The Molecular Weight Characterisation of *Pinus radiata* Bark Condensed Tannins

A thesis submitted in partial fulfilment of the requirements for the degree of Master of Science in Chemistry at the University of Waikato

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ABSTRACT

Crude condensed tannins extracted from *Pinus radiata* bark (sourced from Tokoroa) with various solvents (Hot water (HW), methanol/water/acetic acid (MWA), acetone/water (AW) and hot aqueous 2% sulphite/urea solution (SFU)) were subjected to Sephadex LH-20 column chromatography to purify the condensed tannins by removing carbohydrate and flavonoid components (crude extracts were approximately 70% condensed tannins). The condensed tannins from the HW and MWA extractions were then fractionated on a Sephadex LH-20 column using a stepwise gradient of methanol, water and acetone to separate the condensed tannins according to molecular weight (MW). Fractions were collected and pooled to give eight final fractions (HW 1-8 and MWA 1-8). Purified and fractionated condensed tannin fractions were then analysed with a range of techniques including ^{1}H nuclear magnetic resonance (NMR) spectroscopy, acid/phloroglucinol depolymerisation coupled with high performance liquid chromatography (HPLC) with UV and MS detection, matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS), electrospray ionisation mass spectrometry (ESI-MS) and gel permeation chromatography (GPC).

In the MALDI-TOF MS spectrum of the crude extracts the most intense signal was typically the tetramer peak and the decamer was typically the largest oligomer detected. In the spectra of late-eluting column fractions (*i.e.* HW and MWA 5-7) oligomers greater than the nonamer (DP up to 14) were detected and the most intense signal was shifted from the tetramer to higher MW oligomers. ESI MS showed itself to be better at analysing small MW condensed tannins. The MS results demonstrated that prior separation of the condensed tannins was effective in improving MS detection of higher MW oligomers.

The mDP of the crude and fractionated fractions was obtained by depolymerisation/HPLC. The mean degree of polymerisation (mDP) of the purified condensed tannins ranged between 7.3 (AW) and 9.2 (SFU). The mDP of the fractionated condensed tannins ranged between 2 and 18. The

depolymerisation/HPLC results of the fractionated condensed tannins were used to construct a GPC calibration curve of condensed tannin MW versus GPC retention time. To identify the GPC dimer peak a procyanidin dimer was synthesised by reacting condensed tannins with catechin under acidic conditions. Through the use of various NMR techniques (DEPT135, COSY, HSQC and HMBC experiments) the synthetic dimer was characterised and the ^{1}H and ^{13}C NMR spectra fully assigned. The GPC calibration curve enabled MW profiles for the HW and MWA 2-8 fractions and crude extracts to be obtained. GPC analysis confirmed the presence of larger oligomers that were not detected by MALDI-TOF MS and ESI MS. GPC number average MW results were in agreement with the average MW results obtained from depolymerisation/HPLC results.

Pinus radiata bark condensed tannins from two different geographical locations (Golden Downs (Nelson) and Waimate (South Canterbury)) and different tree heights (0, 10 and 20 m) were analysed using the techniques described above to discover if there was any variability between samples. Trends that were observed between the different condensed tannins were decreases in condensed tannin yield and mDP as bark was obtained from higher up a tree. Extracts from New Zealand native bark (totara, rimu and kauri) were also analysed, showing that Rimu was the only bark of these species that contained significant amounts of condensed tannins. Overall this research has provided additional information on the structure and MW of *P. radiata* condensed tannins.

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LIST OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

AW- Acetone/Water extraction
COSY- Correlated spectroscopy
Da- Daltons
DHB- 2,5-dihydroxybenzoic acid
DP- Degree of polymerisation
ESI- Electrospray Ionisation
GD- Golden Downs (Nelson)
GDP- Gross domestic product
GPC- Gel Permeation Chromatography
HSQC- Heteronuclear single quantum coherence
HMBC- Heteronuclear multiple bond correlation
HPLC- High Performance Liquid Chromatography
HW- Hot Water extraction
IAA- 3-β-indole acrylic acid
LC- Liquid Chromatography
MALDI-TOF- Matrix Assisted Laser Desorption Ionisation/Time of Flight
mDP- Mean degree of polymerisation
mm- Millimeters
MS- Mass spectrum
MW- Molecular weight
MWA- Methanol/Water/Acetic acid extraction
m/z- Mass to charge ratio
NMR- Nuclear magnetic resonance
PC- Procyanidin
PD- Prodelphinidin
PDA- PhotoDiode array
PG- Phloroglucinol
PP- Pilot plant extraction
R_T- Retention time
SFU- Sulphite/Urea extraction
SIC- Selectively ion chromatography
t- Terminal unit
TFA- Trifluoroacetate
THAP- 2,4,6-trihydroxyacetophenone
TIC- Total ion chromatogram
UV- Ultraviolet
u- Extender unit
W- Waimate (South Canterbury)

CHAPTER 1: INTRODUCTION

1.1 FORESTRY IN NEW ZEALAND

Forestry is one of New Zealand's largest industries. In 2005 forestry contributed 4% to the national GDP with over $5 billion in wood products. The major species logged in New Zealand is *Pinus radiata*. The first plantations of *P. radiata* were planted to provide a sustainable timber supply for the future and *P. radiata* forests now cover over 1.83 million hectares of New Zealand's land, which is 18% of New Zealand's forests and 89% of New Zealand's plantation forests. Eighteen million cubic metres of timber is harvested each year (www.maf.govt.nz/forestry, 25/01/06).

Bark is a by-product of the logging of trees and there is yet to be a large market with a profitable use for *P. radiata* bark. The bark represents approximately 10% of the tree's volume, therefore logging operations generate a considerable amount of low value bark. Over 1,000,000 cubic meters of bark are produced annually in New Zealand through logging (www.maf.govt.nz/forestry, 25/01/06). One use of bark is as a fuel at saw mills for drying timber. However the primary reason for which it is burned is as a means of disposal. The high moisture content of bark makes it an inefficient fuel (Coombridge, 1997). Pacific Wide Ltd from Napier uses *P. radiata* bark as a growing media potting mix and as a decorative potting mix (www.pacificwide.co.nz, 07/08/06). Finding a more economic use for *P. radiata* bark would have beneficial outcomes for the New Zealand economy as well as aid in the disposal of an otherwise waste product.

1.2 BARK AND EXTRACTIVES

1.2.1 BARK STRUCTURE

The bark of a tree comprises the phloem (inner bark), rhytidome (outer bark) and periderm (outer bark). The purpose of the phloem or inner bark is to transport sugars around the tree through its conductive tissue and it is therefore essential to the living tree. It is composed of several types of cells, each of which has a significant role in the life of a tree. The phloem lies between the vascular cambium and the outer cork cambium, that is between two layers in which cell division and growth take place, and is derived from the vascular cambium (Prance and Prance, 1993). Figure 1.1 shows a typical cross-section of a *P. radiata* tree trunk.

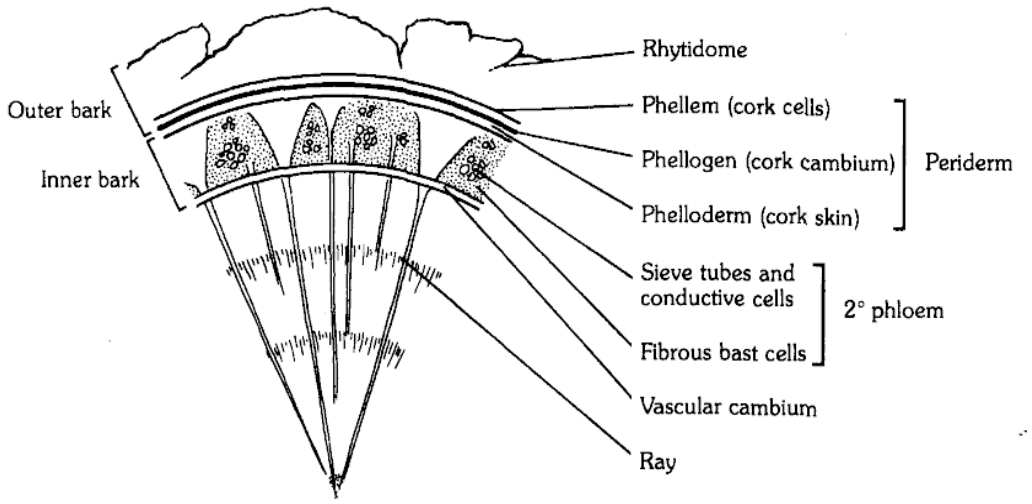


Figure 1.1 Cross section of tree trunk (Prance and Prance, 1993)

The primary function of the rhytidome (outer bark) is to protect the tree. The outer bark has a complex structure and arises from the temporary development of cell division zones. As the tree increases in size the outer layers of the rhytidome are placed under stress, as the dead cells cannot divide. These stresses cause cracks in the bark. At the base of these cracks are tiny pores called lenticels which allow for the diffusion of air into the living tissues under the impermeable bark layer (Kininmonth and Whitehouse, 1991). An additional component of the outer bark is the periderm which consists of three tissue regions the pellogen, pelloderm and phellem (Prance and Prance, 1993).

1.2.2 CHEMISTRY AND COMPOSITION

Conifer bark, including that of *P. radiata* bark contains a higher percentage of extractives than associated wood. Table 1.1 compares the approximate chemical composition of *P. radiata* bark and wood. *P. radiata* bark contains approximately 65% extractable material, compared with 2% in wood (Table 1). The extractives are those materials that can be extracted from the bark using various solvents including those shown in Table 1.2; however the total of the extractives in Table 1.2 do not add up to 65% because Table 1.2 does not include aqueous alkaline extraction.

Table 1.1 Composition of *P. radiata* bark and wood
(Kininmonth and Whitehouse, 1991)

Component	**% Composition**	
	Bark	**Wood**
Extractives	65	2
Lignin	15	27
Cellulose	12	40
Hemicelluloses	6	31
Ash	2	0.2

Table 1.2 also shows the variation in bark extractive yields with tree age. As the tree ages, the total extractives of the bark decrease, and the yield of methanol extractives increases. The reason for the increase in the methanol soluble extracts is due to the increase in the amount of higher molecular weight condensed tannins. Upon ageing, the monomeric flavonoids polymerise to form higher molecular weight condensed tannins and phenolic acids which are insoluble in ethyl acetate and acetone but soluble in methanol (Kininmonth and Whitehouse, 1991).

Table 1.2 Yields of bark extractives obtained by sequential extraction of *P. radiata* with a variety of solvents (Kininmonth and Whitehouse, 1991)

Solvent	**Extractives (% in air-dry bark)**			
	Bark (16 year old)	**Bark (25-30 year old)**	**Bark (40 year old)**	**Approximate composition**
Hexane	2.3	1.7	0.5	terpenes, hydrocarbons
Benzene	2.2	1.7	1.0	resin acids
Ethyl acetate	6.9	4.0	1.3	low M.W. phenolics
Acetone	16.7	13.3	5.2	polymeric tannins
Methanol	4.0	5.0	12.0	polymeric tannins
Total extractives	32.1	25.6	20.0	

1.2.3 FLAVONOIDS

Flavonoids are found in all vascular plants and at least 4000 different structures have been reported (Iwashina, 2000). Flavonoids are known to occur widely in woody plants and are found in both hardwoods and softwoods (Harborne, 1989). In *P. radiata*, bark tissue contains larger amounts of flavonoids than wood (Kininmonth and Whitehouse, 1991). Flavonoids are made up of two aromatic carbon rings, separated by a chain of three carbon atoms. The role of flavonoids is not fully known, but it is believed they have a protective role against fungal attack (Harborne, 1989). The three flavonoids commonly present in *P. radiata* bark are taxifolin (Figure 1.2(a)), catechin (Figure 1.3) and quercetin (Figure 1.2(b)) (Coombridge, 1997).

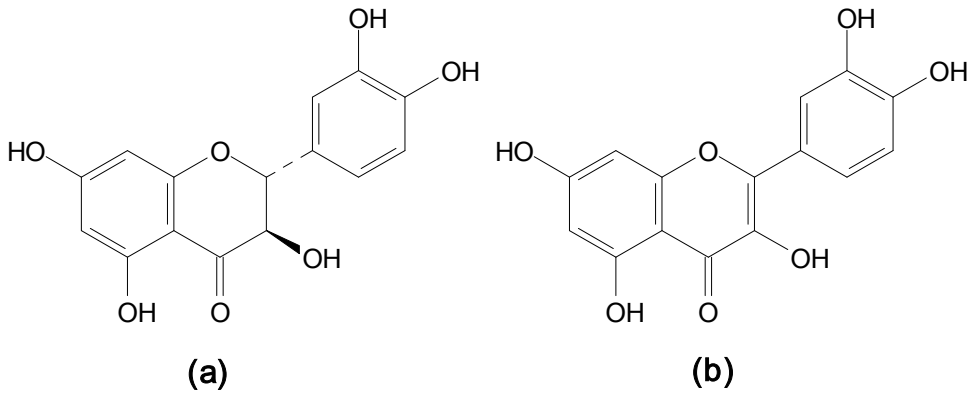


Figure 1.2 Chemical structure of (a) taxifolin and (b) quercetin

1.2.4 CONDENSED TANNINS

Tannins can be conveniently divided into two main groups, hydrolysable and condensed tannins. Hydrolysable tannins are not present in conifers. Condensed tannins, also known as proanthocyanidins, are a group of polyhydroxy-flavan-3-ol oligomers and polymers linked by carbon-carbon bonds between flavanol subunits (Schofield *et al.*, 2001 and Saucier *et al.*, 2001). Condensed tannins are produced *via* condensation of simple phenolics that are secondary metabolites (Chavan *et al.*, 2001).

1.2.4 (a) PROPERTIES

Condensed tannins are mostly soluble in alcohol and acetone, especially if small amounts of water are present, and they form amorphous, non-crystalline materials in the solid state. *P. radiata* condensed tannins have an isoelectric point of pH 2-2.5, and it is accepted throughout the literature that condensed tannins are negatively charged in neutral solution (Coombridge, 1997).

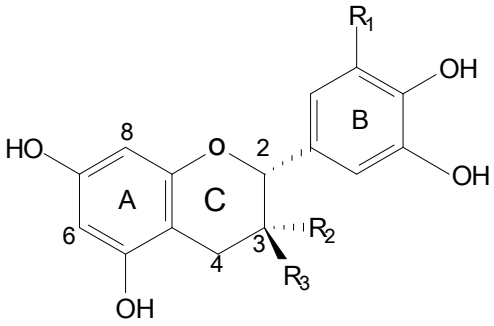
Condensed tannins are the second most abundant natural phenolic after lignin. They are found in bark, stems, phloem, seeds, fruit, fruit pods and wood of most plants (Behrens *et al.*, 2003). The reactivity of condensed tannins with molecules of biological significance has important nutritional and physiological

consequences. Their multiple phenolic hydroxyl groups leads to the formation of complexes with proteins, metal ions and with other macromolecules like polysaccharides (Schofield *et al.*, 2001).

The organoleptic properties (e.g. bitterness and astringency) and the reactivity of condensed tannins, including radical scavenging effects and protein binding ability largely depend on the nature and the number of consecutive units. In particular, the number of active sites, which increases with the degree of polymerisation (DP) (Gross *et al.*, 1999). Therefore determination of tannin structure is a prerequisite to study their chemical properties and eventual commercial uses. Condensed tannins are believed to exhibit anti-oxidant, antiulcer, anticarcinogenic, antimutagenic and antiviral activities (Ashraf-Khorassani and Taylor, 2004). The molecular weight and hydroxyl pattern are the main contributing factors to the effectiveness of condensed tannins to exhibit the above properties (De Bruyne *et al.*, 1999). For example Wang *et al.* (1997) found that increasing the molecular weight of the condensed tannin also increased the anti-oxidant behaviour.

1.2.4 (b) STRUCTURE

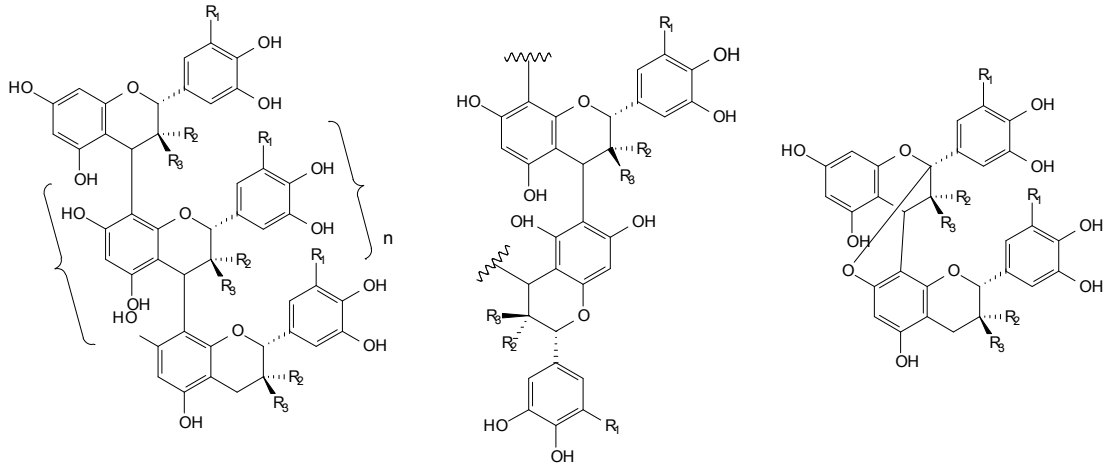
Condensed tannins are composed of flavan-3-ol monomer units. The diversity of condensed tannins arises from the structural variability of the monomer units (Figure 1.3), different hydroxylation patterns of the A and B aromatic rings, different stereochemistry at the chiral centres C2 and C3 and the distinct location and stereochemistry of the interflavonoid bond (Behrens *et al.*, 2003). These variations in the stereochemistry of C2 and C3 of the C ring occur in nature and observations of model compounds suggest that these variations have relatively little effect on most of the reactions used in tannin assays (Schofield *et al.*, 2001). *P. radiata* is comprised of mainly procyanidin and prodelphinidins (Matthews *et al.*, 1996 and Czochanska *et al.*, 1979).



PROCYANIDIN R_1 = H	
Catechin	R_2 = H, R_3 = OH
Epicatechin	R_2 = OH, R_3 = H
PRODELPHINIDIN R_1 = OH	
Gallocatechin	R_2 = H, R_3 = OH
Epigallocatechin	R_2 = OH, R_3 = H

Figure 1.3 Structure of flavan-3-ol unit

Monomer units are linked together intermolecularly by either B-type C4-C8 (Figure 1.4(a)) or C4-C6 (Figure 1.4(b)) bonds or doubly linked with A-type (Figure 1.4(c)) bonds which have an additional ether C2-O-C7 link to form oligomers and high molecular weight polymers (Gross *et al.*, 1999). B-type C4-C8 interflavonoid bonds are the most common linkage. ^{13}C-Nuclear Magnetic Resonance (NMR) spectroscopy and molecular weight studies have shown there is little branching occurring in these polymers (Hemingway and Karchesy, 1989). Thompson and Pizzi (1995) found through NMR spectroscopy that *P. radiata* condensed tannins were mainly B-type C4-C8 linked with some B-type C4-C6 and no A-type linkages.



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

Figure 1.4 Different types of interflavonoid bonding (a) B-type C4-C8 bonding, (b) B-type C4-C6 bonding and (c) A-type C2-O-C7 bonding

1.2.4 (c) USES OF CONDENSED TANNINS

Condensed tannins have historically been employed in a wide range of applications. The most well known use is in the tanning of leather. The use of condensed tannins in this process however became obsolete with the introduction of chromium salts in the 1970's (Prance and Prance, 1993). Chemically modified, condensed tannins have been used as flocculants for the clarification of muddy water reservoirs, as well as in the oil mining industry, to control the viscosity, and water loss of drilling fluids (Roux *et al.*, 1980). A number of potential pharma/nutraceutical uses relate to the properties which were discussed in Chapter 1.2.4 (a).

One of the major areas of tannin research is in adhesive systems. A great deal of research has been carried out on the use of condensed tannins as extenders in phenol-formaldehyde (PF) or phenol-resorcinol-formaldehyde (PRF) resins (Roux *et al.*, 1980). The most expensive components of these resins are resorcinol and phenol. The highly activated phloroglucinol structure of the A-ring in proanthocyanidins may be expected to replace resorcinol, or act as an extender in the PRF system (Warnes and Kerr, 2001).

1.3 CONDENSED TANNINS- EXTRACTION, PURIFICATION, FRACTIONATION AND SYNTHESIS

1.3.1 CONDENSED TANNIN EXTRACTION

In the literature many different solvent systems have been used for the extraction of condensed tannins from plant materials. The efficiency of the solvent at extracting condensed tannin depends on their chemical nature, the solvent system used and extraction conditions (Chavan *et al.*, 2001, Matthews *et al.*, 1996 and Yazaki, 1985). Chavan *et al.* (2001) extracted condensed tannins from beach pea and found that among the solvents tested (water, methanol, acidified methanol, acetone and acidified acetone) 70% (v/v) acetone, containing 1% (v/v) concentrated HCl extracted the largest yield of condensed tannins. Inoue *et al.* (1998) extracted condensed tannins from *P. radiata* and found that extracting condensed tannins with 1% (v/v) NaOH, at 140˚C and a pressure of 10 atm resulted in the highest yield of extracted material.

Different chemicals have been added to the extraction solvent to improve the effectiveness of the solvent. The addition of urea when extracting condensed tannins with water leads to the control of the noncovalent colloidal interactions among condensed tannins from *P. radiata*. This results in minimal phlobaphene (water insoluble compounds formed through the oxidation and polymerisation of procyanidins and flavonoids (Sealy-Fisher and Pizzi, (1992)) formation and precipitation by preventing polyphenol self-condensation (Kim *et al.*, 1996). The addition of sodium sulphite when extracting condensed tannins with water makes comparatively high molecular weight tannins undergo cleavage at the interflavonoid bond to produce oligomeric procyanidin-4-sulfonate derivatives (Kreibich and Hemingway, 1987).

1.3.2 PURIFICATION OF CONDENSED TANNIN

The most widely used method for the purification of condensed tannins from non-proanthocyanidin material, is adsorption chromatography, with Sephadex LH-20

(beaded cross linked hydroxypropylated dextran) as the stationary phase. Sephadex LH-20 has both hydrophilic and lipophilic character depending on the solvent used. The condensed tannin is absorbed onto the Sephadex LH-20 through hydrogen bonding. This method has been used to purify condensed tannins from *P. radiata* (Soto *et al.*, 2001). Sephadex LH-20 can be used for both size fractionation of condensed tannins using alcohol water gradients (see Chapter 1.3.3) and more frequently sample cleanup, to remove sugars, glycosides and monomeric polyphenols from larger proanthocyanidins (Taylor *et al.*, 2003).

The purified polymer is eluted in a narrow band with a small volume of acetone:water (1:1). When methanol is added as the mobile phase, the stationary phase displays hydrophilic character and can hydrogen bond with the condensed tannins. When acetone is added as the mobile phase the stationary phase displays lipophilic character and breaks the hydrogen bonding and releases the condensed tannins. Acetone and methanol are the solvents used because condensed tannins are soluble in them (Ellingboe *et al.*, 1970). Sephadex LH-20 has been used to separate out condensed tannins from many plant materials including blueberries (Prior *et al.*, 2001), hops (Taylor *et al.*, 2003) and plum seeds and skin (Gu *et al.*, 2003).

Another medium that has been used in the separation and clean up of condensed tannins from non-proanthocyanidin material is Toyopearl HW 40-F chromatography medium. Toyopearl HW 40-F is a size exclusion chromatographic material (Jorgensen *et al.*, 2004). Labarbe *et al.* (1999) used Toyopearl HW 40-F for the separation and clean up of grape skin and grape seed condensed tannins. Ethanol/water/trifluoroacetic acid (55.00:44.5:0.5, v/v/v) eluted the unwanted carbohydrates and flavonoids. Acetone/water (60:40, v/v) eluted the proanthocyanidin material.

Ramirez-Coronel *et al.* (2004) used solid-phase extraction with a C-18 Sep-PaK to remove carbohydrates and flavonoids from crude coffee pulp condensed tannins. They removed the carbohydrates with 2.5% acetic acid solution and the proanthocyanidins with aqueous acetonitrile (1:1 v/v). Guyot *et al.* (2001) used a

C-18 Sep-PaK to separate out apple skin procyanidins using the same solvents as Ramirez-Coronel *et al.* (2004).

Another way to purify and fractionate condensed tannins is through ultrafiltration (membrane filtration). This method can be used with large-scale extractions and has been shown to produce uniform quality condensed tannins (Yazaki and Hillis, 1980). Yazaki (1985) applied ultrafiltration methods to *P. radiata* bark and obtained a yield of purified condensed tannins of 50% of the extract or 12.5% of the bark. A major problem with this method is that it is very time consuming (Yazaki and Hillis, 1980).

1.3.3 MOLECULAR WEIGHT FRACTIONATION

The fractionation of condensed tannins according to molecular weight improves MS detection as well as giving a better idea of the true molecular weight distribution of the condensed tannin sample (Taylor *et* al., 2003 and Labarbe *et al.*, 1999). Taylor *et al.* (2003) used Sephadex LH-20 chromatography using a steped gradient of methanol, water and acetone to separate out hop condensed tannins according to molecular weight. Increasing the concentration of acetone led to higher molecular weight condensed tannins eluting through the column. They were able to separate out condensed tannins that had a mean degree of polymerisation (mDP) of 1.8 for the first fraction to 22.2 for the eighth and final fraction.

Labarbe *et al.* (1999) used Pyrex glass microparticles (200-400 µm) as the chromatography medium to fractionate grape seeds and skin condensed tannins according to their mDP. The researchers used a chloroform/methanol gradient and the grape proanthocyanidins were sequentially dissolved by increasing proportions of methanol in the solvent. This method separated proanthocyanidins ranging from mDP of 4.7 to 17.4 in grape seeds and mDP 9.3 to 73.8 in grape skins.

1.3.4 PROANTHOCYANIDIN DIMER SYNTHESIS

Two methods have been used for proanthocyanidin dimer synthesis. In the first method the dimer was synthesised using condensed tannin (Foo and Porter, 1983, Hemingway *et al*., 1983 and Botha *et al.*, 1981) as a starting material and in the second using taxifolin (De Bruyne *et al.*, 1995) as the starting material. The first method relies on the fact that under mildly acidic conditions, condensed tannins can be depolymerised to form monomeric 5,7,3´,4´-tetrahydroxyflavan-3-ol 4-carbocations which can be captured by a nucleophilic (neutral) flavan-3-ol such as catechin (see Figure 1.5 for mechanism) (Foo and Porter, 1983). The second method requires the reduction of taxifolin by sodium borohydride and the product treated with acid to form the flavan-3-ol carbocation which is then captured by the nucleophile (neutral) flavan-3-ol (Foo and Porter, 1983). In both methods the carbocation can be captured stereo- and regio-specifically by a flavan-3-ol unit to produce predominantly B-type C4-C8 linked procyanidin dimers (Botha *et al*., 1981).

In each case after the reaction was completed the dimer was purified. The reaction mixture was extracted with ethyl acetate, which extracts low molecular weight oligomers (i.e. monomers up to tetramers) (De Bruyne *et al.*, 1995) and the dimer was purified using Sephadex LH-20 column chromatography. De Bruyne *et al*. (1995) used a gradient ranging from 75% (v/v) methanol to 100% (v/v) propanol, while Foo and Porter (1983) used an ethanol water gradient. Botha *et al*. (1981) used fast atom bombardment and 2D t.l.c to analyse the fractions collected to identify which contained the higher dimer content. Purified dimers have been analysed in detail by NMR spectroscopy (see Chapter 1.4.2).

1.4 METHODS OF INVESTIGATION

1.4.1 COLOURIMETRIC ASSAYS

The Folin-Ciocalteu assay is an improved version of the Folin-Denis method for measuring tyrosine in proteins. The Folin-Ciocalteu assay is not specific for one particular group of phenolic compounds, but rather serves to quantify the total concentration of phenolic hydroxyl groups in the plant extract of interest (Schofield *et al.*, 2001).

The chemistry behind the assay involves an oxidation-reduction reaction in which the phenolate is oxidised, while phosphotungstic-phosphomolybdic compounds are reduced to produce a blue chromophore; the absorbance is measured at 750 nm. The method involves the production of a catechin calibration curve and the results are calculated as the % total phenolic content (by weight) as catechin equivalent (Schofield *et al.*, 2001). Weston and Foo (1997) recommend that a fresh calibration is produced each time because the method is known to be highly sensitive to moisture, temperature and light.

1.4.2 NMR SPECTROSCOPY

Structural characterisation by NMR spectroscopy of condensed tannins has been performed mainly through solution state ^{13}C and ^{1}H NMR spectroscopic experiments. This structural characterisation is difficult because of the heterogeneous character of condensed tannins (Thompson and Pizzi, 1995 and Porter, 1989). ^{13}C NMR spectral studies can readily provide information on the hydroxylation patterns of the A and B-rings, the stereochemistry of the heterocyclic rings in the chain extender (middle repeating) and terminal (end) units, and in favourable cases can estimate the number average molecular weight (Czochanska *et al.*, 1980, Hemingway and Karchesy, 1989 and Porter, 1989). ^{1}H-NMR spectroscopy can be used to give information on the stereochemistry of condensed tannins and has been used to estimate the mDP of apple condensed tannins (Guyot *et al.*, 2001).

Structural assignment of dimeric condensed tannins using different NMR techniques has been carried out by several groups (De Bruyne *et al.*, 1996, Jacobus *et al.*, 1981 and Foo and Porter, 1983). De Bruyne *et al.* (1996) fully characterised the dimer procyanidin (catechin-(4α-8)-catechin), B3 using solution state NMR through 1D experiments (^{13}C and ^{1}H) and 2D experiments (COSY, LRCOSY, HETCOR, LRHETCOR, HMQC and HMBC).

1.4.3 MASS SPECTROMETRY

1.4.3 (a) MATRIX ASSISTED LASER DESORPTION IONISATION – TIME OF FLIGHT MASS SPECTROMETRY (MALDI-TOF MS)

In recent years Matrix Assisted Laser Desorption Ionisation (MALDI) has been widely utilised as a soft ionisation mass spectrometry (MS) technique which provides spectra of mainly intact quasimolecular ions with little fragmentation. The use of MALDI combined with a time of flight (TOF) is a powerful method for the characterisation of synthetic and natural polymers. MALDI-TOF MS can be used to study intact structures of condensed tannins isolated from various plants (Behrens, 2002 and Ishida *et al.*, 2005). With this technique fragmentation of the target analyte molecule upon laser irradiation can be substantially reduced by co-precipitating it with a matrix which absorbs the laser energy (Behrens, 2002).

According to Reed (2005) the advantages of MALDI-TOF MS are:

1. Compounds of high molecular weight can be detected;
2. The high sensitivity of the technique across a broad range of masses allows detection of oligomeric series of compounds;
3. Only one quasimolecular ion is formed from each parent molecule;
4. Interpretation of isotope patterns allows the detection of oligomers with small differences in mass.

Many reports within the last 10 years have appeared on the application of MALDI-TOF MS for the analysis of condensed tannins. The majority of the research has focused on grape proanthocyanidins (Perret *et al.*, 2003; Yang and Chien, 2000; Krueger *et al.*, 2000; Nonier *et al.*, 2004 and Kennedy *et al.*, 2003). The spectra obtained from grape seed and grape skin extracts show a complex mixture of proanthocyanidins and galloylated (addition of a galloyl group by an ester linkage at C3 (Reed *et al*, 2005)) proanthocyanidins. Other proanthocyanidin-containing materials that have been studied include coffee pulp (Ascencion *et al.*, 2004), apples skin (Ohnishi-Kameyama *et al.*, 1997 and Guyot *et al.*, 1996) and beer hops (Taylor *et al.*, 2003). Apple skins showed some galloylated condensed tannins, while coffee pulp and beer hops contain no galloylation.

Behrens *et al.* (2003) used MALDI-TOF MS to analyse proanthocyanidins from the leaves and needles of willow, spruce, beech and lime trees. Post source decay (PSD) fragmentation was used for a more specific elucidation of individual chains. This technique allowed for the selection of a molecule in a defined mass window and the analysis of its fragments. The authors found that the willow and lime samples yielded only procyanidin units while the spruce and beech samples contain a mixture of procyanidin and prodelphinidin units (Behrens *et al.*, 2003).

Reed, 2005 described several factors that must be optimised in order to obtain good spectra using MALDI-TOF MS. These factors include

1. The selection of an appropriate matrix;
2. Optimal mixing and drying of the sample and matrix;
3. Selection of an appropriate solvent;
4. Optimal selection of an alkali metal salt for spectral enhancement;
5. The appropriate laser strength.

The preferred matrices used by most research for the analysis of procyanidins are 2,5-dihydroxybenzoic acid (DHB) (Ishida *et al.*, 2005, Behrens *et al.*, 2003 and Perrot *et al.*, 2003) and 3-β-indole acrylic acid (IAA) (Krueger *et al.*, 2003; Ohnishi-Kameyama *et al.*, 1997 and Taylor *et al.*, 2003). The most popular solvents used have been various ratios of acetone and water (Pasch *et al.*, 2001),

and methanol and water (Perret *et al.*, 2003). Pasch *et al.* (2001) and Nonier *et al.* (2004) experimented with the addition of NaI as an enhancement ion in the analysis of Mimosa and Quebracho condensed tannins but achieved no spectral enhancement. The addition of K^+, Ag^+ and Cs^+ ions (Krueger *et al.*, 2003) has also been tried in the analysis of ground sorghum grain. The addition of K^+ ions resulted in the formation of cation adducts $[M + K]^+$ but with no spectral enhancement compared with the usual $[M + Na]^+$ ions produced when no enhancement agent was added. The addition of Ag^+ and Cs^+ ions gave spectral enhancement, but in the case of the Ag^+ ion, the analysis of interflavonoid linkages was challenging because Ag^+ has two naturally occurring isotopes differing by 2 amu with similar abundances, resulting in two quasimolecular ions for each parent molecule which complicates the spectrum.

1.4.3 (b) ELECTROSPRAY IONISATION MASS SPECTROMETRY (ESI MS)

Electrospray ionisation (ESI) is a soft ionisation technique and is well suited for analysing high molecular weight compounds. In ESI MS positive or negative mode singly or multiply charged ions are produced. Quasimolecular ions are usually produced by the addition of proton, alkali cation, or ammonium ion in positive ion formation and by subtraction of a proton or cation in negative ion formation (Gross *et al.*, 1999).

Small molecules tend to be detected as singly charged ions, whereas larger ones are generally detected as multiply charged ions. Proanthocyanidin molecules are better detected in the negative ion mode than the positive ion mode (Guyot *et al.*, 1996). The number of negative charges increases as the chain length of the proanthocyanidin molecule increases. This means that condensed tannin oligomers are detected as both singly charged and multiply charged ions up to a DP of 8 and only as multiply charged species beyond this DP (Gross *et al.*, 1999).

A necessary step in the interpretation of proanthocyanidin mass spectra is distinguishing between singly and multiply charged signals. This is done by examination of the distance between isotopic peaks. Isotopic peaks are separated by 1 amu in the case of singly charged species and 0.5 amu for doubly charged ions (Gross *et al.*, 1999).

1.4.4 CHROMATOGRAPHY

1.4.4 (a) GEL PERMEATION CHROMATOGRAPHY (GPC)

Gel Permeation Chromatography (GPC) analysis can give information on molecular weight distributions of condensed tannins, as well as give information on the number average and weight average molecular weights. Analysis of proanthocyanidins has traditionally been carried out on methyl ether or acetylated derivatives. Derivatisation was considered necessary because condensed tannins were too polar to be separated on columns available at the time (Kennedy and Taylor, 2003). Derivatisation helped to minimise interactions between procyanidin phenolic groups and GPC sorbents and also helped to prevent self-aggregation of the procyanidins that can occur in certain solvents. Derivatisation however had its limitations; the recovery of derivatised material was less than quantitative and was also accompanied by side reaction products of unknown composition (Bae *et al.*, 1994).

Kennedy and Taylor (2003) modified the method of Bae *et al.*, (1994) to run underivatised condensed tannins using a mobile phase of *N*,*N*-dimethylformamide (DMF) containing 1% (v/v) glacial acetic acid, 5% (v/v) water and 0.15 mol L^{-1} LiCl. LiCl was added to eliminate the self aggregation of procyanidin oligomers and give retention properties consistent with expectations. Acetic acid was added to reduce the potential for proanthocyanidin oxidation. The method of Bae *et al.* (1994) used a aqueous-DMF 3 mol L^{-1} ammonium formate (99.5:0.5 v/v) as the mobile phase. The main problem with the method of Bae *et al.* (1994) was that the procyanidin oligomers behaved as if they were of higher molecular weight due to self association. The method of Kennedy and Taylor (2003) has many advantageous features including-

1. Prior derivatisation is not required;
2. The analysis provides full molecular mass distribution information;
3. Short run times;
4. Separation occurs under isocratic elution conditions so solvent recycling is possible.

1.4.4 (b) HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

High performance liquid chromatographic (HPLC) methods used for proanthocyanidin analysis can be divided into two sections, those that are used to analyse intact proanthocyanidins (Yanagida *et al.*, 2000, Lazarus *et al.*, 1999 and Prior *et al.*, 2001) and those that are used to analyse the proanthocyanidins following acid-catalysed cleavage (Kennedy and Jones, 2001, Kennedy and Taylor, 2003, Guyot *et al.*, 2001, Labarbe *et al.*, 1999 and Meagher *et al.*, 2004). Various detection methods have been applied in conjunction with HPLC for proanthocyanidin determination. Ultraviolet (UV) detection is the most commonly used. However, this method is not specific for proanthocyanidins in the presence of other polyphenols. Alternative methods that have been used are electrochemical detection and fluorescence detection, but neither has been as successful as the UV detector (Schofield *et al.*, 2001). HPLC ESI MS has also been used (see below).

The analysis of intact proanthocyanidins is carried out by normal phase HPLC. Hammerstone *et al*. (1999) analysed cocoa procyanidins by normal phase HPLC and were able to separate out procyanidins up to the pentamer. Yanagida *et al*. (2000) analysed apple condensed tannins and could also only separate out condensed tannins up to the pentamer. *P. radiata* bark condensed tannins are a complex mixture of oligomers with the majority of the oligomers having a higher DP than the pentamer, which is generally the highest oligomer resolved in normal phase HPLC. Hence normal phase HPLC analyses was not considered to be suitable for the analysis of *P. radiata* bark condensed tannins

Analysis of proanthocyanidins after acid-catalysed cleavage using reversed phase HPLC provides information on their subunit composition and interflavonoid bond location as well as mean degree of polymerisation (Ascencion Ramirez-Coronel *et al.*, 2004). Taylor *et al.* (2003) used reversed phase depolymerisation/HPLC to analyse grape seed and skin condensed tannins and found their mDP to be 7.8. Analysis of grape condensed tannins also showed that they contained catechin and

epicatechin as monomers and as terminal and extension units, while epigallocatechin was found as extension units.

The sub-unit composition of proanthocyanidins may be determined because of the ease with which the interflavonoid bond can be cleaved. Under acidic conditions proanthocyanidins become depolymerised, releasing extension subunits as electrophilic flavan-3-ol intermediates. The electrophilic intermediates can then be trapped by a nucleophilic reagent generating analysable adducts (Kennedy and Jones, 2001). A hypothetical mechanism for this process has been proposed (Figure 1.5).

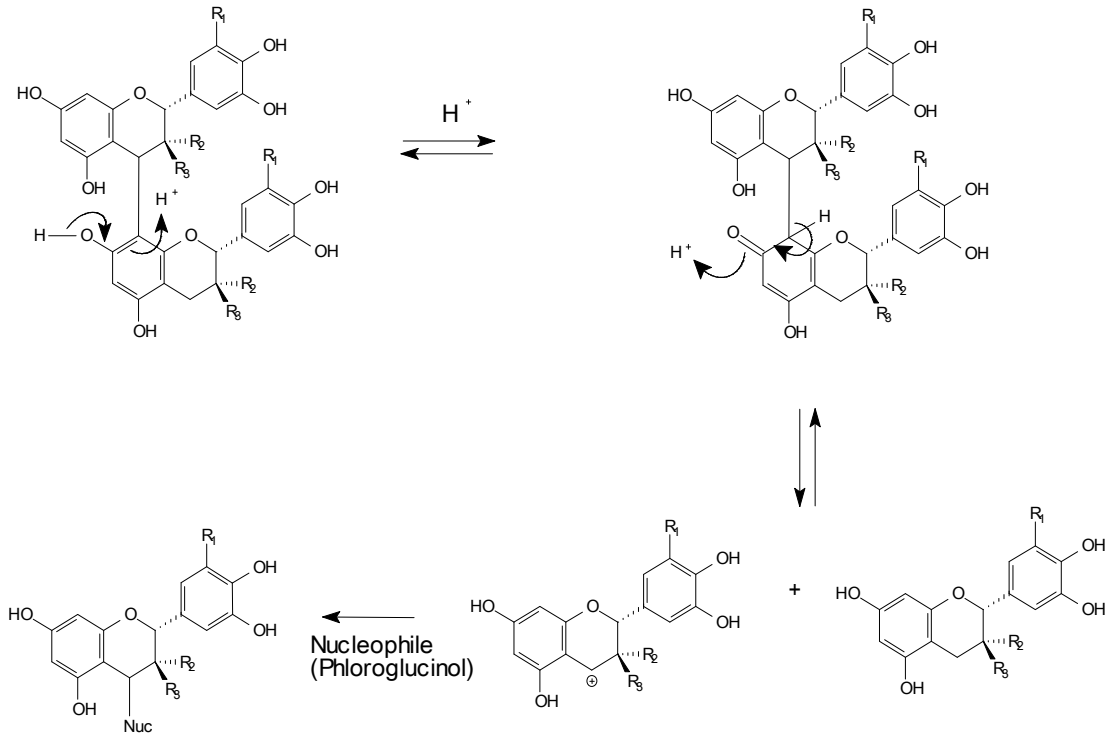


Figure 1.5 Hypothetical reaction mechanism explaining the acid-catalysed cleavage of proanthocyanidins (Kennedy and Jones, 2001)

The two most commonly used nucleophilic reagents are phloroglucinol and benzyl mercaptan (Figure 1.6). Both have been used with success, but both have

their advantages and disadvantages. The advantages of phloroglucinol put forward by Kennedy and Jones (2001) are:

1. Phloroglucinol is odourless, unlike benzyl mercaptan which can only be used in laboratories that have specialised fume hood equipment;
2. There is more selectivity in the formation of 3,4-*trans* adducts from 2,3-*trans* flavan-3-ol extension subunits meaning there will be fewer peaks present in the chromatogram.

