearly to that of natural collagen; while for the vegetable-oxazolidine tanned collagen, although the washing reduces tannin fixation to 50-60%, this does not influence the T_s much. This means covalent fixation of tannin (reaction types I, II) contributes to the tanned collagen's high thermal stability, corresponding to 20-30% of the total amount of tannin used in the vegetable tannage.
- (2) Deamination reduces the fixation of tannin on collagen to 60%, but there is no big reduction in the T_s , but it greatly reduces the T_s of combination tanned collagen to the same temperature as vegetable tanned collagen. The loss of amino groups does not change the vegetable tannage stability substantially, but it makes the cross-linking reaction with oxazolidine impossible, similar to (1).
- (3) Washing the tanned deaminated collagen with acetone strips polyphenols completely and reduces the stability to the same as original collagen. Although the high molecular weight condensation product from polyphenol and oxazolidine can be formed in the collagen fibril structure (reaction type IV), it is unlikely to be the key parameter for high stability.

- (4) For the small molecular weight tanning materials, such as phloroglucinol and green tea polyphenol, high stability can still be achieved in the combination tannage. It is well known that phloroglucinol has no tanning ability and the stability of skin collagen can even be lowered because the presence of phenols will break the hydrogen bonding in collagen. Green tea polyphenol is a mixture of flavanol monomers, in which epigallocatechin gallate is prevalent and it has shown some tanning ability that results in T_s 65-68°C. After washing with 50% acetone, nearly all of the phloroglucinol or tea polyphenol was removed from the collagen and T_s was brought back to the original value. A change occurs after cross-linking with oxazolidine, when both show high T_s and the stability is not changed significantly by the washing process. If the polymerisation hypothesis is correct, the condensation products of small molecular phenols and oxazolidine should be regarded as big molecular polyphenols, so the opposite phenomenon should have been observed. This proves the stability is owing to the cross-linking reaction of oxazolidine between collagen and polyphenols, this is cross-link type II.
- (5) Among the several tannins tested, the gallo catechin tannins pecan and myrica show the strongest synergistic effect in combination tannage. The stability conferred by these two tannins is slightly higher than mimosa and quebracho. We can see from this that the cross-link type IV happens between the polyphenol and the oxazolidine, to benefit the overall stability, but it is not the essential factor.

6.1.4 Summary

In the condensed tannins-oxazolidine combination tannage, 20-30% tannin is immobilised on the collagen fibres by covalent cross-linking. Most of the covalent bond cross-linking happens at side chain amino groups, about two thirds; another one third is formed on guanidyl groups of arginine or other functional groups of collagen. The covalently bonded tannins are responsible for the high stability of leather. The cross-linking between collagen and tannin molecules was formed through covalent bonds from oxazolidine. The sequence of the tannage is

determined by the properties of tannins and oxazolidine. The first vegetable tannage gives the following oxazolidine enough reactive sites to form cross-links between collagen and tannin molecules and between tannin molecules. Therefore, a network is formed throughout collagen.

The other 70-80% of the tannin used in the vegetable tannage is necessary for uniform penetration and distribution. The colloidal properties of tannin solution require the dosage of tannin to be quite high (20g tannin per 100g collagen). This usually causes stiffness and thickness of the vegetable tanned or combination tanned leather. It could be overcome if we choose low molecular weight tannin and weaken its colloidal properties. There is more work needed to be done to reduce the high molecular weight characteristics of normal prodelphinidin polymers²⁵⁶.

Section 6.2

Mechanistic study by hydrothermal isometric tension

6.2.1 Introduction

Hydrothermal stability is one of the most important characteristics of leather, which is mainly from the cross-linking condition among collagenous fibres^{4,5}. Different tanning methods probably involve different cross-linking models that give leather its distinct properties. Traditionally, two methods have been widely applied in the studies of leather thermal stability: one is measuring the shrinkage temperature (T_s) in water or organic solvent directly, when the leather can shrink freely at the denaturation temperature; the other is thermal analysis by differential scanning calorimetry (DSC) to measure the changes in enthalpy, ΔH , at the phase transition^{5,32}. However both methods fail to give direct information about the cross-linking state from various tannages.

Hydrothermal isometric tension (HIT) is used to measure the development and subsequent relaxation of isometric tension during the heating process of polymer materials by preventing contraction^{234,236,237}. This method was first applied in polymer material and biomaterials research about 50 years ago²³⁵⁻²³⁷. From 1987, Alexander and co-workers have transferred this equipment and method into collagen studies and leather science areas^{238-240,246}. In the range 20-95°C, the different phases of isometric tension curves obtained from raw skin have tentatively been attributed to changes in collagen stability and have reflected the extent of cross-linking in collagenous network with ageing²³⁴⁻²⁴⁷.

In this work, over the temperature range 20-125°C, this principle was applied to direct investigation of the cross-linking density and stability of various types of tanned leather. The results provided rapid and precise information of tannage status,

so the mechanisms of different tanning methods can be discussed according to these data, thus a clearer model of tanned collagen molecular structure can be obtained.

6.2.2 Experimental

6.2.2.1 Preparation of leather

All the sheepskin leathers were prepared as described in Appendix I. The leather was shaved to 0.8mm and soaked in Tris-HCl buffer solution at pH 7.4 for 48 hours²³⁴. The specimens, 30x7mm, were stamped out with a press knife.

6.2.2.2 Determination of hydrothermal isometric tension

The wetted leather samples were clamped between two steel jaws and soaked into liquid paraffin and conditioned for 30 minutes before starting the heating process. The tension on the leather samples before heating was adjusted to 1.8 mV, nearly zero tension. The temperature was increased from 20°C to 125°C at a rate of 2°C/min²³⁴. The tension and temperature were recorded by computer.

6.2.3 Results and discussion

The hydrothermal isometric tension curves of skin or different leathers are shown in Fig 6.3, Fig 6.4 and Fig 6.5. The curve shape from raw skin is in good agreement with the literature^{234,244,245}. The curves of leathers show distinct shapes that probably relate to different cross-linking conditions.

Based on the shapes, the curves can be divided into three types: Type I includes the raw skin, vegetable tanned leather, Al or Ti tanned leather and vegetable-Al combination tanned leathers, which all show contraction and relaxation processes before reaching 125°C; Type II is the leather tanned by oxazolidine, the tension remains constant after reaching the maximum tension; Type III is the leathers tanned by chrome or vegetable-oxazolidine combination tanning methods, where the tension increases all the time.

Leather tanned by different amounts of chrome sulfate salt (basity 25%) at 0.25%, 0.5%, 1.0% 1.5% and 2.0% Cr₂O₃ on the weight of pickled pelt. The HIT result is shown in Fig 6.7, in which all the leather samples show similar rate in the contraction process.

6.2.3.1 Implication of hydrothermal isometric tension curves

The HIT curve can be divided into three parts: the first is the tension increasing process from zero to maximum tension; then followed by a relatively constant tension process, if present; finally, the tension will be constant or the relaxation process will occur, due to the gradual destruction of collagen structure or rupture of some cross-linking bonds^{234,236,237,247,258}. The slope of the curve in the tension increasing process accounts for the collagen fibre rigidity, caused by cross-links: the steeper the slope of the contraction curve, the more cross-links should be present in the collagen materials. Relaxation represents the stability of these connecting elements (cross-linking bond): the steeper the rate of relaxation, the more unstable is the cross-linking.

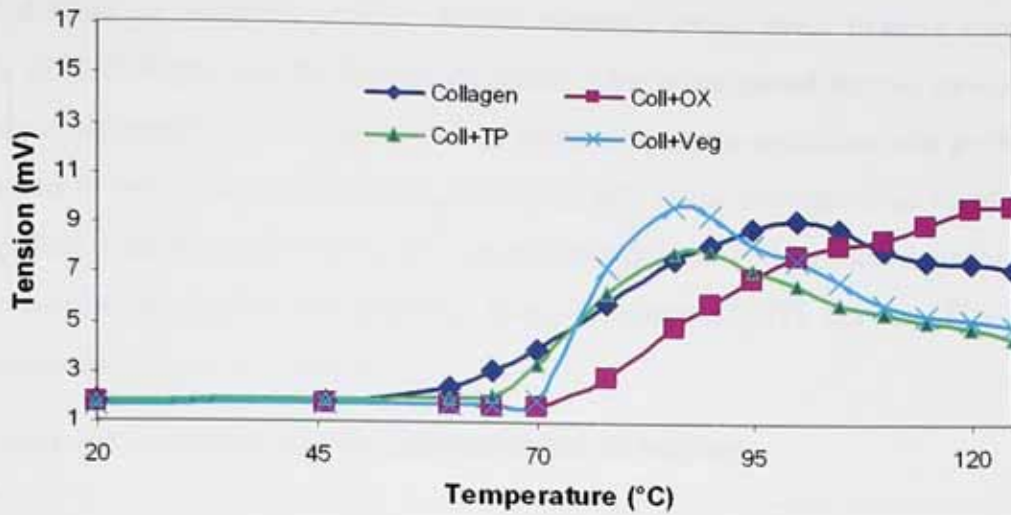


Fig 6.3 The HIT of skins and leathers made by organic tanning methods

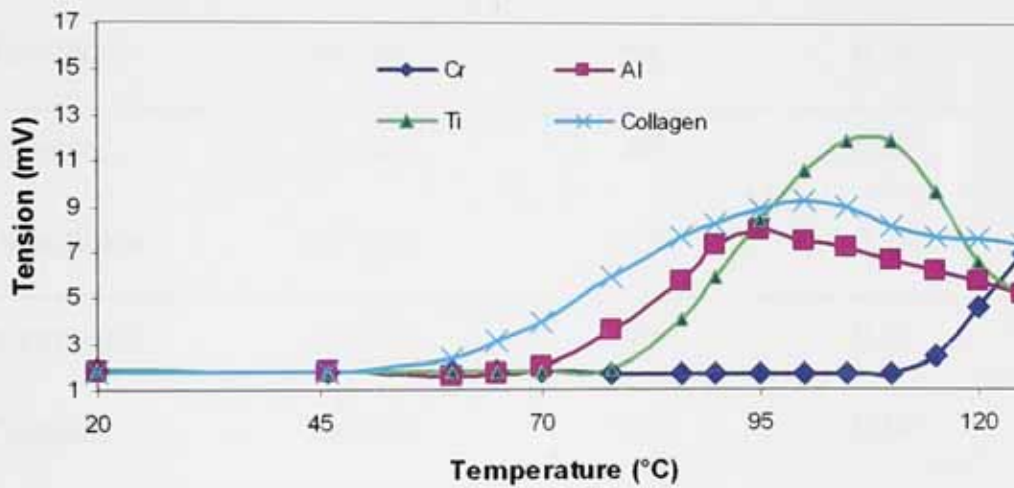


Fig 6.4 HIT of leathers tanned by mineral tanning methods

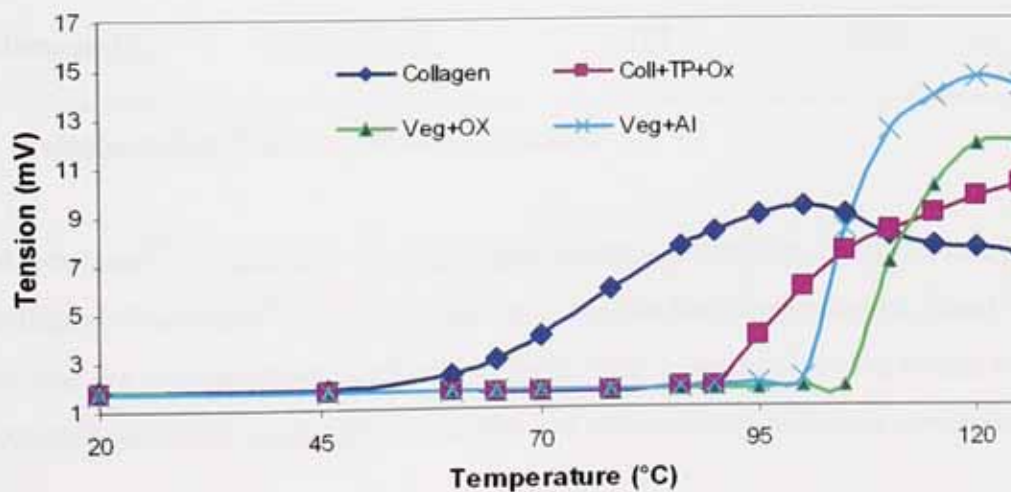


Fig 6.5 HIT curves of leather tanned by combination tanning methods

To date, no mathematical or physical model has been proposed for a full analysis of hydrothermal isometric tension curves obtained under linear heating conditions²³⁴. In 1987, J. Kopp and M. Bonnet proposed a tentative model for the development of isometric tension in collagen²⁴⁴. Unfortunately, these equations are probably only suited to very limited conditions (medium, pH, ionic strength *et al.*) and cannot be applied to these experiments. But we still can get some useful information about the cross-linking density and stability from the slope of HIT curves. The calculated results are shown in Table 6.5.

