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Phenolics and condensed tannins from sulla (*Hedysarum Coronarium*) leaves and their biological significance

This thesis was presented in partial fulfilment of the requirements for the Master of Science degree in Chemistry at Massey University, Palmerston North, New Zealand

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ABSTRACT

The objective of this study was to isolate and characterise condensed tannins (CT) and phenolic compounds from the leaves of the forage legume sulla (*Hedysarum Coronarium*) and evaluate their structure-activity relationships with *in vitro* parasite assays. The study was performed on samples which were collected over different seasons (spring-23/09/02, spring-05/08/02, and summer-21/12/01) from the same site. The effects of processing in different manners, for both freeze-dried and fresh frozen plant material was examined. CT extracts were purified using step and gradient Sephadex LH-20 chromatography methods. The CT fractions obtained were analysed using thiolytic degradation, electrospray ionisation mass spectrometry (ESI-MS), matrix-assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF-MS) and ^{13}C-nuclear magnetic resonance (NMR) spectroscopy.

Acid catalysed degradation of the CT polymer with benzyl mercaptan afforded catechin, epicatechin, gallocatechin and epigallocatechin in both the terminal and extender units. Epigallocatechin was the major extender unit (69%) while gallocatechin was the major terminal unit (54%) with the overall characteristic of a predominantly prodelphinidin-type CT. Sulla CT oligomers and polymers had variable chemical composition with procyanidin:prodelphinidin ratios ranging from 27:73 to 11:89 and *cis:trans* ratios ranging from 56:44 to 82:18. The CT oligomers of gradient LH-20 fractions obtained from fresh frozen material (spring-05/08/02) ranged between 2.9 and 6.9 mean degree of polymerisation (mDP), while CT fractions from the freeze-dried material (summer-21/12/01) varied between 3.1 and 9.1 mDP as determined by thiolysis. The CT polymer from step LH-20 fractions had CTs ranging from 12 to 26 mDP as determined by thiolysis. A medium molecular weight CT with mDP of 46 was identified. No high molecular weight CT (mDP> 50) was obtained. Screening the LH-20 fractions collected in spring (23/09/02) by HPLC-PDA indicated that there was no extractable CT. No seasonal or freeze-drying effects were observed on the chemical composition of CT. ^{13}C NMR provided information on the stereochemistry of the heterocyclic C-ring and the existence of procyanidin and prodelphinidin units in the B-ring. The ^{13}C NMR spectrum confirmed sulla CTs to be predominantly of the *cis*-stereochemistry composed of prodelphinidin units.

Analysis of the CT oligomers from the 100% MeOH fractions from the gradient LH-20 with ESI-MS provided information on the molecular weight distribution and the procyanidin and prodelphinidin unit composition. Singly charged species from dimers to trimers, doubly charged species from tetramers to octamers, and triply charged species from nonamers to undecamers were detected. MALDI-TOF-MS verified the ESI-MS data and fractions were found to contain singly charged ions up to hexamers. The ions consisted of homogenous and heterogeneous CT oligomers, with the overall characteristic of a PD-type CT. This technique demonstrated that the gradient LH-20 method improved separation with fractionation of CT oligomers from polymers.

Investigation of the low molecular weight phenolics (flavonoids) was performed using ESI-MS and atmospheric pressure chemical ionisation mass spectrometry (APCI-MS). Chlorogenic acid, quercetin-7-O-α-L-rhamnosyl-3-O-glucosylrhamnoside, rutin, quercetin-3-O-α-L-rhamnosyl-7-O-glucoside, kaempferol, kaempferol-3-O-β-D-glucoside-dirhamnoside, genistein-7-O-β-D-glucosyl-6”-O-malonate, formononetin-7-O-β-D-glucoside-6”-O-malonate and afrormosin were isolated for the first time from sulla. Chlorogenic acid and rutin were confirmed using authentic standards and by comparison with data from the literature.

Primary metabolites were evaluated by wet chemistry methods including the available carbohydrate (g/100g) content of 12.38 and, 14.11 and individual sugars (g/100g) were quantified; glucose (3.68 and 5.40), fructose (0.98 ad 1.69), galactose and/or rhamnose (0.46 and 0.32), sucrose (1.63 and 5.50) in spring (23/09/02) and summer (21/12/01), respectively. Nutritional composition data (g/100g) by near infrared (NIR) spectroscopy has shown sulla to be a nutritious forage legume with high crude protein (CP; 24.4-25.1), non-structural carbohydrates (NSC; 17.1-19.7), lower neutral detergent fibre (NDF; 12.5-16.4), acid detergent fibre (ADF; 15.5-18.3) and lipid (2.6-3.3). The butanol-HCl assay showed the extractable CT content (g/100g) to be 7.6% and 5.3%, with 2.0% protein bound CT and 0.3% fibre bound CT and 1.3% protein bound CT and had no fibre bound CT, from summer (21/12/01) and spring (23/09/02) respectively.

The effects of LH-20 fractions on egg hatching (EH) and larval development (LD) assays for the nematode, *Trichostrongylus colubriformis* under *in vitro* conditions were investigated. The fractions (freeze-dried summer (21/12/01) step method 50% MeOH eluent) containing flavonoids were effective in inhibiting EH at 500 and 1000 μg/mL, while the CT-containing fractions were not effective. All the fractions from the

fresh frozen material on the step method were not effective in inhibiting EH. However, the CT-containing fractions (LH-20 70% acetone eluent) from the freeze-dried (summer-21/12/01) and fresh frozen (spring-05/08/02) material were effective in inhibiting LD ($p<0.001$) with certain fractions completely inhibiting the LD process. Oligomeric and polymeric CT (gradient LH-20 100% MeOH and 70% acetone eluents) fractions were effective and inhibited the larval development process at 100 μg/mL. The anti-parasitic activity of fractions in the LD and EH assay can be attributed to both the flavonoid and CT content.

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LIST OF ABBREVIATIONS

APCI	atmospheric pressure chemical ionization
AQ	aqueous
BSA	bovine serum albumin
Bu-HCl	butanol hydrochloric acid
C	catechin
CP	crude protein
CT	condensed tannins
COSY	correlated spectroscopy
DHB	2,5-dihydroxy benzoic acid
DCM	dichloromethane
DHQ	dihydroquercetin
DM	dry matter
DP	dietary protein
EC	epicatechin
EGC	epigallocatechin
EHA	egg hatch assay
ESI	electrospray ionization
FAB	fast atom bombardment
GC	gallocatechin
HMBC	heteronuclear multiple quantum coherence
HMQC	heteronuclear multiple bond connectivity
HMW	high molecular weight
HT	hydrolysable tannin
IS	internal standard
LC	liquid chromatography
LCS	lotus corniculatus
LDA	larval development assay
LMI	larval migration inhibition
LMW	low molecular weight
LP	lotus penduculatus
LSI	liquid secondary ionisation
LSU	large subunit
MALDI-TOF	matrix aided laser desorption ionization, time of flight

mDP	mean degree of polymerisation
MMW	medium molecular weight
MS	mass spectrometry
Mw	molecular weight
NDF	neutral detergent fibre
NIRS	near infrared reflectance spectroscopy
NMR	nuclear magnetic resonance
NP-HPLC	normal phase high performance liquid chromatography
NSC	non-structural carbohydrates
ODS	octadecyl silica
OMD	organic matter digestibility
PA	proanthocyanidins
PC	procyanidins
PD	prodelphinidins
PDA	photodiode array
PEG	polyethylene glycol (Mw, 3500)
RP-HPLC	reverse phase high-performance liquid chromatography
RRF	relative response factor
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SIM	selective ion mode
SSU	small subunit
UV-VIS	ultra violet-visible
VFI	voluntary feed intake

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1. Introduction

Phenolics and polyphenolic condensed tannins (CT) or proanthocyanidins (PA) as they are also known, are widely distributed in the plant kingdom, ubiquitous in leaves, barks, flowers, stems, roots and seeds (Bravo, 1998). They are present in other plant derived food substances such as cocoa (Lazarus et al., 1999), green tea (Haenen et al., 2000), red wine (Fulcrand et al., 1999), grapes (Souquet et al., 2000), fruit and vegetables (Scalbert et al., 2000) showing antiviral, superoxide radical scavenging (Bruyne et al., 1999), antioxidant, chelating (Tagliaferro et al., 2002) antimicrobial and antibacterial (Scalbert, 1991) properties. Herbal medicinal plants such as heather, willow herb, bog-rosemarry (Kahkonen et al., 1999), epilobium species (Ducrey et al., 1995), meadowsweet and tree peony (Haslam, 1996) have therapeutic and palliative effects. The active components in these plants are believed to be the polyphenolic substances. Hawthorn (*Crataegus sp*), one of the Europe's traditional herbal medicines has been used as an infusion for the heart and blood circulation, the activity is attributed to the presence of CT (Haslam, 1989).

These plant secondary metabolites are located in cell walls and vacuoles of plants and most notably in angiosperms and gymnosperms (Stafford et al., 1980). They are biologically synthesized through the shikimate and acetate pathway (Kumar et al., 1988). Examples of polyphenolic compounds include: hydrolysable tannins, terpenes, phlorotannins, lignin, alkaloids, flavonoids and CT (Waterman and Mole, 1994).

Sulla (*Hedysarum Coronarim*) is a biennial nutritious forage legume, native to the Mediterranean region (Douglas et al., 1985), that was introduced into New Zealand pastures in early 1949 for soil erosion and re-vegetation control. This forage contains CT in the leaves and flowers (Waghorn et al., 1998) with concentration ranging from 3-12% dry matter (DM), which are attributed to the beneficial nutritional effects in sheep and cattle. CT are present in the leaves of other forage legumes such as birdsfoot trefoil (*Lotus corniculatus,* LCS), big trefoil (*Lotus penduculatus,* LP), erect dorycnium (*Dorycnium rectum)* and sainfoin (*Onobrichus Viciifolia*) (Foo et al., 1983; Terrill et al., 1992a). White clover (*Trifolium re*pens), a forage legume contains CT in the flowers (Foo et al., 2000), but not in the leaves, while weeds including polygonaceae (*Rumex Patientia)* contain CT in the roots (Demirezer et al., 2001). The factors affecting the CT

concentration are not well understood, the content varies with the developmental stages (Bohm et al., 1993), season (Beuselinck et al., 2002), soil fertility (Lowther et al., 1987) drought and temperature (Carter et al., 1999). LP clones were found to have higher concentrations of CT at 30 °C than plant's grown at 20 °C (Lees et al., 1994). The CT and lignin concentrations have a direct relationship as observed from *Lotus* species (Barry et al., 1986).

In order to improve protein utilisation and animal performance in ruminants, alternative forages are urgently required to complement grass, lucerne (*Medicago sativa* L.) and white clover based pastures in New Zealand. One such approach may be to use forages that contain CTs such as sulla. With conventional forages, which contain traces of CT, most of the proteins are lost with the excreta or in the form of ammonia. CT are known to protect plant protein from degradation in the rumen (pH 3.5-7.0) by rumen micro-organisms and dissociate in the abomasum (pH< 3.5), increasing the flow of amino acids in the small intestine (Jones et al., 1976). Amino acids absorption in the small intestine was increased significantly in sheep fed sulla (Bermingham et al., 2001). The increased absorption of amino acids is attributed to CT. Sulla is a drought resistant legume with deep branching tap-root and can thrive in alkaline soils and its establishment in southern Australia to complement lucerne is being investigated (Dear et al., 2003). The fast degradation of microbial protein from grain legumes, peas and beans in the rumen suggest that other feed supplements with slower rates of degradation should be integrated into farming systems (Wilkins et al., 2000). A forage legume such as sulla has a faster rate of degradation, but it can supplement lucerne, ryegrass and white clover pastures (Burke et al., 2002). Mekasha (2002) has shown that cheaper and affordable non-conventional agro-industrial by-products can be used as protein and energy supplements to complement grass based diets. In addition to lower CT, sulla has a high nutritive value with a higher crude protein content (14-18% DM), non-structural carbohydrates (18-25% DM) and lower dietary fibre (Burke et al., 2002). It is highly palatable with high voluntary feed intake (Douglas et al., 1999; Waghorn et al., 1998) and high dry matter yields (Douglas and Foote, 1985). Despite its higher nutritive value, it is not widely used in the farming systems (Waghorn et al., 1998). Improved animal performance was observed in sheep fed sulla compared to ryegrass on time-restricted allocation which is attributed to low CT content, higher intake of CP and high content of soluble carbohydrates (Molle et al., 2003). Sulla can be incorporated into pastoral agriculture through rotational grazing or as an animal feed to complement conventional

pastures and improve animal nutrition and productivity. To further understand the nutritional benefits associated with CTs from legumes, the study of the chemical structure is necessary.

The activity of CT varies depending on the molecular structure, type, composition and concentration of the CT as shown for Lotus spp. (Foo et al., 1996, 1997). Sulla has been shown to have the high activity *in vitro* (Molan et al., 2000a,b) and *in vivo* (Niezen et al., 1995, 2002a) for reduction of nematodes in sheep, cattle, deer and goats (Barry et al., 2001). However, the specific mechanisms have not been elucidated. The mechanism might be attributed to direct or indirect effect of the CT from sulla.

Sulla flowers are a pink to reddish colour. Flower colour pigmentation in plants is attributed to the presence of flavonoids-anthocyanins (anthocyanidin glycosides). Sulla flowers contain peonidin-3-monoglucoside, peonidin-3, 5-diglucoside and malvidin-3, 5-diglucoside, which contribute to predominant colours of red, mauve and violet respectively (Harborne et al., 1983). Anthocyanin pigments are usually associated with reddish colours. The flavonoid content of sulla leaves has not been studied previously.

1.1 Beneficial effects of condensed tannins in ruminant production

In the past, CT were only known to be associated with "anti-nutritional" effects due to their contribution to astringency, which reduces feed intake and digestibility (Kumar et al., 1988) of feedstuffs and complexation with digestive enzymes. Negative effects were observed for feeds containing high concentrations of CT above 8% (80g/kg DM basis). An example of this has been shown in sulla depressing live weight gain in sheep at a concentration of 8.8% DM (Waghorn et al., 1998). Current research has indicated however that CT fed at a low concentration of up to 4% (40g/kg dry matter) in temperate regions may exhibit beneficial effects in ruminant production (McNabb et al., 1998). Tropical forages usually have high concentration of CT, but analysis of CT concentrations from tropical forages (Barry et al., 1996) found them to be similar to those in temperate regions, (7.0-9.0 %CT).

1.1.1 High Performance in Ruminants

The beneficial effects of CT upon lactation performance in cattle and ewes have been studied (Woodward et al., 2000). A significant increase in milk yield of 46% (kg/cow/day) has been observed with cattle, fed the CT-containing forage, LCS with a

low concentration of CT up to 4% (g/100g DM). In another study, 15% milk yield has been observed in lactating ewes (Wang et al., 1996). CT increased the milk yield of Friesian cows grazing LCS (16.5 vs. 10.2 kg/cow/day) as compared to those cows fed ryegrass (Woodward et al., 1999). The milk had low fat concentrations and increased protein content. The low fat milk would be of greater significance in dairy industry, as one of their aims is to decrease fat content in milk which is recommended for improved human nutrition. However, in this study it was also observed that the higher milk output could be as a result of improved pasture quality, higher crude protein and higher DM intake. Other animal performance benefits included, increase in milk concentration of fatty acids C18:2 and C18:3 by increasing their arterial supply to the mammary gland (Reynolds et al., 2000), increase in rate and efficiency of live weight gain, increase in wool growth and quality, reduction of carcass fat depth (Douglas et al., 1999), reduction of dags and flystrike in lambs (Leathwick et al., 1995; Waghorn et al., 1995). Sulla can be used to supplement non-CT containing forages such as white clover and lucerne (Aerts et al., 1999). Supplementation of these feeds reduced rumen ammonia concentrations and decreased the acetate:propionate ratio in lambs. However, CT from LP produced contrary results, with reduced live-weight gain and increased wool growth (Barry 1985; Barry et al., 1999). The mechanism behind high performance is facilitated by protection of microbial proteins from degradation by the rumen micro-organisms and release of proteins in the small intestine leading to absorption of amino acids, and this may also enhance the immune response. This mechanism is attributed to alleviating rumen ammonia concentration (Terrill et al., 1992), which is known to have toxic effects when it exceeds the optimum concentration (McDonald et al., 1995). To further that CT, may be responsible for these benefits, a comparative experiment is performed using the CT-containing forage in the presence of polyethylene glycol (PEG, Mw 3500). PEG has a higher affinity for CT than proteins and therefore deactivates them.

1.1.2 Health Benefits

Bloat is a major problem for cattle in New Zealand and USA pastures dominated by clover rich pastures (Reid et al., 1974), alfalfa and lucerne. Bloat develops when gas formed during the fermentation of feeds is trapped in the rumen in the form of stable foam due to highly soluble proteins. Bloat inhibits eructation of gases formed during fermentation such as carbon dioxide and methane and which results in a very high pressure build-up and failure of belching in the animal, and may lead to death

(McMahon et al., 1999). However, alternative strategies need to be explored through the use of forage legumes which contain CT such as *Lotus* species and sainfoin (widely utilised in USA) which have been indicated in the reduced formation of bloat in cattle (McMahon et al., 1999). The absence of bloat has been observed in cattle grazing temperate and tropical legumes, which is attributed to protein precipitants such as CT (Jones et al., 1971). These polyphenolic compounds complex with soluble leaf proteins which form bloat and reduce protein solubility increasing protein utilisation.

1.1.3 Environmental benefits

Methane is a greenhouse gas produced by ruminants especially in sheep, cattle and deer which is released mainly through their mouths and nostrils. New Zealand has agreed to ratify the Kyoto Protocol at the conference held in 1997, which encourages appropriate measures be taken to mitigate the greenhouse gas emissions contributing towards global warming. Animal trials have shown that cows fed forages containing CT such as LCS and LP have reduced emission of methane gas from sheep and cattle (Waghorn et al., 2001). According to a study conducted by Ulyatt (1992) sheep contribute 58%, dairy production 18%, beef farms produces 21%, deer 2%, and goats 1% of methane gas in New Zealand. The degradation of fibre in the rumen produces hydrogen which is converted to methane by methanogens. Sulla has been shown to reduce methane production from ruminants (Woodward et al., 2002; Waghorn et al., 2002). Methane production was found to be lower for animals fed sulla than ryegrass (19.5g and 24.6g CH_4/ day DM intake respectively). Contributing factors for reduction in emission of methane gas could be attributed to low dietary fibre content, high carbohydrate content and the presence of CT in the diet.

1.2 Condensed tannins

CT should be distinguished from hydrolysable tannins (HT). CT are polymers of flavan-3-ol monomer units, which are linked through C-C bonds. The flavan-3-ol units are usually identified with the use of alphabets A, B and C rings (Figure 1.1). HT (Figure 1.1) are composed of gallic and ellagic acid derivatives (Mammela et al., 2000). They are hydrolysed in acidic media and are associated with toxicity, in interaction with physiological processes in the body (Jansman, 1993).

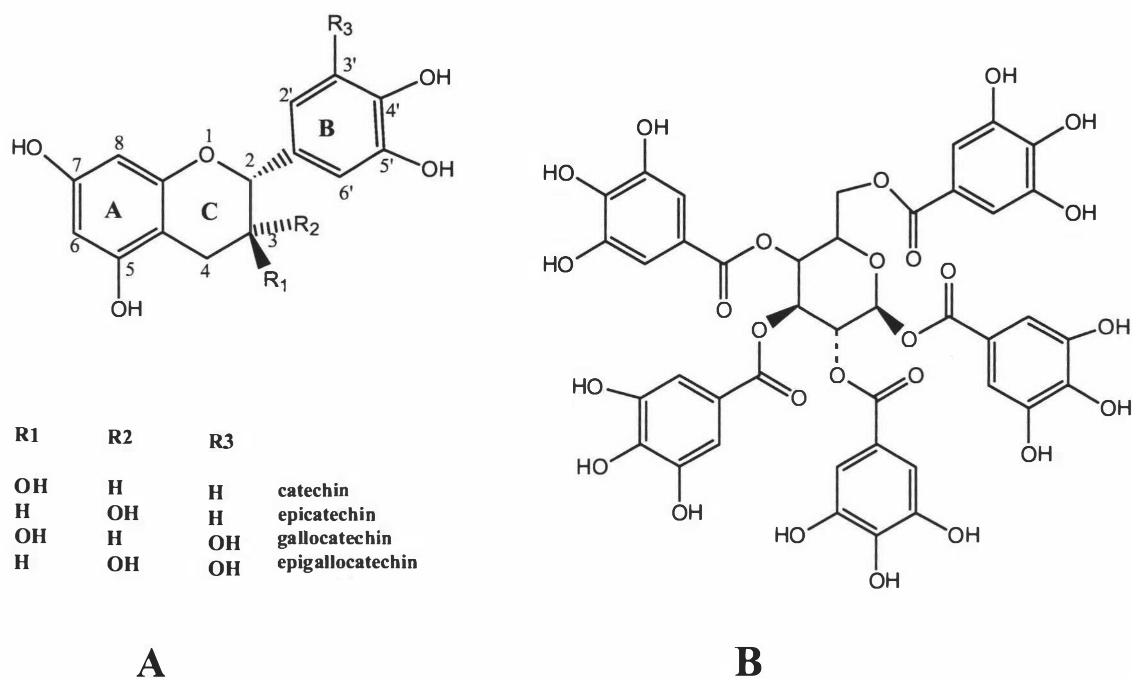


Figure 1.1 (A) Monomeric units of CT polymer, (B) structure of hydrolysable tannin containing gallic acids ester bound to a hexose moiety.

CT is a sub-class of flavonoids possessing C_6-C_3-C_6 flavanol skeleton, but differs from the low molecular weight phenolics in that they are polymeric (Figure 1.2).

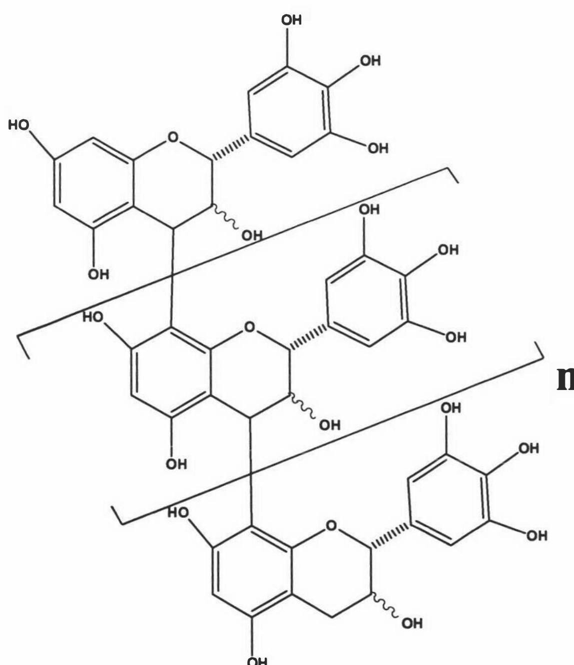


Figure 1.2 Structure of a condensed tannin polymer.

Flavan-3-ols (e.g catechin, epicatechin, gallocatechin and epigallocatechin) are the monomeric constituents of the CT (Halliwell and Gutteridge., 1999). Some CT

contain epifzelechin (R_3= H and C-5' in the B-ring= H) as the monomer unit. Depending on the hydroxylation pattern in the B-ring when R_1= OH, R_2= H and R_3= H the monomer unit is called catechin and epicatechin, having the catechol B-ring. Gallocatechin and epigallocatechin are usually observed for R_1= R_3= OH, having the pyrogalloyl B-ring (Figure 1.1). The heterocyclic C- ring has 3 carbon atoms which are asymmetric and can have different configurations, though the absolute configuration at C-2 is always *R* and rarely changes. However, flavan-3-ols with 2*S* configuration which rarely occurs in nature have been isolated from *Byrsonima Crassifolia* bark (Rimpler et al., 1994). The relative stereochemistry at C2 and C3 may be either *trans* (2*R*, 3S) or *cis* (*2R*, *3R*). The interflavanol C-C linkages, called B-type, can be C4/C8 leading to a linear polymer or C4/C6 producing branched polymer, which exists in grape CT (Cheynier, 1999). A restricted rotation is found in the C-C linkages which makes the polymer flexible and adopts various molecular shapes. Bridged linkages (4β-8, 2β-O-7) have also been isolated (Figure 1.3) and are referred to as A-linkages (Kolodziej et al., 1991; Pieters et al., 1995).

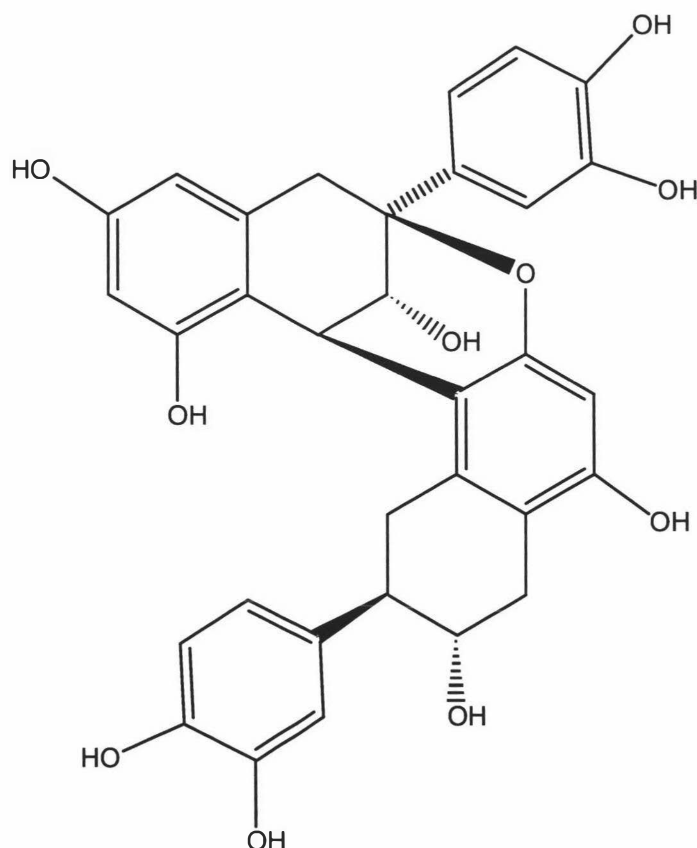


Figure 1.3 A-type linkages for proanthocyanidin epicatechin-(4β→8, 2β→O→7)-catechin.

The CT polymer differs in structure, mean degree of polymerization (mDP), branching and the average molecular weight. The CT oligomers are of chain length 2-10 monomer units, while the chain length greater than 10 monomer units are referred to as CT polymers (Prior et al., 2002). According to the Bate-Smith and Swain (1969) definition, CT are "polyphenolic compounds with a range of molecular weight between 500 and 3000 Da." However, CT with molecular weight above 3000 Da have been isolated from leguminous forages. The CT isolated from forages have molecular masses ranging from 1800-2700 Da comprising from 6 to 9 flavan-3-ol units (Foo et al., 1997).

However, CT may have ester linkages in the C-3 position, especially if the C-ring is functionalised with gallate groups (Figure 1.4) and this may change their biological properties. When considering different monomer units that CT contain, it can be realised that there are an infinite number of structures that CT can take leading to differences in chemical and biological properties (Haslam, 1996).

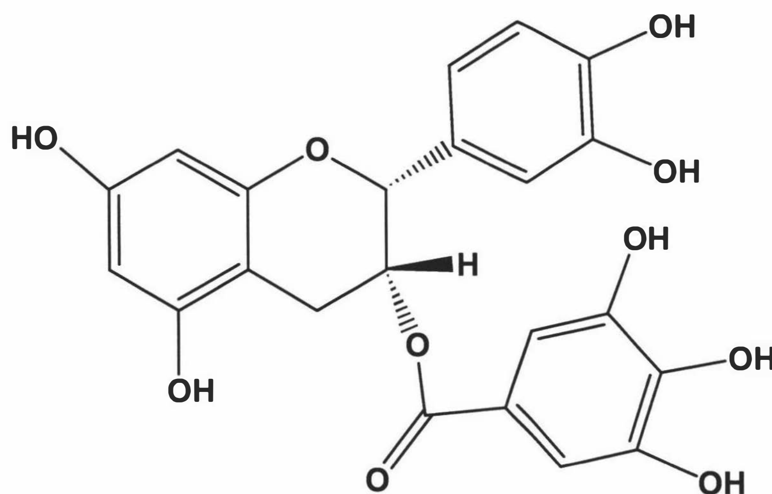


Figure 1.4 Structure of (-) epicatechin-3-O-gallate, a major component in green tea and wine.

CT isolated from green tea, grape seeds and red wine contains (−)-epicatechin-3-O-gallate (De Silva et al., 1991) which exhibits antibacterial properties.

1.3 Structure-activity relationships

The difference in activity, which is usually ascribed to the ability of CT to precipitate protein, varies in forages such as LP, LCS, sainfoin and the affinity for proteins could be attributed to the concentration, type, molecular weight and shape, mDP (Nelson et al., 1997) and the rate of gallolylation (Ricardo-da Silva et al., 1999). Interaction of CT with proteins involves hydrophobic and hydrogen bonding

interactions. The CT from LP and LCS differ markedly in their chemical structure. The average Mw of LP is 2200 which is usually higher than the Mw of LCS which is 1900, and it is observed that reactivity increases with increasing molecular weight (Ferreira et al., 2000; Foo et al., 1996). Hence, high molecular weight CT interacts more strongly with proteins and enzymes.

Condensed tannins can differ in hydroxylation pattern of the A- and B-rings of the flavan-3-ol monomer units. In procyanidin (PC) polymers the constituent flavan-3-ol units are either catechin or epicatechin, while prodelphinidin (PD) polymers contain either gallocatechin or epigallocatechin (Figure 1.1). The monomeric precursors of CTs, do not interact with proteins, but dimers weakly react while trimers strongly react (Artz et al., 1987). The extractable CT from LP has been found to be more effective at reducing protein degradation than LC. The ratio of PC to PD units contributes to the reactivity of CT and therefore reactivity increases with increasing PD content (Foo et al., 1997). It should also be noted that PD units have additional OH's on B-ring which could be contributing to increased activity of CTs containing PD units. It can be concluded that the high concentration of OH groups on the B-ring and functionalisation with esterified gallates contribute to the reactivity (Sarni-Manchado et al., 1999). Gallates isolated from green tea have been observed to exhibit antioxidant properties (Haslam, 1989). The acetone-water extract of sainfoin consists of a mixture of various substances including arbutin, kaempferol, quercetin, rutin, afzelechin, L-tryptophan, (+) pinitol, sucrose and condensed tannins (Marais et al., 2000). It thus becomes difficult to ascertain the compounds that could be responsible for its bioactivity, but its CT is PD predominant. It is not yet clear whether the difference in activity amongst forages legumes could be attributed to increasing Mw of CT, increasing PD content or molecular structure. Further research needs to be undertaken for other CT-containing forages, as characterization has been performed in detail for LCS, LP and sainfoin (Asquith et al., 1986), but not for sulla.

1.4 Effects of CT on inhibition of protein degradation

CT are known to complex reversibly with proteins through hydrophobic and hydrogen bonding interactions and the CT-protein complex is pH dependant (Haslam, 1989). The CT bind to protein at pH range of 5.0-8.0 and dissociate at pH less than 3.5 in the abomasum and at a pH of greater than 8.5 in the small intestine allowing proteins to be fully digested and absorbed in the small intestine. CT reduce the degradation of

proteins in the rumen (Figure 1.5) by protecting proteins from degradation by rumen microflora such as protozoa, fungi and bacteria, that lead to an increase in absorption of amino acids in the small intestine (McNabb et al., 1990). Comparison has been done using other forages like white clover (legume) and perennial ryegrass which contain trace amounts of CT and have low duodenal non-ammonia nitrogen flow which is about 0.75 N of intake (Barry et al., 1984).

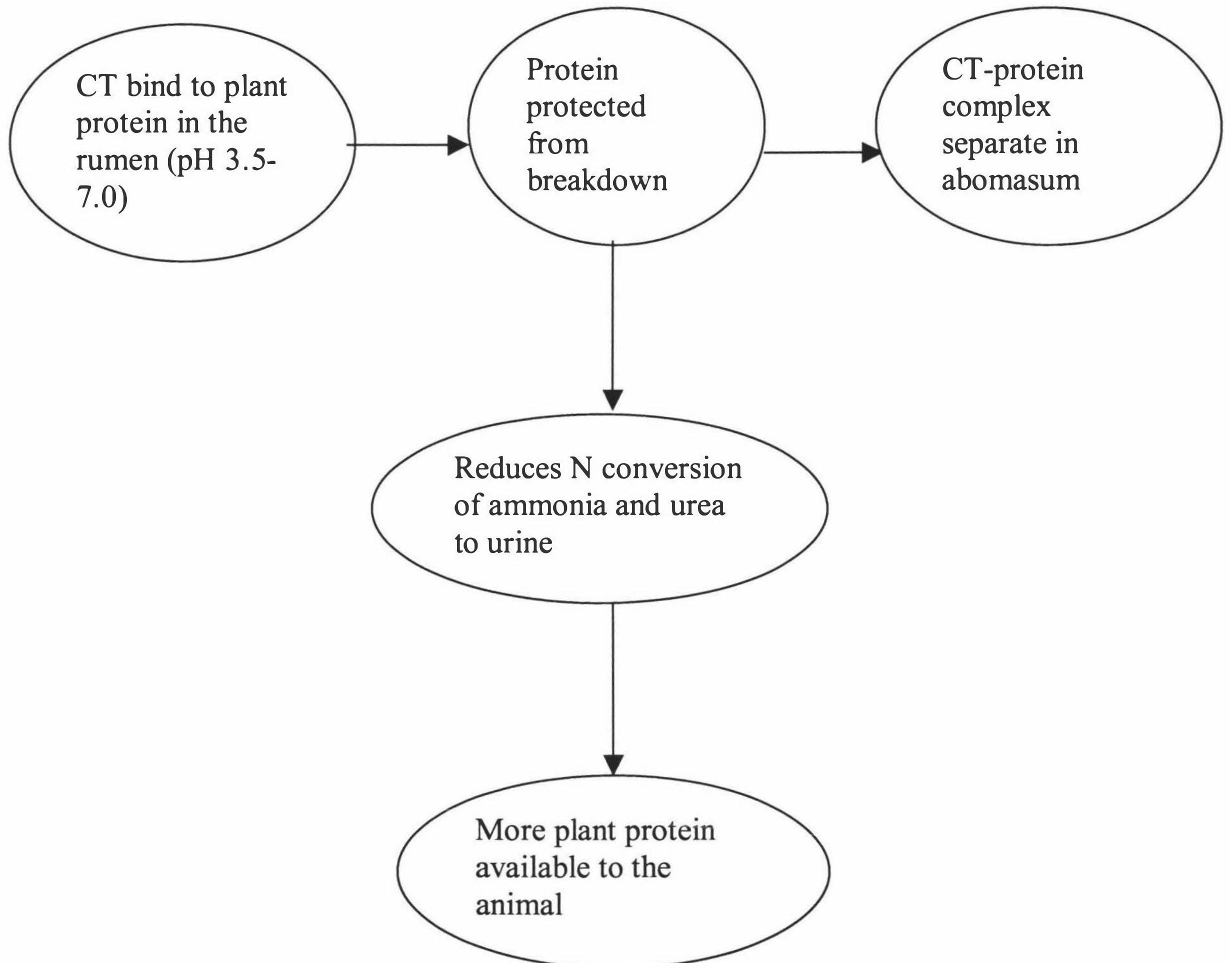


Figure 1.5 A diagrammatic representation of protection of plant protein in the rumen by condensed tannin containing forage.

Protein degradation inhibition assays investigate the effect of CT from leguminous forage such as LP on the digestion of the common leaf protein, ribulose-1,5-biphosphate carboxylase (Rubisco). Rubisco represents 30-50% of total leaf protein in plants and the *in vitro* experiments have shown that CT reduced the digestion of Rubisco in the rumen of sheep fed LP (McNabb et al., 1996) and this was mainly due to the ability of CT to slow down its degradation by rumen micro-organisms without affecting its solubilisation. Bovine serum albumin (BSA) has been employed as a model

protein, but fraction 1 (Rubisco) which is a major component in plants is now commonly used and is more relevant than BSA which has a linear structure. A similar study by Aerts (1999) conducted on LCS and LP, where the quantitative analysis was performed using sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE) and imaging densitometry, showed that at a concentration of 0.89 and 1.79 mg CT mg^{-1} total soluble proteins respectively, the CT from LP were more effective at reducing the degradation of LSU (large subunit) and SSU (small subunit) by rumen micro-organisms than the CT from LCS (Aerts et al., 1999). This indicates that the rate of degradation by rumen microbes vary depending on the molecular structure, composition and type of CT (PC:PD ratio). LP and LCS have similar mean molecular weights, but their flavan-3-ol composition differs. The inhibitory effects of CT are not only due to substrate binding to Rubisco, but also the binding to rumen bacteria and exogenous enzymes and inhibition of the proteolytic activity of some species (Nelson et al., 1997). CTs have got a higher affinity for PEG than proteins and as such protein-CT complexes can be dissociated by PEG which deactivates CT. PEG can be very helpful in reducing high concentrations of CT in tropical forages.

1.5 Extraction and Chromatography

CTs, despite their diverse and large polymeric size are soluble in various solvents and solvent mixtures of methanol, acetone, ethanol and water. This has made the isolation of CT possible. Chromatographic techniques including reverse phase (RP) and normal phase HPLC (Svedstrom et al., 2002) are widely employed for analysis of these compounds.

1.5.1 Plant harvesting and storage

After a plant is harvested, the major task is to preserve the compounds of interest intact prior to extraction and analysis. Freeze-drying (lyophilisation) is considered a safe procedure (Cork et al., 1991) for preservation of phenolics, but the major drawback may arise if after milling the sample absorbs moisture, which may result in decomposition of compounds. Some volatile phenolic compounds are lost during freeze-drying (Waterman and Mole., 1994). Fresh frozen material is considered to be the ideal storage method and extraction should be performed immediately because decomposition can occur when the plant material thaws, reducing the recovery of phenolics and CT.

1.5.2 Extraction and purification

Phenolics can be extracted from the plant material using methanol/water (50:50) and polymeric CT with acetone/water mixture (usually 70:30 or 50:50). The conventional Bate-Smith method employed the use of boiling 50% MeOH mixture for extraction of CT (Foo et al., 1980).

Purification of a crude extract of the plant material through Sephadex LH-20 involves eluting with excess 50% MeOH followed by subsequent elution with a 70% acetone/water mixture. Sugars and low molecular weight CT are eluted with the 50% MeOH and the CT adsorbed to the column in elution with 50% MeOH are eluted with an acetone/water mixture. This shows that 50% MeOH is not a good solvent for elution of the CT polymer. Acetone has the ability to dissociate the CT-protein bonds and inhibit CT-protein interaction. Sephadex LH-20 is a derivatised polysaccharide which is lipophilic and the principle for separation involves adsorption of the CT polymer in the matrix which is then eluted with acetone-water mixture (Bohm, 1998). Terrill (1992) showed that some CTs are free, being extractable with various solvents, while some are protein or fibre bound. Other gels like polyamides, cellulose and silica are ideally suited for low molecular weight CT oligomers and simple phenolic derivatives such as flavonols, isoflavones and methylated flavones (Markham, 1982).

1.5.3 Screening with HPLC

The use of HPLC separation coupled to a photodiode array detector (PDA) is a powerful analytical technique. CT absorb at wavelengths between 269-280nm, while flavonoids can be detected between 240-360nm with absorption maxima in the range 240-285nm (Band II) and 300-350 nm (Band I) reflecting A and B-ring, respectively. The absorption maxima is usually observed around 280 nm in the PDA chromatogram indicative of a CT polymer (Figure 1.6) while sharp peaks are suggestive of flavonoids and CT oligomers.