In early studies the disadvantages of using phloroglucinol were that a large proportion of the bark tannin did not react with phloroglucinol and that yields of adducts were low, between 3 and 10% (Schofield *et al*., 2001). However Kennedy and Taylor's (2001) yields were comparable to benzyl mercaptan because a large excess of phloroglucinol was used.

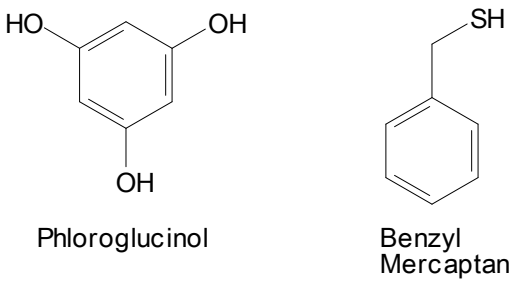


Figure 1.6 Nucleophilic reagents used in depolymerisation reaction

HPLC coupled with ESI MS enabled phloroglucinol adducts to be identified and characterised. Kennedy and Jones (2001) studied grape acid catalysed phloroglucinol adducts using HPLC ESI MS to identify epicatechin-(4β-2)-phloroglucinol, catechin-(4β-2)-phloroglucinol and epicatechin-3-*O*-gallate-(4β-2)-phloroglucinol adducts, which enabled the identification of HPLC-UV peaks. Using HPLC-UV peak areas and published molar absorptivities (Kennedy and Jones, 2001) the relative amount of extender units (detected as phloroglucinol adducts) to terminal units (detected as flavan-3-ol monomers) can be determined.

1.5 PROJECT AIMS

- To use existing methodologies for extraction, purification and fractionation of *P. radiata* condensed tannins and to determine the effects of process conditions, reagents and approaches on the yields and product compositions for *P. radiata* bark extracts.

- To develop an understanding of the chemical structure and molecular weight of the condensed tannins being extracted from *P. radiata* bark.

- To investigate and develop the use of MALDI-TOF MS and GPC in the molecular weight characterisation of condensed tannins.

- To compare condensed tannins from different geographical locations around New Zealand, from different tree heights and from different native New Zealand species

CHAPTER 2: METHODS

2.1 BARK PREPARATION

Pinus radiata bark from 20-25 year old trees used in this study was obtained from the Carter Holt Harvey Kinleith Plywood Mill, Tokoroa on 11/03/05. The bark was first air-dried to <14% moisture content, then subjected to hammer milling with the milled bark being passed through a 2 mm screen. All the bark material was provided by Charles MacIntosh, BME, Scion. All the bark used in the investigation of the different solvents was from the same batch of bark to minimise the variation that occur between samples.

The native bark samples were collected from trees around the Scion campus. Using a hammer and chisel inner and outer bark samples were taken from *Dacrydium cupressinum* – (Podocarpaceae), commonly known as Rimu (R), *Podocarpus totara* – (Podocarpaceae), commonly known as Totara (T) and *Agathis australis* – (Araucariaceae), commonly known as Kauri (K). The bark was dried in a vacuum oven at 40°C overnight and then crushed in a small wiley hammer mill and then passed through a 2 mm screen.

2.2 PARTICLE SIZE

To determine the particle size of the hammer milled bark, approximately 200 g of dry *P. radiata* bark was placed on a sieve with four different sieve sizes (<63, 63–125, 125-250 and >250 μm). The sieve was subsequently shaken for 20 minutes by a mechanical shaker and the amount of bark at each sieve size was weighed. This is a standard procedure used at Scion for finding bark particle size distributions.

2.3 MOISTURE CONTENT

Moisture content of the bark was found by placing for example 1 g of bark in a vacuum oven at 40°C and drying until a constant weight was obtained. The percentage moisture content was then calculated using the following equation.

$$\text{Percentage moisture content} = \frac{\text{Weight loss on oven drying}}{\text{Weight of bark prior to drying}} \times 100$$

2.4 PRE-EXTRACTION

A pre-extraction with hexane and toluene was performed on the *P. radiata* bark to remove hydrocarbons, waxes, carotenoids, terpenes and lipids. This involved soxhlet extraction for 3 hours using hexane (500 mL) followed by toluene (500 mL). The residual solvent was then removed from the bark under reduced pressure and the yields were measured.

2.5 BARK EXTRACTION METHODS

Hot Water (HW) Extraction

Bark (50 g) was placed in a round-bottom flask and distilled water (500 mL) was added. The flask was then placed in an oil bath and heated (90°C). The bark/water mixture was stirred with a magnetic flea (1 hr) and the solution subsequently separated from the residual bark with a Buchner funnel using Whatman #1 filter paper. Any remaining solid was washed with hot water (500 mL). The filtrate and washings were combined and dried on a Virtis freeze dryer.

Bark samples from different geographical locations and tree heights and different New Zealand native tree species were all extracted using the HW method.

Methanol/ Water/ Acetic Acid (MWA) Extraction

Bark (100 g) was placed in a 2 L beaker and methanol/water/acetic acid (1 L, 90:9.5:0.5, v/v) was added. The solution was stirred with a magnetic flea (1 hr). The solution was subsequently separated from the residual bark with a Buchner funnel using Whatman # 1 filter paper and the remaining solid was washed with methanol/water/acetic acid (1 L). The solution and washings were combined and dried under reduced pressure and placed in a vacuum oven at 40°C overnight to remove acetic acid still present.

Acetone/Water (AW) Extraction

Bark (100 g) was placed in a 2 L beaker and acetone/water (1 L, 3:2, v/v) was added. The solution was stirred with a magnetic flea (1 hr). The solution was subsequently separated from the residual bark with a Buchner funnel using Whatman # 1 filter paper and the remaining solid washed with acetone/water (1 L, 3:2, v/v). The solution and washings were combined, the acetone removed under reduced pressure and the water removed by freeze-drying.

Sulphite/Urea (SFU) Extraction

Bark (50 g) was placed in a round-bottom flask and distilled water (500 mL) containing sodium sulphite (2 g) and urea (1 g) was added. The flask was then placed in an oil bath and heated (90°C) with stirring of the bark mixture effected by a magnetic flea (1 hr). The solution was subsequently filtered from the residual bark with a Buchner funnel (using Whatman #1 filter paper) and the remaining solid washed with hot water (500 mL). The solution and washings were then combined and the solvent removed on a Virtis freeze dryer.

2.6 CARBOHYDRATE SEPARATION OF CRUDE CONDENSED TANNINS

Sephadex LH-20 (40 g) was made into a slurry with methanol:water (1:1, v/v) and packed into a glass column (45 x 2.5 cm). The column was then equilibrated with 400 mL of 1:1 methanol/water. An accurately weighed amount of crude tannin (2 g) was dissolved in a minimal volume of 1:1 methanol/water (v/v) solvent and was loaded onto the column. Carbohydrate and tannin fractions were eluted from the column with 400 mL of the following solvents:

1:1 methanol/water	f1	Carbohydrates and non-tannin components rich in flavonoids and phenols
1:1 acetone/water	f2	Condensed tannins
7:3 acetone/water	f3	Condensed tannins

The solvents were forced through under pressure with air to speed up the procedure. For each fraction the methanol and acetone were removed by evaporation under reduced pressure. Following that, the separated material was freeze-dried to remove the water. Mass balances and Folin assays were subsequently performed on all fractions. Fractions f2 and f3 were combined to make the condensed tannin fraction, f2f3. The rationale for combining the two fractions was because both fractions only contained condensed tannins.

2.7 FRACTIONATION OF PURE CONDENSED TANNINS

The procedure for the fractionation of pure (f2f3 fraction) condensed tannins by molecular weight was the same procedure as used by Taylor *et al*. (2001). Sephadex LH-20 (40 g) was made into a slurry with 1:1 methanol:water and packed into a glass column (45 x 2.5 cm). The column was equilibrated with methanol/water (400 mL, 1:1). An accurately weighed amount (0.65 g) of pure condensed tannin (f2f3) was dissolved in a minimal volume of methanol/water (1:1) with 0.5% formic acid and was loaded onto the column. The condensed tannins were eluted with a combination of water, acetone and methanol in different concentrations (Table 2.1).

Table 2.1 Solvent composition for Sephadex fractionation of *P. radiata* condensed tannins according to molecular weight

Solvent Composition	**Volume (mL)**	**Acetone (Volume %)**	**Methanol (Volume %)**	**Water (Volume %)**
A	240	0	60	40
B	240	0	75	25
C	240	0	90	10
D	200	10	80	10
E	200	20	65	15
F	200	30	40	30
G	240	60	0	40

Twenty eight fractions (4 x 60 mL fractions for solvents A, B, C and G and 4 x 50 mL fractions for solvents D, E and F) were collected. For each fraction the

acetone and methanol was removed by evaporation under reduced pressure and the samples were freeze-dried to remove the remaining water. MALDI-TOF MS analyses was done on all the fractions (see Chapter 2.11 for description of the methodology) and fractions were pooled into eight new fractions depending on mass ranges and peak intensities.

2.8 FOLIN-CIOCALTEU ASSAY

Folin-Ciocalteu assays were performed on all separated f1, f2 and f3 fractions to determine the percentage of total phenolics as a catechin equivalent. Fresh Folin-Ciocalteu reagent/water (1:1) and 7.4% Na_2CO_3 (anhydrous) reagents were made up. Seven calibration standards were prepared (Table 2.2) from two stock catechin solutions (50 μg/mL and 100 μg/mL).

Table 2.2 Composition of catechin calibration standards for Folin-Ciocalteu assay

Standard	Stock Catechin Solution (μg/mL)	Volume Stock Solution (mL)	Volume Water (mL)	Catechin Concentration (μg/mL)
1	0	0	0.50	0
2	50	0.04	0.46	4
3	50	0.08	0.42	8
4	100	0.09	0.41	18
5	100	0.15	0.35	30
6	100	0.20	0.30	40
7	100	0.30	0.20	60

The sample (5 mg) was dissolved in 10 mL of MilliQ water (1 mL of 1:1 methanol/water can be used to help dissolve the sample). Sample solution (0.05 mL) was added with MilliQ water (0.45 mL) in a test tube. To the standards and samples 50% Folin-Ciocalteu reagent (0.25 mL) and 7.4% Na_2CO_3 (1.25 mL) solutions were added. Standards and samples were vortex-mixed and left to stand (40 min). The addition of Na_2CO_3 solution was done in one-minute periods to enable the measurement of all samples and standards after 40 minutes. UV/Vis absorbance was measured on a Cary 300 Bio UV-Vis Spectrophotometer at a

wavelength of 750 nm. Samples were analysed in duplicate. Using Beer's law (A = εbc, where A = molar absorbance, ε = molar absorptivity, b = path length of the sample (cm) and c = concentration of compound in solution (mol L^{-1})) to find the concentration of phenolics in the sample; the % total phenolic content as catechin equivalent could be found using the equation below.

$$\text{\% Total Phenolic as Catechin Equivalent} = \frac{\text{Concentration of Phenolics (μg/mL)}}{\text{Concentration of Sample (μg/mL)}} \text{ X } 100$$

The Folin-Ciocalteu assay errors were found through multiple analysis of the AW f1 fraction using different calibration curves. The standard deviation was 15.0% and the 95% confidence interval was ± 17.0%. This error was used for all the Folin-Ciocalteu % total phenolic as catechin equivalent results

2.9 NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY

NMR spectra were recorded on a Bruker Advance 400 NMR spectrometer (^{1}H: 400.13 MHz and ^{13}C: 100.36 MHz), using 5-mm tubes. Condensed tannin samples were dissolved in either CH_3OD and D_2O or acetone-*d6* and D_2O. The spectrometer was locked to the deuterium resonance of the solvent D_2O. Chemical shifts were reported in ppm and the spectra were referenced to the solvent signal (acetone-*d6*, ^{1}H 2.0 ppm, ^{13}C 29.8 ppm and CH_3OD, ^{1}H 3.4 ppm, ^{13}C 49.8 ppm). NMR data was collected and analysed using TopSpin 1.3 software.

1D experiments performed were ^{1}H, ^{13}C and DEPT135 (distortionless enhancement by polarisation transfer). The DEPT135 experiment showed CH and CH_3 signals phased up and CH_2 signals phased down. 2D experiments were carried out on a synthetic condensed tannin dimer so the structure could be elucidated and so that proton and carbon spectra of the dimer could be fully assigned. 2D Experiments carried out for this were gHSQC $^{1}J_{C,H}$ (heteronuclear single quantum coherence), gHMBC $^{2,3}J_{C,H}$ (heteronuclear multiple bond

correlation) and gCOSY $^{3}J_{H,H}$ (gradient correlated spectroscopy). A gradient was used in the 2D experiments to eliminate excess noise and improve resolution.

The theoretical calculation of carbohydrate content of bark extracts from ^{1}H NMR spectra was found using the following method and calculations (Torr, 2006). Spectra were firstly locked on D_2O and the spectra referenced to the solvent signal. Integrals were then measured over the ranges 4.26 – 3.10 ppm (carbohydrates) and 7.56 – 6.36 ppm (condensed tannin B-ring). The integral of the condensed tannin B-ring was calibrated to 1. After determining the tannin to carbohydrate ratio (see below) the carbohydrate content % could then be calculated using the equation below.

Tannin (T):Carbohydrates (C) = (1.0/A) x B:(D-E)/F x G

Divide T and C by C to normalise C to 1

Carbohydrate content (%) = 1/(1 + T) x 100

The equation above used the following values from Torr, (2006)
A = Average number of B-ring H's (T) = 2.8 H
B = Average MW repeating unit (T) = 291 Da
D = Carbohydrate integral (4.26 – 3.1 ppm)
E = Carbohydrate integral for pure condensed tannin prepared by Sephadex LH-20 chromatography = 0.346
F = Average number of H's (C) = 5.85 H
G = Average MW of sugar repeating unit (C) = 156.7 Da

2.10 PROANTHOCYANIDIN DIMER SYNTHESIS

Hot water (HW) extracted *P. radiata* f2f3 condensed tannin (1.0 g) and catechin (1.0 g) was dissolved in ethanol (10 mL) and glacial acetic acid (0.2 mL). The solution was heated to 75˚C and refluxed for 42 hours under nitrogen and stirred with a magnetic flea. Following removal of ethanol by evaporation under reduced pressure, the residue was dissolved in 40 mL of water and extracted in a separating funnel with ethyl acetate (40 mL). The organic ethyl acetate layer was subsequently removed and collected; this procedure being repeated five times. The aqueous solution after the five extractions was discarded and the ethyl acetate solutions combined and dried with Na_2SO_4. Evaporation of the ethyl acetate solution under reduced pressure yielded the crude product as a red/brown solid (0.8 g).

Crude dimer product (0.3807 g) was dissolved in a minimal volume of 1:1 methanol/water and loaded onto a Sephadex LH-20 column (45 x 2.5 cm) to purify the proanthocyanidin dimer. The following gradient was used to elute the sample from the column (Table 2.3). Eleven fractions were collected, with 8 of the fractions being collected when the eluting solvent had compositions between methanol/water (4:1) and methanol/water (9:1). For each fraction, the methanol and acetone were removed by evaporation under reduced pressure and then freeze-dried to remove water. Mass balances were recorded and each fraction was analysed by GPC (see Chapter 2.16 for method) to obtain an indication of the composition.

Table 2.3 Solvent gradient used for the separation of the condensed tannin dimer from the crude dimer product

Fraction	Fractions collected	Solvent	Volume (mL)
f1	1	50 % methanol 50 % water	80
f2	1	70 % methanol 30 % water	80
f3-f6	4	80 % methanol 20 % water	80
f7-f10	4	90 % methanol 10 % water	80
f11	1	50 % acetone 50 % water	100

2.11 MATRIX ASSISTED LASER DESORPTION IONISATION TIME OF FLIGHT MASS SPECTROMETRY (MALDI-TOF-MS)

Matrices that were trialled for MALDI-TOF MS analysis of condensed tannins were 2,5-dihydroxybenzoic acid (DHB), 3-β-indole acrylic acid (IAA), 2,4,6-trihydroxyacetophenone (THAP) and dithranol. The solvent mixes tested were acetone/water (7:3) and methanol/water (6:4). Enhancement salts tried were sodium chloride (NaCl), silver (Ag^+) trifluoroacetate (TFA) and caesium (Cs^+) trifluoroacetate (TFA) (purchased from Sigma).

MALDI-TOF MS spectra were collected on a Bruker Autoflex II mass spectrometer equipped with a nitrogen laser (377 nm). All spectra were obtained in the positive ion linear mode and 2,5-dihydroxybenzoic acid (DHB) was used as the matrix. Both matrix and sample solutions were made to 10 mg/mL in 6:4 methanol/water and were mixed together in a 2:1 ratio of matrix to sample. An aliquot of the sample/matrix mixture was deposited on a stainless steel metal plate and allowed to dry inducing co-crystallisation of the matrix and analyte phases.

Condensed tannin samples were irradiated with the MALDI-TOF laser until the spectrum had a intensity of over 1000 absorbance units. Depending on the ease of ionisation some spectral intensities were recorded as high as 5000 absorbance units. The real-time smoothing function was set to high and the mass spectrometer mass window was set for low molecular weight range molecules (800-4000 Da). Matrix suppression was activated when dealing with the more highly polydisperse samples or when it gave spectral enhancement. The data was collected on Bruker Daltonics flexControl software and was analysed using Bruker Daltonics flexAnalysis software.

2.12 ELECTROSPRAY IONISATION MASS SPECTROMETRY (ESI-MS)

Electrospray ionisation mass spectrometry was performed on a Thermo Finnigan LCQ Deca XP ion trap mass spectrometer in negative ion mode. The tune file used was tuned to catechin. The mass spectrometer had the following settings: sheath gas flow rate was 30 arb, Aux/sweep gas flow rate was 1 arb, I spray voltage was 5 kV, capillary temperature was set at 190˚C, capillary voltage was -6 V and the tube lens offset was -60 V. Samples were dissolved in methanol:water (1:1) and were made to 0.5 mg/mL concentration. Samples were filtered with 0.45 μm PTFE syringe filters and injected into the mass spectrometer by direct infusion using a syringe pump. The spectra were recorded on LCQ Tune software and analysed using Xcalibur software.

2.13 DEPOLYMERISATION METHOD

The procedure used was a modified procedure from Kennedy and Jones, (2001) and Kennedy and Taylor, (2003). A 1 mL solution of 0.1 mol L^{-1} HCl in methanol containing phloroglucinol (50 mg), asorbic acid (10 mg) and the condensed tannin sample (5 mg) was prepared. This mixture was reacted at 50°C for 20 minutes in a heating block and was stirred with a small magnetic flea. Aqueous sodium acetate (5 mL, 40 mmol L^{-1}) was then added to the reacted solution to arrest the depolymerisation reaction. Samples were filtered through 0.45 μm PTFE syringe filters and then analysed by HPLC and LCMS as described below. The reaction is described in more detail in Chapter 1.4.4 (b).

2.14 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Phloroglucinol adducts were analysed by reverse-phase HPLC on a Gilson instrument connected with a Waters™ 717 plus autosampler, 305 pump, 811C dynamic mixer and a 118 UV/Vis detector. The column was an Apollo C18 (particle size 5 μm, 250 x 4.6 mm) purchased from Alltech Associates Inc (New Zealand) and protected by a guard column containing the same material. Data

was collected and analysed using Turbochrom software. The method utilised a binary gradient with water containing 1% (v/v) aqueous acetic acid (mobile phase A) and acetonitrile containing 1% (v/v) acetic acid (mobile phase B). Eluting peaks were monitored at 280 nm. Eluting conditions are shown on Table 2.4.

Table 2.4 HPLC solvent gradient used to separate and elute products from the acid/phloroglucinol condensed tannin depolymerisation reaction

Time (minutes)	%A	%B
0	97	3
5	72	18
15	20	80
20	20	80
30	97	3
55	97	3

Product peaks from the condensed tannin depolymerisation reaction were identified through depolymerisation/LCMS (method described in Chapter 2.15). The mDP of the condensed tannin samples were calculated the following way. The absorbance area of each peak (catechin/epicatechin and phloroglucinol (PG) adducts) were measured and divided by its molar absorption co-efficients shown in Table 2.5. The sum of all the individual responses gave the total response. The total response was then divided by the terminal unit response ((catechin/epicatechin)/3988) to give the mDP (equations below).

Table 2.5 Molar absorptivity coefficient of acid/phloroglucinol condensed tannin depolymerisation compounds (Kennedy and Jones, 2001)

Compound	Molar absorptivity coefficient
Catechin/epicatechin	3988
Procyanidin-PG	4218
Prodelphinidin-PG	1344

$$\text{Total response} = \frac{\Sigma \text{ prodelphinidin-PG}}{1344} + \frac{\Sigma \text{ procyanidin-PG}}{4218} + \frac{\Sigma \text{ catechin/epicatechin}}{3988}$$

$$\text{mDP} = \frac{\text{Total response}}{((\text{catechin/epicatechin})/3988)}$$

The error (95% confidence interval) in the depolymerisation results was found by analysing the HW f2f3 and MWA f2f3 sample three times each. The HW f2f3 sample mDP results were 7.30, 7.90 and 7.38. The standard deviation in the mDP was 0.33 and the 95% confidence interval was ± 0.37. The MWA f2f3 sample results were 8.40, 8.37 and 8.20. The standard deviation in MWA was 0.11 and the 95% confidence interval was ± 0.12. Therefore a conservative approach was taken and all the depolymerisation/HPLC results were assumed to have a 95% confidence interval of ± 0.37.

2.15 LIQUID CHROMATOGRAPHY MASS SPECTROMETRY (LCMS)

Phloroglucinol adducts from the condensed tannin depolymerisation reaction were analysed on an LCQ Deca XP Thermo Finnigan LCMS connected with a Finnigan surveyor PDA detector, Finnigan surveyor autosampler, surveyor MS pump and Thermo Finnigan ion trap mass spectrometer. The data were collected and analysed using Xcalibur software. The column used was a Hypersil Elite C-18 (particle size 5 µm, 150 x 2.1 mm) from Alltech, protected by a Phenomenex C-18 (4 x 2 mm) guard column.

The method utilised a binary gradient, with water containing 0.05% (v/v) aqueous acetic acid (mobile phase A) and acetonitrile containing 0.05% (v/v) acetic acid (mobile phase B). The gradient used is shown on Table 2.6. The gradient sequence runs over 55 minutes and ranges from 0% B to 40% B. Before the samples were injected they were filtered through 0.45 µm PTFE syringe filters. The PDA detector monitored peaks absorbing between 200-600 nm. Eluting peaks of interest were specifically monitored at 280 nm. The flow rate was 0.2 mL min^{-1}.

Table 2.6 LCMS solvent gradient used to separate and elute products from the acid/phloroglucinol condensed tannin depolymerisation reaction

Time (minutes)	%A	%B
0	100	0
10	100	0
33	85	15
38	60	40
42	100	0
55	100	0

The mass spectrometer used a tune file which was tuned to catechin. Depolymerised products were analysed in positive ion mode. The mass spectrometer had the following settings: sheath gas flow rate was 30 arb, Aux/sweep gas flow rate was 1 arb, I spray voltage was 4.65 kV, capillary temperature was set at 190˚C, capillary voltage was 3 V and the tube lens offset was -60 V. Spectra were recorded on LCQ Tune software and analysed using Xcalibur software.

UV 280 nm and total ion chromatograms (TICs) were recorded for each sample. Total UV/Vis and TIC peaks corresponding to phloroglucinol adducts and catechin/epicatechin units were identified and assigned by extracting the selective ion chromatogram (SIC) from the TIC using the appropriate $[M+H]^+$ molecular ion (*i.e.* procyanidin = 291.5, procyanidin-PG = 415.5 and prodelphinidin-PG = 431.5).

2.16 GEL PERMEATION CHROMATOGRAPHY

Purified condensed tannin samples were analysed by gel permeation chromatography on a Polymer Laboratories PL-GPC-50 integrated GPC system with a Var UV detector, WellChrom K-2301 refractive index (RI) detector and a PL-AS RT GPC autosampler. Results were collected on Cirrus™ online software and were analysed using Cirrus™ offline software. The GPC method used to analyse the condensed tannins consisted of two PLgel Mixed-E (300 x 7.5 mm,

3 μm) columns connected in series and protected by a guard column (50 x 7.5 mm) of the same material. The columns were purchased from Polymer Laboratories LTD, Shropshire, UK.

The isocratic method utilised a mobile phase consisting of *N*,*N*-dimethylformamide containing 1% (v/v) acetic acid, 5% (v/v) water and 0.15 mol L^{-1} lithium chloride. Flow rate was maintained at 0.5 mL min^{-1} with a column temperature of 50°C and elution was monitored and recorded at 280 nm. Samples were made to approximately 0.5 mg/mL in the same solvent used above. Samples were filtered through 0.45 μm PTFE syringe filters. The sample injection amount was 40 μg.

The HW and MWA molecular weight fractionated fractions were then used as condensed tannin standards and were used to construct a condensed tannin calibration curve, which would enable condensed tannin mass ranges and distributions to be determined. GPC on the MWA and HW fractions 2, 3 and to a lesser extent 4 separated out the monomers, dimers, trimers and tetramers. The retention times of these resolved oligomers and their corresponding exact weights were used in the calibration. For fractions 5, 6, 7 and 8, due to software limitations the peak maximum was assumed to correspond to the mDP (found from depolymerisation/HPLC), and was used in the calibration.

The errors in the GPC average molecular weights and polydispersity results were found through analysing the HW f2f3 and MWA f2f3 condensed tannins on two calibration curves constructed at different times. The largest confidence intervals from these two sets of data were used as the error for all the GPC data. The number average molecular weight had a 95% confidence interval of ± 70 Da. The weight average molecular weight 95% confidence interval was ± 553 Da and the polydispersity 95% confidence interval was ± 0.48.

CHAPTER 3: EXTRACTION, SEPARATION, FRACTIONATION AND IDENTIFICATION OF CONDENSED TANNINS

Pinus radiata bark contains large amounts of condensed tannin, which under certain conditions can undergo heat-catalysed condensation and polymerisation (Coombridge, 1997). Finding an appropriate method and a suitable solvent are important factors when extracting bark because the extraction may alter the composition and/or the yield of the extracts. Research undertaken in this project investigates the use of different solvents to extract condensed tannins from bark and solvent effects on extract composition and yield. The four different solvents investigated were hot water (HW), methanol/water/acetic acid 90:9.5:0.5 (MWA), acetone/water 60:40 (AW) and sulphite/urea hot water solution (SFU). A hot water pilot plant (PP) extracted crude condensed tannin sample supplied by the Biomaterial Engineering (BME) group at Scion, Rotorua was also analysed.

3.1 BARK PREPARATION

The *P. radiata* bark used for this project had the particle size distribution reported in Table 3.1. Almost half of the crushed bark was less than 63 μm in diameter, while a little over 34% was greater than 250 μm in diameter. Bark used in this investigation had a moisture content of 9.8% (w/w).

Table 3.1 Particle size distribution of *P. radiata* bark used (excluding Chapter 6)

Particle Size (μm)	**Percent (%)[a]**
< 63	47.0
63 - 125	12.1
125 – 250	6.6
> 250	34.4

[a] w/w of dry bark

Pre-extraction of the *P. radiata* bark using hexane and toluene extracted 2.7% and 0.3% of the dry bark weight respectively. ^{1}H NMR spectroscopy was performed

on both the hexane (Figure 3.1) and toluene extracts; this showed that the two solvents had extracted the same type of material. The major signals of the spectrum were from peaks between 0 and 2 ppm. These peaks are most likely due to hydrocarbons such as terpenes, resin acids and waxes. The pre-extraction of the bark was performed to remove these components, which could possibly contaminate the extracted condensed tannins. The yield from the hexane pre-extraction (2.7%) is slightly higher than that previously reported in the literature for a similar extraction. Kininmonth and Whitehouse (1991) obtained a yield of between 1.7 and 2.3% for *P. radiata* bark of a similar age.

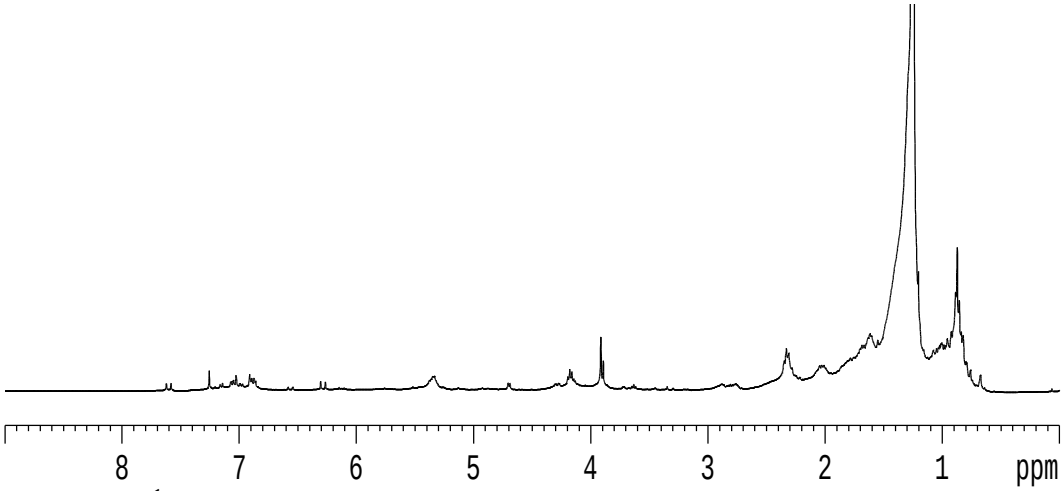


Figure 3.1 ^{1}H NMR spectrum of the hexane extracted product from *P. radiata* bark

3.2 BARK EXTRACTION

The percentage yield of extract obtained from the various bark extractions described in Chapter 2.5 are reported in Table 3.2. The yields for each method ranged from 16.5% for the HW method to 20.7% for the SFU extracted method. The HW extraction yield of 16.5% was slightly low compared to that reported by Matthews *et al*. (1997) (19.6%) for a similar extraction. The MWA yield (18.6%) was also low compared to yields in the literature, Soto *et al*. (2001) extracted a yield of 24% using methanol/water (3:2). The difference between results obtained in this research and those reported in the literature may be due to variations in the *P. radiata* bark used or because of the pre extraction.

Table 3.2 Percentage yields of extractives from *P. radiata* bark using different solvents

Sample	Percentage Yield (%)[a]
HW	16.5
MWA	18.6
AW	18.7
SFU	20.7

[a] w/w of dry bark

3.3 CARBOHYDRATE SEPARATION OF CRUDE CONDENSED TANNINS

Extracted crude condensed tannin contained carbohydrates and flavonoids which had to be separated from the condensed tannins to obtain pure material. In the literature Sephadex LH-20 chromatography is the most widely used technique for the purification of condensed tannins (Taylor *et al.*, 2003 and Soto *et al.*, 2001). The crude extract was purified using the method described in Chapter 2.6 and the percentage yields for the various fractions are reported in Figure 3.2 (w/w of the dried crude condensed tannin).

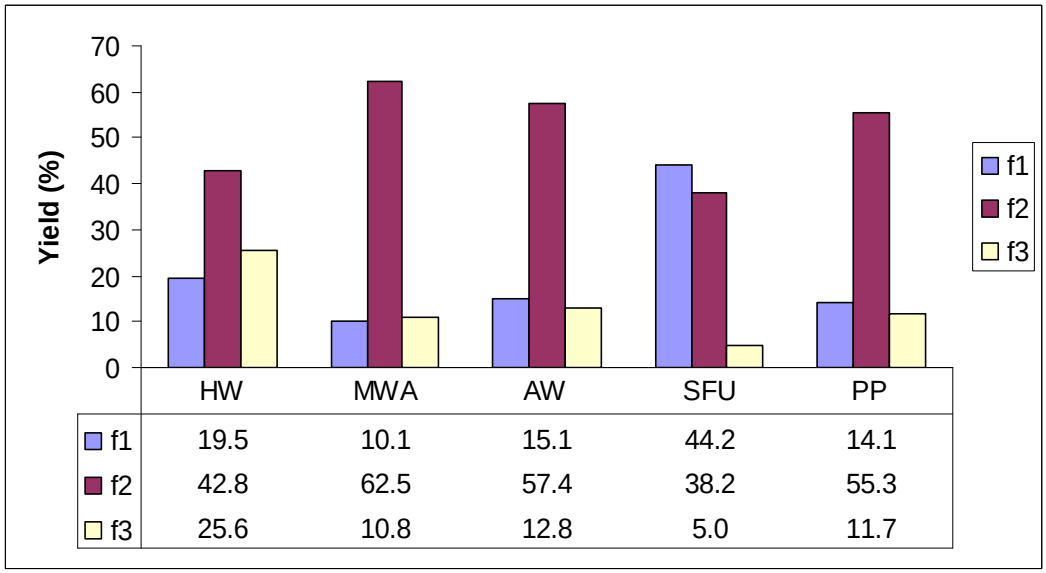


Figure 3.2 Yields of *P. radiata* fractions from extracts from different extraction solvents

With the exception of the SFU extraction the f1 yields ranged between approximately 10 and 20%. Fractions f2 and f3 had slightly varying yields for the different solvents, but when the f2 and f3 fractions were combined, the condensed tannin fraction (f2f3) yields were similar, ranging from approximately 67% for PP to 73% for MWA. In the literature Soto *et al*. (2001), utilising Sephadex LH-20 chromatography, obtained a f1 yield of 70% eluting with methanol/water (8:2) and a f2f3 yield of 30% eluting with acetone/water (1:1) and acetone (100%) (yields do not take into account unrecoverable material). According to the literature the methanol/water (1:1) eluted fractions are expected to contain mainly carbohydrates and flavonoids, while the acetone fractions are expected to contain mainly condensed tannins (Soto *et al*., 2001 and Hemmingway *et al*., 1982).

The fraction yields for the SFU extracts were different to that of the other extraction techniques (Figure 3.2). SFU f1 had a very large yield compared with the f1 fractions from the other techniques. SFU f2 and f3 had low yields compared with the f2 and f3 fractions from other techniques. There are two possible explanations for this result, either the SFU solvent has extracted more carbohydrates and flavonoids and less condensed tannins compared to the other solvents or the urea is disrupting the hydrogen bonding between the condensed tannins and the Sephadex LH-20, thus resulting in material eluting in f1 that would normally elute in the latter fractions.

The unrecovered material was bound irreversibly to the column. Unrecoverable material is composed of high molecular weight condensed tannins and phlobaphenes (Coombridge, 1997). All extraction techniques, other than the PP extracted technique (19% unrecoverable material), exhibited a similar amount of unrecoverable material, ranging between approximately 12% and 14%.

The Folin-Ciocalteu assay results (Table 3.3) gave an indication of the amount of phenolic (condensed tannin and flavonoids) material in each fraction. A weakness of the Folin-Ciocalteu assay is that absolute values are somewhat variable depending on the calibration curve used for different batches of samples (i.e. a new calibration curve had to be constructed for every set of new samples). As discussed in Chapter 2.8 the same sample analysed on separate occasions using

different calibration curves resulted in a large experimental error (95% confidence interval ± 17%). Therefore when interpreting these results it is best to consider the trends between fractions rather than absolute values. Total phenolics (%) results (Table 3.3) showed that f1 fractions consist mainly of non-phenolic material (carbohydrates) and fractions f2 and f3 consisted of predominately phenolics (condensed tannin), which is consistent with the literature (Kennedy and Taylor, 2003).

Table 3.3 Total phenolics (%) of separated fractions

Sample	f1[a]	f2[a]	f3[a]
HW	20.4	96.9	86.0
MWA	47.1	87.1	99.0
AW	49.9	95.6	99.6
SFU	48.7	73.8	46.0
PP	12.9	67.2	73.1

[a] 95% confidence interval of ± 17.0%

The HW and PP f1 fractions had a low total phenolics (%) which is expected as the fraction should mainly consist of carbohydrates. AW and MWA f1 fractions had a higher total phenolics (%) value but compared to there corresponding f2 and f3 fractions the total phenolics (%) values were still low, hence showing that f2 and f3 contained larger amounts of phenolic material (condensed tannin) than the f1 fraction. ^{1}H NMR spectroscopy provided no explanation for why the f1 fractions were high.

^{1}H-NMR spectroscopy was performed on all fractions to identify the composition of each sample and estimate the theoretical carbohydrate content. NMR studies have shown that the majority of the condensed tannin signal lies between 5.82 and 7.56 ppm (due to the aromatic moiety in condensed tannins). Signals between 5.82 and 6.36 ppm correspond to the aromatic A ring and signals between 6.36 and 7.56 correspond to the aromatic B ring. The C ring proton signals lie between 3.8 and 4.6 ppm (De Bruyne *et al*., 1996). The B ring is only used in the estimated carbohydrate calculations because the A ring protons easily undergo deuterium exchange with the NMR solvent (Newman, 2006).

The majority of the carbohydrate signals were between 3.10 and 4.26 ppm (Figure 3.3). Signals between 5 and 5.5 ppm correspond to the anomeric protons. Integration of the carbohydrate and condensed tannin (B ring) signals followed by a simple calculation (see Chapter 2.9) gave an estimate of the carbohydrate content of the sample.

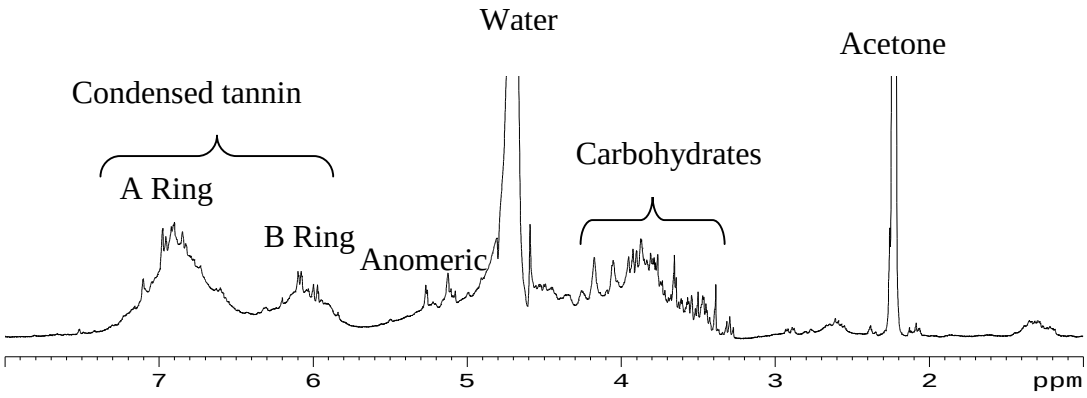


Figure 3.3 ^{1}H-NMR spectrum of crude condensed tannins with peak assignment

The spectra shown in Figure 3.4 of the HW crude, f1 and f2f3 fractions were representative of the spectra of the corresponding fractions for the other extraction methods (see Appendix 1.1) with the exception of SFU. Figure 3.4 shows that the crude fractions consisted of both condensed tannin and carbohydrates. The estimated carbohydrate content for the crude fractions ranged between 10% for the AW extraction and 17% for the PP extraction. All the f1 fractions consisted of mainly carbohydrates with a very small amount of flavonoids. The estimated carbohydrate content of the f1 fractions ranged between 60% for AW and 85% for HW. The f2f3 fractions consisted of mainly condensed tannins. The estimated carbohydrate content of the f2f3 fractions were all approximately 0%.

As discussed in Chapter 1.3.2 condensed tannins were eluted from the column with acetone as this solvent changes the character of the Sephadex LH-20 from hydrophilic to lipophilic, thus disrupting the hydrogen bonding between the condensed tannins (hydroxyl groups) and the stationary phase. Therefore the results obtained from the total phenol content (%) and estimated carbohydrate contents indicated that the composition of the fractions were consistent with what was expected from the literature (*i.e.* f1 carbohydrates and f2 and f3 condensed

tannins). The total phenol content (%) and the estimated carbohydrate content results complement each other and are in good agreement.

