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Condensed tannins in tropical forage legumes: their characterisation and study of their nutritional impact from the standpoint of structure-activity relationships

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SOLANGE, SAMUEL, SANTIAGO *and* ISABELA*:*

Contar con ustedes ha sido la bendicion mas grande de mi vida.

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ABSTRACT

In the tropics, animal production is compromised by the lack of suitable forage for the animals. As a further constraint, low-fertility, acid soils are prevalent in areas where many small and medium size farmers are dependent upon livestock agriculture as their main source of income. This prevents the incorporation of high quality forage legumes (i.e *Leucaena leucocephala, Gliricidia sepium*) into those grazing systems, as these legumes do not perform very well under those conditions. In turn, most of the tropical forage legumes with good adaptation to acidic soils are of low quality and poor acceptability by livestock, which might be associated with their high contents of condensed tannins. However, research on the impact of condensed tannins upon the nutritional value of forage has not provided unequivocal results. This could be related to the variation in structure (molecular weight and monomeric composition) observed among condensed tannins from different legumes. This study investigated the impact of condensed tannins from different tropical legumes on the degradation of different substrates by rumen microorganisms and/or their enzymes under the scope of structure-activity relationships.

In an initial experiment, the chemical composition and *in vitro* degradability of immature and mature forage of seven tropical legumes (*Calliandra calothyrsus*, *Clitoria fairchildiana*, *Desmodium ovalifolium*, *Flemingia macrophylla*, *Leucaena leucocephala*, *Leucaena macrophylla* and *Leucaena pallida*) were determined. Chemical composition varied greatly among the different samples. In turn, gas pool after 144 h of fermentation ranged from 66 to 140 ml and *in vitro* degradability ranged from 230 to 630 g kg^{-1} of dry matter. The concentration of condensed tannins was associated with a decrease in the initial rate, but not with the extent, of dry matter degradation. In particular, the concentration of condensed tannins had significant negative relationship with the maximum rate of gas production observed during the fermentation of these samples ($p \leq 0.0011$). Results from this experiment suggested that condensed tannins differ in nutritional effects and that factors other than condensed tannins affected the *in vitro* degradation of the legumes studied.

In a second set of experiments, condensed tannins purified from all tanniniferous legumes differed in molecular weight, (*number-average* molecular weight = 2360 to 4880 dalton). Condensed tannins also differed in monomeric composition, with some

being mostly composed of procyanidin (up to 86 %), while others were mostly composed of prodelphinidin (up to 87 %). Procyanidin content and molecular weight of condensed tannins were negatively associated with the maximum rate of gas production and with the gas accumulation observed in the first experiment, respectively ($P < 0.01$). Another set of gas production experiments showed that the ability of condensed tannins to inhibit the fermentation of substrates was greater when they were associated to the substrate than when they were present in soluble form. Condensed tannins from *L. leucocephala* were the least effective at inhibiting substrate fermentation, which was related to their low molecular weight. In a final series of experiments, condensed tannins from *L. leucocephala* were also among the least effective at inhibiting the activity of fibrolytic enzymes from the gut anaerobic fungi *Neocallimastix hurleyensis*. Results from this experiment also suggested that the observed degree of inhibition of a given enzyme by condensed tannins is also a function of characteristics of both the enzymes and the substrate they hydrolyse.

A final experiment investigated changes in chemical composition in five different accessions of the tropical legume *D. ovalifolium* grown under different environmental conditions (six sites, two fertilisation levels and two growth seasons) in Colombia, South America. There was considerable variation in chemical composition (condensed tannins included) in response to environmental variables. Likewise, the *in vitro* dry matter digestibility ranged from 250 to 559 g kg^{-1} of dry matter. Differences associated with genotype were comparatively smaller. Content of condensed tannins was inversely related to *in vitro* degradation. However, results from this experiment suggested that *in vitro* degradation of forages is a function of their entire chemical composition. Further studies showed that the chemical structure (molecular weight and monomeric composition) of condensed tannins changed significantly in response to the different environmental variables studied in this experiment. Changes in condensed structure were significantly related to *in vitro* degradability.

It was concluded that whereas condensed tannins in tropical legume could act as anti-nutritive factors, their nutritional impact is a function of their concentration in plant tissue, their association with plant tissue (i.e soluble or associated) and their structure. In addition, condensed tannin structure varies considerably according to plant species and in response to different environmental factors.

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1. INTRODUCTION: ANTI-NUTRITIVE COMPOUNDS IN TROPICAL FORAGE LEGUMES ADAPTED TO ACIDIC, LOW FERTILITY SOILS

A constraint to increasing milk and beef production in the tropics is the lack of suitable forage for the animals during the dry season (NAS, 1979; Schultze-Kraft, 1986). The use of protein-rich leguminous trees and shrubs as protein supplements for low protein, low quality grasses and agricultural by-products may help overcome this limitation (Brewbaker, 1986; Ranjhan, 1986). However, some of the better known, good-quality tropical shrub legumes such as *Leucaena leucocephala*, *Gliricidia sepium*, and *Erythrina poeppigiana* are not well adapted to acid soils (Perdomo, 1991; Sieffert 1992, see Table 1-1). This is a problem, particularly in acid-soil areas where many small and medium size farmers are dependent upon livestock agriculture as their main source of income (Saez, 1991). Thus, there is continued interest in the evaluation and selection of leguminous shrubs and trees adapted to acid soils and also with drought tolerance.

Table 1-1. *Dry matter yields of leguminous shrubs and trees grown in two contrasting soils in Colombia, South America.*

Legume	Accumulated yield, g/plant[1]		Quilichao:
	Quilichao[2]	Palmira[3]	Palmira ratio
Leucaena leucocephala	3	8822	0.0003
Gliricidia sepium	4	3667	0.0011
Erythrina poeppigiana	14	------	-------
Desmodium velutinum	41	1053	0.0389
Cratylia argentea	309	454	0.6806
Flemingia macrophylla	478	1406	0.3400

[1]Accumulated yield from three successive cuttings made at intervals of three months, following a standardisation cut made at the age of 5 months.

[2]Soils characteristics: Ultisol; pH 4.0; Aluminium saturation: 91%; P (Bray II): 5.3 ppm.

[3]Soils characteristics: Vertisol; pH 7.7; Na: 0.2 meq/100 g of soil; P (Bray II): 84.6 ppm.

Source: Perdomo (1991)

Many of the tropical legumes adapted to acid soils might have limited feeding value due to the presence of anti-quality factors. In particular, the presence of high levels of condensed tannins is a very common occurrence among these legumes. Condensed tannins are polyphenols with the ability to bind and precipitate proteins, carbohydrates

and other molecules (Mueller-Harvey and McAllan, 1992). In previous research, high dietary levels of condensed tannins in temperate legumes have been shown to depress intake and interfere with protein and cell wall digestion in ruminants (Barry and Duncan, 1984; Pritchard et al. 1988). Similarly, the presence of high levels of condensed tannins in tropical legumes has been associated with reduced forage quality (Perdomo, 1991, see Table 1-2) and with reduced intake and availability of nutrients in sheep (Carulla, 1994, Barahona et al. 1997).

Table 1-2. *Condensed tannin and crude protein content and* in vitro *dry matter digestibility (IVDMD) of six tropical legumes planted in an acid soil with high aluminium saturation in Colombia, South America.*

Legumes	Condensed tannin, catechin equivalents	Crude protein, % of DM	IVDMD, % of DM
Leucaena leucocephala	0.3	26.5	52.2
Gliricidia sepium	0.3	25.4	50.5
Erythrina poeppigiana	0.3	27.1	48.2
Cratylia argentea	0.2	23.5	48.1
Calliandra grandiflora	15.5	16.6	18.4
Flemingia macrophylla	35.9	17.5	22.3

Source: Perdomo (1991)

Previous research has suggested that condensed tannins from different legumes differ in many characteristics such as molecular weight (Jones et al. 1976; Oh and Hoff, 1979; Kumar and Horigome, 1986; Cano et al. 1994) and chemical structure and composition. Perhaps as a consequence of these differences, condensed tannins from different legumes also differ in astringency (the ability to precipitate protein from solution, as defined by Bathe-Smith, 1973a). Tannin stereochemistry could also affect the biological activity of condensed tannins. For example, despite being of similar average molecular weight, condensed tannins from bitterbrush (*Pursia tridentata*) were more astringent than those of blackbrush (*Coleogyne ramosissima*), perhaps due to differences in stereochemistry at C-3 and C-4 (Clausen et al. 1990).