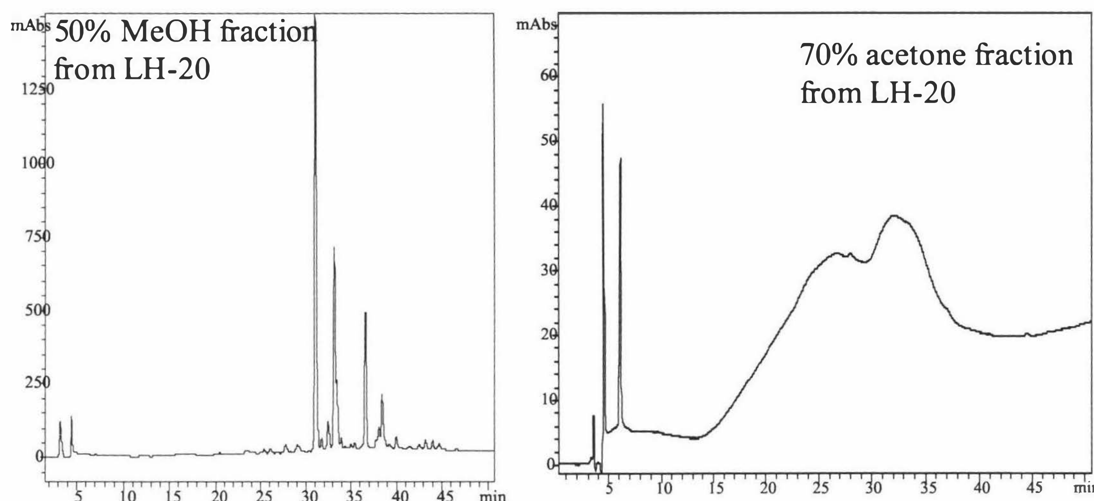


Figure 1.6 HPLC-PDA chromatograms for a 50% MeOH fraction and a 70% aqueous acetone fraction eluted from an LH-20 column from sulla leaves.

The HPLC column routinely used for the analysis of polyphenols is a RP silica employing C18 (octadecyl silica, ODS) and elutes polar compounds more quickly while retaining non-polar compounds (Waterman and Mole, 1994). One of the features of phenolics is that they are soluble in aqueous media and polar solvents. This allows elution to be performed in acetonitrile/water, methanol/water in acidic (formic, acetic and pH buffer adjusted to acidic conditions) conditions that inhibit the presence of ionized forms of the phenolics (Robards et al., 1997).

1.6 Chemical reactions of condensed tannins

Degradation reactions of the C-C interflavanic bond of the CT polymer have assisted in the study of the individual components of the polymer. However, ^{13}C-NMR plays a pivotal role to provide information on the mDP, the PC to PD ratio and on the nature of terminal units in the CT polymers (Czochanska et al., 1980; Foo and Porter., 1980; Foo et al., 2000; Behrens et al., 2000). Since CT molecules are large, long acquisition times are required for analysis of CT fractions.

1.6.1 Acid catalysed degradation with benzyl mercaptan

Quantitative and qualitative determination of thiolysis degradation products have been elucidated through the use of ^{13}C-NMR (Foo et al., 1996) and RP-HPLC (Prior et al., 2002; Koupai-Abyazani et al., 1992). Analysis of CT monomeric units by thiolysis uses reactive nucleophiles such as benzyl mercaptan (Harborne, 1994). The interflavanic C-C bond of the extender units may be cleaved under acidic

conditions in the presence of benzyl mercaptan (Figure 1.7), especially through the use of glacial acetic acid (partial thiolysis) which yields dimers and trimers while dilute HCl (full thiolysis), yielding monomeric benzyl thioether derivatives and free flavan-3-ols (Kennedy et al., 2001). The products of the thiolysis reactions can be analysed using RP-HPLC and ^{13}C NMR to determine the nature of each individual unit, average molecular weight, PC:PD ratio and mDP (Haslam et al., 1989).

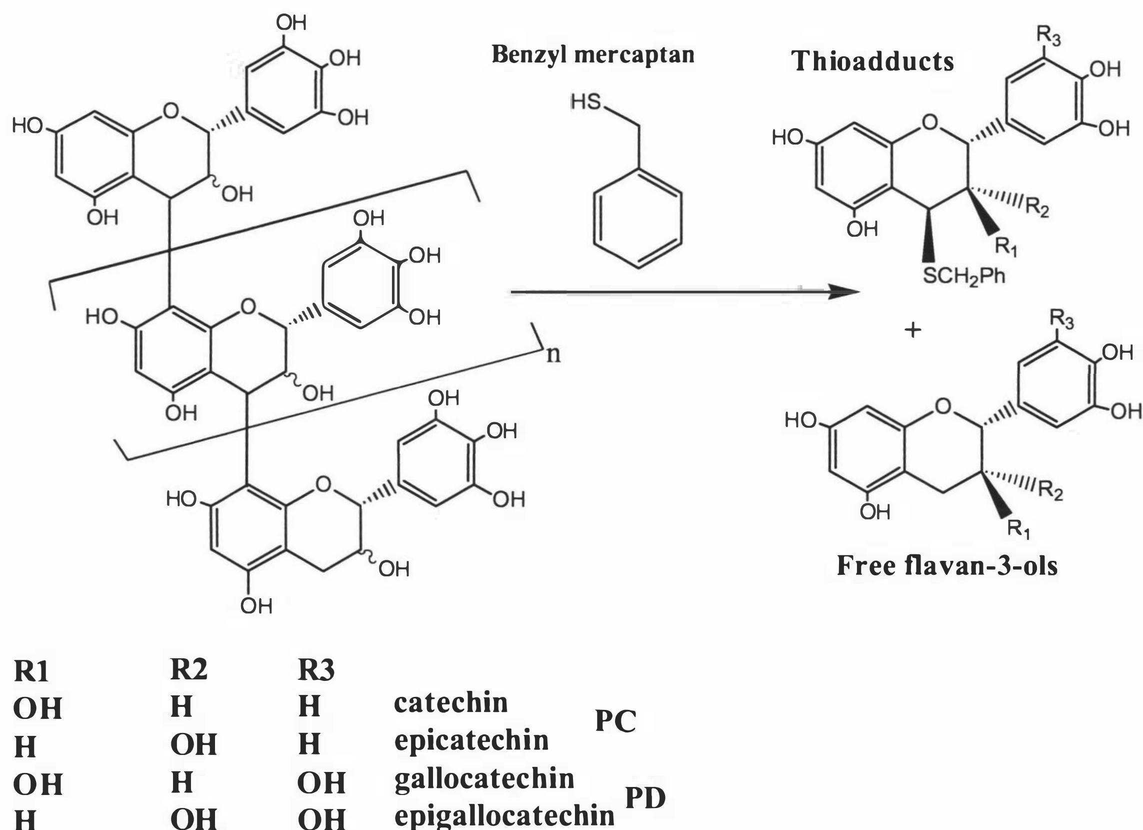


Figure 1.7 Thiolytic degradation of CT polymer using benzyl mercaptan yielding free flavan-3-ols (terminal units) and thioylether derivatives (extender units).

1.6.2 Acid catalysed degradation with phloroglucinol

Reaction of the CT polymer has also been performed with another reactive nucleophile, phloroglucinol and rapid analysis carried using RP-HPLC (Scalbert et al., 1997). However, low yields, incomplete reaction and self-polymerisation have been associated with the use of phloroglucinol while favourable results have been obtained through the use of benzyl mercaptan. Despite its unpleasant odour, toxicity and lachrymatory characteristics, benzyl mercaptan remains a suitable reagent in the analysis of CT polymers. Superior yields have been reported when thiolysis was carried for 4.5 hrs at a temp of 105 °C (Scalbert et al., 1997). However, the advantages of using

phloroglucinol as a trapping reagent are that is odourless, has no special safety requirements (Hemingway et al., 1990) and stereochemistry at C-2 and C-3 positions is preserved (Koupai-Abyazani et al., 1992).

Mixed PC-PD polymers exist in plants (Porter et al., 1982). The products of phloroglucinol scission reaction showed LCS to be a polymer consisting of epicatechin (67%), epigallocatechin (30%) of the extender units (Table 1.1). The polymer is PC predominant (Foo et al., 1996).

Table 1.1 Composition of terminal and extender units of CT fractions from *Lotus corniculatus* and *Lotus penduculatus* (Foo et al., 1996).

	L. Corniculatus		*L. Penduculatus*	
Flavan-3-ols	Terminal %	Extender %	Terminal %	Extender %
Catechin	82	1.5	50	4.4
Epicatechin	16	67	20	18.7
Gallocatechin	0	0	20	13
Epigallocatechin	1.6	30	10	64

The polymer was also glycosylated and with number average Mw in the range 1800-2100 (six to seven flavonoids unit). LP showed to be a heterogeneous polymer with epigallocatechin being the dominant extender unit (Foo et al., 1996). The CT in sainfoin (Ferreira et al., 2000) consists of hetero- and homo-polymers and has a highly variable composition with *cis:trans* ratios ranging from 47:53 to 90:10 and PD to PC ratios from 36:64 to 93:7. Polymers isolated from plant food derived substances such as sorghum bran, cranberry and blueberry have been found to consist predominantly of PC units and through thiolysis, the extender units was predominantly epicatechin while the terminal unit varied between catechin and epicatechin (Prior et al., 2002). The mixture of flavan-3-ol units are also identified in oligomers, not only in polymers. Oligomeric PC isolated from *Craetaegus* leaves and flowers consist of trimer, epicatechin- 4β→8, epicatechin- 4β→6 –epicatechin (Svedstrom et al., 2002). From the literature, sulla is known to be PD predominant (McMahon et al., 1999), but its complete chemical characterization has not been performed.

1.6.3 Acid-butanol assay

Quantitative determination of concentration of CT from plant materials has been accomplished through the use of the colorimetric butanol hydrochloric acid (Bu-HCl) assay (Schofield et al., 2001). It is analogous to thiolysis as it involves acid degradation of the interflavanic C-C bonds, yielding red anthocyanidins (delphinidins and cyanidins) in the presence of traces of metal impurities such as iron salts (Porter et al., 1986). Anthocyanidins absorbs UV light at a wavelength of 525 nm (Barry, 1985). Different varieties of LCS have been analysed using the Bu-HCl assay and these had variable PD:PC ratios of 16:84 to 33:67 amongst seven varieties (Hedqvist et al., 2000). From these ratios it can be deduced that LCS is PC predominant. Other quantitative colorimetric assays include vanillin-HCl, Prussian blue and Folin Ciocalteu. Colorimetric assays have some limitations because they lack selectivity and sensitivity especially in plants with low CT concentrations (Scalbert et al., 1997).

1.7 Characterisation of phenolics with liquid chromatography- mass spectrometry

The coupling of liquid chromatography (LC) with an interface such as electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) to mass spectrometry (MS) has paved a way for simultaneous determination of components within a mixture to obtain structural information.

1.7.1 Electrospray ionization mass spectrometry (ESI-MS) of CT oligomers.

ESI-MS serves as a highly selective detector and provides on-line structural information (Fulcrand et al., 1999). This analytical technique has been applied in the detection of wine CT oligomers (Fulcrand et al., 1999) in a negative ionization mode. Negative mode is employed to measure the mass per charge ratio in a deprotonated form $(M-H)^-$. Singly charged ions were detected from dimers up to pentamers and doubly charged ions from pentamers to heptamers in wine CT. However, as the molecular weight and chain length increases, it becomes increasingly difficult to interpret spectra of CT oligomers and polymers with LC/MS due to multiply charged ions and enormous structural diversity (Souquet et al., 2000). The other drawback is that CT polymers can not be detected by MS due to their inability to ionise. Characterisation of CT compounds from forages has been achieved with increased sensitivity and resolution.

1.7.2 MALDI-TOF-MS

The use of matrix-aided laser desorption ionization time of flight (MALDI-TOF) MS has been utilised to characterize CT oligomers from LC varieties of chain length up

to decamers in positive ion mode (Hedqvist et al., 2000). The MALDI-TOF-MS spectra revealed more heterogeneous CT oligomers than homo-oligomers. The matrix and the solvent play a major role in the crystallisation of the analyte during sample preparation to improve spectra quality in MALDI-TOF-MS. Matrices such as 2,5-dihydroxy benzoic acid (DHB), trans-3-indole acrylic acid (IAA) and 2,4,6-trihydroacetophenone (THAP) were more effective in the analysis of lower and higher CT oligomers from grape seed extracts (Krueger et al., 2000). The CT oligomers were detected up to undecamers in a positive-ion linear mode. Behrens (2003) revealed the presence of CT oligomers up to undecamers in willow and beech leaves using MALDI-TOF-MS. Higher oligomeric CTs up to pentadecamer have been detected from apple extracts using IAA as the matrix (Ohnishi-Kameyana et al., 1997). No molecular ions were produced when sinapinic acid (SA) and α-cyano-4-hydroxycinnamic acid (CHCA) matrices were used. This suggests that the choice of matrix is vital in characterisation of CT polymers. MALDI-TOF-MS is more advantageous than ^{13}C-NMR or other MS techniques because it can elucidate a series of closely related CT oligomers of a mixture (Jian Wong et al., 2000). Future research should focus on coupling MALDI-TOF-MS with HPLC to provide on-line structural information.

1.7.3 ESI/MS and APCI/MS of flavonoids

APCI/MS is a robust technique complemetary to ESI-MS and focuses on fragmentation of compounds, thus providing additional structural information. Fragmentation of CT oligomers especially dimers provides information on the monomer unit composition.

Isolation and characterization of catechins, epigallocatechins, epicatechin gallate and epigallocatechin gallate with *m/z* value of 289, 305, 441 and 457, respectively have been accomplished in tea extracts using LC-ESI-MS (Poon, 1998). Epigallocatechin-3-gallate is the major compound responsible for its biological activity. In other instances, further fragments can be obtained by tandem MS-MS with the use of authentic standards to provide additional structural information. The development of ESI-MS has various advantages with the capability of increased sensitivity, detection of low concentrations of analytes and analysis of large polar compounds (Carreri et al., 1998). In addition, no derivatisation is required (Hakkinen, 1998). ESI-MS has found many applications in the analysis of flavonoids from medicinal plant extracts, foods and fruits. The most commonly use of these techniques has been in the characterization of

flavonoids over a selected mass range. Soft ionization and sensitive ESI-MS provided analysis of flavonoids such as rutin, quercetin, and genistein from tomato puree (Mauri et al., 1999). Quercetin, kaempferol and arbutin are present in the forage legumes LC, LP. Further information can be attained by fragmentation of flavonoid adducts such as sugars and acyl adducts which are not UV absorbing (Graeyer, 2000). However, when ESI-MS and APCI-MS were employed in a negative ionization mode, pseudomolecular ions were obtained while more fragmentation were found in a positive mode and hence more structural information were obtained in analysis of phenolics from olive extracts (McDonald et al., 2001). Mass spectrometric techniques such as MALDI-TOF, MS/MS, FAB-MS, LSI-MS, have been explored in analysis of flavonoids in forages and recent research focuses on APCI-MS and ESI-MS due to better sensitivity and detection limit. It is important to validate the mass spectral data with NMR and of recent, it has been shown that on-line information can be obtained by coupling HPLC to NMR (Wolfender et al., 2001).

1.8 Sulla as an anthelmintic forage for parasite control

Diseases caused by parasites contribute enormous losses to the animal industry in New Zealand and worldwide. Due to the problem of anthelmintic resistance (Pritchard, 1990; Al-Qarawi et al., 2001) and rising consumer concerns about chemical residues in animal products, alternative non-chemical strategies are required. Recent surveys have shown that internal parasites in New Zealand have become resistant to anthelmintic drugs in sheep and cattle (Pritchard, 1994). A high level of resistance has been reported to benzimidazole drenches (Jackson et al., 1999). Some common parasites present in New Zealand sheep are collated in Table 1.2.

One alternative strategy to drenching may be to use CT-containing forages such as sulla (Waghorn et al., 1998). Alternatively, the solution may be to focus on reduction of the contamination of pasture with infective larvae rather than treatment of the infection. Unlike anthelmintic drugs, CT are not absorbed from the gastrointestinal tract (Terrill et al., 1994), so eggs shed by adult worms will be exposed to the effects of CT throughout their development. Polymeric CT may be carried intact throughout the digestive system and become eliminated with the faecal matter due to stable C-C linkages (Rogler et al., 1994). The focus is to reduce the build-up of infective larvae on pasture. If this is achieved, dependence on anthelmintic drugs may be minimized.

Table 1.2 Parasite checklist of nematodes of sheep in New Zealand

Location	Type
Abomasum	*Haemonchus contortus* *Ostertagia circumcinta* *Ostertagia trifucata* *Trichostrongylus axei*
Small intestine	*Cooperia curticei* *Nematodirus fillicollis* *Mematodirus spathige* *Trichostrongylus colubriformis* *Trichostrongylus vitrinus*
Large intestine	*Tricums Ovis*

(Adapted from Vlassof et al., 1994)

The economically important abomasal nematodes in New Zealand are *Haemonchus contortus*, *Ostertagia spp.* and *Trichostrongylus spp.*(Vlassoff and Bisset 1991) and these are the most resistant to anthelmintic drugs in Australia (Donald et al., 1978). In the small intestine, *Trichostrongylus colubriformis* are the most economically important parasitic nematode in New Zealand.

1.8.1 Life cycle of gastrointestinal nematode *Trichostronglus colubriformis*

Larvae on pasture become ingested by the sheep during grazing and migrate to the abomasum or small intestine depending on the type of nematode. The process involves adult worms in the gut laying eggs which pass into animal's faeces and fall onto pasture. The first stage larvae develop into egg and the eggs in the faeces hatch into larvae L1 (Figure 1.8) as they feed on bacteria in the faeces and is a non-infective stage. The cycle continues with L1 larvae feeding and growing into second stage larvae (L2), the L2 larvae feeds and grows into third stage larvae (L3) and the L3 larvae is ensheathed and is the infective stage. It is at this stage that animals consume the larvae in the grass or pasture. In the animal, L3 larvae develop to L4 larvae and to immature adult worm (L5) in the intestine (Bruere and West, 1993). Egg hatch (EH) and larval development (LD) assays are routinely used to evaluate the effects of CTs from forage legumes on the gastrointestinal nematodes of sheep *in vitro*. The CTs from forages can disrupt their life cycle by reducing the number of eggs hatched and inhibiting the development of the first stage larvae (L1) to the third infective stage (L3) on pasture. The purpose of this investigation with sulla fractions using EH and LD is to arrest the developmental stages of the eggs and larvae, hence reducing contamination on pasture with viable eggs and infective larvae.

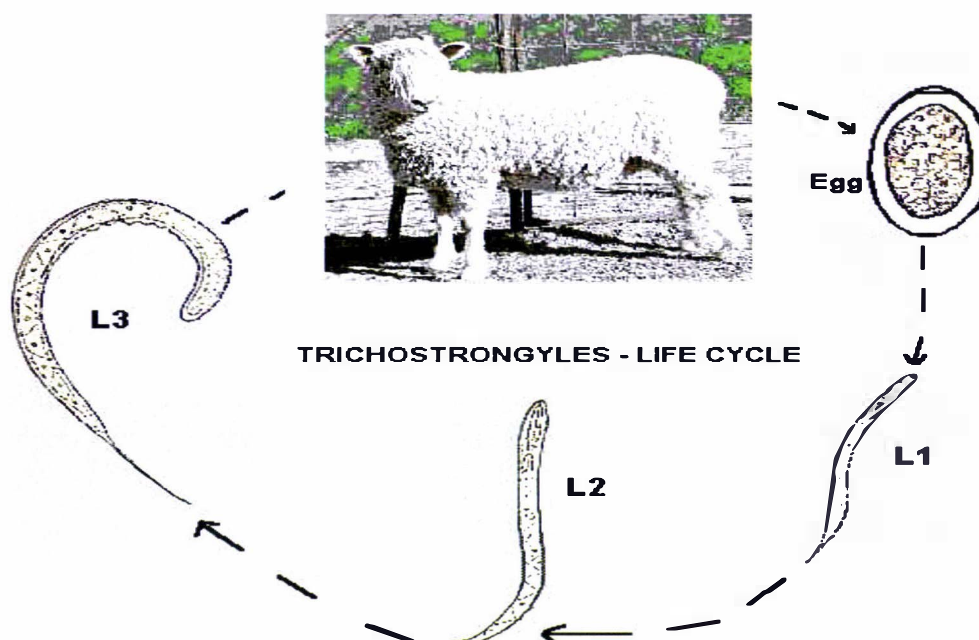


Figure 1.8 The basic life cycle of *Trichostrongylus colubriformis* nematode in sheep (Bruere and West, 1993).

In New Zealand the damp moderate climate is very favourable for the development and survival of the free living stages of nematodes on pasture. The eggs require warm, moist conditions to develop successfully. Below 10 °C, the development is slow and most eggs fail to hatch (Vlassof, 1991).

1.8.2 Effects of nematodes on animal nutrition

Gastrointestinal nematodes usually referred to as roundworms, are found in the gastrointestinal tract (abomasum and the small intestine), pose a major threat to the animal industry in New Zealand and are responsible for high production losses, clinical diseases and mortality (Sykes, 1982). Parasites cause damage to the small intestine and the abomasum (Sykes et al., 1982; Bruere and West, 1993) and affect digestibility and absorption of nutrients. Hence, nutrients will be channelled for protein repair rather than skeletal and body development.

1.8.3 Effects of CT on GI *in vitro* parasite assays

CT may have direct or indirect effect on resistance and resilience of ruminants to gastrointestinal nematodes (Kahn and Diaz-Hernandez., 1999). *In vitro* studies, using EH and LD bioassays have shown that Sephadex LH-20 70% acetone fractions containing CT from forage legumes may reduce contamination on pasture with infective larvae and viable eggs by breaking the life cycle of sheep nematodes, *T. colubriformis* (Molan et al., 1999). CT fractions from LP, LCS, sainfoin and sulla

were found to inhibit egg hatching and development of L1 larvae to L3 larvae. Sainfoin and LP had the highest inhibitory effects compared to CT fractions from sulla and LC. The anthelmintic effect of a commercially available CT from quebracho was evaluated in sheep (Athanasiadou et al., 2001) and the results showed that the level of GI was not affected. However, in another study direct toxicity of CT on larvae has been observed using dietary quebracho extract (Butler et al., 2001) which led to reduction of worm numbers and involved a direct effect of CT on parasites. *In vitro* assays using EH and LD have been performed by Molan (2002b). The study showed that Sephadex LH-20 70% acetone fractions containing CT from seven forages, LP, LCS, sulla, sainfoin, dock, *Dorycnium pentaphylum*, and *D. rectum* significantly inhibited the development of eggs into L3 larvae over a concentration range of 200 μg/mL and 400 μg/mL. The addition of CT fractions after hatching also inhibited the development of L1 larvae to L3 at a concentration of 200 μg/mL. A similar trend was observed that % hatching and % LD (Figure 1.9) decreased with the increasing concentrations of the CT fractions.

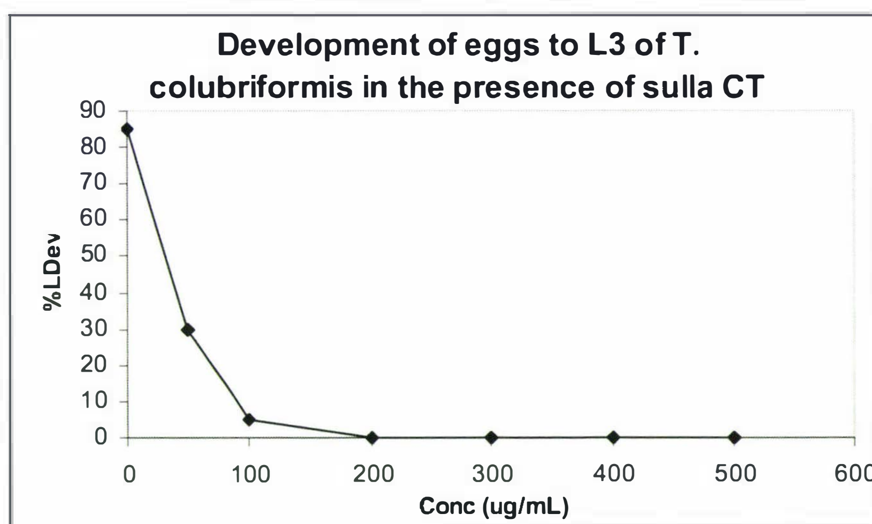


Figure 1.9 Inhibition of development of *T. colubriformis* eggs to L3 in the presence of CT (Adapted from Molan et al., 2002, with permission from A.L. Molan).

Differences in activity amongst the seven CT-containing forages could be attributed to the molecular structure, type and composition of CT. The mechanism behind inhibition of egg hatching is not known, but it is believed that CTs may deactivate the hatching enzymes such as lipases, chitinases, β-glucosidases, and proteases (Molan et al., 2002). The CT fractions from sulla, LP, LCS, sainfoin may also cause paralysis to the L1 larvae, therefore preventing their development to L3 by

interacting with the cuticle of the larvae or during feeding the larvae may swallow the CT which paralyses the pharyngeal muscles and inhibits further feeding (Molan et al., 1999). The LH-20 70% acetone fractions containing CT used in the EH and LD assays were extracted from sulla and other forages using 70% acetone and purified through Sephadex LH-20 chromatography (Molan et al., 1999; 2002).

1.8.4 Effects of CT on GI using larval migration inhibition assay

The larval migration inhibition assay (LMI) is the measure of the ability of the test material to immobilise infective larvae to prevent them from passing through 20 μm mesh sieves. This assay measures the reductive capacity *in vitro* of the L3 larvae and may mimic the *in vivo* conditions as the larvae squeeze themselves through in order to penetrate the lining of abomasum or small intestine. Molan (2000a) showed that incubation of L3 larvae in the rumen and abomasal fluid in sulla extract reduced the migration of larvae compared to that in control incubations. In another study, seven CT containing forages were evaluated (Molan et al., 2000b). At a concentration of 100 μg/mL CT from sulla, LP, LCS, sainfoin, *D. rectum*, dock and *D. pentaphylum* inhibited 20%, 10%, 15%, 25%, 28%, 32% and 27% of larvae, respectively to pass through the sieves compared to controls. Inhibitory effects measured by LMI showed that CT from LP was more potent in preventing the migration of *T. colubriformis* larvae *in vitro*. Activity was also found to be different among the CT-containing forages.

1.8.5 *In vivo* parasite activity of sulla

Parasitised lambs have been observed to have improved performance when fed forages which contain CT relative to conventional forages. Feeding of infected lambs with lucerne increased worm numbers of *T. colubriformis* and *O. circumcinta* while infected lambs fed sulla had reduced worm burdens and lower faecal egg count (Niezen et al., 1995; 2002a). The mechanism for high performance for infected animals is still not well understood, but CT are known to protect plant protein from degradation by rumen organisms and increase the flow of amino acids in the small intestine. It is well understood that animals given increased planes of nutrition can resist diseases and infections. It was noted that there was enhanced immunological response attributed to the sulla diet. Dietary protein supplied from fish meals and protein meals have been associated with enhancement of immune system in parasitised animals (Van Houtert et al., 1996). Alternatively, the CT may interact directly with the internal parasites in the

gastrointestinal tract. In another study by Niezen (1998) infected lambs fed *Lotus* (LP), which contains CT had lower *O. circumcinta* burdens, lower faecal egg counts and increased growth rates relative to lambs fed perennial ryegrass. However, unexpected results were obtained because the *T. colubriformis* nematodes were not affected. *In vitro* assays have shown LH-20 70% acetone fractions from sulla to be effective in inhibiting EH and reducing the development of eggs to L3 (Molan et al., 1999; 2002b). It has been observed that the host diet play a major role in reduction of egg hatching and larval development of *T. colubriformis* in the laboratory and on pasture (Niezen et al., 2002b). The number of larvae recovered from the faeces placed on pasture was found to be higher when the lambs were fed lucerne than sulla. The CT in the diet was found to be responsible for observed differences in egg hatch and larval development. The effects measured *in vitro* gave underestimates to effects measured under field conditions.

1.9 Aims of the Thesis

The aim of the thesis is to extract, separate and chemically characterise phenolics and CT from sulla (*Hedysarum coronarium*) leaves from both freeze-dried and fresh frozen plant material. The isolation of fractions is to be performed using chromatography on Sephadex LH-20 and RP-HPLC with structural elucidation carried out employing ^{13}C-NMR, ESI-MS and MALDI-TOF-MS spectroscopy and acid catalysis thiolytic degradation with benzyl mercaptan. Comparison of the CT composition; PC:PD ratio, stereochemistry and the mDP can be obtained with these techniques. The CT content is to be investigated using the Bu-HCl assay. Flavonoids are to be characterised using ESI-MS and APCI-MS.

The anti-parasitic effects of the fractions from sulla leaves, purified through Sephadex LH-20 are to be investigated using the EH and LD *in vitro* assays. The aims of the project are to study the structure-activity relationship of phenolics and CT fractions from sulla, in relation to the gastrointestinal nematodes *T. colubriformis* in sheep, which is economically important to New Zealand's agricultural industry.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Plant material

Fresh sulla (*Hedysarum coronarium*) plants were collected on different dates from Aorangi, an AgResearch farm near Palmerston North in New Zealand. Sulla flowers and leaves were harvested from a plant stand approximately 24 months old, 80 cm tall, 4 months old re-growth (frost damaged) in summer, 21st of December 2001 by Garry Waghorn. It was stored in a cool room overnight and transferred to plastic bags after removal of flowers and was kept frozen at -20 °C. Fresh frozen material was later freeze-dried and milled.

A second batch of sulla leaves were collected by Matt Deighton in spring, 5th of August 2002. The material was kept in the freezer prior to extraction.

The third batch of sulla leaves was also collected from Aorangi by Peter Schreurs in spring, 23rd of September 2002. The remaining fresh frozen material of spring (23/09/02), 1.54 kg coded OTA03501 was freeze-dried and milled.

The last batch was collected from Aorangi in summer, 12th of December 2002 and kept fresh frozen prior to extraction.

2.1.1 Plant extraction for freeze-dried material

Extraction was carried out according to the procedure previously used by Foo (1996). Lyophilised and milled sulla plant material (100 g), summer (21/12/01) was transferred into a 3L volumetric flask, followed by addition of 2L of 70% acetone (Australasian Solvents and Chemical Company, AR) containing 1 g/L ascorbic acid (BDH, England) and stirred for 2 hours. The solution was strained through three layers of a cheese cloth and the filtered extract was retained. The plant residue was further extracted using 70% acetone for 2 hours. The first and second filtered extract were pooled and concentrated under a reduced pressure using a Rotavapor (Buchi Rotavapor R-114) at 40 °C and 500 mL for each extract was recovered. Each concentrated extract solution was washed four times with dichloromethane (DCM) 500 mL. The DCM washings were discarded. The brown defatted aqueous layer was concentrated under reduced pressure for 30 minutes to remove residual DCM. Extract 1 and 2 were pooled and about 600 mL of the extract was partitioned with 3×200 mL ethyl acetate. The aqueous layer was rotavaped to remove residual ethyl acetate and was placed in a small

plastic bag (OTA00201). The ethyl acetate layer was concentrated *in vacuo* and a minimal amount of water added. The extract was freeze-dried (OTA00202). An emulsion layer was formed during washing which could be attributed to small amounts of acetone. An intense red colour was observed and could be attributed to anthocyanins from flowers. About 8×100 g batches for plant extraction were performed for sulla plant, summer (21/12/2001).

Table 2.1 Batches for sulla plant extraction yielding ethyl acetate and aqueous layer extracts.

Sample name	Date collected	Aqueous layer	Weight (g)	Ethyl acetate layer	Weight(g)
Summer	21/12/01	OTA00201	20.62	OTA00202	0.52
		OTA00301	19.94	OTA00302	0.33
		OTA00501	21.83	OTA00502	0.56
		OTA00701	17.77	OTA00702	0.54
		OTA00901	24.10	OTA00902	0.46
		OTA01101	23.65	OTA01102	0.55
		OTA01701	23.38	OTA01702	0.47
		OTA01901	24.24	OTA01902	0.43
Spring	23/09/02	OTA09601	17.36	OTA09602	
Spring	05/08/02	OTA01301	25.44	OTA01302	0.56
		OTA01501	62.06	OTA01502	0.64
Spring	23/09/02	OTA03502	25.14	OTA01302	0.164
Summer	12/12/02	OTB02901	15.29	OTB02902	0.14

2.1.2 Plant extraction for fresh frozen material

Fresh frozen sulla plant material 1kg (3×330 g), collected in spring, 5th of August 2002, was ground and extracted in a blender (Hallde VCM62 Varning, AB Hallde Maskiner, Kista, Sweden) with 1.8 L (3×600 mL) of 70% acetone containing 1 g/L ascorbic acid for 10 minutes. The mixture was filtered through two layers of cheese cloth to remove the plant material. The mixture was washed 4 times with equal amounts of DCM (500 mL). After concentrating under a reduced pressure, partitioning was carried out using ethyl acetate (3×150 mL). The aqueous layer and ethyl acetate layer were freeze-dried. The colour of the aqueous layer was found to be light brown, which was different from the previous extractions. This might have been attributed to leaving the plant acetone/water mixture in the fridge for 2 days, which might have led to oxidation or the CT concentration was low.

2.2 Purification with Sephadex LH-20 Column

2.2.1 Packing of the LH-20 Column

Sephadex LH-20 gel (128g) purchased from Pharmacia, Sweden was treated with 700 mL of 50% methanol and allowed to swell before loading onto a 25×1000 mm

column. The column was connected to a Pharmacia GradiFrac system and was equilibrated with 600 mL of 50% MeOH before the sample solution could be injected.

2.2.2 Step fractionation method with LH-20

The freeze-dried aqueous CT crude extract (5 g) was dissolved in 30 mL of 50% aqueous methanol and loaded onto a Sephadex LH-20 column (Pharmacia, SK25×300 mm, Sweden) equilibrated with 50% MeOH, connected to the Pharmacia GradiFrac system according to a procedure adapted from Foo (1996). Elution occurred at a flow rate of 5 mL/min. Four 50% MeOH fractions (total volume of 600 mL) were obtained; I (OTA02801, 3.5 g), II (OTA02802, 175 mg), III (OTA02803, 51.1 mg), IV (OTA02804, 28.8 mg) (Table 2.2). The column was further eluted with 70% acetone (600 mL) to obtain three fractions; V (OTA02805, 78.1 mg), VI (OTA02806, 369 mg) and VII (OTA02807, 7.25 mg) (Figure 2.1). All the fractions were concentrated under reduced pressure at 40 °C and freeze-dried. Three batches (5g/30 mL loaded onto Sephadex LH-20) were performed for this sample. Small volumes of the fractions were screened with HPLC-PDA.

Table 2.2 Step LH-20 fractionation for crude extracts from freeze-dried and fresh frozen sulla material. Abbreviations; FD- freeze-dried, FF- fresh frozen

Sample name	Date Collected	1st batch	Weight (mg)	2nd batch	Weight (mg)	3rd batch	Weight (mg)	Eluting solvent
Summer FD	21/12/01	OTA02801	3500	OTA03101	3420	OTA03701	3250	50% MeOH
		OTA02802	175	OTA03102	353	OTA03702	491	50% MeOH
		OTA02803	51.1	OTA03103	68.6	OTA03703	70.5	50% MeOH
		OTA02804	28.8	OTA03104	33.3	OTA03704	32.2	50% MeOH
		OTA02805	78.1	OTA03105	96.3	OTA03705	69.3	70% acetone
		OTA02806	369	OTA03106	361	OTA03706	386	70% acetone
		OTA02807	7.25	OTA03107	5.76	OTA03707	4.63	70% acetone
Spring FF	05/08/02	1st batch for elution of OTA01301		2nd batch for elution of OTA01501				
		OTA03801	3350	OTA04501	2220			50% MeOH
		OTA03802	249	OTA04502	66.34			50% MeOH
		OTA03803	45.6	OTA04503	44			50% MeOH
		OTA03804	17.3	OTA04504	17.3			50% MeOH
		OTA03805	25.9	OTA04505	91.9			70% acetone
		OTA03806	241	OTA04506	112.5			70% acetone
		OTA03807	4.1	OTA04507	4.37			70% acetone

The colour of fraction 1 was intense brown. Fraction 2-4 was light yellow while fraction 5 and 6 were light brown and fraction 7 was colourless. The same procedure from above

was used for fresh frozen sulla (spring-23/09/02) and two batches were obtained for OTA01301 and OTA01501 aqueous crude extract loaded onto LH-20, respectively.

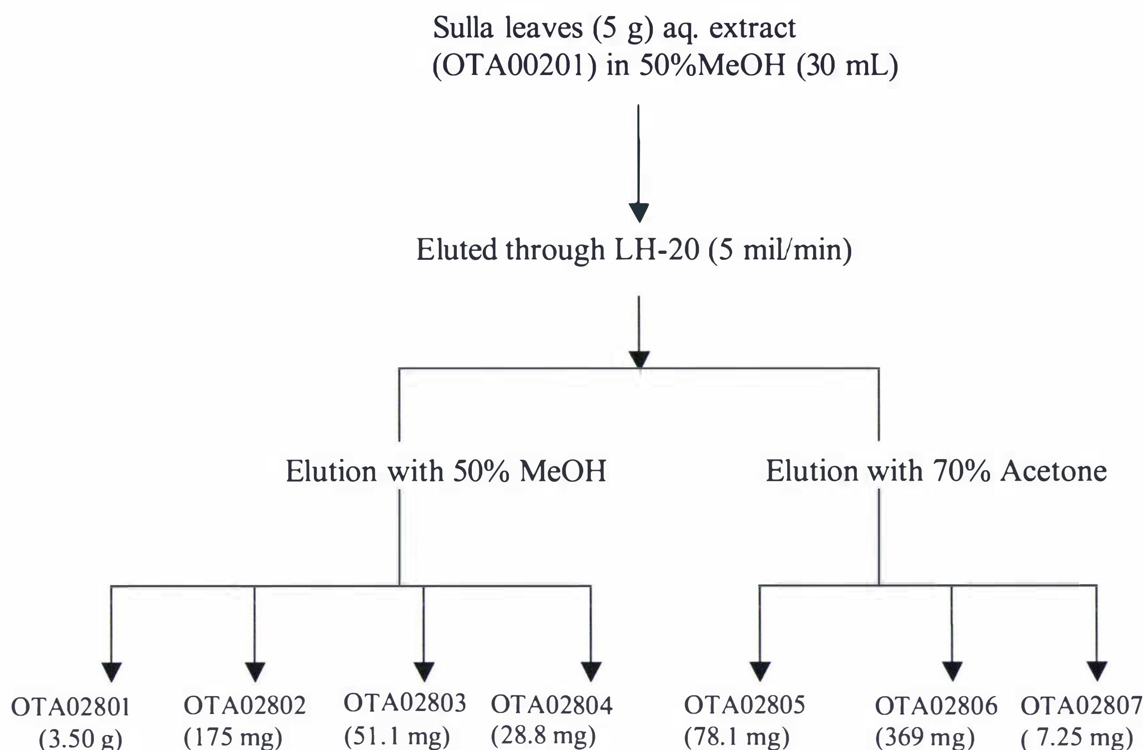


Figure 2.1 Flow chart for separation of aqueous crude extract through Sephadex step LH-20 column chromatography.

2.2.3 Gradient fractionation method with LH-20

Sulla aqueous crude extracts (17 g, OTA01301 and OTA01501 from spring 23/09/02) was dissolved in milliQ-water (100 mL) and loaded onto a Sephadex LH-20 column (25×1000 mm Pharmacia, Sweden) packed with 50% MeOH and connected to the Pharmacia GradiFrac system. Elution was performed at a flow rate of 7 mL/min. The gradient involved elution with water and then increasing concentration of MeOH (25% MeOH, 50% MeOH, 75% MeOH, 100% MeOH) and finally 70% acetone (Table 2.3). Two water fractions (650 mL); I (OTA04301, 10.67 g), II (OTA04302, 2.84 mg), one 25% MeOH fraction (350 mL); III (OTA04303, 68.8 mg), one 50% MeOH fraction (500 mL); IV (OTA04304, 171 mg), one 75% MeOH fraction (500 mL); V (OTA04305, 191 mg), four 100% MeOH fractions (1200 mL); VI (OTA04306, 28.4 mg), VII (OTA04307, 40.8 mg), VIII (OTA04308, 29.6 mg), IX (OTA04309, 41.5 mg), and two 70% acetone fractions (1200 mL); X (OTA04310, 378 mg), XI (OTA04311,

26.8 mg) were obtained. All the fractions were concentrated under reduced pressure at 40 ℃ and freeze-dried. Two batches (17g/100 mL loaded onto LH-20) were performed for this sample and a similar method was used for other sulla extracts (Table 2.3). Small volumes of the fractions were kept and run by HPLC-PDA.