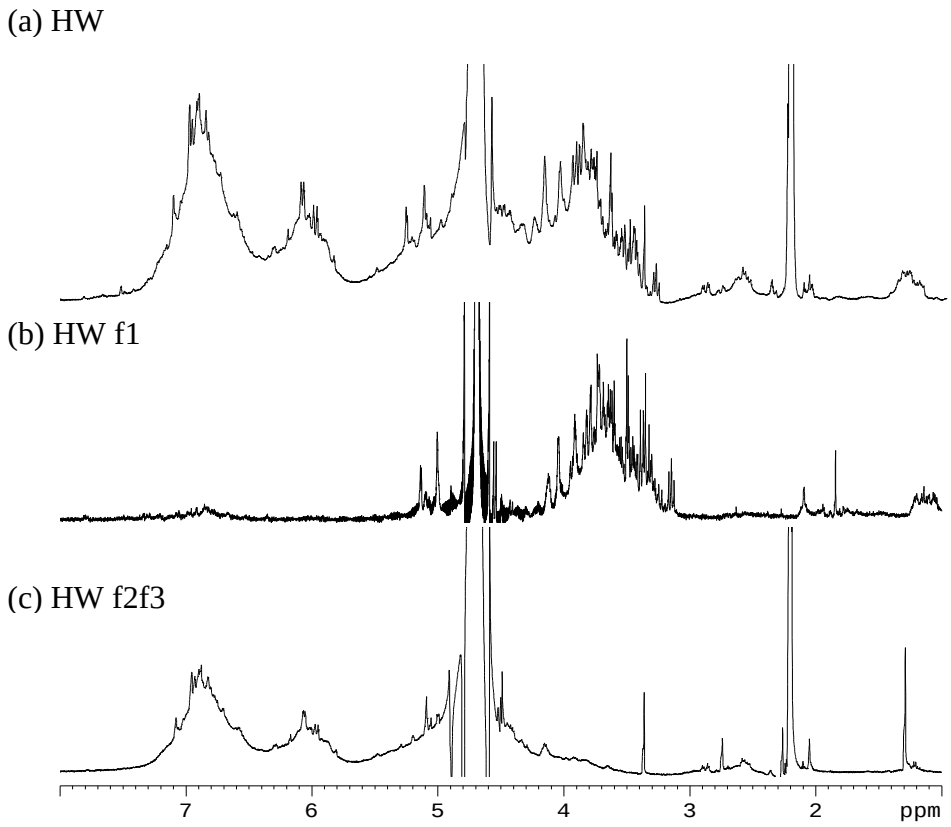


Figure 3.4 ^{1}H-NMR spectrum of (a) HW crude extract, (b) HW f1 column fraction and (c) HW f2f3 combined column fractions

Using a Sephadex LH-20 column, Hemmingway *et al*., (1982) and Foo and Porter, (1980) found that methanol/water (1:1) eluted carbohydrates, flavonoids and small molecular weight condensed tannins (i.e. dimers and trimers). In this research, methanol/water (1:1) eluted very little flavonoids and small molecular weight condensed tannins. There could be several possible reasons for this: firstly, the moment when to stop collecting one fraction and start collecting the other was visually estimated, secondly, the ease at which the carbohydrates came off the column in methanol/water meant they were preferentially eluted before small molecular weight condensed tannins and thirdly, *P. radiata* bark doesn't contain small molecular weight condensed tannins.

^{1}H NMR spectroscopy of the SFU crude sample (Figure 3.5 (a)) showed that it consisted of both condensed tannins and carbohydrates. The estimated carbohydrate content of the crude sample was 14% carbohydrates. The NMR spectrum of the SFU f1 fraction showed that a considerable amount of condensed tannin had been eluted in this fraction (Figure 3.5 (b)). The SFU f1 fraction estimated carbohydrate content was 39% carbohydrates. This provides a explanation why the SFU f1 fraction yield and total phenolics (%) were high and the SFU f2 and f3 fraction yields were low compared with the other solvents. The NMR evidence supports the theory that the urea from the SFU extraction is disrupting the hydrogen bonding between the condensed tannins and Sephadex LH20, thus resulting in the condensed tannins eluting in the earlier fraction. The f2f3 fraction ^{1}H NMR spectrum, (Figure 3.5 (c)) was estimated to contain no carbohydrates.

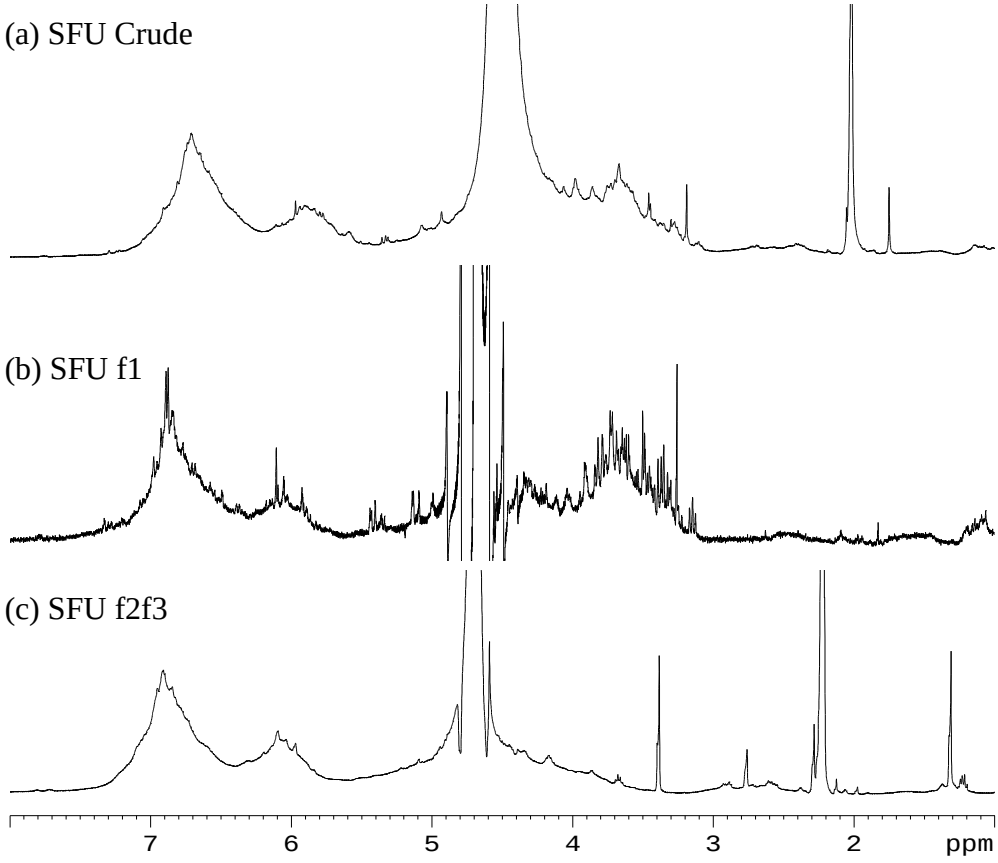


Figure 3.5 ^{1}H-NMR spectrum of (a) SFU crude extract, (b) SFU f1 column fraction and (c) SFU f2f3 combined column fractions

3.4 FRACTIONATION OF PURE CONDENSED TANNINS

Pure HW and MWA condensed tannins were fractionated according to their molecular weight utilising separation methods based on the relative solubility of proanthocyanidins of different molecular sizes in different solvents, as well as the differing hydrophilic and lipophilic behaviour of the column (see Chapter 1.3.2 for detailed explanation) in different solvents (Ellingboe *et al.*, 1970). As the proanthocyanidin molecular weight increases, the interactions between the condensed tannin and Sephadex LH-20 increase. These interactions are broken with acetone-water solution. The technique used was developed by Taylor *et al.* (2003).

The twenty eight fractions collected and the subsequent yields were noted (Figure 3.6). The letter describes the portion of the solvent gradient used (see Chapter 2.7 for definitions), while the number identifies it as the 1st, 2nd, 3rd or 4th fraction collected for that solvent mixture).

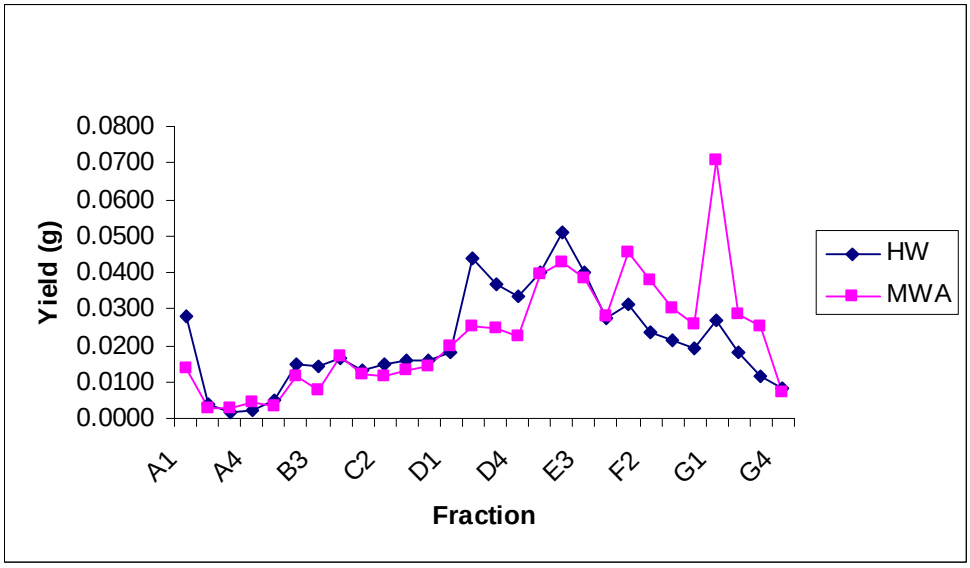


Figure 3.6 Yields of HW and MWA fractions from fractionation of pure condensed tannins

MALDI-TOF MS of the 28 fractions collected (see Appendix 1.2 for spectra) enabled the fractions to be pooled into 8 fractions according to MALDI-TOF MS distributions and peak intensities. Table 3.4 describes the makeup and yields of these new pooled fractions. The yields do not add up to 100% as there was some

unrecoverable material that did not elute from the column (i.e. HW 8.5% and MWA 5.1%).

Over 63% of the total MWA yield was contained in the pooled fractions 6, 7 and 8, compared with 47% for the HW extraction method. Correspondingly, less than 19% of the MWA yield was contained in fractions 1, 2 and 3, compared with 24% for the HW extraction method. This may indicate that the MWA extraction technique may have extracted higher molecular weight condensed tannins compared with the HW extraction technique. The description of the analysis of each of the pooled fractions is discussed further in Chapter 4.

Table 3.4 Yields and composition of pooled HW and MWA fractions from molecular weight fractionation

Fraction	**Hot Water**		**Methanol/Water/Acetic Acid**	
	Included in fraction	**Yield %**	**Included in fraction**	**Yield %**
1	A1,A2	5.3	A1,A2	2.6
2	A3,A4,B1,B2,B3,B4	9.0	A3,A4,B1,B2,B3,B4	7.4
3	C1,C2,C3,C4	10.0	C1,C2,C3,C4	8.1
4	D1,D2,D3	16.5	D1,D2	7.1
5	D4,E1	12.3	D3,D4,E1	13.9
6	E2,E3,E4	19.7	E2,E3,E4	17.4
7	F1,F2,F3,F4	16.0	F1,F2,F3,F4	22.3
8	G1,G2,G3,G4	10.8	G1,G2,G3,G4	21.1

3.5 SYNTHESIS AND CHARACTERISATION OF PROANTHOCYANIDIN DIMER

The method for the synthesis of the proanthocyanidin dimer is described in Chapter 2.10. GPC analysis on the crude dimer (before purification) produced a chromatogram showing that the sample still contained some unreacted catechin as well as a small amount of condensed tannin oligomers larger than the dimer,

which had not been depolymerised (see Appendix 1.3 for GPC chromatogram of crude dimer products).

Purification of the proanthocyanidin dimer on a Sephadex LH-20 column utilising a methanol/water gradient (Table 2.3) yielded eleven fractions. GPC chromatograms of each dimer fraction are shown in Appendix 1.3. Fraction f1 and f2 contained mainly unreacted catechin. Fraction f3 and f4 contained mainly dimer with a small amount of trimer and flavonoids. Fractions f5-9 contained mixtures of dimer and trimer. Fractions f10 and f11 contained condensed tannin oligomers that had not been fully depolymerised.

GPC showed fraction f4 (figure 3.7) to be the most pure dimer fraction. The GPC chromatogram shows that the fraction was approximately 65-70% pure (as estimated through integration of the GPC chromatogram). The dimer could have been further purified using preparative normal phase HPLC, however it was considered sufficiently pure for NMR characterisation and the purpose of this study. Analysis of fraction f4 by ESI MS (Appendix 1.4) indicated that the major peak in the GPC chromatogram was the condensed tannin dimer (m/z = 577)

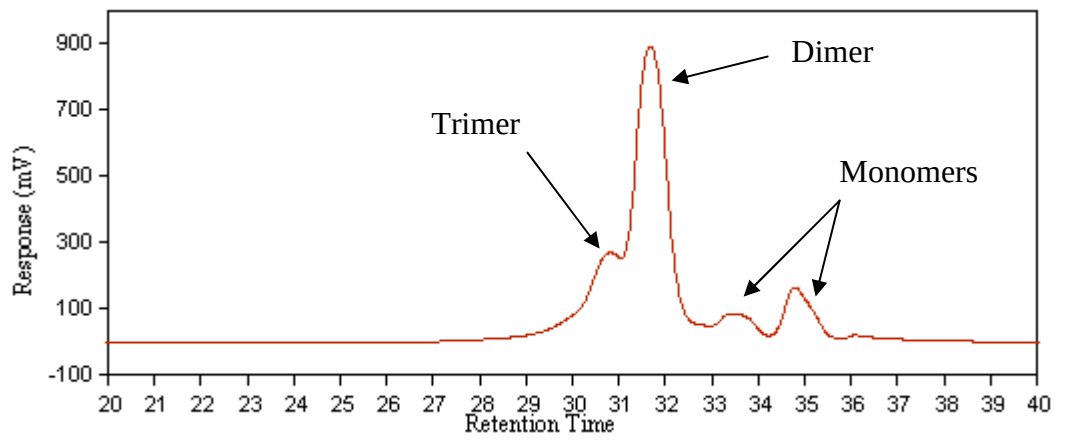


Figure 3.7 GPC of dimer fraction f4 showing the composition of the fraction.

The dimer fraction (f4) was then analysed by NMR spectroscopy. Using a combination of COSY, HSQC, HMBC and DEPT135 experiments, the ^{1}H and ^{13}C spectra were fully assigned and the structure fully elucidated (Table 3.5). The 2D and DEPT NMR spectra are shown in Appendix 1.5. When assigning the full

structure, the dimer was assumed to be C4-C8 linked and the extender (*u*) and terminal (*t*) units to be both catechin (Figure 3.8).

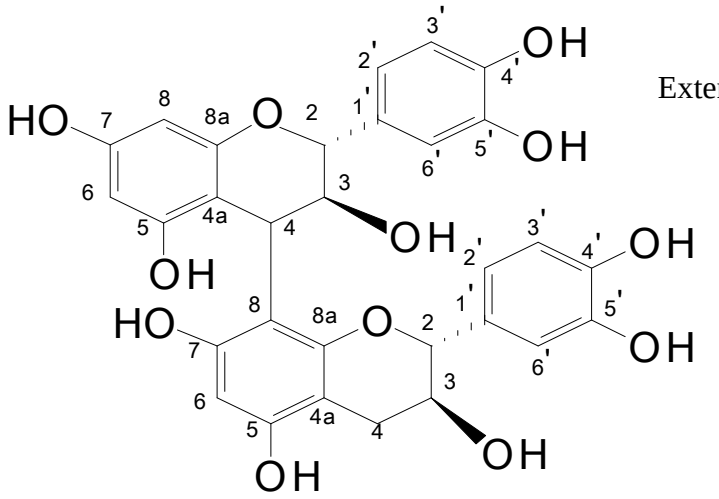


Figure 3.8 Proanthocyanidin dimer structure

In the ^{13}C spectrum (Figure 3.9) the signals that range between 27.9 and 83.0 ppm arise from the aliphatic carbons 2, 3 and 4 (refer to Figure 3.8 for structure) on both the extender and terminal units. Through the NMR DEPT135 experiment the C4*t* was shown to have two attached protons as this is the only peak that is phased down in the spectrum (27.9 ppm). Signals that lie between 96.2 and 131.7 ppm arise from aromatic carbons 1', 2', 4a, 5', 6 and 8 without attached hydroxyl groups (for both *u* and *t* units). Signals ranging between 144.8 and 157.7 ppm arise from aromatic carbons 3', 4', 5, 7 and 8 that are joined to an oxygen atom through a ether bond or hydroxyl group.

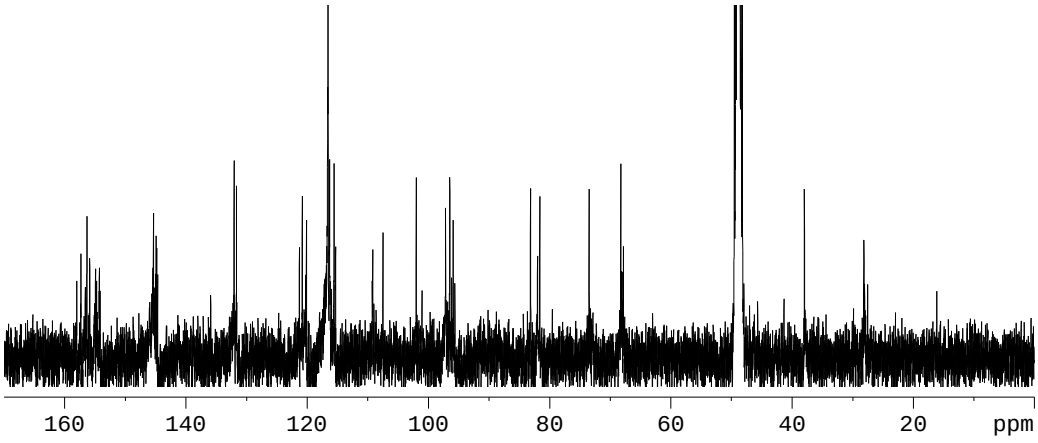


Figure 3.9 ^{13}C NMR spectrum of synthetic proanthocyanidin dimer f4

In the ^{1}H spectrum (Figure 3.10) there are three distinct regions of peaks. Signals observed at 2.62 and 2.89 ppm are due to the two protons at 4*t*, these display a large geminal coupling of 15.9 Hz. Signals ranging between 3.96 and 4.67 ppm arise from the aliphatic protons from 2, 3 and 4. The last range of signals is between 5.86 and 6.98 ppm and arises from the aromatic protons from both the A and B ring. The NMR data obtained is consistent with that described in the literature (De Bruyne *et al*., 1995).

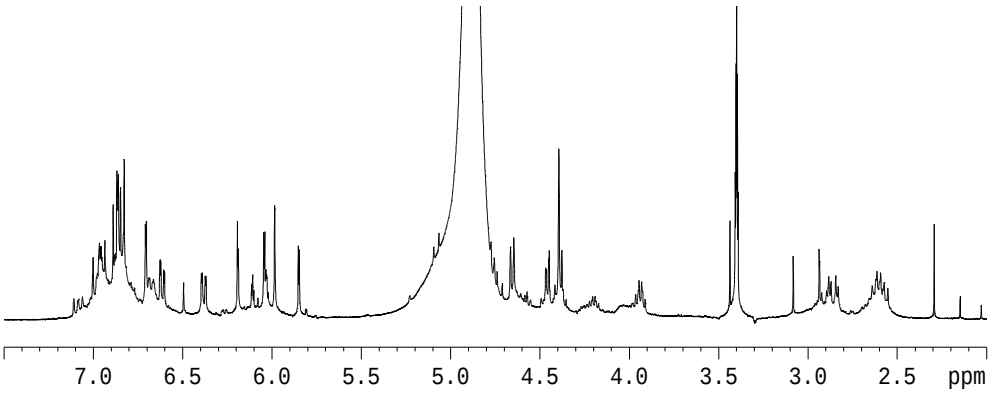


Figure 3.10 ^{1}H NMR spectrum of synthetic proanthocyanidin dimer f4

Starting from the C-4*t* carbon the two H-4*t* protons were assigned through correlations from the HSQC experiment and through the DEPT135 spectrum (discussed above). The C3*t* carbon had two HMBC correlations with the two H-4*t* protons and the corresponding proton (H-3*t*) was assigned through the HSQC spectrum and through its COSY correlation with the H-4*t* protons (see Appendix 1.5). C-2*t* had a HMBC correlation with H-3*t* and from there the rest of the B*t* ring was assigned (Table 3.5). C-4a*t* has a strong short range (^{2}J) correlation with the two H-4*t* protons, while C-8a*t* has a weak long range (^{3}J) correlation to the two H-4*t* protons. The C-4a*t* carbon then had a long range HMBC correlation (^{3}J) to the H-6*t* proton. The C-6*t* carbon had an HSQC correlation with the H-6*t* proton, while C-5*t* and C-7*t* had two short range HMBC correlations (^{2}J) with H-6*t* proton. The C-8*t* carbon also had a long range correlation (^{3}J) with H-6*t* proton. The C-8*t* carbon had a HMBC correlation with the H-4*t* proton on the extender unit. From there the rest of the extender unit was assigned in a similar manner (see Table 3.5). The correlations are shown visually in Figure 3.11.

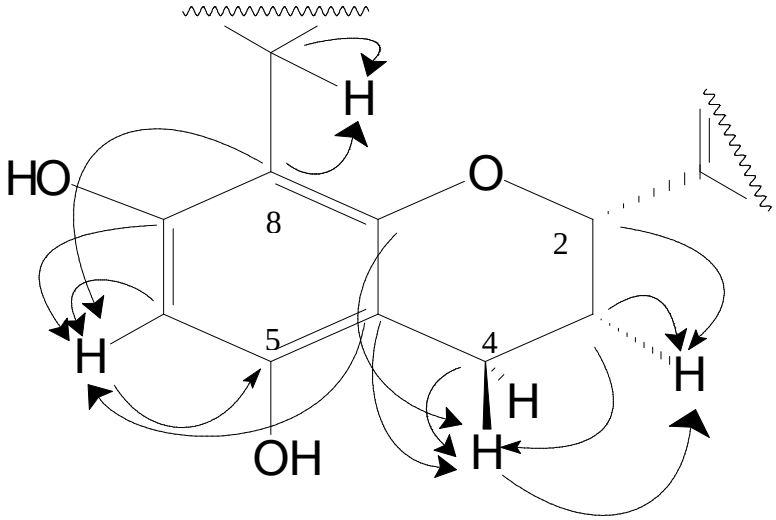


Figure 3.11 NMR correlations showing how part of the dimer structure was assigned.

Table 3.5 NMR assignments and correlations for proanthocyanidin dimer (fraction f4)

Signal	**^{13}C**	**HSQC**	**HMBC**	**^{1}H**	**gCOSY**
4*t*	27.9	2.62,2.89	4.67	α 2.89 (5.4 Hz)	2.62, 3.95
				β 2.62 (8.2 Hz)	2.89, 3.95
4*u*	37.7	4.48	4.40	4.48 (6.5 Hz)	4.40
3*t*	68.0	3.95	2.62, 2.89, 4.60	3.95	2.62, 2.89, 4.65
3*u*	73.4	4.40	4.38, 4.48	4.40	4.38, 4.48
2*t*	81.7	4.67	6.98, 6.83, 2.89, 2.62	4.67 (6.7 Hz)	3.95
2*u*	83.0	4.38	4.40, 6.64, 6.87	4.38 (7.6 Hz)	4.40, 4.48
6*t*	96.2	6.21		6.21	
8*u*	96.3	5.86	6.06	5.86	6.06
6*u*	96.9	6.06	5.86	6.06	5.86
4a*t*	101.6		2.62, 2.89, 3.95, 6.21		
4a*u*	107.4		4.48, 6.06		
8*t*	109.0		4.40, 4.48, 6.21		
5'*u*	115.3	6.72	6.64	6.72 (2 Hz)	6.64
5'*t*	115.4	6.74	4.67, 6.83	6.74 (2 Hz)	6.83
2'*t*	116.3	6.98	4.67	6.83 (8 Hz)	6.98
2'*u*	116.5	6.87	4.38, 6.64	6.87 (8 Hz)	6.64
6'*t*	119.52	6.83	4.67, 6.74	6.98 (2 Hz)	6.74
6'*u*	120.1	6.64	4.38, 6.72	6.64 (2 Hz)	6.72
1'*t*	131.1		4.67, 6.98		
1'*u*	131.7		4.38, 4.48, 6.87, 6.64		
4'*u*	144.8		6.72		
4'*t*	144.9		6.98, 6.83		
3'*u*	145.0		6.72, 6.87		
3'*t*	154.2		6.74		
5*t*	154.4		2.62, 2.89, 6.21		
7*u*	155.5		5.86, 6.06		
8a*t*	156.0		2.62, 2.89		
7*t*	156.4		4.48, 6.21		
5*u*	156.4		4.48, 5.86, 6.06		
8a*u*	157.7		4.38, 4.48, 4.67, 6.21		

CHAPTER 4: MASS SPECTROMETRY

Both ESI MS and MALDI-TOF MS are suited for analysing condensed tannins. However, mass spectra for both ESI and MALDI-TOF are usually dominated by lower molecular weight species. This occurs even when other chemical methods have shown that large condensed tannins are present in significant quantities (Taylor *et al*., 2003), thus mass spectrometry of condensed tannins may not reflect the actual abundance but the relative ease of ionisation of the molecules. Prior size separation may be beneficial to mass spectral analysis of condensed tannins (McEwen *et al*., 1996 and Byrd and McEwen, 2000).

Both ESI MS and MALDI-TOF MS are capable of detecting non-fragmented molecular ions of up to 100,000 Da. ESI MS is best suited for the analysis of monodispersed polymers and oligomers because of complications in the interpretation due to the formation of multiply charged ions (Fulcrand *et al*., 1999). Alternatively, MALDI-TOF MS is suited to characterising polydisperse oligomers. The MALDI-TOF MS is the method of choice when analysing condensed tannins with varying types of repeating units (Reed *et al*., 2005)

4.1 MALDI-TOF MS METHOD DEVELOPMENT

Crucial factors that affect the quality of MALDI-TOF MS spectra are the crystallisation of the analyte during sample preparation and the behaviour of the matrix during laser irradiation (Behrens *et al.*, 2002 and Onishi-Kameyama *et al.*, 1997). No one matrix suits all analytes; therefore a suitable matrix for *P. radiata* condensed tannins had to be found. Several matrices including 2,5-dihydroxybenzoic acid (DHB), 3-β-indole acrylic acid (IAA), 2,4,6-trihydroxyacetophenone (THAP) and dithranol were trialled. In agreement with Behrens *et al.*, (2003)'s work, the use of dithranol as a matrix did not result in reasonable spectra. The best spectra were achieved using DHB, IAA and THAP. Overall DHB spectra gave the best signal to noise ratio and allowed for the detection of the largest range of condensed tannins.

For further improvement of the crystallisation process, two different solvent mixes were tested, namely acetone/water (7:3) (Behrens *et al.*, 2003) and methanol/water (6:4) (Krueger *et al.*, 2003). Both solvents were suitable, but methanol/water (6:4) produced a spectrum with the best signal to noise ratio.

Other researchers have shown the addition of certain ions can enhance the spectrum of proanthocyanidins (Onishi-Kameyama *et al*., 1997 and Krueger *et al*., 2003). Salts tested were sodium chloride (NaCl), silver (Ag^+) trifluoroacetate (TFA) and caesium (Cs^+) trifluoroacetate (TFA). No apparent spectral enhancement was gained with any of the added ions/salts. The addition of Na^+ produced a spectrum which was exactly the same as when no salts were added. The Ag^+ and Cs^+ ions gave unassignable spectra. This may be due to the salts forming ion clusters, which is known to occur in the presence of acidic matrices (Krueger *et al*., 2003).

The use of both negative and positive ion mode MALDI-TOF MS was tested (Appendix 2.1). Positive mode gave better resolution and detected condensed tannins of a larger MW, therefore all the samples were analysed in positive ion mode. MALDI-TOF MS analysis of condensed tannins was also performed in linear and reflectron mode (Appendix 2.2). Reflectron mode gave increased resolution of smaller MW condensed tannins but was not suitable for larger MW condensed tannins. A lower sensitivity of the larger MW ions in reflectron mode is due to the breakdown of the ions; which is in turn due to a longer flight path and the post acceleration process (Yang and Chien, 2000). Therefore it was decided to analyse all the samples in linear mode. MALDI-TOF MS of bark (Appendix 2.3) produced an analysable spectrum showing that condensed tannins do not have to be extracted from bark before analysis by MALDI-TOF MS. The optimum MALDI-TOF MS conditions are shown below.

Optimum Conditions

Matrix:	Dihydroxybenzoic acid (DHB)
Solvent:	Methanol/water (6:4)
Added salt:	None
Ion mode:	Positive
Linear or reflectron:	Linear

4.2 MALDI-TOF MS OF CONDENSED TANNINS

MALDI-TOF MS was performed on the condensed tannin f2f3 fractions and the molecular weight fractionated HW and MWA fractions. Figure 4.1 shows a typical MALDI-TOF MS spectrum of an f2f3 fraction (HW f2f3). MALDI-TOF MS of the pure bark gave a similar-looking spectrum (Appendix 2.3).

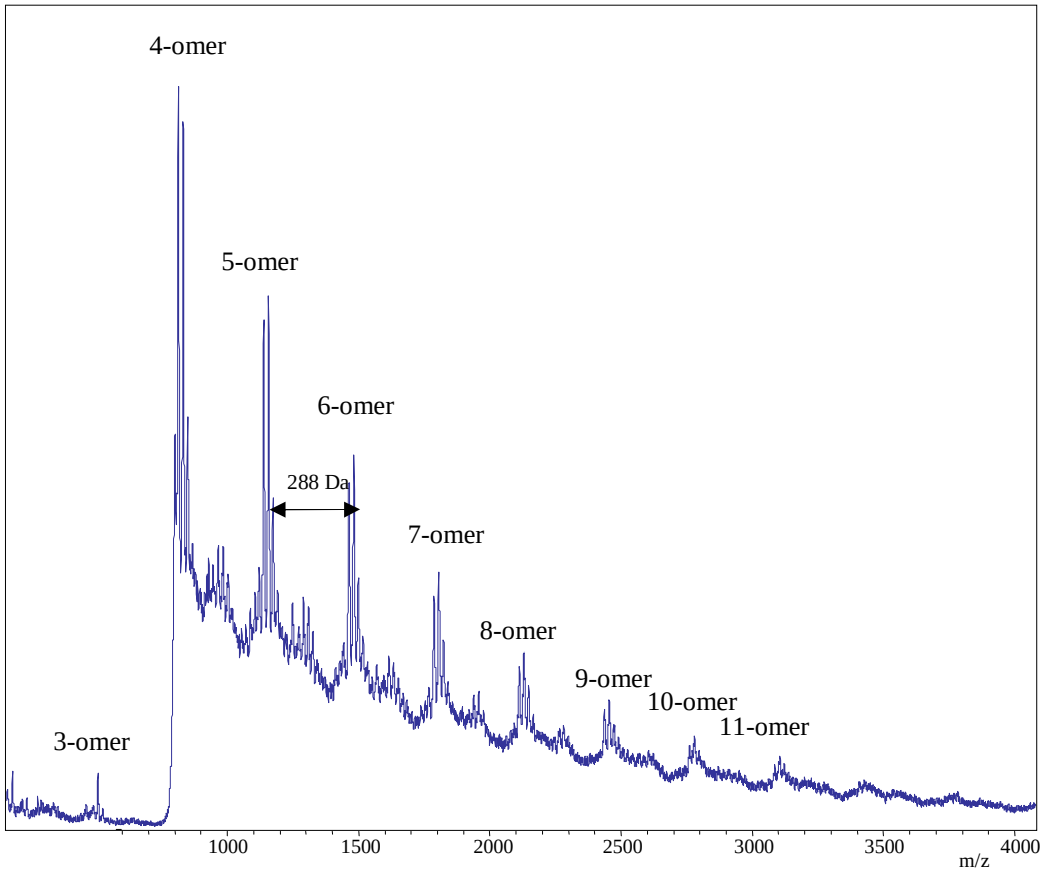


Figure 4.1 MALDI-TOF MS spectrum of HW f2/f3 fraction

MALDI-TOF MS analysis of the unfractionated HW f2f3 sample detected condensed tannin oligomers between a DP of 3 and 11. The large increase in intensity from the 3-omer to the 4-omer (in Figure 4.1) is due to matrix suppression being set up to 1000 m/z. This prevents smaller ions (typically from the matrix) from reaching the mass spectrometer, which enables larger condensed tannins to be detected. The MALDI-TOF MS spectra of *P. radiata* condensed tannins are characterised with a series of peaks separated by 288 Da, which corresponds to the mass of one catechin/epicatechin (procyanidin) unit (See Figure 1.3).

Magnification of the 6-omer peak (Figure 4.2) from the above spectrum demonstrates the good resolution of the MALDI-TOF MS technique and confirms the presence of prodelphinidin (gallocatechin/epigallocatechin) units. These units were also identified by HPLC analysis of acid-catalysed depolymerisation products of condensed tannins (see Chapter 5). Corresponding with the all procyanidin peaks at each DP are ion peaks 16 and 32 Da higher. As the atomic weight of oxygen is 16 Da, the additional peaks 16 and 32 Da higher than the parent ion can be presumed to be the ions of condensed tannins containing one or two prodelphinidin units. *P. radiata* condensed tannin spectra also exhibit small signals characterised by peaks 16 Da lower than the parent ion, which may be indicative of oligomer chains containing units with only one hydroxyl group on the A or B aromatic ring e.g. properlargonidin (1 less OH on the B ring) or profisetinidin (1 less OH on the A ring). These peaks are very small and therefore the presence of properlargonidins or profisetinidins is very minor.

All the f2f3 fractions from the different extraction solvents produced similar MALDI-TOF MS spectra. The condensed tannins observed ranged from DP 3 to DP 11 for the HW f2f3 fraction and DP 3 to DP 9 for the AW and SFU f2f3 fractions (AW, PP, and MWA spectra are in Appendix 2.4). In all the spectra of the f2f3 fractions the most intense peak always corresponded to either the tetramer or pentamer, which arises as an artefact of the matrix suppression. MALDI-TOF MS of fraction SFU f2f3 produced peaks corresponding to condensed tannins and not condensed tannin sulphonates (discussed in ESI MS results (Chapter 4.3)).

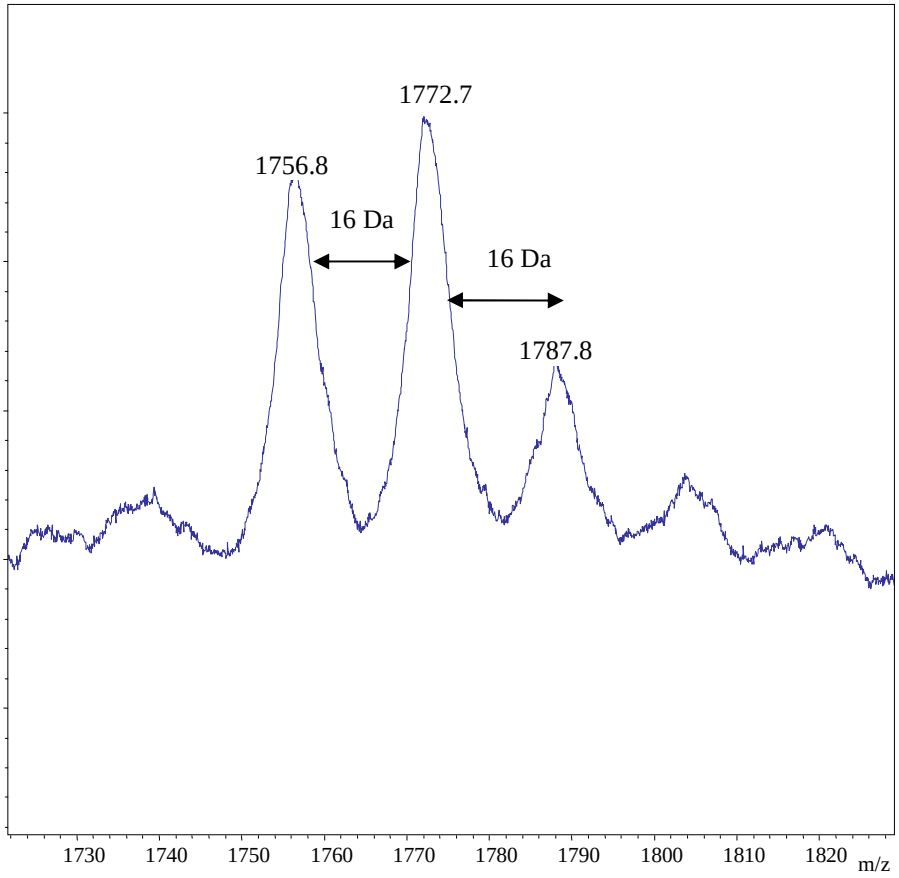


Figure 4.2 Magnification of 6-omer peaks showing the 16 Da difference corresponding to one and two prodelphinidin units

Observed and calculated masses of condensed tannins for HW f2f3 are shown below in Table 4.1. The expected masses were calculated using the equation below (Behrens *et al*., 2003 and Ishida *et al*., 2005).

$$m/z = 288.26\,n + 304.26x + 23 + a$$

288.26 = Procyanidin MW

n = Number of procyanidin units

304.26 = Prodelphinidin MW

x = Number of prodelphinidin units

23 = Sodium adduct MW

a = number of terminal protons = 2

For all peaks the observed *m/z* was slightly different to the expected *m/z* (up to 1 Da). This is due to the natural abundance of different H, C and O isotopes in the condensed tannin oligomers. The expected masses did not take into account different isotopes (equation took into account the most abundant isotope).

Table 4.1 Expected and Observed $[M + Na]^+$ Quasimolecular Ions in MALDI-TOF MS of HWf2/f3

DP	PD	Expected m/z	Observed m/z
3	0	889.8	890.9
	1	905.8	906.8
	2	921.8	922.4
4	0	1178.0	1179.6
	1	1194.0	1195.7
	2	1210.0	1211.1
5	0	1466.3	1467.9
	1	1482.3	1483.9
	2	1498.3	1499.7
6	0	1754.6	1755.5
	1	1770.6	1771.6
	2	1789.6	1788.6
7	0	2042.8	2043.4
	1	2058.8	2059.7
	2	2074.8	2075.4
8	0	2331.1	2332.3
	1	2347.1	2348.3
	2	2363.1	2364.1
9	0	2619.3	2620.6
	1	2635.3	2636.9
	2	2651.3	2652.5
10	0	2923.6	2924.1
	1	2939.6	2940.1
11	0	3211.8	3211.7, 3213.8
	1	3227.8	3226.6

Molecular weight fractionated HW and MWA fractions were analysed by MALDI-TOF MS to learn more about the molecular weight distribution of

P. radiata condensed tannins and to see if prior fractionation improved the detection of larger MW condensed tannins. Figure 4.3 compares the MALDI-TOF MS spectra of the HW 2-7 fractions (Appendix 2.5 contains the MALDI-TOF MS spectra of the MWA 2-7 fractions). As the fraction number increased the range of detected condensed tannins increased and the most intense peak shifted to higher MW. The spectrum of HW 7 confirms that larger MW condensed tannins (DP 13) can be detected when *P. radiata* condensed tannins are fractionated according to molecular weight prior to MALDI–TOF MS analysis.