Table 6.5 Isometric tension measurement on leathers

Leather	Onset temperature (°C) ^a	Temperature at maximum tension (°C)	Relative slope in contraction	Relative slope in relaxation
Raw Skin	53 (52)	100	0.25	-0.13
Mimosa	80 (78)	86	0.67	-0.20
Oxazolidine	80 (78)	>125	0.28	0.00
Aluminium	75 (74)	95	0.27	-0.20
Titanium	90 (88)	105	0.50	-0.52
Chrome	110 (106)	>125	0.29	—
Mimosa+Al	105 (103)	110	1.23	-0.05
Mimosa+Ox	110 (105)	>125	0.93	—

^a Ts obtained from DSC also showed in brackets

Macfarlane²³⁷ found that restraint significantly delays the denaturation process of collagen; Snowdon²⁴⁷ also provided evidence for this phenomenon. From Table 6.5, we can see the shrinkage temperature from DSC is lower than the values of the **HIT** extrapolated onset temperature, but the two temperatures exhibit a consistent pattern.

The temperature of the maximum tension point is also shown in Table 6.5, which should be the starting temperature for unstable cross-links to be ruptured. Vegetable tannins and aluminium tanned leather both show lower maximum tension temperatures than raw skin, which may indicate less stable cross-links are formed in these tanning process, while oxazolidine, chrome and vegetable-oxazolidine combination tannage all produce more stable cross-linking bonds.

6.2.3.2 Tanning mechanism study

As discussed above, the slopes of the HIT curves at different stages represent the cross-linking density or rigidity of collagen fibres and the stability of these cross-links. By setting the cross-linking density in raw skin at 1.0, then the relative cross-linking in leathers can be calculated, as shown in Fig 6.6.

6.2.3.2.1 Relationship between shrinkage temperature and cross-linking density

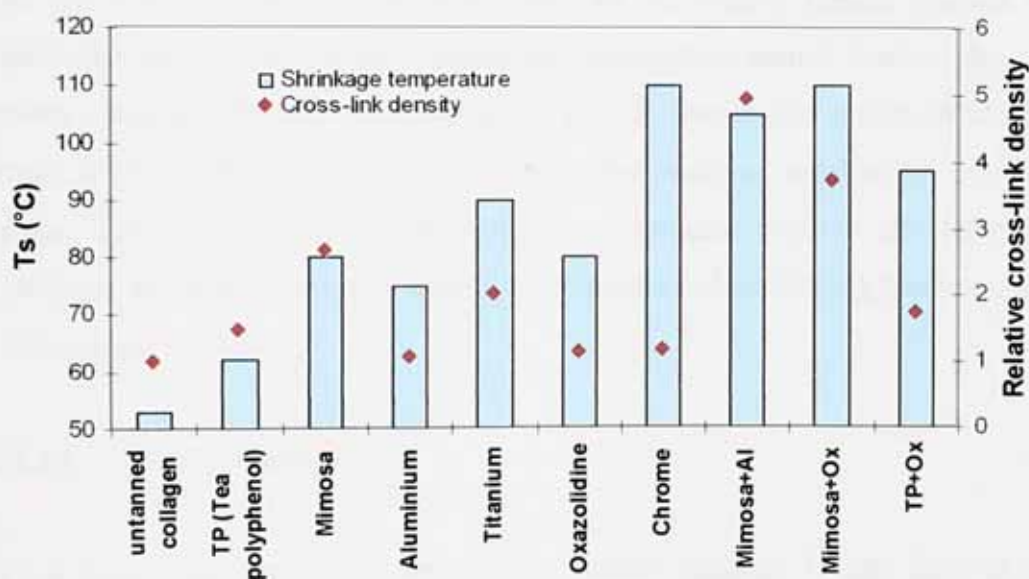


Fig 6.6 Relationship between shrinkage temperature (T_s) and relative cross-linking density

The results of HIT show that different tanning methods confer distinct cross-linking density and stability. For some tanning agents, such as vegetable tannins and titanium, the higher the cross-linking density, the higher the thermal stability. But,

for some other tanning agents such as chrome and oxazolidine, the thermal stability of leather is determined more by the cross-linking stability rather than the cross-linking density. When comparing chrome and titanium, chrome tanning did not produce high cross-linking density, as anticipated. With high cross-linking density, titanium only gave T_s 95°C.

6.2.3.2.2 Vegetable tannage

For vegetable tanned leathers, the high cross-linking density comes from the high molecular weight of polyphenols with a large number of weak bond cross-links (hydrogen bonds and hydrophobic bonds), which cannot produce high stability. After reaching the maximum tension, the tension reduces quickly, to even lower than raw skin in the relaxation stage, which indicates the stability of these bonds is low and will be destroyed by the continued heating.

6.2.3.2.3 Oxazolidine tannage

After reaching the maximum tension point, all the organic tanned leathers except oxazolidine show a relaxation process; for oxazolidine tanned leather, the tension remains constant after the first increasing period, due to the stable covalent bond formed in the collagen fibres. This supports the accepted mechanism of aldehyde tannage. The soft cross-link chains from the oxazolidine will not affect the rigidity of collagen fibres significantly; therefore the process of increasing tension is similar to the original collagen.

6.2.3.2.4 Mineral tannage

Fig 6.4 shows the results of some mineral tanned leathers. Unlike chrome tanned leather, Al(III) and Ti(IV) show an apparent relaxation process after the maximum tension point. Aluminium tanned leather has a similar HIT curve shape to raw material, but titanium tanned leather shows an HIT curve more like vegetable tanned leather, which may be due to the hydrogen bonding of titanium in the leather and its filling function. The interaction between titanium and collagen is more electrostatic

interaction rather than complexation. The relaxation process for titanium tanned leather is fast compared to the other leathers; the reason, whether from the destruction of collagen or the rupture of cross-links or both, is unknown.

Chrome tanned leather from an industry tannery (usually tanned to contain 4% Cr_2O_3) shows a slow process of increasing tension up to 115°C. Due to the heating medium used in the experiments, we cannot obtain the full HIT curve, but the curves appear to increase up to 130°C, which indicates the cross-linking structure (if there is one) in collagen is much more stable than aluminium or titanium tanned leather. Fig 6.7 has more information about chrome combination reaction on collagen fibres. Leather tanned by 0.25%, 0.5% and 1.0% Cr_2O_3 all showed similar slope rates to raw materials in the contraction process, although the slope rate slightly increases with increasing offer of chrome. This indicates there is very little so-called effective cross-linking formed in the chrome tanned leather. Then this begs the question: what causes the increasing of hydrothermal stability of chrome tanned leather, if it is not cross-linking?

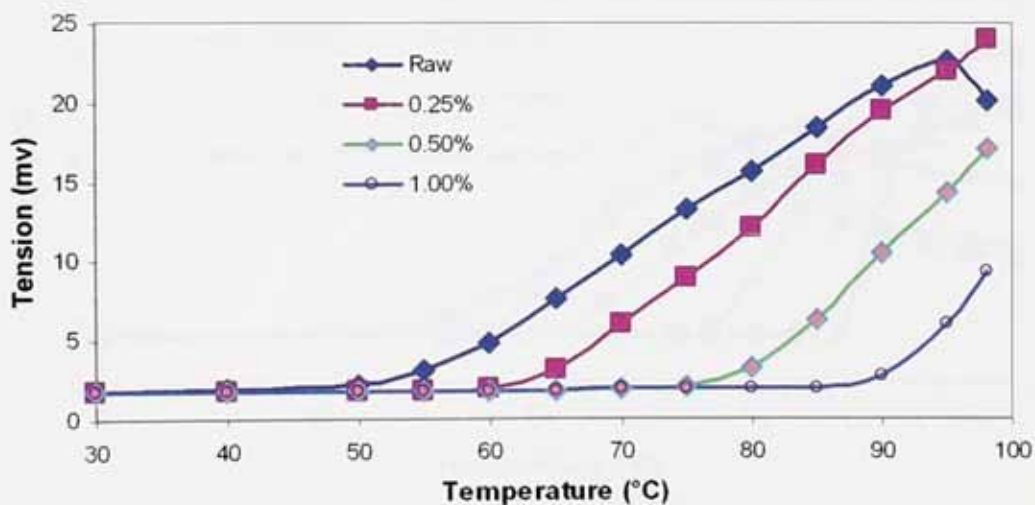


Fig 6.7 Hydrothermal isometric tension of leather tanned by different offers of chrome salt (% Cr_2O_3)

In the tanning theory proposed by Covington^{5,32}, the supramolecular water layer around the collagen fibres may be displaced or changed in the tanning process. Due to the different chemical or physical changes in different tanning reactions, this results in distinctly different shrinkage temperatures. In other words, it may not be

necessary to form cross-linking in chrome tanning; the high shrinkage temperature from chrome tannage is due to a changing of the supramolecular water layer of collagen.

6.2.3.2.5 Combination tannage

In Fig 6.5, vegetable-aluminium and vegetable-oxazolidine tanned leather, have steep slopes in the contraction stage, which could be explained in the same way as vegetable tanning. After reacting with aluminium or oxazolidine, the higher molecular weight condensation products', filling function will make the collagen fibres more rigid than in vegetable tanned leather. The difference between these two tanning methods is shown in the relaxation processes of the HIT curves. Vegetable-aluminium combination tanned leather has a relaxation above 120°C, which indicates the combination bond is not stable at high temperature with continued heating, but the tension in vegetable-oxazolidine tanned leather remains constant (Fig 6.8).

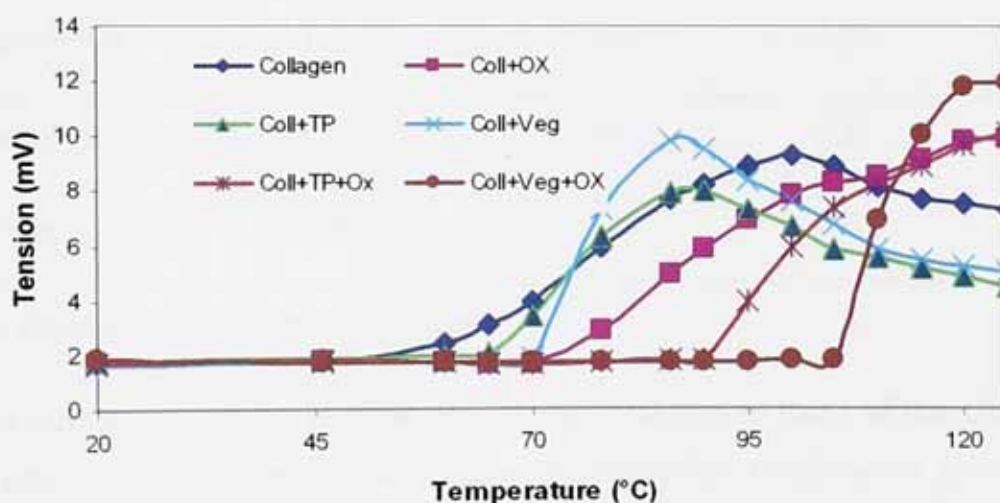


Fig 6.8 Hydrothermal isometric tension curve of vegetable tanned and combination tanned collagen

In the semi-alum process, Al(III) forms complexation products with the hydroxyl bonds of tannins and (possibly) the carboxyl groups of collagen, so the cross-linking is similar to aluminium tannage, which has been shown not to be stable at high temperature⁵⁷. On the other hand, in the condensed tannin-oxazolidine process, the

vegetable tannins are cross-linked to collagen by covalent bonds through oxazolidine and this cross-linking is stable even at high temperature, which is similar to oxazolidine tannage. If cross-linking only happened between polyphenol molecules, the HIT result should be similar to pure vegetable tanned leather, so the hydrogen bonds or hydrophobic bonds in the combination system would be destroyed at high temperature. In other words, there should be a relaxation process if there is no covalent bonding.

Table 6.6 Difference between green tea-polyphenol and mimosa tannins combination tannage

Leather	Onset Temperature (°C) ^a	Temperature at maximum tension (°C)	Slope in contraction	Slope in relaxation
Raw Skin	53 (52)	100	0.25	-0.13
Mimosa	80 (78)	86	0.67	-0.20
Mimosa+Ox	110 (105)	>125	0.93	—
Tea polyphenol	65 (64)	90	0.37	-0.18
TP+Ox	98 (95)	>125	0.40	—

^a Ts obtained from DSC shown in brackets

This further supports the covalent bond theory, proposed in Part 1 of this Chapter. It is note worthy that green tea polyphenol-oxazolidine combination tannage has shown different properties to traditional vegetable tannage. (Table 6.6) The slope of contraction in TP tanned leather is much lower than vegetable tanned leather, which indicates the rigidity of the collagen fibres is much lower, due to the low molecular weight of the polyphenol. It further confirms that high cross-linking density might not lead to high stability, but effective cross-linking should be from, properly reacted tanning agent. This means we can use low molecular weight condensed

tannins and oxazolidine to produce higher stability leather and the problems from traditional vegetable tannins could be avoided.