Table 2.3 Sulla gradient LH-20 fractions from freeze-dried and fresh frozen plant material

Sample name	Date collected	1st batch	Weight (mg)	2nd batch	Weight (mg)	Eluting solvent
Summer	21/12/01	OTA03901	3000	OTA09801	9720	H_2O
		OTA03902	1150	OTA09802	1550	H_2O
		OTA03903	80.7	OTA09803	85.1	25% MeOH
		OTA03904	164	OTA09804	255.9	50% MeOH
		OTA03905	223	OTA09805	283	75% MeOH
		OTA03906	55.3	OTA09806	60.9	100% MeOH
		OTA03907	41.5	OTA09807	84.3	100% MeOH
		OTA03908	51.9	OTA09808	72.5	100% MeOH
		OTA03909	65.2	OTA09809	106.9	100% MeOH
		OTA03910	479	OTA09810	431.7	70% acetone
		OTA03911	45.4	OTA09811	35.5	70% acetone
Spring	5/08/02	OTA04301	10670			H_2O
		OTA04302	2.84			H_2O
		OTA04303	68.8			25% MeOH
		OTA04304	171			50% MeOH
		OTA04305	191			75% MeOH
		OTA04306	28.4			100% MeOH
		OTA04307	40.8			100% MeOH
		OTA04308	29.6			100% MeOH
		OTA04309	41.5			100% MeOH
		OTA04310	378			70% acetone
		OTA04311	26.8			70% acetone

2.3 Chromatographic screening techniques

2.3.1 Liquid chromatography-PDA

Screening fractions by HPLC-PDA was performed using a Shimadzu LCMS QP8000 alpha equipped with a Shimadzu SPD-M10A VP PDA detector and connected to an Alltech reverse phase column (C18), Alltima column (250 × 2.1 mm, 5 μm) with 10 μL injections. A solvent gradient was used for elution: A = 0.1% formic acid in water, H_2O; B = 0.1% formic acid in acetonitrile, CH_3CN at a flow rate of 0.2 mL/min using the following conditions: 0-7 min, 10% B; 7-23 min, 18% B; 23-28 min, 23% B; 28-69 min, 41% B; 69-72 min, 95% B; 72-80 min, 10% B; 80-95 min, 10%B and detection with PDA detector to acquire UV spectrum at a range of 240-360 nm.

2.3.2 Thin layer chromatography

TLC was run for the following fractions; OTA02801-06, OTA03105-06, OTA04301-11, aqueous crude and ethyl acetate extracts (Table 2.2 and 2.3). Each fraction (2 mg) was dissolved in methanol (0.5 mL) and spots were applied on aluminium backed silica TLC plate. Elution of the spots was carried out using a mixture of ethyl acetate: formic acid: water (18:1:1). After separation the solvent front was quickly marked with a pencil and the chromatogram viewed under UV-light at 320 nm. The components were sprayed in the TLC plate using vanillin-HCl spray reagent (prepared by dissolving 0.8 g vanillin in 20 mL MeOH and adding 5 mL of concentrated HCl in a 100 mL Erlenmeyer flask). The chromatogram was heated using a hair-dryer and some coloured spots were seen. Various colours were observed clearly after scanning. The spray reagent reacts with all flavonoids possessing a phloroglucinol A-ring.

2.4 Further Purification

2.4.1 Rechromatography with the gradient LH-20 fractionation method

Further purification of OTA07010, first fractions of 50% MeOH (12 g of OTA02701, OTA02801, OTA03101, OTA03701 pooled) was performed. OTA07010 (12 g) was dissolved in milliQ-water (80 mL) loaded onto the Sephadex LH-20 column (25×1000 mm). Elution was performed (7 mL/min) with water, five fractions (total volume 250 mL) were obtained. The column was further eluted with 50% methanol to obtain two fractions (total volume 500 mL). Two fractions were obtained for elution with 70% acetone (total volume 1 L). All the fractions were concentrated under reduced pressure at 40 °C and freeze-dried. Small volumes of the fractions were retained and run by HPLC-PDA.

2.4.2 Reverse-phase (C18) flash chromatography

The column (C18 35/20 C-Kontes) was loaded with (160×30 mm) of C18 silica gel (60 g, size 60 Å, surface area: 500 m^2/g, average particle size: 15-40 μm purchased from Sigma, St Louis, USA). The solvent was flashed with gaseous nitrogen under pressure. The column was equilibrated with 50% MeOH, 30% MeOH, water and 10% MeOH. Pooled OTA02803 and OTA03103 (120 mg), which had similar PDA profiles were dissolved in (3 mL of 10% MeOH). The sample was loaded onto the column and gradient elution performed from 10% MeOH up to 100% MeOH, fractions (10-20 mL) were collected. Yellow bands on the column were observed when eluting with 10%

MeOH, and 50% MeOH eluted all the yellow components. The red band might be attributed to anthocyanins. Three additional yellow bands were observed after elution of the first yellow band. The fractions were concentrated *in vacuo* and freeze-dried. Fractions were run for PDA.

2.4.3 Solid phase extraction (SPE)

The C18 Sep-pak cartridges (Phenomenex) were preconditioned with MeOH (5mL) followed by milliQ-water (5 mL). The sample (OTA04303, 5.02 mg in 3 mL H_2O) was loaded onto the pre-conditioned column. The impurities were washed from the column with water (5 mL), 50% MeOH (5 mL) and then 75 % MeOH (5 mL). The fractions were concentrated *in vacuo* prior to freeze-drying. The fractions were run by HPLC-PDA and LC-MS.

2.5 Chemical characterisation

2.5.1 LC-MS of CT oligomers

Mass spectra were acquired through Shimadzu LCMS QP8000 alpha with ESI-MS in scan mode (*m/z* 250 to 1400) and detection in the negative ion mode using the same conditions as outlined above for HPLC analysis.

LC-MS was performed for the following fractions; OTA02801, OTA02805, OTA02806, OTA03701, OTA03705, OTA03706, OTA09806, OTA09807, OTA09808, OTA09809, OTA09810, OTA03801, OTA03806, OTA04301, OTA04306, OTA04307, OTA04308, OTA04309, OTA04310, OTA07001, OTA07008, OTB00301 and OTB00306.

2.5.2 Selective ion monitoring- mass spectrometry (SIM-MS)

SIM-MS was performed for selected ions (Table 2.4) to identify CT hetero-oligomers PC/PD, or homo-oligomers PC and PD. SIM was performed for two fractions, OTA09807 and OTA09808. The same conditions described above for LC-MS were used.

Table 2.4 Ions for SIM-MS for the determination of CT oligomers

m/z	PC	PD	Charge	Ions
576	4	0	2	PC_4^{2-} tetramer
577	2	0	1	PC_2^{1-}dimer
584.1	3	1	2	PC_3PD^{2-} tetramer
592.1	2	2	2	$PC_2PD_2^{2-}$ tetramer
593.1	1	1	1	$PCPD^-$ dimer
600.1	1	3	2	$PCPD_3^{2-}$ tetramer
609.1	0	2	1	PD_2^- dimmer
652.1	4	0	2	gPC_4^{2-} tetramer
720.1	5	0	2	PC_5^{2-} pentamer
736.1	3	2	2	$PC_3PD_2^{2-}$ pentamer
745.1	1	1	1	$gPCPD^-$ dimer
865.1	3	0	1	PC_3 trimer
881.1	2	1	1	PC_2PD trimer
897.1	1	2	1	$PCPD_2^-$ trimer
913.1	0	3	1	PD_3^- trimer

2.5.3 Matrix assisted laser desorption ionisation -time of flight mass spectrometry (MALDI-TOF-MS) of CT oligomers.

2.5.3.1 Preparation of the MALDI target plate

The target plate was rinsed with milliQ-water and HPLC grade acetonitrile then allowed to dry. The plate was soaked in 100 ml 2% Decon and sonicated for 30 minutes to remove solid deposits. The detergent was washed off with milliQ-water then oven-dried at 80 °C.

2.5.3.2 Analysis of CT oligomers with MALDI-TOF-MS

The matrix was prepared according to the method of Ohnishi-Kameyama (1997) by dissolving 10 mg of 2,5-dihydroxy benzoic acid (DHB)) in 1 mL acetone and 10 mg of DHB in 1 mL acetonitrile. The LH-20 70% acetone and 100% MeOH fractions were prepared by dissolving 0.5 mg in 1mL milliQ-water. The following fractions were used in the analysis; OTA02805 (70% acetone), OTA04309 (100% MeOH), OTA09806 (100% MeOH), OTA09807 (100% MeOH), OTA09808 (100% MeOH) and OTA09809 (100% MeOH). The sample was mixed with the matrix in a 1:1 ratio (50 µL sub-sample of each fraction and 50 µL of DHB in acetonitrile/acetone) in Eppendorf tubes. The DHB in acetone was used with 70% acetone fractions and DHB in acetonitrile was used

with the 100% MeOH fractions. One drop of the mixture was applied to the target plate made of stainless steel. The spots were air-dried and left to co-crystallise.

The MALDI-TOF mass spectra were recorded using a Bruker MALDI-TOF (Micromass MALDI TM) in the positive-ion reflectron mode. The nitrogen laser was set at 337 nm, source voltage; 15 kV, pulse voltage; 2470 V and reflectron voltage; 2000 V. Sensitivity was improved by taking multiple laser shots. No spectra were obtained for the 70% acetone fractions which contain CT polymers.

2.5.4 APCI-MS and ESI-MS of flavonoids

Mass spectra were acquired through Shimadzu LCMS QP8000 alpha connected to Phenomenex reverse phase column (C18) Prodigy column (150 × 4.6 mm, 5 μm) for APCI in scan mode (*m/z* 250 to 1000) with detection in the positive ion mode using quadrupole mass analyser. Elution and detection were performed under the following conditions; solvent gradient- 5 min- 5%B, 10 min- 10%B, 30 min- 25%B, 40 min- 35%B, 48 min- 45%B, 53 min- 97%B, 58 min- 97%B, 65 min- 5%B, flow rate- 1 mL/min, APCI temp; 400 °C, CDL temp; 250 °C, detector voltage; 1.6 kV, pirani gauge; 1.9×10^{-2} Pa, ion gauge; 7.8×10^{4} Pa. ESI mass spectra were obtained in the negative ion mode in scan mode (*m/z* 250 to 1400). The same solvent gradient conditions above for APCI-MS were used for ESI-MS using an Alltech Alltima C18 column (150 × 2.1 mm, 5 μm) with a flow rate of 0.2 mL/min, CDL temp; 250 °C. Detection for acquiring UV spectrum was performed in the range 220-420 nm. LC-MS was performed for the following selected fractions from SPE and C18 flash chromatography (C18F); OTB08801 SPE, OTB08802 SPE, OTB08401 C18F, OTB08403 C18F, OTB08408 C18F, OTB08411 C18F, OTB08412 C18F, OTB08609 C18F, OTB08611 C18F, OTB08612 C18 F.

2.5.5 ^{13}C and ^{1}H NMR analysis

2.5.5.1 ^{13}C-NMR analysis of CT polymers

Fractions (50 mg) OTA02806 (70% acetone), OTA07008 (70% acetone) and OTA07001 (H_2O) were dissolved in 0.5 mL acetone-d_6 and added a minimum of D_2O (0.5 mL) solvent and transferred into an NMR tube. The sample was insoluble in acetone-d_6, but became soluble after addition of a small amount of D_2O. The ^{1}H NMR spectrum was obtained after analysis and the sample was run overnight for ^{13}C-NMR spectrum at 90 MHz using a Bruker 400 MHz NMR. OTA07008 was dark brown which might indicate a concentrated solution with a high molecular weight. The sample

solution was then transferred into an NMR tube for analysis after filtering through glass micro-fibre filters.

2.5.5.2 ^{13}C NMR analysis of flavonoids

Fractions (OTA04303 (25% MeOH), OTA10305 (75% MeOH) and OTA03804 (50% MeOH), 40 mg) were dissolved in methanol-d_4 and the sample was run overnight using 400 MHz Bruker NMR.

2.6 Chemical reactions

2.6.1 Thiolysis

A method based on that described by Guyot (1998) was utilised to perform thiolysis. CT containing fractions (20 mg) (Table 2.2 and 2.3) were weighed and diluted with 5 mL 100% MeOH in a 5 mL volumetric flask. 50 µL of each CT containing fraction was transferred into a glass vial (polypropylene insert, Fisher Scientific, Boston, MA) followed by 50 µL of 3.3% HCl (0.2 M) and 100 µL of benzyl mercaptan (5%). The reaction was carried out for 30 minutes at 40 °C in a heating block and cooled to room temperature. An internal standard, dihydroquercetin (DHQ), about 100 µL of DHQ (4.8 mg in 10 mL milliQ-water and 1 ml of MeOH) was added. The mixture was then centrifuged for 5 minutes at 13400 rpm. 200 µL of the sample was transferred into an insert placed in a brown glass vial. The reaction was carried out with and without internal standard due to co-elution of some compounds with the internal standard. The sample was then analysed immediately to avoid epimerisation. Analysis of thiolysis adducts was acquired using the same conditions as outlined for HPLC analysis and UV detection at 280 nm. 10 µL of each fraction was injected. The crude extracts did not completely dissolve in methanol, but dissolved nicely in water however, thiolysis cannot be performed using water as two layers form. The 50% MeOH fractions did not dissolve completely in methanol. However, the supernatant liquid was used. Calibration was performed using commercially available standards (flavan-3-ols and benzyl thioether standards, Table 2.5). Thiolysis was performed for the following fractions; OTA02805, OTA02806, OTA07008, OTA03805, OTB00306, OTA03806, OTA04310, OTA04311, OTA04308, OTA04309, OTA00701, OTA03705, OTA03706, OTA04306, OTA04307, OTA09808, OTA09809, OTA09810, OTB00306, LMA04205, LMA04206

2.6.1.1 Preparation of standards

Standards (catechin, epicatechin and epigallocatechin) were obtained from Sigma Chemicals, St Louis, Mo USA. Flavan-3-ol benzyl thioethers were prepared by Suba Sivakumaran (AgResearch, New Zealand).

Table 2.5 Commercially available standards used for calibration in analysis of thiolysis adducts and free flavan-3-ols.

Compounds	Amount per ml of solvent
Catechin (C)	2.6 mg/5mL 100% MeOH
Epicatechin (EC)	2.6 mg/5 mL 100% MeOH
Epigallocatechin (EGC)	1.1 mg/2 mL 100% MeOH
Epigallocatechin-thio (EGC-thio)	1.1 mg/2 mL 100% MeOH
Catechin-thio (C-thio)	1.1 mg/2 mL 100% MeOH
Epicatechin-thio (EC-thio)	1.1 mg/2 mL 100 % MeOH
Dihydroquercetin (DHQ)	4.8 mg/50 mL milliQ water

Mixed standards consisting of catechin (100 µL), epicatechin (100 µL), epigallocatechin (100 µL), epigallocatechin-4β-benzyl thioether (100 µL), catechin-4-benzyl thioether (100 µL), taxifolin (100 µL) and 200 µL milliQ-water were prepared in duplicate and used in the analysis.

2.6.2 Acid butanol assay for CT content

The butanol-HCl assay was performed according to the method of Porter (1986). Freeze-dried, ground sulla material (500 mg) (summer-21/12/01 and spring-23/09/02) were placed into 50 mL falcon tubes, in duplicate. 20 mL of 70% acetone containing 0.1% ascorbic acid and 10 mL of diethyl ether were added. This was then mixed thoroughly using a vortex mixer. The mixture was centrifuged at 2000 rpm for 15 minutes. The supernatant liquid was poured into a 250 mL round bottom flask. The residue was extracted twice with 70% acetone and subsequently with diethyl ether and the supernatants pooled. The combined supernatants separated into two phases. The organic layer was separated from the aqueous layer using a separating funnel. The organic layer containing acetone and diethyl ether was discarded. The aqueous layer was poured into a round bottom flask and rotary evaporated to remove the residual acetone-diethyl ether at 40 °C. The aqueous layer containing the CT was transferred into 35 mL centrifuge tubes (Oakridge) and centrifuged at 15 000 rpm under refrigeration (Sorvall RC-5 Superseed Refrigerated) for 15 minutes to remove all the flocculated material. The supernatant was diluted with water to 50 mL in a volumetric flask. The stock solution was prepared by using about 5 mg of purified tannin in 10 mL volumetric

flask and diluted to the mark with distilled water. Working standards were prepared from the stock solution (500 ppm) as shown in Table 2.6 below.

Table 2.6 Working standards prepared from the stock solution using butanol-HCl method in determination of sulla CT concentration.

Amount in ml taken from stock solution	Water	Concentration (ppm)
0.00	5.00	0
0.25	4.75	25
0.50	4.50	50
1.00	4.00	100
1.50	3.50	150
2.50	2.50	250
3.75	1.25	375

Each sample solution (1mL) was taken into 15 mL Kimax glass test tubes with Srew-caps in duplicate. Butanol-HCl solution (6 mL) was added to one tube and butanol-H_2O solution added to the other. A reagent blank was prepared using 1 mL of distilled water and 6 mL of butanol-HCl. The reaction mixture was mixed on a vortex mixer and placed in a water bath at 95 °C for 75 minutes. The same treatment was applied to all the standards. The samples were left to cool. The readings for absorbance were read at 550 nm using UV/Vis-spectrophotometer (GBC UV/VIS 918). The instrument was zeroed with the reagent blank. The sample blanks were also read and their values were subtracted from sample absorbance to obtain corrected sample absorbance. Data was compared with the published values from the literature.

2.7 Near infrared spectroscopy (NIRS) for nutritional content

Freeze-dried sulla plant samples were sent to Feedtech, AgResearch Grasslands for near infrared reflectance spectroscopy (NIRS systems 6500) analysis to determine the nutritional composition (**CP**, **NSC**, **NDF**, **ADF**, **Lipid**, **Ash** and **OMD**). Data was compared with the published values from the literature.

2.8 Analysis and identification of soluble sugars

Freeze-dried sulla leaves (summer-21/12/01 and spring-23/09/02) were analysed using HPLC Shimadzu LC-4A, column-Aminex HPX-87, BioRad using anion cation exchange with deionised water as the mobile phase by Wilhelmina Martin, Analytical Laboratory, Nutrition and Behaviour, AgResearch, Palmerston North.

2.9 Parasite Bioassays

2.9.1 Egg Extraction

Lambs with monospecific infections of *T. colubriformis* were housed indoors, fed lucerne chaff and pellets, and given free access to water. Faeces containing eggs were collected using harnesses and collecting bags. Egg extraction was performed according to the procedure previously used by Molan et al. (2002). The faecal pellets were crushed against a 250 μm mesh sieve, placed in a container containing ice cold water (4 °C) for inhibition of eggs from hatching. The suspension in the container was passed through a 60 μm mesh sieve to remove most of the debris and further placed on a 20 μm sieve to trap the eggs. The eggs were cleaned from the 20 μm using ice-cold distilled water. The solution in the beaker was transferred into 20 μm mesh sieve again and washed thoroughly using cold 20% (w/v) magnesium sulphate ($MgSO_4$) solution (BDH Laboratory Supplies, England). The $MgSO_4$ solution was used to separate the eggs from the debris and the eggs are expected to float in the solution while with the use of water the eggs settle at the bottom with the debris making it difficult to separate. The solution was then transferred into falcon tubes and centrifuged for 15 minutes at a speed of 6000 rpm. The supernatant liquid was poured into a 20 μm mesh sieve and $MgSO_4$ was then washed away through the sieve and eggs were removed and transferred into a small beaker. 100 μL of the liquid containing eggs was estimated to contain 106 eggs. The eggs extracted were used for egg hatch assay (EHA) and larval development assay (LDA).

2.9.2 Egg hatch assay

Stock solutions of CTs were prepared (8 mg of CT dissolved in about 800 μL distilled water). The assay was carried out at three different concentrations, 100, 500 and 1000 μg/mL CT for each fraction in duplicate prepared from stock solution, in a 48-well cultured tissue (Costar) with about 50 μL containing approximately 50 eggs and topped up to 500 μL. The volumes used for CT concentrations were 5, 25 and 50 μL litre, respectively. The eggs in the distilled water were used as the controls. The eggs were incubated for 26 hrs at 24 °C in plastic containers containing sponges soaked in water to provide 100% relative humidity and prevent plates from drying. At the end of the incubation period, the number of unhatched eggs and L1 larvae were counted and % hatching was calculated. The % inhibition of hatching for each concentration of tested compound was estimated relative to the number of eggs hatched in the control wells. *In vitro* egg hatch assay was performed for the following fractions; aqueous crude extracts,

ethyl acetate extracts, LH-20 fractions OTA02801-06, OTA03801-06, OTA04301-11 (Table 2.2 and 2.3).

2.9.3 Larval development assay (LD)

The LD assay was performed at three concentrations of 25,100, 200 µg/mL CT using same fractions used in the egg hatch assay. The assay was carried out using about 100 eggs in duplicates in a 96-well micro-litre plate. About 150 µL/well of the artificial medium consisting of 20 mL nutritive media (Y-1000, Sigma) and antifungal (Gentamycin), 240 µL of antibiotic (Amphotericin) and 2 mL of *E. coli* were added. The procedure for the preparation of the artificial medium is shown in Appendix 4. The plate assays were incubated at 24 °C for seven days. At the end of the incubation period, the unhatched eggs, L1, L2 and L3 larvae were counted to determine the exact number of eggs per well. The % development to L3 larvae and the % inhibition for each concentration of the tested compounds were calculated relative to the development in controls.

2.9.4 Calculations and statistical analysis

The % inhibition of egg hatching (EH assay), % inhibition of larval development (LD assay) were calculated using the following equation (Rabel et al., 1994):

% inhibition = (A-B)/ A ×100,

where A = the number of eggs hatched (EH assay) or the number of hatched larvae that managed to develop into L3 larvae (LD assay) in control incubations, and B = the number of eggs hatched or the number of L3 larvae in LD assay containing different concentrations of LH-20 fractions.

CHAPTER THREE

CONDENSED TANNINS

3.1 Introduction

Plant secondary metabolites, especially CTs have raised much research interest in animal nutrition due to their ability to complex with proteins. Characterisation of the structure of extractable CT from herbaceous legumes (Foo et al., 1996; 1997) and non-forage sources (Guyot et al., 1998; Taylor et al., 2003) has been achieved through acid catalysed reaction with phloroglucinol and benzyl mercaptan, mass spectrometry and ^{13}C NMR analysis. Thiolysis is performed through acid catalysis to degrade the CT in the presence of reactive nucleophiles such as benzyl mercaptan. The thiolysis adducts are analysed using RP-HPLC or NP-HPLC (Svedstrom et al., 2002; Koupai-Abyazani et al., 1992). The data from NMR and thiolysis provide information on the mDP, mean molecular weight, PC:PD ratio and the stereochemistry of the heterocyclic C-ring. CT oligomers ($n<10$) are well characterised using MS techniques. The MS techniques do not provide information on CT polymer ($n>10$) as it becomes increasingly difficult to characterise these large molecules.

One of the chemical methods that have been used to quantify CT content directly in forage plants is Bu-HCl. Fibre-bound and protein bound CT in addition to extractable CT can be quantified. It is a colorimetric technique which provides information on the CT content, but does not provide any information on the CT structure (Schofield et al., 2001). A modified Bu-HCl method has been devised by Terrill (1992), which measures extractable, protein bound and fibre-bound CT (Mangan, 1988). The nutritional composition can be obtained using NIRS technique which uses a monochromator absorbing in the range 1100 to 2500 nm. Prediction of the nutritional composition and chemical constituents of plant material is achieved using calibration equations and unknowns can be predicted (Xiccato et al., 2003). Feed samples, such as pasture, ryegrass, lucerne, white clover, sulla, red clover and LP have been analysed using NIRS to predict their nutritive value (Waghorn et al., 2002; Burke at al., 2002; Woodward et al., 2002).

Soluble sugars and available carbohydrate are present in fruits, vegetables and legumes. They contribute to the nutritive value of forages. Forages with high non-structural carbohydrates, with soluble sugars as some of their components, are associated with high voluntary feed intake (Burke et al., 2002; Waghorn et al., 2002). It

has been noticed that the high concentrations of water soluble carbohydrates such as glucose, fructose, sucrose and other carbohydrate fractions increases voluntary feed intake. The increase in herbage intake will in turn improve performance in ruminants (Ciavarella et al., 2000). Sucrose is believed to be the major sugar component in forage legumes (Marais et al., 2000). Soluble sugars have been identified and quantified with different analytical methods such as chromatographic methods and enzymatic methods (Lopez-Hernandez et al., 1994). The solvents routinely used for their extraction are hot 80% ethanol and methanol (v/v) (Lopez-Hernandez et al., 1994). HPLC and gas chromatography (GC) MS are the widely used techniques in analysis of sugars from plant samples (Sanchez-Mata et al., 1998). One of the advantages of HPLC compared to other analytical techniques is that is faster, simpler, provides good accuracy, allows the quantification of sugars within a complex mixture in a single chromatographic run and no derivatisation is required. GC requires the sample be derivatised prior to analysis and this is time consuming and involves many tedious preparation steps (Sanchez-Mata et al., 2002).

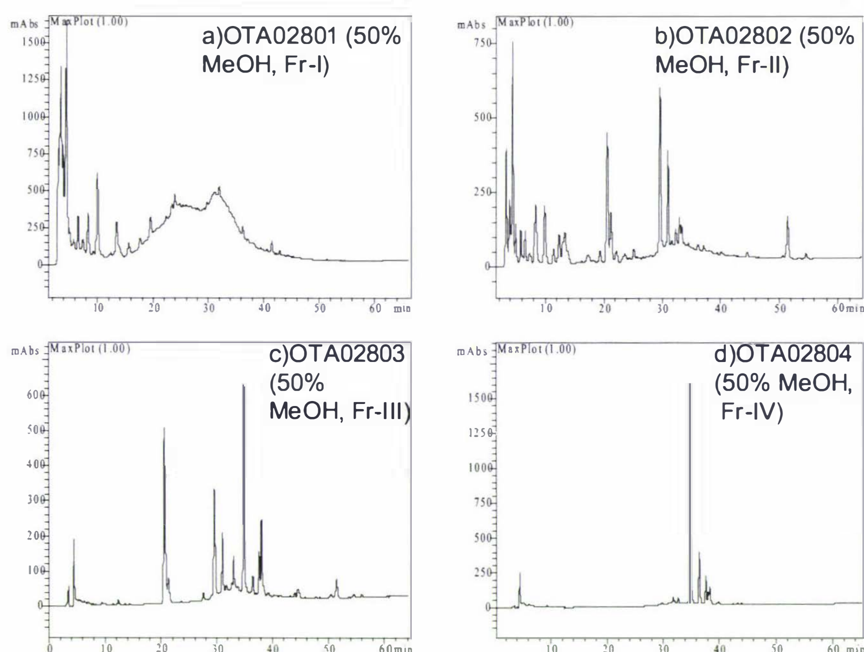
3.2 Results

3.2.1 Liquid chromatography-PDA

3.2.1.1 PDA chromatograms for step LH-20 fractionation

RP-HPLC chromatography with PDA detection can be used to monitor fractions for phenolic compounds. Chromatograms for the 50% MeOH LH-20 fractions from sulla collected in summer (21/12/01) exhibited sharp peaks which were indicative of low molecular weight phenolics, notably flavonoids (Figure 3.1). Detection was performed in the range 240-360 nm, with flavonoids absorbing in the range 240-285 nm (Band II), which is attributed to the cinamomyl B-ring system. Band II (300-550 nm) absorption is attributed to benzyl A-ring system (See Introduction, Section 1.2). Fraction OTA02801 (50% MeOH) contained sharp peaks and a small hump (Figure 3.1a). A small hump with unresolved peaks was depicted in the UV spectrum at λ_{max} 280 nm in the first fraction (OTA02801) of the 50% MeOH which was indicative of the CT polymer. However, this pattern was not observed in the 50% MeOH fractions (OTA03801 and OTA04501) from sulla plants collected in spring on 23rd Sept 2002. In the past, the 50% MeOH was discarded, it was never investigated to ascertain if it contains CT. However, in this study we endeavoured to isolate and characterize the

phenolic compounds (flavonoids and CT) that could be present in the 50% MeOH fractions. The PDA chromatogram for fraction II (OTA02802) differed from that of fraction I (OTA02801) with no indication of the presence of any absorbance at 280 nm. The PDA chromatogram for OTA02803 had sharp peaks which were indicative of flavonoids. Fraction IV (OTA02804) also had sharp peaks with compounds eluting between 30 and 40 minutes. Sugars are prevalent among the first 50% MeOH fractions making the fractions sticky. They were identified and quantified from sulla by HPLC. After elution of sugars and low molecular weight phenolics, a brown band, notably CT polymer was adsorbed to the stationary phase and was eluted with 70% acetone.



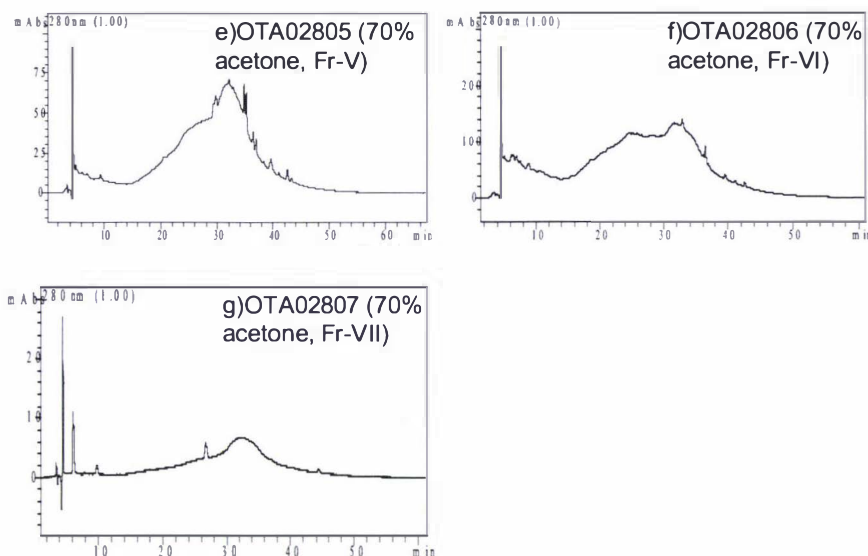


Figure 3.1 PDA chromatograms for step LH-20 fractions OTA02801-04 (at 240-360 nm) and OTA02805-07 (at 280 nm) from the freeze-dried sulla harvested summer (21/12/01).

From the PDA spectrum, it was realised that the CT-containing fractions (V-VII, OTA02805-7) had a distinctive UV spectrum with one maxima at 280 nm. This broad peak for the 70% acetone fractions resembles the presence of polymeric substance like CT (Figure 3.1e-g). Fraction V (OTA02805) contained CT though the last fraction (OTA02807) constituted only trace amounts of CT. The signal intensity of the hump for the second 70% acetone fraction (OTA02806) was bigger than the hump for the first 70% acetone fraction (OTA02805). The CT oligomers are expected to be eluted in the first 70% acetone fraction. Two more batches from the same aqueous crude extract were fractionated on the LH-20 column giving fractions OTA03101 to OTA03107 and OTA03701 to OTA03707. Similar PDA profiles for these fractions were observed with fractions OTA02801 to OTA02807. Similar PDA profiles were observed for fraction OTA03801-07, OTA04501-07 from the fresh frozen material plant material collected in spring on 23rd Sept 2002 (Appendix 6). However, fractions OTA03801 and OTA04501 had no absorbance attributable to CT.

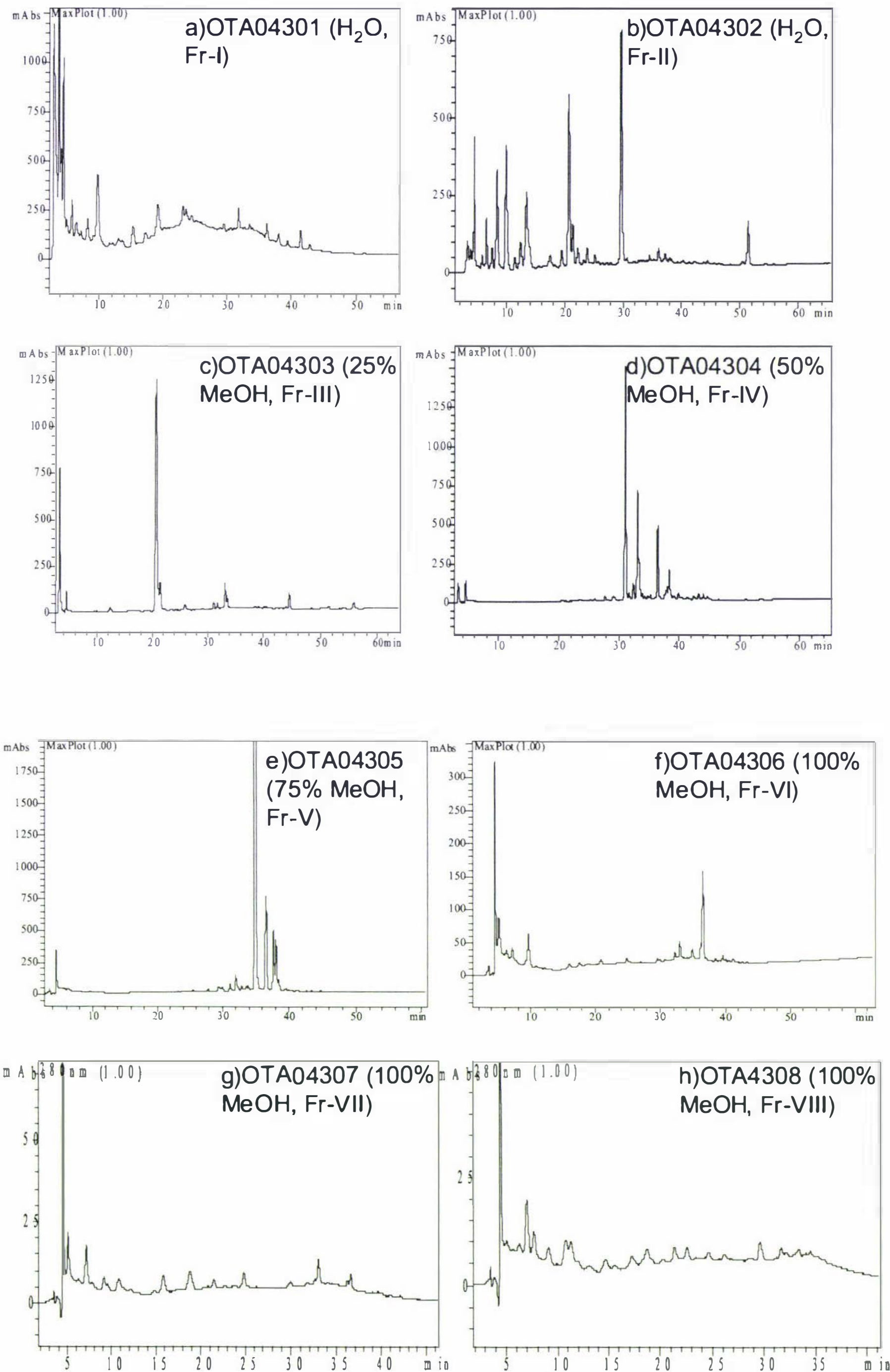
A RP-HPLC column eluted more polar compounds first and the less polar compounds eluted later. In this separation, the analyte has to interact with the stationary

phase and the mobile phase with gradient elution conducted using acetonitrile and and water. This is a reliable diagnostic tool for the presence of the polyflavan-3-ol structure.

3.2.1.2 PDA chromatograms for the gradient LH-20 fractionation

Gradient LH-20 fractionation from the method of Meagher (2003) involves elution with water and ascending concentrations of MeOH; 25% MeOH, 50% MeOH, 75% MeOH, 100% MeOH and 70% acetone. A series of fractions were obtained from the aqueous crude extract (OTA01301) from fresh frozen sulla collected in spring (23/09/02). The PDA, Fraction I (OTA04301) depicted sharp peaks which were indicative of flavonoids (Figure 3.2a). Small hump was found at 280 nm and was more vivid in the absorption maxima between 240-360 nm. The fractions were sticky probably due to the presence of sugars. Fraction II (OTA04302) showed absorbance representative of flavonoids and no indication of the presence of CT. Fraction III (OTA04303) was fairly pure with few sharp peaks (Figure 3.2c). It had outstanding peak eluted at a retention time of 20 minutes. UV spectrum suggested the presence of flavonoids. Fraction IV (OTA04304) had a PDA chromatogram with sharp peaks. In fraction V (OTA04305), a PDA chromatogram different from that of fraction IV was observed. Two absorption maxima for most of the peaks were observed at 350 nm and 257 nm. Fraction VI (OTA04306) (Figure 3.2f) had sharp peaks with lower signal intensity indicative of CT oligomers and an intense peak with a retention time (t_R) of 36.5 minutes suggesting the presence of flavonoids. Small peaks were observed at 280 nm for the 100% MeOH fractions which were suggestive of CT oligomers. Fractions VIII (OTA04308) and IX (OTA04309) also appeared to be polymeric. Fraction VII (OTA04307) and VIII (OTA04308) had similar PDA profiles. Fraction IX (OTA04309) was polymeric with a broad hump. It shows the presence of CT oligomers and possibly CT polymers. The size of the hump increased from the first 100% MeOH fraction (OTA04306) to the last 100% MeOH fraction (OTA04309). These 100% MeOH fractions are usually expected to contain CT oligomers with chain lengths varying between 2 and 10. Fraction X (OTA04310) and XI (OTA04311) had a big and clean hump around 280 nm which was indicative of CT polymer and small hump were found for fraction XI (OTA04311). Fraction XI contained trace amounts of CT. The chromatogram only had a big hump with few small peaks which indicates that this fraction was well purified with less sugars and flavonoids. This indicates that this method is very effective in separating CT oligomers from polymers.

Similar PDA profiles were observed for fraction OTA03901-11, OTA09801-11 from the freeze-dried plant material collected in summer (21/12/01) (Appendix 6). However, fraction OTA03901 possessed a bigger hump than OTA04301.



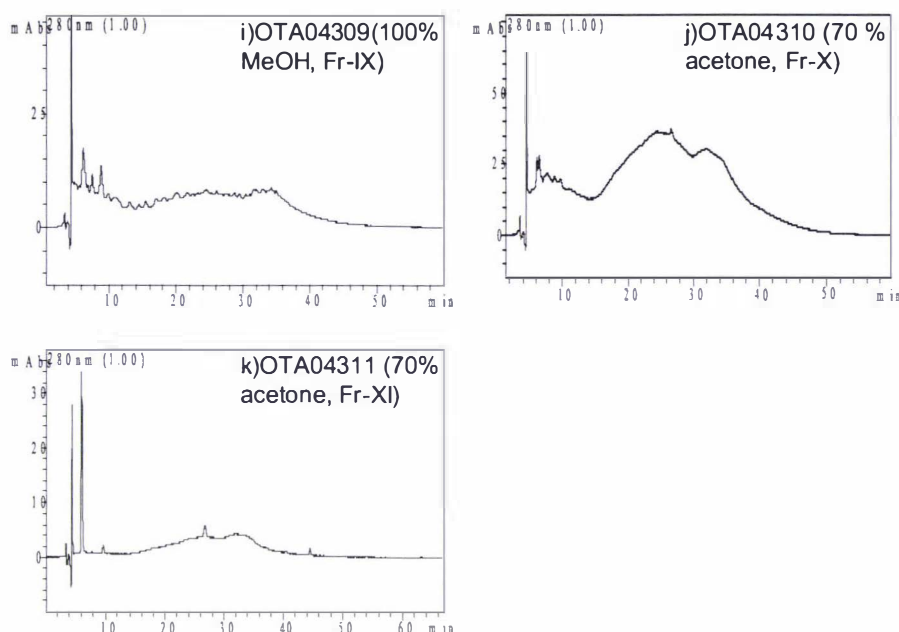


Figure 3.2 PDA chromatograms for the gradient LH-20 fractions OTA04301-05 (at 240-360 nm) and OTA04306-11 (at 280 nm) from fresh frozen plant material collected in spring (23/09/02).