It has also been shown that condensed tannins can either be in loose interaction with other molecules and as a result they can be easily extracted with the use of an organic solvent (extractable condensed tannin fraction) or they can be so tightly bound to other molecules so that they cannot be extracted even with powerful solvents such as 70%

aqueous acetone (bound condensed tannin fraction). Research with tropical legumes has shown that that the distribution of condensed tannins between these two fractions varies from legume to legume. For example, Jackson et al. (1996a) observed that content of bound condensed tannins, as a proportion of total tannin content, ranged from 0% in *Sena velutina* to 99.8% in *Gliricidia sepium* (see Table 1-3).

Table 1-3. *The distribution (as a proportion of total condensed tannin content) of extractable and bound condensed tannins from tropical legumes grown in South America and Northern Australia.*

Plant species	Total CT g/kg of DM	Extractable CT (% of total CT)	Bound CT (% of total CT)
Grown in South America			
Calliandra calothyrsus	155.4	84.9	15.1
Desmodium ovalifolium	191.5	82.0	18.0
Flemingia macrophylla	167.4	59.0	41.0
Senna velutina	54.0	100.0	0.0
Grown in Northern Australia			
Calliandra calothyrsus	63.9	86.9	13.1
Gliricidia sepium	37.2	0.3	99.8
Leucaena leucocephala	57.3	90.7	9.3
Leucaena pallida	66.0	69.2	30.8

Source: Jackson et al. (1996a)

Furthermore, condensed tannin concentration in plant tissue has been shown to vary with species, plant part, plant maturity, drought, CO_2 concentration and soil fertility (Foo et al., 1982; Barry and Forss, 1983; Barry, 1989; Anuraga et al., 1993, Carter et al., 1995). Additionally, the method of forage collection and handling may also have a significant effect on the values obtained (Chiquette et al., 1988; Ahn et al., 1989; Terrill et al., 1989; Cano et al., 1994; Barahona et al., 1996).

Condensed tannin activity is a function of tannin concentration in plant tissue and tannin structure. Structure-activity relationships are important in defining the nutritional impact of condensed tannins. At present, it is difficult to predict the extent of the nutritional impact that condensed tannins from a given legume will have when fed to ruminant livestock. Adequate understanding of the mechanisms by which some tannins elicit depressions in intake and digestibility in ruminants is required for the development of screening procedures aimed to identify high quality leguminous shrubs and trees adapted

to acid soils. Such knowledge would also be extremely useful to develop appropriate feeding strategies for tanniniferous legumes.

The work reported in this thesis was aimed to provide insight into some of the key mechanistic factors that influence the nutritional impact on condensed tannins from tropical legumes. To that end, the bulk of the work was conducted using stem-free samples of mature and immature leaves from seven tropical legumes (*Desmodium ovalifolium, Flemingia macrophylla, Calliandra calothyrsus, Clitoria fairchildiana, Leucaena Leucocephala, L. pallida* and *L. macrophylla*). It is not an intention of this study rank these legumes in terms of their forage quality. Rather, the legumes were selected to provide a unique set of measurable characteristics in order to try to explain differences in forage quality in light of differences in these characteristics.

Additionally, a null hypothesis was assumed with regard to the effects of tannins on forage digestion. Thus, unless the converse was demonstrated by experimentation, condensed tannins and other secondary metabolites from tanniniferous legumes were assumed to have no effect(s), either positive or negative, on forage digestion. Another distinction was made between nutritional and microbial and enzymatic effects. This approach was considered necessary to enhance the scientific rigor of this study and to avoid the often-made assumption that all tannins have anti-nutritional effects in ruminant feeding systems. The three main components of this study are:

Objective 1: To determine the effects of chemical composition on the fermentation kinetics of a range of tropical forage legumes.

The chemical composition of leaves from the seven tropical legumes listed above was determined and related to the *in vitro* degradation of leaf dry matter as determined by gas production experiments and by the technique of Tilley and Terry (1963). Determinations included non-starch polysaccharides, amino acid composition and condensed tannin content.

Objective 2: To study structure-activity relationships in condensed tannins from a range of tropical forage legumes.

Condensed tannins from leaves of tropical legumes were isolated in bulk and purified for analysis of tannin structure including molecular weight determination and proanthocyanidin ratios. The effects of the purified tannins on the fermentation

characteristics of non-lignified primary cell walls from cell suspension cultures of grasses were evaluated, together with other biological properties including concentration effects on condensed tannin binding to cell walls. Additionally, the activity of enzymes collected from rumen anaerobic fungal cultures was investigated in the presence or absence of condensed tannins extracted from the range of tropical legumes used in objective 1. In addition the effect of condensed tannins on the digestion of non-lignified primary cell walls (from cell suspension culture) by cellulolytic and hemicellulolytic enzymes was investigated.

Objective 3: To study the effects of plant development under different environmental conditions on the production of secondary metabolites.

This part of the work concentrated on determining the environmental and genotypical effects on the condensed tannins of *Desmodium ovalifolium.* This investigative effort was aimed at establishing structure-activity relationships for these secondary metabolites, as well as complementing other research conducted to define agronomic practice, climatic restrictions and feeding protocols for this tropical legume.

Chapter Two of this thesis describes ruminant production in the tropics in the face of tannins, and pays particular attention to those aspects of tannin chemistry and nutritional effects of condensed tannins that might be of relevance to this particular study. Chapters Three to Five describe experimental work undertaken under the objectives listed above. In concluding this study, Chapter Six discusses and integrates the findings of these experiments.

2. THE INFLUENCE OF CONDENSED TANNINS ON RUMINANT PRODUCTION IN TROPICAL AREAS

2.1 THE USE OF LEGUMINOUS SHRUBS AND TREES AS FORAGES IN TROPICAL RUMINANT PRODUCTION SYSTEMS

2.1.1 Introduction

In the tropics, the efficiency of forage-based ruminant production systems is limited by forage quality and forage quantity during the dry season (NAS, 1979). In most tropical regions, native grasses from permanent pastures constitute the most important feed resource for ruminants. Unfertilised and unmanaged native grasses generally have poor nutritive value: 2.5-7.0 % crude protein (dry matter basis) and low dry matter digestibility (40-50 %, McIvor and Chen, 1986; Ranjhan, 1986).

Table 2-1. *Percentage of different tissues in cross-sectional area of the lamina of tropical and temperate grasses.*

Tissue	Tropical (C_4)	Temperate (C_3)	Relative tissue digestibility
Epidermis	33.0 ± 2.1	28.7 ± 1.5	Intermediate
Mesophyll	34.6 ± 2.4	61.0	High
Bundle sheath	19.3 ± 2.1	------	Low
Vascular bundle	7.6 ± 0.4	5.5 ± 0.4	Low
Sclerenchyma	5.0 ± 1.1	3.4 ± 0.4	Low

Adapted from Humphreys (1991).

During the dry season, which ranges from two to six months, the dry matter availability from these pastures diminishes dramatically. Furthermore, the low levels of crude protein and minerals in tropical grasses tend to decline rapidly during the dry season. As a result, cattle lose weight and milk production drops (NAS, 1979; Ranjhan 1986; Schultze-Kraft, 1986).

During the wet season, the protein level in most grasses is lower (< 7 %, dry matter basis) than the level necessary for adequate animal growth (McIvor and Chen, 1986). In addition, the low (< 50 %) digestibility of tropical grasses can limit animal production, being on average 13 % less digestible than temperate grasses (Minson and McLeod, 1970). This has been related to differences in anatomical structures (Table 2-1)

associated with the different photosynthetic pathways and the higher temperatures at which tropical grasses are normally grown (Minson, 1990; Humphreys, 1991). Rapid maturation, decreased leaf-stem ratio, and increased production of vascular tissue as the season progresses are also factors associated with the lower digestibility of tropical grasses (Humphreys, 1991).

Table 2-2. *Benefits of the use of legumes as supplements for ruminants fed a variety of basal diets*

Animal species	Basal diet	Legume supplement	Significant increase vs. basal diet
(i) Cattle	*Digitaria decumbens*	*Leucaena leucocephala*	Milk production
(ii) Cattle	*Cynodon pleystostachius*	*Leucaena leucocephala*	Milk production, liveweight gain
(iii) Cattle	African star grass + concentrate	Grass + *Erythrina poeppigiana*	Similar net profits in milk production
(iv) Cattle	African star grass	*Arachis pintoi*	Milk production
(v) Goats	Napier grass	*Leucaena leucocephala*	Liveweight gain
(vi) Goats	King grass	*Erythrina poeppigiana*	Milk production

Sources: (i) Suarez et al. (1987); (ii) Saucedo et al. (1980); (iii) Abarca (1989); (iv) Gonzalez et al. 1996; (v) Devendra (1982); (vi) Esnaloa and Rios (1986)

2.1.2 Benefits of the incorporation of leguminous shrubs and trees into tropical ruminant production systems

Although not yet fully exploited, the strategy of incorporating forage legumes into feeding schemes such as cut and carry systems or protein banks has enormous potential towards solving the severe nutritional limitations that ruminants face within tropical production systems (Ranjhan, 1986; Devendra, 1990). Indeed, improved animal performance (see Table 2-2) has been frequently reported in response to the use of high quality forages such as *Leucaena leucocephala* and *Gliricidia sepium* as supplements for ruminants fed low-quality roughage diets. Perhaps, the main reason for this is that the protein content of these legumes in usually high (12-30 %) compared with mature tropical grass (3-10 %). Another reason for this is because, at the same level of digestibility, intakes for legumes are usually greater than those observed for grasses. This is associated with shorter retention of legume material in the rumen and a greater packing density for legume forage in the rumen (Humphreys, 1991). Furthermore because they are deep rooting, the extent of nutrient decline with maturation is less in

legumes than in grasses (McIvor and Chen, 1986) and legumes are able to retain green leaves during the dry season (NAS, 1979).