6.2.4 Summary

The covalent bond combination theory in condensed tannin-oxazolidine tannage has been confirmed by hydrothermal isometric tension studies. The high thermal stability of this tannage is mainly determined by the covalent bonding formed between collagen and polyphenol via oxazolidine.

Compared with traditional methods for studying leather stability, hydrothermal isometric tension can provide additional information about the tanning mechanism of leather, quantitatively and qualitatively. Most of the results are in good agreement with the present understanding of tanning chemistry, except condensed tannin-oxazolidine combination tannage, when cross-linking between polyphenol and collagen is the main reason for the high stability: unlike the traditional theory, which proposed that cross-linking only occurs between polyphenols.

Section 6.3

New theory of tanning

Tanning is a process to change putrescible raw skin or hide into a stable material, which is resistant to heat (dry or wet) and microbial attack. In the history of tanning, surprisingly few changes in the technology of tanning have been made, except for the "chrome revolution", which was introduced around 130 years ago and still dominates the current leather tanning industry^{5,32,264}. The same is true concerning the understanding of the origin of the tanning effect; very little information is available. Whenever tanning theory has been talked about, the mechanism normally distinguishes between different tanning methods, such as chrome tanning, vegetable tanning, aldehyde tanning or other tanning methods, but without taking an overview. The mechanisms for these tanning methods have been reviewed in Chapter 1. In the conventional theory for each tanning method, the possible reactions on collagen side chains are discussed, but without concerning how this is going to change the collagen structure. Therefore, the question still remains: what happened to the collagen structure at the molecular level after tanning?

Due to the complexity of collagen chemistry, this is a difficult question to answer directly. But useful information can be obtained through modern analytical techniques. In 1989, by employing ²⁷Al-NMR spectrometry, Covington and co-workers proved only hydrogen bonds are broken in the leather shrinking process, regardless of the tanning method. This is consistent with the enthalpy change observed in DSC measurements²⁶, but it appears to be in conflict with traditional tanning theory, which explained the stability of leather in terms of the stability of the cross-linking bonds formed between collagen and the tanning agent. More recently, by using extended X-ray analysis fine structure (EXAFS), new insights into the chrome tanning reaction have been obtained. The results showed that the average bound chromium species is a linear chain of four chromium ions, linked by oxy bridges, but no evidence was found that the sulphate counterion is bound to chromium²⁶⁴. However, it is true that sulphate ions are important in the chrome tanning process; Komsa-Penkova²⁷³ reported that high concentration of sulphate

ions alone could significantly increase the denaturation temperature of soluble collagen. Therefore there must be some kind of interaction between the chromium species, collagen and sulphate ions, which produces a synthetic function and contributes to the high shrinkage temperature of basic chromium(III) sulphate tanned leather. This then is the question for the traditional chrome tanning theory: how is the high shrinkage temperature produced?

From the results of the hydrothermal isometric tension measurements in this work, it has been shown that conventional cross-linking is not a prerequisite for high hydrothermal stability, whether in organic tanning or inorganic tanning. Without the so-called cross-linking, then from where does the increased stability come? In fact this is the same question proposed at the beginning of this Section: what happens to the collagen structure? This can be explained, as proposed by Covington³² in the 2000 Atkin Memorial Lecture, "the remaining rule for high hydrothermal stability: the creation of a stable, rigid matrix around the collagen, preferably by displacing the supramolecular water, but may incorporate some of the supramolecular water as part of the matrix structure". It is widely believed that the stability of the collagen triple helix is mainly based on hydrogen bonding^{1,8-13,35,36,264,272}, in which hydroxyproline together with water molecules are believed to play an important role. Collagen peptide chains and how they are structured with the functional groups have been reviewed before^{32,272}. It is also true that the presence of water is critical to collagen stability^{276,277}; Rochdi¹¹ and Grigera²⁷¹ have reported that bound water molecules play an important role in the denaturation process of collagen, this has been recently confirmed by Berman's group^{277,279-281}, although Holmgren²⁷⁸ proposed a unlikely new theory, that collagen stability comes from the inductive effect rather than hydrogen bonding. When the supramolecular water around the collagen fibres is modified, it also significantly affects its thermal stability¹¹. This could be the answer to the high stability of the chrome sulphate tanning system, which the sulphate counterion probably plays a role in the way the supramolecular water layer is changed.

In the tanning process, the collagen supramolecular water layer may be disrupted when a new matrix structure is formed around the collagen and the functional

groups are modified; for example, the carboxyl groups in mineral tanning and the basic groups in aldehyde tanning. In condensed tannin-oxazolidine combination tanning, the proanthocyanidin molecules are fixed on the collagen peptide chains through oxazolidine, so the covalent bonded polyphenol structure may have an important effect on the original hydrogen bonding of collagen. This relatively stable "intruder" is where the high hydrothermal stability comes from. Similar changes happen in the chrome tanning process, although the whole picture of collagen-chromium combination still needs to be established.

From this argument, there is no difference between organic and inorganic tanning mechanisms. Hydrothermal stability depends on the stability of the newly formed matrix structure and of the bonding directly formed between the collagen and the tanning agent.

Chapter 7

Main conclusions and future work

7.1 Conclusions

From the present investigation on the cross-linking reaction in the three component system, condensed tannin, collagen and oxazolidine, the following conclusions have been drawn:

- With the new understanding of the tanning mechanism, new high stability tanning methods can be developed in a more effective way, by monitoring the key parameters, which are known to lead to the desired result. For this purpose, more chemistry knowledge is necessary, both organic and inorganic, to understand the choices for selecting the parameters to be studied. Better understanding of collagen physical chemistry, especially the relationship between its hydration structure (supramolecular water layer) and properties is equally important for new tanning methods' development.
- From progress in chrome tanning studies using EXAFS³², the new understanding of the mineral tanning mechanism, especially chrome tanning, will help to improve this well established technology. It could lead to the elimination of the pollution problem caused by sulphate ion, by employing alternative, environmentally friendly counterions instead. Similar procedures might be used to improve other mineral tanning methods, such as aluminium, titanium and zirconium.
- In the search for high stability organic tanning methods, as alternatives to chrome tanning, the matrix structure should be considered first. It has to comply with two conditions: first, the organic tanning agent must fit into the collagen structure and form a matrix around the collagen fibres; second, the interaction between the tanning agent and collagen must include some covalent bonds or similarly stable interaction. Vegetable tannins contain multiple hydrophilic hydroxyl groups and hydrophobic benzene ring structures, which provide a suitable base for multiple hydrogen bonding and hydrophobic bond formation. But the covalent bonding interaction has to be introduced by another cross-linking agent. In the case of condensed tannins,

because of the presence of strong nucleophilic sites, oxazolidine has high reactivity towards both collagen amino groups and condensed tannins, so high stability is obtained. For hydrolysable tannins, because of the lack of nucleophilic sites in the tannin molecules, oxazolidine cannot form linkages between the tannin and collagen. The alternative strategy is to use aluminium salts to polymerise the vegetable tannin in situ: high stability is still achievable, although clearly this is not a pure organic tanning method. Nevertheless, exactly the same principles apply.

- In the model studies of the combination tanning reaction, it is a surprise to find that leathers tanned with small phenol molecules, such as phloroglucinol or green tea polyphenol (mainly proanthocyanidin monomers) with oxazolidine showed high stability. These low molecular weight phenols are normally regarded as non-tannins and make little contribution to the vegetable tanned leather stability: usually, non-tans have been treated as a byproduct obtained from the extraction of vegetable tannins. But in this work, it is clearly shown we can make a full use of this “waste” to produce high stability leather. More importantly, this approach also eliminates the penetration problem faced in traditional vegetable tanning, which can introduce defects to the vegetable tanned leather. Even the high molecular weight tannins might be degraded to lower molecular weight phenols to be used in this combination tannage. Because the so-called “tannins” usually have high average molecular weight, this does limit their use in this new process.

7.2 Future work

Based on the new understanding of tanning theory, it is possible to improve current tanning methods, but, before that, more evidence is needed to know how this could be done. First of all, for chrome tanning, a more clear vision is needed concerning the combination of chromium(III) with collagen carboxyl groups, chromium(III) species with the sulphate ions and the relationship between the supramolecular water layer and the sulphate ions. Modern analytical techniques and a comprehensive computer model for collagen might be helpful to solve this problem.

In the search for high stability organic tanning methods, the small gallo catechin molecules, including monomers, dimers, even trimers, are worthy of further investigation, then a better understanding about this combination tanning process could be achieved and even higher stability might be obtained. Searching for a more suitable cross-linker is as important as the prodelphinidin tannin itself. This could be based on the oxazolidine structure, although some modifications may bring completely different effects to the combination tanning.

In this study, HIT has been proved to be a useful method to study tanning mechanism, although more work needs to be done to establish a physical-mathematical model for tanning processes and their consequences for hydrothermal stability, then the new tanning theory can be better understood.

Concerning the reaction of polyphenol with oxazolidine, the reaction pathway or the mechanism need to be elucidated in more detail, by separating the reaction intermediates and determining the ratios of different intermediates. The next step will be to establish the effect of conformation, by using prodelphinidin oligomers. Concerning the three component system, amino acid, simple phenol and oxazolidine, more information might be obtained for the composition of the precipitate formed in the model reaction system by using solid state NMR, although it could be difficult.

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Appendix I
Leather tanning methods (all start from pickled sheep skin)

1 Vegetable tannage

Operation	Chemicals	Offer (%)	Temp. (°C)	Running time (mins)	Comments
Depickling	Sodium chloride	8	20	20	
	Water	100			
	Sodium sulphite	0.2	20	30	
	Sodium bicarbonate	0.2	20	60	Dissolved in 10 times water and added in four times, final pH 6.0
Drain Tanning	Vegetable tannin	10	20	90	
	Vegetable tannin	10	20	120	Stand overnight, next day, run for another 90 minutes
	Water	100	40	90	
	Formic acid	0.1	25	30	pH 4.0
	Water	200	25	10	
Drain					Horse overnight

2 Chrome tannage

Operation	Chemicals	Offer (%)	Temp. (°C)	Running time (mins)	Comments
Tanning	Sodium chloride	8			
	Water	100	20	20	
	Cr ₂ O ₃	1.5	20	90	pH 3.1
	Sodium bicarbonate	0.2	20	30	pH 4.0, Stand overnight, next day, run for another 30 minutes
Drain	Water	100	40	90	pH4.0 Horse overnight

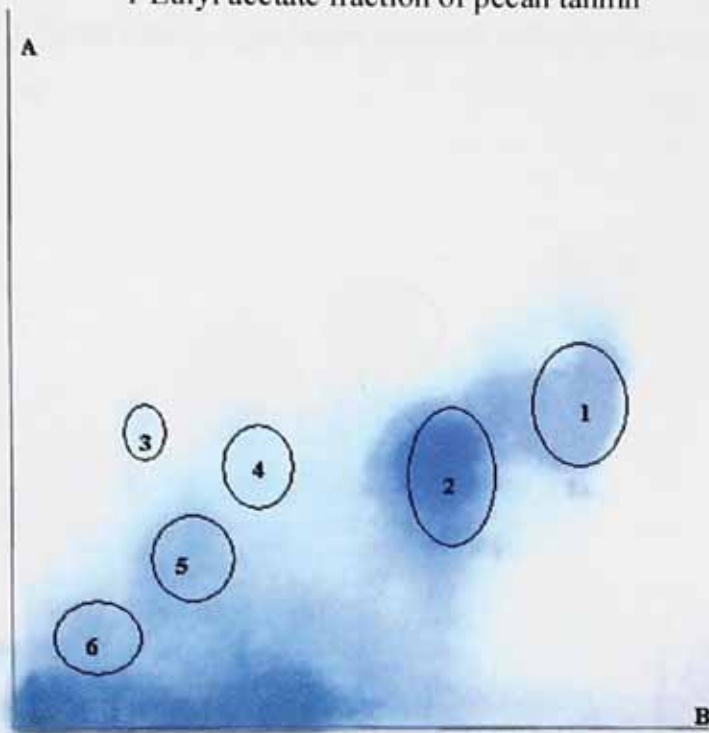
Similar methods were employed for Al(III) and Ti(IV) tannage