3.2.1.3 TLC

The presence of CT and flavonoids were further verified using one dimensional silica TLC for sulla aqueous extracts and step LH-20 fractions. Vanillin-HCl reagent complexes with flavonoids possessing a phloroglucinol A-ring (Introduction, section 1.2) producing yellow spots. Blue spots were observed for the 50% MeOH fractions under UV-light at 320 nm which were suggestive of the presence of flavonoids. The CT can be identified from the reddish and purplish spots in the chromatogram. The aqueous crude extract was expected to contain flavonoids, CT oligomers and polymers since is a crude fraction, but it was not well separated (Figure 3.3). The ethyl acetate extract was well separated indicating the presence of CT oligomers from dimers, trimers, tetramers with red spots and flavonoids with yellow spots. Nothing was observed on the TLC from fraction OTA02801 (50% MeOH), the first fraction from the LH-20 column. Subsequently, fractions OTA02802-OTA02804 (50% MeOH) produced yellow spots indicating the presence of flavonoids. The 70% acetone fractions (OTA02805-07 and OTA03105-7) produced reddish and purplish spots which were deduced to be dimers, trimers, tetramers, pentamers and polymers. The final fractions OTA02807 and OTA03107 contained immobile spots. They contained neither CT oligomers nor flavonoids. The CT polymer can be easily identified because of virtually immobile

intense red spots in fractions (OTA02805-07 and OTA03107) which resemble the presence of a polymerised CT (Sun et al., 1998). Green spots were observed in fraction OTA02802 and OTA03102 which might indicate the presence of other compounds which require further investigation. TLC can only provide qualitative information on CT.

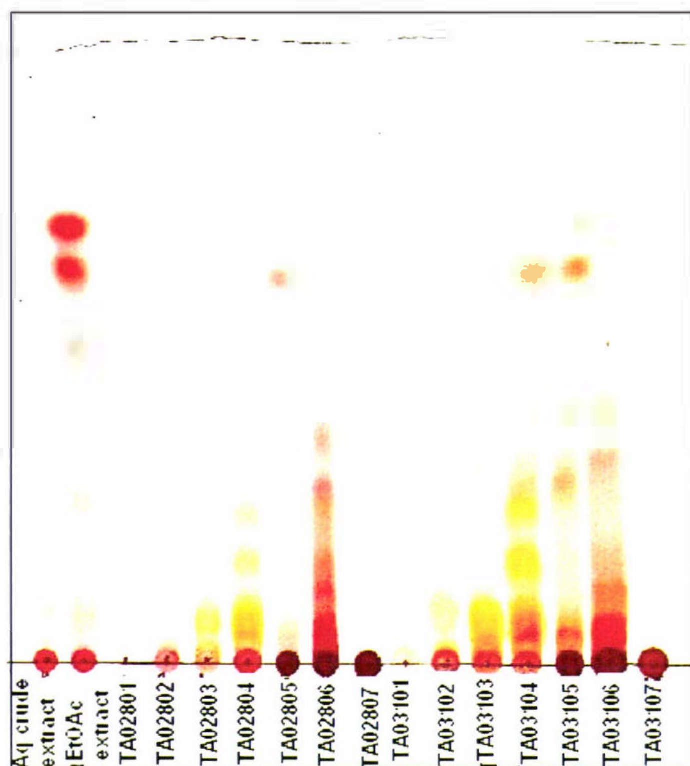


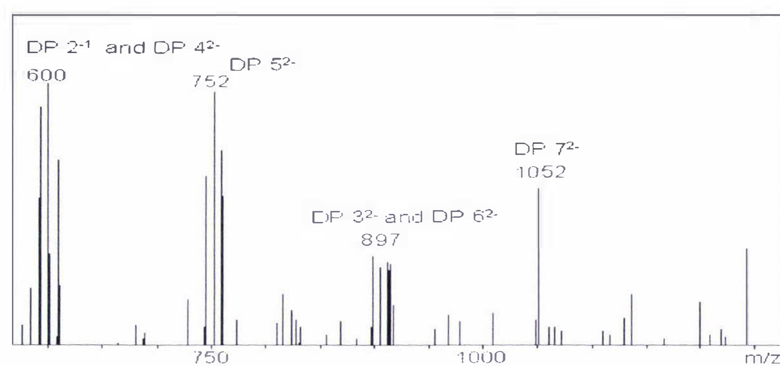
Figure 3.3 TLC chromatogram for the aqueous (Aq) crude extract, ethyl acetate extract, 50% MeOH fractions (OTA02801-4, OTA03101-4), 70 % acetone fractions (OTA02805-7, OTA03105-7) from sulla collected in summer (21/12/01).

3.2.2 LC-ESI-MS of CT oligomers

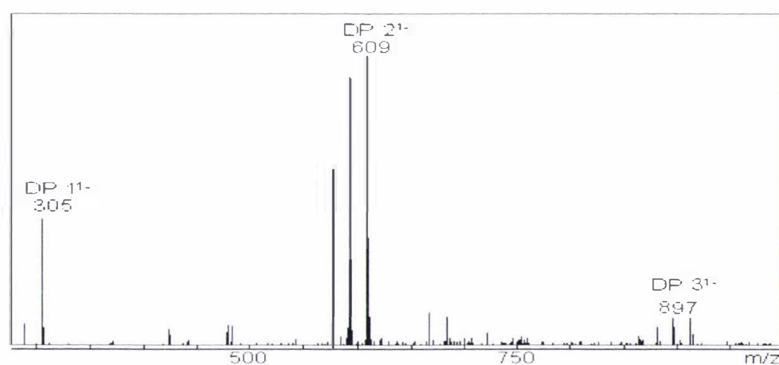
Condensed tannin oligomers were characterised by LC-ESI-MS for the first time from sulla within the range 250-1400 *m/z* in a negative ionisation mode. Monomers with singly charged ions were identified from the step LH-20 70% acetone fraction (OTA03705) with *m/z* 305 and 289 for gallocatechin and catechin, respectively. Unexpected results were obtained for fraction OTA02805, the first fraction eluted with 70% acetone because it was poorly ionised and no mass spectrum was obtained. The CT oligomers are expected to be eluted in this fraction and the highly polymerised CT to be present in fraction OTA02806. The step LH-20 fraction (OTA02806), second fraction eluted with 70% acetone yielded a low molecular weight CT (LMWCT) polymer with a

DP range of 2 to 7 from the MS. Hetero-oligomers containing both PC and PD (Figure 3.4a) were detected from this fraction as singly charged species *m/z* 593 $(PCPD)^-$, 881 $(PC)_2(PD)^-$, 897 $(PC)(PD)_2^-$ and doubly charged species *m/z* 600 $(PC)(PD)_3^{2-}$, 744 $(PC)_2(PD)_3^{2-}$, 752 $(PC)_2(PD)_4^{2-}$, 896 $(PC)_2(PD)_4^{2-}$ and 1052 DP_7^{2-}. The mass spectrum for the first 100 % MeOH fraction (OTA04306) was complex and no mass spectral data was obtained. When the sulla aqueous crude extract is fractionated on LH-20 using the gradient method a series of oligomers were obtained, the second LMWCT eluting with 100% MeOH, OTA04307 with a DP range 2 to 3 and singly charged species *m/z* 577 $(PC)_2^-$, 593 $(PC)(PD)^-$, 609 $(PD)_2^-$, 881 $(PC)_2(PD)^-$, 897 $(PC)(PD)_2^-$ were detected. The third sulla fraction (OTA04308) eluted with 100% MeOH with a DP range of 2 to 3, but the mass spectra (Figure 3.4c) is unexpected in that it exhibits only few ions with singly charged species *m/z* 593 $(PC)(PD)^-$, 609 $(PD)_2^-$, or (doubly charged species 608 $(PD)_4^{2-}$), 881 $(PC)_2(PC)^-$, 897 $(PC)(PD)_2^-$, 913 $(PD)_3^-$. The doubly charged species are more evident in the last 100% MeOH fraction with a DP range of 2 to 5. The complete series of doubly charged pentamers (DP_5^{2-}) (Figure 3.4d) with ^{13}C isotope ratios are observed: the PC homo-oligomer *m/z* 720 $(PC)_5^{2-}$, all the possible hetero-oligomers *m/z* 728 $(PC)_4(PD)^2$, 736 $(PC)_3(PD)_2^{2-}$, 744 $(PC)(PD)_5^{2-}$, and the PD homo-oligomer *m/z* 760 $(PD)_5^{2-}$. The same patterns of ions are observed for dimers (DP_2^-) through to pentamers (DP_5^{2-}) (Figure 3.4d). The last fraction eluting with 70% acetone (OTA04310) is composed of a series of ions observed for tetramers (DP_4^{2-}) through to octamers (DP_8^{2-}) (Figure 3.4e). Fraction OTA04310 with a DP range of 4 to 11 indicates that it contains polymeric substance with multi-charged ions. Besides tetramers and pentamers, doubly charged hexamer or triply charged nonamer (DP_9^{3-}), triply charged decamer (DP_{10}^{3-}), doubly charged heptamer (DP_7^{2-}) and doubly charged octamer (DP_8^{2-}) corresponding to *m/z* 912, 1008, 1056, 1209 were detected, respectively (Figure 3.4e). It was observed from the mass spectra that the low molecular weight CT oligomers produced the most abundant ions with high signal intensity. There is also a possibility of gallolylation, *m/z* 729, 745 and 761 which might be attributed to galloylated PC dimer, PD dimer and PCPD dimer were detected in fraction OTA09808, respectively. In the mass spectrum, the signal intensity for galloylated dimers was lower. This was also evidenced in thiolysis with a compound eluting at a retention time of 63 minutes which may suggest the presence of flavan-3-ol gallates. Most of the peaks identified were in the form of envelopes or clusters.

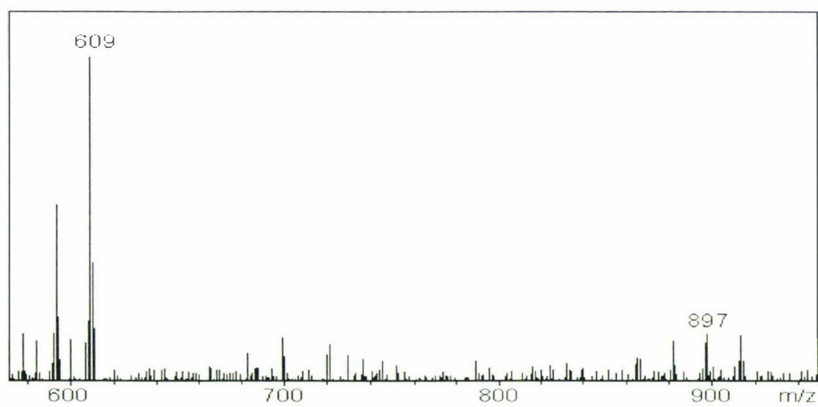
a) Fraction OTA02806 (70% acetone, Fr-6)



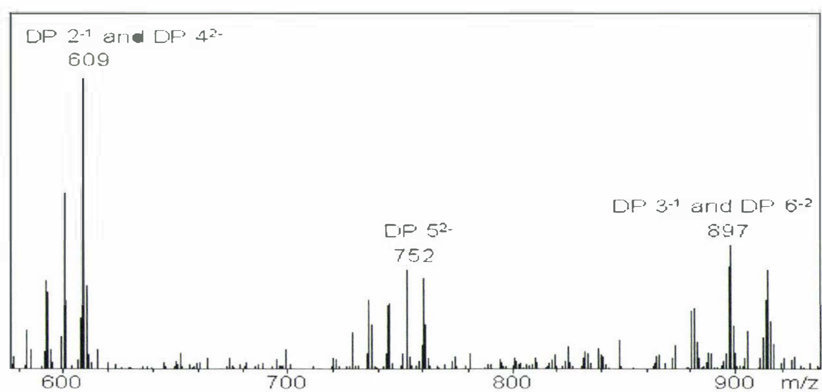
b) Fraction OTA04307 (100% MeOH, Fr-7)



c) Fraction OTA04308 (100% MeOH, Fr-8)



d) Fraction OTA04309 (100% MeOH, Fr-9)



e) Fraction OTA04310 (70% Acetone, Fr-10)

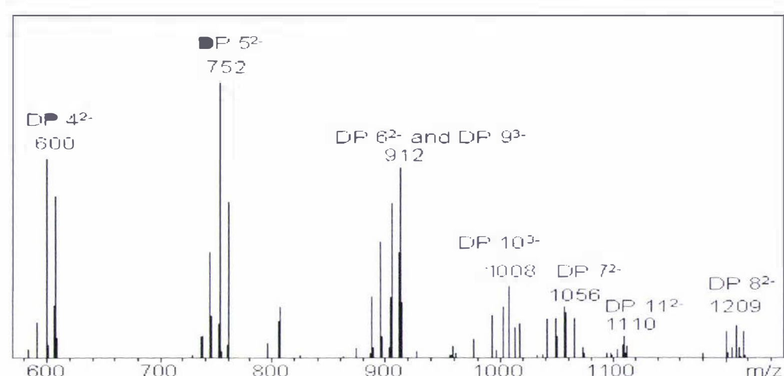


Figure 3.4 Mass spectra obtained for CT oligomers using LC-ESI-MS in a negative ion mode for the LH-20 gradient fractionation a)OTA02806 (70% acetone), b-d)OTA04307-OTA04309 (100% MeOH) and e)OTA04310 (70% acetone).

3.2.3 Selective ion mode (SIM) mass spectroscopy for homogeneous and heterogeneous oligomers

The presence of CT oligomers was confirmed with SIM-mass spectrometry. SIM was carried out after selecting ions from the previous mass spectra acquired in scan mode. In this mode, sensitivity is increased because specific compounds are targeted unlike scanning which involves detection of all compounds present across a defined mass range. Time spent on the target ion is longer, hence increasing sensitivity. The selected ions for homogeneous (PC and PD) and heterogeneous (PCPD) oligomers are shown in Table 2.4 (Materials and methods). Dimers with *m/z* 579, 593.1 and 609 corresponding to PC_2, PCPD and PD_2 were detected from fraction OTA09807 (Appendix 1), respectively. Trimers with *m/z* 865, 881 and 897 were also detected respectively corresponding to PC_3, PC_2PD and PD_2PC. Similar spectra were obtained for fraction OTA09807. The UV spectra for all the retention times of the peaks from the PDA chromatograms were observed and were CT-like with absorption maximum ranging between 269-280 nm. Due to complexity with multiply charged ions, dimers and trimers with singly charged ions were detected (Appendix 1).

3.2.4 MALDI-TOF mass spectrometry

Spectra were acquired in a positive-ion reflectron mode for singly charged ions and the spectra have shown that the *m/z* comprised of Na^+ and K^+ adducts. Masses corresponding to $(M+H)^+$ were not identified in the spectra. The peaks were well resolved and came out as clusters. Assignment of the observed peaks from MALDI-TOF mass spectra was based on observed *m/z* as shown in Table 3.1. Fractions containing CT oligomers which were obtained after elution with 100% MeOH were well characterized using MALDI-TOF. Fraction OTA09806 (Figure 3.5b) with a DP

range of 3 to 4 by MALDI-TOF was found to consist of homogeneous and heterogeneous trimers with *m/z* values of 889, 905, 920 and 937 corresponding to PC_3Na^+, PC_3K^+, $PCPD_2Na^+$ and $PCPD_2K^+$, respectively. Singly charged heterogeneous tetramers with *m/z* 1225 and 1241 were detected and correspond to PD_3PCNa^+ and PD_3PCK^+, respectively. These ions were also present in fraction OTA09807 and OTA09808 (Figure 3.5c and d). Singly charged pentamers and hexamers were detected from fraction OTA09809 with *m/z* 1546, 1562, 1834, 1850, 1866 corresponding to PD_5Na^+, PD_5K^+, PD_5PCNa^+, PD_5PCK^+/PD_6Na^+ and PD_6K^+, respectively (Figure 3.5e).

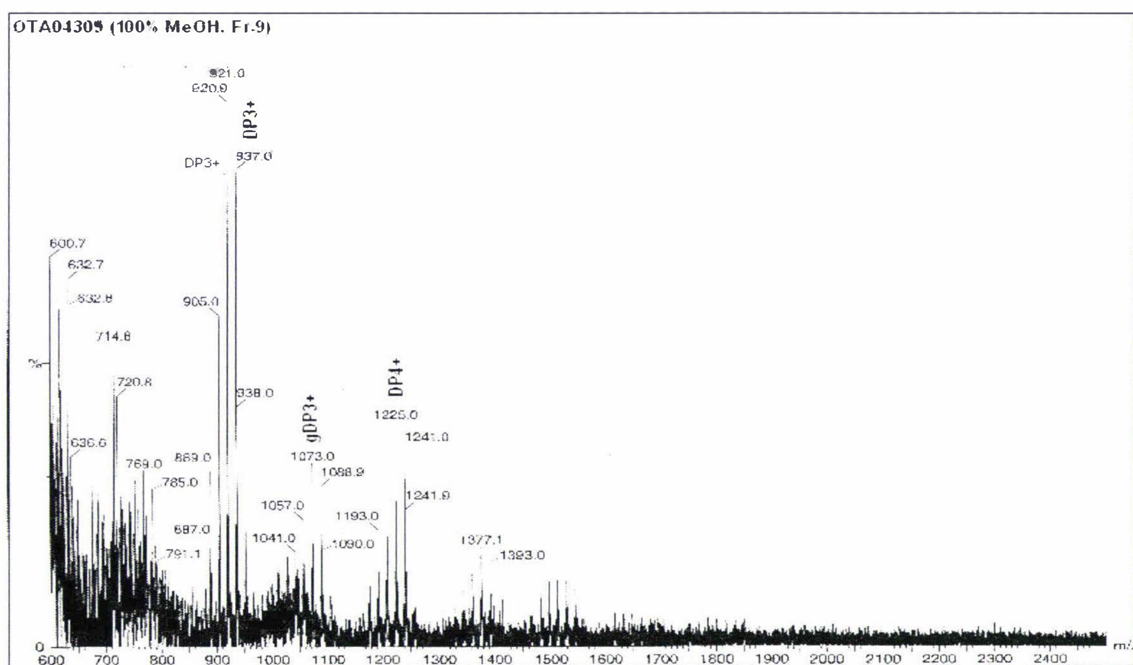
Table 3.1 Observed and expected $[M+Na]^+$ and $[M+K]^+$ ions from MALDI-TOF-MS employed in a positive reflectron mode from the 100% MeOH fractions.

DP	PD	PC	Expected *m/z*	Observed *m/z*	Expected *m/z*	Observed m/z
			$[M+Na]^+$		$[M+K]^+$	
3	0	3	889	889.0, 888.573, 888.511,	905	905.0, 904.652, 904.508, 904.673
	1	2	921	920.9, 921.0, 920.568, 920.522, 920.627, 920.554	937	936.573, 937.561, 937.0, 936.573, 937.603
4	3	1	1225	1224.599, 1224.503, 1224.671	1241	1240.614, 1241.583, 1240.263,1240.586
5	5	0	1546	1544.578, 1545.576, 1546.576	1562	1560.553, 1561.585
6	5	1	1834	1833.574	1850	1850.568
	6	0	1850	1850.568	1866	1865.563

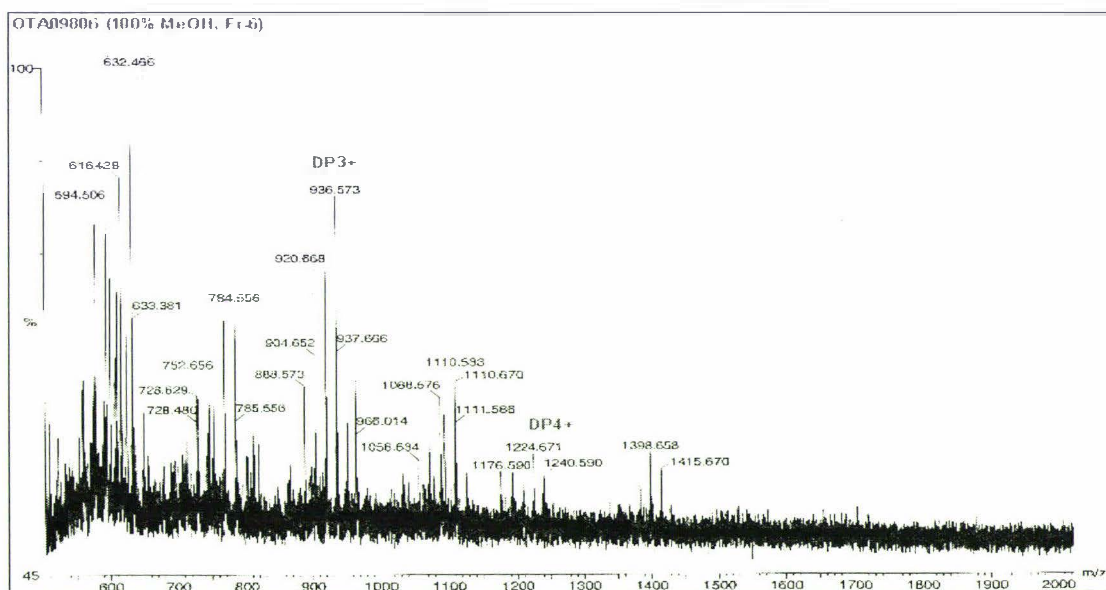
Abbreviations: DP=degree of polymerisation, g=gallate, PC=procyanidin, Mw=289, PD=prodelphinidin, Mw=305, *m/z*=mass per charge ratio.

It was observed that the signal intensity decreased with increasing molecular weight (Figure 3.5a). There is a possibility of gallolylation as ions with *m/z* 1041 and 1057 were detected which corresponds to gPC_3Na^+ and gPC_3K^+, respectively in fraction OTA04309 (Figure 3.5a).These gallated molecules were not detected in the other 100% MeOH fractions.

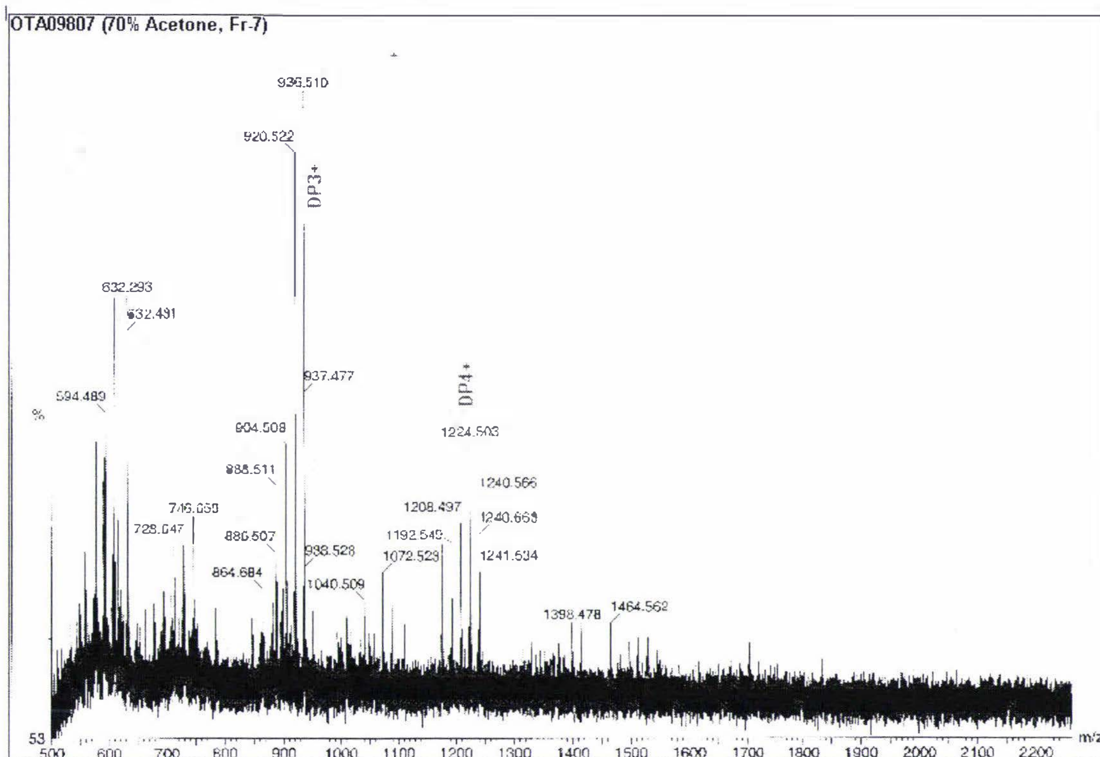
a) Fraction OTA04309 (100% MeOH, Fr-9)



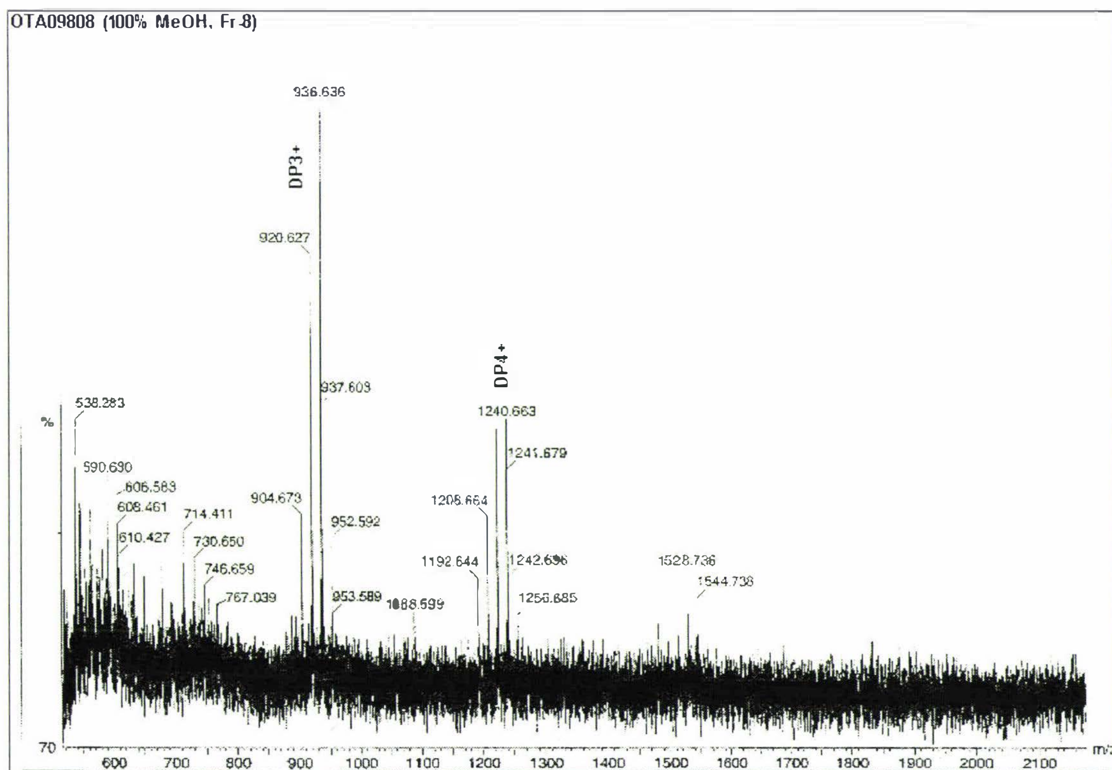
b) Fraction OTA09806 (100% MeOH, Fr-6)



c) Fraction OTA09807 (100% MeOH, Fr-7)



d) Fraction OTA09808 (100% MeOH, Fr-8)



e) Sulla OTA09809 (100% MeOH, Fr-9)

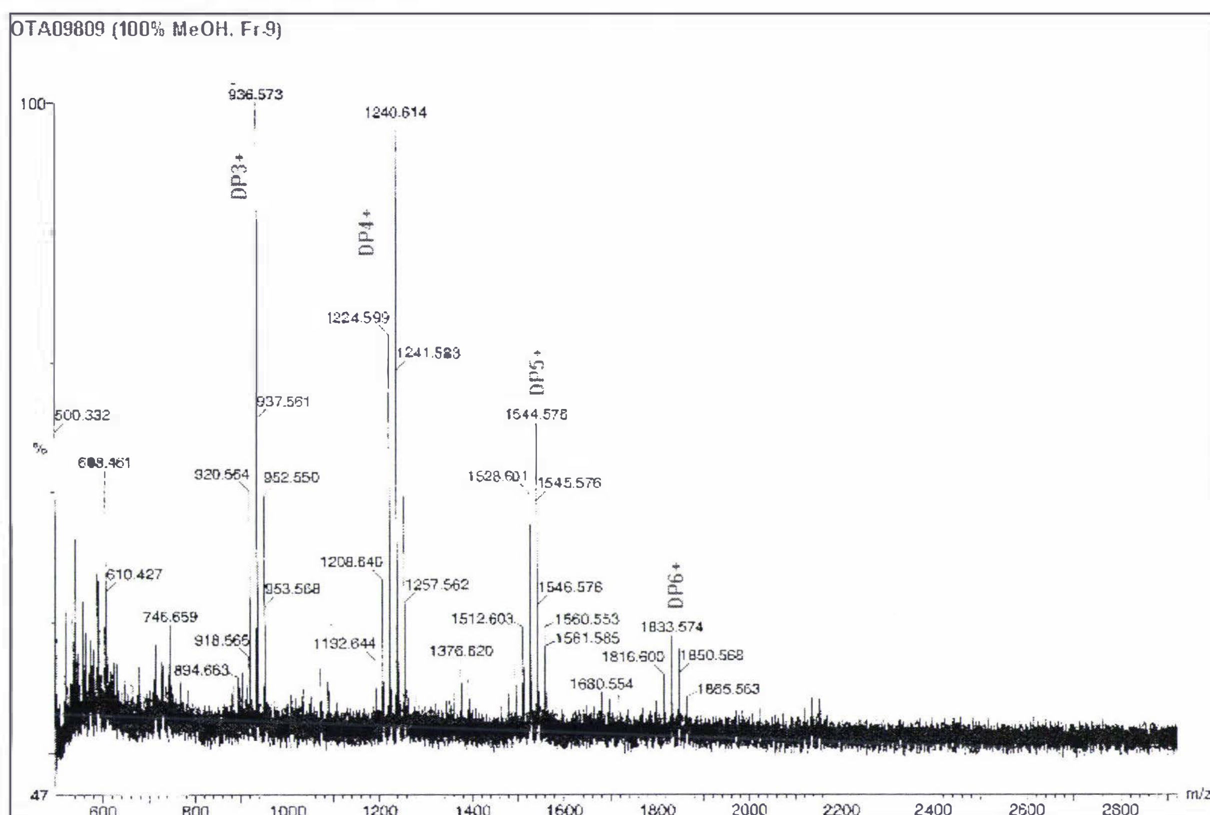


Figure 3.5 MALDI-TOF-MS spectra acquired in a positive reflectron mode for CT oligomer fractions: a) OTA04309 b) OTA09806 c) OTA09807 d) OTA09808 and e) OTA09809.

3.2.5 Thiolysis

This is the first report of evaluation of purified extractable CT from sulla by thiolysis. These results provided quantitative information on the chemical composition of sulla on PC:PD ratio, mDP and the *cis:trans* ratio. EGC is the dominant extender unit (69%) while GC is the dominant terminal unit (54%), with the overall characteristic of a PD-type CT (Figure 3.6). The peaks in the HPLC chromatograms were identified by calibration with authentic standards. Thiolysis for each fraction produced C, EC, GC, EGC in the terminal units and catechin thioylether (C-thio), epicatechin thioylether (EC-thio), gallocatechin-thioylether (GC-thio) and epigallocatechin-thioylether (EGC-thio) from the extender units. The order of elution of free flavan-3-ols and thiolysis adducts was as follows; GC> EGC> C> EC> GC-thio> EGC- thio> C-thio> EC-thio (Figure 3.6) as shown for a series of 70% acetone eluted fractions OTA02805, OTA02806, OTA03706 and OTA07008. Quantitative analysis of the eluted components were based on the use of relative response factor (RRF) with taxifolin also known as

dihydroquercetin (DHQ) used as the internal standard (Figure 3.6), which eluted at 35 minute and did not interfere with the compounds of interest.

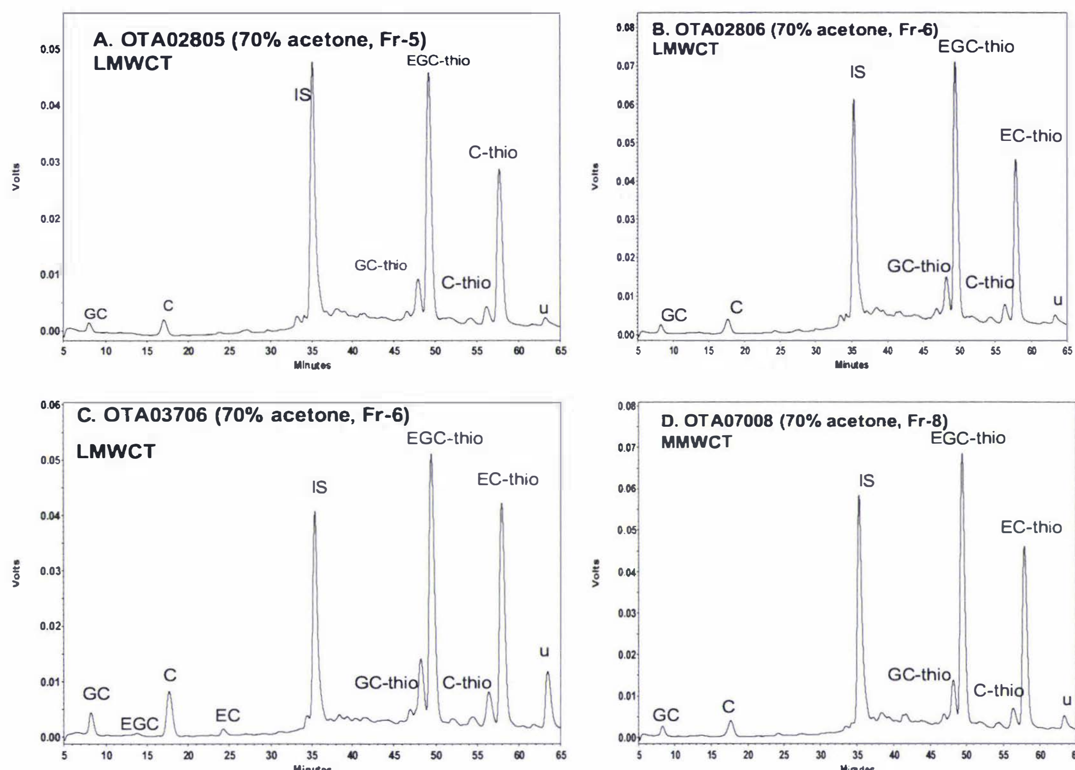


Figure 3.6 HPLC-UV chromatograms (at 280 nm) for elution of free flavan-3-ols and thioylether derivatives obtained through thiolysis reaction for 70% acetone fractions a) OTA02805 b) OTA02806 c) OTA03706 and d) OTA07008 Abbreviations: U-unknown, IS-internal standard.

Fraction OTA02805 consists of 24% C, 3% EC, 52% GC and 22% EGC in the terminal unit and 2% C, 10% EC, 16% GC and 72% EGC in the extender unit. GC was the major terminal unit while EGC was the major extender unit (Table 3.2). PC: PD ratio was 13:87, which indicates that sulla CT is PD predominant. This fraction also contained more *cis* than *trans* in the ratio of 80:20 and the mDP of 26. Fraction OTA02806 had similar composition with the fraction constituting of 27% C, 3% EC, 53% GC and 17% EGC in the terminal unit and 3% C, 11 % EC, 20% GC and 66% EGC in the extender unit. EGC-thio had the highest peak, which confirms that EGC is the major extender unit (Figure 3.6). The fraction had more *cis* than *trans* units in the ratio 74:26 and a PC:PD ratio of 15:85. The mDP was 14 (Table 3.2), which was lower than that of the first 70% acetone fraction. Similar results were obtained for fraction OTA03705 and OTA03706. The results were fairly comparable to previous step LH-20 70% acetone fractions (Table 3.2), LMA04205 and LMA04206 with a mDP of 21 and 11, respectively (Meagher, 2003). The average *cis:trans* ratio and PC:PD ratio for the

step LH-20 fractions is 77:23 and 14:86, respectively. The CT composition for LH-20 fractions obtained from the plants collected on summer 21/12/01. However, contrary results were obtained for fraction OTA03805, which had a mDP of 13 compared to 26 obtained from the fractions OTA02805 and OTA03705. The PC:PD and *cis:trans* ratios for OTA03805 were similar to those for fraction OTA02805 and OTA03705. Fraction OTA07008 contained a medium molecular weight CT (MMWCT) with a mDPof 46. The *cis:trans* ratio increased from 56:44 for the first 100% MeOH fraction (OTA04306) to 69:31 for the last 100% MeOH fraction (OTA04306) from the gradient LH-20 method. The mDP varied between 2.9 and 6.9. Fraction OTA04310 and OTA04311 had similar composition to the 70% acetone fractions from the step LH-20.

It was also noticed that some fractions had an unknown compound (U) which eluted last at a retention time of 63 minutes (Figure 3.6 above). Viable results were not obtained for crude extracts, first fractions eluted with the 50% MeOH and water fractions probably due to poor solubility in methanol. Thiolysis is never performed in water, but the supernatant liquid was used. The results obtained were not quantitative, probably due to interference with other substances.

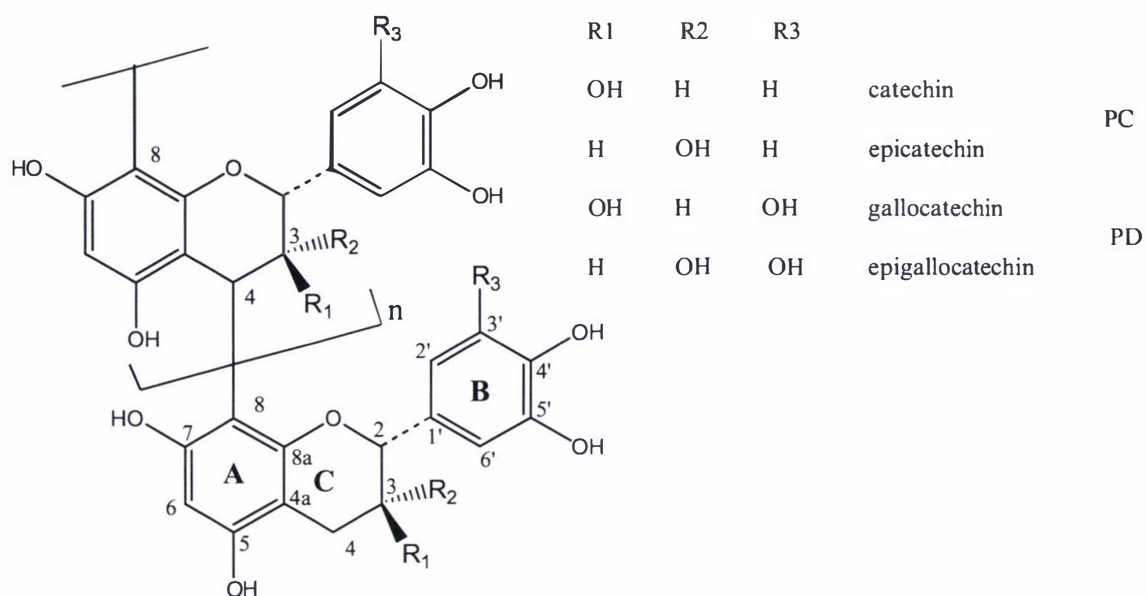
Table 3.2 Comparison of % extender and % terminal units of proanthocyanidin polymers, average degree of polymerization (mDP), *cis:trans* ratio from sulla LH-20 fractions by thiolysis.