There many other advantages to the use of legumes as supplements for low quality diets in smallholder production systems, as summarised by Devendra (1990):

- Availability on the farm
- Accessibility
- Provision of variety in the diet
- Reduction in the requirements of purchased concentrates
- Reduced cost of feeding
- Improvement of soil fertility due to the nitrogen fixation ability of legumes (Brewbaker, 1986), which results in higher quality of the grass in grass-legume mixed swards

Much attention has been focused on herbaceous and shrub legumes due to their nitrogen fixing ability and the high protein content of the edible forage (Brewbaker, 1986). Species from many genera (*Leucaena, Acacia, Desmanthus, Gliricidia, Calliandra, Prosopis, Erythrina, Desmodium, Flemingia, Senna, Centrosema, Cratylia, Stylosanthes, Zornia,* and *Arachis)* have been evaluated (NAS, 1979, 1983, 1984; Brewbaker 1986; Schultze-Kraft, 1986; Pezo et al. 1990).

Among these genera, *Leucaena leucocephala* is by far the most commonly cultivated species of tree legumes. It is used in subsistence farming around the world, where livestock is raised in small numbers by smallholder farmers, with trees being commonly planted in hedgerows or on unused land as protein banks. Supplementation of grasses or crop residues with *Leucaena sp.* and *Gliricidia sp.* has proved to be an effective way of improving animal gain and/or milk production (Devendra, 1990). Reports by Izham et al. (1982) and Jones and Jones (1984) showed the beneficial effects of *Leucaena leucocephala* when used as protein banks for liveweight gain by cattle. Likewise, Saucedo et al. (1980) and Suarez et al. (1987) have reported large responses in milk yield with cows supplemented with *L. leucocephala*. Reports from Kass and Abarca (1988) and from Vargas et al. (1988) have shown that *Erythrina spp*. can be an effective

protein supplement for animals fed low-quality roughage diets, resulting in increased milk production and liveweight gain.

2.1.3 Leguminous shrubs and trees for acidic, low fertility soils in the tropics

Most of the well-known tropical shrub legumes are not particularly well adapted to the acid-infertile soils prevalent in many areas with large cattle inventories. For example, in the acid soils of Quilichao, Colombia, Perdomo (1991) found that dry matter yields of *G. sepium*, *E. poeppigiana* and *L. leucocephala* were extremely low in successive cuttings (see Table 1-1). Additionally, Saez (1991) reported the need for heavy liming for the successful establishment of *L. leucocephala* in the acid soils prevalent in the Cerrados of Brazil. Likewise, Sieffert (1982) reported no increases in liveweight gain in response to the small amounts of forage provided by *L. leucocephala* grown in those acid soils.

Recognition of the lack of suitable forage legume species for acidic soils in some of the major cattle producing areas of the tropics has focused attention on the need for collection and evaluation of new germplasm. Early results from the Centro Internacional de Agricultura Tropical (CIAT) in Cali, Colombia, showed that some shrub legumes perform extremely well in acid soils with high aluminium saturation: (*Flemingia macrophylla* Kuntze ex Merrill, *Cratylia argentea* (Desvaux) Kuntze and *Calliandra grandiflora* (L'Her) Bentham; Perdomo, 1991). Since then several other legumes with good agronomic performance in acid soils have been identified. Some of these legumes such as *Arachis pintoi* and *Cratylia argentea* have received a great deal of attention by researchers in South America.

However, none of these legumes is problem-free. In the case of *A. pintoi*, for example, despite being an outstanding legume in terms of agronomic performance and forage quality, adoption by farmers has been low. This is due to high establishment costs for this legume, associated with the high seed requirement (4 kg of seed/hectare). Seed expenditures of about 100 US dollars/hectare are needed for the establishment of *A. pintoi*, and this amount is well beyond the reach of most small and medium size farmers in less developed countries.

In the case of *C. argentea,* Aroeira and Xavier (1991) recorded a voluntary intake of only 1.8% of liveweight, which is low compared to the reported intake of such legumes as *C. calothyrsus*, *G. sepium* (2.6 % of liveweight) and *L. leucocephala* (3.2% of

liveweight; Norton, 1994). Moreover, Raaflaub and Lascano (1995) showed that intake of immature *C. argentea* forage was significantly lower than that of the mature forage (Table 2-3). It is probable that the low acceptability of the immature forage is related to the presence of non-protein amino acids in young leaves of *C. argentea*, as exceptionally high levels of crude protein (42%) were observed in the immature forage (CNPGL-EMBRAPA, Brazil). Additionally, the condensed tannin content in young leaves of five different accessions of *C. argentea* was higher (up to 2.5 times) than in mature leaves (R. Barahona and A. Brooks, personal communication).

Table 2-3. *Effect of the post-harvest treatment of leaves from* Cratylia argentea *on intake by sheep housed in metabolic crates*.

Type of forage	Intake of dry matter, g/hour	
	Immature forage	Mature forage
Fresh forage	84	291
Wilted for 24 hours	157	376
Wilted for 48 hours	183	---
Sun-dried	160	359

[2]Intake measured in 20-minute periods.

Source: Raaflaub and Lascano (1995).

Table 2.4. *Voluntary intake, dry matter (DM), neutral detergent fibre (NDF) and nitrogen (N) digestibility and N absorption by sheep fed a low quality grass supplemented with different levels of* Cratylia argentea.

Experiment:	Level of supplementation, % of DM	DM intake, g/animal/day	Digestibility, g/kg of constituent			Absorption of N, g/kg of N intake
			DM	NDF	N	
Fässler[1]	0	457	447	523	308	----
	50	476	438	490	617	----
Quiñonez [2]	0	818	576	637	581	4.8
	10	899	557	601	605	6.1
	20	924	551	586	634	7.3
	40	945	530	543	659	8.2

[1]Fässler and Lascano (1995); sun-dried *C. argentea* offered as a pure supplement during the morning feeding only.

[2]Wilson Quiñonez, pre-graduate thesis in Lascano (1996); fresh and mature *C. argentea* offered mixed with the grass during the morning and afternoon feeding.

Yet another characteristic that might limit the intake of *C. argentea* is its high content of indigestible neutral detergent fibre. Thus, even when the substitution *Brachiaria dictyoneura* with *C. argentea* resulted in higher intakes, dry matter and neutral detergent fibre digestibility of the mixed diet decreased almost linearly as the proportion of *C. argentea* in the diet increased (Fässler and Lascano. 1995; W. Quiñonez as cited by Lascano, 1996; Table 2-4). Another characteristic that might limit the use of *C. argentea* as a forage, it that the protein from *C. argentea* is rapidly degraded by rumen microorganisms. Although this results in high ammonia concentrations in the rumen which might be beneficial in terms of the degradation of fibre (Lascano, 1996), it would also lead to wasteful usage of rumen available protein, especially when *C. argentea* is fed in combination with slowly-degradable roughages. This could be related to lack of synchrony between the release of protein and energy within the rumen.

Table 2-5. *Effect of the supplementation with* Cratylia argentea *and other legumes on milk production by cows grazing either* Brachiaria dictyoneura *or* Brachiaria decumbens *pastures*.

Pasture	Legume supplement	Legume offered[1]	Legume intake[1]	Milk production, kg/cow
Dry season:				
B. dictyoneura	Control	---	---	6.2
	F. macrophylla	8.4	3.0	6.4
	C. argentea	8.1	6.3	7.0
Wet season:				
B. dictyoneura	Control	---	---	6.8
	C. macrocarpum	13.5	10.5	7.0
	C. argentea	11.3	7.8	6.9
B. decumbens	Control	---	---	9.3
	C. macrocarpum	11.4	8.1	10.1
	C. argentea	11.3	7.8	10.0

[1]Expressed in g of forage dry matter per kg of liveweight per day.

Source: Lascano (1996).