3 Combination tannage

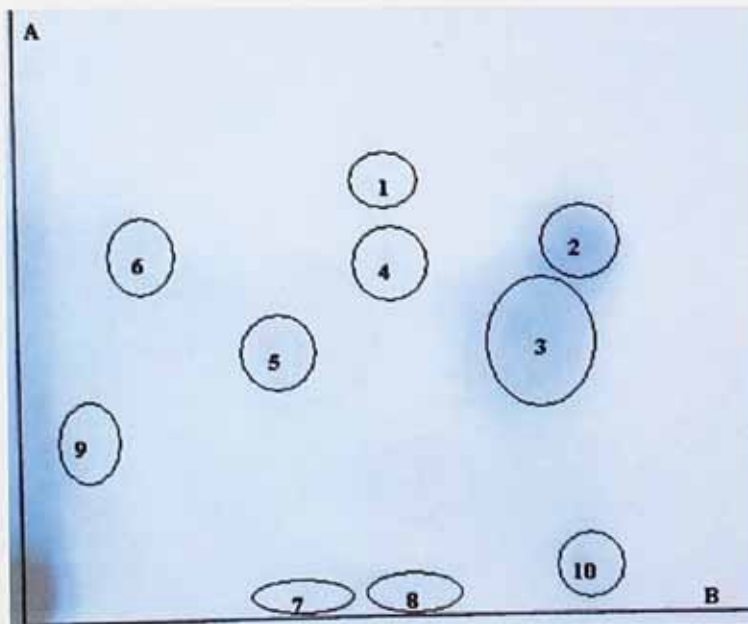
Operation	Chemicals	Offer (%)	Temp. (°C)	Running time (mins)	Comments
Depickling	Sodium chloride	8	20	20	
	Water	100			
	Sodium sulphite	0.2	20	30	
	Sodium bicarbonate	0.2	20	60	Dissolved in 10 times water and added in four times, final pH 6.0
Drain Tanning	Vegetable tannin	10	20	90	
	Vegetable tannin	10	20	120	Stand overnight, next day, run for another 90 minutes
	Water	100	40	90	
	Formic acid	0.1	25	30	pH 4.0
	Water	200	25	10	
Drain Washing					Horse overnight
	Water	300	25	5	
Bleaching	Water	100			
	Sodium sulphite	0.5	25	30	
	Water	100			
Retanning	Neosyn TP	1	25	45	
	Oxazolidine	4-10%	25	180	After 1 hour, the temperature increased to 60°C for another one hour running
Washing	Water	200%	25	30	Horse overnight

Appendix II Ethyl acetate fraction of pecan and myrica tannins

1 Ethyl acetate fraction of pecan tannin

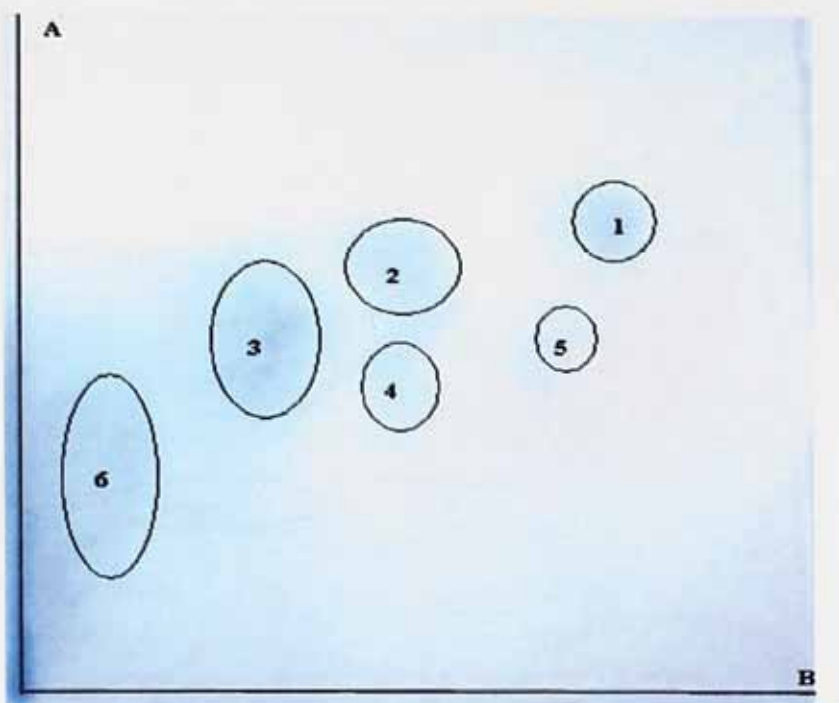


2 Ethyl acetate fraction of myrica tannin

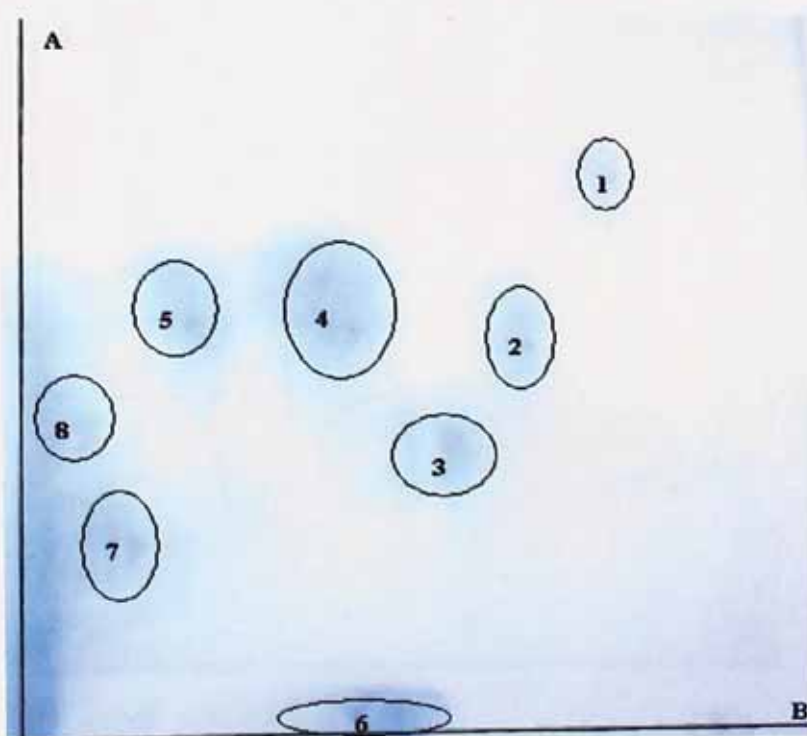


Appendix III Degradation products of pecan and myrica tannins with phloroglucinol