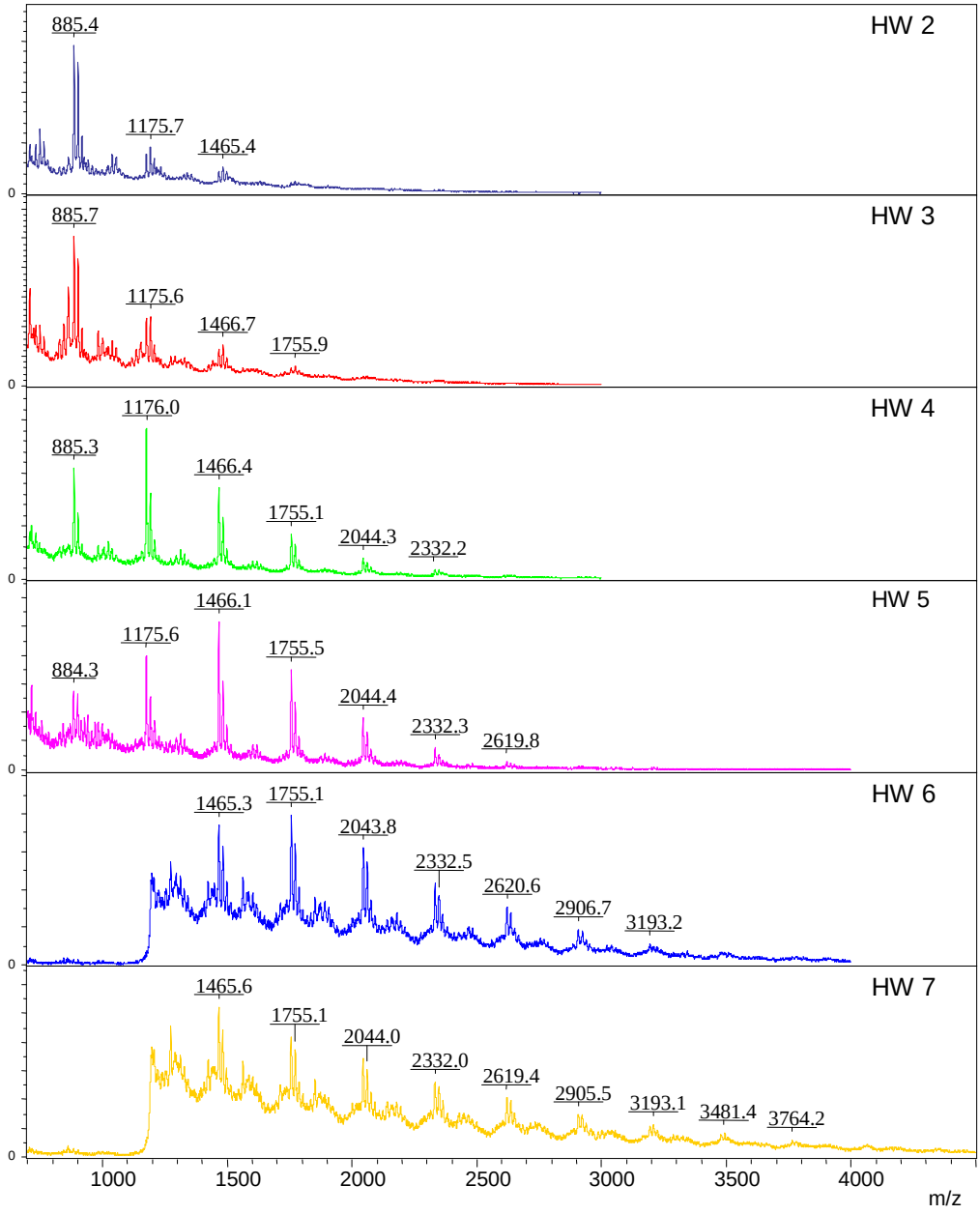


Figure 4.3 Comparison of HW 2-7 fractions analysed by MALDI-TOF MS

Fractions HW 2-5 in Figure 4.3 were analysed with the matrix suppression off because with the matrix suppression on there was no spectral enhancement and the smaller condensed tannins were not observed (these fractions were expected to contain large amounts of smaller condensed tannins). Fractions HW 6 and 7 were analysed with matrix suppression on because it enabled larger condensed tannins to be detected and secondly, without matrix suppression the spectra had a poor signal to noise ratio.

Fraction 1 was not analysed by MALDI-TOF MS because it did not dissolve in either acetone/water (7:3) or methanol/water (6:4) solvents. Fraction HW 8 was analysed by MALDI-TOF MS, but an analysable spectra that could be interpreted could not be obtained because the condensed tannins were too large and polydisperse.

4.3 ESI MS OF CONDENSED TANNINS

ESI MS was performed on both the fractionated HW and MWA condensed tannins and the f2f3 samples. A typical ESI negative ion spectrum for an f2f3 fraction is shown in Figure 4.4.

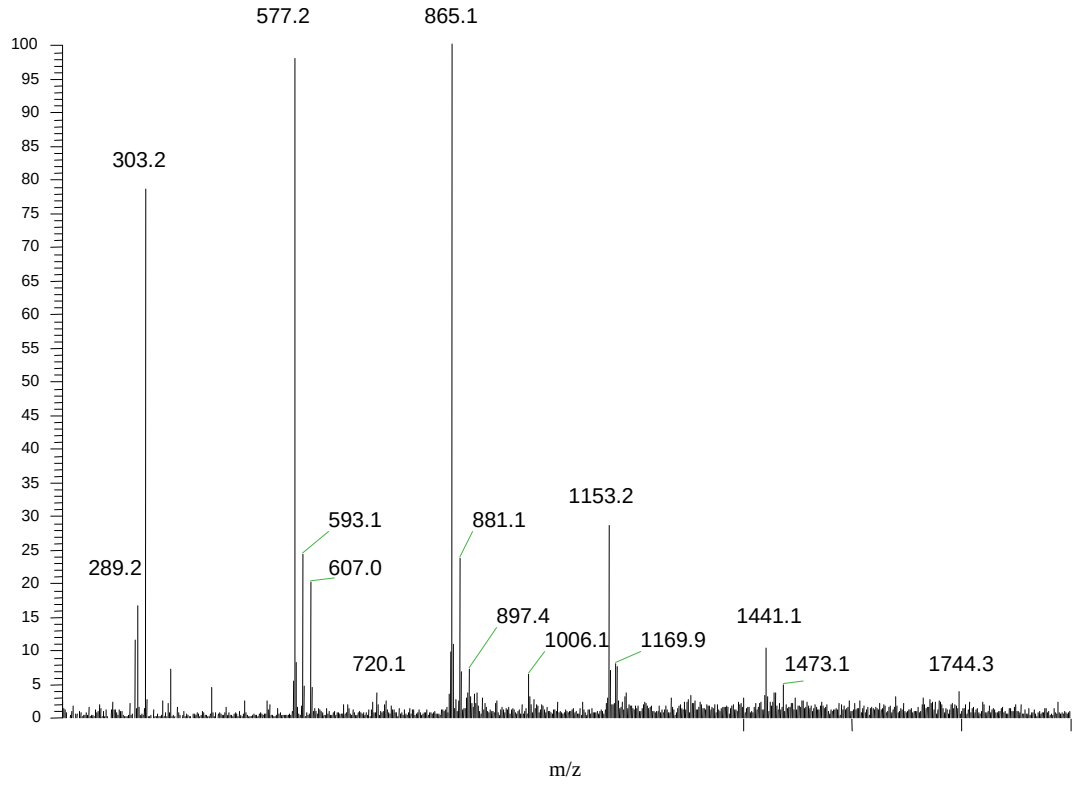


Figure 4.4 ESI MS of HW f2f3 fraction

Like the MALDI-TOF MS results, ESI MS of HW f2f3 shows that there is a series of peaks separated by a *m/z* of 288 Da, which corresponds to the MW of one catechin/epicatechin (procyanidin) unit. As observed in the MALDI-TOF MS spectra each procyanidin peak was accompanied by ion peaks 16 and 32 Da higher which are presumed to be the ions of condensed tannins containing one or two prodelphinidin units.

Literature has shown that ESI MS of low molecular weight condensed tannins ions tend to produce singly charged ions while larger condensed tannins tend to produce doubly charged ions (Fulcrand *et al.*, 1999). Doubly charged species can be identified in two ways; through groups of peaks that are separated by 144 Da

and peaks within individual groups that are separated by 8 Da. Small peaks in Figure 4.4 at *m/z* of 720 and 1006 were identified as doubly charged species. These peaks correspond to the doubly charged pentamer and heptamer respectively. The HW f2f3 spectra indicates that the major flavonoid extracted from *P. radiata* bark is taxifolin (*m/z* = 303) with some catechin/epicatechin (*m/z* = 289). Table 4.2 shows the mass of expected singly and doubly charged ions as well as the masses observed through ESI MS. In agreement with the literature (Taylor *et al*., 2003), smaller condensed tannins (DP 1 to 6) were generally observed as singly charged ions, while larger condensed tannins (DP 5 to 8) were generally observed as doubly charged ions.

Table 4.2 Expected and Observed Ions in ESI MS for HW f2f3 condensed tannins

		Expected m/z		
DP	**PD**	**$[M-H]^-$**	**$[M-2H]^{2-}$**	**Observed m/z**
1	Catechin	289.3		289.2
	Taxifolin	303.3		303.2
2	0	577.5		577.2
	1	593.5		593.1
3	0	865.8	432.4	865.1
	1	881.8	440.4	881.1
	2	897.8	448.4	897.4
4	0	1154.1	576.6	1153.2
	1	1170.1	584.6	1169.9
	2	1186.1	592.6	1184.1
5	0	1442.3	720.7	1441.1, 720.1
	1	1458.3	728.7	1458.3, 729.1
	2	1474.3	736.7	1473.1
6	0	1729.6	864.3	1729.2, 863.2
	1	1745.6	872.3	1744.3
7	0	2017.8	1008.4	1006.1
	1	2033.8	1016.4	1016.0
8	0	2305.0	1152.0	1152.2
	1	2321.0	1160.0	1159.9
	2	2337.0	1168.0	1167.1

ESI MS of the HW f2f3 fraction produced a spectrum with peaks ranging from DP 1-8, with the most intense peak being at DP 3. ESI MS of the other f2f3 fractions (MWA, AW and PP (Appendix 2.6)) produced spectra with peaks ranging from DP 1-5, with the most intense peak attributable to the flavonoid, taxifolin. ESI MS of SFU f2f3 fraction (Figure 4.5) produced a spectrum with a series of procyanidin peaks 288 Da apart (*m/z* = 577.3, 865.3 and 1153.5) as well as a series of condensed tannin sulphonates peaks (*m/z* = 657.3, 945.3, 1233.4 and 1521.4) which were 80 Da more than the procyanidin peaks.

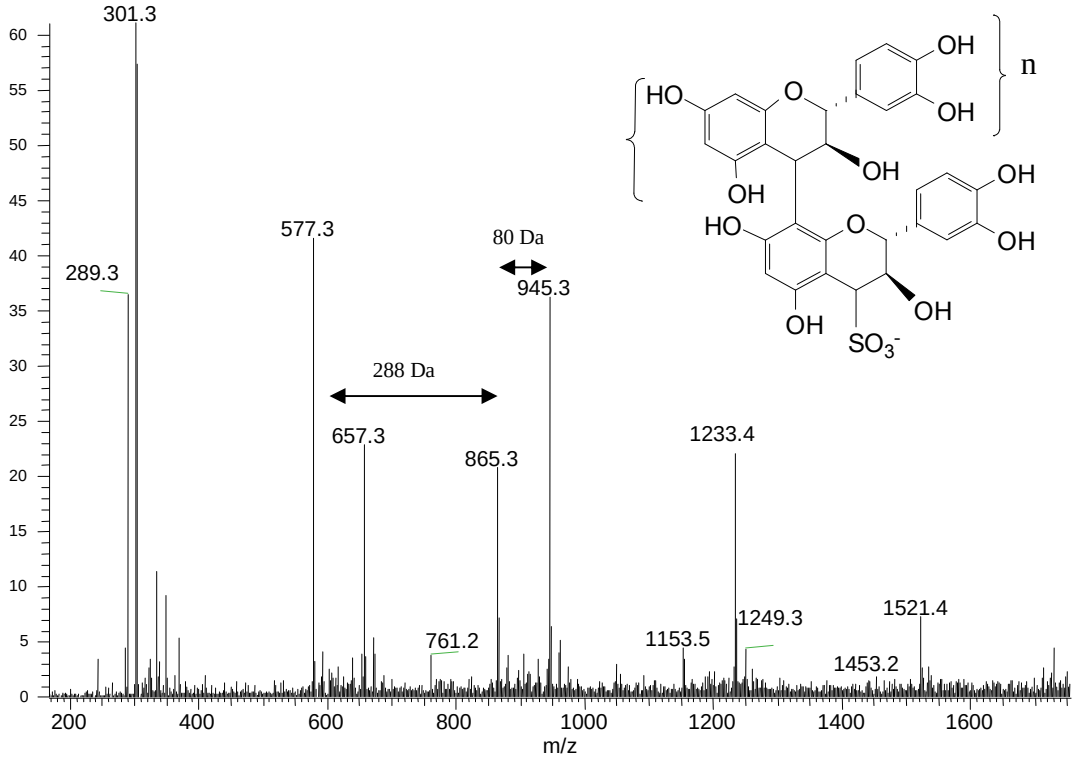


Figure 4.5 ESI MS of SFU crude fraction and condensed tannin sulphonate structure

These condensed tannin sulphonates result from the SFU extraction process where the sodium sulphite depolymerises the condensed tannin and adds SO_3^- onto the C-4*t* carbon (Kreibich *et al*., 1987). This is consistent with other work, which has been reported in the literature, using this extraction technique (Kreibich *et al*., 1987 and Karchesy *et al*., 1989).

ESI MS of the HW molecular weight fractionated condensed tannins (Figure 4.6) showed that as the fraction number increased the range of condensed tannins

detected and the *m/z* of the most intense peak increased. Fractionation of HW condensed tannins extended the detection range by 1 DP. Fractionation of the MWA condensed tannins similarly extended the detection range by 4 DP. An analysable spectrum that could be interpreted could not be obtained for the HW 7 and 8 fractions as the condensed tannins were too large and polydisperse. Fraction HW 1 was not analysed because it did not dissolve in a suitable solvent. Appendix 2.7 shows the ESI MS spectra of MWA fractions 2 to 6.

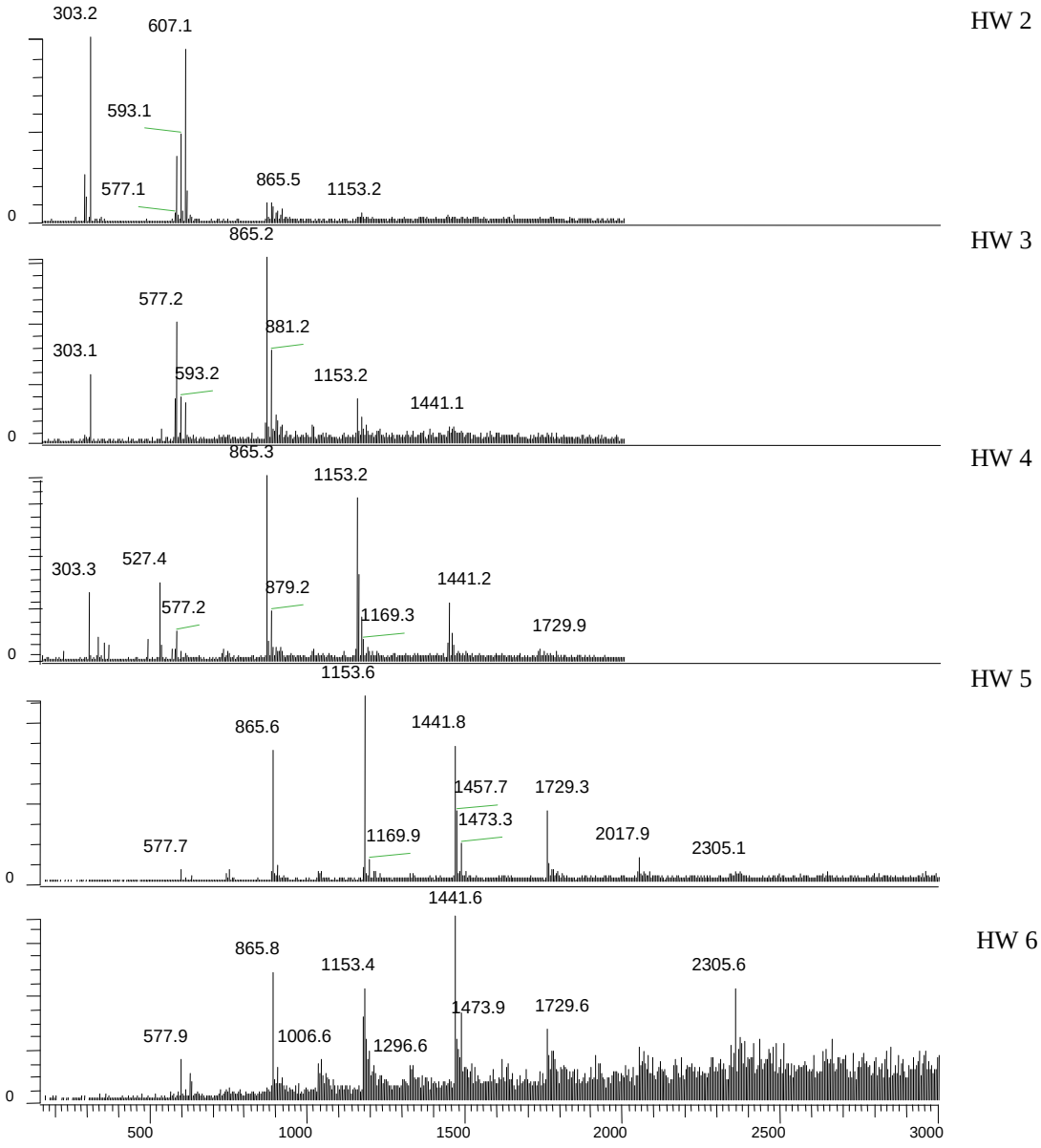


Figure 4.6 ESI MS of fractionated HW 2-6 condensed tannins

4.4 COMPARISON OF MS METHODS

In both MALDI-TOF MS and ESI MS condensed tannins of lower mass appear in more abundance in the mass spectra. When HW f2f3 was fractionated according to molecular weight, 26.8% of the mass was obtained in fractions 7 and 8 and 75.3% of the mass was obtained in fractions 4,5,6,7 and 8. This indicates that MALDI-TOF MS and ESI MS were incapable of detecting the high MW condensed tannins (> 4000 Da) proven to exist through depolymerisation/HPLC and GPC (see Chapter 5). The reason for this is due to the ease with which smaller MW condensed tannins are ionised compared with larger MW condensed tannins, and not due to their relative abundances (as indicated by the analysis undertaken in Chapter 5). These results are consistent with the results of other researchers (Taylor *et al*., 2003).

MALDI-TOF MS and ESI MS analysis of *P. radiata* condensed tannins produced similar results. Both MALDI-TOF MS and ESI MS singly charged ions showed a series of peaks corresponding to oligomers of catechin/epicatechin (procyanidin) units. Also at each series of peaks there were peaks indicating that one or two epigallocatechin/gallocatechin (prodelphinidin) groups were included in the oligomer. ESI MS also showed peaks separated by 144 Da which corresponded to a doubly charged species.

Table 4.3 summarises the ranges and the most abundant species detected in both MALDI-TOF MS and ESI MS. When the MS data is compared with mDP data obtained in Chapter 5 (found through depolymerisation/HPLC) the following observation was found. As the molecular weight increases the range and most intense peak observed become further removed from the mDP. Table 4.3 also shows that fractionation of the f2f3 sample allowed, larger MW condensed tannins to be detected in both MALDI-TOF MS and ESI MS compared with the analysis of the unfractionated f2f3 samples.

Analysis of the different f2f3 samples by ESI MS (excluding HW f2f3) produced a spectrum that was very similar for each sample, while analysis by MALDI-TOF MS produced spectra with slight variations between samples. Analysis by ESI

MS produced spectra (excluding HW f2f3) with a DP range of 1-5 with the most intense peak being at a DP of 1. The MALDI-TOF MS spectrum of the different f2f3 samples showed the ranges of peaks detected were different between samples, for example the HW f2f3 spectra contained peaks ranging from DP 3 to DP 11, while in the spectrum of the AW fraction peaks were detected ranging from DP 3 to DP 9. The most intense peak for all the f2f3 samples corresponded to condensed tannin with a DP of 4 or 5.

Table 4.3 Comparison of mass range estimates by ESI and MALDI-TOF mass spectrometry

Sample	Oligomer range by ESI	Most intense Oligomer in ESI	Oligomer range in MALDI	Most intense Oligomer in MALDI
HW 2	1-3	1	3-5	3
HW 3	1-5	3	3-6	3
HW 4	1-5	3	3-8	4
HW 5	2-8	4	3-9	5
HW 6	2-8	5	5-11	6
HW 7			5-13	5
MWA 2	1-4	1	3-2	3
MWA 3	1-5	3	3-4	3
MWA 4	1-6	3	3-8	4
MWA 5	2-8	4	3-10	5
MWA 6	2-9	5	5-12	6
MWA 7			5-14	5
HW (f2/f3)	1-8	3	3-11	4
MWA (f2/f3)	1-5	1	3-10	5
AW (f2/f3)	1-5	1	3-9	5
SFU (f2/f3)	1-5	1	3-9	5
PP (f2/f3)	1-5	1	4-10	4

A direct comparison between the MALDI-TOF MS and ESI MS of the fractionated HW 5 fraction is shown in Figure 4.7. The differences of the *m/z* in MALDI-TOF MS and ESI MS spectra is due to MALDI-TOF MS detecting positive sodium adduct ions $[M+Na]^+$ and ESI MS detecting negative $[M-H]^-$ ions. The MALDI-TOF MS detected larger MW condensed tannins up to a DP of 10 while ESI MS only detected condensed tannins up to a DP of 8. The most intense peak from the MALDI-TOF MS spectrum (pentamer) is also closer to the mDP determined for this fraction by depolymerisation/HPLC (see Chapter 5) compared with the most intense peak observed by ESI MS (tetramer). Therefore MALDI-TOF MS gives a better indication of the oligomeric composition of the condensed tannin sample than ESI MS, although the true composition is not accurately reflected (see Chapter 5).

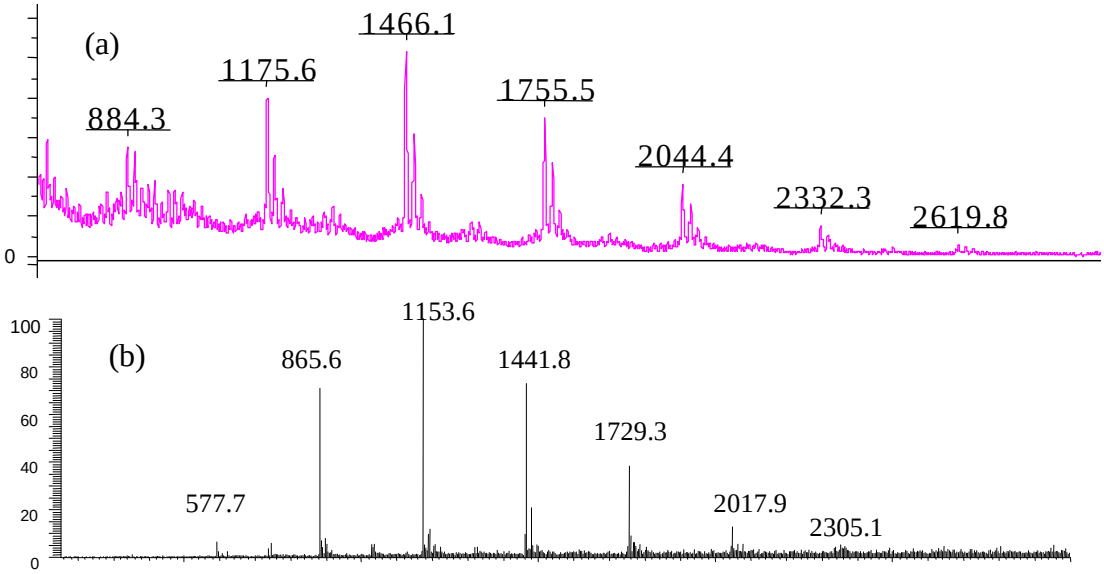


Figure 4.7 HW 5 spectra from (a) MALDI-TOF MS and (b) ESI MS

ESI MS was shown to be better for analysing smaller condensed tannins compared with MALDI-TOF MS as there is no matrix interference. ESI was able to prove that taxifolin was the most abundant flavonoid in our *P. radiata* condensed tannin samples. Alternatively, MALDI-TOF MS was shown to be better at analysing larger MW condensed tannins compared with ESI MS as there is no double charging. The lack of double charging makes the spectra less complex and easier to interpret. MALDI-TOF MS also enables higher molecular weight condensed tannins (up to DP of 14) to be detected.

CHAPTER 5: CHROMATOGRAPHY

Various chromatography techniques have been reported in the literature for the separation of condensed tannins. Reversed phase HPLC has been used to separate the different products produced by acid-catalysis depolymerisation of condensed tannin in the presence of excess phloroglucinol (Kennedy and Jones, 2001). Alternatively, GPC has been used to separate condensed tannins of different molecular weights to obtain information on molecular weight distributions (Kennedy and Taylor, 2003). This chapter describes the use of various chromatography techniques to find molecular weight information. Depolymerisation/LCMS was used to separate and identify products from the acid-catalysed depolymerisation reaction. Depolymerisation/HPLC was used to determine the mDP of condensed tannin samples and GPC was used to provide additional MW information such as average MW's, polydispersity and MW profiles.

5.1 DEPOLYMERISATION/LC-MS

Analysis of the products from the acid-catalysed phloroglucinol depolymerisation reaction (Chapter 2.13) by LCMS (Figure 5.1) was used to identify products and their order of elution. Identifying the peaks in the chromatogram enabled the mDP of the sample to be determined using HPLC with UV detection (Chapter 5.2).

Analysis of the resulting TIC, SIC (selective ion chromatogram) and UV (280 nm) chromatogram (Figure 5.1) showed that the peaks at retention time (R_T) ~ 10.5, 17.0 and 22.2 minutes correspond to epigallocatechin/gallocatechin-phloroglucinol adducts (m/z = 430.5). Peaks at R_T ~ 26.2, 27.4, 28.3 and 32.6 minutes correspond to epicatechin/catechin-phloroglucinol adducts (m/z = 415.5) and the peak at R_T ~ 31.5 minutes corresponds to catechin (m/z = 290.5). With this information the mDP could be determined for condensed tannin oligomers by HPLC with UV detection (Chapter 5.2).

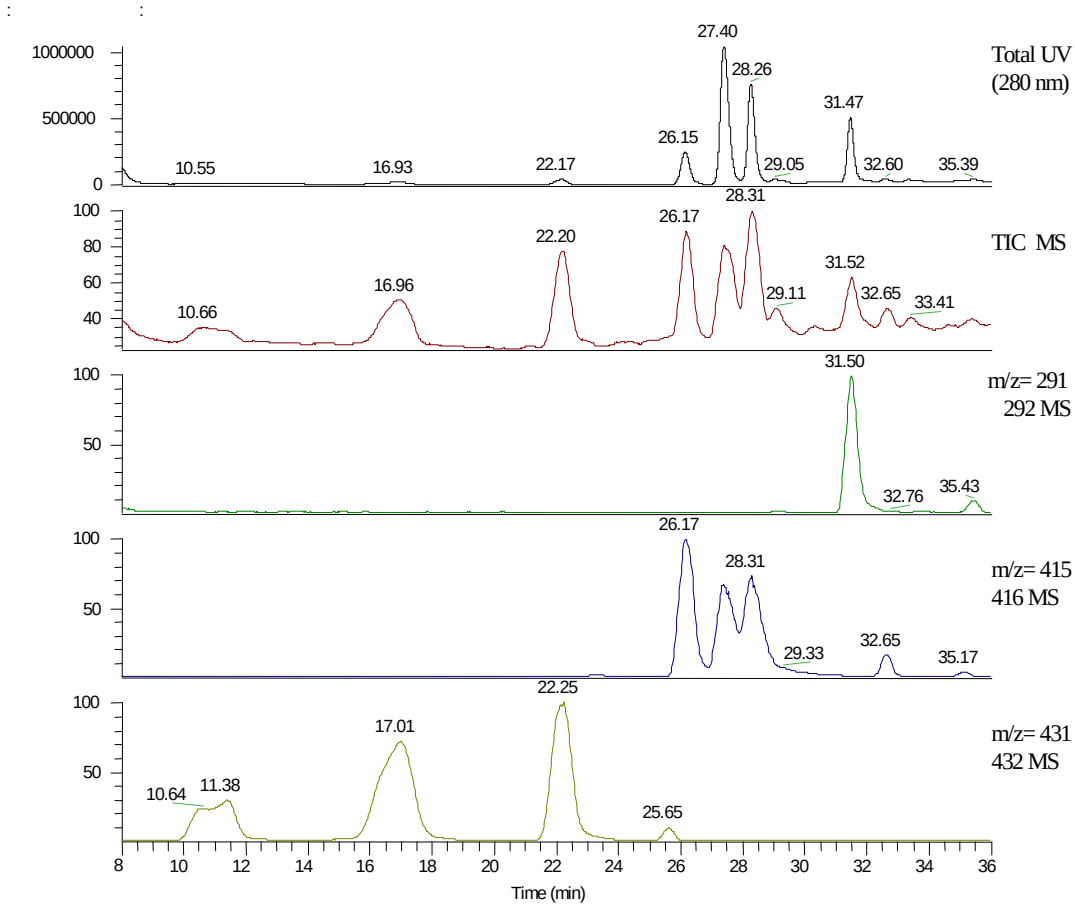


Figure 5.1 LC-MS (TIC and SIC) and UV chromatogram of acid-catalysed depolymerisation products

Pinus radiata proanthocyanidins are complex mixtures of oligomers and polymers with a large range of molecular weights (Kininmonth and Whitehouse, 1991). Depolymerisation/LCMS showed that in *P. radiata* condensed tannins, catechin occurs as both extension and terminal units (*i.e.* catechin is detected as itself and as a phloroglucinol adduct). Catechin is the only observed terminal unit (*i.e.* the only species detected without a phloroglucinol adduct). Epicatechin and gallocatechin occur as extension units (*i.e.* detected as a phloroglucinol adducts) in the polymeric proanthocyanidins. No evidence for galloylation (addition of a galloyl group by an ester linkage at C3 (Reed *et al.*, 2005)) of the *P. radiata* condensed tannins was observed. Galloylation of condensed tannins is common in grape seed condensed tannins (Yang and Chien, 2000). These results are consistent with the literature (Jerez *et al.*, 2007).

Czochanska *et al*. (1979) using ^{13}C NMR found that both catechin and epigallocatechin occurred as terminal groups in *P. radiata* condensed tannins. However, through depolymerisation/LCMS there were no ions detected at *m/z* 307, indicating there were no epigallocatechin terminal groups in the *P. radiata* condensed tannins analysed in this study. HPLC-UV was used to determine mDP and not LCMS because of software limitations on the LCMS.

5.2 DEPOLYMERISATION/HPLC

Utilising the chromatographic peak areas corresponding to extension and terminal units, depolymerisation/HPLC enabled the mean degree of polymerisation (mDP) and the composition of the condensed tannins to be determined. A typical depolymerisation/HPLC chromatogram with peaks assigned (as determined by LC-MS of the acid-catalysed depolymerisation products) is shown in Figure 5.2. An initial analysis of the products formed in the presence of excess phloroglucinol revealed four major products and 7 peaks. These products were catechin (R_T~ 21.5 min), catechin-PG (R_T~ 18.2 and 19 min), epicatechin-PG (R_T~ 22 min) and gallocatechin-PG (R_T~ 9.3, 12.5 and 14.5 min). The first three major peaks on the chromatograms represent the excess phloroglucinol (R_T~ 7.5 min) and ascorbic acid (R_T~ 2.5 and 4 min).

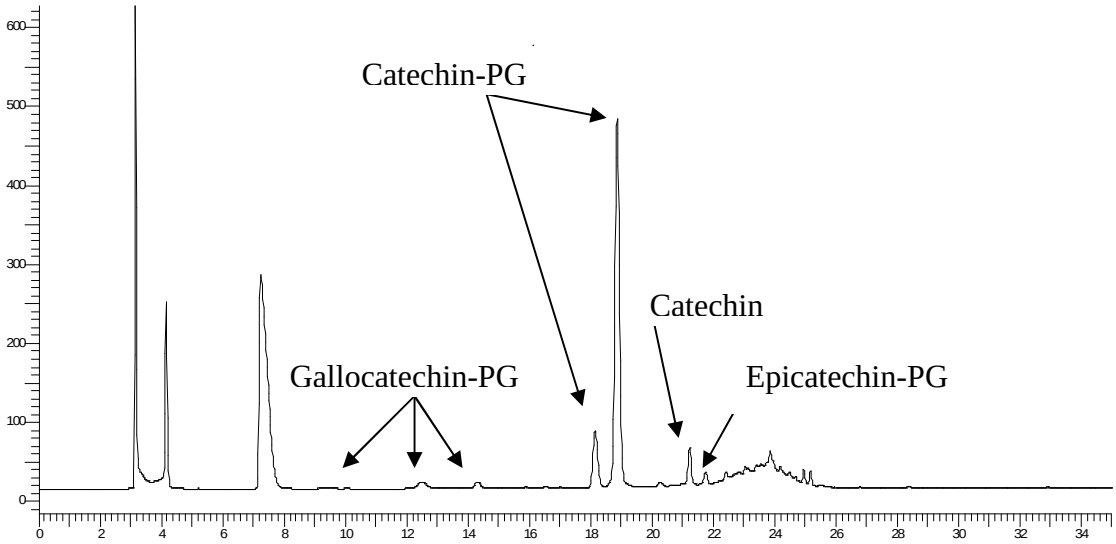


Figure 5.2 Typical depolymerisation/HPLC chromatogram showing peak assignments

From the LCMS (Figure 5.1) the selected ion chromatogram for *m/z* = 415 (catechin/epicatechin-PG) showed four distinctive peaks which were all present in the UV (280 nm) chromatogram. In the HPLC UV chromatogram there are only three peaks assigned to this product. This is because we believe that the two peaks at retention time 27.40 and 28.26 minutes in the LCMS (Figure 5.1) were not resolved on the HPLC column, hence the reason for one large peak (R_T~ 19 min) in the HPLC chromatogram instead of two similar sized peaks. Catechin and epicatechin were assigned from authentic samples (purchased from Sigma) which were run as standards. Resulting chromatograms showed epicatechin (R_T~ 22.8 min) to have a longer retention time than catechin (R_T~ 21.5 min). It was presumed that the catechin-PG and epicatechin-PG products behaved in a similar manner (Figure 5.2).

The reason for the existence of more than one peak per species is due to the existence of multiple chiral centres in some of the species and therefore the existence of diastereoisomers. For example catechin-PG (Figure 5.3) and epicatechin-PG have three chiral carbons and therefore could comprise of a series of 8 diastereoisomers. Only four diastereoisomers exist and they are the two for catechin-PG (Figure 5.3) and the two for epicatechin-PG.

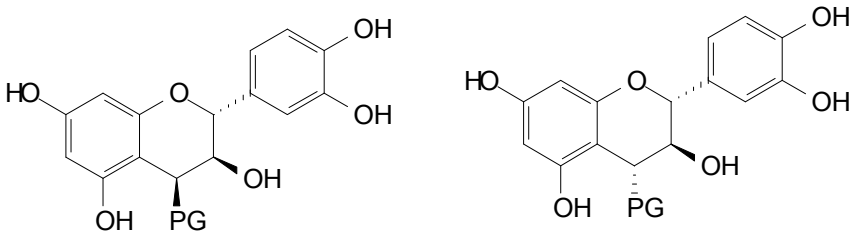


Figure 5.3 The two diastereoisomers of catechin-PG

The depolymerisation/HPLC chromatograms enabled the mean degree of polymerisation (mDP) and the ratio of prodelphinidin to procyanidin of the condensed tannin samples to be determined (Table 5.1). This methodology for determining mDP was proven accurate through depolymerisation/HPLC analysis of the dimer fraction f4 (see Chapter 3.5). The dimer f2 fraction was shown to have a mDP of 2.6 which is close to what was expected as GPC analysis (Figure

3.7) showed this fraction consisted of approximately 65-70% dimer with some catechin and trimer.

Table 5.1 Results of depolymerisation/HPLC for molecular weight fractionated condensed tannins and f2f3 fractions

Sample	Sample Yield (%)	mDP by acid catalysis[a]	Mean Molecular Weight[a]	Ratio of PD/PC [a, b]
HW 1	5.3	15.5	4467	nd[c]
HW 2	9.0	2.2	637	0.156
HW 3	10.0	4.6	1314	0.131
HW 4	16.5	7.8	2248	0.096
HW 5	12.3	10.3	2960	0.174
HW 6	19.7	12.8	3677	0.199
HW 7	16.0	15.9	4580	0.247
HW 8	10.8	18.7	5386	0.265
MWA 1	2.6	15.7	4525	nd[c]
MWA 2	7.4	2.3	657	0.208
MWA 3	8.1	4.1	1185	0.101
MWA 4	7.1	6.0	1732	0.108
MWA 5	13.9	8.9	2574	0.118
MWA 6	17.4	11.0	3182	0.134
MWA 7	22.3	14.6	4208	0.181
MWA 8	21.1	18.7	5389	0.184
MWA (f2/f3)		8.3	2392	0.121
HW (f2/f3)		7.6	2190	0.221
AW (f2/f3)		7.3	2092	0.156
SFU (f2/f3)		9.2	2654	0.211
PP (f2/f3)		8.9	2572	0.209

[a] 95% confidence interval of mDP = ± 0.37, mean MW = ± 107 Da and PD/PC ratio = ± 0.03

[b] Ratio PD/PC = prodelphinidins/procyanidins. [c] nd = not determined, peaks were too small to be accurately estimated

Depolymerisation/HPLC results (Table 5.1) showed that the HW molecular weight fractionated condensed tannins (HW 2 - HW 8) had a mDP ranging from 2.2 to 18.7, while the MWA molecular weight fractionated condensed tannins (MWA 2 - MWA 8) had a mDP ranging from 2.3 to 18.7. The mDP for each fraction increased approximately 2-4 DP with each increasing fraction number, which is larger than the experimental error of ± 0.37. The depolymerisation/HPLC results give further evidence (in addition to the MALDI-TOF and ESI MS spectra, discussed in Chapter 4) that the *P. radiata* condensed tannins have been successfully separated into different molecular sizes by fractionation with Sephadex LH-20 chromatography. Taylor *et al*. (2003) using a similar fractionation technique was able to fractionate hops (*Humulus lupulus* L. cv. Willamette) condensed tannins (mDP = 7.8) into 8 fractions ranging from mDP 1.8 to 22.2.

The HW 1 and MWA 1 fractions had an unusually high mDP. Both samples only partially dissolved in the reaction solvent and only small chromatographic peaks were obtained for the depolymerisation products relative to the other fractions. A hypothesis for this is that some of the condensed tannin had bound with some polysaccharides. Polysaccharides that are not dried by lyophilisation become "horny" and become difficult to re-dissolve due to internal H-bonding (Dea, 1989). A suitable solvent could not be found to dissolve the sample which meant it could not be analysed by NMR spectroscopy.

For most of the fraction numbers the mDP was significantly higher (difference was larger than the experimental error) for the HW fractions compared with the corresponding MWA fractions. Alternatively there are larger yields (%) for the latter fractions in the MWA fractions compared with the HW fractions. This is why the MWA f2f3 mDP (8.3) was larger than the HW f2f3 mDP (7.6), thus proving the MWA method had extracted more larger MW condensed tannins compared with the HW method.

Comparison of the depolymerisation/HPLC results with the MALDI-TOF and ESI MS (Chapter 4) spectra showed that the higher the fraction number the further away from the mDP the most intense ion peak is. This indicates that mass

spectrometry of condensed tannin does not give a true representation of the whole sample, only part of the sample (smaller MW condensed tannins).

Condensed tannin f2f3 samples from the different extraction techniques had mDP's ranging from 7.3 for AW to 9.2 for SFU (Table 5.1). SFU has a larger mDP compared with that of the other extraction methods (all outside the 95% confidence interval except PP). The most likely reason for this is some small molecular weight condensed tannins were eluted in the f1 fraction (as indicated by the ^{1}H NMR spectrum shown in Figure 3.5). This point is discussed further in Chapter 5.3.

The prodelphinidin to procyanidin ratio (PD/PC) ranges between 0.121 and 0.220 for the f2f3 samples. This indicates that the solvent used has an effect on the ratio of prodelphinidins to procyanidins extracted. The increase in the proportion of prodelphinidins in the higher MW fractionated fractions (Table 5.1) is most likely to be an artefact of the separation process and not a real correlation with mDP. The number of hydroxyls in condensed tannins can increase by increased DP and by a increased fraction of prodelphinidin units. The more hydroxyl groups in the condensed tannin the stronger the interactions (hydrogen bonds) with the Sephadex stationary phase (Taylor *et al*., 2003). Therefore prodelphinidins have stronger interactions with the Sephadex LH-20 than procyanidins and are eluted with solvents with higher concentrations of acetone.

5.3 GEL PERMEATION CHROMATOGRAPHY (GPC)

Gel permeation chromatography (GPC) performed on unmodified *P. radiata* condensed tannins provided information on average molecular weights and molecular weight distributions. Using the methodology described in Chapter 2.16 both HW and MWA fractions 2-8 were analysed by GPC. The chromatograms of the MWA fractions (overlaid over each other) are shown below in Figure 5.4. The chromatograms of the HW fractions are shown in Appendix 3.1.