However, as depicted in table 2-5, the use of *C. argentea* as a supplement for dairy cows can have beneficial effects in terms of milk production (Lascano, 1996). This benefit, however, was confined to the dry season, exemplifying that during this period production of animals raised in tropical systems is limited due to nutrient deficiencies in their diets. Table 2-5 also shows that supplementation with *C. argentea* resulted in similar dry matter intakes and production of milk as those observed with the high quality

legume *Centrosema macrocarpum*. Results in this experiment suggest a possible loss of rumen ammonia in these animals, due to the lack of an adequate energy supply for the synthesis on microbial protein.

Table 2-6. *The condensed tannin content of some tropical legumes with good adaptation to acidic soils*.

Legume Species	Total condensed tannin, g/kg of dry matter	Method of condensed tannin determination	Source
Acacia mangium	100.4	Butanol-HCl[1]	Jackson et al. (1996a)
Arachis pintoi	33.6	Butanol-HCl[1]	Jackson et al. (1996a)
Calliandra sp.	125.7	Butanol-HCl[1]	Longland et al.(1995)
Calliandra sp.	155.4	Butanol-HCl[1]	Jackson et al. (1996a)
Cassia sp.	93.2	Butanol-HCl[1]	Jackson et al. (1996a)
Codariocalyx gyroides	97.0	Folin-Dennis[2]	Lascano and Carulla, (1992)
Cratylia argentea	19.0	Folin-Dennis[2]	Lascano and Carulla, (1992)
Dendrolobium sp.	95.0	Butanol-HCl[3]	Lascano et al. (1995)
Desmodium ovalifolium	48.0	Butanol-HCl[1]	Cano et al. (1994)
Desmodium ovalifolium	191.5	Butanol-HCl[1]	Jackson et al. (1996a)
Desmodium velutinum	15.0	Folin-Dennis[2]	Lascano and Carulla, (1992)
Dioclea guianensis	132.7	Butanol-HCl[1]	Longland et al.(1995)
Dioclea guianensis	256.0	Butanol-HCl[1]	Cano et al. (1994)
Flemingia macrophylla	373.0	Butanol-HCl[1]	Cano et al. (1994)
Flemingia macrophylla	83.0	Folin-Dennis[2]	Lascano and Carulla, (1992)
Flemingia macrophylla	154.0	Butanol-HCl[3]	Lascano et al. (1995)
Flemingia macrophylla	166.8	Butanol-HCl[1]	Longland et al.(1995)
Flemingia macrophylla	167.3	Butanol-HCl[1]	Jackson et al. (1996a)
Phyllodium sp.	192.5	Butanol-HCl[1]	Cano et al. (1994)
Senna velutina	54.0	Butanol-HCl[1]	Jackson et al. (1996a)
Tadehagi sp.	160.0	Butanol-HCl[1]	Cano et al. (1994)
Tadehagi sp.	183.0	Folin-Dennis[2]	Lascano and Carulla, (1992)
Tadehagi sp.	230.0	Butanol-HCl[3]	Lascano et al. (1995)
Tadehagi sp.	161.0	Butanol-HCl[1]	Longland et al.(1995)
Tadehagi triquetrum	145.6	Butanol-HCl[1]	Jackson et al. (1996a)

[1]Tannin determination by the method of Terrill et al. (1992b).
[2]Determination of total phenols by the method of Swain and Hills (1959).
[3]Determination of tannins by the method of Porter et al. (1986).

2.1.4 Tanniniferous shrub and tree legumes adapted to acidic, low fertility soils in the tropics

Most of the leguminous shrubs and trees adapted to acid soils have been shown to have high levels of condensed tannins (Lascano and Carulla, 1992; Cano et al. 1994, Lascano et al. 1995; Jackson et al. 1996a, see Table 2-6). The presence of high levels of condensed tannins in tropical legumes might limit their use as forages. High levels of condensed tannins in temperate legumes have been associated with reductions on forage intake, dry matter digestibility, and nitrogen utilisation by ruminants (Donnelly and Anthony, 1969, 1970; Reed et al. 1982, Barry and Duncan, 1984; Barry and Manley, 1984; Pritchard et al. 1988). On the other hand, low levels of condense tannins in temperate legumes are considered beneficial in terms of protein utilisation (Driedger and Hatfield, 1972; Waghorn et al. 1987; Wang et al. 1994) and bloat prevention (Jones and Mangan, 1977; Barry, 1989). These latter results are explained by the fact that condensed tannins form complexes with dietary proteins and that these complexes are not of an irreversible nature. Work by Jones and Mangan (1977) demonstrated that the condensed tannin-protein complex is stable and insoluble at pH 3.5-7.0, but dissociates and releases protein at pH < 3.5.

Desmodium ovalifolium is a herbaceous legume that has been studied by researchers in grass-legume associations (CIAT 1983; 1984 and 1985) given that it has demonstrated a good nitrogen-fixation capability (CIAT, 1981), is shade-tolerant (Suarez, 1985) and it grows well in acid, low fertility soils (Grof, 1982). When grown in association with very aggressive grasses, such as those from the genera *Brachiaria* and *Cynodon*, *D. ovalifolium* was very persistent, even under high stocking rates (Gonzalez et al. 1996). Lascano et al. (1991) attributed this to the poor palatability of *D. ovalifolium*, which in turn, has been associated with the presence of high levels of condensed tannins in this legume (Lascano and Salinas, 1982; Carulla, 1994; Lascano et al. 1995). Perhaps this explains why cows grazing *Cynodon nlemfuensis-D. ovalifolium* pastures did not show greater milk production than those grazing *C. nlemfuensis*-only pastures (Gonzalez et al. 1996). Likewise, the presence of *D. ovalifolium* in association with *Brachiaria dictyoneura* did not contribute to the liveweight gains of cattle (Vela Alvarado and Flores Mere, 1996).

It should be recognised that most of the nutritional work with *D. ovalifolium* has been made using two accessions (CIAT numbers 350 and 13089). The world-wide collection, with 160 accessions, has a much broader genetic base, although many of them are morphologically very similar (Schultze-Kraft, 1997). Additionally, it has been observed that fertilisation, especially with sulphur, improves the nutritive quality of *D. ovalifolium* (Salinas and Lascano, 1983; Pérez, 1997; Table 2-7). Furthermore, Schultze-Kraft and Benavides (1988) observed great variation in the condensed tannin content among accessions grown under the same environmental conditions, all of which is indicative of the existence of genotype-environment interactions in *D. ovalifolium* (Schmidt et al. 1997). Changes in *D. ovalifolium* quality in response to different growth environments might explain why in the Colombian Orinoquia, the association *Brachiaria-D. ovalifolium* resulted in improved animal performance (Table 2-7; Pérez, 1997).

Table 2-7. *Liveweight gains by steers grazing* Brachiaria (B.) *pastures with or without* Desmodium ovalifolium (D.o.) *at 15% or less of the total pasture.*

Pasture	Liveweight gain			
	kilogram/animal/day		kilogram/year	
	Dry season	Wet season	/animal	/hectare
B. decumbens	0.160	0.520	135	405
B. decumbens + *D.o.*	0.440	0.680	171	513
B. decumbens + *D.o.* + sulphur[1]	0.540	0.670	187	561
B. humidicola	0.230	----	----	----
B. humidicola + *D.o.*	0.418	----	----	----
B. dictyoneura + *D.o.*	0.448	----	----	----

[1]Application of 20 kg of sulphur per hectare at pasture establishment.

Source: Pérez (1997).

The herbaceous legume *D. ovalifolium* and the shrub legume *Flemingia macrophylla* have very high condensed tannin content and are therefore used as models to study the nutritional effects of condensed tannin in tropical legumes. Work by Carulla (1994) showed that reduction of solvent-extractable condensed tannins from forage of *D. ovalifolium* (CIAT 350) by the use of polyethylene glycol (PEG) resulted in an increase in dry matter intake by sheep. Additionally, in this same study, the presence of high levels of condensed tannins in *D. ovalifolium* was related to increased nitrogen flow and absorption from the small intestine of sheep, as has been shown to occur with temperate legumes (Barry et al., 1986, Waghorn et al., 1987). Reduction of extractable condensed

tannin content from *F. macrophylla* and *D. ovalifolium* CIAT (350) using PEG resulted in increases of dry matter intake of 11 and 6.5 %, respectively (Barahona et al. 1997). Additionally, reduction of extractable condensed tannin content was also associated with an increase in the rumen digestibility of protein, dry matter, neutral and acid detergent fibre.

Thus, it appears that under some conditions, condensed tannin in tropical legumes can have negative effects on the nutritional status of ruminants. Indeed, work conducted with tropical shrub legumes selected for acid soils has shown a negative correlation between tannin content and *in vitro* dry matter digestibility (IVDMD; Lascano and Carulla; 1992, Lascano et al. 1995) and *in vitro* gas accumulation (Longland et al. 1994).