1 Pecan tannin degradation products with phloroglucinol

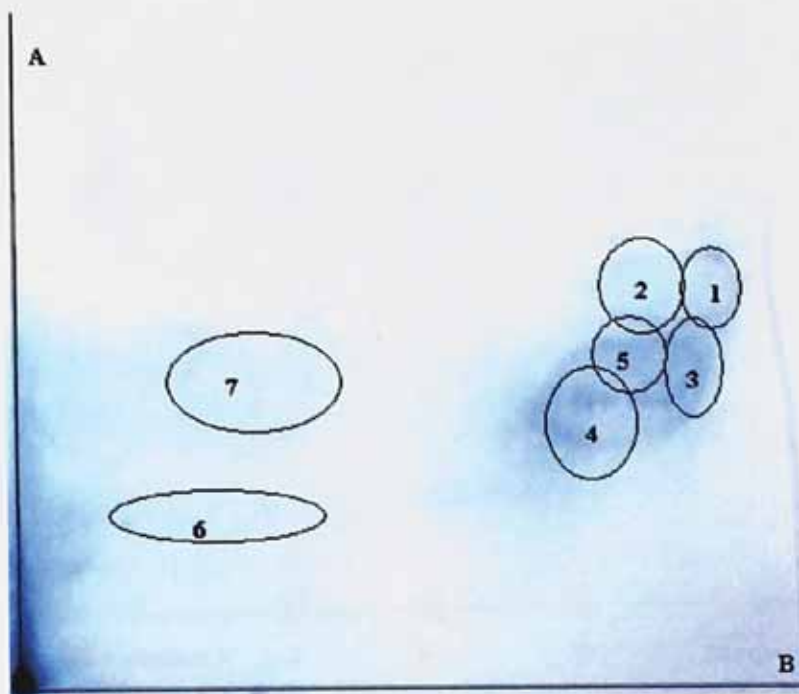


2 Myrica tannin degradation products with phloroglucinol

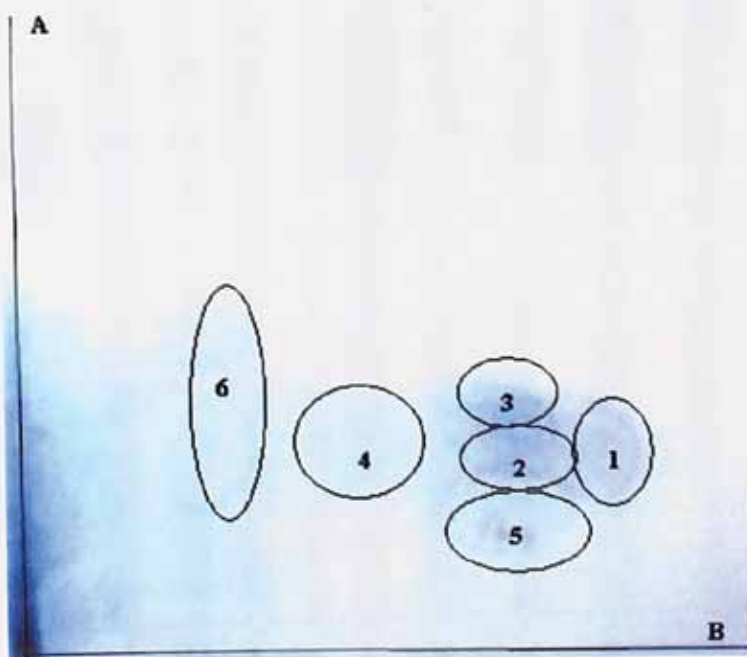


Appendix IV Degradation products of pecan and myrica tannins with toluene-thiol

1 Pecan tannin degradation products with toluene-thiol

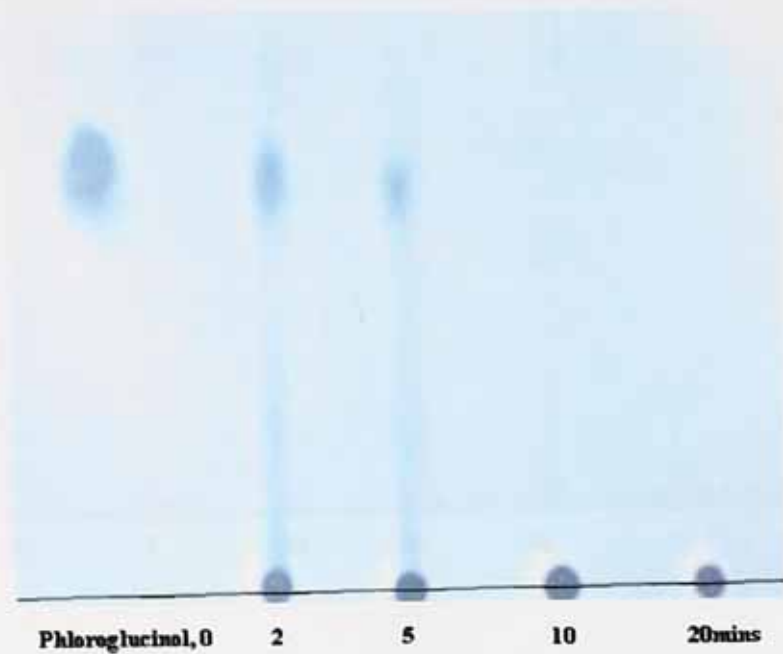


2 Myrica tannin degradation products with toluene-thiol

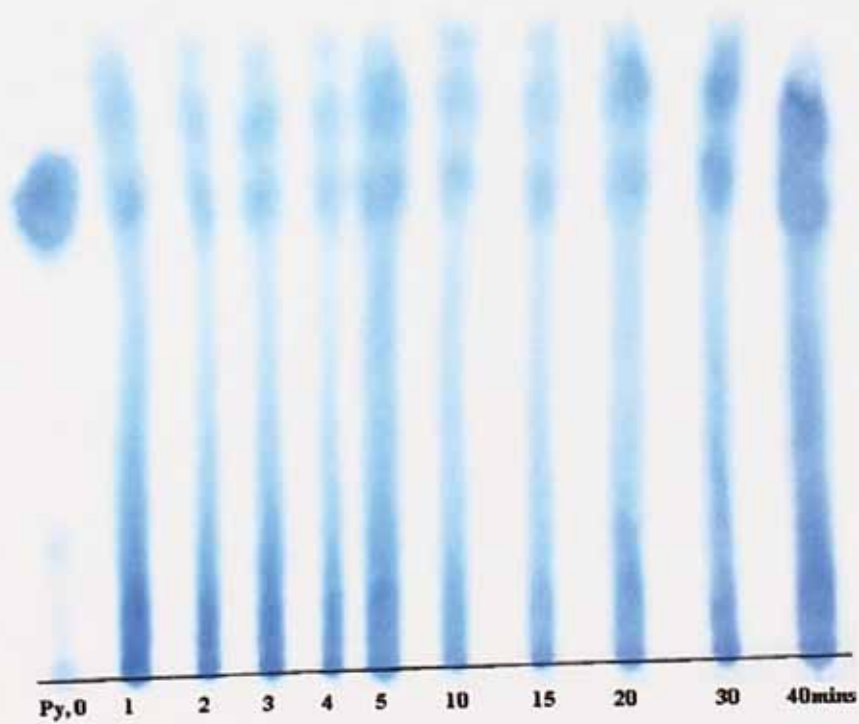


Appendix V Reaction of phloroglucinol and pyrogallol with oxazolidine

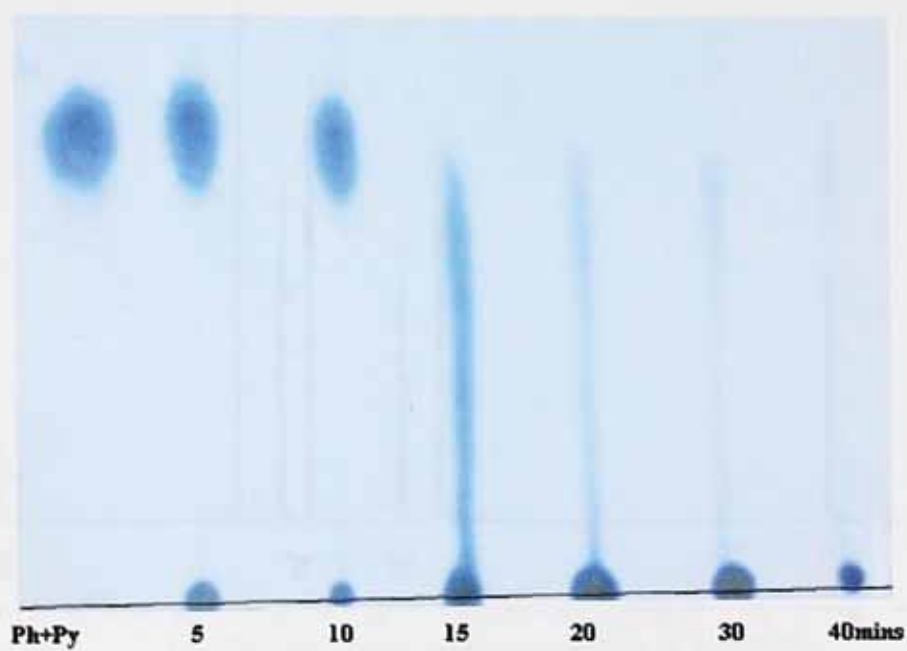
1 Phloroglucinol with oxazolidine



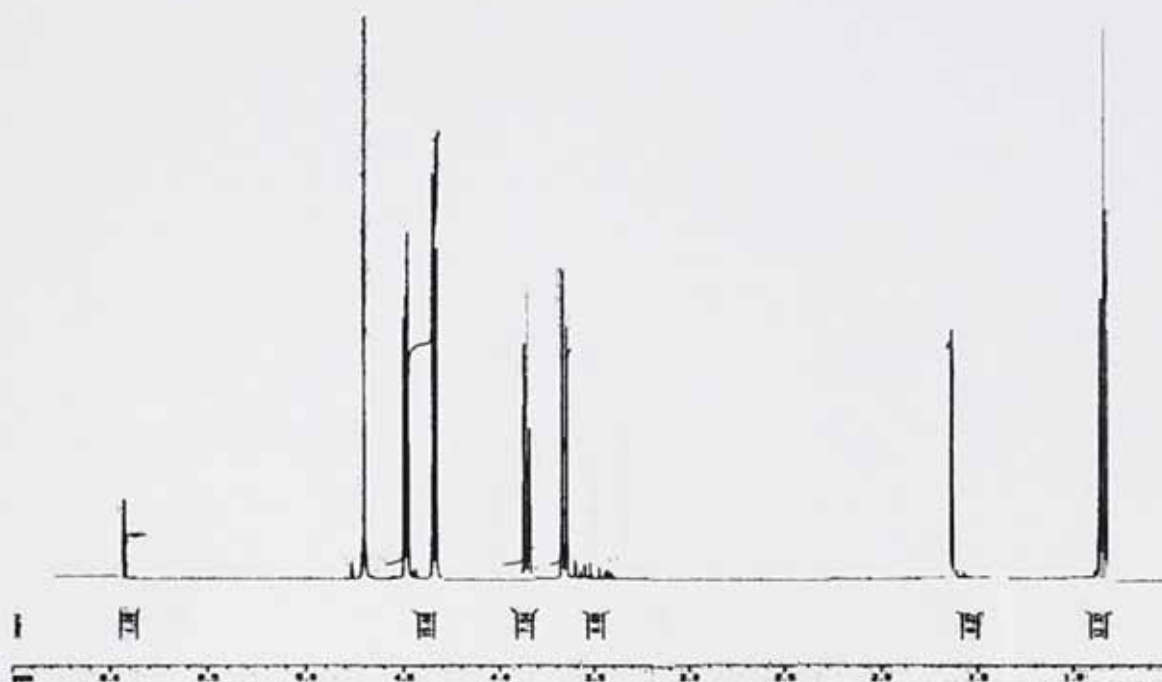
2 Pyrogallol with oxazolidine



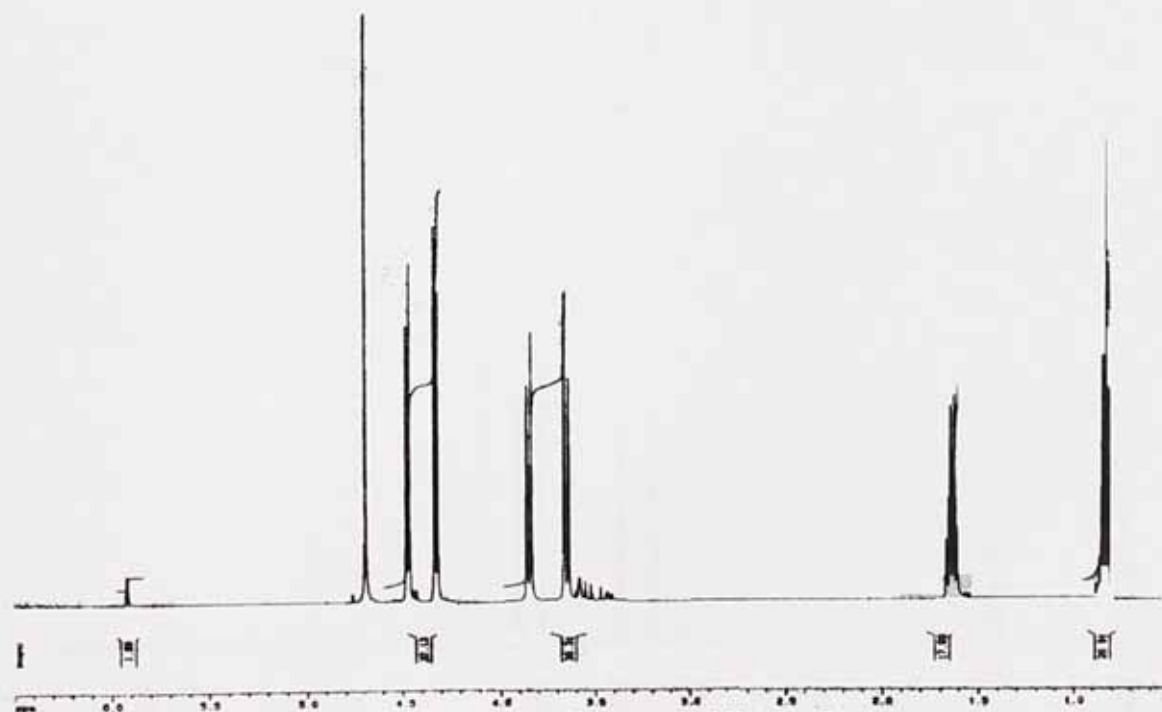
3 (Phloroglucinol+ pyrogallol) with oxazolidine



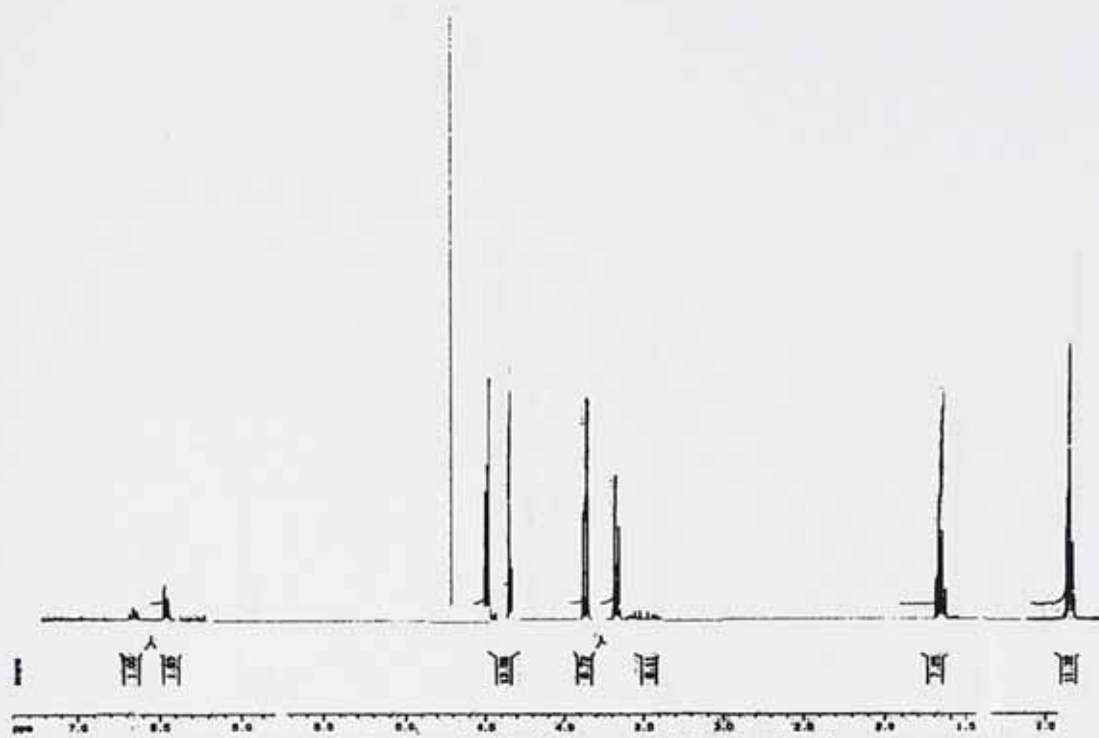
Appendix VI $^1\text{H-NMR}$ spectra of simple phenols with oxazolidine



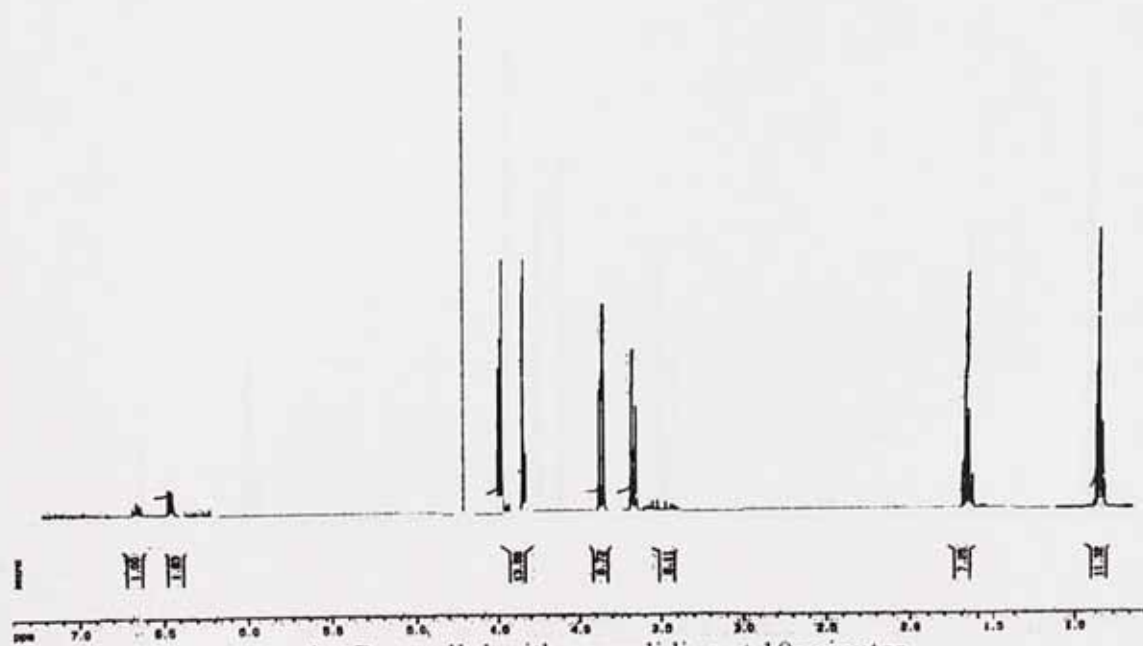
1. Phloroglucinol with oxazolidine at 2 minutes