			Terminal units				Extender units				DP	PC:PD
ID	Eluent	*Cis:trans*	C	EC	GC	EGC	C	EC	GC	EGC		
OTA02801	50% MeOH	52:48	26	31	0	43	42	0	8	50	1	43 57
OTA02805	70% Acetone	80:20	24	3	52	22	2	10	16	72	25.8	13 87
OTA02806	70% Acetone	74:26	27	3	53	17	3	11	20	66	14.2	15 85
OTA03705	70% Acetone	80:20	25	3	58	13	2	11	15	72	26	13 87
OTA03706	70% Acetone	74:26	27	4	53	16	3	12	20	65	12.9	16 84
OTA07008	70% Acetone	82:18	28	1	63	7	1	10	15	74	46	11 89
OTA03805	70% Acetone	75:25	26	0	56	17	3	13	17	67	12	16 87
OTA03806	70% Acetone	73:27	25	3	66	7	3	12	19	66	13	16 84
OTB00306	70% Acetone	72:28	26	2	66	6	3	8	20	69	11.7	12 88
OTA04301	H_2O	89:11	8	1	0	91	51	4	9	36	0.1	12 88
OTA04306	100% MeOH	56:44	30	5	54	12	8	17	23	53	2.9	27 73
OTA04307	100% MeOH	62:39	32	6	51	11	7	18	21	55	4.1	27 73
OTA04308	100% MeOH	63:37	29	5	50	16	5	18	23	54	5.0	25 75
OTA04309	100% MeOH	69:31	32	4	51	13	5	17	19	59	6.9	24 76
OTA04310	70% Acetone	74:26	26	2	59	13	3	11	20	67	14.9	14 86
OTA04311	70% Acetone	82:18	27	2	59	12	2	9	14	75	20.7	12 88
LMA04205	70% Acetone	79:21	24	2	68	6	2	6	16	76	21.2	9 91
LMA04206	70% Acetone	69:31	21	2	67	10	3	7	23	66	11.8	11 89

Abbreviations: C=catechin, EC=epicatechin, GC=gallocatechin, EGC=epigallocatechin, mDP=mean degree of polymerisation.

3.2.6 ^{13}C-NMR of condensed tannin polymers

^{13}C-NMR was run for the LH-20 70% acetone fractions containing CT polymers (n>10). The signal assignment was performed according to Foo (1980) and Behrens (2003). Broad peaks were detected in the ^{13}C-NMR spectrum for fraction OTA02806 (70% acetone), but useful information can be obtained. PC/PD, *cis/trans* distinctions can be made and the mDP can be determined. The ^{1}H NMR spectrum has broad peaks and could not be interpreted. The ^{13}C-NMR spectrum (Figure 3.7) shows a distinct signal at 146 ppm which might be attributed to the two carbons in the pyrogalloyl group, C3'and C5'. The resonance signal at 146 ppm is an indication of the presence of prodelphinidin units. The resonance signal at 145 ppm is an indication of C3' and C4' for catechol PC units. The relative proportions of PC and PD may be estimated from the signals centred around 145-146 ppm. The presence of PD is confirmed by the signals at 106.7 and 108.0 ppm corresponding to C2' and C6'. The C4' (PD) and C1' (PD) resonates downfield at 132.0 and 131.3 in the B-ring, respectively. Small signal resonating at 116 ppm depicts C3' in the B-ring signal can be seen which indicates the presence of PC hydroxy. The upfield region between 65 and 85 ppm is sensitive to the stereochemistry of the heterocyclic C-ring. The signal at 71.8 ppm was assigned to C3 extender and C3 terminal resonated at 67 ppm. The C3 terminal unit usually resonates at 67 ppm, but from the spectrum only a small peak signal was observed. So it is possible to determine the molecular weight, since the signal intensity of C3 terminal relative to that of the signal of C3 extender can be used to determine the polymer chain length. The mDP of the polymer can be integrated from the ratios of the areas of the two C-3 signals giving extension: terminal flavan-3-ol group. In addition, the ratios of the C2 *cis* and C2 *trans* for extender and terminal unit could be used for the calculation of the *cis:trans* ratio. The signal at 75.8 ppm is attributable to C2 *cis* while at 84 ppm is attributable to C2 *trans*. This is also an indication that this fraction has more *cis* than *trans*. The signal intensity for the C2 *trans* was low, and is usually expected to resonate at 84 ppm. The C4 signal is shown upfield at 36.7 ppm. The A-ring signals are observed downfield at 155.1 ppm and 153.9 ppm for C5, C7 and C8a.



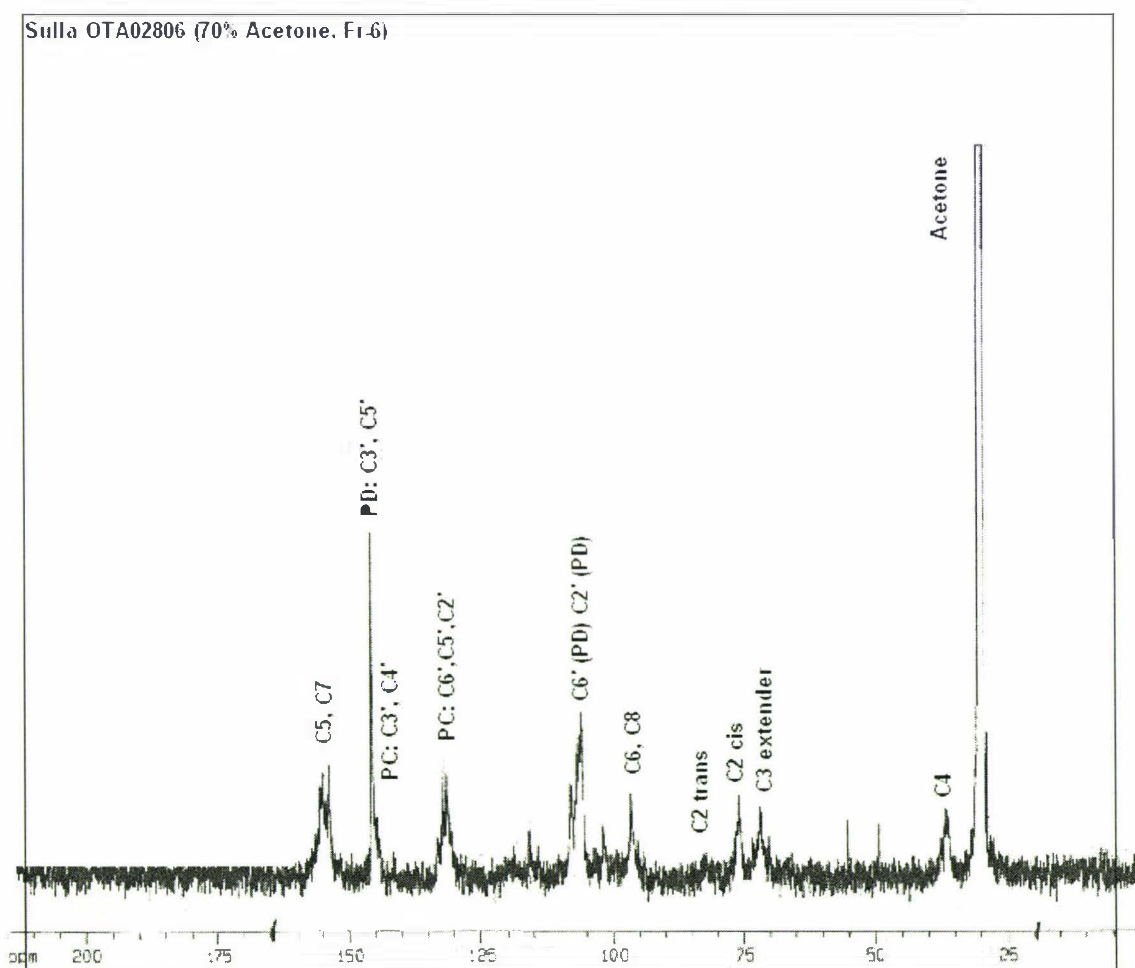


Figure 3.7 a) Chemical structure of CT polymer and b) ^{13}C-NMR spectra for CT polymer (OTA02806, 70% acetone, Fr-6).

Similar ^{13}C-NMR spectrum was obtained for fraction OTA07008 (70% acetone) with a mDP of 46. The spectrum had higher signal intensity (Appendix 5). However, the PC:PD, *cis:trans* ratio and average molecular weight were not integrated.

3.2.7 Acid butanol assay

Results for fibre and protein bound CT content were determined by wet chemistry methods. In this assay, freeze-dried and ground sulla summer (21/12/01) and spring (23/09/02) were found to contain total CT concentrations of 7.6 and 5.0%, respectively (Table 3.3). Summer (21/12/01) contained the highest free CT content of 5.3% while spring (23/09/02) contained 3.7%. Summer (21/12/01) had protein-bound CT with a concentration of 2% while spring (23/09/02) contained CT content of 1.3 %. Fibre-bound CT content of 0.3% was only detected in spring (23/09/02).

Table 3.3 Concentrations of free, protein bound and fibre bound CT (g/100g) from sulla plants measured by the butanol-HCl method (Terrill et al. 1992) with literature comparisons.

Season and date of harvesting	% free CT (g/100g)	Protein bound CT (g/100g)g	Fibre bound CT (g/100g)	Total extract CT (g/100g)
Summer (21/12/01)	5.3	2.0	0.3	7.6
Spring (23/09/02)	3.7	1.3	ND	5.0
Data from the literature				
Burke et al. (2002)	-	-	-	5.6
Waghorn et al. (2002)	-	-	-	6.8
	-	-	-	3.5
Woodward et al. (2002)	-	-	-	2.7
Bermingham et al. (2001)	-	-	-	6.4

The standard curve (Figure 3.8) was plotted and absorbances from the samples were substituted in the equation to determine CT concentration in ppm and converted to %CT (g/100g). The LH-20 70% acetone fraction obtained from sulla plant material collected summer 21/12/01 was used as the standard.

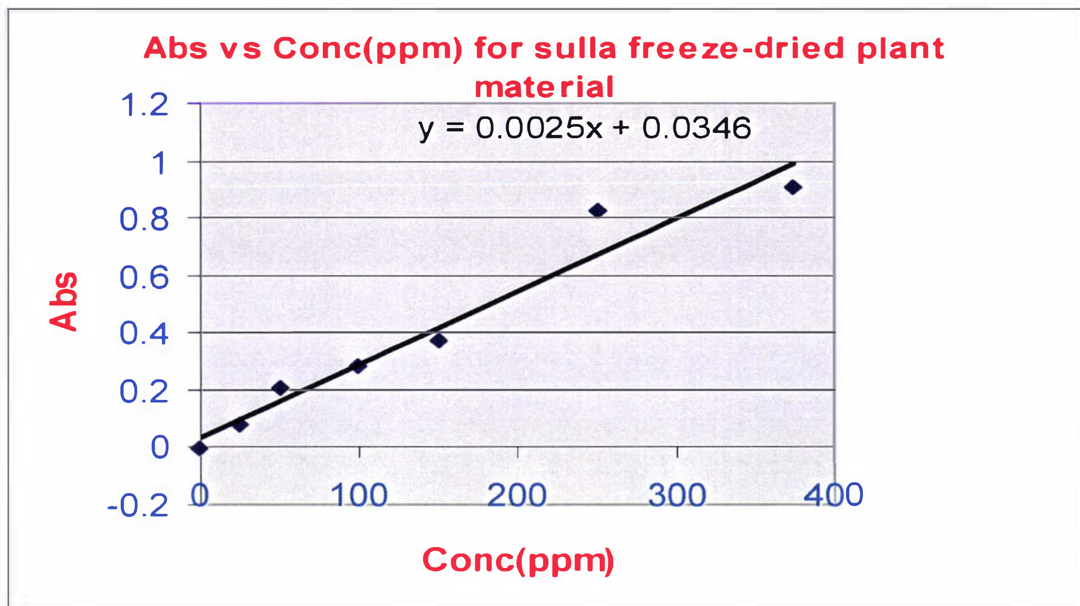


Figure 3.8 Calibration curve for the standards in the determination of CT concentration of plant material.

3.2.8 NIRS data

The nutritional composition results show that sulla has a higher CP content (%DM) of 24.4, 25.7 and 25.1 for sulla summer (21/12/01), spring (23/09/02) and summer (12/12/02), respectively (Table 3.4).

Table 3.4 Nutritional composition (g/100g) of sulla plants collected on different dates and analysed using NIRS with literature comparisons.

Sample name	Date of collection	CP %DM	NSC %DM	Lipid %DM	NDF %DM	ADF %DM	Ash %DM	OMD %DM
Summer	21/12/2001	24.4	17.1	2.6	14.8	18.3	11.7	83.8
Spring	23/09/2002	25.7	19.4	3.3	16.4	15.5	11.7	>85
Summer	12/12/2002	25.1	19.7	3.3	12.5	15.8	11.4	>85
Data from the literature								
Burke et al. (2002)		19.2	21.8	2.2	21.5	-	-	-
Waghorn et al. (2002)		17.5	23.2	-	20.2	-	-	-
		19.7	17.6	-	22.6	-	-	-
Woodward et al. (2002)		26.6	19.2	3.4	14.7	14.5	-	-

Abbreviations: DM, dry matter; CP, crude protein; ADF, acid detergent fibre; NDF, neutral detergent fibre; NSC, non-structural carbohydrates; OMD, organic matter digestibility.

Dietary protein, which is generally referred to as crude protein (CP) is defined as the nitrogen (N) content x 6.25. The nutritional composition results from NIRS analysis depict sulla to contain high non structural carbohydrates (17.1-19.7% DM). The lipid

content was found to be 2.6-3.3%, which is within the normal range. The NDF measures structural components in the cell wall consisting of cellulose, hemicellulose and lignin and are less degradable. The NDF (%DM) was 13.8, 16.4 and 12.5 for summer (21/12/01), spring (23/09/02), and summer (12/12/02), respectively. Sulla collected in summer (12/12/02) contained the lowest NDF content. Organic matter digestibility is a prediction and was higher for all the samples and of good quality. Sulla summer (21/12/01) had a lower CP, lipid, NSC and NDF content compared to spring (23/09/02) and summer (12/12/02) (Table 3.3).

3.2.9 Soluble sugars and available carbohydrate

Results for the identification of sugars were provided by Wilhelmina Martin, Analytical Laboratory, Nutrition and Behaviour, AgResearch, Palmerston North. Retention times of the external standards were compared to those of the samples. We are not aware of any report on analysis of individual sugars by HPLC and available carbohydrate from sulla leaves in the literature, so this is the first report on identification and quantification of soluble sugars. The following sugars were identified from freeze-dried sulla leaves in comparison with commercially available reference standards; monosaccharides (fructose, glucose, xylose, galactose, rhamnose, arabinose) and disaccharide (sucrose) (Table 3.5). It was observed from the chromatogram that rhamnose co-eluted with galactose.

Table 3.5 Soluble sugars content and available carbohydrates (g/100g) from sulla leaves.

Sample name	Date of collection	Sucrose (g/100g)	Glucose (g/100g)	Xylose (g/100g)	Galactose and/or rhamnose (g/100g)	Fructose (g/100g)	Available carbohydrate (g/100g)
Spring	23/09/02	1.63	3.68	1.87	0.46	0.98	12.38
Summer	21/12/01	5.50	5.40	1.45	0.34	1.69	14.11

The eluted sugars were quantified by peak areas comparison, using mixed standards of fructose, galactose, rhamnose, sucrose and xylose. Glucose (3.68%) was the major sugar present, followed by xylose (1.87%) and sucrose (1.63%). Galactose and rhamnose (0.46%) and fructose (0.98%) were minor sugars present from sulla spring (23/09/02) (Table 3.5). Sucrose (5.50%) was higher for sulla summer (21/12/01). Glucose (5.40%) was also the major sugar present. Fructose and xylose were also the

minor sugar present in both sulla plants (Table 3.5). The available carbohydrate was higher for sulla summer (21/12/01). Available carbohydrate content (%) for sulla summer (21/12/01) and spring (23/09/02) were fairly close with a content of 14.11 and 12.38, respectively.

3.3 Discussion

3.3.1 LC/MS of CT oligomers

CT oligomers, but not polymers from LH-20 fractions were characterised using LC-ESI-MS. The presence of CT oligomers was also confirmed in the UV spectrum with absorption maxima in the range 269-280 nm. The response of the polyphenols is better in a negative ion mode $(M\text{-}H)^-$ than in a positive ion mode $(M+H)^+$ (Fulcrand et al., 1999). Several experiments have been performed and have revealed poor ionisation in instances where the MS is performed in a positive ion mode, but works better for CT containing PCs only (Svedstrom et al., 2002). ESI–MS is a relatively soft ionisation technique with less or minimal fragmentation and as such parent molecules can be detected. These data indicate that sulla contains hetero-oligomers CT containing C/EC and GC/EGC units in the same CT molecule characteristic of PD-type.

Analysis with LC-ESI-MS has shown sulla fractions from the step LH-20 fractionation to contain CT oligomers from dimers up to heptamers which were detected in negative ionisation mode. Poor ionisation of the OTA02805 fraction might suggest that it is constitutive of a high molecular weight CT polymer. Fraction OTA02806 was expected to be less well ionised as compared to fraction OTA02805 since it was the second 70% acetone fraction eluted as most of the CT oligomers are expected to be eluted in the first 70% acetone fraction. There is also a possibility that these two fractions were somehow interchanged. However, a similar trend was observed in other fractions from the same batch and from thiolysis results performed in duplicate.

For the gradient LH-20 fractionation method, the first 100% MeOH fraction (OTA04306) consisted of flavonoids and CT oligomers and as such a complex spectrum was obtained. Unexpected results were obtained for fraction OTA04308 in that it exhibited few ions. Doubly charged species up to octamers were detected from fraction OTA09809, which were below the mDP of 9.1 obtained from thiolysis (Table 3.6 and Appendix 7). The low molecular weight (LMWCT) oligomer from fraction OTA04309 had the highest *m/z* 897 ion for a doubly charged hexamer, while for the HMWCT polymer from OTA04310, a doubly charged octamer with *m/z* 1209 and triply charged

undecamer with *m/z* 1110 were detected. The highest *m/z* ions detected from the LH-20 fractions from sulla by ESI-MS corresponds to the mass of doubly charged octamer which is just below the value of the mDP (14.9) which was estimated by thiolysis (Table 3.6). However, larger polymers were expected as previously found or detected in other plant derived food substances (Souquet et al., 2000). The study was carried out to investigate the presence or absence of mixed CT (containing both PC and PD). No homo-PD or PC oligomers were detected in sulla leaves. The ESI-MS results for the gradient LH-20 70% acetone fractions are consistent with the increase in chain length. For example, fraction OTA04309 had multiply charged ions up to hexamers which is consistent with its mDP from thiolysis of 6.9. As the molecular weight increases it becomes increasingly difficult to detect the ions due to overlapping of multicharged species and increasing number of possible isomers without the use of a high resolution MS. This study indicates the mDP to be between 2 and 11. The low molecular weight CT oligomers were detected as singly charged ions, but the high molecular weight CT oligomers were detected as doubly and triply charged ions. The high molecular weight CT oligomers were evidenced from fraction OTA09807 to OTA09809 obtained from the freeze-dried sulla (Table 3.6).

The MS spectra for the first fractions of the 50% MeOH from step LH-20, water fractions from gradient LH-20 and aqueous crude extracts were not viable due to interferences from flavonoids and sugars. Alternatively, the mass spectra were not obtained because the ions present were outside the mass range of the instrument, or not carrying sufficient charge to be detected. It is not known if some of the molecules detected are real or they arise from the fragmentation of large molecules and as such there may be other possibilities. The step LH-20 fractions eluted with 70% acetone are constitutive of a mixture of CT oligomers and polymers. The gradient LH-20 method yielded a series of CT oligomers with increasing chain length separated from CT polymers. This indicates that the gradient LH-20 fractionation method is efficient at separating CT oligomers and polymers. In general, signal intensity decreases as the mDP increases. Moreover, beyond tetramers, oligomers mainly occur as multicharged ions, implying that the probability to find them existing as singly charged ions decreases. The CT oligomers were isolated for the first time from sulla.

To further validate these data, analysis of the CT oligomers was performed using MALDI-TOF-MS employed in a positive reflectron mode. It is a soft ionisation technique with less fragmentation which aids in detecting a series of CT oligomers

within a mixture. No multicharged ions were detected in this technique. Thus, from the results, sulla oligomeric fractions contained a mixture of homo and hetero-oligomers units. Hetero-oligomers appear to be predominant amongst most of the fractions. Pure PCs and PDs were also detected.

The lack of chemical studies of large CT molecules is due to their polymeric nature, structural diversity and insolubility as the molecular weight increases. One of the advantages of ESI-MS is that it can be easily interfaced with LC to permit on-line analysis of the components within a mixture. Thus, this technique has been largely employed in analysis of polar molecules such as CT. However, several polymers consisting of PD only have not been identified, unlike in other instances where PC polymer exist especially in plant derived food substances (De Silva et al., 1991). The results of ESI-MS for sulla LH-20 fractions are presented herein. There is also a possibility that some of the simple monomers might be functionalised with ester gallates. This pattern has been observed in other fractions. These results are in good agreement with those of Fulcrand (1999). The LC/MS does not provide information on the CT polymer (n>10), but useful information can be obtained from NMR.

Table 3.6 Comparison of mDP by acid catalysis using benzyl mercaptan and the mass range estimates by MALDI-TOF and ESI-MS.

Fraction ID	mDP by thiolysis	Mass range by MALDI-TOF	Mass range by ESI-MS
OTA02806 (70% acetone)	14.2	-	2-7
OTA04306 (100% MeOH)	2.9	-	-
OTA04307 (100% MeOH)	4.1	-	1-3
OTA04308 (100% MeOH)	5.0	-	1-3
OTA04309 (100% MeOH)	6.9	3-4	2-6
OTA04310 (70% acetone)	14.9	-	4-11
OTA09806 (100% MeOH)	3.8	3-4	-
OTA09807 (100% MeOH)	5.7	3-4	2-3
OTA09808 (100% MeOH)	6.9	3-4	4-6
OTA09809 (100% MeOH)	9.1	3-6	4-8

It is clearly shown from the SIM-MS chromatograms that signal intensity decreases as the number of PD units increases. Pure PD oligomer is more polar and was eluted first, followed by PC/PD mixed dimers and pure PC dimer later. The existence of these species was confirmed using SIM-MS which is very sensitive as specific ions were targeted. It was noted that the trihydroxylated units were eluted earlier than dihyroxylated species since they were more polar. Singly, doubly and triply charged

ions were detected. Finally, doubly charged octamers and triply charged undecamers were the highest *m/z* detected. Sensitivity decreases as the molecular weight increases.

3.3.2 MALDI-TOF of CT oligomers

MALDI-TOF-MS is a widely used, sensitive, fast and soft ionisation technique with little or no fragmentation. This technique made it possible to detect low and higher molecular weight oligomers in complex mixtures from sulla. In this analysis, singly charged ions were released and no multiply charged species were detected like ESI-MS and APCI. The matrix DHB which was used has been shown to be ideal in the analysis of CT oligomers. No spectra were obtained for the analysis of CT polymers which could be attributed to the behaviour of laser irradiation with the matrix and homogeneity of the sample. All these points may become the limiting factors for the ionisation of the sample. The principle involves the laser radiation irradiating the matrix which desorbs and ionises the sample. The purpose of the matrix is also to protect the sample from fragmentation by absorbing the incoming laser radiation and also to aid in ionising the sample. Since its inception, MALDI-TOF has found a wide application especially in characterisation of large molecules such as CT from food samples (Ohnishi-Kameyana et al., 1997). It is able to detect CT that have structural variations and are highly heterogeneous.

The last 100% MeOH fraction from the gradient LH-20 method is expected to possess high molecular weight oligomers close to the mDP of 9.1 from thiolysis. However, poor ionisation of the higher oligomers might be a contributing factor. The largest CT oligomers detected in the mass spectrum were hexamers which were below the DP of 9.1 from thiolysis. Fraction OTA04309 with a DP of 6.9 from thiolysis was expected to have a similar spectrum as OTA09809 with a DP of 9.1 from thiolysis. But from the mass spectrum, it appears to be consisting of oligomers with a chain length ranging between 3 and 4. However, the results from the mass spectrum show these oligomers to be below the mDP 6.9 acquired by thiolysis (Table 3.6). The MALDI-TOF results for fraction OTA09809 are below the mass range of 4 to 8 obtained from the ESI mass spectrum (Table 3.6 and Appendix 7). The ESI and MALDI-TOF-MS polymer size estimates were lower than the DP from thiolysis (Table 3.6). This shows that MS may not give the actual abundance of CT polymer with different DP, but just the relative ease of ionisation of the compounds.

The MALDI-TOF spectrum provided information in the analysis of CT oligomers when it was employed in a positive-ion reflectron mode. In this mode,

MALDI-TOF is associated with a good sensitivity and resolution. Sodium and potassium adducts were observed whilst no protonated molecular ions $(M+H)^+$ were detected. One of the advantages of MALDI-TOF as compared to ^{13}C-NMR spectroscopy and other MS techniques is that it can elucidate a series of a mixture of closely related CT oligomers reasonably well (Jian Wong et al. 2000). From the MALDI-TOF results, it can be noted that the CT oligomers were PD predominant. This is also supported by thiolysis and LC-ESI-MS results. Therefore, the MALDI-TOF spectra suggest the existence of heterogeneous and homogeneous polymer with PD as the predominant unit. MALDI-TOF revealed that CT from sulla are a mixture of oligomers ranging from trimers to hexamers, consisting of PC (catechin/epicatechin) and PD (gallocatechin/epigallocatechin) units. No dimers were detected in any of the fractions. The peaks were well resolved and the highest oligomers observed were hexamers. Detection of high molecular weight CT was tried using acetonitrile/water mixtures (1:1) and ionisation performed in a linear mode, but no spectrum was obtained. To improve spectra quality and crystallisation process, analysis can be performed using various solvent mixtures which are usually employed in extraction and purification of CT (Bohm, 1998).

It should also be noted that the choice of the matrix is crucial in characterization of CT oligomers as other matrices are incapable of ionising the sample. Different matrices such as 2,5-dihydroxy benzoic acid (DHB), trans-3-indole acrylic acid (IAA) and 2,4,6-trihydroacetophenone (THAP) have been employed in the analysis of CT oligomers and have shown to be effective and efficacious. Other matrices such as sinapinic acid (SA) and α-cyano-4-hydroxycinnamic acid (CHCA) have been tried but do not produce any mass spectra (Krueger et al., 2000). Detection of higher molecular weight compounds can be attained by using the IAA and THAP matrix. These matrices were not available. DHB was the only matrix used in the analysis. No spectra were obtained for CT with a higher molecular weight above hexamers. There is still a possibility of detecting CT oligomers beyond hexamers. They could not be identified because sensitivity decreases as the chain length increases. It is speculated that the low molecular weight oligomers saturate the detector and as such reduce the detection of high molecular weight oligomers. Higher oligomeric CT up to undecamer has been detected using MALDI-TOF from apple extracts using trans-3-indole acrylic acid as the matrix (Ohnishi-Kameyana et al., 1997). Other articles have shown the characterization of *Lotus spp*. CT oligomers, also a legume species, yielding up to decamers (Hedqvist et

al., 2000). This study attempts to report for the first time the characterisation of a series of CT oligomers from sulla using MALDI-TOF-MS.

3.3.3 Thiolysis

The degradation of CT polymers from sulla with benzyl mercaptan and analysis with RP-HPLC has indicated that it contains all four flavan-3-ol monomers; C, EC, GC and GC as both terminal and extender units. A late eluting peak in the RP-HPLC chromatograms may be an indication of possibile galloylation.

CT containing homogenous PCs can be separated using NP-HPLC (Svedstrom et al., 2002), but this technique can not be employed for heterogeneous CT. CT with pure PCs are mainly prevalent in plant derived food substances (Prior et al., 2002), but also present in white clover flowers (Foo et al., 2000). Chromatograms of the 70% acetone fractions had big humps (Figure 3.4), which after the degradation of the CT polymer with benzyl mercaptan produced well resolved peaks (Figure 3.10). After thiolytic degradation, these compounds were converted into monomer units (free flavan-3-ols and flavan-3-ol thioether derivatives). Detection was performed at 280 nm, so this might have led to a lower signal intensity of CT containing PD. The PD absorb around 269-272 nm, but the detection was performed at 280 nm which is favourable for the PC which have absorption maxima at 278 nm (Guyot et al., 1998).

The retention times for the gallocatechin/epigallocatechin units are shorter than those of catechin/epicatechin units. The gallocatechin/epigallocatechin thioylether derivatives were eluted earlier. The order of elution might also be attributed to the stereochemistry at the C-3 position (Bohm et al., 1992). Gallocatechin with 2R: 3S configuration has a shorter retention time than 2R: 3R configuration. This is also shown by catechin eluting before epicatechin. Determination of the constituent units of CT from sulla was performed by degradation with dilute HCl in the presence of reactive nucleophile, benzyl mercaptan. The stereochemistry at C3 is known to be preserved (Hemingway and Karchesy, 1990). Although benzyl mercaptan has an unpleasant smell, it has more advantages in obtaining a well resolved spectra and very effective in capturing the carbocations. Phloroglucinol was not used in the analysis of sulla CT due to its ineffectiveness in trapping the carbocations released and is associated with low yields (Scalbert et al., 1997). The results obtained have shown that 30 minutes reaction time was sufficient for complete degradation of the heterogeneous sulla CT polymer. EGC was found to be the major extender unit while gallocatechin was found to be the major terminal unit, with overall characteristic of PD predominance. All the fractions

from plant samples harvested on different dates have similar PC:PD ratio, *cis:trans* ratio and mDP. For example, fraction OTA02805 from summer (21/12/01) has PC:PD ratio of 13:87 while OTA03805 from spring (05/08/02) has a PC:PD ratio of 16:84.

The PC:PD ratio varies amongst the forage legumes. LP is known to be PD predominant (Foo et al., 1996), sainfoin to be PD predominant (Ferreira et al., 2000) and LC to be PC predominant (Foo et al., 1997). Units with 2,3-*cis* stereochemistry are predominant in sulla and this is apparently normal because this phenomena is observed in most of the forage legumes. From the literature LC, LP and sainfoin leaves are *cis* predominant with the *cis:trans* ratio of 92:8, 75:25 and 90:10, respectively (Foo et al., 1982). From the results in Table 3.2, it can be observed that *cis:trans* ratio increases as the chain length increases.

Besides CT concentration, the biological activity is also attributed to the chemical structure, mDP and the PC and PD sub-units (Foo et al., 1996; 1997). Thiolysis results have shown sulla to be PD and *cis* predominant, has CT oligomers with chain length varying between 2.9 and 6.9. In addition, it has LMWCT polymers varying between 12 and 26 and a MMWCT polymer of 46. Sulla does not appear to contain any HMWCT as it has been depicted in other forages like *D. rectum* which has mDP of 138 (Meagher et al., 2003, unpublished). The mDP of fractions OTA09806 to OTA09809 from freeze-dried sulla material was higher (Table 3.6). However, this is not an indication that freeze-drying extends the chain length. The increase of the CT hump from PDA for fraction OTA04306 to OTA04309 or OTA09806 to OTA09809 is consistent with the increase in chain length.

The additional hydroxyl groups in the polyflavan-3-ol structures could be responsible for its antiparasitic activity since reactivity increases with increasing PD content. The *cis/trans* stereochemistry also affects reactivity and most of the forages with higher activity are associated with the predominance of *cis* stereochemistry. The predominance of *cis* stereochemistry in CT from sulla could be a contributing factor to its antiparasitic activity. Further research is required to provide convincing evidence on the structure-activity relationship using other forages which are known to contain CT. The effect of increasing molecular weight of CT on parasites needs further investigation.

Freeze-drying does not appear to have any significant effect on the chemical composition. However, some variation was observed on average chain lengths. Fraction

OTA03805 from fresh frozen sulla had a chain length of 12 while OTA02805 from a freeze-dried sulla had a chain length of 25. This may be an indication that OTA02805 contains a higher molecular weight CT. The PDA results for the LH-20 70% acetone fractions from fresh frozen and freeze-dried sulla spring (23/09/02) indicated that the plant contained no CT. So no data was obtained to make comparison on the effect of freeze-drying on chemical composition between fresh frozen and freeze-dried LH-20 fractions from sulla collected on the 23rd Sept 2002. Seasonality does not appear to have any effect on PC:PD ratios, *cis:trans* ratios and mDP. Further work is still required to investigate the effect of seasonality on the chemical composition of CT.

3.3.4 Acid butanol assay

The Bu-HCl assay is a colorimetric technique which does not provide any information on the chemical structure of CT, only the CT concentration. The assay is a diagnostic tool for the presence of the polyflavan structure. From the results obtained, the total CT content from the freeze-dried sulla plants collected in spring (23/09/02) and summer (21/12/01) were 5.0 and 7.6% CT (g/100g), respectively. The effect of freeze-drying on CT content was to be investigated, but since freeze-dried and milled plant material was used, no comparison could be made with fresh frozen material which was not available. Fresh frozen material could not be used in the quantitative analysis of CT. CT content was lower during spring (23/09/02) than summer (21/12/01). This might suggest that there is a possibility that seasonal effects contributed to low CT content during spring.

In an attempt to investigate the effect of freeze-drying, a fresh frozen sulla spring (23/09/02) leaves were freeze-dried and fractions from the LH-20 were run by HPLC-PDA. Apparently no hump was observed in the PDA chromatogram for the 70% acetone fractions, which indicates that the plant had no CT or had trace amounts of CT. This raises many questions on the authenticity of the butanol-HCl method in the determination of the CT content.

Insoluble CTs are protein and fibre-bound, so a modified Bu-HCl method was used for their determination (Terrill et al., 1992b). These bound CT are nutritionally beneficial to ruminants as in these low concentrations could increase the absorption of essential amino acids in the small intestine. CT-protein complexes are pH dependant, in that dissociation of the complex occurs at pH<3.5 in the small intestine. “Free CT” can be detrimental to ruminants especially in high concentrations because of their ability to inactivate microbial enzymes. The results obtained indicate that sulla has protein bound

and fibre-bound CT in low concentrations. This may indicate that the low concentrations of CT (5.0% from spring-23/09/02) determined from sulla could be of beneficial nutritional effects in ruminant production, as CT in these low concentrations protect protein from degradation and therefore increase the flow of essential amino acids in the small intestine. From the literature, the concentration of CT in sulla is known to be in the range 3-12% DM (g/100g) (Waghorn et al., 1998). When the CT concentration is above 6% DM, animal performance is impaired due to reduction in rumen carbohydrate digestion (Barry et al., 1996), protein digestibility and reduced feed intake (Kumar et al., 1988), but is also dependent on the chemical attributes of CT (Burke et al., 1990; Waghorn et al., 1990; Douglas et al., 1999). A trial undertaken to study the effects of high concentrations of CTs in sulla (8.8% DM) showed that animal performance was not impaired (Douglas et al., 1999). Therefore, the higher CT concentrations of sulla spring-05/08/02 (7.6% DM) may have beneficial effects in ruminant production. Increase in the flow of amino acids in the small intestine was observed when sheep were fed sulla with a CT content of 6.44% (Bermingham et al., 2001) (Table 3.3). It should also be understood that nutritious forages with high dietary protein supply such as sulla may increase performance and enhance the immune system. These low concentrations of CT from sulla could reduce carcass fatness in growing lambs, reduce methane emissions from ruminants (Woodward et al., 2002) and may help the ruminants to counteract the effects of parasitism (Molan et al., 2002) in temperate regions. The low concentrations of CT from LC have been associated with increase in milk yield (Wang et al., 1996) while CT from sulla are associated with mitigation of worm numbers (Niezen et al., 2002). The antiparasitic effects of LP CT might be attributed to the chemical structure, mDP and the PC and PD sub-units (Foo et al., 1996). Sulla has been found to be PD predominant, possessing low molecular weight CT oligomers, LMWCT and MMWCT polymer.

Sulla is known to have variable CT content. In the study conducted by Woodward et al. (2002), the CT concentration (%DM) was found to be as low as 2.7% (Table 3.3). It is not understood why sulla behaves like this as some forages such as LP, LC have a stable CT concentration, alternatively the variation might be attributed to soil fertility and other environmental factors.

The refined LH-20 70% acetone fraction from sulla plant material was used as the standard. This helps to reduce overestimates and underestimates, especially as catechin, delphinidin and quebracho tannin are widely used as standards (Schofield et

al., 2001). To mitigate these drawbacks, standards from the plant materials are recommended (Waterman and Mole., 1994). The Bu-HCl assay is used to quantify the CT but because of some insoluble CT, underestimations may not be avoided. The CT from LP has also been used as the standard. It has to be utilised with caution because CT from forages differ in chemical structure and mean degree of polymerisation (Foo et al., 1996). This present study attempts to report a more reliable determination of CT content from sulla using CT isolated from sulla as the standard.

The choice of the correct solvent for CT extraction is crucial. The boiling MeOH/water (1:1) has been used as the extracting solvent (Foo and Porter., 1980), but this method is questionable considering the lability of CT. But a 70% acetone/water mixture (7:3) has emerged as an efficient solvent for the extraction of CT because it has the ability to break the CT-protein associations much better than methanol. Porter et al. (1989) suggested that 70% acetone is the best solvent and most efficient extraction solvent. This has been proved to be a reliable method because when the crude extract is loaded onto LH-20 column, the CT adsorb to the stationary phase and can only be eluted more efficiently with 70% acetone than methanol.

3.3.5 NIRS data

Based on these chemical composition data obtained from the NIRS, sulla has a high nutrient value for ruminants with a higher CP, NSC and lower dietary fibre. From the results, sulla was found to contain CP (%DM) in the range 24.4-25.7. The CP content is higher than those obtained by Burke et al. (2002) and Waghorn (2002) in which the CP content ranged from 17.5-19.2 %DM. The CP results correlate with those of Woodward (2002) with CP content of26.6% DM. The high CP of this level may be associated with high performance (Waghorn et al., 1998). The high performance in ruminants fed sulla might be ascribed to high CP. Alternatively, CT binds to proteins in the rumen and dissociates in the abomasum and therefore increase the flow of amino acids in the small intestine.

It is not known whether sulla can be used as a sole diet or it would have detrimental effects on animal performance over a long period of time (Burke et al., 2002). The nutritional composition results from NIRS analysis depict sulla to contain high non structural carbohydrates (17.1-19.7% DM). This is not within the normal range which is usually 10-15%. These results are fairly comparable with those obtained from the literature in which NSC were found to be 21.8% DM (Burke et al., 2002). The NDF measures structural components in the cell wall consisting of cellulose, hemicellulose

and lignin and are less degradable. The mean NDF content was 14.3% DM. The chemical composition of these samples agrees favourably well with those found by Burke et al. (2002) though NDF is lower. The findings obtained by Waghorn (2002) indicated a higher NDF content in sulla (Table 3.4). These data agree with those of Woodward (2002) in which sulla was found to contain 26.6% CP, 3.4% Lipid, 14.5% ADF and 19.2% NDF content (Table 3.4). These results suggest that sulla has a lower fibre content which is not within the normal range. However, the lower fibre content in sulla is believed to play a major role in reduction of methane emissions from ruminants. Dietary fibre is broken down during digestion in the rumen producing hydrogen which is converted to methane gas by methanogens (Woodward et al., 2002). So, forages with higher fibre content would release high methane emissions compared to sulla with a lower fibre content. The lower fibre content may increase feed intake, as it was observed in lambs fed lucerne supplemented with sulla (Burke et al., 2002).

3.3.6 Soluble sugars and available carbohydrate

Soluble sugars were identified and analysed by HPLC from sulla and found to have variable concentrations. This study has shown that sucrose, glucose and fructose were the major components of soluble sugars. Forages containing high concentrations of water soluble carbohydrates have been implicated to play a major role in diet selection by ruminants (Ciavarella et al., 2000). Forages such as sulla containing high concentrations of water soluble carbohydrates are linked with high performance in lambs (Molle et al., 2003). The high concentrations of soluble sugars and available carbohydrate determined from sulla could contribute to high voluntary feed intake and better performance (Douglas et al., 1999; Terrill et al., 1992a). Acceptability is a crucial factor for stock maintenance, especially when feed is in short supply. It is actually not known which soluble sugar component may be responsible for the high voluntary feed intake and better performance. The sugars are ubiquitous in forages. Sucrose has been identified as the major water soluble carbohydrate from sainfoin (Marais et al., 2000). The concentration of soluble sugars is known to vary with season (Marais et al., 2000; Ulyatt et al., 2002), and the low concentration of sucrose from sulla collected on summer (21/12/01) may be attributed to seasonality, as this plant material was harvested during summer. The sugar content of glucose, xylose, fructose, galactose and rhamnose from samples collected in summer (21/12/01) and spring (23/09/02) did not differ significantly. Another sugar component was eluted at the same time with the alcohol peak, which might be arabinose and could not be quantified.