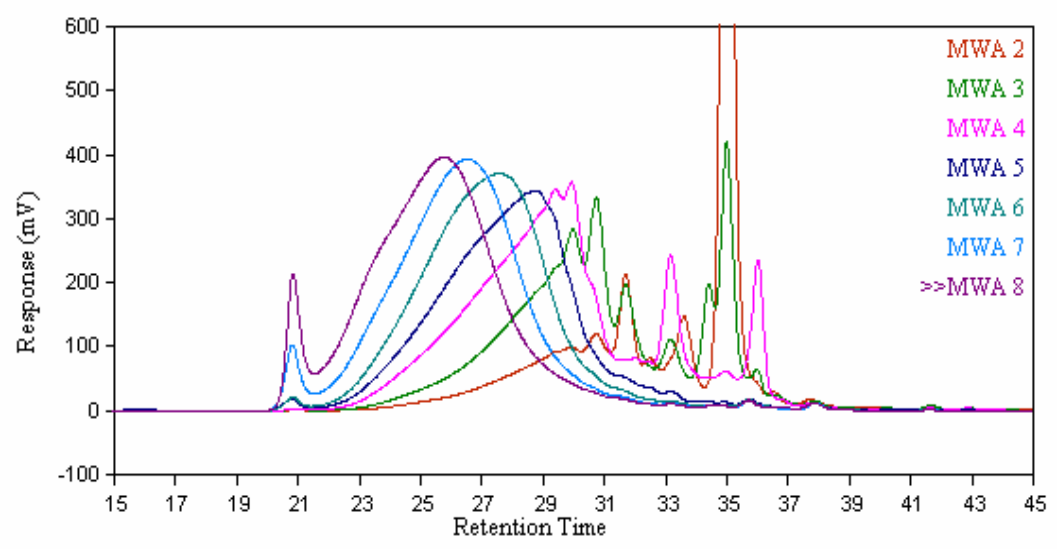


Figure 5.4 GPC chromatograms of MWA 2-8 molecular weight fractionated condensed tannins

A calibration curve for condensed tannin was constructed using the fractionated HW and MWA condensed tannins (see Chapter 2.16 for method). Resolved flavonoids and low MW oligomers were identified and assigned as follows. ESI MS of the condensed tannin samples found taxifolin (*m/z* = 303) to be the most abundant flavonoid present and enabled the assignment of taxifolin at R_T~ 35 minutes (Figure 5.4). Catechin monomer was assigned by comparison with a sample of pure catechin run as a standard (R_T~ 33.5 min). Through ESI MS and NMR analysis of the synthesised dimer f4 fraction it was shown that this fraction contained large amounts of dimer. GPC of the f4 dimer fraction produced a large peak at R_T~ 31.5 minutes. This indicates that the peaks at 31.5 minutes on the chromatogram (Figure 5.5) correspond to the condensed tannin dimer (*m/z* = 578). Peaks at R_T~ 30.5 and 29.5 minutes were assumed to be the trimer and tetramer respectively. Because the dimer, trimer and tetramer peaks were well resolved in

the GPC chromatograms the MW's and retention times of the oligomers were directly used in the calibration. For fractions HW and MWA 5-8 the mean MW (as determined from the mDP obtained by depolymerisation/HPLC) was assigned to the peak maximum and was also used in the calibration (see discussion Chapter 2.16).

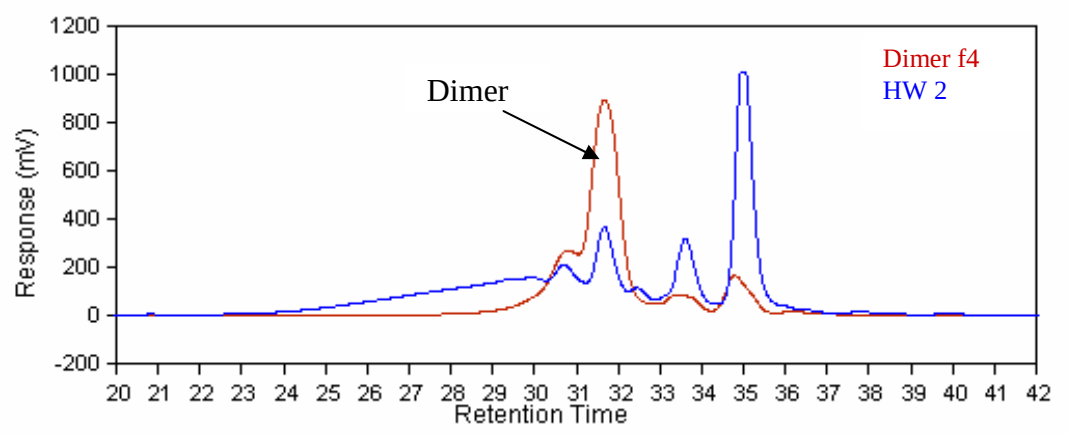


Figure 5.5 GPC chromatograms of dimer f4 and HW 2 proving that the dimer peak eluted at 31.5 minutes

The condensed tannin calibration curve (Figure 5.6) demonstrates the good correlation between condensed tannin molecular weight and the retention time. The calibration had a R^2 value of 0.985. Kennedy and Taylor (2003) using a similar technique obtained a condensed tannin calibration curve with a $R^2 = 0.996$ from grape seed condensed tannins.

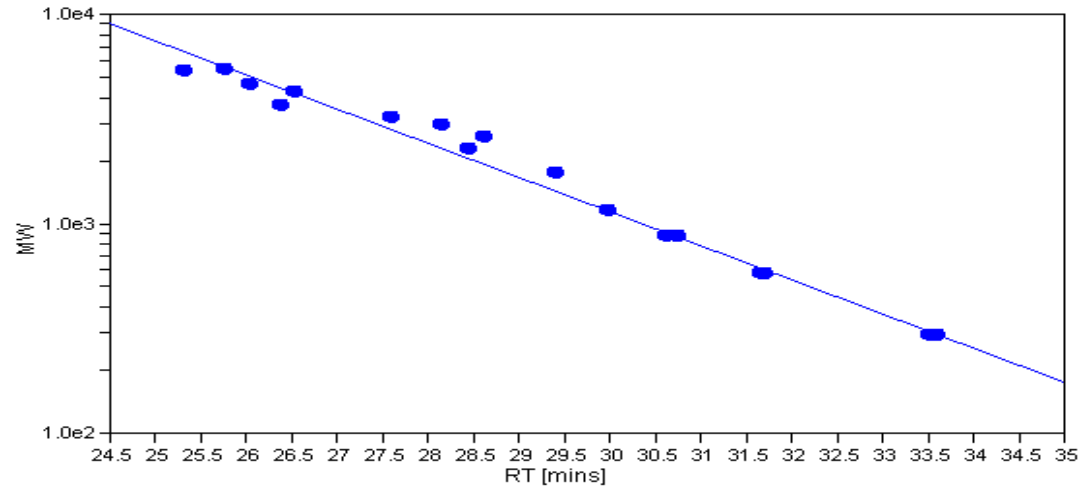


Figure 5.6 Calibration plot of molecular weight versus retention time using HW and MWA fractions 2-8 as standards

A calibration curve enabled the MW distribution (Figure 5.7) and MW information of the samples to be calculated. Figure 5.7 demonstrates through plots of the molecular weight distribution (A) and MW versus percentage cumulative height (B) that the average molecular weight of the condensed tannins increases with the order of elution from the Sephadex LH-20 fractionation. This is consistent with what was observed by mass spectroscopy (Chapter 4) and depolymerisation/HPLC (*i.e.* larger molecules came off the column in later fractions with increasing acetone concentration).

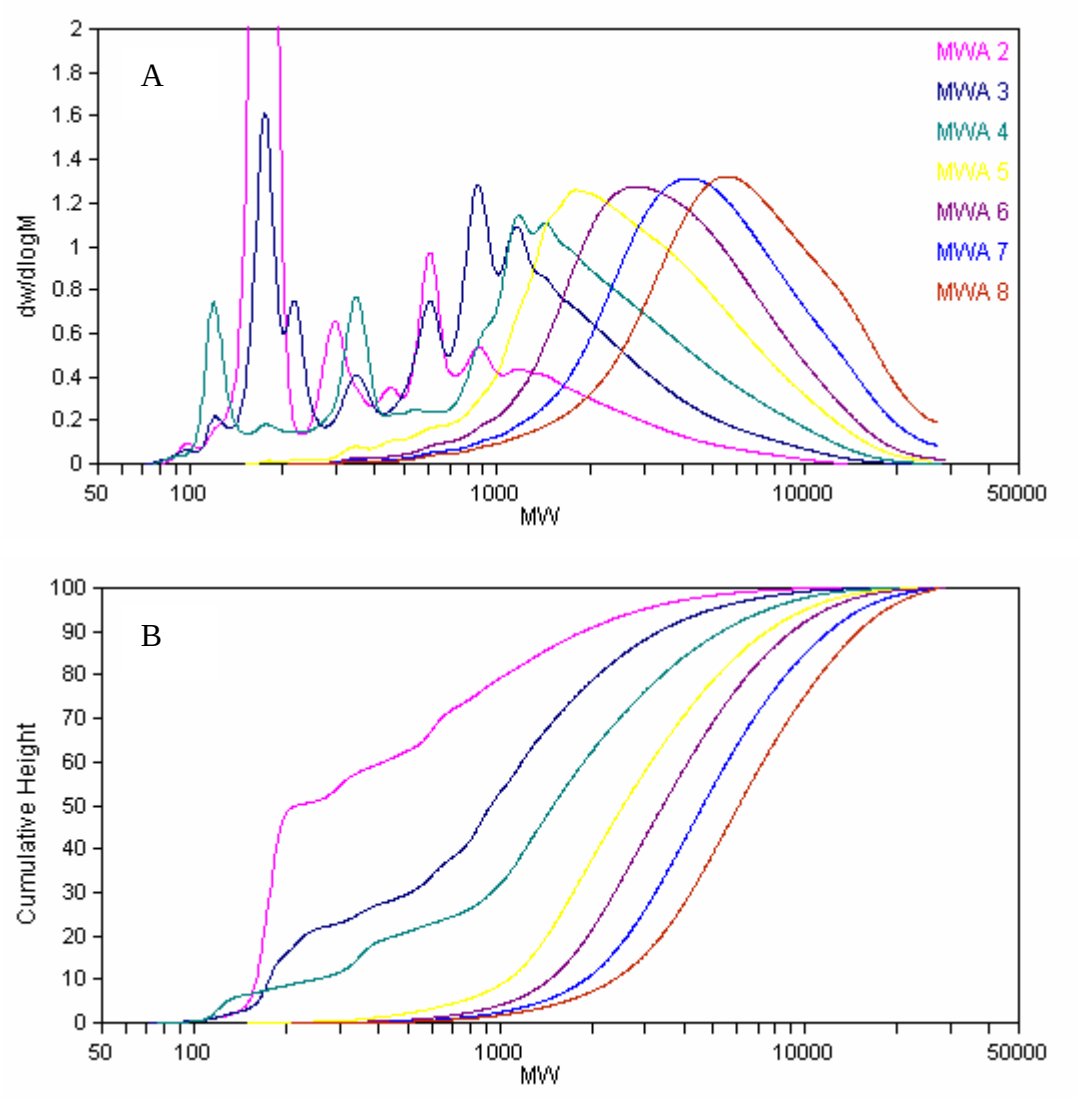


Figure 5.7 GPC plots of (a) Molecular mass distribution of MWA fractions (b)Percentage cumulative height versus MW for MWA fractions

GPC analysis provides information about the whole MW distribution of each sample unlike mass spectrometry and depolymerisation/HPLC which only gave partial MW information. The percentage cumulative height versus MW (Figure 5.7 (b)) shows that approximately 50% of fraction MWA 2 has a MW less than 300 Da (indicating that 50% of the fraction is made up of flavonoids), while approximately 50% of fraction MWA 3 has a MW of under 1000 Da. Ninety percent of the condensed tannins in fraction MWA 7 have a MW over 2000 Da, while 90% of the condensed tannins in fraction MWA 8 have a MW over 3000 Da.

The average MW and polydispersity calculations in Table 5.2 were based on the whole condensed tannin sample, which includes the flavonoids. The number average molecular weight ranged from approximately 350 to over 5000 Da for the HW fractionated condensed tannins and ranged from approximately 270 to 4400 Da for the MWA fractionated condensed tannins. The weight average molecular weight ranged between approximately 1100 and 8100 Da for HW fractionated condensed tannins and 750 and 7500 Da for the MWA fractionated condensed tannins. The number average molecular weight increases by approximately 800 Da with every fraction number. Correspondingly, the weight average molecular weight increases by approximately 1000-2000 Da with every fraction number. Both the number average and weight average MW increases for each fraction were larger than there corresponding experimental error.

Polydispersity is a measure of the distribution of molecular weights in a polymer sample. As the fraction number increases the polydispersity of the sample decreases (Table 5.2), which shows that the higher the fraction number the smaller the MW range of condensed tannins *i.e.* HW 2 has a polydispersity of 3.2 while HW 8 has a polydispersity of 1.6.

Table 5.2 Average MW and polydispersity calculations of molecular weight fractionated condensed tannins

Sample	Number average molecular weight (M_n)[a]	Weight average molecular weight (M_w)[a]	Polydispersity[a]
HW 2	347	1115	3.21
HW 3	668	1940	2.90
HW 4	1847	4140	2.24
HW 5	2679	5210	1.94
HW 6	3450	6184	1.79
HW 7	4266	7144	1.67
HW 8	5027	8122	1.62
MWA 2	269	750	2.78
MWA 3	487	1475	3.03
MWA 4	660	2294	3.48
MWA 5	1941	3572	1.84
MWA 6	2676	4505	1.68
MWA 7	3512	5938	1.69
MWA 8	4407	7495	1.70

[a] 95% confidence interval for Mn = ± 70 Da, Mw = ± 553 Da, Polydispersity = ± 0.48

A typical GPC chromatogram of a condensed tannin f2f3 fraction is shown in Figure 5.8. The chromatogram shows that the two columns used enabled the resolution of flavonoids (taxifolin and catechin monomers) and low MW condensed tannin oligomers (up to the tetramer). Separated flavonoids and low MW oligomers were identified and assigned as discussed earlier. All the condensed tannins are eluted between a R_T of 20 and 36 minutes.

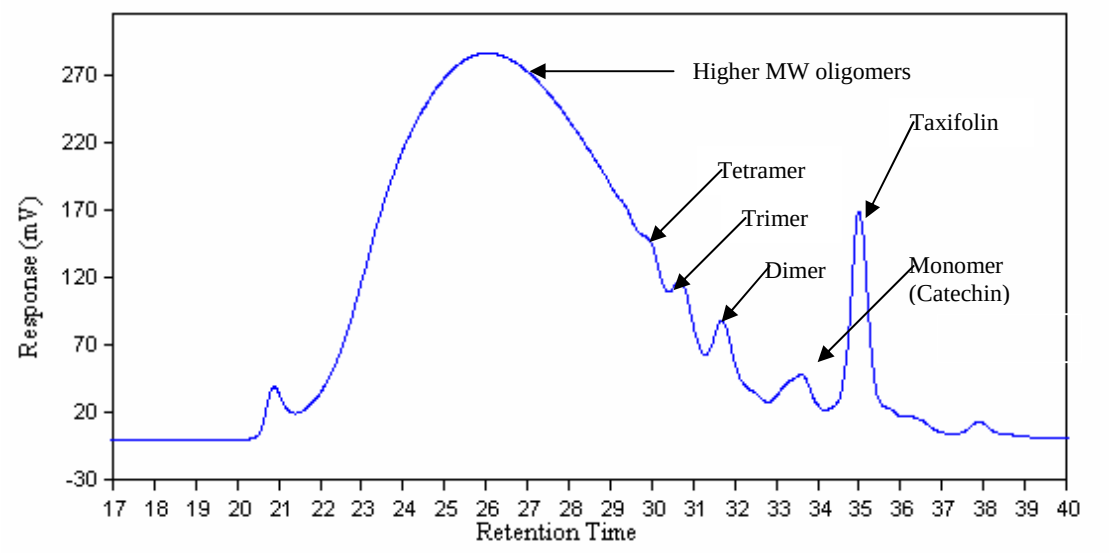


Figure 5.8 GPC chromatogram of HW f2f3 showing individual chromatogram features

GPC proved itself to be an excellent technique for comparing MW distributions of condensed tannins obtained from different extraction solvents. Figure 5.9 compares the MW distributions of HW and AW extracted condensed tannins. Assuming that each extraction solvent has extracted material of similar composition the results show that the HW extraction has extracted higher MW condensed tannins than the AW extraction. This agrees with results obtained from depolymerisation/HPLC which showed the AW extracted condensed tannins had a mDP of 7.3 and HW extracted condensed tannins had a mDP of 7.6. Kennedy and Taylor (2003) used this technique to compare mass information of condensed tannins from grape seeds picked earlier than and later than normally harvested.

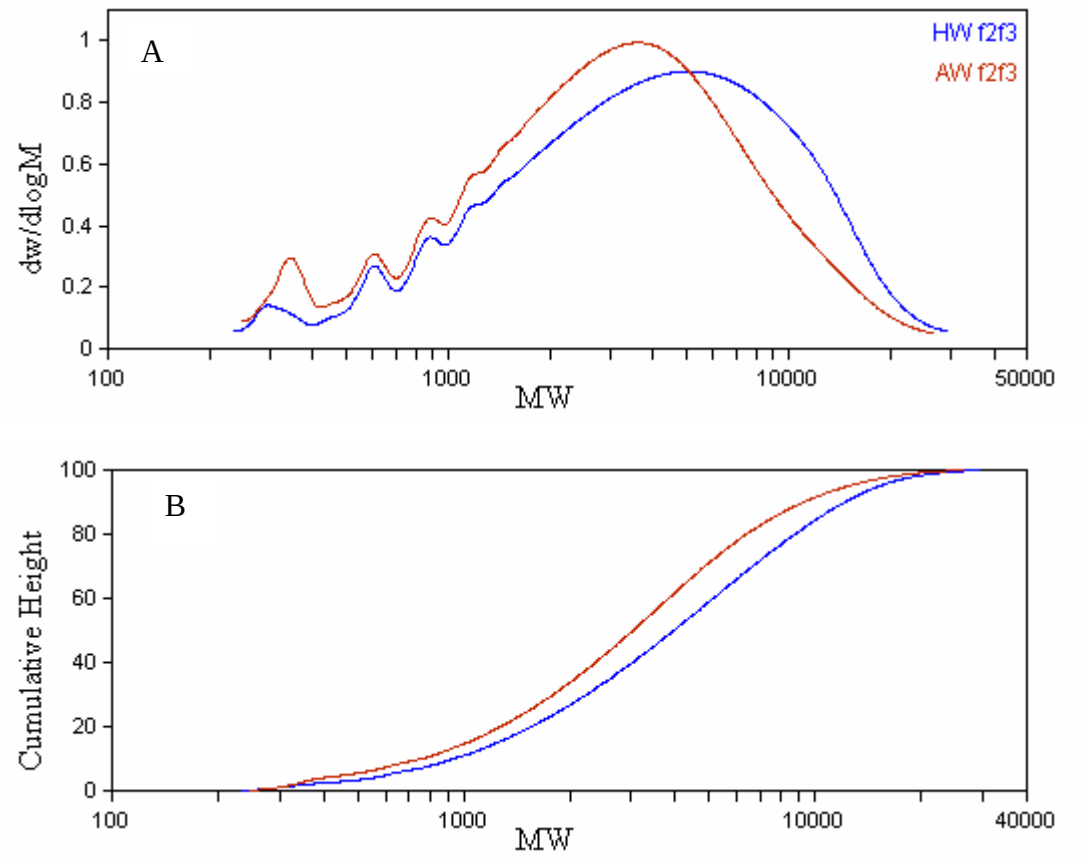


Figure 5.9 Comparison between HW f2f3 and AW f2f3- (a) MW distribution plot and (b) Percentage cumulative height versus MW

A comparison of the average MW and polydispersity for the different extraction methods (f2f3 fractions) are shown in Table 5.3. It shows that the AW extraction method has extracted on average the smallest MW condensed tannins (M_n 950 Da and M_w 3850 Da), while the PP extraction method has extracted the largest MW condensed tannins (M_n 1750 Da and M_w 6000 Da). Not taking into account the SFU extraction method (discussed below) the polydispersity were all within the experimental error and ranged between 3.45 for the PP method and 4.43 for the MWA method. Appendix 3.2 contains the GPC chromatograms of MWA f2f3 and PP f2f3.

Table 5.3 Average MW and polydispersity calculations of f2f3 fractions from different extraction techniques

Sample	Number average molecular weight (M_n)[a]	Weight average molecular weight (M_w)[a]	Polydispersity[a]
HW (f2f3)	1292	5150	3.99
MWA (f2f3)	1019	4519	4.43
AW (f2f3)	952	3858	4.05
SFU (f2f3)	1692	4782	2.83
PP (f2/f3)	1752	6040	3.45

[a] 95% confidence interval for Mn = ± 70 Da, Mw = ± 553 Da, Polydispersity = ± 0.48

GPC was performed on the SFU f1 fraction to gain an understanding of the distribution of condensed tannins that were eluted in this fraction (proven through ^{1}H NMR spectroscopy (Figure 3.5)) and to compare this fraction with the SFU f2f3 fraction. The SFU f2f3 is not consistent with the results obtained for the HW, MWA and AW extraction methods. The chromatograms of SFU f1 and f2f3 (not shown) show that the two fractions contain condensed tannins of different molecular distributions.

The MW distributions (Figure 5.10 (a)) and cumulative height versus MW (Figure 5.10 (b)) plots show that the fraction SFU f1 contained a larger proportion of lower molecular weight condensed tannin (MW~ 400-2000 Da), compared to fraction SFU f2f3 which consisted of more higher molecular weight condensed tannins (MW 2000-20000 Da). This is the reason for the SFU f2f3 fraction having a higher than expected mDP as determined by depolymerisation/HPLC and a larger than expected f1 yield.

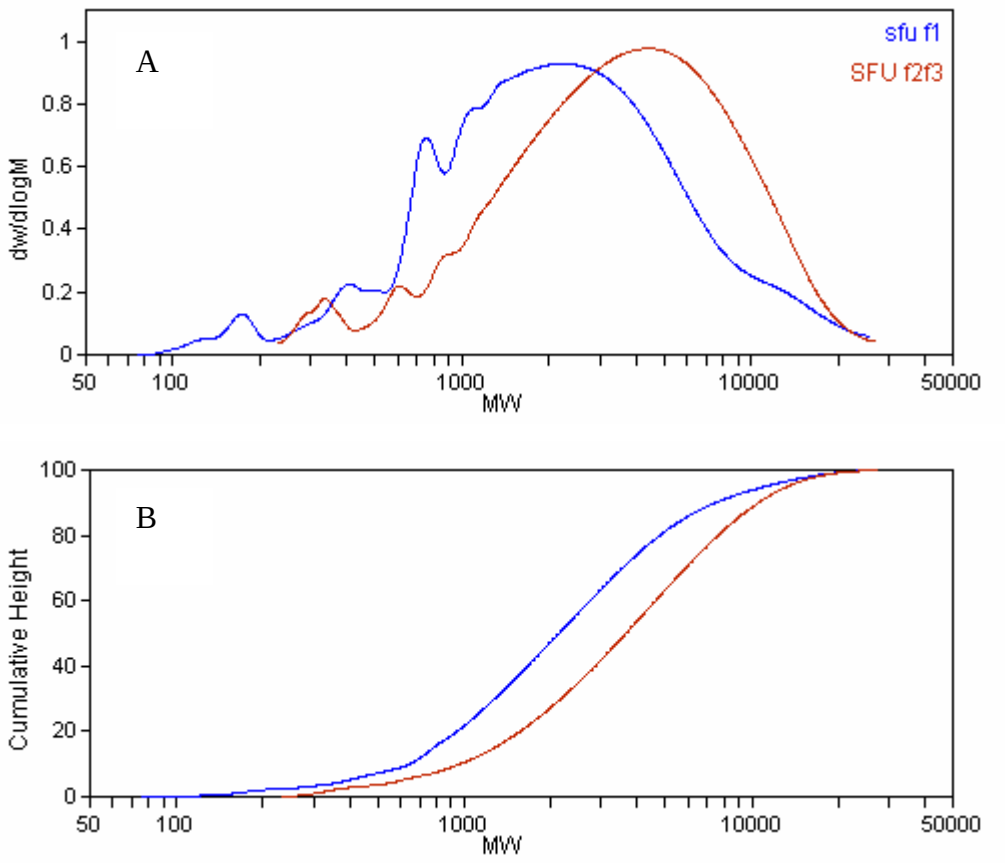


Figure 5.10 Comparison of SFU f1 and SFU f2f3 by (a) MW distribution plot and (b) percentage cumulative height versus MW

A comparison of the average MW values and polydispersities calculated from the GPC data of the SFU fractions is shown in Table 5.4. The results confirm that the condensed tannins separated in f1 are of significantly smaller MW (number average MW of 1250 Da and a weight average MW of 3400 Da) compared with those condensed tannins separated in the f2f3 fraction (number average MW of 1700 Da and a weight average MW of 4800 Da).

Table 5.4 Comparison of MW and polydispersity values obtained from GPC data for SFU f1 and SFU f2f3

Sample	Number average molecular weight (M_n)[a]	Weight average molecular weight (M_w)[a]	Polydispersity[a]
SFU (f1)	1246	3351	2.69
SFU (f2f3)	1692	4782	2.83

[a] 95% confidence interval for Mn = ± 70 Da, Mw = ± 553 Da, Polydispersity = ± 0.48

Kennedy and Taylor (2003) used similar methods to fractionate and analyse grape skin, grape seed and hop condensed tannins by depolymerisation/HPLC and GPC. The behaviour of the grape seed condensed tannins behaviour was inconsistent with that of the grape skin and hop condensed tannins, indicating that separate condensed tannin calibrations are necessary for condensed tannins from different sources.

5.4 COMPARISON OF DEPOLYMERISATION/HPLC AND GPC RESULTS

When comparing the GPC and depolymerisation/HPLC results (Table 5.5) it was important that, with the exception of catechin and epicatechin, all flavonoids (principally taxifolin) were excluded from the GPC calculations and plots because these flavonoids were not accounted for in the depolymerisation/HPLC calculations.

Table 5.5 Comparison of HPLC and GPC results excluding all flavonoid monomers (*e.g.* taxifolin) except catechin/epicatechin

Sample	**Average molecular weight by depoly/HPLC[a]**	**Number average molecular weight (GPC)[a]**	**Weight average molecular weight (GPC)[a]**
HW 2	637	688	1388
HW 3	1314	1187	2164
HW 4	2248	1847	4140
HW 5	2960	2679	5210
HW 6	3677	3450	6184
HW 7	4580	4266	7144
HW 8	5386	5027	8122
MWA 2	657	690	1401
MWA 3	1185	998	1845
MWA 4	1732	1177	2447
MWA 5	2574	1941	3572
MWA 6	3182	2676	4505
MWA 7	4208	3512	5938
MWA 8	5389	4407	7495
HW (f2f3)	2392	2139	5435
MWA (f2f3)	2190	2054	4957
AW (f2f3)	2092	1736	4221
SFU (f2f3)	2654	2078	4870
PP (f2/f3)	2572	2588	6230

[a] 95% confidence interval for deploy/HPLC MW = ± 107 Da, Mn = ± 70 Da, Mw = ± 553 Da

The result obtained from depolymerisation/HPLC and GPC (when taxifolin is excluded) complement each other. The molecular weight by depolymerisation/HPLC for HW fractionated condensed tannins agreed within 8% of the number average molecular weight by GPC. The corresponding MW values obtained for the MWA fractionated tannins did not agree with each other as well as the HW fractionated condensed tannins. The reason for the difference between the MWA depolymerisation/HPLC and GPC results is unknown. The difference between the two sets of results for the MWA fractions were outside the 95% confidence intervals and the difference ranged from 5% for MWA 2 up to 23% for MWA 5. Other than SFU f2f3 fraction, depolymerisation/HPLC and GPC molecular weight values for all f2f3 fractions were in good agreement ranging from a difference of 0.6% for PP f2f3 to 20% for SFU f2f3.

MW studies in the literature have been mainly on grape skin and seed condensed tannins (Kennedy and Taylor, 2003; Labarbe *et al.*, 1999 and Perret *et al.*, 2003). However some studies have been carried out on *P. radiata* condensed tannins. For instance, Williams *et al*., (1982) analysed peracetylated condensed tannin derivatives from *P. radiata* needles by GPC. By utilising a THF mobile phase with a mixture of polystyrene and small MW condensed tannin peracetates as standards, they found the number average MW to be 3000 Da and the weight average MW to be 10700 Da. In contrast, Matthews *et al*. (1996), using the same GPC method on bark condensed tannins, found the number average MW to be 7500. These results are larger than those obtained in the present study due to the possibility of derivatisation side reactions or the use of polystyrene standards for the calibration curve (Bae *et al*., 1994).

Yazaki and Hillis (1980) using ultrafiltration methods found that more than 50% of the *P. radiata* bark condensed tannins extracted in water had a molecular size larger than that of a 12000-14000 molecular weight dextran. The large difference between the MW data reported in the present research and that of Yazaki and Hillis (1980) is likely due to the self-aggregation of their condensed tannins in aqueous solutions so they are detected as apparent large MW condensed tannins. This aggregation is due to intermolecular interactions of hydroxyl groups.

Czochanska *et al*. (1979) estimated by ^{13}C NMR that the mean molecular weight of *P. radiata* bark condensed tannin extracted with hot water was approximately 1740 Da, while Kininmonth and Whitehouse (1991) using NMR and GPC reported the average molecular weight to range between 1800 and 2600 Da. These results are comparable with the results obtained through this research by depolymerisation/HPLC (2392 Da) and GPC (2139 Da) (Table 5.5).

Jerez *et al*. (2007) found through acid-catalysed depolymerisation/HPLC that *P. radiata* bark condensed tannins extracted with ethanol had a mDP of 5.3 (MW 1526). All the extraction solvents used in this research have extracted condensed tannins with a larger mDP. Matthews *et al.* (1996) extracted *P. radiata* bark condensed tannins with a mDP of 7.8 using methanol/water, which is similar to the mDP 8.3 achieved in this study.

CHAPTER 6: APPLICATION OF ANALYTICAL METHODS TO DIFFERENT BARK SAMPLES

6.1 *PINUS RADIATA* SAMPLES

Pinus radiata bark samples from different geographical locations and tree heights were analysed using the techniques described in previous chapters. Bark samples were sourced from two different locations in New Zealand; Waimate (W) in South Canterbury and Golden Downs (GD) in Nelson. Bark samples from these locations were collected from 0, 10 and 20 metres up the trees relative to ground level. Bark was collected from different geographical locations around New Zealand and from different heights up the tree to discover if there was any variation between the extracts.

6.1.1 EXTRACTION, SEPARATION AND IDENTIFICATION

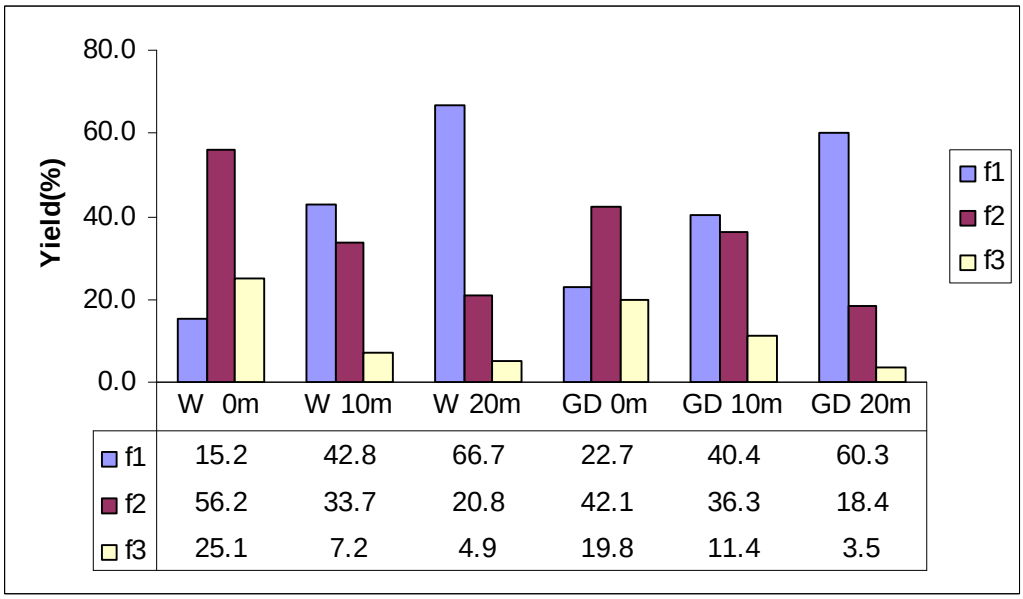
The bark samples from Waimate and Golden Downs were extracted using the hot water (HW) method (see Chapter 2.5) and yields are reported in Table 6.1. Extraction yields ranged from 15.6% to 24.2%. The Waimate yields decreased as bark from higher up the tree was extracted. The Golden Downs yields were similar for all the tree heights (ranging between 17 and 18%). These yields were slightly higher than the yield obtained from the HW extraction of the standard *P. radiata* bark sample, which was 16.5%. This is most likely due to the standard *P. radiata* bark being subject to a pre-extraction with hexane and toluene.

Table 6.1 Percentage yields of HW extracts from bark from different geographical locations and tree heights

Sample	**Percentage Yield (%)[a]**
Waimate 0 m	24.2
Waimate 10 m	18.3
Waimate 20 m	15.6
Golden Downs 0 m	16.9
Golden Downs 10 m	17.7
Golden Downs 20 m	17.2

[a] w/w of dry bark

The crude condensed tannin samples also contained significant amounts of carbohydrates and flavonoids. Utilising the method and gradient described in Chapter 2.6, the carbohydrates and flavonoids (fraction f1) were separated from the condensed tannins (fraction f2f3). The following yields were obtained (Figure 6.1).



	W 0m	W 10m	W 20m	GD 0m	GD 10m	GD 20m
f1	15.2	42.8	66.7	22.7	40.4	60.3
f2	56.2	33.7	20.8	42.1	36.3	18.4
f3	25.1	7.2	4.9	19.8	11.4	3.5

Figure 6.1 Fraction yields of Golden Downs and Waimate bark extracts from tree heights of 0, 10 and 20 metres

The f1 yield increased by approximately 20% at each sample height as bark is obtained from higher up the tree. This indicated that the bark contained a larger concentration of flavonoids and/or carbohydrates higher up the tree. Three hypotheses for the f1 yield increasing are, firstly there could be an increase in flavonoids higher up the tree. In younger bark the flavonoids are less likely to have reacted to form condensed tannins compared with older bark. Secondly there could be an increase in the concentration of carbohydrates higher up the tree and thirdly there could be a increase in both carbohydrates and flavonoids higher up the tree. There was only a small difference between the yields of fractions from the same tree heights at the different geographical locations (Figure 6.1).

Yields of material unrecoverable from the Sephadex LH-20 column (assigned to high molecular weight tannins and phlobaphenes) varied between samples. The

percentage yield of unrecoverable material ranged between 3.7 and 17.8%. There was no trend of unrecoverable material between the different tree heights or locations. The unrecoverable material from the standard HW *P. radiata* extract described in Chapter 3.3 was 12.1%.

The Folin-Ciocalteu assay results (Table 6.2) gave an indication of the total amount of phenolic (condensed tannins and flavonoids) material in each fraction. The results indicated that all the f1 fractions had low total phenolic (%) (between 0 and 4.8%) content, indicating there was very little condensed tannins and/or flavonoids in these fractions. The f2 and f3 fractions had high total phenolic (%) contents, 61-98% (w/w) and 71-90% (w/w) respectively, showing that they had high concentrations of condensed tannins.

These results are consistent with the literature, that is methanol/water will elute mainly carbohydrates and flavonoids and acetone/water will elute condensed tannins from a Sephadex LH-20 column (Kennedy and Taylor, 2003). The Folin-Ciocalteu assay was not carried out on two f3 fractions (W 20 m and GD 20 m) because there was insufficient material to perform the assay accurately. The total phenolic content (%) results of the standard HW *P. radiata* sample were also added to Table 6.2 as a comparison.

Table 6.2 Total phenolic content (%) of fractions from different geographical locations and tree heights

Sample	**f1**	**f2**[a]	**f3**[a]
W 0 m	nd	94.3	80.6
W 10 m	4.8	88.2	70.5
W 20 m	0	60.0	nd
GD 0 m	4.6	67.7	90.2
GD 10 m	0	93.2	80.1
GD 20 m	0	98.1	nd
HW	20.4	96.9	86.0

[a] 95% confidence interval of ± 17.0%, nd- not determined

^{1}H-NMR spectroscopy performed on all the aforementioned fractions helped identify the composition of each sample. As no hexane and toluene pre-extraction was done on these barks, additional peaks to the carbohydrates and condensed tannins between 0 and 2 ppm were observed in the ^{1}H NMR spectra of the crude, f1 and f2f3 fractions. These peaks were possibly due to hydrocarbons such as terpenes and waxes. The estimated carbohydrate content was not found because the hydrocarbons were not included in the estimated carbohydrate calculation (Chapter 2.9).

The ^{1}H NMR spectra of the f1 fractions show that they mainly consist of carbohydrates with a small amount of flavonoids and hydrocarbons. The f2f3 fractions mainly consist of condensed tannins with a very small amount of carbohydrates and hydrocarbons. These results are consistent with the total phenolic content (%) results found through the Folin-Ciocalteu assay. The ^{1}H NMR also showed that the reason for the decrease in the condensed tannin yield from bark higher up a tree is due to the increase in the amount of carbohydrates rather than in the amount of flavonoids.

6.1.2 MALDI-TOF MASS SPECTROSCOPY

MALDI-TOF MS spectra showed very little difference between the f2f3 fractions from different geographical locations and tree heights. MALDI-TOF MS spectra of GD condensed tannins (f2f3 fractions) from the different tree heights are shown in Figure 6.2. All the MALDI-TOF MS spectra showed oligomers ranging from 4-10 units and the tetramer as the most intense peak. The spectra like all the previous *P. radiata* condensed tannin samples (Chapter 4.2) showed a series of peaks 288 Da apart (one catechin/epicatechin group), as well as additional peaks 16 and 32 Da higher (one and two prodelphinidin groups). Because the MALDI-TOF spectra were very similar between samples it was decided that it was not worthwhile performing ESI MS on these samples as the spectra obtained would more than likely show little variation in appearance between samples.

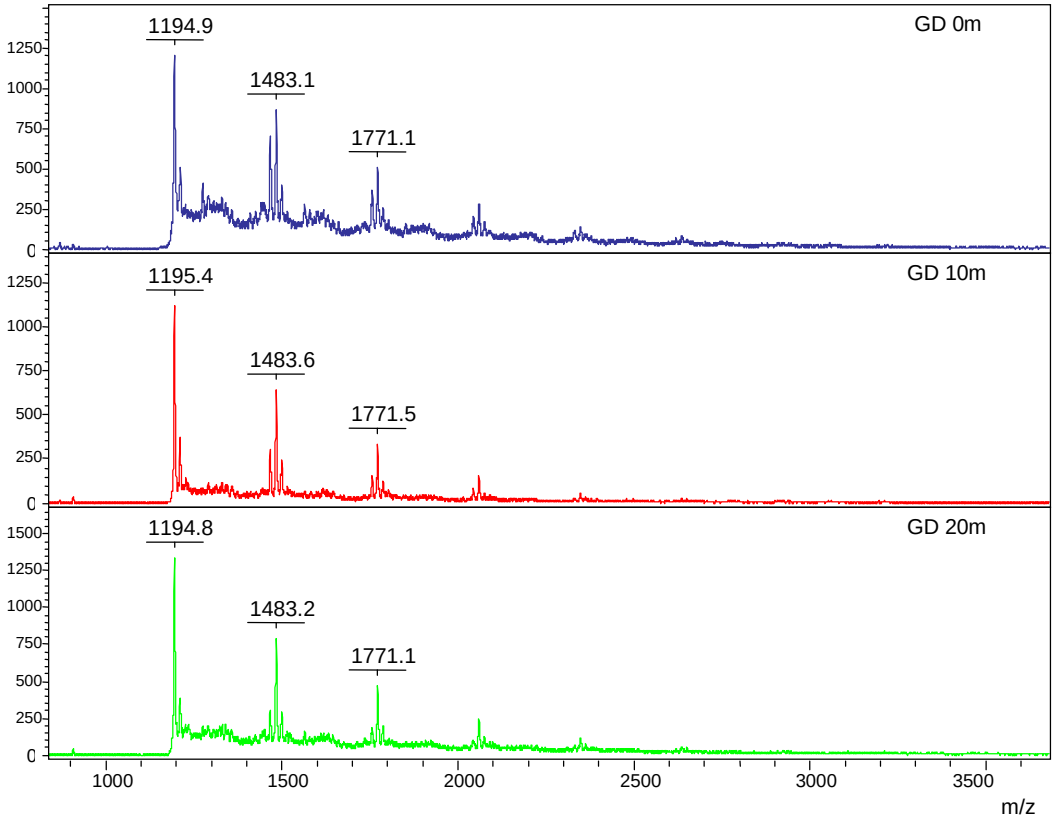


Figure 6.2 MALDI-TOF MS spectra of GD condensed tannins (f2f3 fractions) from 0 m, 10 m and 20 m

6.1.3 DEPOLYMERISATION/HPLC AND GPC

Condensed tannins extracted from bark (that was sourced from different geographical locations and tree heights) were presumed to be of the same composition as the standard *P. radiata* HW f2f3 condensed tannins (*i.e.* with catechin as the only terminal unit and catechin, epicatechin and epigallocatechin as extension units) as there were no additional peaks in the depolymerisation/HPLC chromatograms.

The mDP (calculated through acid-catalysed depolymerisation/HPLC (Table 6.3)) decreased as condensed tannins were extracted from higher up a tree. The mDP of Waimate condensed tannins ranged from 6.6 at 0 m to 5.3 at 20 m. The mDP of Golden Downs condensed tannins ranged from 7.7 at 0 m to 5.5 at 20 m. This is what one might expect as bark higher up a tree is younger and has not had as much time to form condensed tannins as in the case of older bark nearer the base

of the tree. The standard *P. radiata* HW f2f3 sample had a significantly higher mDP compared with the mDP of condensed tannins from different geographical locations and tree heights (excluding GD 0 m).

Condensed tannins from Golden Downs appeared to have a lower ratio of prodelphinidins to procyanidins (0.10) compared with the Waimate condensed tannins (0.15) however when the experimental error was considered the values were within the 95% confidence limits of each other. There was no difference between the ratios of prodelphinidins to procyanidins from different tree heights. The standard *P. radiata* HW f2f3 sample had a higher ratio of prodelphinidins to procyanidins compared with condensed tannins from different geographical locations and tree heights (Table 6.3).