However, there are difficulties to the elucidation of the true role played by condensed tannins in the nutritive value of tropical forage legumes. This is because, in addition to condensed tannins, other secondary products in plants can limit their degradation in the rumen. Indeed, Barahona et al. (1997) suggested that when evaluating the quality of tanniniferous tropical legumes there is the need to examine the full array of chemical constituents. Additionally, as condensed tannins from different plants species are likely to differ greatly in structure, there is a strong likelihood that their biological activity would also differ accordingly. Thus, it would be ideal to include structure-activity considerations when evaluating the nutritional impact of condensed tannins.

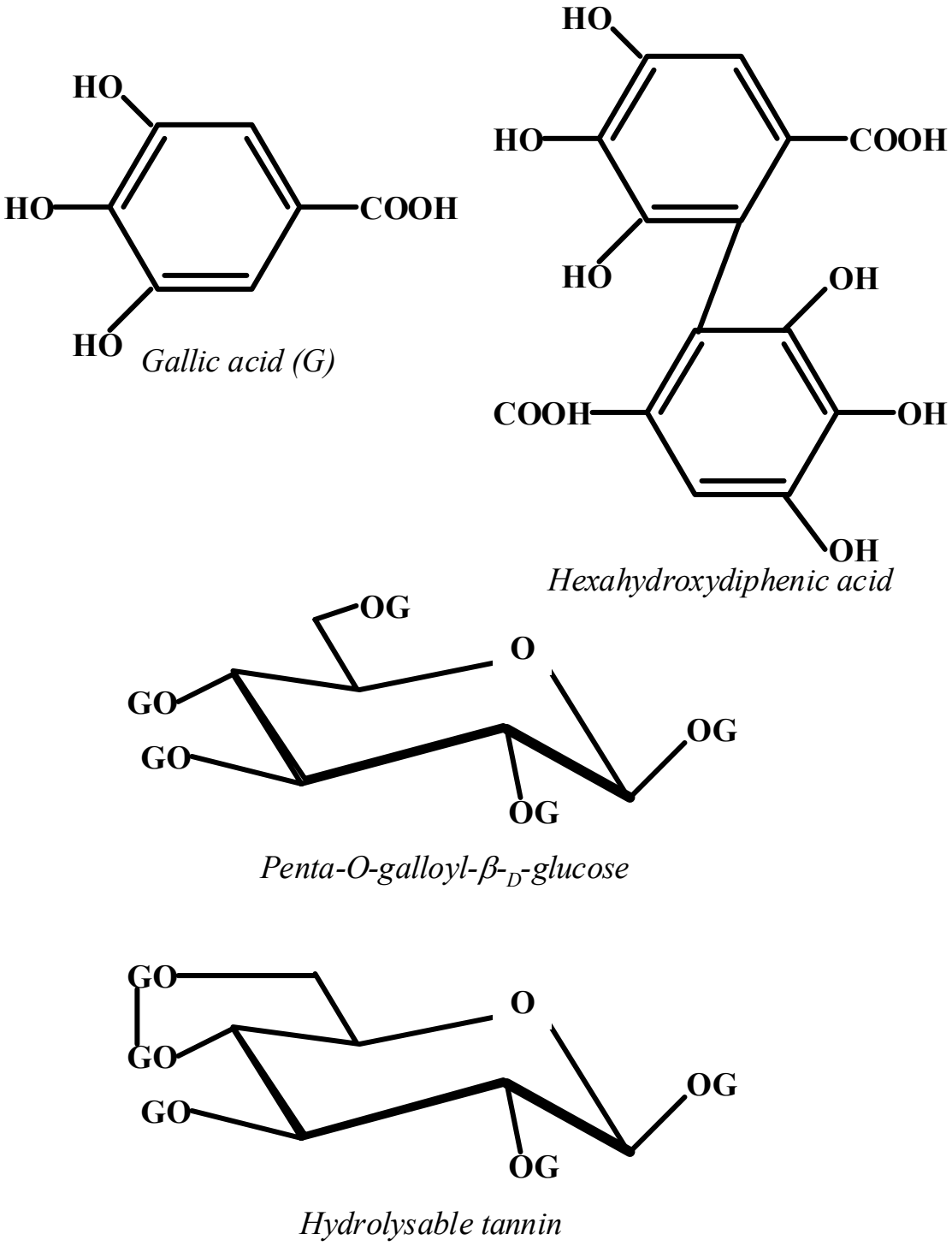


Figure 2-1. *An example of hydrolysable tannins and related compounds; G = gallic acid. Adapted from Mangan (1988) and Mueller-Harvey and McAllan (1992).*

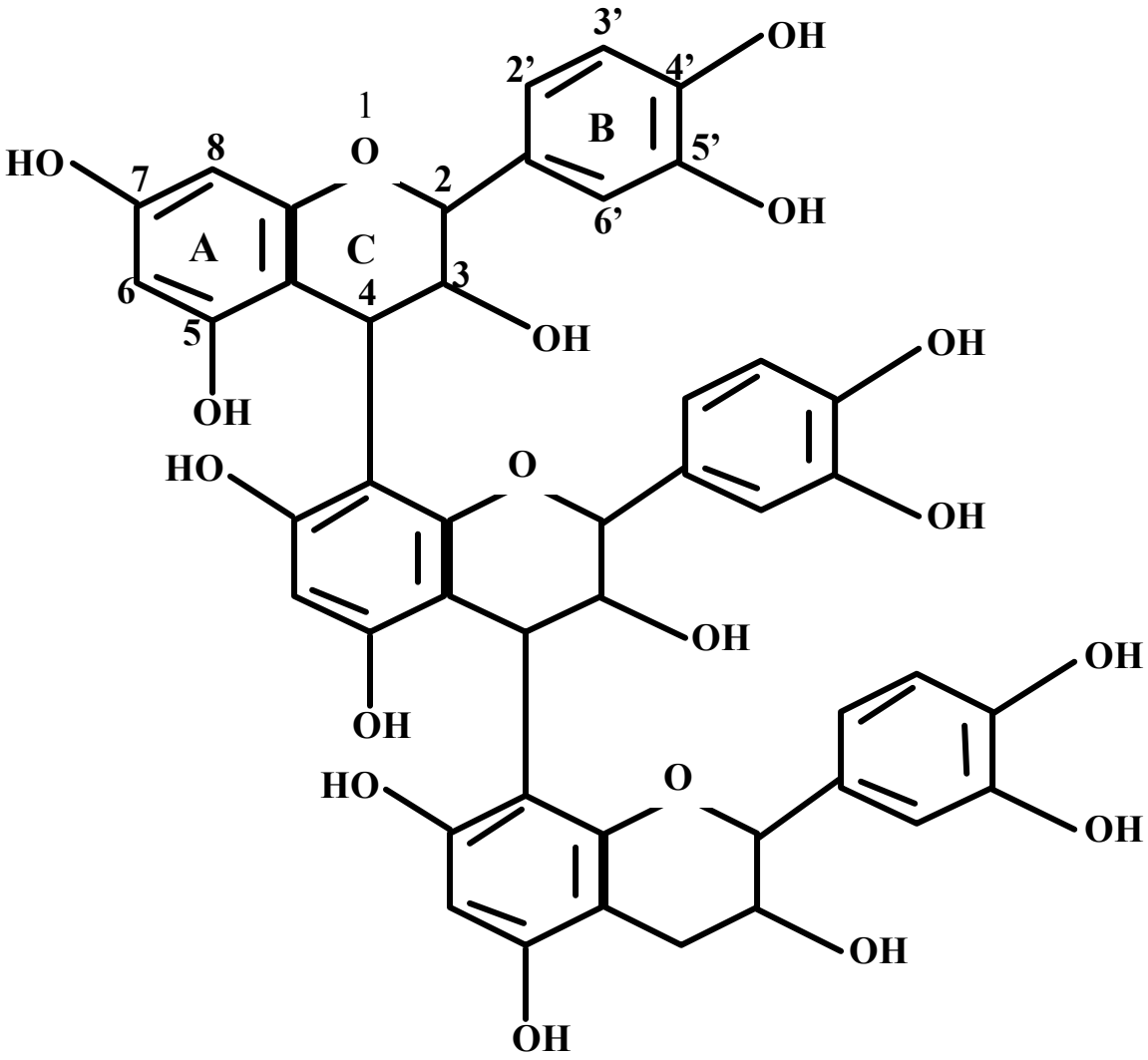


Figure 2-2. *An example of a condensed tannin. Adapted from Mueller-Harvey and McAllan (1992)*

2.2 CONDENSED TANNINS

2.2.1 Definition of tannins

Plant tannins are ubiquitous in nature and although a lot of attention has been given to their study, the term "tannin" continues to be difficult to define precisely. Indeed, whereas related phenolic compounds such as simple phenolics, neolignans and flavonoids are characterised and classified according to their chemical structure, tannins are a diverse group of compounds that are related primarily in their ability to complex with proteins (Fahey and Jung, 1990). Thus, tannins are usually defined as water-soluble polyphenolic substances that have high molecular weight and that possess the ability to precipitate proteins. Furthermore, researchers proposed that the molecular weight of tannins should lie between 500 to 3000, permitting the tannin molecule to easily orientate itself between the protein chains, but having sufficient phenolic groups to crosslink efficiently with protein (Goldstein and Swain, 1963; McLeod, 1974). However, the definition of tannins is regularly modified in the light of new findings (Mueller-Harvey and McAllan, 1992). As a consequence, the list of polymers bound by tannins has been extended to include polysaccharides (cellulose, hemicellulose and pectin) and nucleic acids, steroids, alkaloids, and saponins (Haslam, 1986). While this working definition is useful in describing some chemical and physical characteristics of tannins, it is nevertheless vague and could obfuscate the research on tannins (Ayres et al. 1997).