3.4 Conclusions

The separation of the CT was achieved using step and gradient Sephadex LH-20 methods and this enabled information to be obtained using spectroscopic techniques. TLC and HPLC-UV chromatography with a PDA detector were useful analytical techniques which provided qualitative information for the detection and identification of CT and flavonoids from sulla leaves. The gradient LH-20 method improved fractionation of CTs and helped us obtain a better understanding of the large structural diversities existing in the CT from sulla. This indicates that better and new fractionation methods for the CT polymers are still needed to better study the relationships of the molecular size to their biological properties.

The acid thiolytic degradation reaction with subsequent analysis of the thiolysis products using RP-HPLC enabled the quantitative analysis of CT to study the chemical characteristics of CT from sulla leaves. ^{13}C-NMR provided information on the stereochemistry of the heterocyclic C-ring and the existence of PC and PD sub-units in the B-ring. However, no information could be obtained on the C-C interflavan linkages of the CT polymer. The results revealed that sulla CT consist of a considerable variable average chain lengths, mean molecular weight, PC:PD ratio and *cis/trans* stereochemistry. CT from sulla were found to be predominantly *cis* and PD-type. Seasonality and freeze-drying appeared to have no effects on the sulla CT composition.

CT oligomers were well characterised using ESI-MS and singly charged ions up to trimers, doubly charged ions up to octamers and triply charged species up to undecamers consisting of homogenous and heterogeneous CT characteristic of PD-type were detected. MALDI-TOF mass spectra recorded using DHB matrix, complemented ^{13}C-NMR, ESI-MS and thiolysis data by providing average chain lengths and molecular weight distribution of mixtures of closely related CT oligomers from sulla. However, high resolution mass spectrometry techniques are required to help characterise the high molecular weight CT oligomers and polymers, as results showed the mass spectral data to be dominated by lower molecular weight species.

Individual soluble sugars have been identified for the first time from sulla leaves. HPLC analysis has assisted in the identification of soluble sugars. The applied method of HPLC allowed separation, identification and quantification of six soluble sugars.

The butanol-HCl assay indicated that sulla contained total CT (g/100g) of 7.6% and 5.3% CT. It has shown sulla to contain extractable (free), fibre-bound and protein bound CT. The results obtained for nutritional composition of sulla have shown sulla to have higher CP, higher non-structural carbohydrates and lower dietary fibre. The higher nutritive value of sulla and the presence of CT indicate it can be incorporated into pastoral agriculture to complement conventional forages and improve animal nutrition and productivity.

CHAPTER FOUR

CHARACTERISATION OF FLAVONOIDS

4.1 Introduction

Flavonoids are polyphenolic plant secondary metabolites which are widespread in the plant kingdom. The basic core structure of flavonoids possesses C_{15} flavone nucleus (Figure 4.1).

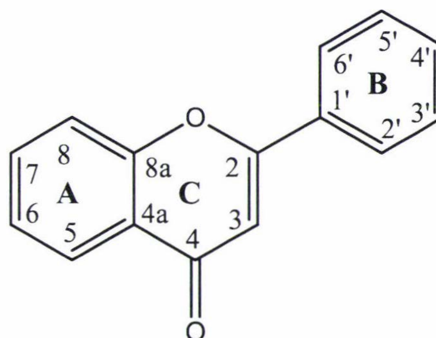


Figure 4.1 Flavonoid core structure (adapted from Markham, 1982).

Further functionalisation of the flavone skeleton with additional hydroxyl groups, methylations of hydroxyl groups or of the flavonoid nucleus and glycosylation produce a diverse variety of flavonoids (Markham, 1982). Other flavonoids (Figure 4.2) isolated from plants include flavones (e.g apigenin), flavonols (e.g quercetin, kaempferol), isoflavone (e.g formononetin, genistein), flavanone (e.g naringenin), and anthocyanidins (delphinidin, cyanidin). Flavonoids commonly occur as C- or O-glycosides, in which one of the carbons or hydroxyl is bound to a sugar moiety such as glucose, galactose and rhamnose (Choong et al., 1994; Bilia et al., 1993). The sugar substitution pattern in flavonoids is favourable at the seventh and third position on the A and C-rings, respectively (Figure 4.1). Glycosylation can have an enormous effect on the flavonoids rendering them more water soluble. Identification of these low molecular weight phenolics has been made possible by the use of LC-PDA, LC-ESI-MS, APCI-MS and NMR techniques. APCI and ESI are the widely used MS interfaces. Flavonoid glycosides release protonated molecules and ions corresponding to the loss of sugar fragments can be seen clearly using APCI in a positive mode $[M+H]^+$.

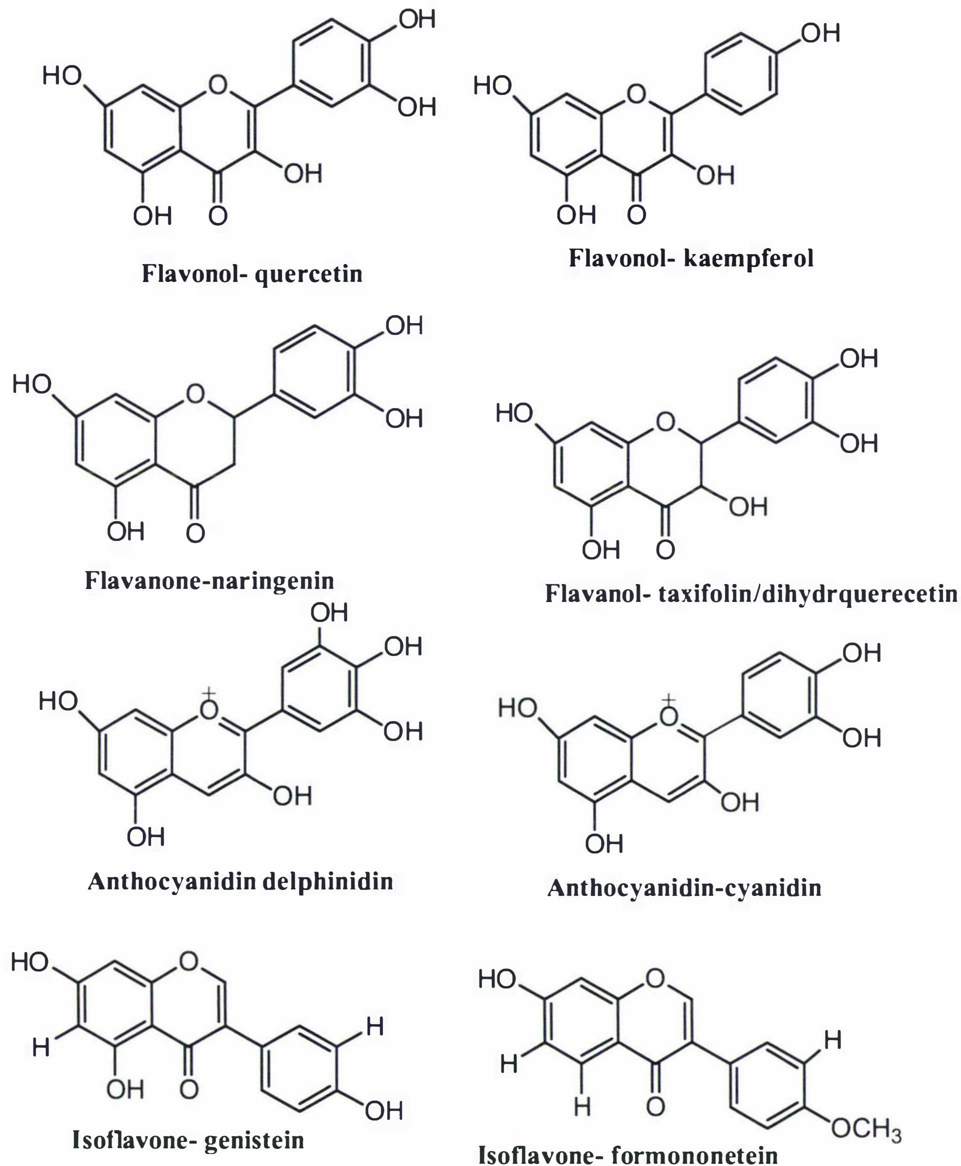


Figure 4.2 The main classes of flavonoids found in plants.

MALDI-TOF has also been employed successfully in the analysis of flavonoids (Wang and Sporns., 2000). The low molecular weight phenolics, have been identified in a variety of forage legumes, flavonoid aglycones quercetin, kaempferol, afzelin and flavonoid glycosides, i.e rutin have been identified in sainfoin (Marais et al., 2000). White clover flowers contain cinnamic acid derivatives, myricetin, quercetin and kaempferol derivatives (Foo et al., 2000).

Isoflavone glycoside malonates (genistein, formononetin and quercetin) were found prevalent in flowers and leaves of red clover (Lin et al., 2000; Klejedus et al., 2001). Phytoestrogens in herbage and their metabolites in the ruminant have deleterious effects on reproduction, particularly in sheep (Wyleet al., 1993) and to a limited extent

in cattle. Pastures dominated by red clover have been shown to be highly oestrogenic to ewes (Kelly et al., 1976). Formononetein, the main isoflavone present in red clover (Niezen et al., 1992) is implicated in these reproductive problems. It is not oestrogenic itself but it is metabolized to equol in the rumen, and equol is oestrogenic. Forage legumes with low phytoestrogens have been the long term plant breeders. They have been found to play an important role in cancer prevention and reduction of pre- and post-menopause symptoms in women (Klejedus et al 2001).

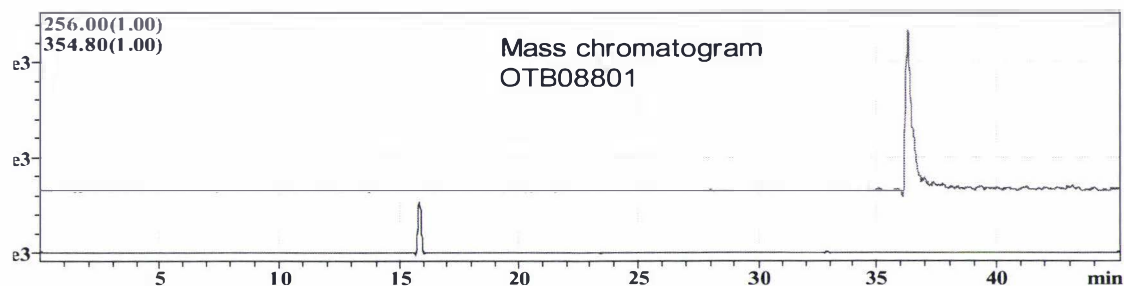
Flavonoids are of no known nutritional benefits in ruminants but they have been isolated from plant derived food substances and have shown to possess various health benefits for humans, such as antioxidant (Mauri et al., 1999), radical scavenging (Tagliafero et al., 2002) and anti-carcinogenic (Robards and Antolovich., 1997) properties. Current research is been geared towards how these compounds in the Western diet can reduce coronary heart disease (Halliwell and Gutteridge., 1999).

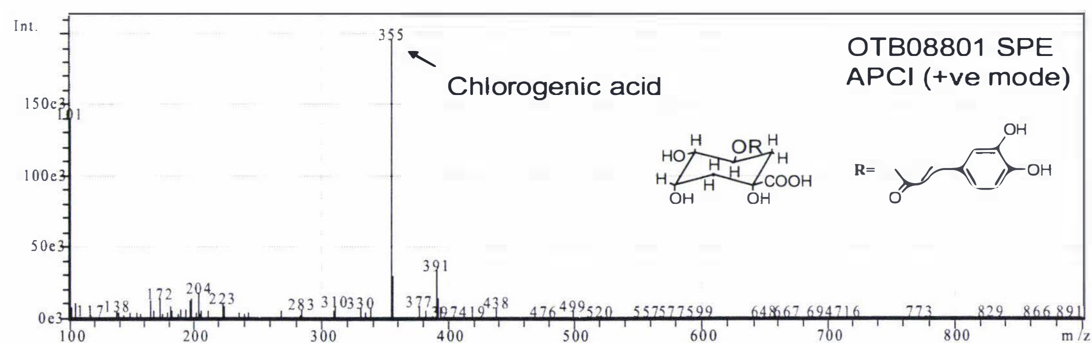
4.2 Results and discussion

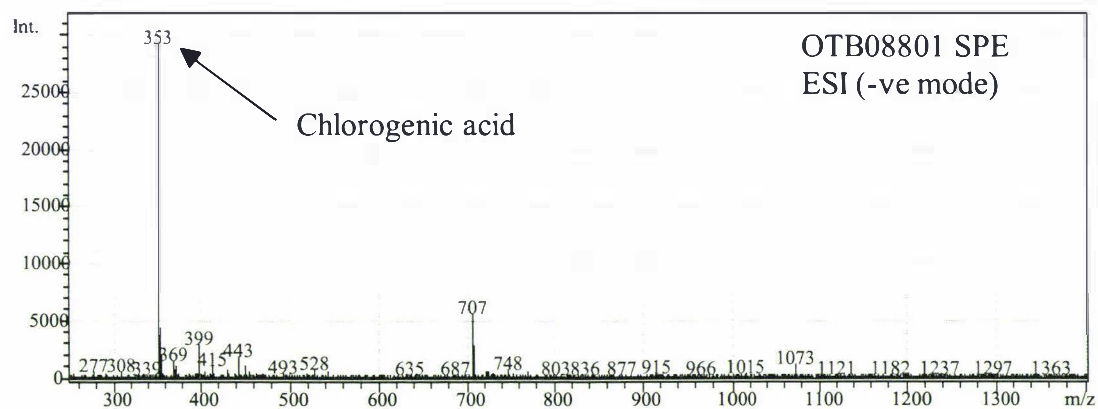
4.2.1 APCI-MS and ESI-MS for flavonoids

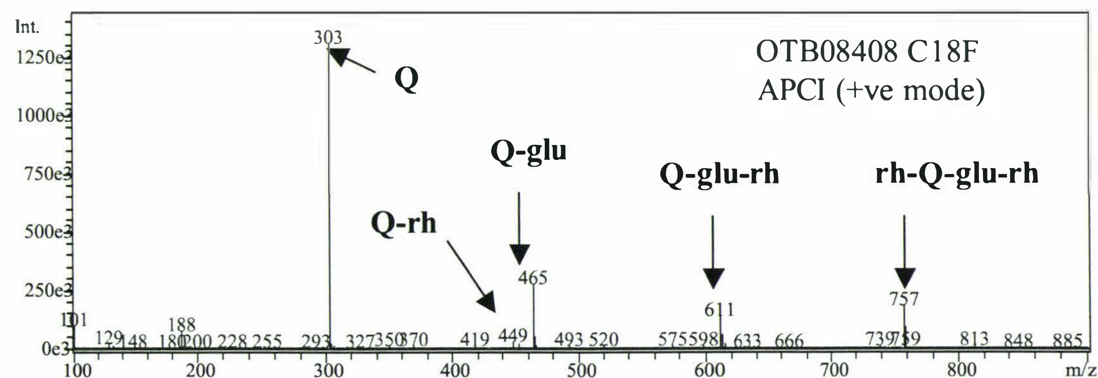
The mass spectra for flavonoids were detected using APCI-MS in the range 200-1000 *m/z* using a quadruple mass analyser. APCI can allow flow rates of 1 mL/min. From the APCI-MS data, it can be deduced that sulla contains chlorogenic acid derivatives and glycosylated flavonoid derivatives. Elucidation of these compounds has been confirmed by UV spectra and through the use of authentic standards which had identical UV spectra and retention times (Table 4.1). Due to the presence of UV chromophores and extensive electron delocalisation in flavonoids, this enables them to be UV-absorbing. Parent molecules were detected using ESI-MS in a negative ion mode. APCI-MS and UV spectra suggest the presence of chlorogenic acid in fraction OTB08801, OTB08802 and OTB08403 with *m/z* 355 (Table 4.1). The UV spectral data suggest the presence of ferulic acid derivatives which has absorption maximum at 326 nm, but the mass spectra suggest the possibility of chlorogenic acid. It is doubtful that this compound could be ferulic because no base fragment was detected in the mass spectrum which was produced as a result of the loss of glucose or galactose. However, contrary results were found for fraction OTB08801 and OTB08803 with *m/z* 295 and 591 using ESI-MS. It was thus difficult to assign the *m/z* values from the mass spectrum. Chlorogenic acid derivatives appear to be the main compounds in sulla. Major peak for quercetin-7-O-α-L-rhamnosyl-3-O-β-D-glucosyl-rhamnoside with *m/z*

757 ion was identified with other fragments (Figure 4.3) from fraction OTB08408 and OTB08609. ESI-MS showed that the parent ion had m/z 755 which showed the results to be consistent with those of APCI-MS.









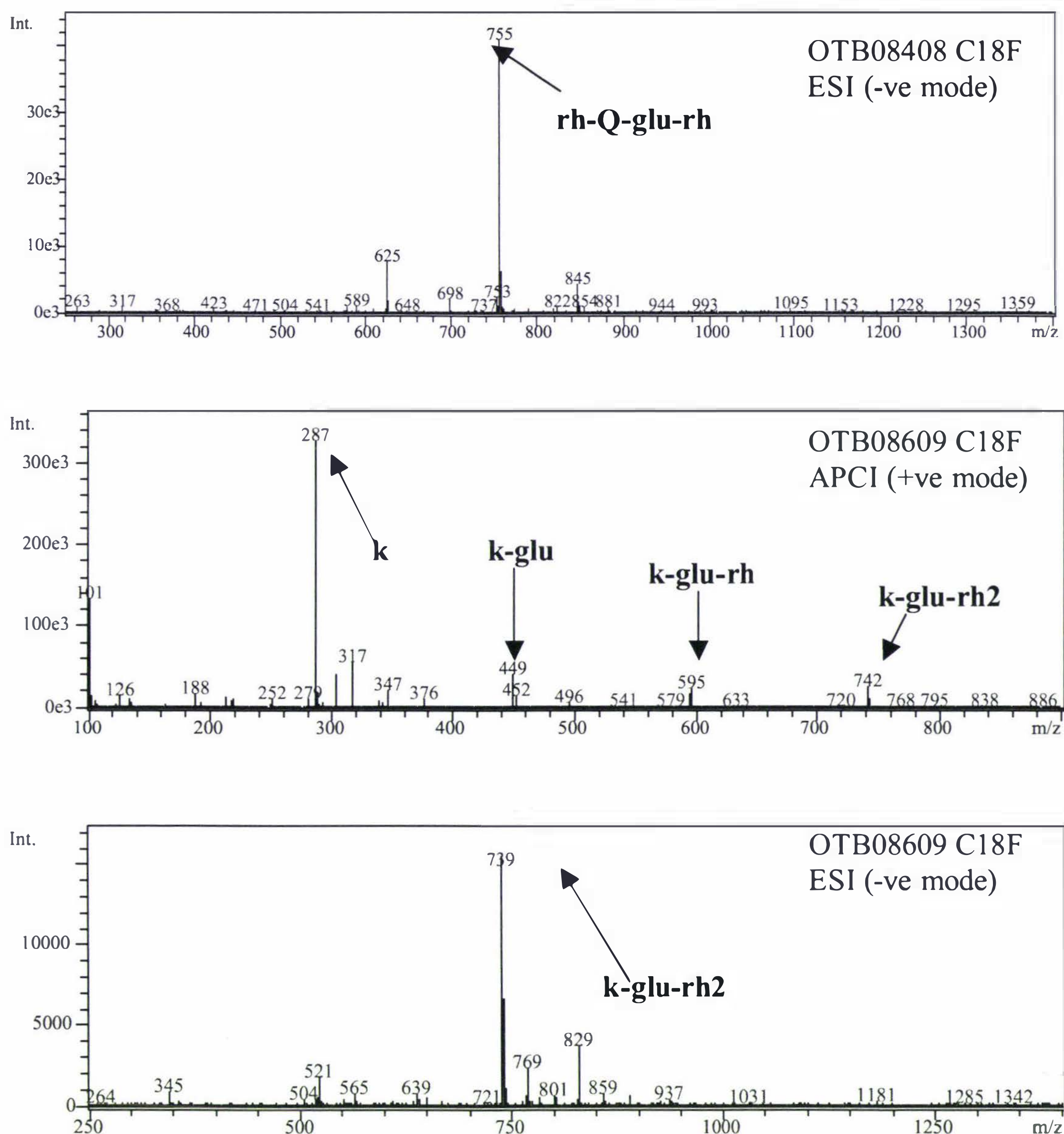


Figure 4.3 Mass spectral data for OTB08801 SPE and OTB08408 RP-C18 fractions characterized using APCI $[M+H]^+$ and ESI $[M-H]^-$.
Abbreviations; Q-quercetin, k-kaempferol, glu-glucose and rh-rhamnose.

Since the scanning range was extended to 1400 *m/z* in ESI-MS, it can be noticed that there were no additional sugars attached or the ions may be above this range. The position of the attachment of sugars is not known. Sugar positions in the aglycon moiety can be determined through analysis of the fractions with NMR. Due to small amounts of some fractions obtained, the fractions were not sufficient to be run with NMR. The *m/z* value 449 corresponding to quercetin-7-O-α-L-rhamnosyl-3-O-glucoside (Figure 4.3) may indicate that another rhamnose was attached on the other side (i.e. position 7) rather than on the same site. The loss of the first rhamnose led to *m/z* 611 after loss of 146. The loss of second rhamnose produced a fragment with *m/z* 465. Glucose was also

found to be present because of loss of 162 releasing *m/z* of 303 for quercetin. The difference between the two masses, *m/z* 465 and 303 is 162 amu, which indicates the loss of a glucose sugar moiety. The *m/z* ratio of 303 is indicative of the base component quercetin. This is confirmed from the UV spectrum with λ_{max} 255 and 355 nm which is suggestive of quercetin derivative. It is difficult to distinguish glucose from galactose since they have the same molecular weight. So glucose was ascribed to be the main sugar present. Derivatisation and enzymatic hydrolysis can help in this regard. The parent molecule for quercetin-3-O-β-D-glucosyl-rhamnose with *m/z* 611 was also present in fraction OTB08411 and it was the most common among the impure LH-20 fractions (data not shown). The loss of rhamnose released *m/z* 465. This indicates the loss of 146 *m/z* from the rhamnose moiety. The low abundance of the aglycon moiety may indicate that the parent molecule was not well fragmented and the cleavage of the sugar molecule was limited. Similar patterns were observed for other flavonoids. Isoflavone derivatives (Figure 4.4 and Appendix 2) were found to be present in fraction OTB08411, OTB08611 and OTB08612. Fraction OTB08411 had *m/z* 519 corresponding to genistein-7-O-β-D-glucosyl-6”-O-malonate.

MS in a negative mode. This aglycon was also found from ESI-MS with *m/z* 269. The parent molecules for the isoflavone derivatives were not detected in ESI (-mode), probably due to poor ionisation. Isoflavones are known to ionise well in ESI (+mode), but this mode was not employed in analysis of these fractions. Formononetin-7-O-β-D-glucoside-6”-O-malonate corresponding to *m/z* 517 was present and this was clearly shown after the loss of malonate and glucose (M_w =248), with the aglycon *m/z* 269. The UV spectrum for the formononetin is well distinguished from those of other isoflavonoids through the possession of a “nipple” at 248 nm (Figure 4.5).

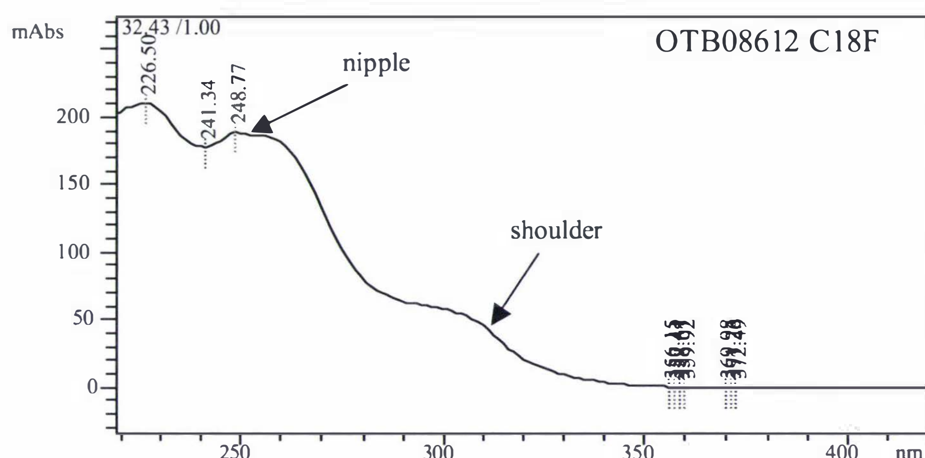


Figure 4.5 UV spectrum for formononetein characterised from fraction OTB08612 C18F.

In addition, the UV spectra of the isoflavones are completely distinct from those of flavonoids, especially with the presence of a shoulder around 300 nm. Afrormosin with *m/z* 299 was detected in APCI-MS and *m/z* 297 in ESI-MS. This compound is also confirmed from the UV data with two absorption maxima at 258 and 319 nm. Afromorsin is a derivative of formononetin with the attachment of an extra $-OCH_3$ group. The sugars can not be detected in MS because they are released as neutral molecules. Kaempferol derivative (kaempferol-3-O-β-glucoside-dirhamnoside) with *m/z* 741 and 739 was also detected in APCI and ESI-MS, respectively (Appendix 2). The UV spectrum with absorption maxima 265 and 346 nm is typical of kaempferol derivative. These compounds isolated from sulla have also been isolated from red clover (Lin et al., 2000; Klejedus et al., 2001). These flavonoids are the first to be isolated from sulla.

Table 4.1 Identification of flavonoids from C18 solid phase extraction (SPE) fractions and reverse phase C18 flash (C18F) chromatography fractions using APCI-MS and ESI-MS.

Sample Number	t_R (min)	APCI $[M+H]^+$ (*m/z*)	Fragment Ion (*m/z*)	UV λ_{max} (nm)	Possible compound	ESI $[M-H]^-$ (m/z)
OTB08801SPE	15.8	355	Undetected	326	Chlorogenic acid	352.8
		391				369
		256				707
OTB08802 SPE	15.8	354.9	Undetected	326	Chlorogenic acid	352.9
		534.9				443
		256				707
		284				
		287			Kaempferol	
OTB08403 C18F	16.2	354.9	Undetected	326	Chlorogenic acid	353
		256				440
		284				466
OTB08408 C18F	21.2	757	303,449,465,611	254, 353	Quercetin-glycosyl-dirhamnose	755
OTB08411 C18F	29.6	519	271,433,475	260, 310sh	Genistein-7-O-β-D-glucoside-6''-O-.malonate	269
	23.9	611	303,449,465	255, 354	Q-3-glu-rh	609
OTB08609 C18F	21.8	757	303,449,465,611	255, 354	Quercetin-glucoside-dirhamnose	755
From OTA04303						
	22.5	741	287,595,449	265, 346	Kaempferol-glucoside-dirhamnoside	739
OTB08611 C18F	32.4	517	269,299,431,473	249, 300sh	Formononetin-7-O-β-D-glucoside-6''-O-malonate	267
OTB08612 C18F	32.4	517	269,299,431,473	250, 300sh	Formononetin-7-O-β-D-glucoside-6''-O-malonate	267

Abbreviations; Q-quercetin, glu-glucose, rh-rhamnose, sh-shoulder, t_R-retention time.

The reference standards were analysed by LC-ESI-MS and APCI-MS, to determine their retention times, UV and MS spectra. Confirmation of the existence of chlorogenic acid (t_R=15.8 min) and rutin (t_R=23.9 min) was accomplished through comparison of the data from the sulla fractions with those of the reference standards (Appendix 4). These compounds had similar retention times, similar parent ions both in APCI and ESI spectra with those of the standards.

4.2.2 ^{13}C-NMR of flavonoids

The ^{13}C-NMR signals for fraction OTA04303 were assigned according to Cheminat (1988). The results obtained for this fraction are in good agreement with those of Cheminat (1988). The signals from this fraction resemble those of the caffeoyl

moiety. The presence of chlorogenic acid was confirmed by APCI-MS and ESI-MS. The results from the ^{13}C-NMR and ^{1}H NMR are consistent with the structure of chlorogenic acid. From APCI and ESI, this compound was found to be chlorogenic acid, but the quinic acid moiety signals are not depicted in the ^{13}C-NMR spectrum. The signal at 169.5 ppm is attributable to an ester carbon. The signals also indicate the presence of substituted and unsubstituted carbons in the benzene ring. The ^{1}H NMR spectrum of this fraction exhibits signals belonging to a caffeoyl moiety with two *trans* olefinic protons; 7.60 ppm (d, 15.9 Hz) and 6.35 ppm (d, 15.9 Hz). Most of the signals from ^{1}H NMR were broad and not easy to interpret. The broad peaks from the ^{1}H NMR spectrum indicated the presence of sugars but they were not shown in the ^{13}C-NMR spectrum. Further work should be performed using authentic standards.

^{13}C-NMR signals for fraction OTA10305 were assigned according to Huang (2003) and Foo (2000) and they resembled those of a quercetin moiety. The ^{13}C-NMR signals for the quercetin moiety are shown in Table 4.2. The signals indicate the presence of sugars which resonated around 100 ppm and 75 ppm. The structure of quercetin is shown in Figure 4.4. The results from the ^{13}C-NMR and ^{1}H-NMR are consistent with the structure of quercetin derivative. The signal at 179.1 ppm confirms the presence of a carbonyl carbon. The signals also indicate the presence of A- and B-ring signals. Further work ought to be performed using authentic standards and COSY NMR. Analysis with COSY NMR (HMQC, HMBC and DEPT) can help correctly assign the signals attributed to the substituents in the aglycone quercetin. Many peaks were observed which indicates the presence of a substituent group.

Table 4.2 ^{13}C-NMR spectral data (δ) of quercetin moiety for fraction OTA10305 (75% MeOH).

Carbon number	Chemical shift
2	158.0
3	135.3
4	179.1
4a	104.3
5	159.3
6	100.0
7	165.4
8	95.1
8a	158.0
1'	123.7
2'	-
3'	145.3
4'	149.4
5'	116.1
6'	117.7

The signal intensity for fraction OTA03804 was low and therefore difficult to assign the signals, but the results indicated the presence of a quercetin moiety. A carbonyl carbon signal was observed at 179 ppm, which might be attributed to C3 carbon in the quercetin aglycone. The ^{13}C-NMR spectrum was similar to that of OTA10305 (75% MeOH).

4.3 Conclusions

The aims of the thesis were to isolate and characterise flavonoids from sulla leaves which exhibited antiparasitic properties. Characterisation of flavonoids from sulla using LC-ESI-MS and APCI-MS has indicated chlorogenic acid, quercetin derivatives, kaempferol derivatives and isoflavone (formononetein and genistein) malonates to be present. The existence of rutin and chlorogenic acid was confirmed through the use of standards. The study has revealed that LC-ESI-MS and APCI-MS were suitable and effective techniques for identification of polar compounds from sulla leaf extracts employed in a negative and positive ion mode, respectively.

CHAPER FIVE

PARASITES ASSAYS

5.1 Introduction

Sulla is a leguminous plant and is known to contain CT. CTs from sulla have been shown to interact with the gastrointestinal nematodes directly and indirectly in ruminants and exhibit antiparasitic properties (Niezen et al., 1995, Molan et al., 2000, 2002b). Indirect mechanisms have been associated with increase in protein supply, hence increasing the flow of amino acids (McNabb et al., 1990). CT dissociate from proteins in the abomasum and amino acids will be ready for absorption in the small intestine and then enhance the animal resistance to nematode infection. *In vitro* studies have the ability to decrease hatch ability of nematode eggs and inhibit the hatching larvae from attaining full development to infective larvae (Molan et al., 1999). These assays have shown that CT from sulla may reduce the build-up of infective larvae on pasture by disrupting the life cycle of nematodes. Anthelmintic resistance to all currently available drench families has been reported in sheep, cattle and goats (Vlassoff and McKenna., 1994). Therefore, non-chemical strategies are required which would focus on reduction of infective larvae on pasture rather than treatment of the infection. If this is achieved, dependence on anthelmintic drugs as the main parasite control would be minimised. In comparison to other forages, sheep fed sulla have been associated with reduced worm numbers (Niezen et al., 1995). CT from other forages and browses have indicated to reduce nematode numbers in sheep (Molan et al., 2002b) and deer (Molan et al., 2002a). However, specific mechanisms for the antiparasitic activity of this forage have not been elucidated.

The fate of CT during digestion is not well understood. Unlike anthelmintic drugs, CT are not absorbed from the gastrointestinal tract (Terrill et al., 1994). Jimenez-Ramsey et al. (1994) have also shown that CT were not absorbed in the digestive tract of chickens. Hence the eggs laid by adult worms would be exposed to the negative effects of CT throughout their development. Some studies, however, claim that CT are absorbed in the digestive tract (Perez-Maldonado and Norton., 1996). Further research is still required in this area.

5.2 Results

5.2.1 *In vitro* egg hatch assay (EHA) for LH-20 fractions

The aim of this study was to investigate the effects of LH-20 fractions isolated from sulla leaves on egg hatching for *T. colubriformis* under *in vitro* conditions using the egg hatch assay (EHA). In this assay, aqueous crude extracts, LH-20 fractions (OTA02801-06, OTA03801-06 and OTA04301-11) from freeze-dried (summer-21/12/01) and fresh frozen sulla leaves (spring-23/09/02) were used. In control incubations, about 73 % of the eggs hatched (Figure 5.1), while in the presence of increasing concentrations of the fractions, the hatching process decreased and some fractions (OTA02801-04) inhibited the hatching process completely (Table 5.1).

Table 5.1 Effect of CT fractions from sulla step LH-20 fractionation on inhibition of the hatching rate and development to L3 larvae of *T. colubriformis in vitro* compared with the chemical composition as determined by thiolysis.

ID Fraction	Eluent	%PD term	%PD ext	Cis	DP	%EH (500 µg/mL)	%LD (100 µg/mL)
Sulla							
OTA02801	50% MeOH	0	0	0	0	96	37
OTA02802	50% MeOH	0	0	0	0	100	9
OTA02803	50% MeOH	0	0	0	0	99	65
OTA02804	50% MeOH	0	0	0	0	97	66
OTA02805	70% Acetone	73	88	80	26	19	100
OTA02806	70% Acetone	70	88	74	14.2	13	87
Sulla							
OTA03801	50% MeOH	0	0	0	0	0	100
OTA03802	50% MeOH	0	0	0	0	0	34
OTA03803	50% MeOH	0	0	0	0	11	98
OTA03804	50% MeOH	0	0	0	0	8	87
OTA03805	70% Acetone	73	84	75	12	0	97
OTA03806	70% Acetone	73	85	73	13	27	90

Abbreviations: PD=prodelphinidin; DP=degree of polymerisation; term=terminal, ext=extender.

The 50% MeOH fractions (OTA02801-04) from LH-20 extracts were very effective and completely inhibited egg hatching while LH-20 fractions (OTA03801-04) from the fresh frozen plant material were ineffective. All the CT-containing fractions (OTA02805-07 and OTA03805-07) with an average %PD terminal of 72, 86% PD extender units and 76% *cis* stereochemistry (Table 5.1) from the fresh frozen and freeze-dried plant material were ineffective.

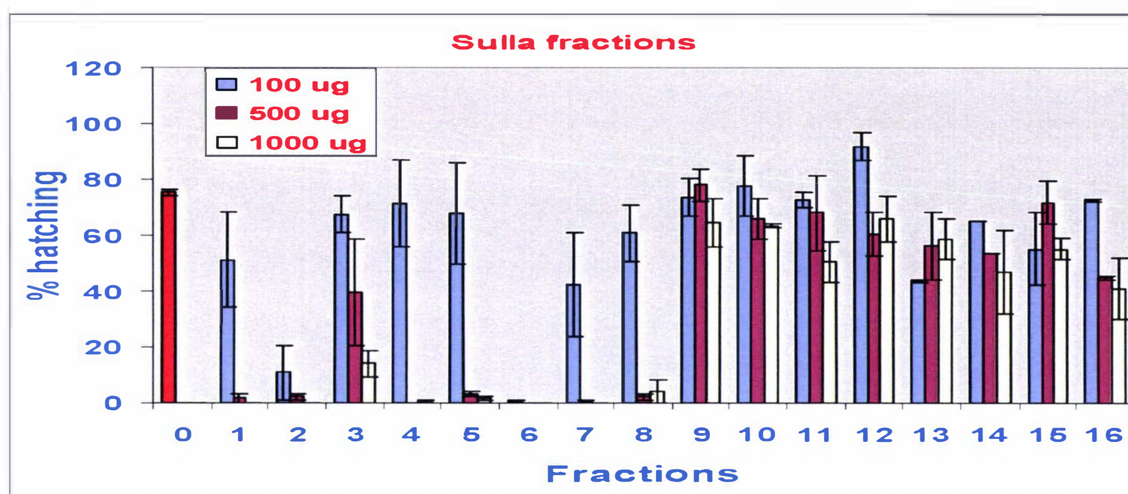


Figure 5.1 The effect of sulla step LH-20 fractions with increasing concentrations (100 to 1000 µg/mL) on hatching of *T. colubriformis* eggs incubated for 24 hrs at 26 °C.
(0= control, 1-4= crude extracts, 5-10= OTA02801-06, OTA0285-06, 11-16= OTA03801-06). Means of duplicate incubations were used for each treatment.

At 100 µg/mL, most of the fractions were not effective at decreasing the hatching process and the highest anti-parasitic activity was observed at a concentration of 500 and 1000 µg/mL.

Table 5.2 Effect of CT fractions from sulla leaves from gradient LH-20 fractionation on the hatching rate and development to L3 of *T. colubriformis* eggs *in vitro* compared with chemical composition as determined by thiolysis.

ID fraction	Eluent	%PD term	%PD ext	Cis	DP	%EH inhibition at 500 ug/mL	%LD inhibition at 100 µg/mL
Sulla							
OTA04301	Water	0	0	0	0	18	61
OTA04302	Water	0	0	0	0	6	21
OTA04303	25% MeOH	0	0	0	0	1	39
OTA04304	50% MeOH	0	0	0	0	1	47
OTA04305	75% MeOH	0	0	0	0	12	67
OTA04306	100% MeOH	66	76	56	2.9	7	94
OTA04307	100% MeOH	62	76	62	4.1	10	100
OTA04308	100% MeOH	66	77	63	5	3	100
OTA04309	100% MeOH	64	78	69	6.9	11	100
OTA04310	70% Acetone	72	87	74	14.9	9	100
OTA04311	70% Acetone	85	88	80	20.7	12	65

Abbreviations: PD=prodelphinidin; DP=degree of polymerisation; term=terminal, ext=extender

For fractions OTA04301-11, only the OTA04301 (H_2O) fraction was effective in decreasing egg hatching (Figure 5.2) at a concentration of 1000 µg/mL. The percentage hatching decreased with increasing concentration of the fractions.

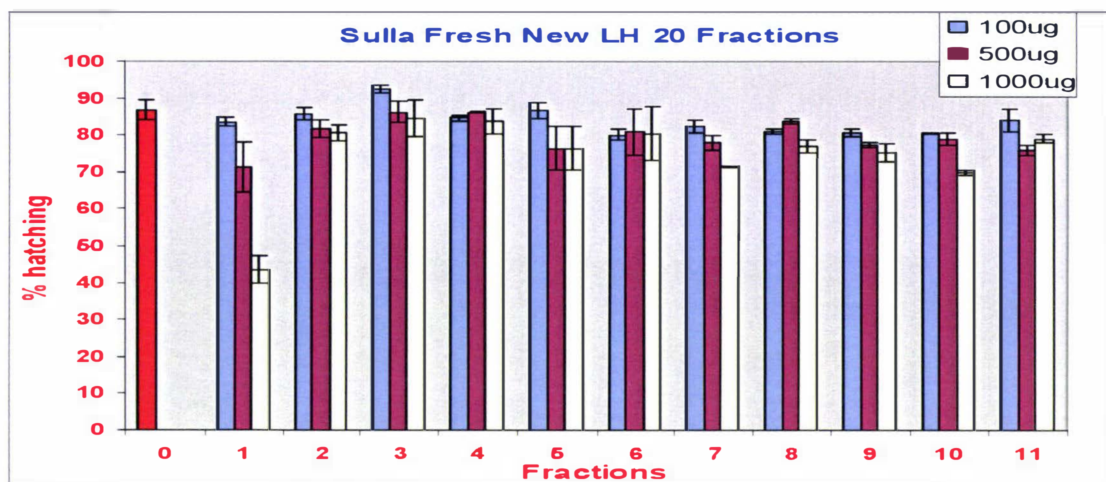


Figure 5.2 The effect of sulla gradient LH-20 fractions (100 to 1000 µg/mL) on hatching of *T. colubriformis* eggs incubated for 24 hrs at 26 °C.
(0=control, 1-11 represents fractions OTA04301-11). Means of duplicate were used for each treatment.