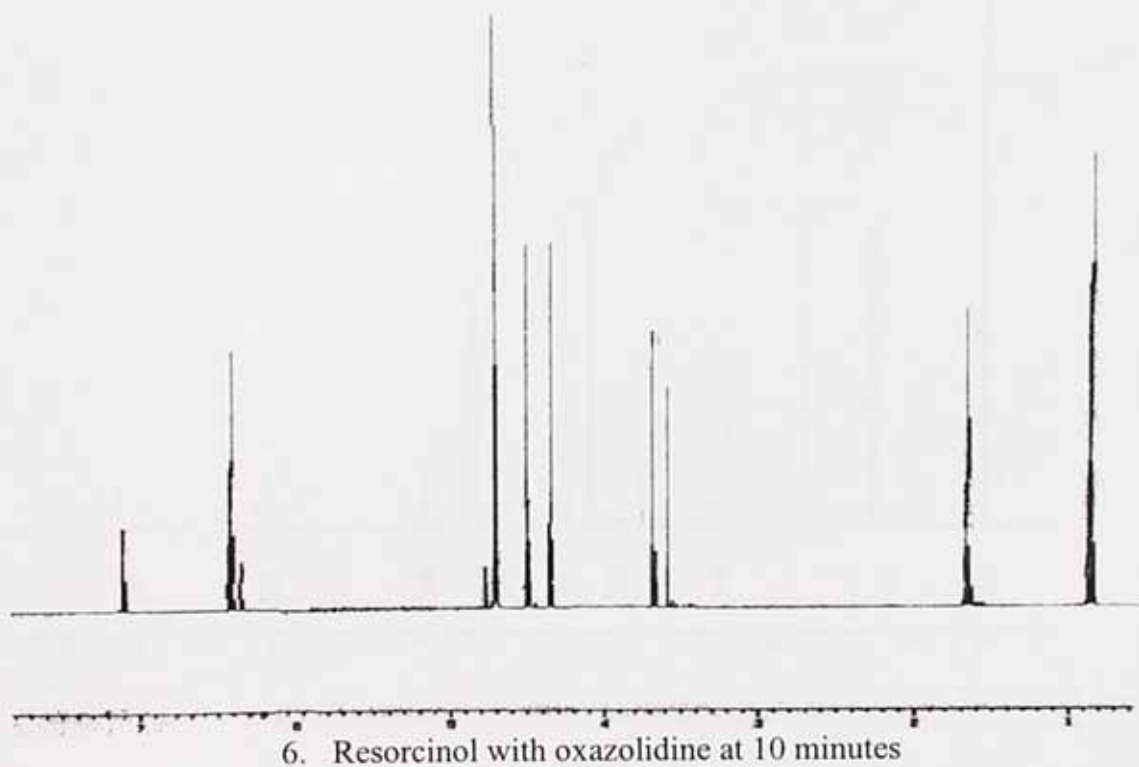
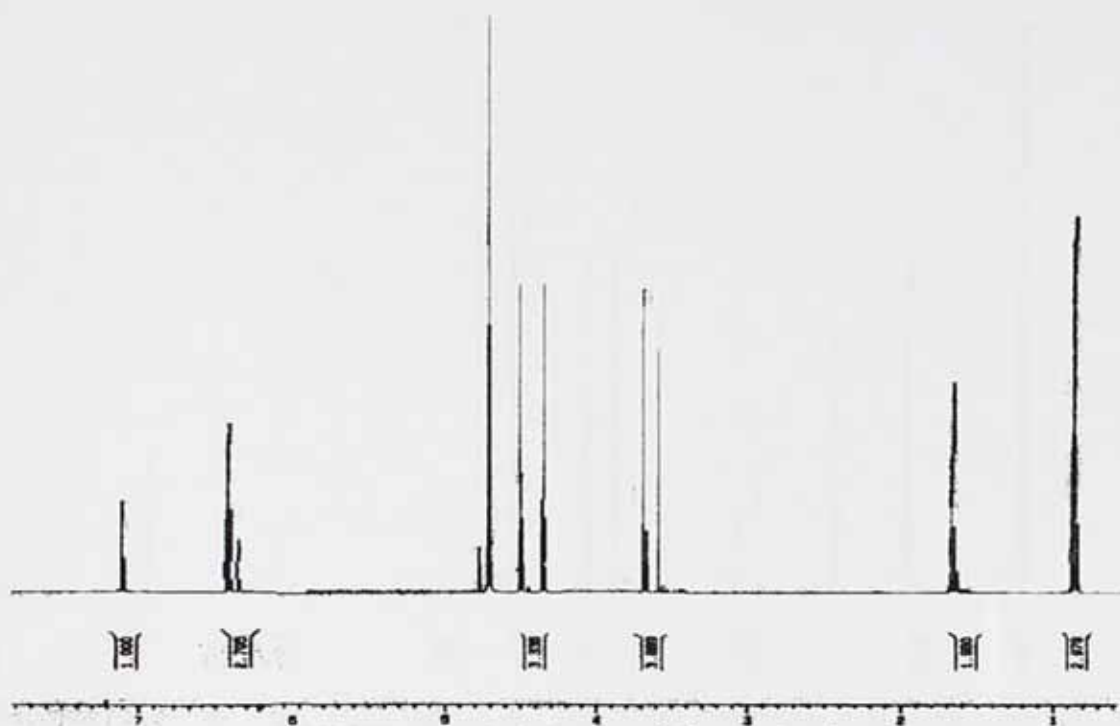
2. Phloroglucinol with oxazolidine at 5 minutes



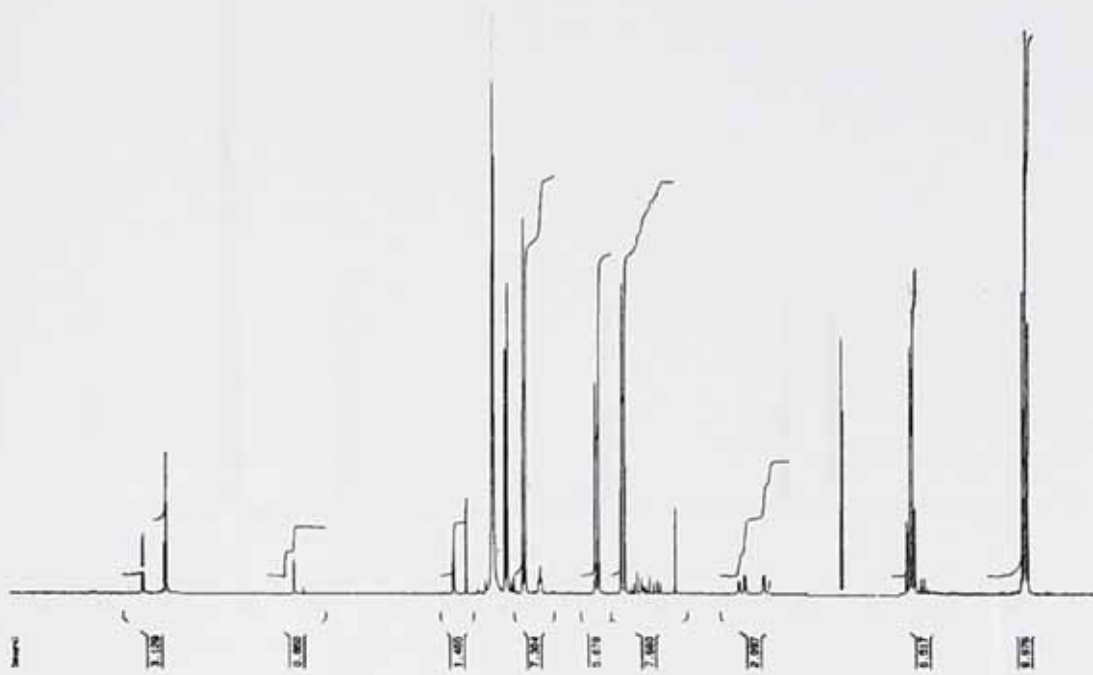
3. Pyrogallol with oxazolidine at 5 minutes



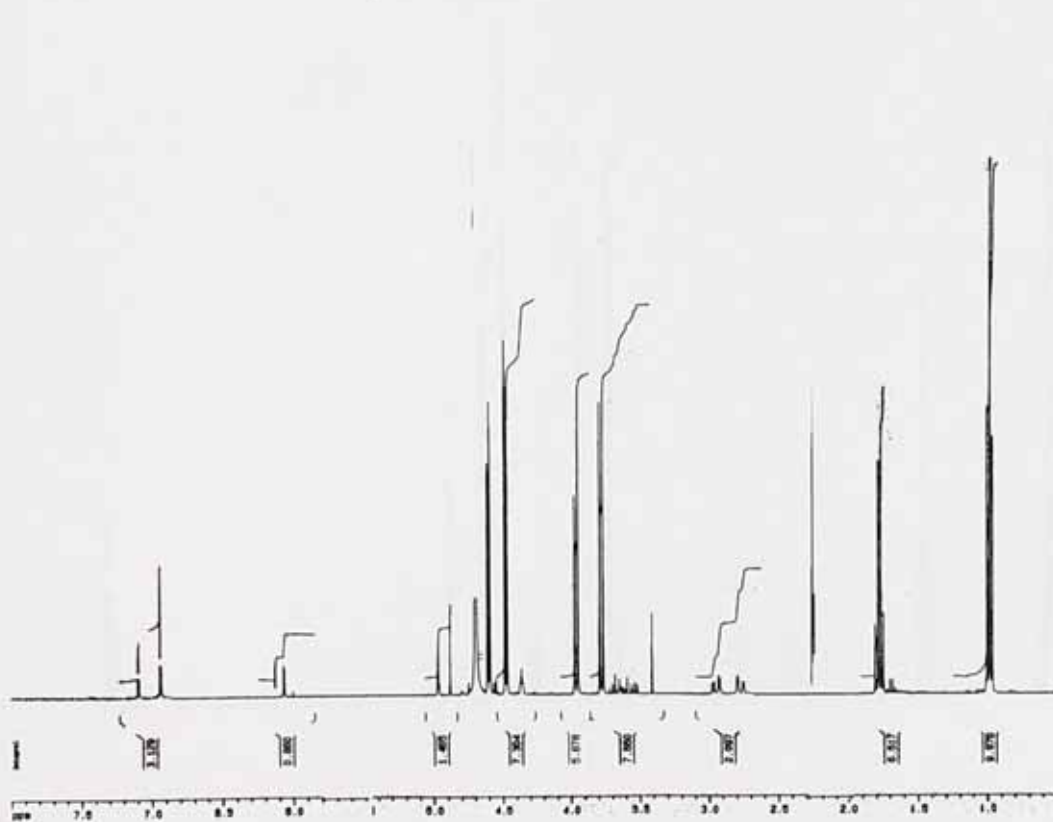
4. Pyrogallol with oxazolidine at 10 minutes