Table 6.3 Results of depolymerisation/HPLC of condensed tannins from bark sourced from different geographical locations and tree heights

Sample	mDP by depoly/HPLC[a]	Mean Molecular Weight (Da)[a]	Ratio of PD/PC[a]
W 0 m	6.6	1909	0.152
W 10 m	6.1	1754	0.152
W 20 m	5.3	1529	0.150
GD 0 m	7.7	2209	0.117
GD 10 m	6.3	1817	0.104
GD 20 m	5.5	1581	0.107
HW f2f3	7.6	2190	0.221

[a] 95% confidence interval mDP = ± 0.37, mean MW = ± 107 Da and PD/PC ratio = ± 0.03

GPC analysis of the condensed tannin (f2f3) samples (Appendix 4.1) obtained from different geographical locations and tree heights (Table 6.4) showed no noteworthy difference between average MW's and polydispersities calculated for the samples. The number average MW (M_n) of the W condensed tannins were between 1000 and 1100 Da, while the M_n of the GD condensed tannins were between 900 and 1260 Da. The weight average MW ranged from approximately 2700 to 3300 Da for the GD condensed tannins and from 2900 to 3700 Da for the

W condensed tannins. Polydispersities of the condensed tannin samples were similar (all within the 95% confidence interval) and ranged between 2.62 and 3.46 and appear to be generally lower than the standard *P. radiata* HW f2f3 sample from Chapter 5. Like the depolymerisation/HPLC results the GPC results showed that condensed tannins from different locations and tree heights were lower than that of the standard *P. radiata* bark sample.

Table 6.4 Average molecular weights and polydispersity of condensed tannin samples obtained from bark from different geographical locations and tree heights (including flavonoids)

Sample	Number average molecular weight (M_n)[a]	Weight average molecular weight (M_w)[a]	Poly dispersity[a]
W 0 m	1068	3693	3.46
W 10 m	1001	2893	2.89
W 20 m	1074	3402	3.17
GD 0 m	914	2686	2.94
GD 10 m	1264	3316	2.62
GD 20 m	993	2986	3.01
HW f2f3	1292	5150	3.99

[a] 95% confidence interval for Mn = ± 70 Da, Mw = ± 553 Da, Pplydispersity = ± 0.48

When comparing the depolymerisation/HPLC and GPC results, the flavonoids (except the catechin/epicatechin) were not included in the GPC calculations (Table 6.5) for the reasons discussed in Chapter 5. This made the number average molecular weights calculated by GPC comparable with those calculated by depolymerisation/HPLC. The GD 0 m sample was the only sample which did not have a good agreement between the two techniques (23% difference). For the remainder of the condensed tannins, the depolymerisation/HPLC and GPC results agreed within 8% of each other (inside error limits).

Excluding flavonoids (mainly taxifolin) from the GPC calculations meant the number average molecular weight for the Waimate condensed tannin samples

ranged from approximately 1900 Da at 0 m to 1650 Da at 20 m. Processing the data in this way meant that the Waimate GPC results agreed with the depolymerisation/HPLC results and showed a decrease in the molecular weight of the condensed tannin as you go higher up the tree. The Golden Downs GPC results showed no trend between condensed tannins extracted from bark from different tree heights.

Table 6.5 Comparison of depolymerisation/HPLC and GPC results of condensed tannins from different geographical locations and tree heights, excluding flavonoid monomers (*e.g.* taxifolin) except catechin/epicatechin

Sample	**MW by depoly/HPLC[a]**	**Number average molecular weight (GPC)[a]**	**Weight average molecular weight (GPC)[a]**
W 0 m	1909	1908	5097
W 10 m	1754	1685	4123
W 20 m	1529	1663	4179
GD 0 m	2209	1794	4087
GD 10 m	1817	1956	4256
GD 20 m	1581	1602	3969
HW (f2f3)	2392	2139	5435

[a] 95% confidence interval for deploy/HPLC MW = ± 107 Da, Mn = ± 70 Da, Mw = ± 553 Da

Overall the analysis of condensed tannins isolated from bark from different geographical locations showed that there is no significant difference between condensed tannins from two South Island locations (Nelson and South Canterbury), but they were different to the Central North Island standard sample. Analysis of condensed tannins from different tree heights (0, 10 and 20 m) showed that, in the case of the Waimate samples, the MW of the condensed tannins decreases as you go higher up the tree.

6.2 NATIVE SPECIES SAMPLES

Bark from three New Zealand native species were analysed *Dacrydium cupressinum* – (Podocarpaceae), commonly known as rimu (R), *Podocarpus totara* – (Podocarpaceae), commonly known as totara (T) and *Agathis australis* – (Araucariaceae), commonly known as kauri (K). These native trees were selected for bark analysis because they are species which have been investigated by Scion with respect to their commercial viability.

6.2.1 EXTRACTION, SEPARATION AND IDENTIFICATION

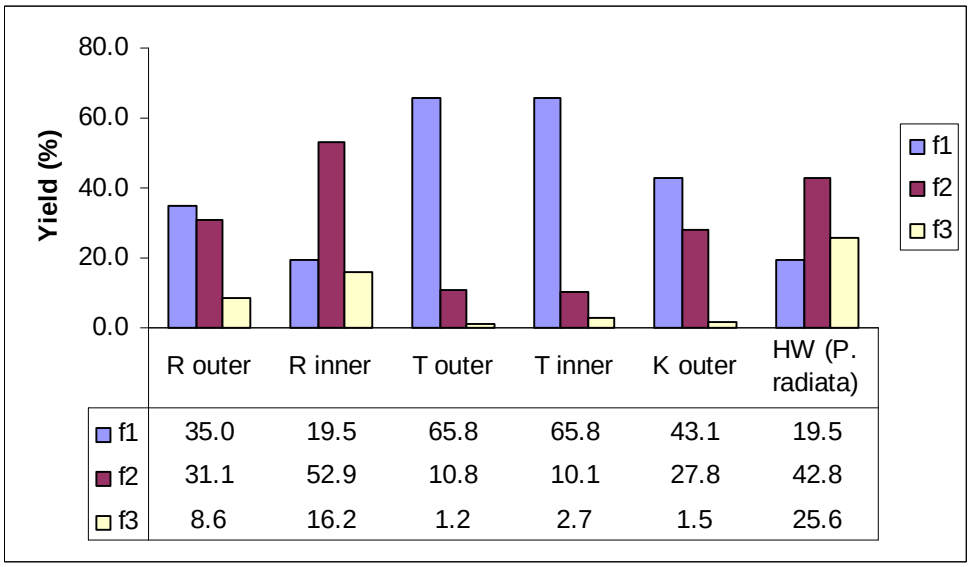
Extraction percentage yields of the native bark samples using the HW method (Chapter 2.5) are reported in Table 6.6. Rimu inner bark contained a large amount of extractives (13.7%) compared to the other native samples, which ranged between 1.3 and 3.0%. All the native barks had small yields of extractives compared to *P. radiata* bark. The reason for the large difference between the rimu inner and outer yields is unknown and further research is needed to discover why this occurred.

Table 6.6 Percentage yields of HW extractives from different native bark samples

Sample	**Percentage Yield (%)[a]**
Rimu Outer	2.5
Rimu Inner	13.7
Totara Outer	2.0
Totara Inner	1.3
Kauri Outer	3.0

[a] w/w of dry bark

Like the *P. radiata* samples the crude condensed tannins from the native samples were expected to contain large amounts of carbohydrates and flavonoids which had to be separated from the condensed tannins to obtain pure material. The percentage yields obtained for each fraction obtained by Sephadex LH-20 chromatography (Section 2.6) are reported in Figure 6.3. The HW *P. radiata* sample is presented as a comparison for the native samples.



	R outer	R inner	T outer	T inner	K outer	HW (P. radiata)
f1	35.0	19.5	65.8	65.8	43.1	19.5
f2	31.1	52.9	10.8	10.1	27.8	42.8
f3	8.6	16.2	1.2	2.7	1.5	25.6

Figure 6.3 Fractionated yields of native barks

The fraction yields of the extractives varied between the native species. Totara inner and outer bark samples had very high f1 yields (both 66%) and low f2 (~10.5%) and f3 yields (~2%) compared with the other species. The combined yield for the rimu inner fractions f2 and f3 (69%) was comparable to the yield for the *P. radiata* f2 and f3 fractions (68%). Rimu inner had a considerably larger f2 and f3 yield compared with the other native bark samples. The fraction yields for the kauri outer sample were comparable with the totara fractions with a large f1 yield (43%) and smaller f2 (28%) and f3 yields (2%).

Other than rimu inner all the native samples had a very high yield of unrecoverable material which ranged between 21.4 and 27.8%. The rimu inner bark unrecoverable material yield of 11% is comparable with the unrecoverable material of the standard *P. radiata* HW sample (12%). The reason for the high unrecoverable yields is unknown and was not investigated further as it was not an integral part of this research.

The total phenolics content (%) for each fraction are shown in Table 6.7. The totara fractions analysed (f1 and f2 (inner and outer)) had low total phenolic contents. This indicated that the totara bark contained very little condensed tannin material. The total phenolic (%) results for the kauri outer sample were similar to that of the totara fractions with the f1 and f2 fractions having low total phenolic

content. The total phenolic (%) contents determined for the rimu fractions was similar to those observed for the *P. radiata* HW sample (Table 6.7). The f1 yields for both the inner and outer samples were low and the f2 and f3 fractions were high, indicating fraction f2 and f3 had a high concentration of condensed tannins.

Table 6.7 Total phenolics (%) of fractions from different native bark samples

Sample	f1	f2[a]	f3[a]
R outer	3.8	69.5	nd
R inner	2.3	81.6	76.8
T outer	2.0	34.4	nd
T inner	0.0	nd	nd
K outer	2.6	19.2	nd
HW (*P. radiata*)	20.4	96.9	86.0

[a] 95% confidence interval of ± 17.0%, nd- not determined

^{1}H-NMR spectra of totara inner f1 and combined f2f3 fractions are shown in Figure 6.4 (totara outer spectra were very similar). The spectra of the f1 totara fraction showed that it consisted of mainly carbohydrates with some hydrocarbons (maybe terpenes and resin acids) and a very small amount of aromatic material. The combined f2f3 fraction contained some aromatic and carbohydrate material as well as a large amount of hydrocarbons at 0 to 2 ppm. These hydrocarbons were not observed in the standard *P. radiata* fractions (Chapter 3) because this sample was subjected to pre-extraction with hexane, which would have removed any of this material that was present.

^{1}H-NMR spectroscopy of the kauri fractions (Appendix 4.2) showed that very little condensed tannins were extracted. ^{1}H-NMR spectrum of the f1 fraction showed that it consisted of mainly carbohydrates and some hydrocarbons. The ^{1}H-NMR spectrum of the f2f3 fraction shows it contains of a large amount of hydrocarbons and carbohydrates with only a small amount of aromatic material, possibly condensed tannins.

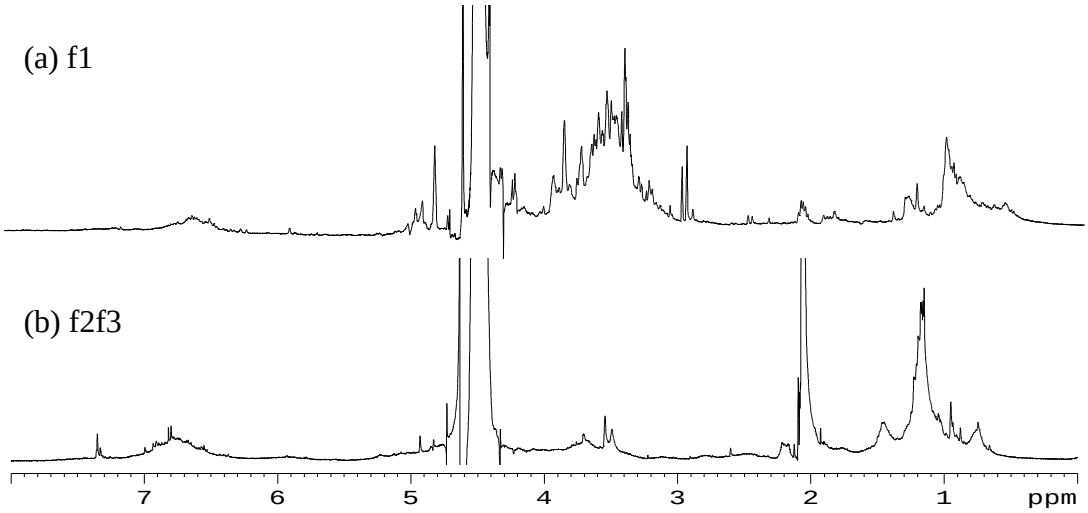


Figure 6.4 ^{1}H NMR spectra of totara inner (a) f1 and (b) f2f3 fractions

The ^{1}H-NMR spectrum of the Rimu inner f1 fraction (not shown) showed it contained mainly carbohydrates and hydrocarbons with a little condensed tannin, while the spectrum of the f2f3 fraction (Figure 6.5) indicated that mainly condensed tannins with some hydrocarbons were present. The rimu samples were shown through ^{1}H-NMR spectroscopy to be the only native samples to contain a significant amount of condensed tannins.

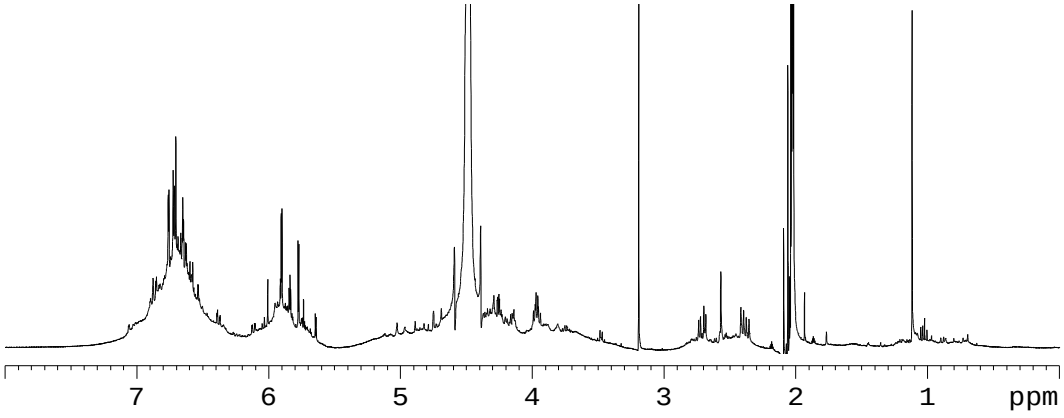


Figure 6.5 ^{1}H-NMR spectrum of rimu inner f2f3 fraction

The ^{1}H-NMR spectra of the rimu f2f3 fractions shows that the condensed tannin peaks were well resolved compared to the *P. radiata* f2f3 fractions. This may indicate that the rimu condensed tannins are not as polydisperse as the *P. radiata* condensed tannins or that there are more monomers present (discussed further in Chapter 6.2.2).

6.2.2 MALDI-TOF AND ESI MASS SPECTROSCOPY

As discussed in Chapter 6.2.1 only rimu inner and outer extracts contain significant amounts of condensed tannins. Therefore as expected MALDI-TOF MS analysis of the totara inner, totara outer and kauri outer samples resulted in spectra with no resolved condensed tannin peaks. MALDI-TOF MS analysis of the rimu outer f2f3 fraction resulted in a spectrum with a poor signal to noise ratio and therefore was not included. In contrast, MALDI-TOF MS analysis of rimu inner f2f3 fraction resulted in very good spectra (Figure 6.6) with resolved peaks between DP 4 and DP 11. The increase in intensity of the base line after the matrix suppression cut off at 1000 Da was also small when analysing this sample, which may indicate that the rimu inner condensed tannins are not as polydisperse as the *P. radiata* condensed tannins. This arises because the base line intensity after the matrix suppression cut off is reported to decrease with decreased polydispersity (McEwen *et al*., 1997).

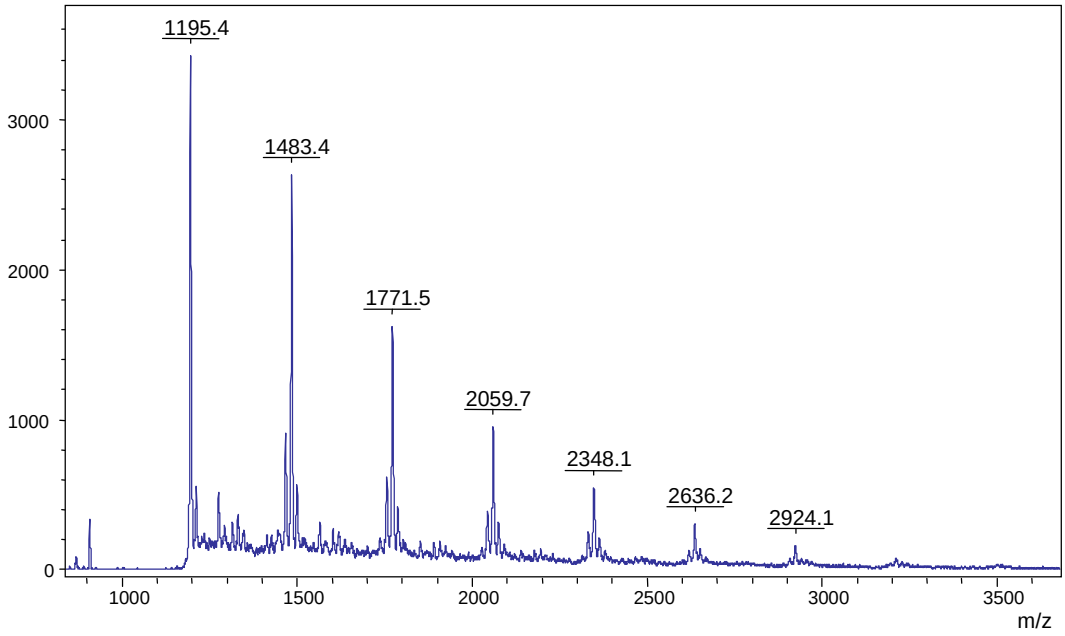


Figure 6.6 MALDI-TOF MS spectra of rimu inner fraction f2f3 condensed tannins

ESI MS of the rimu inner sample resulted in a spectrum of condensed tannin ions ranging from DP 1-4, with the most intense peak being the dimer (Appendix 4.3). ESI MS also indicated that the major flavonoid present with the rimu condensed tannins is catechin or epicatechin.

6.2.3 DEPOLYMERISATION/LCMS (AND HPLC) AND GPC

As indicated through ^{1}H NMR spectroscopy (Chapter 6.2.1) only the rimu bark samples contained significant amounts of condensed tannin, therefore they were the only native sample to be analysed by depolymerisation/LCMS and depolymerisation/HPLC. Depolymerisation/LCMS (Figure 6.7) of the rimu inner sample gave an indication of what products were produced from the acid-catalysed depolymerisation reaction with excess phloroglucinol. The only difference between the *P. radiata* samples and the rimu samples was that there was a large peak at R_T~ 34.8 minutes. This peak had a *m/z* of 291 and was proven (through the analysis of pure epicatechin (purchased from Sigma) run as a standard) to be depolymerised epicatechin or a epicatechin monomer. Epicatechin now had to be taken into account as a terminal group when computing the mDP for the rimu inner condensed tannins.

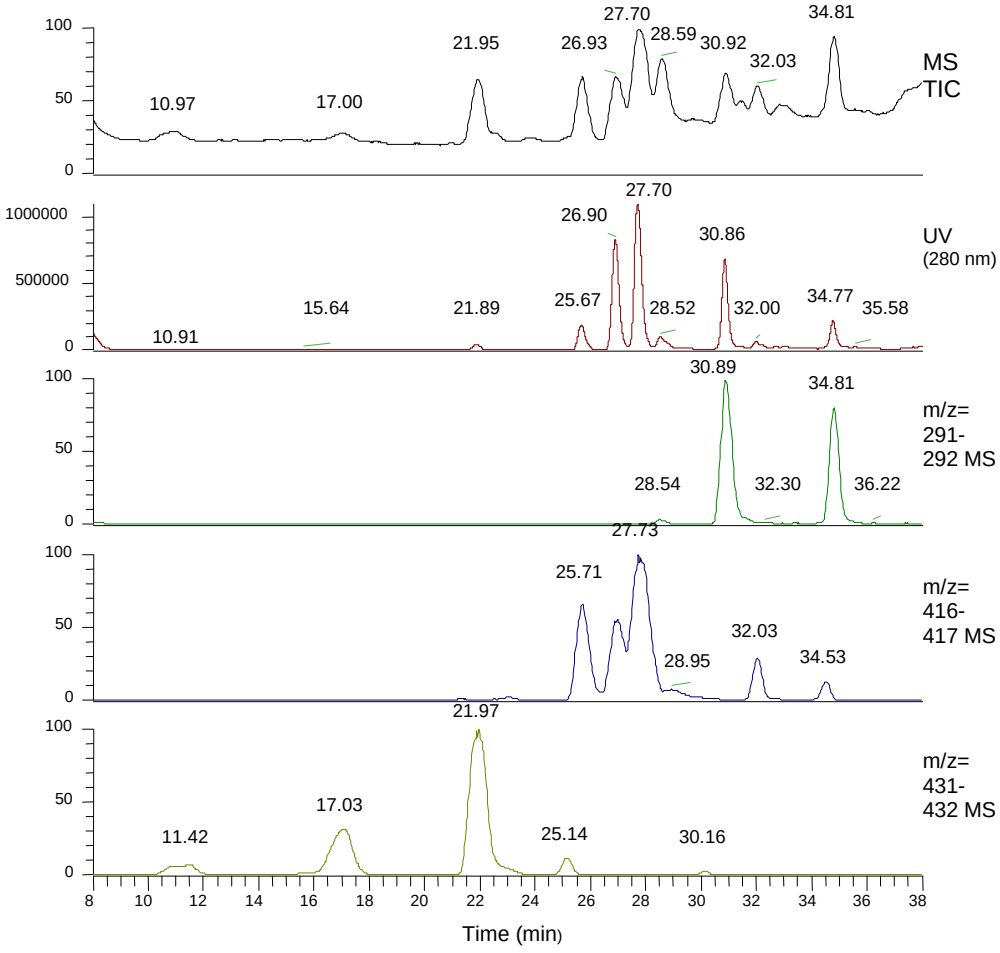


Figure 6.7 LC-MS (TIC and SIC) and UV chromatogram of acid-catalysed depolymerisation products from Rimu condensed tannins

Depolymerisation/LCMS indicates that in rimu condensed tannins, catechin and epicatechin occur as both extension and terminal units. Catechin and epicatechin are the only observed terminal units (unlike *P. radiata*, which only had catechin as terminal units). Epigallocatechin/gallocatechin (prodelphinidins) occur as extension units in the polymeric proanthocyanidins. No evidence of galloylation at C3 (see Chapter 5.1 for explanation) was observed in Rimu condensed tannins.

A comparison of the *P. radiata* and rimu inner depolymerisation/HPLC results is shown on Table 6.8. Taking epicatechin as a terminal group into account the mDP for rimu inner was found to be 4.5. Hence compared with *P. radiata* condensed tannins (mDP 8) the mDP is significantly smaller. Rimu inner condensed tannins had a PD/PC ratio of 0.080 which shows that there were very little prodelphinidins present, especially compared to *P. radiata* which had a PD/PC ratio of 0.221. The ratio of epicatechin to catechin terminal units was 0.43 showing that catechin was the major terminal unit and epicatechin was the minor terminal unit.

Table 6.8 Comparison of depolymerisation/HPLC results for *P. radiata* and rimu inner condensed tannin samples

Sample	mDP deploy/HPLC[a]	Mean Molecular Weight[a]	Ratio of PD/PC[a]
Rimu inner	4.5	1296	0.080
P. radiata (HWf2f3)	7.6	2190	0.221

[a] 95% confidence interval of mDP = ± 0.37, mean MW = ± 107 Da and PD/PC ratio = ± 0.03

GPC performed on rimu inner and outer f2f3 samples are compared with *P. radiata* HW f2f3 in Figure 6.8. The chromatogram agrees with the ESI results that the main flavonoid present in the rimu condensed tannin samples is catechin or epicatechin, compared with taxifolin in *P. radiata* condensed tannin samples. The lower molecular weight condensed tannins in the rimu inner sample were well resolved with monomers, dimers, trimers and tetramers clearly evident.

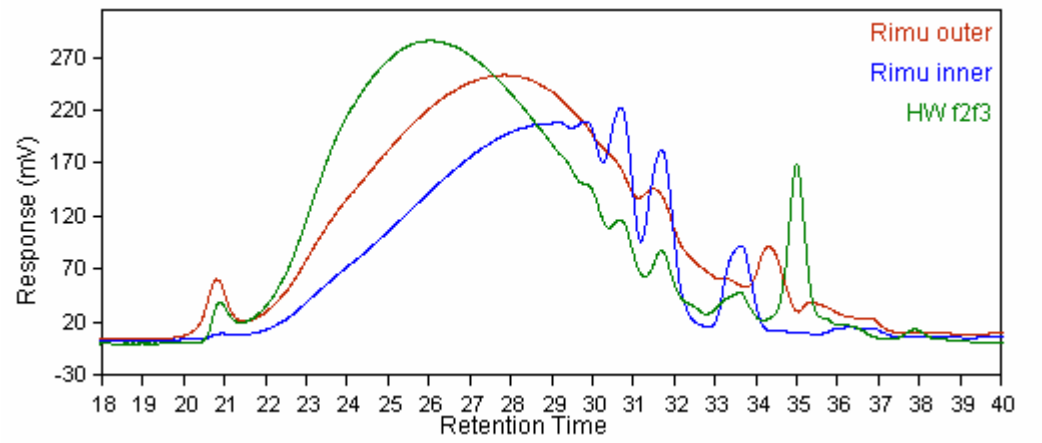


Figure 6.8 GPC chromatogram of rimu inner, rimu outer and HW f2f3 samples

Comparison of the GPC average MW's with the depolymerisation/HPLC calculations (Table 6.9) showed there is a very good agreement (within experimental error) between the number average molecular weight by GPC (1299 Da) and the molecular weight by depolymerisation/HPLC (1296 Da). The weight average MW of rimu inner and outer condensed tannins was 3300 and 4000 Da respectively. These values were significantly lower than what was observed for the *P. radiata* HW f2f3 standard sample.

Table 6.9 Comparison of depolymerisation/HPLC and GPC results of condensed tannins from rimu inner, rimu outer and *P. radiata* (HW f2f3) bark, excluding flavonoids (*e.g.* taxifolin) except catechin/epicatechin

Sample	MW by Depoly/HPLC[a]	Number average molecular weight (GPC)[a]	Weight average molecular weight (GPC)[a]
Rimu inner	1296	1299	3312
Rimu Outer	nd	1523	3981
HW (f2f3)	2392	2139	5435

[a] 95% confidence interval for deploy/HPLC MW = ± 107 Da, Mn = ± 70 Da, Mw = ± 553 Da

nd- not determined

The rimu outer sample contained on average larger molecular weight condensed tannins compared with the rimu inner sample. This may be because rain has extracted the more water soluble small MW condensed tannins from the outer bark, resulting in a higher percentage of larger condensed tannins or that UV light

exposure has caused the small MW condensed tannins to react to form larger MW condensed tannins. The average MWs for both the rimu inner and outer condensed tannins were outside the 95% confidence interval of the average MWs of the *P. radiata* HW f2f3 sample showing that the rimu samples are of smaller MW compared to the *P. radiata* HW f2f3 condensed tannins.

Plots of the molecular weight distribution and cumulative height versus MW are shown in Figure 6.9, comparing the rimu and *P. radiata* samples. The plots provide additional evidence that the rimu inner sample contained more of the smaller MW condensed tannins than the rimu outer sample and that rimu condensed tannins are generally smaller in size than the *P. radiata* (HW f2f3) condensed tannins. Approximately 50% of the rimu inner condensed tannins are above 2000 Da, while approximately 50% of the *P. radiata* condensed tannins are above 4000 Da (Figure 6.9).

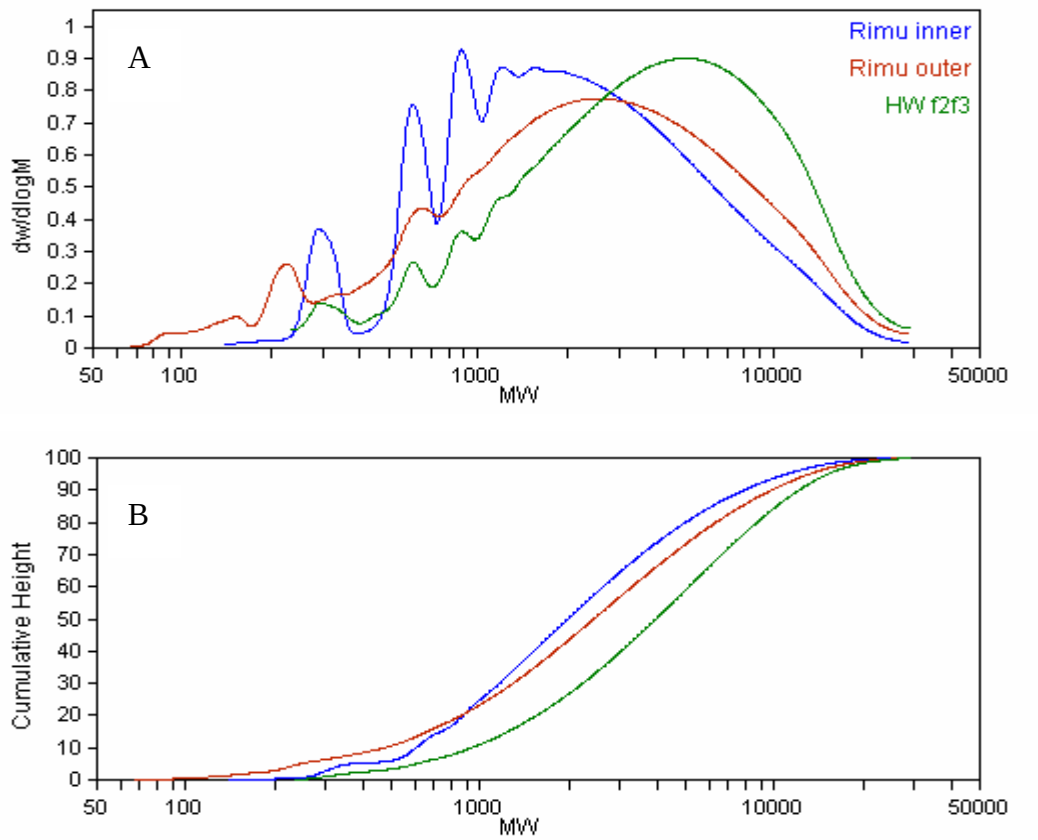


Figure 6.9 Comparison of rimu inner, rimu outer and *P. radiata* condensed tannins through (a) MW distribution and (b) cumulative height versus MW plots

The literature on bark extractives from New Zealand natives is very limited (Webby *et al*., 1987). To the author's knowledge this is the first known research that investigates condensed tannins in the bark of rimu, totara and kauri. Of the native barks analysed, *Dacrydium cupressinum* (rimu) bark was shown to be the only native bark to have significant amount of condensed tannins and these condensed tannins were shown to be of low MW relative to *P. radiata* (approximately 1300 Da). This may be because of the slow maturation of rimu compared to *P. radiata*.

CHAPTER 7: CONCLUSIONS

Research into the use of different solvents to extract *P. radiata* bark condensed tannins showed that each solvent also extracted variable amounts of carbohydrates and flavonoids. Condensed tannins were separated through Sephadex LH-20 chromatography and crude extracts from all the solvents were shown to comprise of approximately 70% condensed tannins. The hot water sulphite/urea extraction extracted the largest amount of crude material.

The use of Sephadex LH-20 (absorption) chromatography utilising a water, methanol and acetone gradient is a good way to achieve molecular weight fractionation of condensed tannins. The condensed tannin separation was broad and there was considerable molecular weight overlap. This research followed a technique that uses standard laboratory equipment in the quantitative separation of condensed tannins for either analytical or preparative purposes. Improvements that could be made to this technique include ways to decrease the time it takes to fractionate the condensed tannins on the column thus making it a less tedious process so it can be routinely used, a more accurate and efficient way of pooling the fractions and a new stationary phase which would decrease the amount of unrecovered material.

The molecular weight fractionation of *P. radiata* condensed tannins has resulted in more information being gained from each of the analytical methodologies used in this research. In the case of MALDI-TOF MS, the detection of high molecular weight condensed tannins (>3500 Da) was improved. There was also an improvement in the detection of condensed tannins of greater than 2300 Da molecular weight by ESI MS. MALDI-TOF MS proved to be better at detecting larger molecular weight condensed tannins as there was no double charging, while ESI MS proved to be better at detecting smaller molecular weight condensed tannins as there is no matrix interference. The improvements in molecular weight detection achieved with MALDI-TOF MS were not sufficient enough to allow complete analysis of the entire molecular weight distributions. Further improvements in MALDI-TOF MS may be possible through further optimisation

of the ionisation process (*i.e.* MALDI-TOF MS ionisation could be improved through research into alternative matrices and optimisation of silver and caesium as enhancement ions which seemed to produce ion clusters in this research, but in other research has shown to increase detection of larger MW condensed tannins).

Research into the construction of a calibration curve for GPC indicates that there is a good correlation between the mDP (found through depolymerisation/HPLC) and the GPC retention time of the molecular weight fractionated condensed tannins. The use of molecular weight fractionated condensed tannins to construct a calibration curve for GPC analysis of condensed tannins was an important step in the analysis, as it has been established that molecular weight GPC standards should be of similar composition to the samples being tested. The construction of this calibration curve coupled with the implementation of a literature GPC method has established a quick and accurate method of analysing the MW of *P. radiata* bark condensed tannin samples.

In agreement with the literature, MALDI-TOF MS, ESI MS, depolymerisation/LCMS, depolymerisation/HPLC and GPC has indicated that *P. radiata* bark condensed tannins are a mixture of proanthocyanidin oligomers, comprised of catechin terminal units and catechin, epicatechin and epigallocatechin/ gallocatechin extension units with a prodelphinidin to procyanidin ratio of approximately 0.2. Taxifolin was shown to be the most abundant flavonoid.

This research also shows that there is little variation in the yields of *P. radiata* bark condensed tannins sampled from different geographical locations around New Zealand. Bark condensed tannins sampled from different tree heights (0 m, 10 m, and 20 m) up a tree showed several trends. As bark was sampled higher up a tree the f1 yield (carbohydrates) increased, while the condensed tannin yield decreased. At one location (Waimate) the mDP of the condensed tannins also decreased higher up the tree the bark was sampled. This research could be expanded through the sampling of more bark samples from different climates *i.e.* northland and southland, or *P. radiata* bark from different countries *e.g.* Chile.

Bark samples from New Zealand native trees (rimu, kauri and totara) showed that Rimu bark was the only native bark to contain significant amounts of condensed tannins. The composition of the rimu condensed tannins was similar to that of *P. radiata* condensed tannins except epicatechin is also observed as a terminal unit. The mDP of rimu condensed tannins (4.5) was lower than that found for *P. radiata* bark condensed tannin. This research could be expanded by sampling and analysing other native bark samples as well as analysing other non-native species to compare how the condensed tannins differ.

This research has increased the body of knowledge on *P. radiata* bark condensed tannins as well as developing techniques and methods (*e.g.* MALDI-TOF MS and GPC) that can be used in the future to analyse condensed tannins. Aspects of this research have been presented as a poster at the 2006 New Zealand Institute of Chemistry conference in Rotorua (see Appendix 5.1).