5.2.2 *In vitro* larval development assay (LDA)

Most of the fractions were effective at inhibiting the development of eggs to L3 larvae. The results (Figure 5.3) indicate that the CT-containing fractions significantly ($P<0.001$) inhibited the development of eggs to L3 larvae. Percentage larval development decreased with increasing concentrations of the fractions (Figure 5.3). In the absence of the sulla fractions (control incubations), about 82.2% of the eggs developed to L3. The 50% MeOH fractions, OTA02803 and OTA02804 inhibited about 66% of the eggs from hatching. About 94% of the eggs were inhibited from developing to L3 as observed for all the step LH-20 70% acetone fractions with an average %PD terminal of 72, 86% PD extender unit and 76% *cis* configuration (Table 5.1). There is no clear variation between the structure-activity correlation (PC:PD ratio, *cis:trans* ratio) and increase in mDP regarding the inhibitory effects. The highest activity was observed at a concentration of 100 and 200 µg/mL. In contrast, low activity was associated with fraction OTA03801 and OTA03802 with 37% and 34% of the eggs inhibited from developing to L3, respectively. The CT-containing fractions were more potent in inhibiting the larval development than egg hatching.

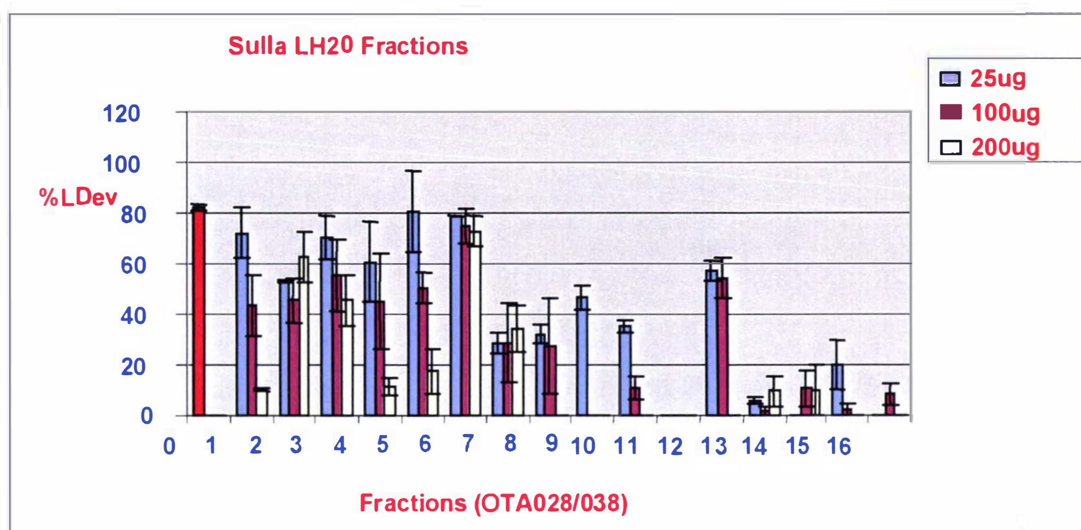


Figure 5.3 The effect of sulla step LH-20 fractions (25 to 250 µg/mL) on larval development of *T. colubriformis* eggs incubated for seven days at 24 °C.
(0= control, 1-4= crude extracts, 5-10= OTA02801-06, 11-16= OTA03801-06). Means of duplicate incubations were used for each treatment.

The first water fraction (OTA04301) was effective while the second fraction (OTA04302) was not effective. The 100% MeOH fractions with average %PD term of 65, 77% PD extender units and 63% *cis* and the 70% acetone fractions with average %PD terminal units of 70, 88% PD extender units and 77% *cis* inhibited about 100% of the eggs from developing to L3 (Figure 5.4). Low inhibitory effects were observed for fraction OTA04303 and OTA04304 with 39% and 47% LD inhibition, respectively.

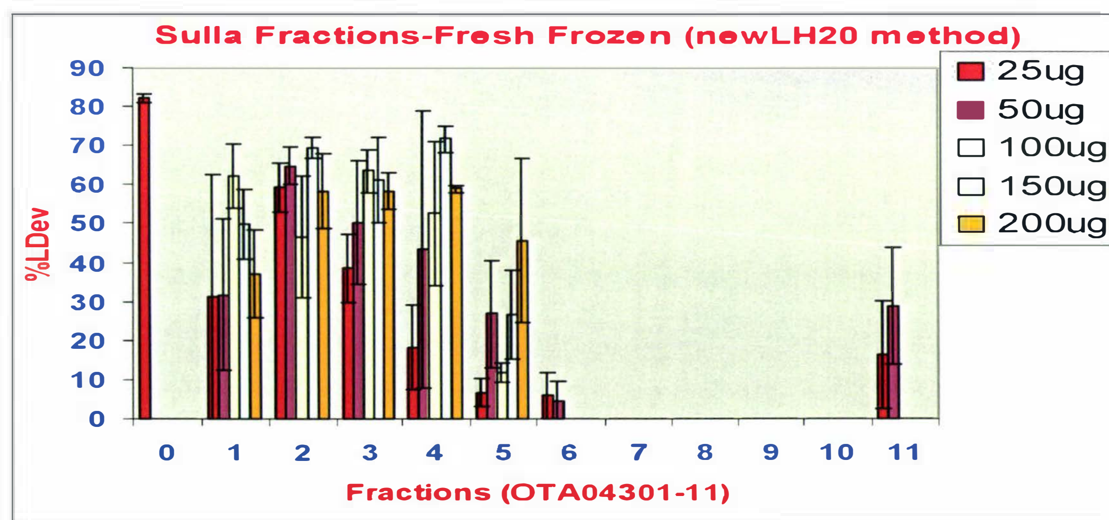


Figure 5.4 The effect of sulla gradient LH-20 fractions (25 to 200 µg/mL) on larval development of *T. colubriformis* eggs incubated for seven days at 24 °C.
(0=control, 1-11 represents fraction OTA04301-11). Means of duplicate were used in each treatment.

5. 3 Discussion

The results of this study showed that exposure of *T. colubriformis* to LH-20 fractions from sulla leaves can disrupt the life cycle of nematodes by preventing eggs from hatching and the hatching larvae from developing to L3 larvae under *in vitro* conditions. At 500 μg/mL, fractions OTA02805, OTA02806, OTA03805 and OTA03806 inhibited 19%, 13%, 0% and 27% of the eggs from hatching (Table 5.1). However, these fractions inhibited 85 to 100% of the hatched eggs to attain full development to L3 larvae. These figures are lower than those obtained previously by Molan (2002b) using step LH-20 70% acetone fractions. In their results, the CT from sulla were found to have higher inhibitory activity. At 200 μg/mL, they inhibited 28% of eggs from hatching and 36% at 400 μg/mL (Molan et al., 1999). The inhibitory activity on larval development was similar to that obtained by Molan (1999). The lower inhibitory effects obtained in this study could be attributed to different methods of extraction and different times of harvesting the forage. The inhibitory effect of the fractions tested in this study was found to be concentration-dependant.

Methanolic fractions from step LH-20 were found to be active at inhibiting egg hatching and the development of hatched larvae to L3. These have not been evaluated in parasite assays previously (Molan et al., 1999; 2002b). PDA results indicated that the methanolic fractions contain flavonoids. Compounds such as chlorogenic acid, quercetin, kaempferol, genistein and formononetein derivatives have been characterised using APCI-MS and ESI-MS for the first time from this plant. These compounds are components of the fractions which exhibited antiparasitic activity. A comparative study is required with authentic standards because there is no published data on these compounds in regard to anti-parasitic activity or interactions with hatching enzymes and larvae. It is not known if the attachment of sugars (glucose, galactose and rhamnose) might be enhancing their activity. The first fraction (OTA02801) of the 50% MeOH eluent contains a MMWCT polymer. There is no convincing evidence that the activity is ascribed only to CT or the activity is best suited within a mixture of CT with low molecular weight phenolics. It is not known if the MMWCT with a mDP of 46 isolated from this fraction might be interacting with the eggs and the larvae or the activity is attributed to other low molecular weight phenolics. The effect of increasing mDP on egg hatching and larval development needs to be investigated. The other 50% MeOH

fractions (OTA02802-04) contain flavonoids which may be responsible for inhibiting LD.

CT-containing fractions from sulla were more potent in inhibiting larval development, especially among the 70% acetone than methanolic fractions. The mechanism for this inhibiting may be attributed to the interaction of these polyphenolic substances with the body musculature of the larvae. The CT-containing fractions were more potent in inhibiting larval development than egg hatching. It is also speculated that the shell of the eggs is hard and the CT can not penetrate the shell to inactivate the enclosed larvae. This might explain why the potency of CT in larval development assay was higher than that in egg hatching assay. The incubation period may also be a contributing factor. The incubation period for EHA is 24 hrs. LDA is performed for seven days, hence the L1 and L2 larvae may be exposed to the test compounds for a longer time. On the other hand, the L1 and L2 larvae are feeding stages and their bodies are soft and the CT may easily interact with the body musculature and hence paralyse the larvae. During LD assay experiment, it was observed that the L1 larvae in controls were active while the larvae in wells containing the test material (sulla fractions) were sluggish, immobile and inactive. It is also believed that the CT have a direct effect on parasites because they either bind to the proteins in the body musculature or the larvae swallow the CT during feeding which interacts with pharyngeal muscles and kill them (Molan et al., 2002).

Similar anti-parasitic activity, both for EHA and LDA were observed for fractions OTA02805-06 and OTA03805-06 from freeze-dried and fresh frozen sulla leaves, respectively. These results indicate that freeze-drying had no effect on CT activity. The inactivity of the 70% acetone fractions extracted and purified from the freeze-dried and fresh frozen sulla leaves against egg hatching may be attributed to the low concentrations of CT. However, all the 70% acetone fractions were effective at inhibiting larval development. Analysis of the CT content from sulla leaves by the butanol-HCl method showed the CT content to be within the normal range for beneficial nutritional effects in ruminants. The concentrations used in these assays (about 100 to 1000 μg/mL) were ten times lower than the CT concentrations (Molan et al., 2002b) in the digesta (2000 to 8000 μg/mL). The CT added to the *in vitro* incubations bind only to the larvae and the components in the medium whereas in the digesta and faeces they bind to many particulate materials.

For the gradient LH-20 fractions, fraction OTA04301 was the only one effective in inhibiting egg hatching. Fraction I (OTA04301) may contain a HMWCT. The HMWCT from this fraction may penetrate the egg shell and interact with the enclosed larvae. The CT oligomers and CT polymers fractions were separated using the gradient LH-20 method and were effective in inhibiting the larval development. The HMWCT were less effective in LD, while the CT oligomers and LMWCT polymers inhibited completely the larval development process. There is still no clear trend of a structure-activity relationship since all the CT oligomeric fractions and CT polymeric fractions inhibited completely the development of eggs to L3 larvae. Despite the fact that the 100% MeOH fractions had lower mDP, lower *cis* and lower PD composition than the 70% acetone fractions, the anti-parasitic activity appeared to be the same. Structure-activity relationships need to be evaluated in other forages as the activity of the CT is usually attributed to molecular weight, chemical structure and PC:PD ratio. Characterisation of the structure of CT using ^{13}C-NMR and thiolysis has been performed for other leguminous forages. For example, LP and sainfoin CT were found to be PD and *cis* predominant (Foo and Porter., 1980) and had the highest activity against nematodes (Molan et al., 1999). Thiolysis processes indicated that sulla is PD and *cis* predominant and it is clear that the high PD content may be responsible for its antiparasitic activity. Lowest activity of LCS CT (Molan et al., 1999) may be attributed to PC predominance (Foo et al., 1996). Previous studies using EH and LDA have shown that forages with *cis* predominant configuration tend be more effective in inhibiting egg hatch and larval development (Molan et al., 2002b). This was also evidenced in the study conducted to investigate the effects of flavan-3-ols on *in vitro* EH, LD and LMI assays (Molan et al., 2003). Sulla was found to be *cis* predominant and this stereochemistry may be contributing to its activity.

Hence, sulla CTs may disrupt the life cycle of nematodes by preventing the *in vitro* development of *T. colubriformis* eggs and first stage larvae L1 to L3 larvae. Unlike anthelmintic drugs, CT are not absorbed from the digestive tract (Terrill et al., 1994). Hence the eggs shed by adult worms will be exposed to the harmful effects of CT throughout their development. The decreased number of viable eggs and infective larvae on pasture may reduce dependence on anthelmintic drugs.

Functionalisation of flavan-3-ol monomers with gallates has been shown to increase the antiparasitic activity (Molan et al., 2003). Sulla LH-20 fractions were found to contain galloylated oligomers by analysis with MALDI-TOF-MS. Bioassays were not

performed on the purified compounds that were isolated, but rather semi-purified fractions from the LH-20. Recently, Molan (2003) found that commercially available flavan-3-ol monomer gallates were effective in inhibiting the development of egg to L3 (Molan et al., 2003) and these low molecular weight phenolics were tested for the first time against nematodes. Flavan-3-ol gallates were active against parasites, while free flavan-3-ols were inactive. This may indicate that additional hydroxyl groups from gallates increase their biological activity (Schofield et al., 2001).

5.4 Conclusions

The study has shown that step and gradient LH-20 fractions containing CT and flavonoids, from sulla leaves inhibited egg hatching and larval development of *T. coubriformis* as demonstrated *in vitro*. This indicates that CT from sulla may reduce contamination on pasture with viable eggs and infective larvae by disrupting their life cycle. Results from previous studies, together with results from this current study have shown that CT may reduce dependence on anthelmintic drugs which are associated with anthelmintic resistance. Some fractions known to contain flavonoids were effective in inhibiting egg hatching and larval development. The 70% acetone fractions containing CT were effective in inhibiting LD. The CT from sulla were shown to be *cis* and PD predominant and contain considerable variation in chain lengths. Therefore, the antiparasitic activity from this forage could be attributed to its chemical structure. This study suggests that forages such as sulla which contains CT may be used as alternatives for control of parasites in farming systems. The study aims at reducing the population of infective larvae on pasture rather than treatment of the infection.

CHAPTER SIX

SUMMARY AND FUTURE WORK

6.1 Summary and future work

Fractionation of the sulla crude extracts by Sephadex LH-20 chromatography and subsequent analysis of the fractions with RP-HPLC allowed more information to be obtained through a number of analytical methods. For future work, improvement of fractionation using counter current chromatography (Marais et al., 2000) is necessary to better link the biological activity with the chemical structure.

^{13}C-NMR, acid catalysis thiolytic degradation, LC-ESI-MS and MALDI-TOF-MS techniques complemented each other in characterisation of CT oligomers and polymers. Acid catalysis in the presence of benzyl mercaptan yielded all the four flavan-3-ol units; catechin, epicatechin, gallocatechin and epigallocatechin both in the extender and terminal units. Epigallocatechin was found to be the major extender unit, while gallocatechin was the major terminal unit. Thiolysis revealed that sulla CT contain variable chain lengths ranging from 2.9 to 46 mDP, *cis:trans* ranging from 56:44 to 82:18 and PC:PD ratios ranging from 27:73 to 11:89. The results have shown the attributes of sulla CT to be PD and *cis* predominant. CT polymer (n>10) was further characterised successfully by ^{13}C-NMR. ^{13}C-NMR indicated the presence PC:PD, *cis/trans* from the heterocyclic C-ring.

The thiolysis adducts require further investigation, especially with NMR to confirm the presence of gallates. It was shown from the MALDI-TOF and ESI-MS data that sulla CT are galloylated. CT from sulla were extracted and purified by Sephadex LH-20 chromatography using a step and gradient LH-20 fractionation. The gradient LH-20 method efficiently separated the CT oligomers from CT polymers and this was clearly evidenced from the HPLC-PDA chromatograms. LC-ESI-MS and MALDI-TOF-MS verified that sulla CT contain a heterogenous and homogeneous CT with varying mDP and PD as the predominant unit. The lower molecular weight CT up to hexamers and undecamers were observed in analysis with MALDI-TOF-MS and LC-ESI-MS, respectively. Further investigation of CT with other matrices such as trans-3-indole acrylic acid (IAA) and 2,4,6-trihydroacetophenone(THAP) which have been found to be efficacious by others should be explored. In this study, IAA and THAP

were not used because they were not available. Different solvents should be tried to improve crystallisation of the sample with the matrix. More information on molecular weight distribution and type of CT, especially of higher molecular weight species may be achieved by high resolution mass spectrometry, MS-MS and gel permeation chromatography. These techniques were not employed in this study.

The linkages between flavan-ol monomer units in the CT polymer have not been established in this study. Partial thiolysis could be utilised to probe the natural characteristics of the interflavonoid bonds for CT polymers. Thiolysis with acetic acid should lead to formation of thiolyl dimer and possibly trimer derivatives. These could be separated on RP-HPLC or by flash chromatography and subquently characterised using established NMR techniques.

The LH-20 methanolic fractions were purified by RP-C18 flash chromatography, C18 Sep-pak cartridges and semi-preparative HPLC. The purified fractions were successfully characterised using LC-ESI-MS and APCI-MS, yielding chlorogenic acid, isoflavone and flavone derivatives. Verification of the low molecular weight phenolics isolated from sulla should be performed using 2-D correlated spectroscopy (COSY) NMR and derivatisation. Future work should focus on how CT and low molecular weight phenolics can be of beneficial effects in humans. Recent studies have revealed that formononetein can be used to prevent pre- and post-menopausal problems in women and prostrate enlargement in men. This compound and its derivatives have been identified in sulla. Compounds like quercetin are widely known to possess antioxidant and radical scavenging properties.

In addition to secondary metabolites, primary metabolites such as soluble sugars were identified and quantified. Terrill (1992) have shown that some CT are fibre and protein-bound and this was evidenced in sulla. CT content is known to vary with seasonality and soil fertility. The sulla samples used in the analysis were collected from the same site, further study is still needed to find out if soil fertility, temperature and developmental stages have any effect on sulla CT content and composition. Another area of interest may be to investigate the distribution of CT and low molecular weight phenolics in roots, leaves, stems and flowers.

Anthelmintic resistance, consumer awareness and rising concerns about chemical residues in animal products suggest alternative strategies are urgently required to mitigate contamination on pasture with infective larvae. Forages such as sulla, which contains CT may offer such an alternative. Parasite assays using egg hatch and larval

development have shown that CT from freeze-dried and fresh frozen sulla may disrupt the life cycle of *T. colubriformis* nematodes by reducing contamination on pasture with viable eggs and infective larvae. Further *in vivo* investigation is needed to validate the effects of CT-containing forages on egg hatch and larval development under field conditions. The major challenge is to find ways in which CT-containing forages can be integrated into farming systems. Sulla may be used to supplement pasture or be used as a silage, animal feed and medicine. Flavonoids identified from sulla were components of fractions which exhibited antiparasitic activity. Therefore, further investigation should be performed using flavonoids standards. Comparative study is required with authentic standards because there is no published data from the literature about these compounds being antiparasitic. It would be too early to conclude that the antiparasitic and nutritional benefits from sulla plant are attributed only to CT, or who knows if the activity performs better within a mixture of low molecular weight phenolics and primary metabolites.

In conclusion, conventional forages dominated by grass and white clover based pastures, need to be supplemented or complemented with forages which contain CT to fully utilise plant protein and improve animal nutrition and productivity. In the use of conventional forages which contain traces of CT, proteins which are needed for growth and maintenance are lost as the excreta or in the form of ammonia in the rumen.

REFERENCES

Aerts, R.J., McNabb, W.C., Molan, A., Brand, A., Barry, T.N., Peters, J.S. 1999. Condensed tannins from *Lotus corniculatus* and *Lotus penduculatus* exert different effects on the *in vitro* rumen degradation of ribulose-1, 5- bisphosphate carboxylase/oxygenase (Rubisco) protein. *Journal of Science Food and Agriculture*., 79, 79-85

Al-Qarawi, A.A., Mhmoud, O.M., Sobaih, M.A., Haroun, E.M., Adam, S.E.I. 2001. A preliminary study on the activity of *Calotropis procera Latex* against *Haemonchus contortus* infection in Najdi sheep. *Veterinary Research Communications*., 25, 61-70

Artz, W.E., Bishop, P.D., Dunker, A.K., Schanus, E.G., Swanson, B.G. 1987. Interaction of synthetic proanthocyanidin dimer and trimer with Bovine serum albumin and purified bean globulin fraction G-1. *Journal of Agricultural and Food Chemistry*., 35, 417-421

Asquith, T.N., Butler, L.G. 1986. Interactions of condensed tannins with selected proteins. *Phytochemistry*., 25, 1591-1593

Athanasiadou, S., Kyriazakis, I., Jackson, F., Coop, R.L. 2001. Direct anthelmintic effects of condensed tannins towards gastrointestinal nematodes of sheep: *in vitro* and *in vivo* studies. *Veterinary Parasitology*., 99, 205-219

Barry, T.N. 1985. The role of condensed tannins in the nutritive value of *Lotus penduculatus* for sheep: rates of body growth and wool growth. *British Journal of Nutrtion*., 54, 211-217

Barry, T.N., and Manley, T.R. 1984. The role of condensed tannins in the nutritional value of *Lotus penduculatus* for sheep. *British Journal of Nutrition*., 51, 493-504

Barry, T.N., Jackson, F.S. 1996. The extractable and bound condensed tannin content of leaves from tropical tree, shrub and forage legumes. *Journal of Science Food and Agriculture*., 71, 103-110

Barry, T.N., Manley, T.R. 1986. Interrelationships between the concentrations of total condensed tannins, free condensed tannin and lignin in *Lotus* sp. and their possible consequences in ruminant nutrition. *Journal of Science Food and Agriculture*., 37, 248-254

Barry, T.N., McNabb, W.C. 1999. The implications of condensed tannins on the nutritive value of temperate forages fed to ruminants. *British Journal of Nutrition*., 81, 263-272

Barry, T.N., Mcneill, D.M., McNabb, W.C. 2001. Plant secondary compounds; their impacts on forage nutritive value and upon animal production. *Proceedings of the XIX International Grassland Congress*., 445-452

Behrens, A., Maie, N., Knicker, H., Kogel-Knabner, I. 2003. MALDI-TOF spectrometry and PSD fragmentation as means for the analysis of condensed tannins in plant leaves and needles. *Phytochemistry.*, 62, 1159-1170

Bermingham, E.N., Hutchinson, K., Revell, D.K., Brookes, I.M., McNabb, W.C., 2001. The effect of condensed tannins in sainfoin (*Onobrychis viciifolia*) and sulla

Beuselinck, P.R., Gebrehiwot, L., Roberts, C.A. 2002. Seasonal variations in condensed tannins concentration of three *Lotus* species. *Agronomy Journal.*, 94, 1059-1065

Bilia, A.R., Palme, E., Marsili, A., Pistelli. L., Morelli, I. 1993. A flavonol glycoside from *Agrimonia eupatoria. Phytochemistry.*, 32, 1078-1079

Bohm, B.A. 1998. Extraction, purification and identification: *Introduction to Flavonoids.*, Harwood academic publishers, Canada, 193

Bohm, B.A., Koupai-Abyazani, M.R., McCallum, J., Muir, A.D., Towers, Gruber M.Y. 1993. Developmental changes in the composition of proanthocyanidins from leaves of sainfoin (*Onobrychis viciifolia* Scop.) as determined by HPLC analysis. *Journal of Agricultural and Food Chemistry.*, 41, 1066-1070

Bravo, L. 1998. Poplyphenols: Chemistry, dietary sources, metabolism, and nutritional significance. *Nutrion Reviews.*, 56, 317-333

Bruere, A.N., and West, D.M. 1993. Gastrointestinal nematodes of sheep: *In The Sheep: Health, Disease and Production.*, Veterinary Continuing education, Massey University, Pp 150-151

Brundson, R.V. 1970. Seasonal changes in the level and composition of nematode worm burdens in young sheep. *New Zealand Journal of Agricultural Research.*, 13, 126-48

Brundson, R.V. 1980. Principles of helminth control. *Veterinary Parasitology.*, 6, 185-215

Burke, J.L., Brookes, I.M., Waghorn, G.C. 2002. An evaluation of sulla (*Hedysarum coronarium*) with pasture, white clover and lucerne for lambs. *Proceedings of the New Zealand Society of Animal Production.*, 62, 152-156

Butler, N.L., Buttery, P.J., Dawson, J.M., Wakelin, D. 2001. Effect of dietary condensed tannins on gastrointestinal nematodes. *Journal of Agricultural Science Cambridge.*, 137, 461-469

Carreri, M., Mangia, A., Musci, M. 1998. Overview of the applications of liquid chromatography-mass spectrometry interfacing systems in food analysis: naturally occurring substances in food. *Journal of Chromatography A.*, 794, 263-297

Carter, E.B., Theodoruo, M.K., Morris, P. 1999. Responses of *Lotus corniculatus* to environmental change. 2. Effect of elevated CO_2, temperature drought on tissue digestion in relation to condensed tannin and carbohydrate accumulation. *Journal of Science Food and Agriculture*., 79, 1431-1440

Cheminat, A., Zawatzky, R., Becker, H., Broullard, R. 1988. Caffeoyl conjugates from *Echinacea* species: structures and biological activity. *Phytochemistry*., 9, 2787-2794

Cheynier, V. 1999. Tannins in grape and grape products: Brooker, J.D (ed). *In* Tannins in livestock and human nutrition, *Proceedings of an International Workshop*, Adelaide, Australia. ACIAR Proceedings 92, Pp91

Ciavarella, T.A., Dove, H., Leury, B.J., Simpson, R.J. 2000. Diet selection by sheep grazing *Phalaris aquatica* L. pastures of differing water soluble carbohydrate content. *Australian Journal of Agricultural Research*., 51, 757-764

Choong, E.T., Syahbirin, G., Achmadi, S., Hemingway, R.W. 1994. Catechin-3-O-rhamnoside chain extender units in polymeric procyanidins from Mangrove bark. *Phytochemistry*., 35, 217-219

Cork, S.J., and Krockenberger, A.K. 1991. Methods and pitfalls of extracting condensed tannins and other phenolics from plants: insights from investigations on Eucalyptus leaves. *Journal of Chemical Ecology*., 17, 123-134

Czochanska, Z., Foo, L.Y., Newman, R.H., Porter, L.J. 1980. Polymeric proanthocyanidins: stereochemistry, structural units, and molecular weight. *Journal of Chemical Society*., Perkin I. 2278-2286

Dear, B.S., Moore, G.A., Hughes, S.J., 2003. Adaptation of potential contribution of temperate perennial legumes to the southern Australian wheatbelt. *Australian Journal of Experimental Agriculture*., 43, 1-18

De Bruyne, T., Pieters, L., Witvrouw, M., De Clercq, E., Berghe, D.V., Vlietinick, A.J. 1999. Biological evaluation of proanthocyanidin dimers and related polyphenols. *Journal of Natural Products*., 62, 954-958

De Freitas., V.A.P., Glories, Y., Bourgeois, G., Virty, C. 1998. Characterisation of oligomeric and polymeric procyanidins from grape seeds by liquid secondary ion mass spectrometry. *Phytochemistry*., 49, 1435-1441

De Silva, J.R.M., Rigaud, J., Cheynier, V., Cheminat, A., Motounet, M. 1991. Procyanidin dimers and trimers from grape seeds. *Phytochemistry*., 30, 1259-1264

Demirezer, L.O., Kuruuzum-Uz, A., Bergere, I., Schiewe, H.J., Zeeck, A. 2001. The structures of antioxidant and cytotoxic agents from natural source: anthraquinones and tannins from roots of *Rumex patientia*. *Phytochemistry*., 58, 1213-1217

Donald, A.D., Southcott, W.H., Dineen, J.K. 1978. *The Epidemiology and Control of Gastrointestinal Parasites of Sheep in Australia*: Commonwealth Scientific and Industrial Research Organisation, Australia, 80, 1094-1117

Douglas, G.B., Foote, A.G. 1985. Dry matter and seed yields of sulla (*Hedysarum coronarium L*). *New Zealand Journal of Experimental Agriculture.*, 13, 97-99

Douglas, G.B., Stiennezen, M., Waghorn, G.C., Foote, A.G. 1999. Effect of condensed tannins in birdsfooot trefoil (*Lotus corniculatus*) and sulla (*Hedysarum coronarium*) on body weight, carcass fat depth, and wool growth of lambs in New Zealand. *New Zealand Journal of Agricultural Research.*, 42, 55-64

Foo, L.Y., Newman R., Waghorn, G., McNabb, W.C., Ulyatt, M.J. 1997. Proanthocyanidins from *Lotus penduculatus. Phytochemistry.*, 2, 617-624

Foo, L.Y., and Newman, R., Waghorn, G., McNabb, W.C., Ulyatt, M.J. 1996. Proanthocyanidins from *Lotus corniculatus. Phytochemistry.*, 2, 617-624

Foo, L.Y., and Porter, L.J. 1980. The phytochemistry of proanthocyanidin polymers. *Phytochemistry.*, 19, 1747-1754

Foo, L.Y., Jones, L.J., Porter, L.J., Williams, V.M. 1982. Proanthocyanidin polymers of fodder legumes. *Phytochemistry.*, 21, 933-935

Foo, L.Y., Lu, Y., Molan, A.L., Woodfield, D.R., McNabb, W.C., Scalbert, A., Mila, I., Deprez, S. 2000. The phenols and prodelphinidins of white clover flowers. *Phytochemistry.*, 54, 539-548

Fulcrand, H., Remy, S., Souquet, J.M., Cheynier, V., Moutounet., M. 1999. Study of wine tannin oligomers by on-line liquid chromatography ionization mass spectrometry. *Journal of Agricultural and Food Chemistry.*, 47,1023-1028

Graeyer, R.J., Kite, G.C., Abou-Zaid, M., Archer, L. J. 2000. The application of atmospheric pressure chemical ionization liquid chromatography-mass spectrometry in the chemotaxonomic study of flavonoids from *Ocimum gratissimum* var. gratissimum. *Phytochemical Analysis.*, 11, 257-267

Guyot, S., Marnet, N., Laraba, D., Sanoner, P., Drilleau, J. 1998. Reverse phase HPLC following thiolysis for quantitative estimation and characterisation of the four main classes of phenolic compounds in different tissue zones of a French cider apple variey (*Malus domestica* Var. Kermerrien). *Journal of Agricultural and Food Chemistry.*, 46, 1698-1705

Hakkinen, S., Auriola, S. 1998. High-performance liquid chromatography with electrospray ionization and diode array ultraviolet detection in the identification of flavonol aglycones and glycosides in berries. *Journal of Chromatography A.*, 829, 91-100

Halliwell, B., and Gutteridge, J.M.C. 1999. Plant phenols: *In Free Radicals in Biology and Medicine*, Oxford university press, New York, 3rd Edition., 228

Harborne, J.B. 1994. Flavans and proanthocyanidins: *In The Flavonoids*. Chapman and Hall, London, 1st Edition, 46

Harborne, J.B., and Chriki, A. 1983. Anthocyanins of *Hedysarum Coronarium* and their contribution to flower colour variation. *Phytochemistry.*, 22, 2322-23

Haslam, E. 1996. Natural polyphenols (vegetable tannins) as drugs: possible mode of action. *Journal of Natural Products.*, 59, 205-211

Haslam, E. 1989. Polyphenol complexation: *In Chemistry and Pharmacology of Natural Products, Plant Polyphenols and Vegetable Tannins Revisited.*, Cambridge University Press, 71, 159

Hedqvist, H., Murphy, M., Mueller-Harvey, I., Reed, J.S., Krueger, C.G. 2000. Characterisation of tannins and in vitro protein digestibility of several *Lotus corniculatus* varieties. *Animal Feed Science and Technology.*, 87, 41-56

Hemingway, R.W., and Karchesy, J.J. 1990. Reactions at the interflavonoid bond of proanthocyanidins: *In Chemistry and Significance of Condensed Tannins*, Plenum press, New York, 265

Jackson, R.A., Townsend, K.G., Pyke, C., Lance, D.M. 1999. Isolation of oxfendazole resistance Cooperia oncophora in cattle. *New Zealand Veterinary Journal.*, 35, 187-189

Jansman, A.J.M. 1993. Tannins in feedstuffs for simple-stomached animals. *Nutrition Research Reviews.*, 6, 209-236

Jones, W.T., Lyttleton, J.W., 1971. Bolat in cattle (XXXIV). A survey of forage legumes that do and do not produce bloat. *New Zealand Journal of Agricutural Research.*, 14, 101-107

Jones, W.T., Richard, B., Broadhurst, B., Lyttleton, J.W., 1976. The condensed tannins of pasture legume species. *Phytochemistry.*, 15, 1407-1409

Kahkonen, M.P., Hopia, A.I., Vuorella, H.J., Rauha, J.P., Pihlaja, K., Kujala, T.S., Heinonen, M. 1999. Antioxidant activity of plant extracts containing phenolic compounds. *Journal of Agricultural and Food Chemistry.*, 47, 3954-3962

Kahn, L.P., Diaz-Hernandez, A. 1999. Tannins with anthelmintic properties: Brooker, J.D (ed). In tannins in livestock and human nutrition, *Proceedings of an International Workshop*, Adelaide, Australia. ACIAR Proceedings 92, Pp 130-131

Kelly, R.W., Allison, A.J., Shirley, D.K. 1976. Interactions between phyto-oestrogens and stereoids in the cervical mucus and uterine weight responses to ewes. *Australian Journal of Agricultural Research*., 27, 101-107

Kennedy, J.A., 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, 1740-1746

Klejdus, B., Vitamvasova-Sterbova, D., Kuban, V. 2001. Identification of isoflavone conjugates in red clover (*Trifolium pratense*) by liquid chromatography-mass spectrometry after two-dimensional solid phase extraction. *Analytica Chimica Acta*., 450, 81-97

Kolodziej, H., Sakar, M.K., Burger, J.F., Engleshowe, R., Ferreira, D. 1991. A-type proanthocyanidins from prunus spinosa. *Phytochemistry*., 30, 2041-2047

Koupai-Abyazani, M.R., McCallum, J., Bohm, B. 1992. Identification of the constituent flavanoid units in sainfoin proanthocyanidins by reverse-phase high performance liquid chromatography. *Journal of Chromatography*., 594, 117-123

Krueger, C.G., Dopke, N.C., Treichel, P.M., Folts, J., Reed, J.D. 2000. Matrix assisted laser desorption/ionization spectrometry of polygalloyl polyflavan-3-ols in grape seed extract. *Journal of Agricultural and Food Chemistry*., 48, 1663-1667

Kumar, R., Horigome, T., Okamoto, K. 1988. Effects of condensed tannins prepared from leaves of fodder plants on digestive enzymes *in vitro* and in the intestine of rats. *British Journal of Nutrition*., 60, 275-285

Lazarus, S.A., Adamson, G.E., Mitchell, A.E., Prior, R.L., Cao, G., Jacobs, P.H., Kremers, B.G., Hammerstone, J.F., Rucker, R.B., Ritter, K.A., Schimtz, H.H. 1999. HPLC Method for the quantification of procyanidins in cocoa and chocolate samples and correlation to total antioxidant capacity. *Journal of Agricultural and Food Chemistry*., 47, 4184-4188.

Leathwick, D.M., Atkinson, D.S. 1995. Dagginess and flystrike in lambs grazed on *lotus corniculatus* or ryegrass. *Proceedings of the New Zealand Society of Animal Production*., 55, 196-198

Lees, G.L., Hinks, C.F., Suttill, N.H. 1994. Effect of high temperature on condensed tannin accumulation in leaf tissues of big trefoil (*Lotus uliginosus* Schkur). *Journal of Science Food and Agriculture*., 62, 415-421

Lin, L., He, X., Lindenmaier, M., Yang, J., Cleary, M., Qiu, S., Cordell, G. A. 2000. LC-ESI-MS study of the flavonoid glycoside malonates of red clover (*Trifolium pratense*). *Journal of Agricultural and Food Chemistry.*, 48, 354-365

Lopez-Hernandez, J., Gonzalez-Castro, M.J., Vazquez-Blanco, M.E., Vazquez-Oderiz, M.L., Simal-Lozano, J. 1994. HPLC determination of sugars and starch in green beans., *Journal of Food Science.*, 59, 1048-1049

Lowther, W.L., Manley, T.R., Barry, T.N. 1987. Condensed tannin concentrations in *Lotus corniculatus* and *Lotus penduculatus* cultivars grown under low soil fertility conditions. *New Zealand Journal of Agricultural Research.*, 30, 23-25

Mammela, P., Savolainen, H., Lindroos, L., Kangas, J. 2000. Analysis of oak tannins by liquid chromatography-electrospray ionization mass spectrometry. *Journal of Chromatography A.*, 891, 75-83

Mangan, J.L. 1988. Nutritional effects of tannins in animal feeds. *Nutrition Research Reviews.*, 1, 209-23

Marias J.P.J., Ferreira, D., Brandt, E.V., Harvey, I.M. 2000. Polyphenols, condensed tannins and other natural products in *Onobrychis viciifolia* (Sainfoin). *Journal of Agricultural and Food Chemistry.*, 48, 3440-3447

Markham, K.R. 1982. Isolation and analytical techniques: *In Techniques of Flavonoid Identification.*, Academic press, New York, Pp 28

Mauri, P.L., Iemoli, L., Gardana, C., Riso, P., Simonetti, P., Porrni, M., Pietta, P.G. 1999. Liquid chromatography/electrospray ionization mass spectrometric characterization of flavonol glycosides in tomato extracts and human plasma. *Rapid Communications in Mass Spectrometry.*, 13 924-931

McDonald, Edwards, Greenhalgh, Morgan 1995. Digestion: *In Animal nutrition*, 5th Edition, Longman Singapore publishers, 167.

McDonald, S., Prenzler, P.D., Antolovich, M., Robards, K. 2001. Phenolic content and antioxidant activity of olive extracts. *Food Chemistry*, 73, 73-84

McMahon, L.R., McAllister, T.A., Berg, B.P., Majak, W., Achrya, S.N., Popp, J.D., Coulman, B.E., Wang, Y., Cheng, K.J. 1999. A review of the effects of forage condensed tannins on ruminal fermentation and bloat in grazing cattle. *Canadian Journal of Plant Science*, 469-485

McNabb, W.C., Peters, J.S., Foo, L.Y., Waghorn, G.C., Jackson, F.S., Scalbert, A., Santos-Buelga, C. 1998. Effect of condensed tannins prepared from several forages in the in vitro precipitation of Ribulose-1, 5 bisphosphate carboxylase (Rubisco) protein and its digestion by trypsin (EC 2.4.21.4) and chymotrypsin (EC 2.4.21.1. *Journal of Science, Food and Agriculture.*, 77, 201-212

McNabb, W.C., Shelton, I.D., Jones, W.T., Waghorn, G.C. 1990. Condensed tannin and the nutritive value of herbage. *Proceedings of the New Zealand Grassland Assoiciation.*, 52, 171-176

McNabb, W.C., Waghorn, G.C., Peters, J.S., Barry, T.N. 1996. The effect of condensed tannins in *Lotus penduculatus* on the solubilisation and degradation of ribulose-1,5- biphosphate carboxylase (EC 4. 1.1.39; Rubisco). *British Journal of Nutrition.*, 76, 535-549

Meagher, L., Molan, A.L., Spencer, P. 2003. AgResearch, Confidential report to Meat and Wool Innovation, New Zealand.