Although tannins are chemically a diverse and ill-defined group it is usual to divide them into two main classes: (a) the hydrolysable and (b) the condensed tannins (Mangan, 1988). *Hydrolysable tannins* are polyesters of phenolic acids such as gallic acid, *m*-digallic acid (gallotannins) or hexahydroxydiphenic acid (ellagitannins) and D-glucose or quinic acid, the latter serving as a polyalcohol core (Mueller-Harvey and McAllan, 1992; see Figure 2-1). *Hydrolysable tannins* receive their name because they are readily cleaved by enzymes (i.e. *Penicillium* tanninase; EC 3.1.1.20) as well as by dilute acid to give a sugar such as glucose and a phenolcarboxylic acid such as gallic acid (Strumeyer and Malin, 1975). On the other hand, *condensed tannins* are composed of flavan-3-ols linked via carbon-carbon bonds that produce anthocyanidins upon treatment with acidic alcohol (Porter et al. 1986; see Figure 2-2). However, some newly identified compounds such as the phlobatannins, flavanol glycosides and flavanol gallates do not fit into either of these two categories (Mueller-Harvey and McAllan, 1992).

2.2.2 Occurrence of tannins

Tannins occur commonly in fodder shrubs and trees. A survey by Bate-Smith and Lerner (1954) showed the distribution of condensed tannins in over 500 species of plants. Likewise, Bate-Smith and Metcalf (1957) observed that 80% of woody perennial dicots and 15% of annual and herbaceous dicots contain tannins. Other surveys of the distribution of tannins have shown that tannins occur commonly in legumes (Bate-Smith, 1973b, 1975, 1977, 1978, 1981; Jones et al. 1973; Marshall et al. 1979; Foo et al. 1982). In a review article, Kumar and Vaithiyanathan (1990) reported the existence of tannins in the leaves of 46 different species of fodder trees and stated that generally tree leaves and browse contain both types of tannins (hydrolysable and condensed).

In what refers to the occurrence of tannins in tropical shrubs and trees, Rittner and Reed (1991) reported the presence of proanthocyanidins (i.e. condensed tannins) in 72 West African fodder trees. Work by Perdomo (1991), Lascano and Carulla, 1992, Cano et al. 1994, Longland et al. 1994, Lascano et al. 1995 and Jackson et al. (1996a) suggests that condensed tannins commonly occur in tropical legumes, and at very high concentrations, especially in those legumes adapted to acid soils (see Table 2-6).

According to Carulla (1994) the most common type of tannin found in the tropical legume collection of CIAT is of the condensed type. Even where hydrolysable tannins are present, the high concentration of condensed tannins in these legumes warrants further investigation of their impact when fed to ruminants.

2.2.3 Condensed tannin biosynthesis

Tannins are the products of secondary metabolism of plants. Gottlieb (1990) provided an excellent and succinct summary of plant metabolism when he stated: "The basic metabolism of autotrophic plants combines photosynthesis with respiration, leading from CO_2 via the sugars of the Calvin cycle, pyruvic acid and acetic acid either to the fatty acids of the Lynen spiral (a reversible process) or to the simple aliphatic acids of the Krebs cycle and thence back to CO_2. Connected by mostly reversible pathways are some essential intermediates such as the Krebs-cycle-derived aliphatic amino acids, the purines and the pyrimidines, the acetic acid-derived mevalonic acid, the sugar-derived glycerol and the sugar-plus pyruvic acid-derived shikimic acid, the latter functioning as a precursor to the aromatic amino acids."

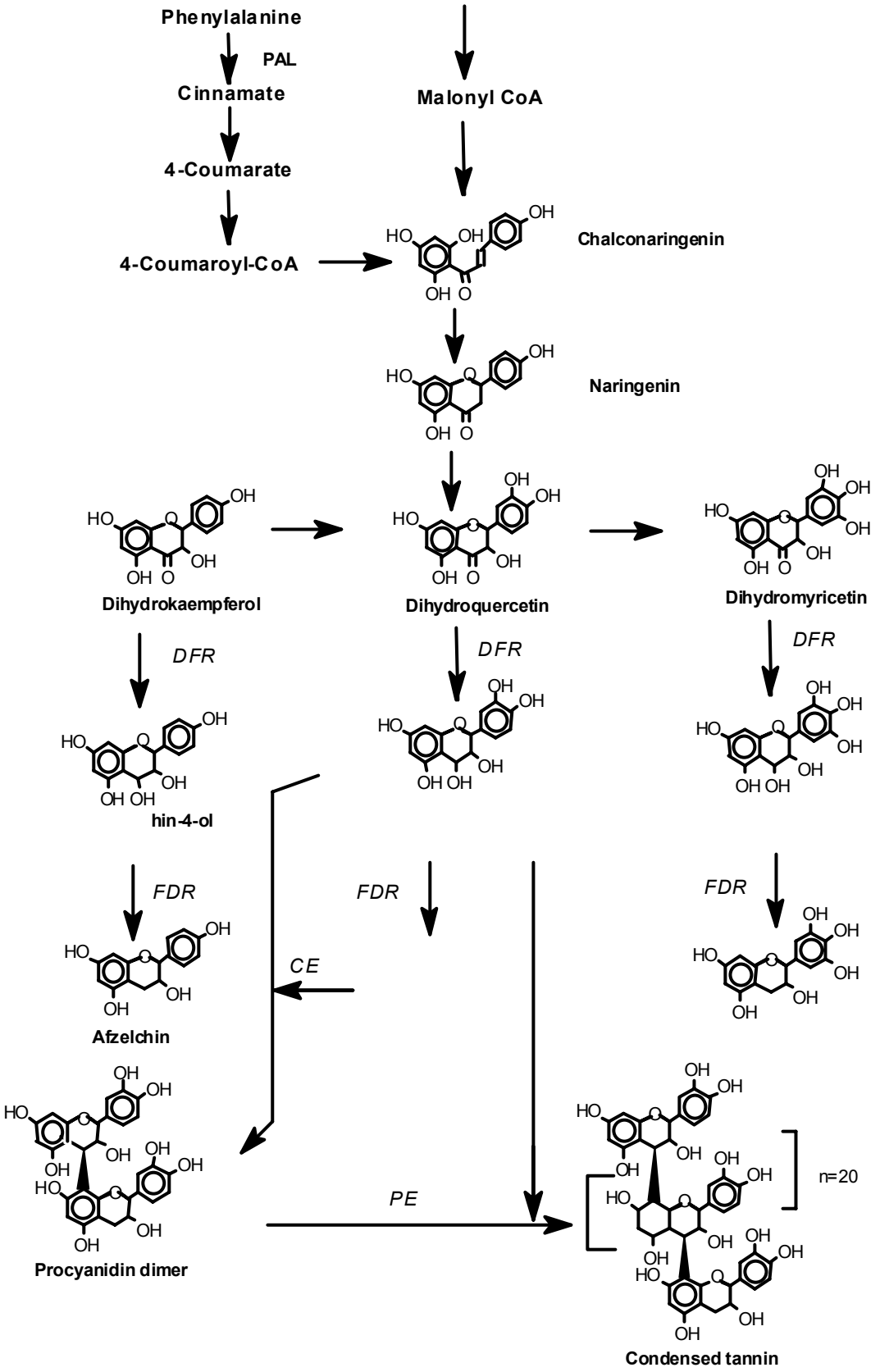


Figure 2-3. *Generalised biosynthetic pathway to condensed tannins in forage legumes. PAL, phenylalanine ammonia lyase; CoA, coenzyme A; CHS, Chalcone synthase; CHI, chalcone isomerase; F3OH, (2S)-flavanone-3-hydroxylase; F3'OH, flavanoid-3'-hydrolxylase; F3'5'OH, flavanoid-3'5'-hydrolxylase; DFR, dihydroflavanol reductase; FDR, flavan-3.4-cis-diol-4-reductase; CE, condensing enzyme; PE, polymerising enzyme. Source: Morris and Robbins (1997).*

"Basic and intermediary (jointly designed primary) metabolism forms an integrated system, a first metabolic division, which extends branches into two further broad divisions of metabolites, a general one and a special (usually called secondary) one. General metabolism transforms primary metabolites into macromolecules, particularly amino acids into proteins, purines and pyrimidines plus sugars into nucleic acids, sugars into polysaccharides and glycerol plus fatty acids into lipids. Special metabolism can be separated into two subdivisions: a herbaceous one and a woody one. The woody subdivision comprises shikimic acid-derived lignoids and benzylisoquinoline alkaloids, as well as classes of mixed biosynthetic origin such as the indole alkaloids (shikimic acid plus mevalonic acid) and the *flavonoids* synthesised from shikimic acid and acetic acid".