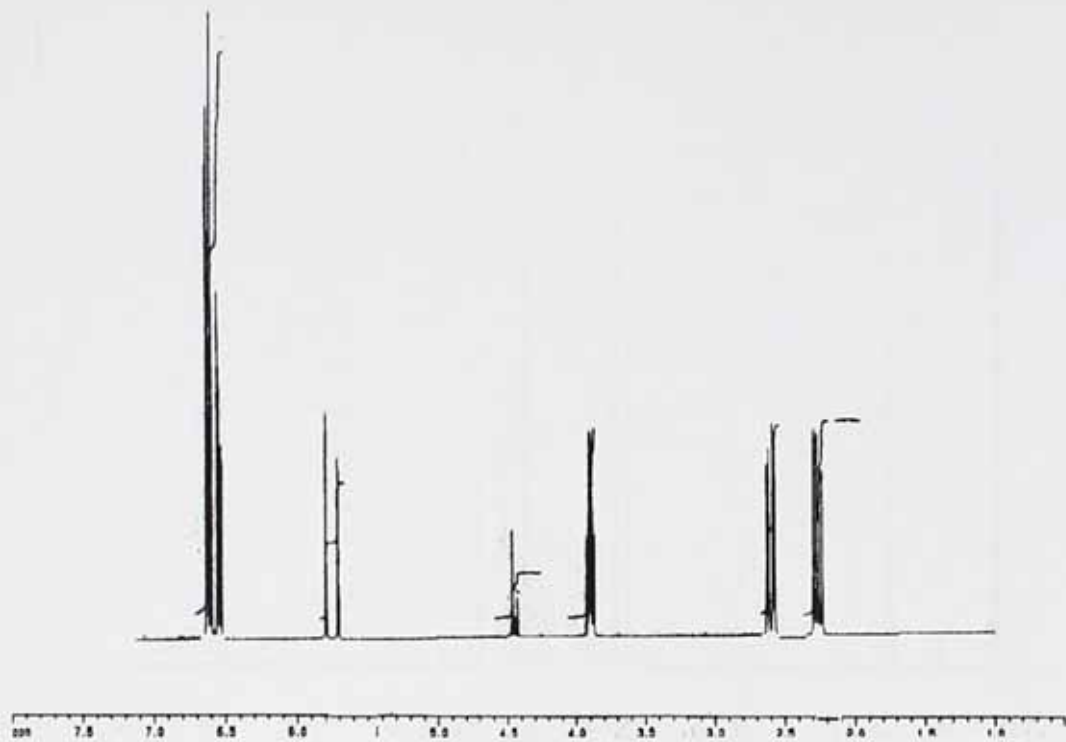
Appendix VII $^1\text{H-NMR}$ spectra of monomer reactions with oxazolidine



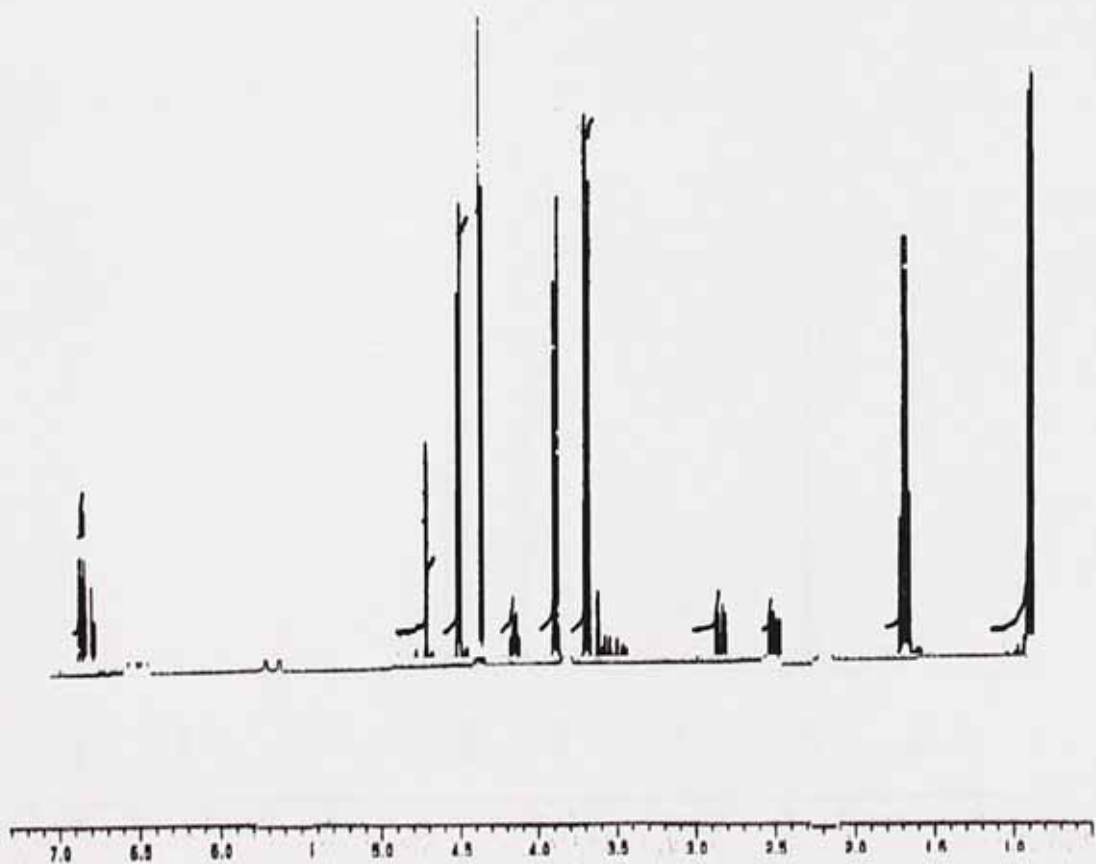
1. Epicatechin with oxazolidine at 5 minutes



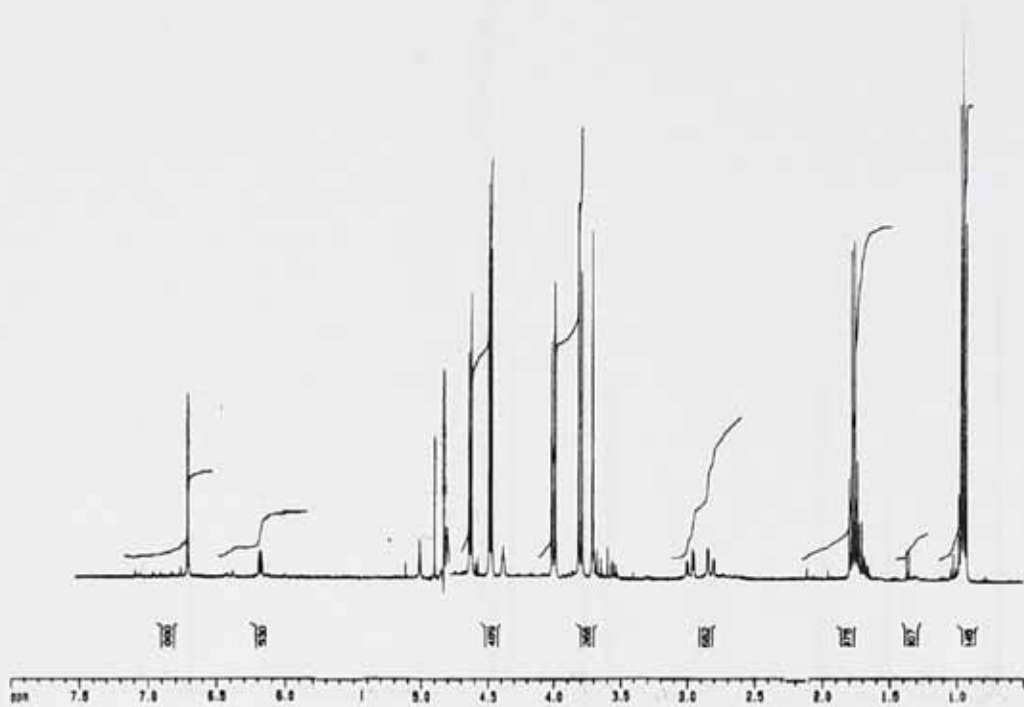
2. Epicatechin with oxazolidine at 10 minutes



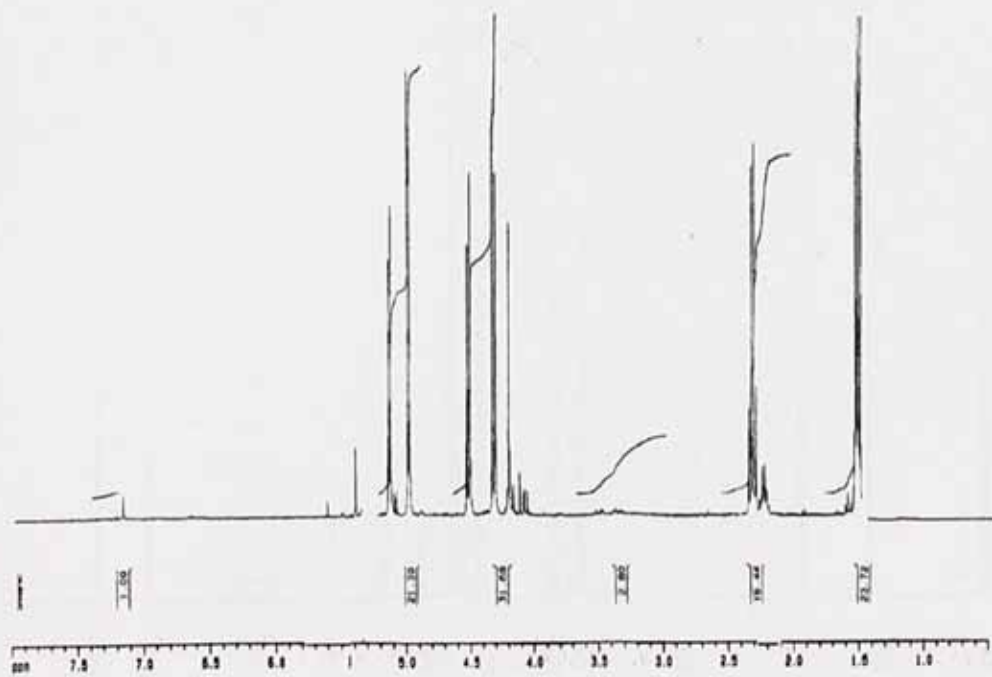
3. Catechin



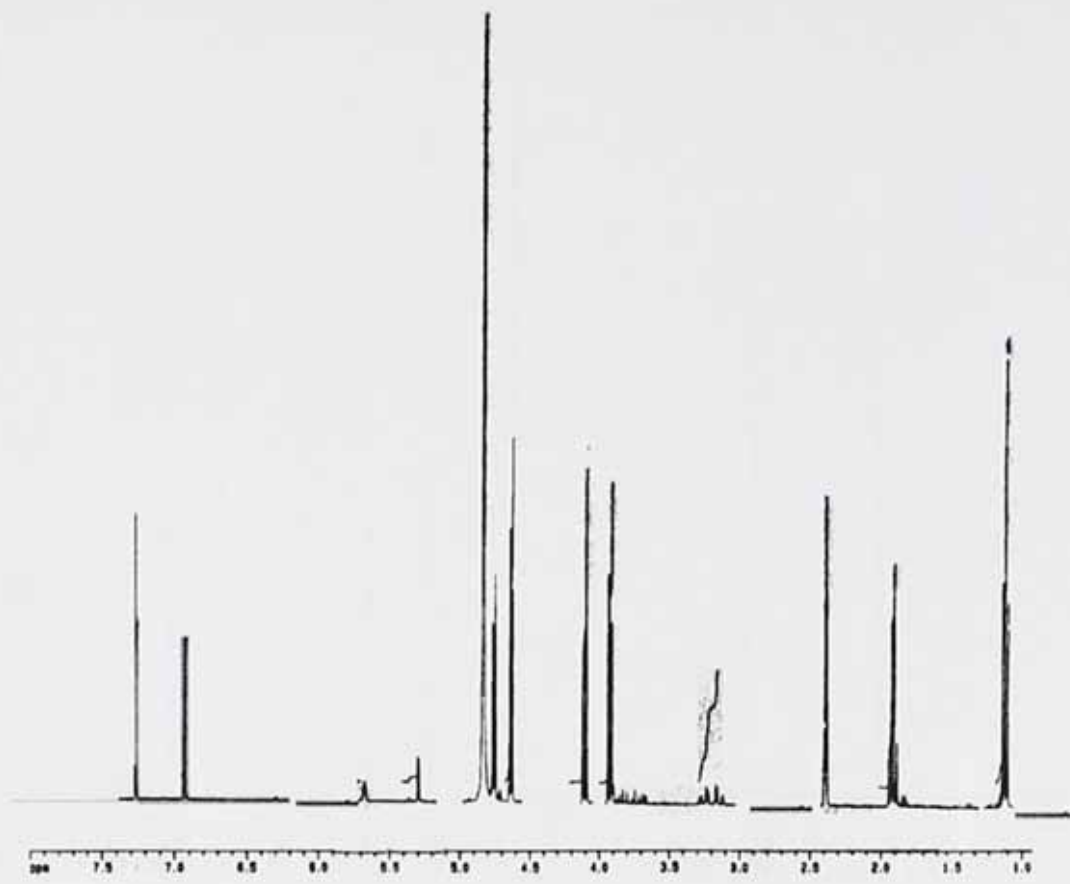
4. Catechin with oxazolidine at 10 minutes