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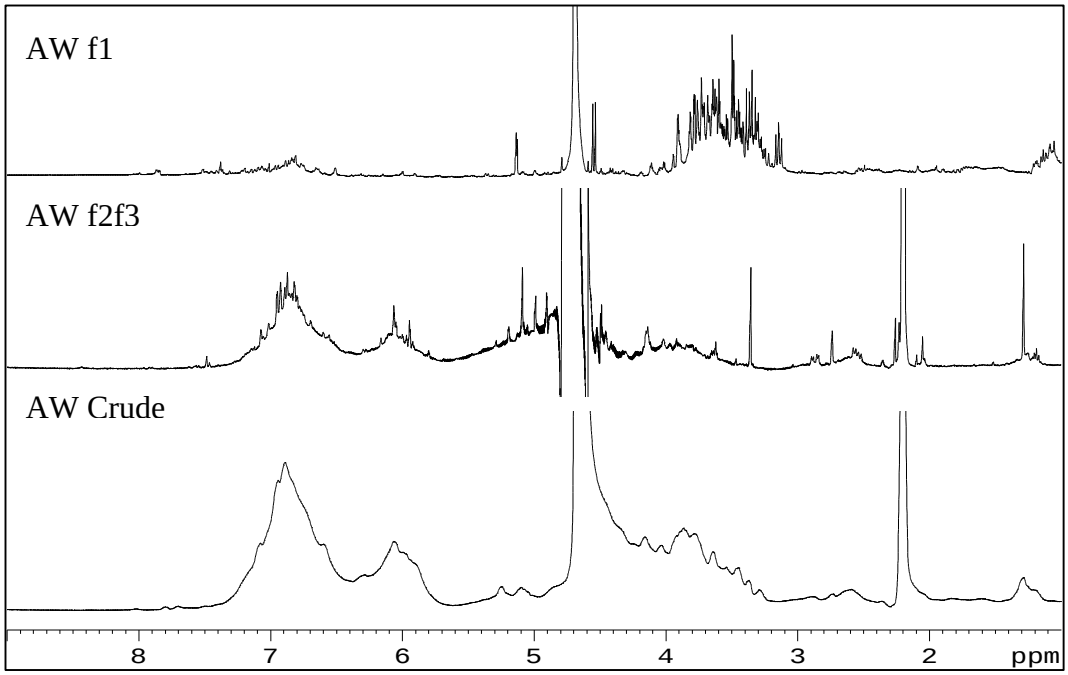
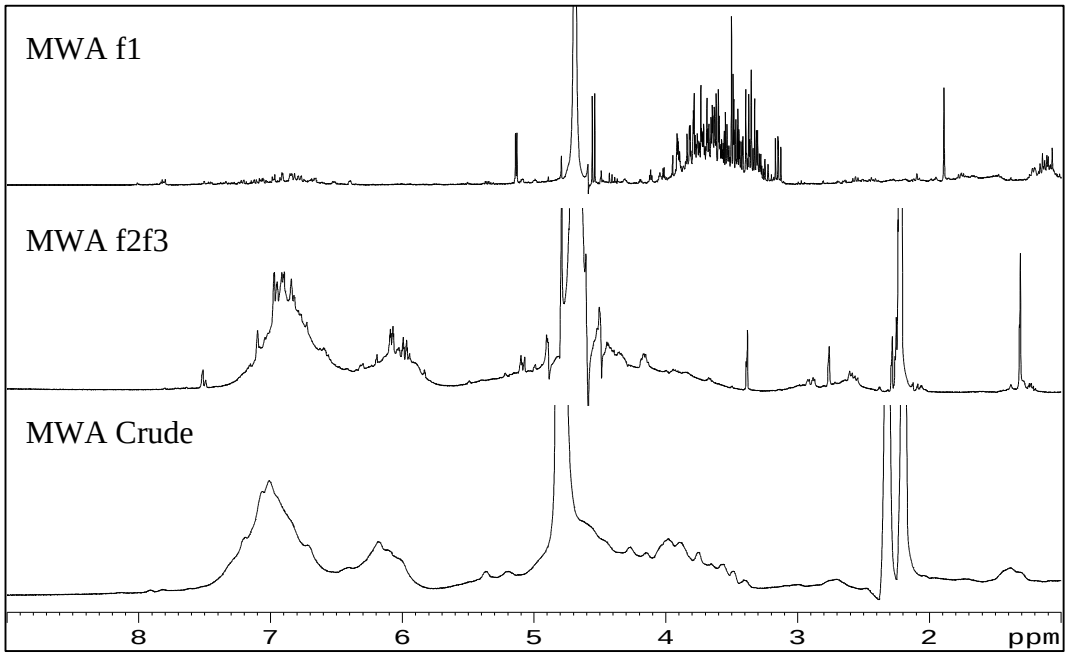
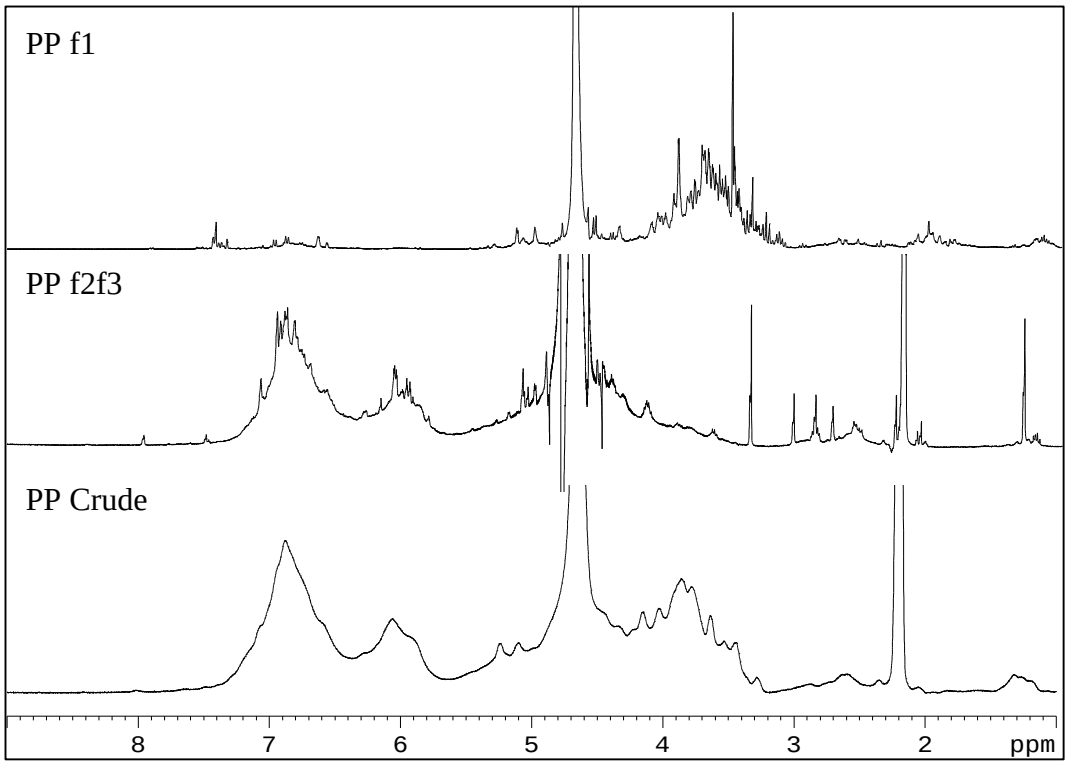
APPENDICES

APPENDIX 1.1: ^{1}H NMR SPECTRA OF FRACTIONS f1, f2f3 AND CRUDE FOR AW, MWA AND PP EXTRACTION

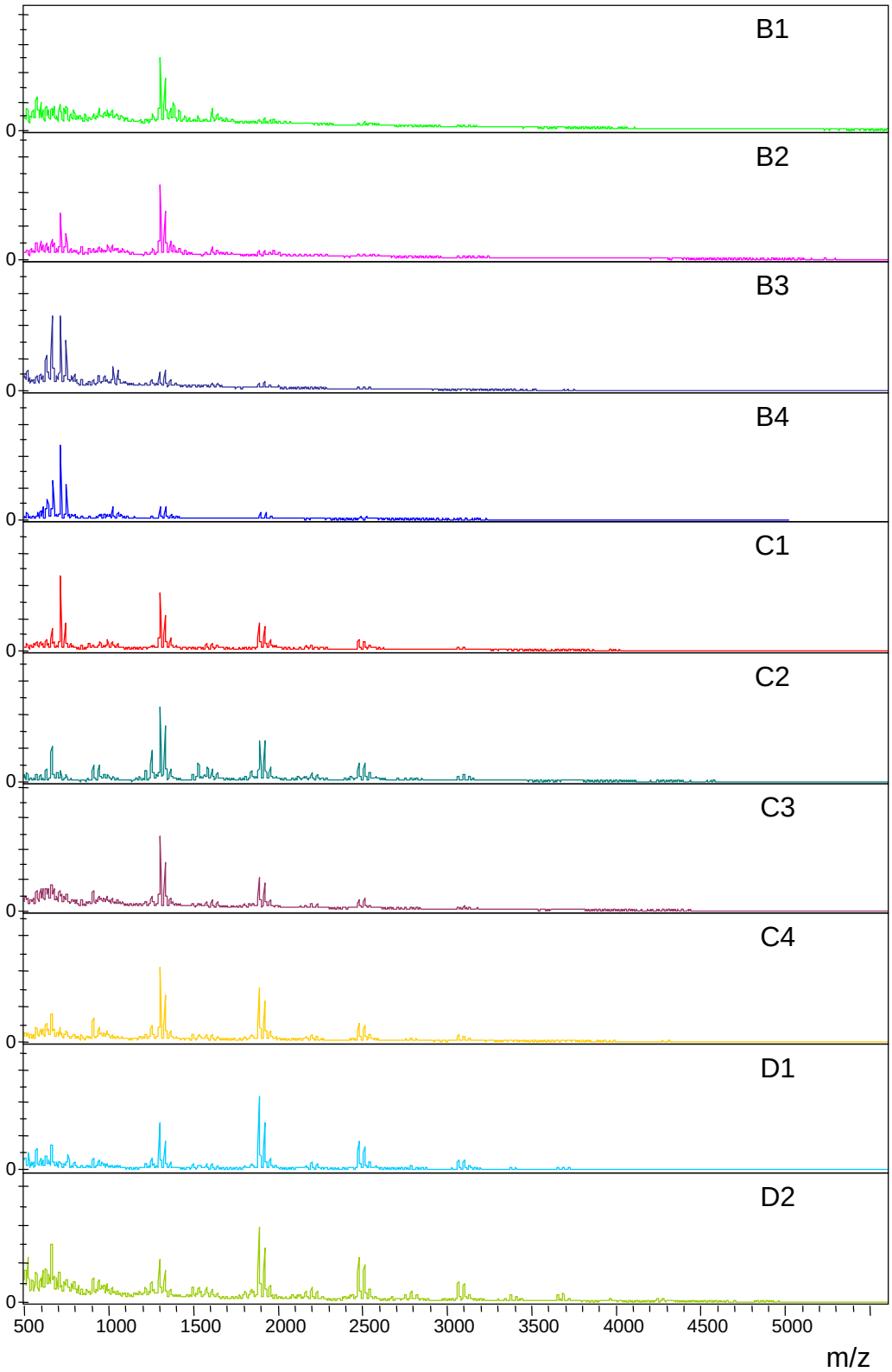


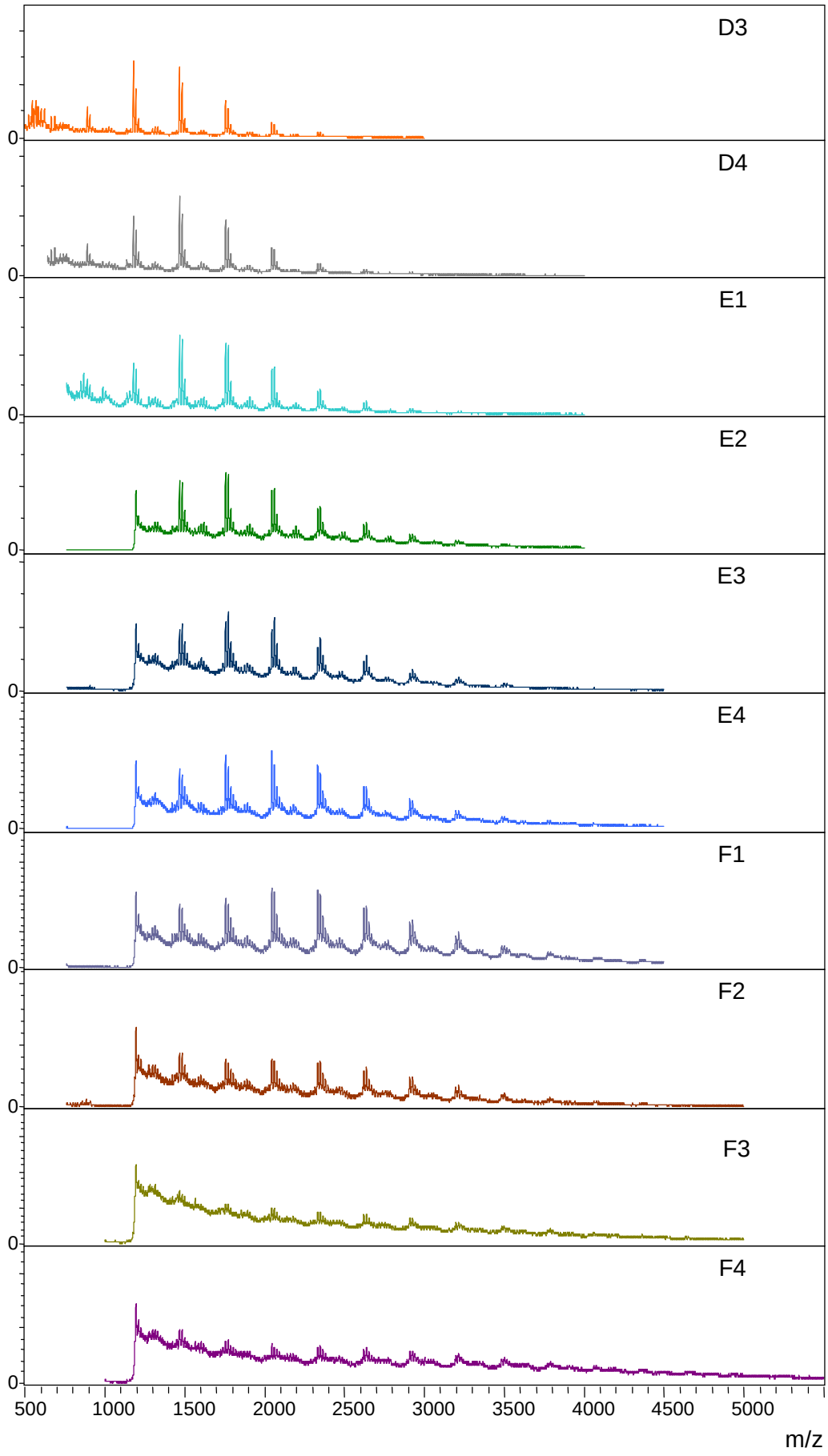



PP f1
PP f2f3
PP Crude
8
7
6
5
4
3
2
ppm

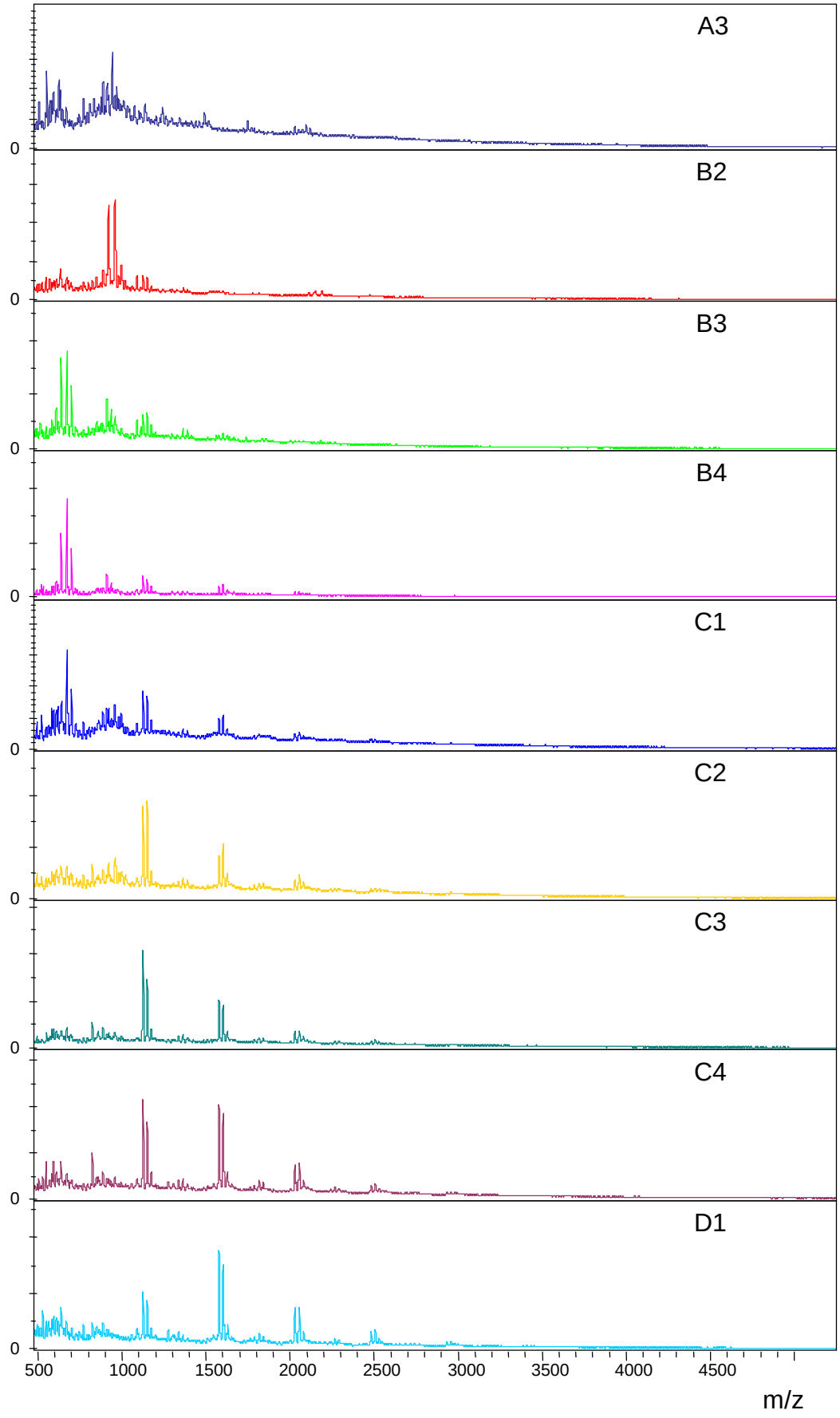
APPENDIX 1.2: MALDI-TOF MS SPECTRA OF FRACTIONATED HW AND MWA CONDENSED TANNINS (NON POOLED)

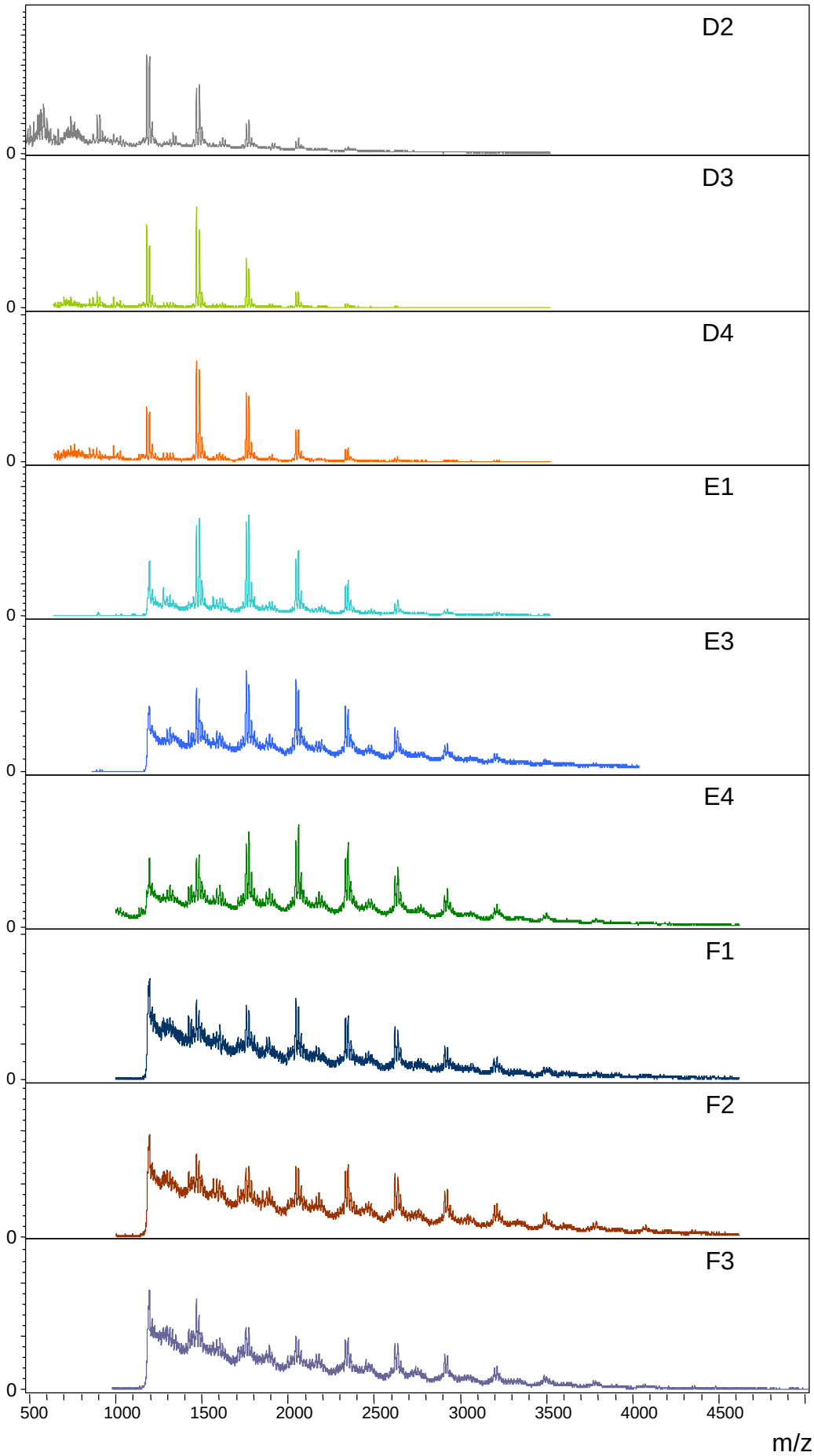
APPENDIX 1.2A: HW NON POOLED FRACTIONS



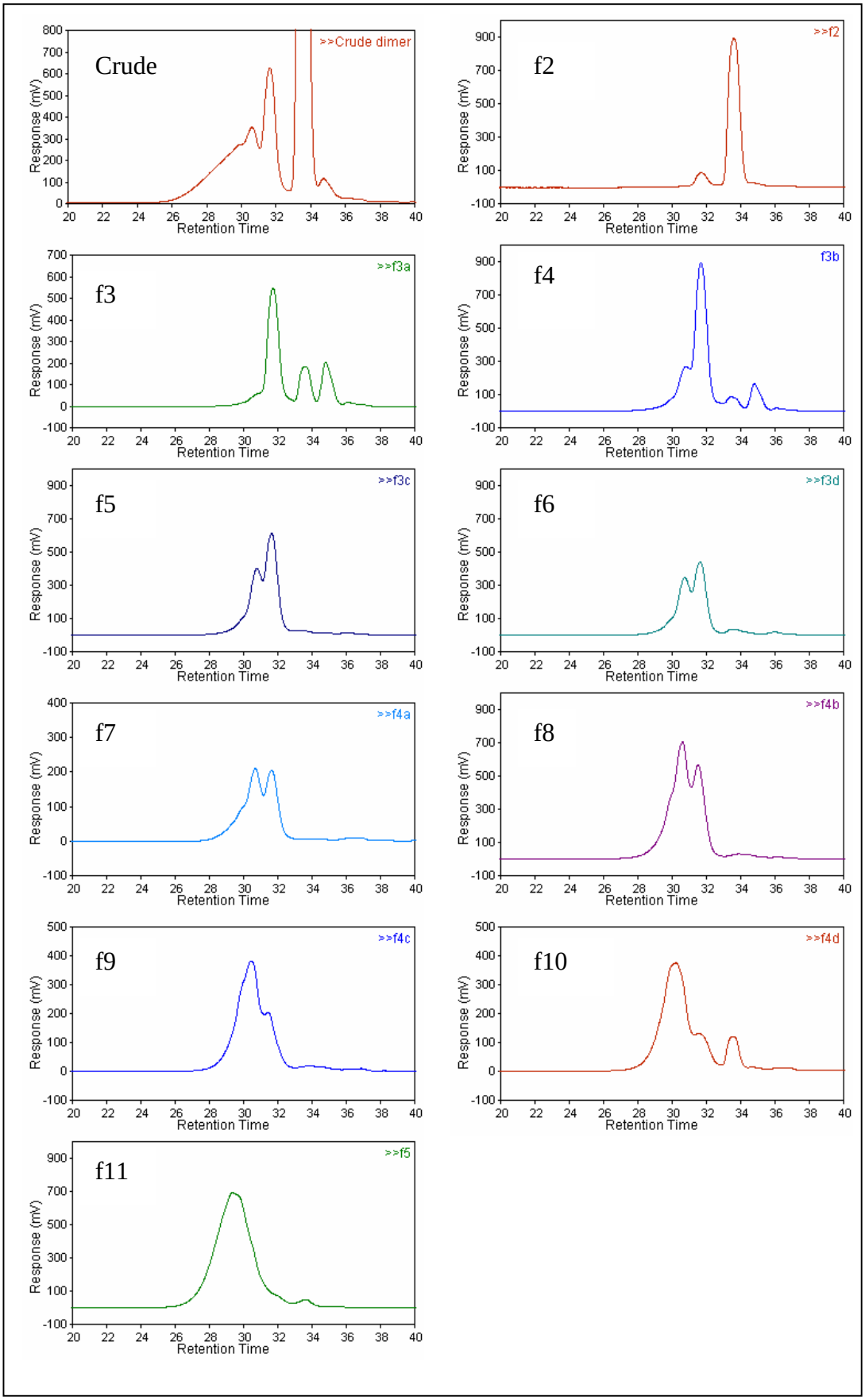

D3
D4
E1
E2
E3
E4
F1
F2
F3
F4
0
500
1000
1500
2000
2500
3000
3500
4000
4500
5000
m/z

APPENDIX 1.2B: MWA NONPOOLED FRACTIONS

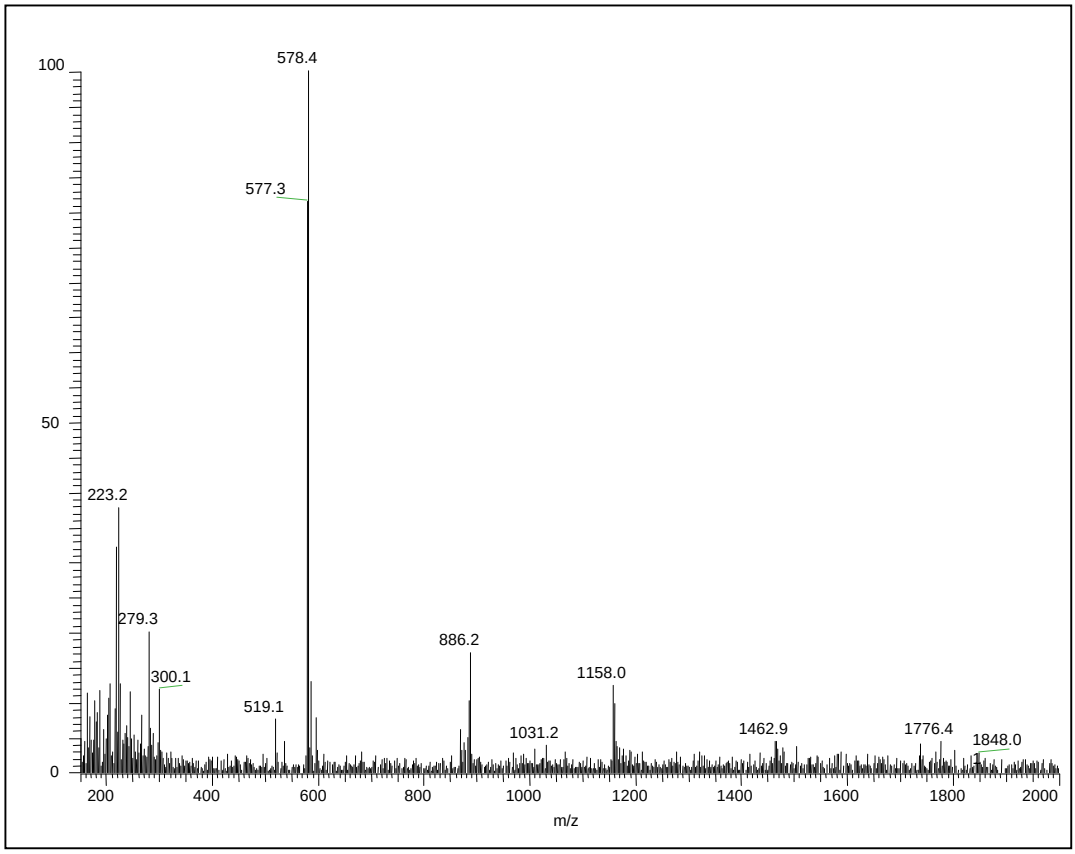



D2
D3
D4
E1
E3
E4
F1
F2
F3
0
500
1000
1500
2000
2500
3000
3500
4000
4500
m/z

APPENDIX 1.3: GPC CHROMATOGRAMS OF DIMER FRACTIONS

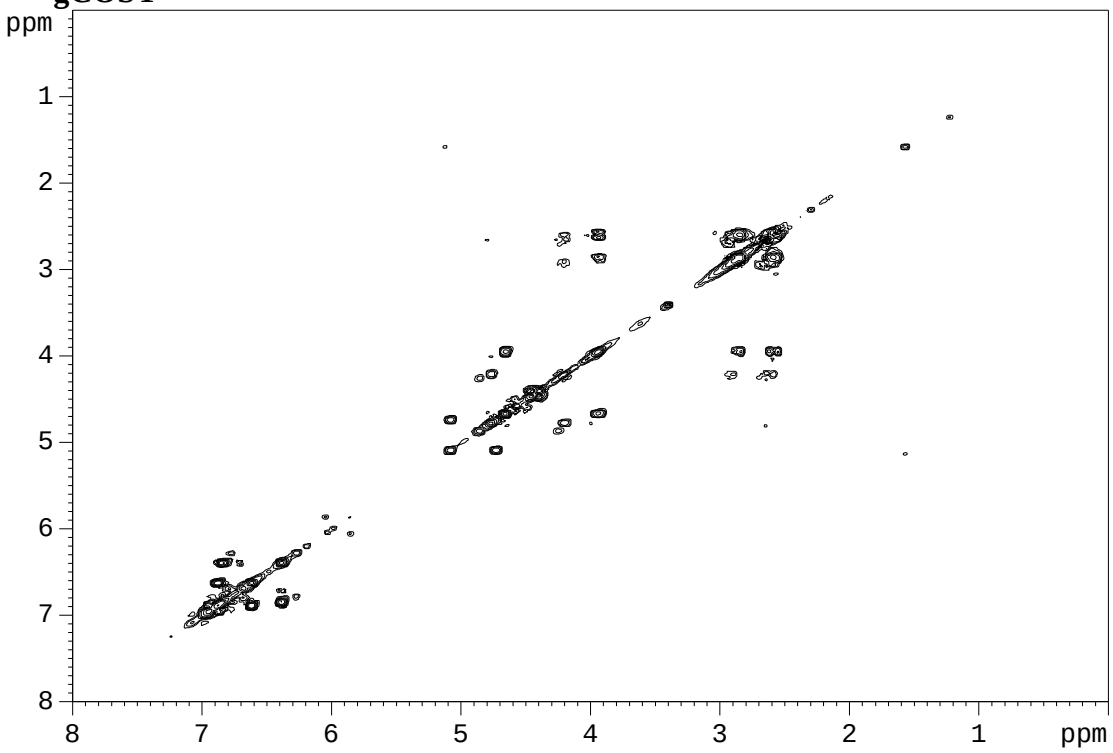


APPENDIX 1.4: ESI SPECTRUM OF DIMER FRACTION f4

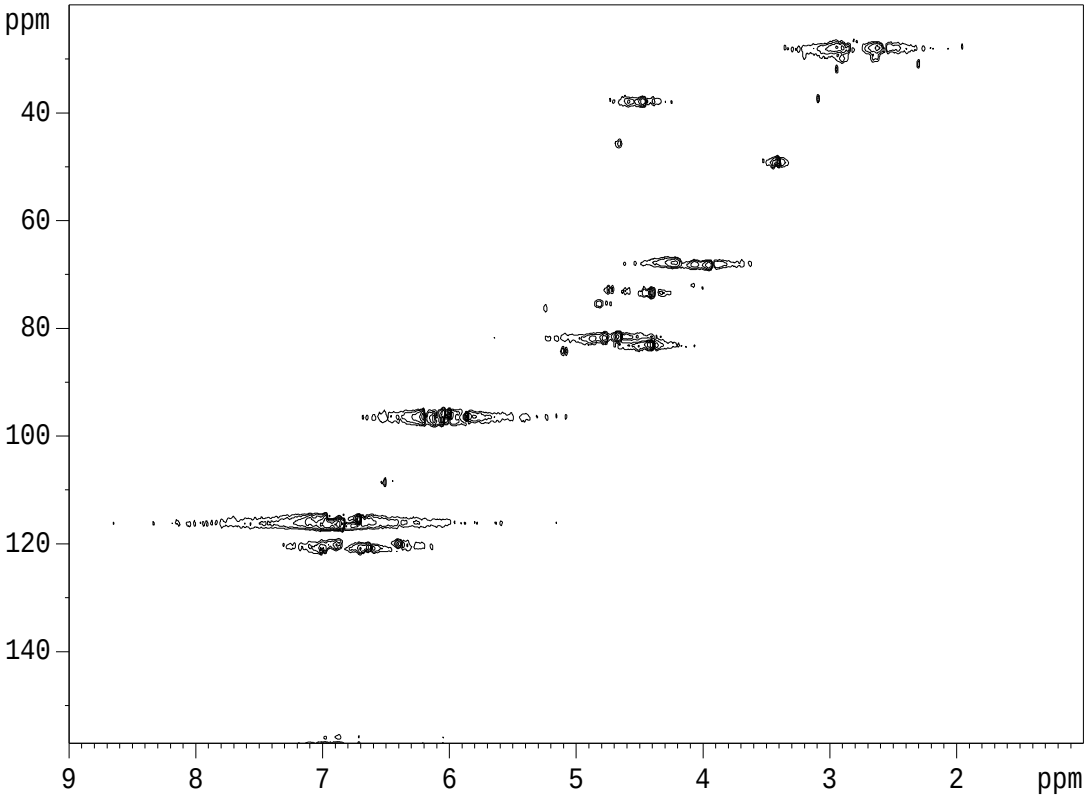


APPENDIX 1.5: NMR SPECTRUM OF DIMER f4

gCOSY



HSQC


ppm
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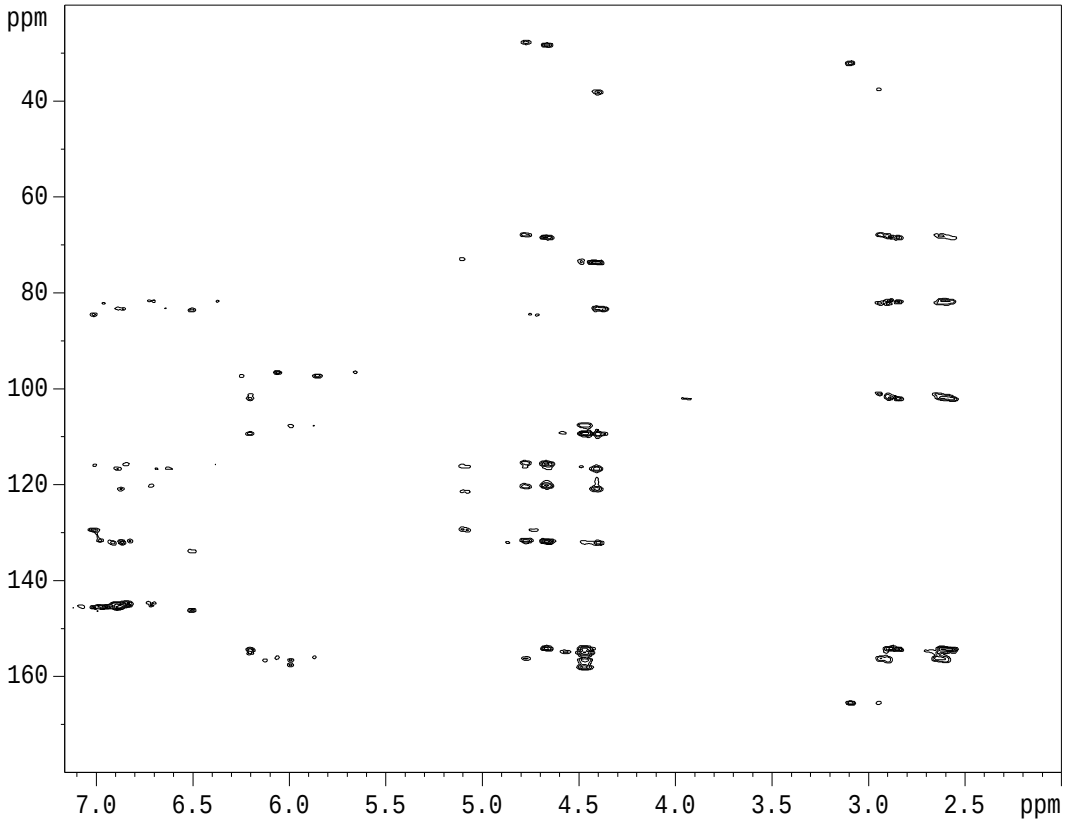
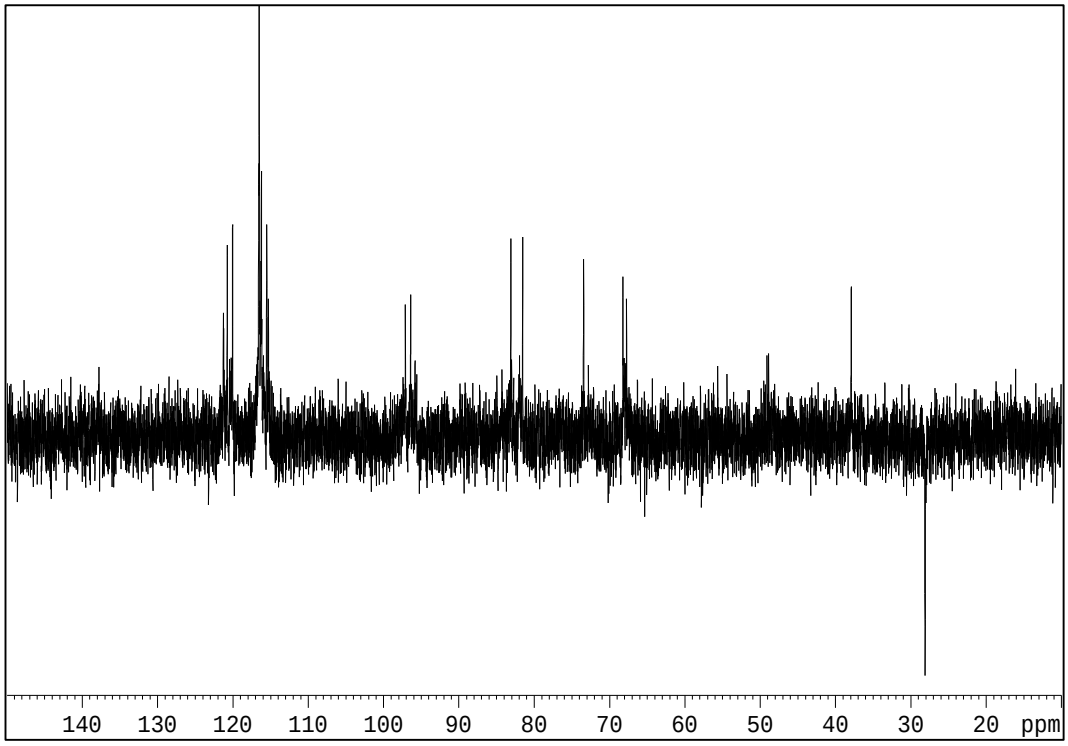
60
80
100
120
140
9
8
7
6
5
4
3
2
ppm

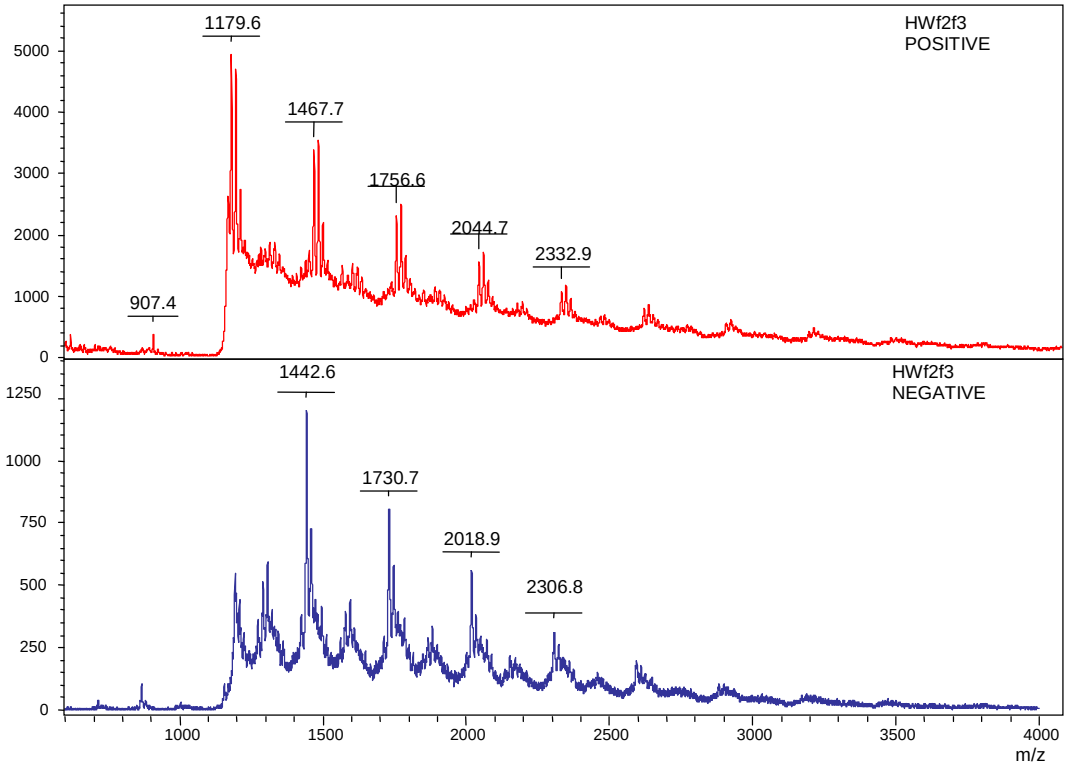
HMBC


ppm
40
60
80
100
120
140
160
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
ppm

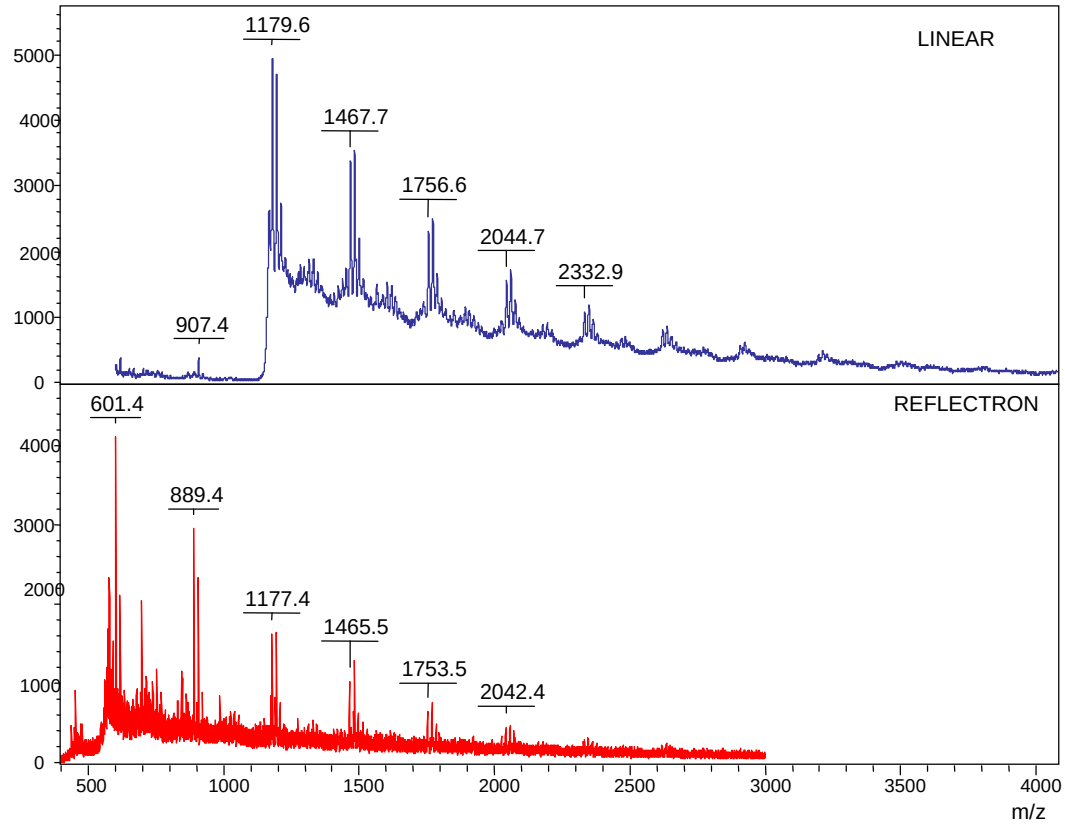
DEPT135



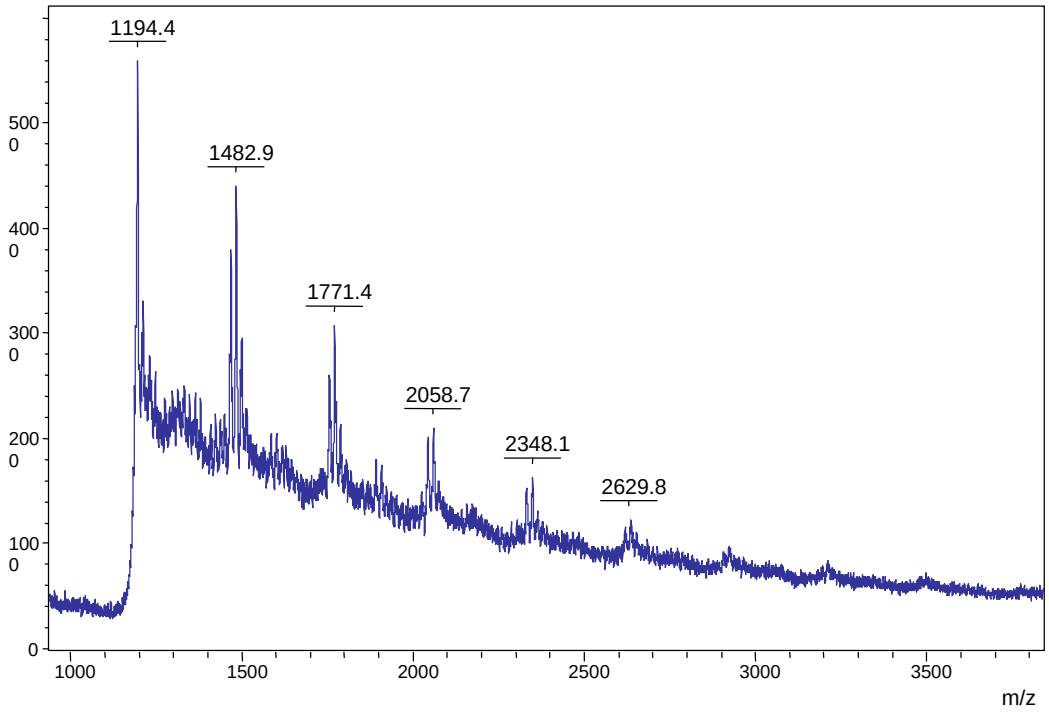
APPENDIX 2.1: MALDI-TOF MS SPECTRA OF HW f2f3 IN NEGATIVE AND POSITIVE ION MODE