Indeed, the shikimic acid and acetate-malonate pathways are the major metabolic routes of polyphenolic synthesis in plants (Van Soest, 1982; Jung and Fahey, 1983). According to Heller and Forkmann (1988), two precursors are necessary for flavonoid synthesis, acetate and phenylalanine (See Figure 2-3). While the A ring carbons (see Fig. 2-2) are derived from three acetate units, the B and C ring carbons originate from phenylalanine. For the synthesis of condensed tannins, a flavan-3,4,-diol (i.e. leucopelargonidin in Figure 2-3) is one of the immediate precursors and the other is usually a flavan-3-ol acting as a nucleophile (Stafford, 1990). A similar pathway was suggested earlier by Haslam (1977), but with the minor change of having cinnamate as a precursor instead of phenylalanine (both these compounds are derived from the shikimic pathway). In tracer studies with a range of fruit-bearing plants using a variety of labelled cinnamate precursors, this author showed that the C6-C3 carbon skeleton of the cinnamate precursor was incorporated intact into the flavan units. A further significant observation in these studies was that the two identical structural fragments, which condense to create a procyanidin dimer, were labelled to different extents. This was interpreted to show that the two flavan-3-ol units in a procyanidin molecule are derived from different metabolic entities. Porter et al. (1986) reported this same observation and it was thereby proposed that the distinct metabolic pools may provide the electrophilic (chain elongating: top, middle and junction units) and the nucleophilic units (chain terminating; bottom units) necessary for the condensation of oligomeric condensed tannins.

Many factors can affect condensed tannin biosynthesis in plants. Foo *et al*. (1982) suggested that because polymers isolated from the leaves and roots of the same plant

show structural variation, biosynthesis of tannins may be under different control in the two tissues. They reported two independent processes of tannin biosynthesis in mutants of *L. pedunculatus*: (a) light mediated and occurring in the apical meristem, and (b) nutritional and occurring in the root system. Biosynthesis of condensed tannins in leaves was controlled by light quality, and in the roots by stressing the plants by applying conditions of nitrogen deficiency.

Condensed tannin concentration in plant tissue has been shown to vary with many factors. These include plant species (Jackson et al. 1996a), plant part (Foo *et al*., 1982, Barahona et al. 1997), plant maturity (Lees et al. 1995), growing season (Clarke et al. 1939; Donnelly, 1959, Feeny, 1970) and soil fertility (Barry and Forss, 1983; Barry, 1989). The concentration of condensed tannins in a normal and a low-tannin sericea lespedeza (*Lespedeza cuneata*) cultivar grown under a warm (32/24°C) controlled environment was significantly higher in both cultivars than when grown under a cool (22/17°C) environment (Fales, 1984). Anuraga et al. (1993) observed that a combination of moisture stress and high temperature resulted in an increase in the concentration of condensed tannins in *Lotus pedunculatus*. A dramatic change in condensed tannin concentration in *Lotus pedunculatus* (cv. Grasslands 'Maku') in response to changes in soil fertility was reported by Barry and Forss (1983, see Table 2-8).

Table 2-8. *Concentration of condensed tannins (CT) and soluble N in primary growth leaf + stem tissue of vegetative lotus (VL), mature lotus (ML), white clover (WC) and red clover (RC) as influenced by growing the plants under conditions of high and low levels of soil fertility.*

			Soluble N				Soluble N	
	CT	Total N	% total N		CT	Total N	% total N	
Plants	% DM	% DM	-PEG	+PEG	% DM	% DM	-PEG	+PEG
VL	3.2	4.59	6.6	31.2	7.80	3.74	7.3	32.9
ML	2.0	2.70	7.4	31.6	5.10	2.70	7.5	19.5
WC	ND	4.71	42.6	43.8	0.12	3.84	38.0	41.7
RC	ND	ND	ND	ND	0.03	3.33	55.3	60.4

ND = not determined.

Effects attributable to condensed tannins were assessed by adding polyethylene glycol (PEG), MW 4000, which displaces protein from the insoluble tannin: protein complex and forms a PEG: tannin complex.

Source: Barry and Forss (1983).

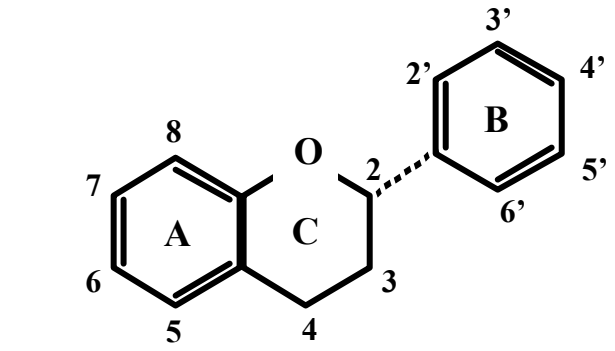
Bell et al. (1992) reported that the content of condensed tannins increases progressively in each new leaf of cotton (*Gossypium* spp.) until plateau concentrations were reached in

leaves developed during early fruiting. Feeny and Bostock (1968) found that condensed tannins were virtually absent in oak (*Quercus robur*) leaves in the early spring and did not appear until late May. In apples (*Malus domestica*), large quantities of catechin were formed in the early fruit, but after a few weeks declined sharply to reach a more steady level at plant maturity (Mosel and Hermann, 1974). On the other hand, Brandon et al. (1982) observed that the concentration of proanthocyanidins (i.e. condensed tannins) constituents in barley remains about constant throughout the growth season, although there was reduction in the relative amount of a procyanidin trimer as barley matured. Lees et al. (1995) speculated that in sainfoin (*Onobrychis viciifolia*) a finite amount of condensed tannins was formed in the two subepidermal layer of the new leaves at different stages of early leaf development, did not increase during the mature phase, and was catabolized in older leaves and during senescence. These differences may indicate that the dynamics of condensed tannin accumulation in plant tissue might be regulated by mechanisms that are species-dependent.

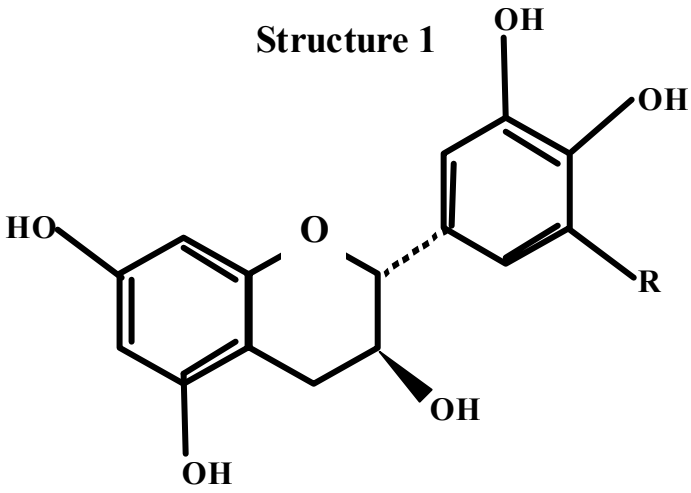
It has been suggested that accumulation of secondary compounds in plants is dependent upon photosynthetic capacity (Mooney et al. 1975). However, Lees et al. (1995) observed large amounts of condensed tannins during the very early stages of sainfoin seedling leaflet development, when there was very little leaf area and a corresponding low photosynthetic capacity. This may be related to the ready availability of both carbon and nitrogen before leaf initiation (Dement and Mooney, 1974). This could also imply that in mature plants there is active mobilisation of pre-formed condensed tannin molecules from mature and senescing leaves to the young, developing leaves.