Mekasha, Y., Tegegne, A., Yami, A., Umunna, N.N. 2002. Evaluation of non-conventional agro-industrial by-products as supplementary feeds for ruminants: in vitro and metabolism study with sheep. *Small Ruminant Research.*, 44, 25-35

Molan, A.L., Barry, T.N., Schreurs, N.M., Lopez-Villalobos, N., McNabb, W.C. 2002a. Effects of grazing undrenched weaner deer on chicory or perennial ryegrass/white clover pasture on the viability of gastrointestinal nematodes and lungworms. *Veterinary Record.*, 151, 1930(01-10)

Molan, A.L., McNabb, W.C., Brookes, I.M., Alexander, R.A. 2000a. Effects of an extract from sulla (*Hedysarum Coronarium*) containing condensed tannins on the migration of three sheep gastrointestinal nematodes in vitro. *Proceedings of the New Zealand Society of Animal Production.*, 60, 21-25

Molan, A.L., Sivakumaran, S., Meagher, Spencer, P. 2003 (in press). Effect of flavan-3-ols on *in vitro* egg hatching, larval development and viability of infective larvae of *Trichostrongylus colubriformis. International Journal for Parasitology*

Molan, A.L., Waghorn, G.C., McNabb, W.C. 1999. Condensed tannins and gastrointestinal parasites in sheep. *Proceedings of the New Zealand Grassland Association.*, 61, 57-61

Molan, A.L., Waghorn, G.C., McNabb, W.C., Min, BR. 2000b.The effect of condensed tannins from seven herbages on *Trichostrongylus colubriformis* larval migration *in vitro. Folia Parasitologica.*, 47, 39-44

Molan, A.L., Waghorn, G.C., Molan, A.L. 2002b. Effect of condensed tannins on egg hatching and larval development of *Trichostrongylus colubriformis in vitro. Veterinary Records.*, 150, 65-69

Molle, G., Decandia, M., Fois, N., Ligios, S., Cabiddu, A., Sitzia., 2003. The performance of Mediterranean dairy sheep given access to sulla (*Hedysarum coronarium* L.) and annual ryegrass (*Lolium rigidum* Gaudin) pastures in different time proportions. *Small Ruminant Research.*, 49, 319-328

Nelson, K.E., Pell, A.N., Doanne, P.H., Ginger-Chavez, BI., Schofield, P. 1997. Chemical and biological assays to evaluate bacterial inhibition by tannins. *Journal of Chemical Ecology.*, 23, 1175-1194

Niezen, J.H., Barry, T.N., Wilson, P.R., Lane, G., Beuselinck, P.R., Gebrehiwot, L., Roberts, C.A. 1992. Red urine from red clover grazed on pure red clover swards. *New Zealand Veterinary Journal.*, 40, 164-167

Niezen, J.H., Chareston, W.A.G., Robertson, H.A., Shelton, D., Waghorn, G.C., Green, R. 2002a. The effect of feeding sulla (*Hedysarum coronarium*) or lucerne (*Medicago sativa*) on lamb parasite burdens and development of immunity to gastrointestinal nematodes. *Veterinary Parasitology.*, 105, 229-245

Niezen, J.H., Waghorn, G.C., Charleston, W.A.G. 1998. Establishment and fecundity of *Ostertagia circumcincta* and *Trichostrongylus colubriformis* in lambs fed lotus (*Lotus penduculatus*) or perennial ryegrass (*Lolium perenne*). *Veterinary Parasitology.*, 78, 13-21

Niezen, J.H., Waghorn, G.C., Graham, T., Carter, J.L., Leathwick, D.M. 2002b. The effect of diet fed to lambs on subsequent development of *Trichostrongylus colubriformis* larvae *in vitro* and on pasture. *Veterinary Parasitology.*, 105, 269-283

Niezen, J.H., Waghorn, T.S., Charleston, W.A.G., Waghorn, G.C. 1995. Growth and gastrointestinal nematode parasitism in lambs grazing either Lucerne (*Medicago sativa*) or sulla (*Hedysarum coronarium*) which contains condensed tannins. *Journal of Agricultural Science, Cambridge.*, 125, 281-289

Ohnishi-Kameyama, M., Yanagida, A., Kanda, T., Nagata, T. 1997. Identification of catechin oligomers from apple (*Malus pumila* cv. Fuji) in matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry and fast atom bombardment mass spectrometry. *Rapid Communications in Mass Spectrometry.*, 11, 31-36

Perez-Maldonado, R.A., Norton, B.W. 1996. Digestion of ^{14}C-labelled condensed tannins from *Desmodium intortum* in sheep and goats. *British Journal of Nutrition.*, 76, 501-513

Pieters, L., Balde, A.M., Bruyne, T.S., Kolodziej, H., Berghe, D.V., Claeys, M., Vlietinck, A. 1995. Oligomeric proanthocyanidins possessing doubly linked structure from Pavetta Owariensis. *Phytochemistry.*, 38, 719-723

Poon, G.K. 1998. Analysis of catechins in tea extracts by liquid chromatography-electrospray ionization mass spectrometry. *Journal of Chromatograophy A.*, 794, 63-74

Porter, L.J., Hrstich, L.A., Chan, B.G. 1986. The conversion of procyanidins and prodelphinidins to cyanidins and delphinidins. *Phytochemistry*., 25, 223-230

Porter, L.J., Newman, R.H., Foo, L.Y., Wong, H., Hemingway, R.W. 1982. Polymeric proanthocyanidins. ^{13}C N.M.R. Studies of procyanidins. *Journal of Chemical Society Perkins* 1., 1217-1221

Prior, R.L., Vannozzi, S., Cunningham, D., Beecher, G., Hammerstone, J.F., Kelm, M., Gu, L. 2002. Fractionation of polymeric procyanidins from lowbush blueberry and quantification of procyanidins in selected foods with an optimized normal-phase HPLC-MS fluorescent detection method. *Journal of Agricultural and Food Chemistry*., 50, 4852-4860

Pritchard, R. 1994. Anthelmintic resistance. *Veterinary Parasitology*., 54, 259-268

Pritchard, R.K. 1990. Anthelmintic resistance in nematodes: extent, recent and understanding directions for control and research. *International Journal for Parasitology*., 20, 515-523

Rabel, B. Dough, P.G.C., McGregor, R., 1994. Improved bioassay for estimation of inhibitory effects of ovine gastrointestinal mucus and anthelmitics on nematode larval migration. *International Journal for Parasitology*., 24, 671-676

Reid, C.S.W., Ulyatt, M.J., Wilson, J.M. 1974. Plant tannins, bloat and nutritive value. Proceedings of New Zealand Society of Animal production. *Journal of Agricultural and Food Chemistry*., 82-93

Reynolds, G.W., Knight, T.W., Roy, N.C., Deighton, M.H., Death, A.F., Sinclair, B.R., Peters, J.S., McNabb, W.C. 2000. The effect of condensed tannins in fresh sulla (*Hedysarum coronarium*) on the net flux of fatty acids across the mammary gland and their secretion in the milk of lactating ewes. *Proceedings of the New Zealand Society of Animal Production*., 62, 231-235

Ricardo-da Silva, J.M., Cheynier, V., Souquet, J.M., Moutounet, I., Georgi, J.R. 1991. Interaction of grape seed procyanidins with various proteins in relation to wine fining. *Journal of Science Food and Agriculture*., 57, 111-125

Rimpler, H., Geiss, F., Heinrich, M., Hunkler, D. 1994. Proanthocyanidins with (+) epicatechin units from *Byrsonima crassifolia* bark. *Phytochemistry*., 39, 635-643

Robards, K., Antolovich, M. 1997. Analytical chemistry of fruit bioflavonoids. *Analyst*., 122, 11R-34R

Rogler, J.C., Jimenez-Ramsey, L.M., Housley, T.L., Butler, L.G., Elkin, R.G. 1994. Absorption and distribution of ^{14}C-labelled condensed tannins and related phenolics in chickens. *Journal of Agricultural and Food Chemistry.*, 42, 963-967

Rolando, C., Donnelly, D.M.X. 1997. Method for estimation of proanthocyanidins based on their acid depolymerisation in the presence of nucleophiles. *Journal of Agricultural and Food Chemistry.*, 45, 1195-1201

Sanchez-Mata, M.C., Camara-Hurtado, M., Diez-Marquies, C. 2002. Identification and quantification of soluble sugars in green beans by HPLC. *European Food Research and Technology.*, 214, 254-258

Sanchez-Mata, M.C., Penuela-Teruel, J., Camara-Hurtado, M., Diez-Marues,C., Torija-Isasa, M.E. 1998. Determination of mono-, di-, and oligosaccharides in legumes by High-Performance Liquid Chromatography using an amino-bonded silica column HPLC. *Journal of Agricultural and Food Chemistry.*, 43, 377-380

Scalbert, A. 1991. Antimicrobial properties of tannins. *Phytochemistry.*, 30, 3875-3883

Scalbert, A., Matthews, S., Pollet, B., Lapierre, C., Herve du Penhoat, C.L.M., Rolando, C., Donnelly, D.M.X. 1997. Method for estimation of proanthocyanidins based on their acid depolymerisation in the presence of nucleophiles. *Journal of Agricultural Food Chemistry.*, 45, 1195-1201

Scalbert, A., Santos-Buelga, C. 2000. Proanthocyanidins and tannin-like compounds- nature, occurrence, dietary intake and effects on nutrition and human health. *Journal of Science, Food and Agriculture.*, 80, 1094-1117

Souquet, J.M., Labarbe, B., Le Guerneve, C., Cheynier, V., Moutounet, M. 2000. Phenolic composition from grape stems. *Journal of Agricultural and Food Chemistry.*, 48, 1076-1080

Stafford, H.A., Cheng, T.Y. 1980. The procyanidins of Douglas fir seedlings, callus and cell suspension cultures derived from cotyledons. *Phytochemistry.*, 19, 131-135

Sun, B., Leandro, C., Ricardo da Silva, J.R.M., Spranger, I. 1998. Separation of grape and wine proanthocyanidins according to their degree of polymerisation. *Journal of Agricultural and Food Chemistry.*, 46, 1390-1396

Svedstrom, U., Vuorella, H., Kostiainen, R., Tuominen, J., Kokkonen, J., Rauha, J.P., Laakso, I., Hiltumen, R. 2002. Isolation and identification of oligomeric procyanidins from Crataegus leaves and flowers. *Phytochemistry.*, 60, 821-825

Sykes, A.R. and Poppi, D.P. 1982: Effects of Parasitism on metabolism in sheep. In Ross, A. D. (ed) *Control of Internal Parasites in Sheep*. Lincoln College, Canterburry, New Zealand. Pp 25-36

Sykes, A.R., 1982: Parasitism in adult sheep. In Ross, A. d. (ed) *Control of Internal Parasites in Sheep*. Lincoln College, Canterburry, New Zealand. Pp 37-41

Schofield, P., Pell, A.N., Mbugua, D.M. 2001. Analysis of condensed tannins: a review. *Animal Feed Science and Technology*., 91, 21-40

Tagliaferro, A.R., Bobilya, D.J., Heim, K.E. 2002. Flavonoids antioxidants: chemistry, metabolism and structure-activity relationships. *Journal of Nutritional Biochemistry*., 13, 572-584

Taylor, A.W., Barofsky, E., Kennedy, J., Deinzer, M.L. 2003. Hop (*Humulus lupulus* L.) Proanthocyanidins characterised by mass spectrometry, acid catalysis, and gel permeation chromatography. *Journal of Agricultural and Food Chemistry*., 51, 4101-4110

Terrill, T.H. Barry, T.N., Wilson, G.F., Foote, A.G., Douglas, G.B. 1992a. Effect of condensed tannins upon body growth, wool growth and rumen metabolism in sheep grazing sulla (*Hedysarum Coronarium*) and perennial pasture. *Journal of Agricultural Science Cambridge*., 119, 265-273

Terrill, T.H., Rowan, A.M., Douglas, G.B., Barry, T.N. 1992b. Determination of extractable and bound condensed tannin concentrations in forage plants, protein concentrate meals and cereal grains. *Journal of Science Food and Agriculture*., 58, 321-329

Terrill, T.H., Waghorn, G.C., Woolley, D.J., McNabb, W.C., Barry, T.N. 1994. Assay and digestion of ^{14}C-labelled condensed tannins in the gastrointestinal tract of sheep. *British Journal of Nutrition*., 72, 467-477

Ulyatt, M.J., Betteridge, K., Knapp, J., Baldwin, R.L. 1992. Methane production by New Zealand ruminants: *In Global Change, Impact on Agricuture and Forestry*., 89

Ulyatt, M.J., Lassey, K.R., Shelton, I.D., Walker, C.F. 2002. Seasonal variation in methane emissions from dairy cows and breeding ewes grazing ryegrass/white clover pasture in New Zealand. *New Zealand Journal of Agricultural Research*., 45, 217-226

Van Houtert, M.F.J., Sykes, A.R., Foo, L.Y., Newman, R., Waghorn, G. 1996. Implications of nutrition for the ability of ruminants to withstand gastrointestinal nematode infections. *International Journal for Parasitology*., 26, 1151-1168

Vlassoff, A., and Bisset, S.A. 1991. Basic Principles of parasite epidemiology: *In Proceedings of Sheep and Cattle Society of NZVA*. 134: 5-11

Vlassoff, A., McKenna, P.B. 1994. Nematodes parasites of economic importance in sheep in New Zealand. *New Zealand Journal of Zoology*., 21, 1-8

Waghorn, G.C., Douglas, G.B., Niezen, J.H., McNabb, W.C., Foote, A.G. 1998. Forages with condensed tannins-their management and nutritive value for ruminants. *Proceedings of the New Zealand Grassland Association*., 60, 89-98

Waghorn, G.C., Niezen, J.H., Robertson, H.A., Charleston, W.A.G. 1995. The effect of six herbages on liveweight gain, wool growth and faecal egg count of parasitized ewe lambs. *Proceedings of the New Zealand Society of Animal Production*., 55, 199-201

Waghorn, G.C., Tavendale, M.H., Woodfield, D.R. 2002. Methanogenesis from forages fed to sheep. *Proceedings of the New Zealand Grassland Association*., 64, 167-171

Waghorn, G.C., Woodward, S.L., Ulyatt, M.J., Lassey, K.R. 2001. Early indications that feeding *Lotus* will reduce methane emissions from ruminants. *Proceedings of the New Zealand Society of Animal Production*., 38, 23-26

Wang, Y., Douglas, G.B., Waghorn, G.C., Barry, T.N., Foote, A.G. 1996. Effect of condensed tannin in *Lotus corniculatus* upon lactation performance in ewes. *Journal of Agricultural Science, Cambridge*., 126, 353-362

Wang, J., Sporns, P. 2000. MALDI-TOF-MS analysis of food flavonol glycosides. *Journal of Agricultural and Food Chemistry.,* 48, 1657-1662

Waterman, P.G., and Mole, S. 1994. Qualitative and quantitative separation methods. In *Analysis of Plant Phenolic Metabolites*, Blackwell Scientific Publications, Oxford, 159

Wilkins, R.J., Jones, R. 2000. Alternative home-grown protein sources for ruminants in the United Kingdom. *Animal Feed Science and Technology*., 85, 23-32

Wolfender, J. L., Ndjoko, K., Hostettmann, K. 2001. The potential of LC-NMR in phytochemical analysis. *Phytochemical Analysis*., 12, 2-22

Woodward, S.L., Auldist, M.J., Laboyrie, P.J., Jansen, E.B.L. 1999. Effect of *Lotus corniculatus* and condensed tannins on milk yield and milk composition of dairy cows. *Proceedings of the New Zealand Society of Animal Production*., 59, 152-155.

Woodward, S.L., Laboyrie, P.J., Jansen, E.B. 2000. *Lotus corniculatus* and condensed tannins-Effects on milk production by dairy cows. *Asia-Australasian Journal of Animal Science*., 13, 521-525

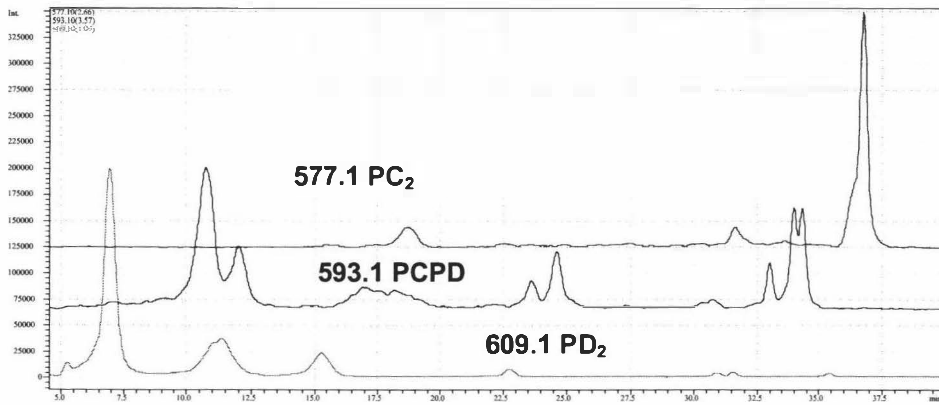
Woodward, S.L., Waghorn, G.C., Lassey, K.R., Laboyrie, P.G. 2002. Does feeding sulla (*Hedysarum coronarium*) reduce methane emissions from dairy cows? *Proceedings of the New Zealand Society of Animal Production*., 62, 227-230

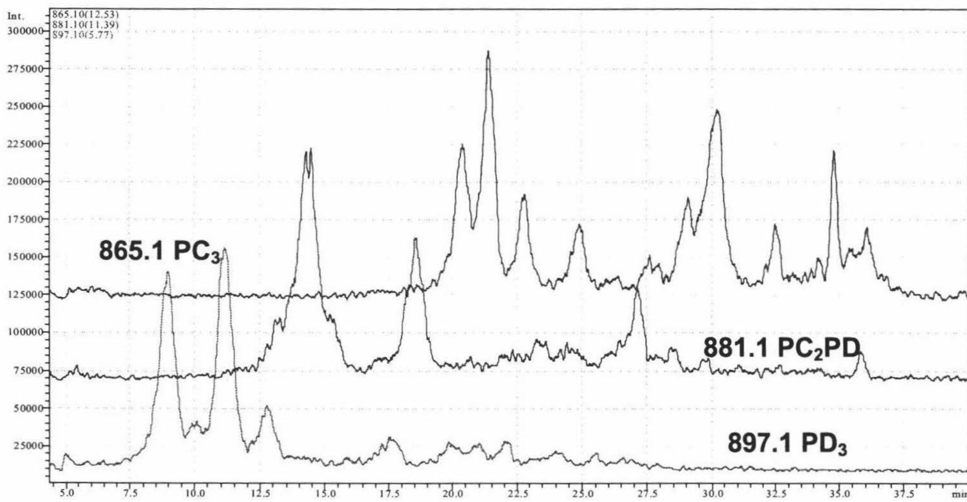
Wyle, J.G., Shakel, GH., Kelly, R.W. 1993. Effects of prolonged exposure of ewes to oestrogenic pasture and occurence of abnormalities of the external genitalia and altered mating performance. *New Zealand Journal of Agricultural Research.*, 36, 459-464

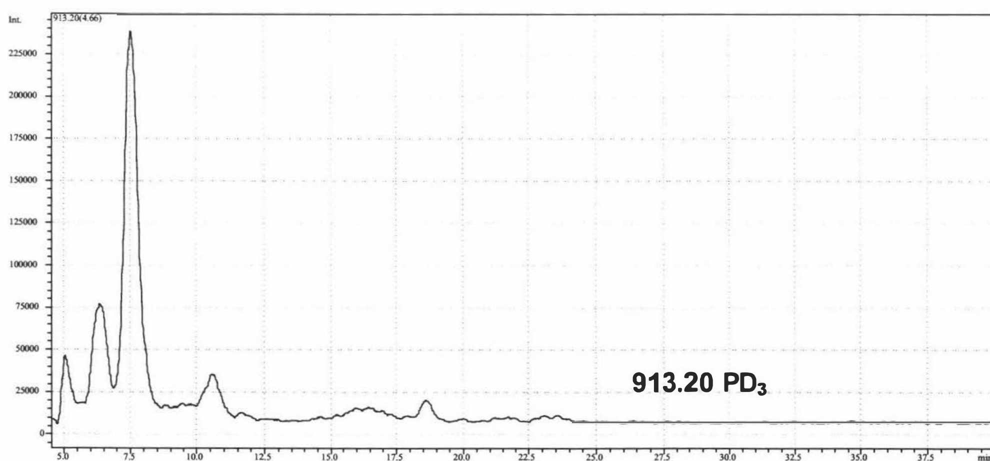
Xiccato, G., Trocino, A., De Boever, J.L., Maertens, L., Carabano, R., Pascual, J.J., Perez, J.M., Gidenne, T., Falcao-E-Cunha, L. 2003. Prediction of chemical composition, nutritive value and ingredient composition of European compound feeds for rabbits by near infrared spectroscopy (NIRS). *Animal Feed Science and Technology.*, 104, 153-168

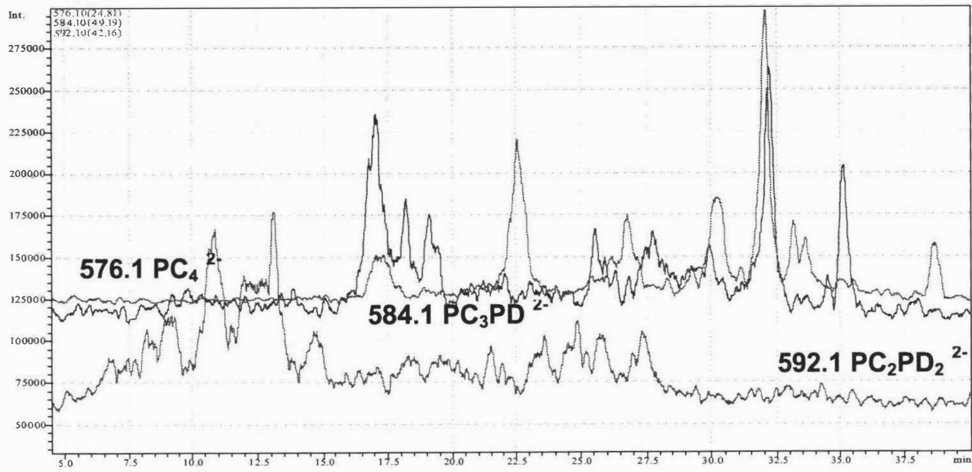
Appendix 1

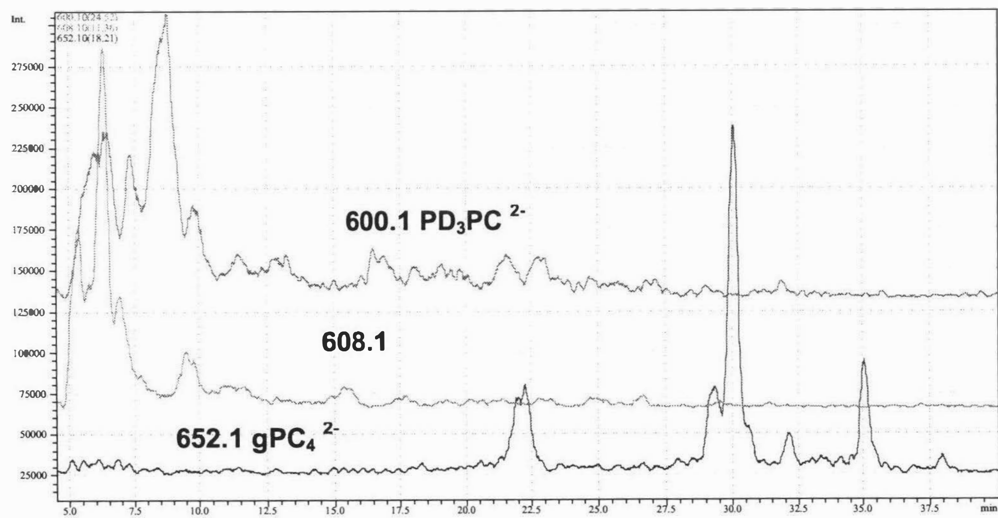
SIM-MS chromatograms of CT oligomers from OTA09807 (100% MeOH, Fr-7) for detection of dimeric to pentameric PC, and PD, homo-oligomers and PC:PD hetero-oligomers.

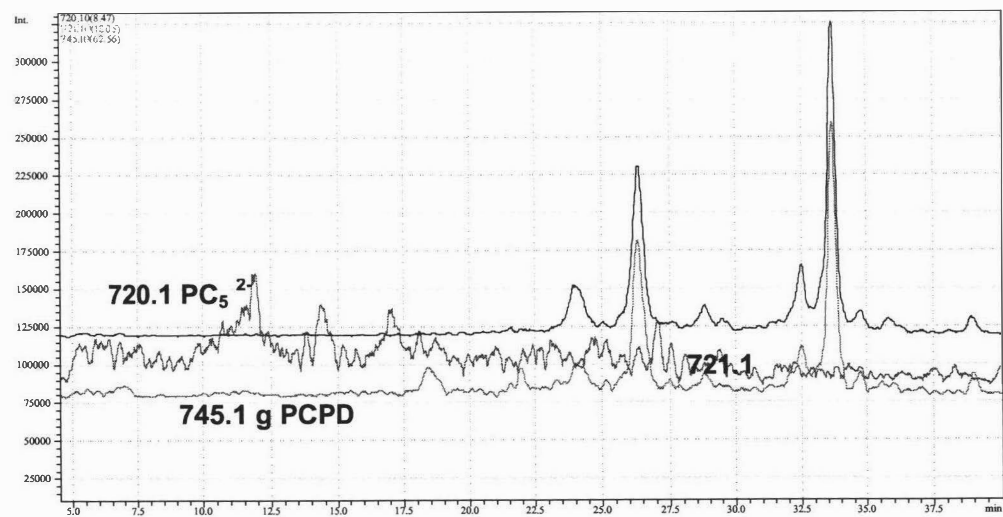






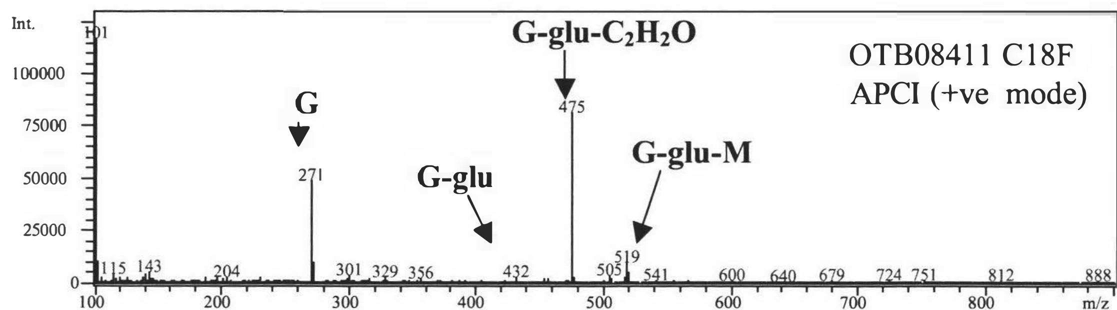

576.1 PC_4 2-
584.1 PC_3PD 2-
592.1 PC_2PD_2 2-

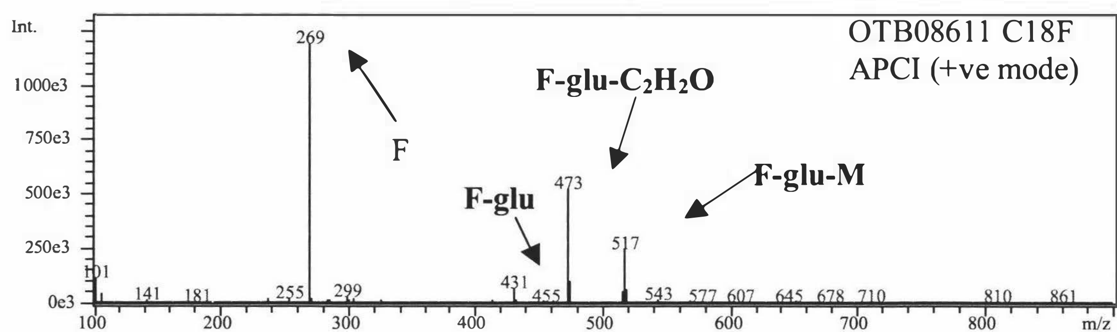

600.1 PD_3PC 2-
608.1
652.1 gPC_4 2-

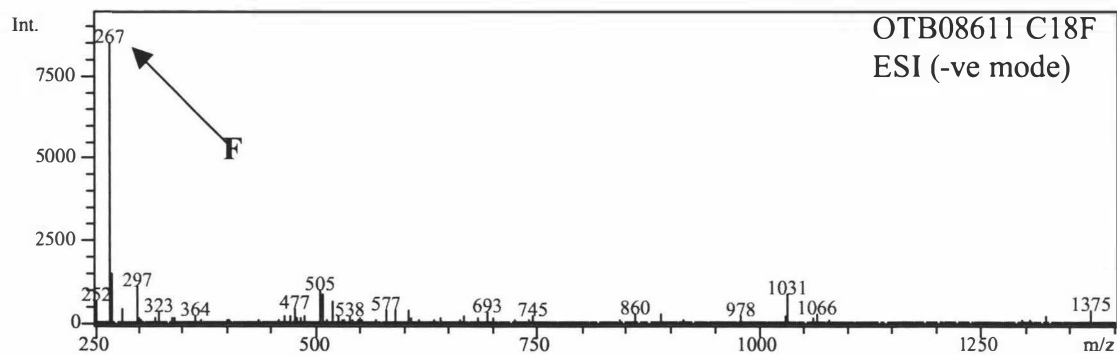

720.1 PC_5 2-
721.1
745.1 g PCPD

Appendix 2

APCI-MS (+ve ion mode) and ESI-MS (-ve ion mode) spectra for fractions OTB08411, OTB08611, and OTB08612.







Appendix 3

The retention times (t_R) fragment ions, APCI-MS $(M+H)^+$, ESI-MS $(M-H)^-$ ions and UV absorption maxima of the reference standards for flavonoids.

Compound	Retention time (min)	APCI (+ve mode) Fragments	APCI Parent ion	ESI-MS (-ve mode)	UV λmax (nm)
Rutin	23.069	465 303	611	609	255, 355
Chlorogenic acid	15.657	355	355	353	Overloaded
Q-3-O-Rh-7-Glu_2	16.009	611 627 465 303	773	771	255, 355
Q-3 (2,6-$(Rh)_2$Glu)	20.478	611 465 303	757	755	Overloaded
Quercetin-3-O-β-D-galactoside	23.811	303	465	463	248, 366

Abbreviations: Q–quercetin, Rh-rhamnose, Glu-glucose

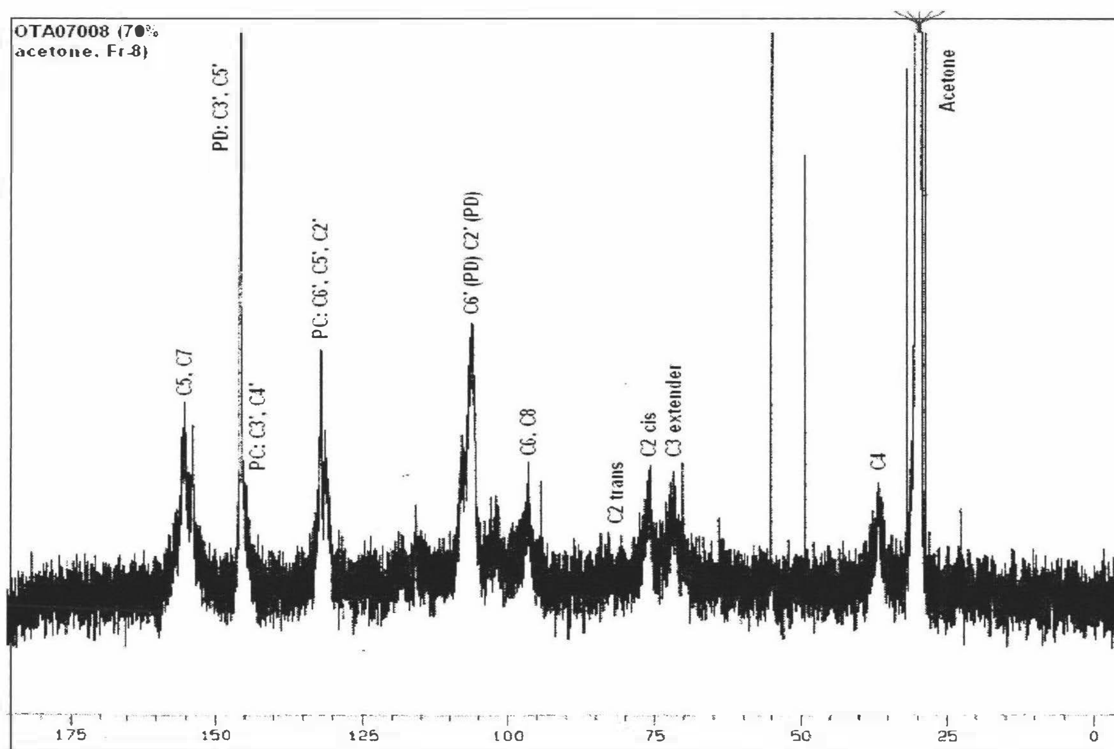
Appendix 4
Preparation of artificial medium for the larval development (LD) assay

1. 2.5 g Bacto peptone
2. 3.0 g NaCl
3. 1 mL of 5 mg/mL cholesterol in 100% ethanol. If cholesterol is not available then an egg yolk will do although this is a little unpleasant.
4. Water to 975 mL and autoclave
5. Then add 1mL of
   - 1 M $MgCl_2$ (sterilised)
   - 1 M $CaCl_2$ (sterilised)
   - 25 mL of pH 6.5 K_3PO_4 buffer (sterilised)

This can be converted to agar by adding 17 g/l prior to autoclaving.

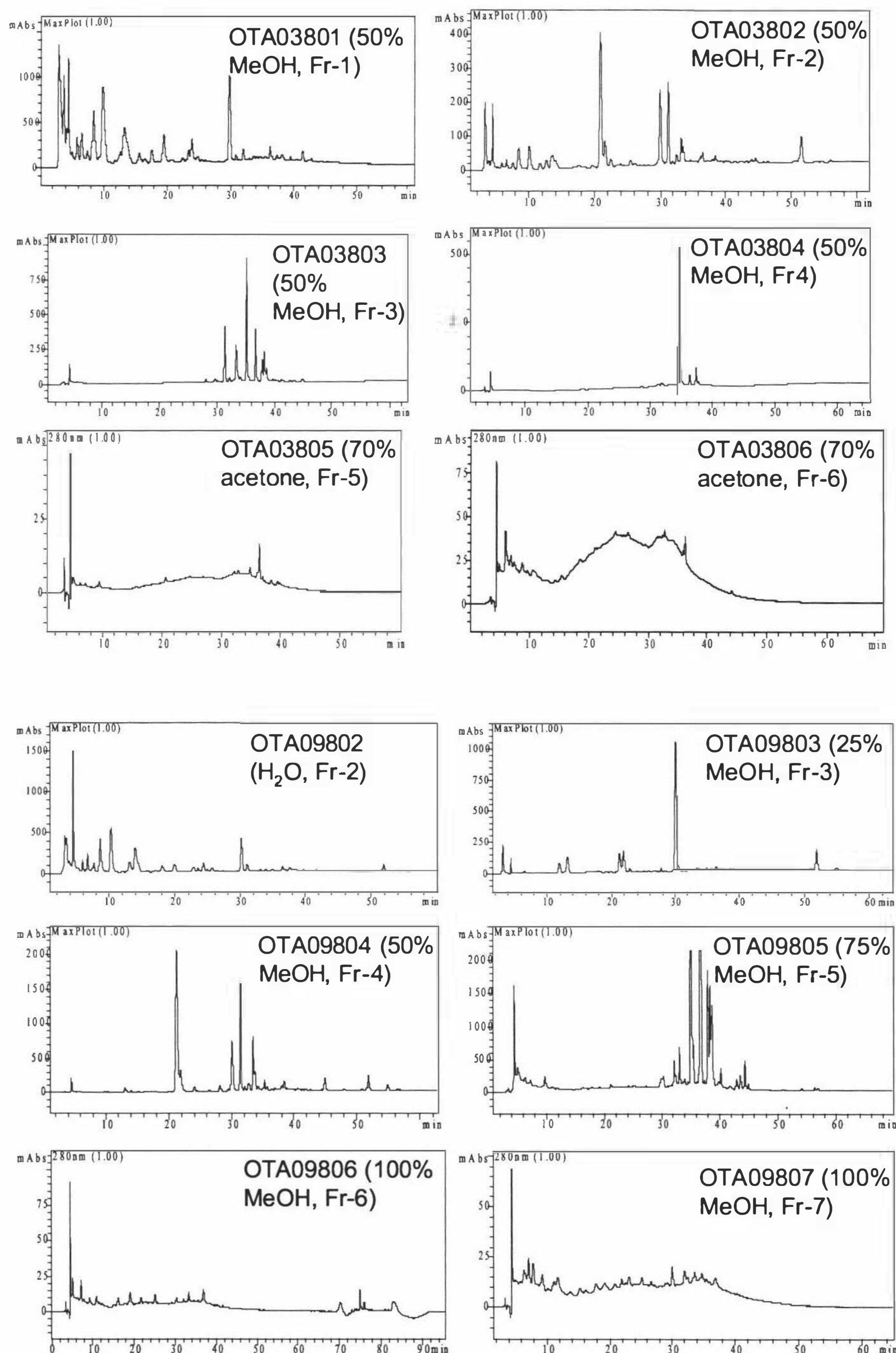
6. Before use, add 1/10 volume of 10% w/v *E. coli* (HB101 strain was used but any regular laboratory strain should be satisfactory) and gentamycin (50 µg/mL final). Fungizone may be added if trouble with fungi or yeast is anticipated

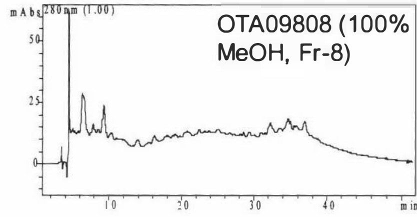
Appendix 5 ^{13}C-NMR spectrum for CT polymer fraction (OTA07008, 70% acetone, Fr-8).

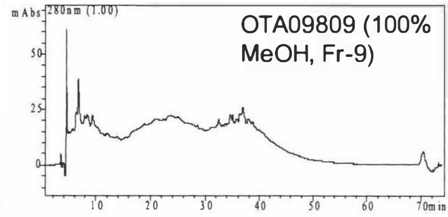


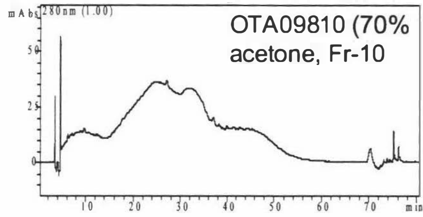
Appendix 6

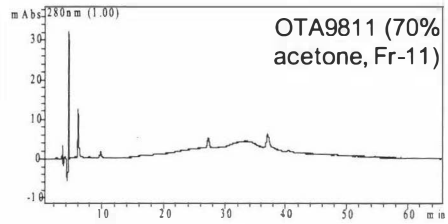
PDA chromatograms recorded for LH-20 fractions (OTA03801-06 (LMA07301 (05/08/02) and OTA09802-11 (LMA03104-21/12/01). PDA chromatograms shown for CT fractions (at 280 nm) list and for non CT-containing fractions list (at 240-360 nm).




mAbs
280nm (1.00)
OTA09808 (100%
MeOH, Fr-8)
50
25
0
10
20
30
40
min


mAbs
280nm (1.00)
OTA09809 (100%
MeOH, Fr-9)
50
25
0
10
20
30
40
50
60
70min


mAbs
280nm (1.00)
OTA09810 (70%
acetone, Fr-10
50
25
0
10
20
30
40
50
60
70
min


mAbs
280nm (1.00)
OTA9811 (70%
acetone, Fr-11)
30
20
10
0
-10
10
20
30
40
50
60
min

Appendix 7

Mass spectra obtained for CT oligomers using LC-ESI-MS in a negative ion mode for the LH-20 gradient fractionation a) OTA09807 (100% MeOH), b) OTA09808 (100% MeOH) and OTA09809 (100% MeOH).