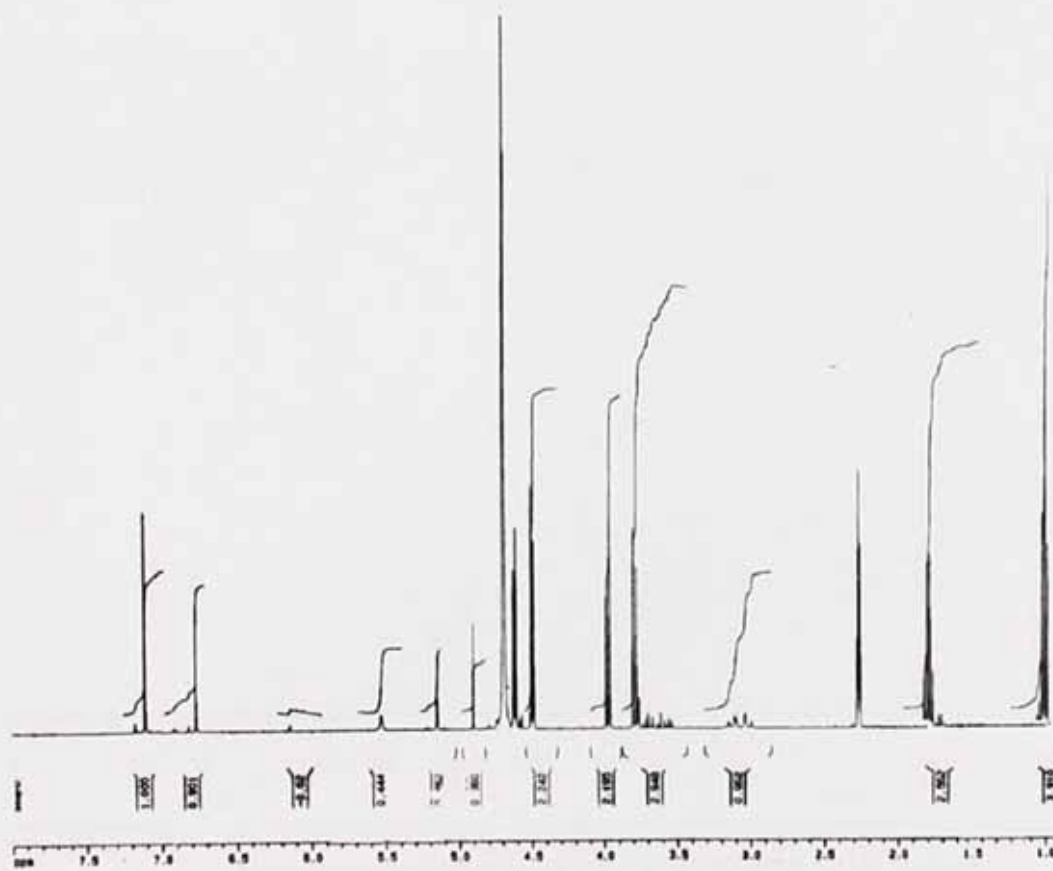
5. Epigallocatechin with oxazolidine at 5 minutes



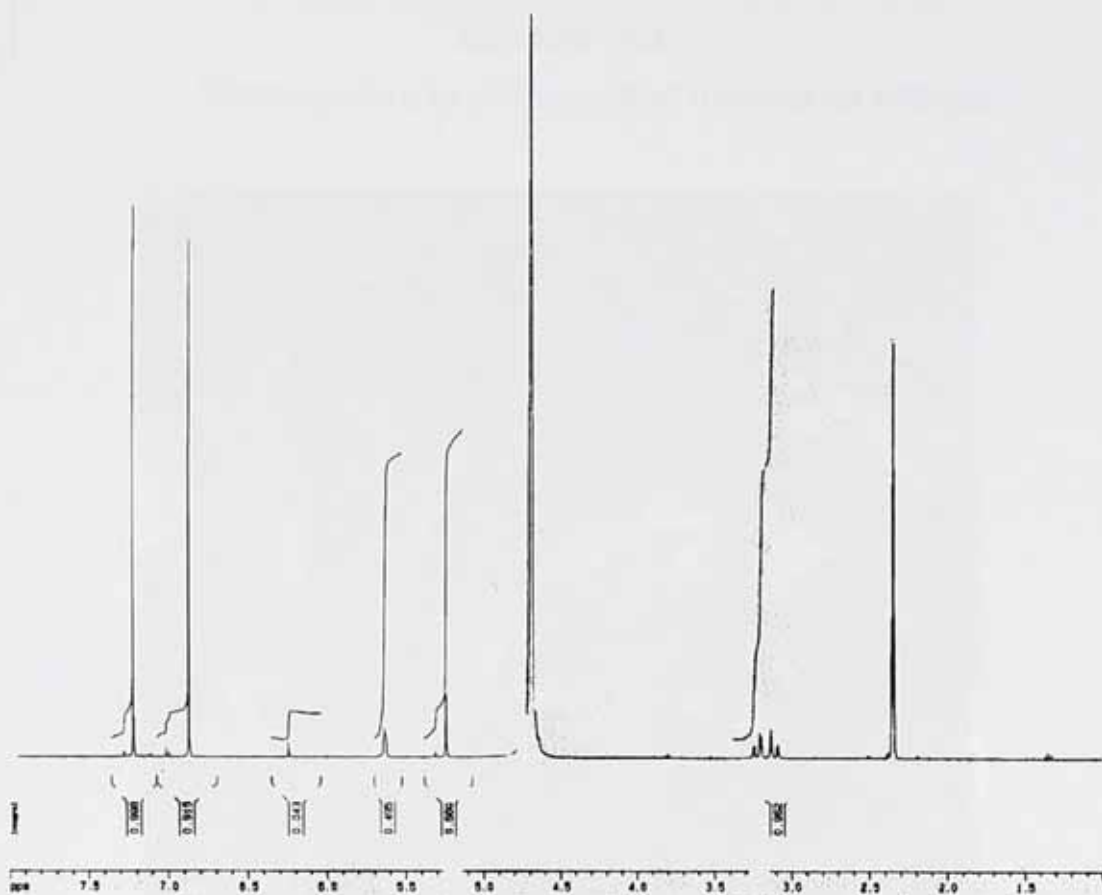
6. Epigallocatechin with oxazolidine at 10 minutes



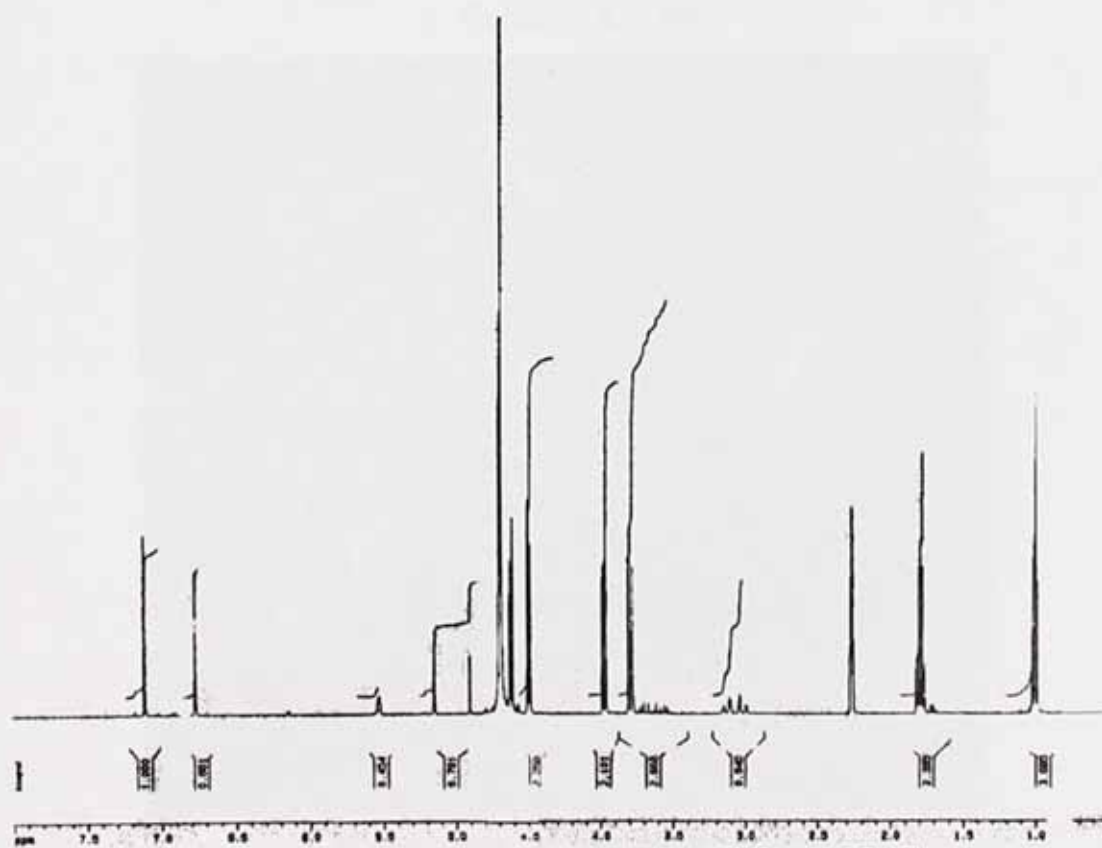
7. Gallocatechin with oxazolidine at 5 minutes



8. Gallocatechin with oxazolidine at 10 minutes



9. Epigallocatechin-gallate with oxazolidine at 5 minutes



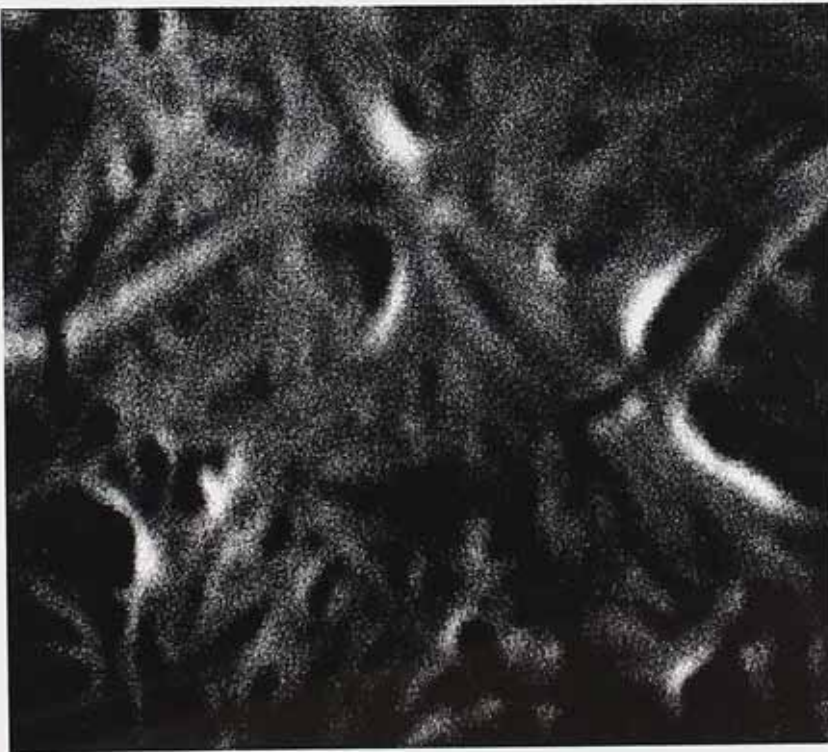
10. Epigallocatechin-gallate with oxazolidine at 10 minutes

Appendix VIII

Scanning electron micrograph of freeze-dried collagen



A Collagen X 30000



B Cross-linked Collagen X 30000

Appendix IX

Publications

1. A. D. Covington and L. Song, Crosslinking-what crosslinking? Studies on the general mechanism of tanning, XXVII Congress of International Union of Leather Technologist and Chemists Society, May 28, 2003, Mexico, (voted the best paper in the congress by the organisers)
2. A. D. Covington and L. Song, New insight into combination organic tanning-condensed plant polyphenols and oxazolidine, XXVII Congress of International Union of Leather Technologist and Chemists Society, May 28, 2003, Mexico, (voted the third best paper in the congress by the organisers)
3. A. D. Covington and L. Song, New insights into combination tanning, *Leather*, 2003, 205(4738), 38-44
4. A. D. Covington and L. Song, Cross-linking-what cross-linking, *Leather*, 2003, 205(4739), 28-32

Oral Presentations

1. L. Song, Cross-linking reaction of phenols with oxazolidine, at the Northern group meeting of the Society of Leather Technologists and Chemists, September, 2000, Bradford, UK
2. L. Song, Reaction between collagen, prodelphinidins and oxazolidine, 97th of the Society of Leather Technologists and Chemists Annual conference, September, 2002, Harrogate, UK