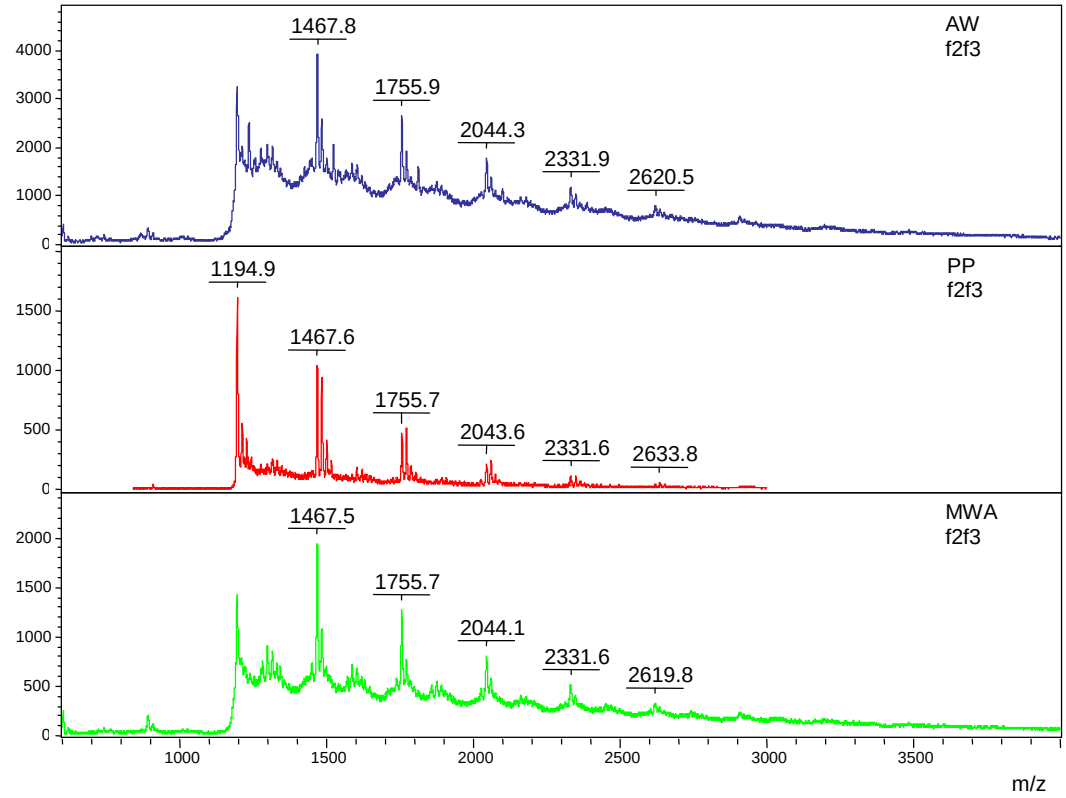
APPENDIX 2.2: MALDI-TOF MS SPECTRA OF HW f2f3 IN LINEAR AND REFLECTRON MODE



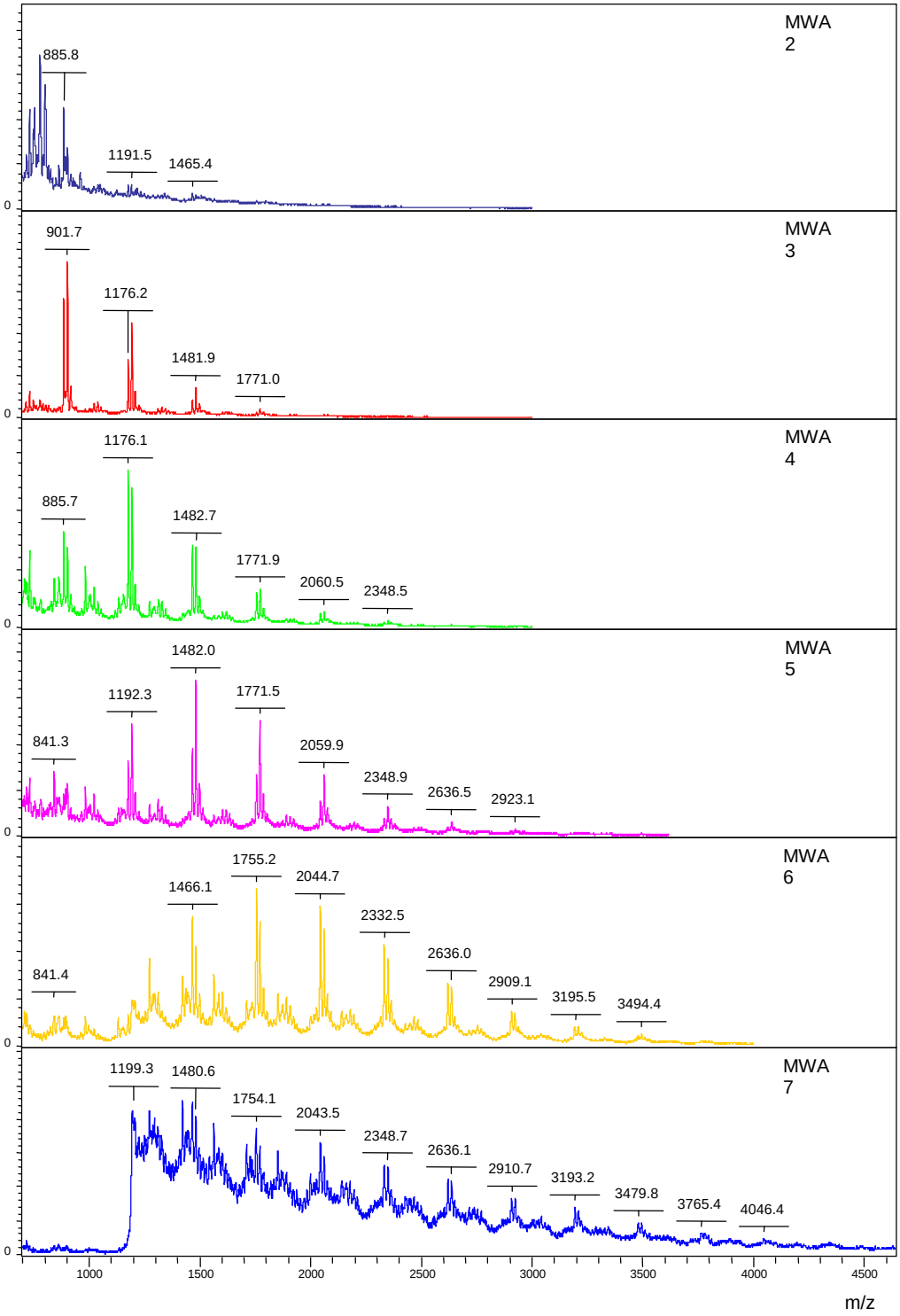
APPENDIX 2.3: MALDI-TOF MS SPECTRUM OF PURE BARK



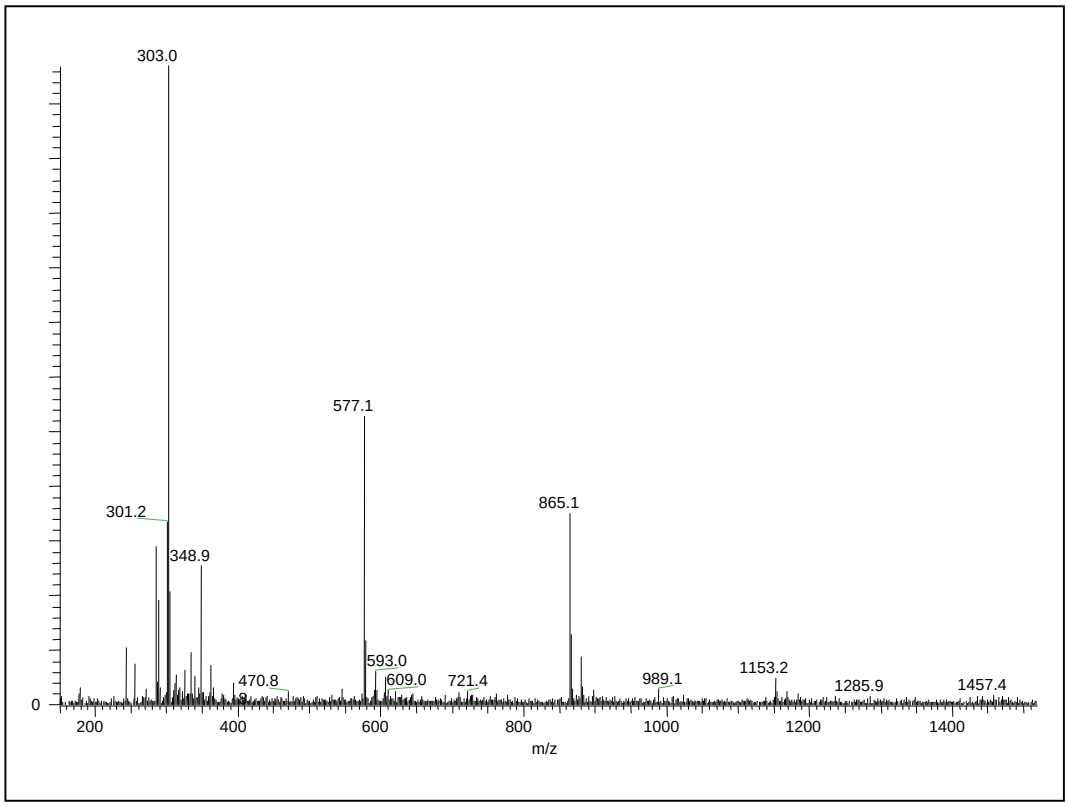
APPENDIX 2.4: MALDI-TOF MS SPECTRA OF f2f3 FRACTIONS



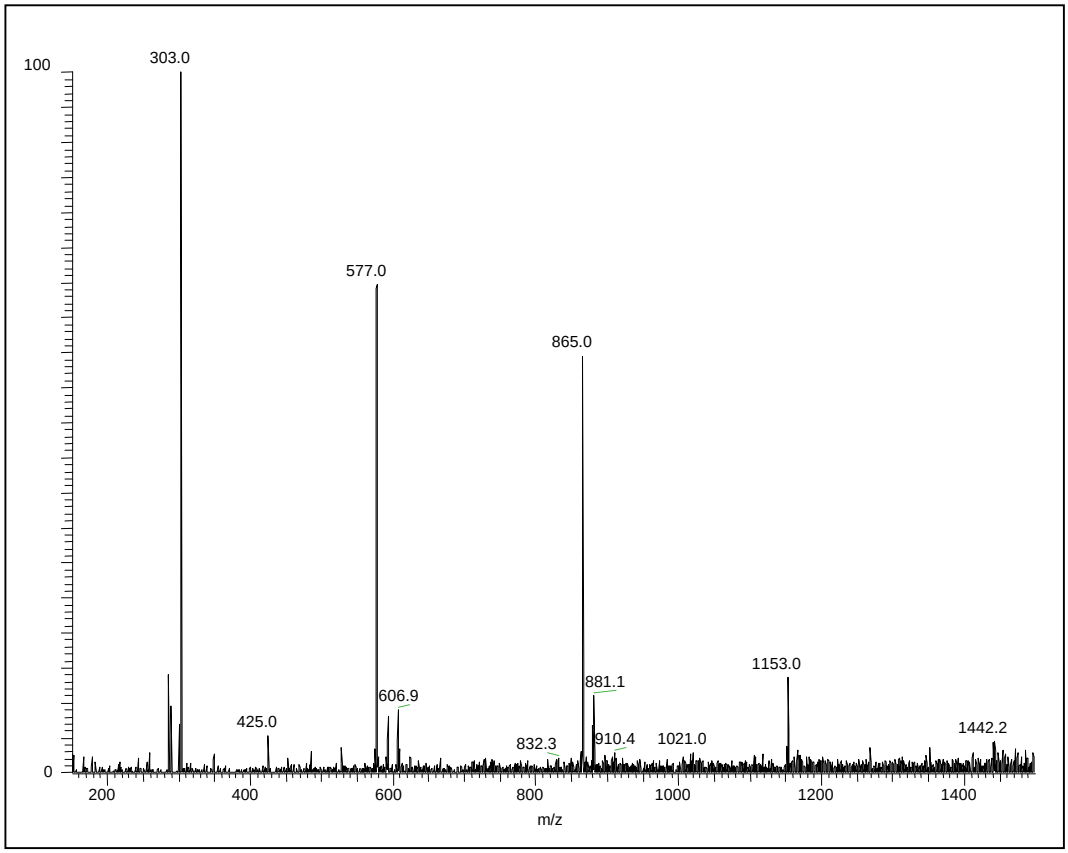
APPENDIX 2.5: MALDI-TOF MS SPECTRA OF MWA 2-7



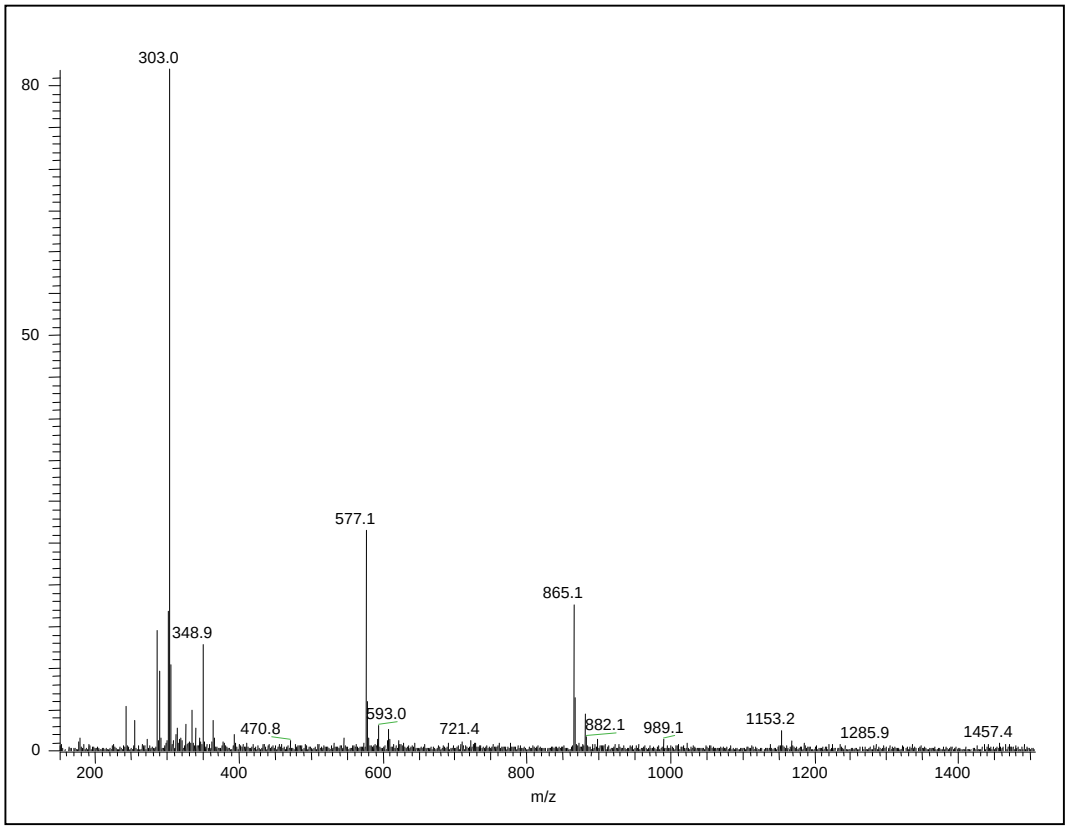
APPENDIX 2.6: ESI MS SPECTRA OF f2f3 FRACTIONS
AW f2f3



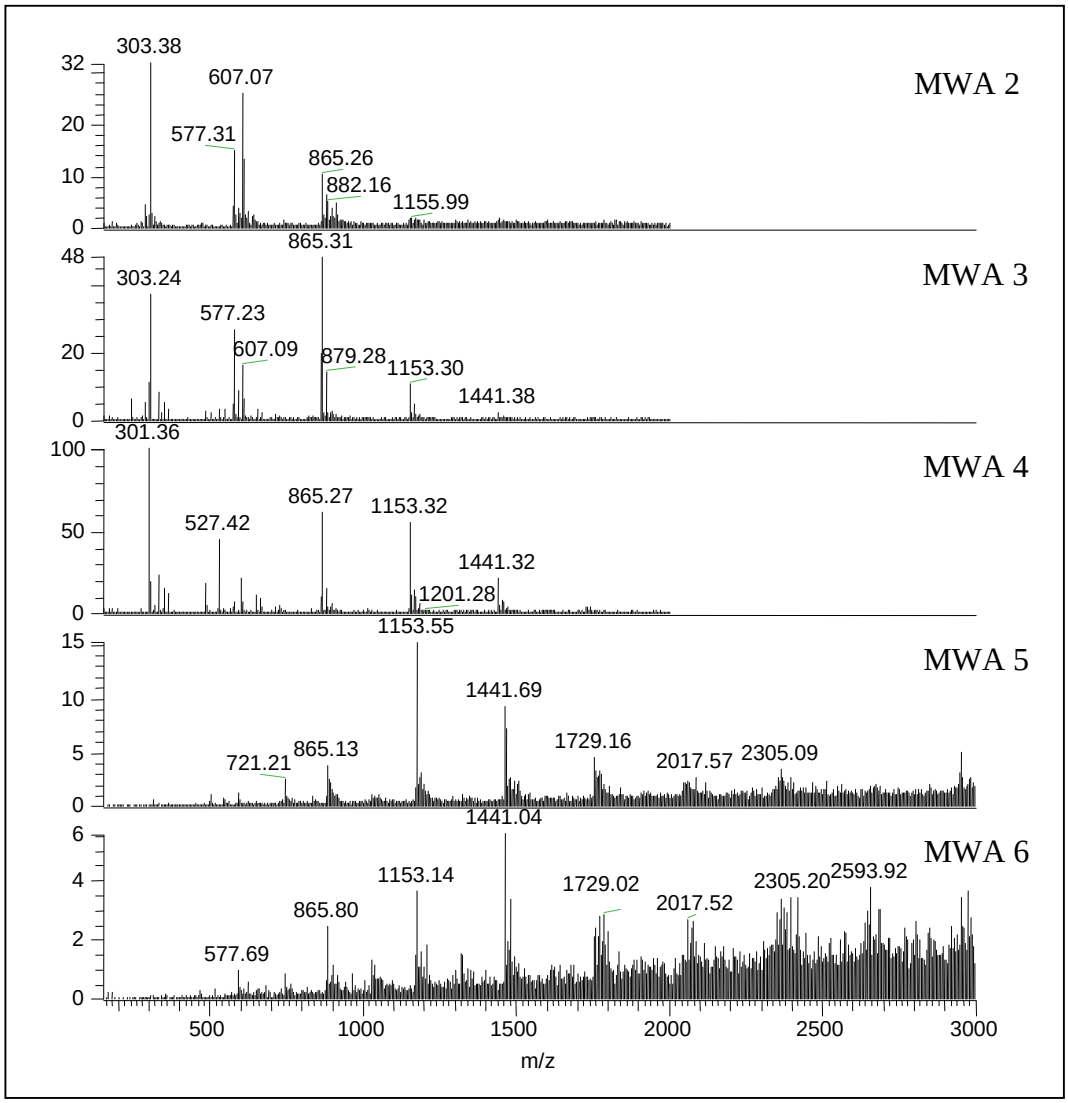
MWA f2f3



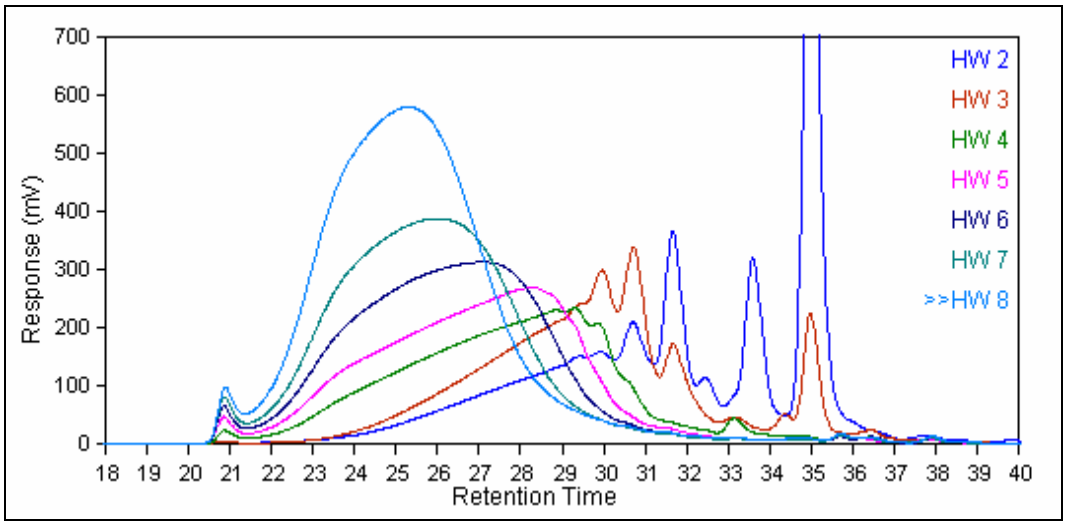
PP f2f3


303.0
80
50
577.1
865.1
348.9
0
593.0
470.8
721.4
882.1
989.1
1153.2
1285.9
1457.4
200
400
600
800
1000
1200
1400
m/z

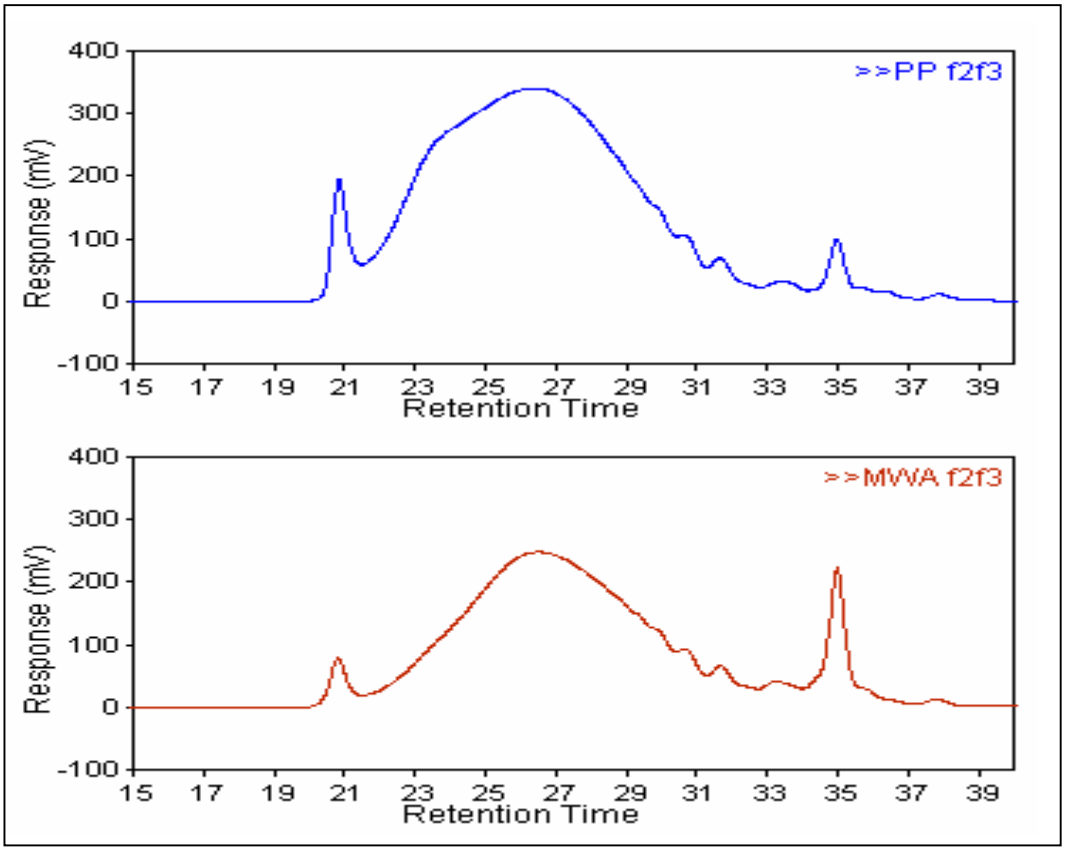
APPENDIX 2.7: ESI MS SPECTRA OF MWA 2-6



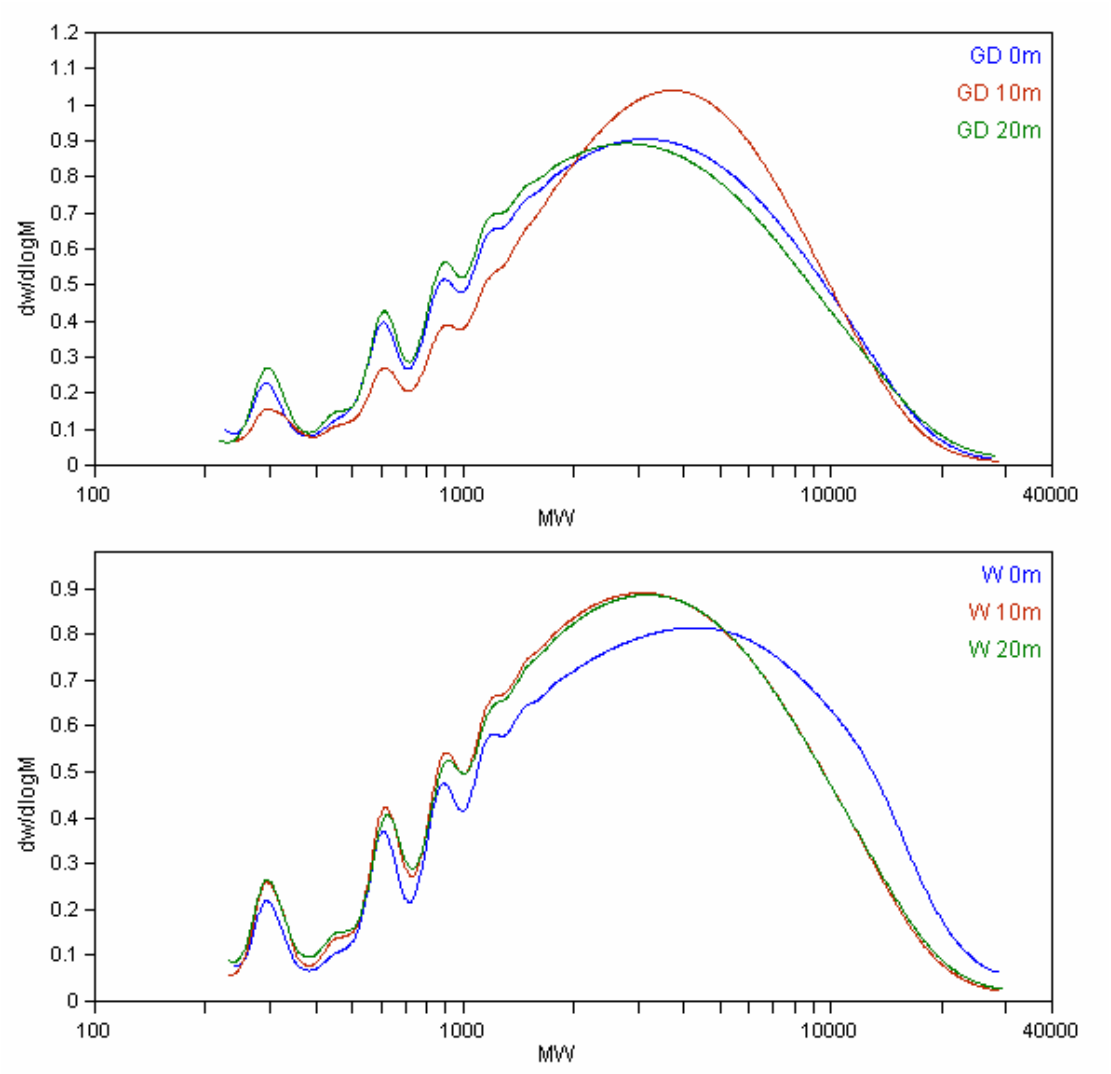
APPENDIX 3.1: GPC CHROMATOGRAMS OF HW 2-8



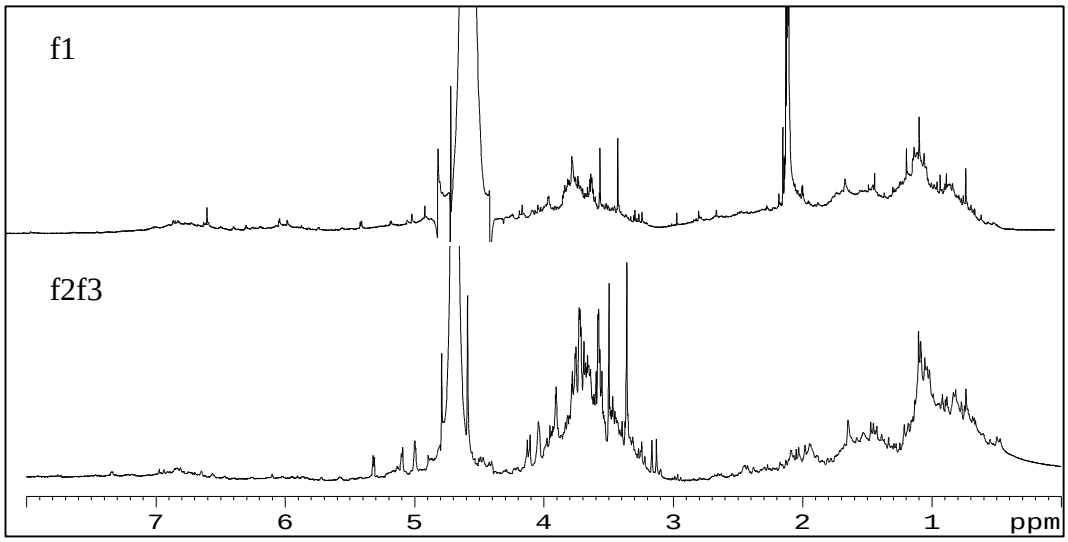
APPENDIX 3.2: GPC CHROMATOGRAMS OF MWA f2f3 AND PP f2f3



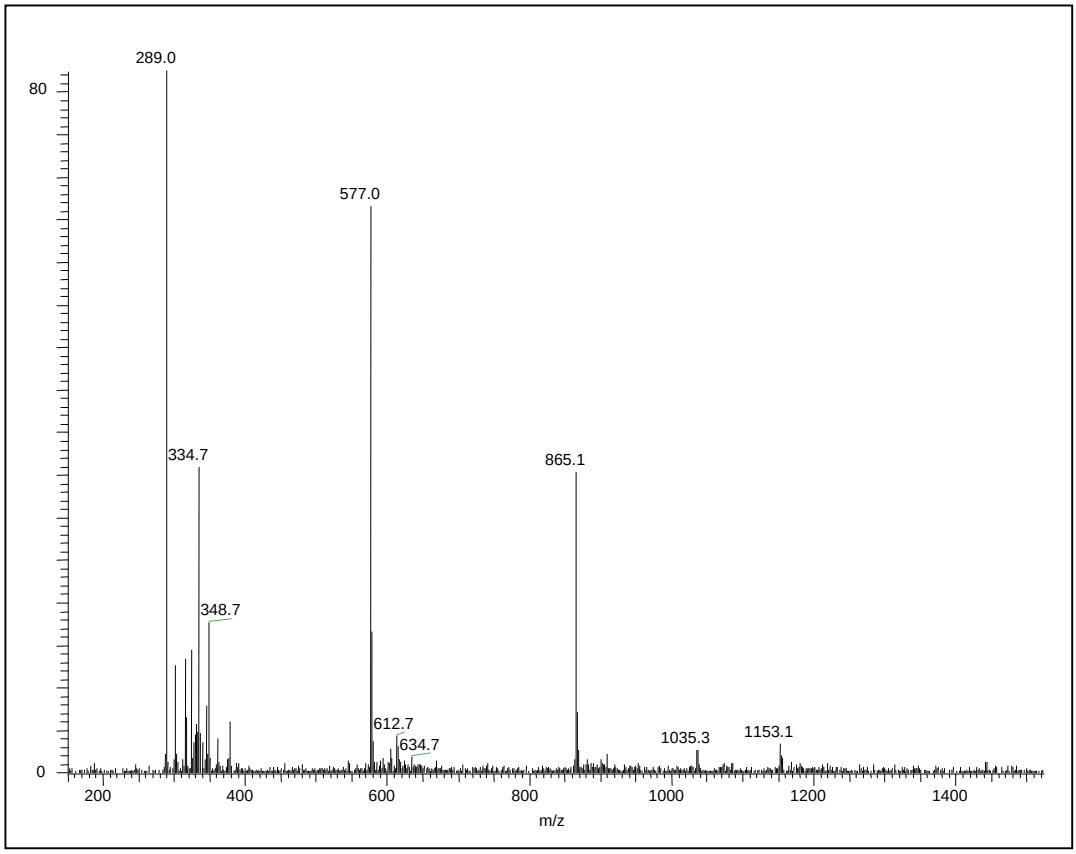
APPENDIX 4.1: MOLECULAR WEIGHT DISTRIBUTIONS OF GOLDEN DOWNS AND WAIMATE CONDENSED TANNINS USING GPC



APPENDIX 4.2: ^{1}H NMR SPECTRA OF KAURI f1 AND f2f3 FRACTIONS



APPENDIX 4.3: ESI MS SPECTRUM OF RIMU INNER f2f3



APPENDIX 5.1: NZIC CONFERENCE 2006 POSTER

Molecular Weight Characterisation of *Pinus radiata* Bark Condensed Tannins

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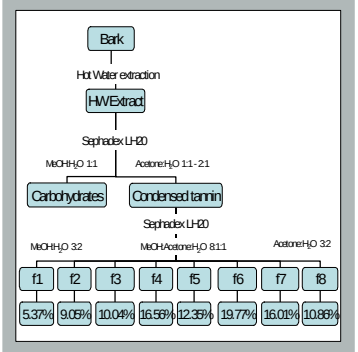
Abstract

Condensed tannins extracted from *Pinus radiata* bark with hot water (90˚C, 1hr) were subjected to Sephadex LH-20 column chromatography using a stepwise gradient of methanol, water and acetone. Fractions were collected and pooled to give eight final fractions (f1-f8). These fractions were analysed by acid/phloroglucinol depolymerisation coupled with high performance liquid chromatography (HPLC), matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) and gel permeation chromatography (GPC).

The mean degree of polymerisation (mDP) of the condensed tannins in the crude bark extract was determined to be 7.6, however in the MALDI-TOF MS spectrum of this extract the most intense signal was the tetramer peak and the decamer was the largest oligomer detected. In the spectra of late eluting column fractions (*i.e.* f5-f7) oligomers greater than the nonamer (DP up to 14) were detected and the most intense signal was shifted from the tetramer to higher molecular weight (MW) oligomers. These results demonstrated that prior separation of the tannins was effective in improving MS detection of higher oligomers.

The mDP of the eight fractions, which was obtained from depolymerisation/HPLC results, was used to calibrate the GPC analysis. GPC analysis produced condensed tannin MW profiles for the eight fractions and crude extract and confirmed the presence of larger oligomers that were not detectable by MALDI-TOF MS.

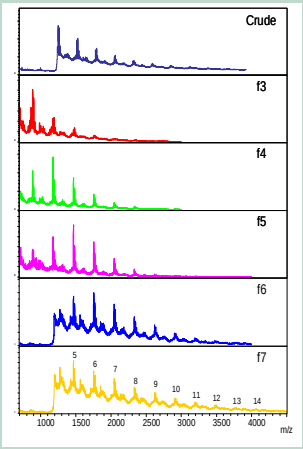
Methods



- The fractionation method used was modified from Taylor *et al.*, 2003.
- The acid/phloroglucinol depolymerisation coupled with HPLC method was modified from Kennedy and Jones, 2001.
- The GPC method was modified from Kennedy and Taylor, 2003.

Results

MALDI-TOF MS



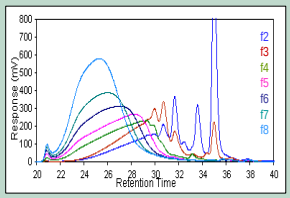
MALDI-TOF MS spectra of crude bark extract and condensed tannin fractions 3-7 showing $[M+Na]^+$ ions

- With increasing fraction number:
  - Higher condensed tannins were detected.
  - The most intense peak was shifted to a higher MW.
- Condensed tannins up to DP 10 were detectable in the crude bark extract by MALDI-TOF MS. Column chromatography fractionation enabled condensed tannins up to DP 14 to be detected.
- MALDI-TOF MS was found to be superior to ESI MS for the detection of condensed tannin molecules larger than the hexamer due to a lack of multiple charging.

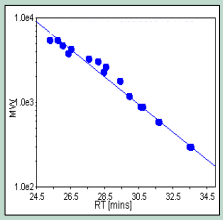
MALDI-TOF MS OPTIMUM CONDITIONS

Matrix: 2,5 dihydroxybenzoic acid
Solvent: Methanol/Water (6:4)
Salt added: None
Ion mode: Positive
Time of Flight: Linear

GPC AND DEPOLYMERISATION/HPLC



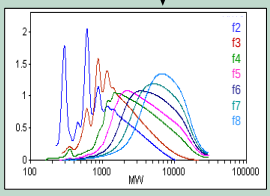
GPC chromatograms of seven fractionated condensed tannins



Condensed tannin calibration curve R^2=0.985

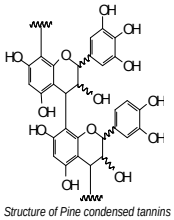
Sample	Average Molecular Weight (Depoly/HPLC)	Number Average Molecular Weight (GPC)	Weight Average Molecular Weight (GPC)
f2	637	688	1388
f3	1314	1187	2164
f4	2248	1847	4140
f5	2960	2679	5210
f6	3677	3450	6184
f7	4580	4266	7144
f8	5386	5027	8122

Table comparing the depolymerisation/HPLC and GPC results



MW distribution of fractions 2-8

The fractionated condensed tannins were used as calibration standards for GPC analysis. The mDP (and therefore average MW) of each fraction was determined by depolymerisation/HPLC analysis and assigned to GPC peak maxima in the case of f5-f8. Monomers to tetramers were resolved by GPC in f1-f4 and hence exact MWs could be assigned to these retention times. Using these values a calibration curve was constructed to enable number average MW and weight average MW, and MW distributions to be determined for various condensed tannin samples.



Structure of Pine condensed tannins

Conclusions

Molecular weight fractionation of *Pinus radiata* condensed tannins by Sephadex LH-20 chromatography facilitated their characterisation by MALDI-TOF MS, acid/phloroglucinol depolymerisation coupled with HPLC and GPC. Fractionation enabled condensed tannin molecules of DP>10 to be detected by MALDI-TOF MS. Additionally the fractionated condensed tannins proved useful calibration standards in establishing a GPC methodology for MW characterisation of *Pinus radiata* condensed tannins.

REFERENCES
- Kennedy, J.A., and Jones, G.P., 2001, Analysis of Proanthocyanidin Cleavage Products Following Acid-catalysis in the presence of Excess Phloroglucinol, Journal of Agricultural and Food Chemistry, 49, pg 1740-1746.
- Kennedy, J.A. and Taylor, A.W., 2003, Analysis of proanthocyanidins by high-performance gel permeation chromatography, Journal of Chromatography A, 995, pg 99-107.
- Taylor, A.W., Bandiky, E., Kennedy, J.A., and Deinzer, M.L., 2003, Hop (*Humulus lupulus L.*) Proanthocyanidins Characterized by Mass Spectrometry, Acid Catalysis, and Gel Permeation Chromatography, Journal of Agricultural and Food Chemistry, 51, pg 4101-4110.