2.2.4 Chemical structures of tannins

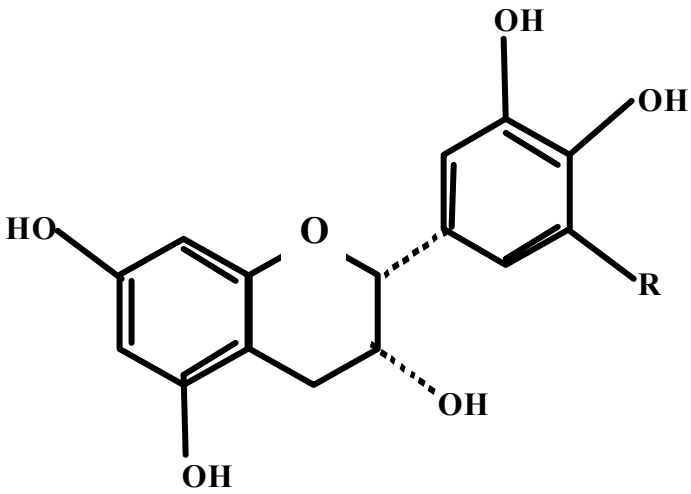
Work carried out towards completing this thesis dealt primarily with condensed tannins, and as a consequence, we will limit our discussion on the chemical structures of tannins to that observed for the condensed tannins. It is generally believed that the biological activities of condensed tannins are directly related to their chemical structures. For example, the molecular weight of the condensed tannin molecules has been shown to influence their astringency (Bate-Smith, 1973a; Kumar and Horigome, 1986). Likewise, condensed tannin stereochemistry at C-2 and C-3 has been shown to influence the astringency of condensed tannins and diet acceptability by rabbits (Clausen et al. 1990).



Structure 1



Catechin : R = H
Gallocatechin : R = OH



Epicatechin : R = H
Epigallocatechin : R = OH

Figure 2-4. *An illustration of the basic structure of flavan-3-ols and some of the most common flavan-3-ols found in nature. From Koupai-Abyazani et al. (1992)*

It would be ideal that any study aimed at elucidating the nutritional effects of condensed tannins should be also concerned with obtaining some information on the chemical structures of the condensed tannins studied. Unfortunately, that has been the exception rather than the rule, with many of the nutritional papers published being severely limited by the use of inadequate analytical methods (Mangan, 1988).

2.2.4.1 *Nomenclature*

Two different systems of nomenclature are in use for naming condensed tannins. The IUPAC (1979) system, widely used by chemists, provides a systematic approach to the naming of chemical structures. However, the IUPAC rules are rather awkward when applied to condensed tannins (Mueller-Harvey and McAllan, 1992). A simpler naming system has been proposed that use trivial names for describing the flavanols (Hemingway et al. 1982; Porter, 1988; see Table 2-9). Interflavanoid linkages are named in a manner analogous to the carbohydrates, in that the C-4 of the flavan unit is considered to be analogous to the C-1 anomeric sugar carbon (Brandon et al. 1982). The configuration at C-4 is shown by α (substituent below C ring) or β (above C ring). As it should be noted in Figure 2-4, the C ring of flavan-3-ols has three chiral centres (carbons 2, 3 and 4).

Table 2-9. *Flavan-3ol units found in naturally occurring oligoflavanols (i.e. condensed tannins).*

Monomeric	Oligomeric	Substitution pattern[1]						
Flavan-3-ol	Flavan-3-ol	3	5	7	8	3’	4’	5’
Catechin	Procyanidin	OH	OH	OH	H	OH	OH	H
Gallocatechin	Prodelphinidin	OH	OH	OH	H	OH	OH	OH
Guibourtinidol	Proguibourtinidol	OH	H	OH	H	H	OH	H
Fisitinidol	Profisetinidin	OH	H	OH	H	OH	OH	H
Afzelechin	Propelargonidin	OH	OH	OH	H	H	OH	H
Robinetinidol	Prorobinetinidin	OH	H	OH	H	OH	OH	OH
Prosopin	Promelacacinidin	OH	H	OH	OH	OH	OH	H

[1]Numbers refer to C atoms as labelled in Structure 1, Figure 2-4, opposite page.

Source: Porter (1988), Heller and Forkmann (1988).

Stereochemistry of C-3 relative to C-2 determines whether a monomeric flavan-3-ol has a “normal” name (i.e. catechin) or an epi-preceded name (i.e. epicatechin; see Figure 2-4). It should also be noted that C-4 is always *trans* to C-3. All flavan-3-ols depicted in

Figure 2-4 and most of those occurring in nature are in a 2*R* configuration (Ellis et al. 1983). Those with a 2*S* configuration should be distinguished by the enantio-prefix (Hemingway et al. 1982). Thus, the absolute configuration of catechin can be described as 2*R*: 3*S* and that of epicatechin as 2*R*: 3*R*.

2.2.4.2 *Primary structures of condensed tannins*

As was stated earlier, proanthocyanidins (synonymous condensed tannins) consist of flavan-3-ols units linked together through C-4→C-8 and C-4→C-6 bonds (Czochanska et al. 1980). This means that condensed tannins are only linked via their C and A ring. However, there have been reports of the existence of C → B, A → A, B → A and B → B ring linkages (Mueller-Harvey and McAllan, 1992), although these have been the exception rather than the rule. The great majority of the work with condensed tannins has shown that the prevalent linkage in most of the cases occurs between C-4→C-8 and C-4→C-6 bonds (C and A rings). Yet another type of linkage (A type) has been often reported where the C and A rings are doubly linked through (C2-O-C7) and C-4→C-8 (Bathe-Smith, 1975; Porter, 1988).

Work by Fletcher et al. (1977) suggested that the ratio of C-4–C-8 to C-4–C-6 isomeric pairs was relatively high (8: or 9: 1). However, Hemingway et al. (1983) observed that this ratio ranged from 1.6: 1 to 3.3: 1 and averaged 2.4: 1 in samples from a number of loblolly pine phloem tannins. These differences reflect the great structural variation that can exist among proanthocyanidins, which could occur from dimers and trimers to more complex oligomers and polymers depending on the nature of the interflavanoid linkage, hydroxylation pattern and stereochemistry at carbons 2, 3 and 4. For example, using reversed-phase HPLC coupled with photodiode-array detection, Bartolome et al. (1996) observed 24 different peaks in the chromatograph of an extract from grape seeds *(Vitis vinifera* var. airen), which corresponded mostly to dimers, flavanol-gallates and trimers. Similarly, Foo et al (1996) isolated more than 13 different compounds among monomers and dimers of flavan-3-ols from ground leaves of *Lotus pedunculatus*.

Indeed, a great diversity of flavan-3-ols has been isolated from different plants. For example, Brandon et al. (1982) identified the dimers catechin (4α→ 8) catechin and gallocatechin (4α→ 8) catechin and the trimers gallocatechin (4α→ 8) gallocatechin (4α→ 8) gallocatechin, and catechin (4α→ 8) catechin (4α→ 8) catechin in barley ear

samples. In turn, Hemingway et al (1983) detected the presence of the procyanidins epicatechin (4β→ 8) catechin, epicatechin (4β→ 6) catechin, catechin (4α→ 8) catechin, epicatechin (4β→ 8) epicatechin (4β→ 8) catechin, epicatechin (4β→ 6) epicatechin (4β→ 8) catechin and epicatechin (4β→ 8) epicatechin (4β→ 6) catechin in the phloem of *Pinus taeda*.

The isolation of more exotic flavan-3-ols is continually being reported. Demello et al. (1996) reported on the isolation of prorobinetinidins from *Stryhnodendron adstringens* which included robinetinidol (4β→ 8) epigallocatechin, robinetinidol (4α→ 8) epigallocatechin, robinetinidol (4α→ 6) gallocatechin and robinetinidol (4α→ 6) epigallocatechin. The natural occurrence of the first oligomeric profisetinidins with (2R, 3R)-2,3-cis-epifisetinidol chain extender units was demonstrated by Steynberg et al. (1997) in the bark of *Pithecellobium dulce*. In turn, Ishimaru et al. (1995) reported the isolation of (+)-afzelechin (+)-catechin from the roots of *Fragaria* x *ananassa.*

Using reverse-phase HPLC, Kuopai-Abzayani (1993) observed that in all condensed tannins extracted from 26 accessions of sainfoin (*Onobrychis*), gallocatechin, epicatechin and epigallocatechin are both extension and terminal units while catechin is a terminal unit only. Additionally, it was observed that epigallocatechin was the major extension unit in all sainfoin samples studied, providing 52-63% of the polymer constituents. A similar pattern was observed by Foo et al. (1996) in condensed tannins from *L. pedundulatus*, with epigallocatechin being the major extender unit (64%). In contrast, in the same study, condensed tannin polymers from *L. corniculatus* had epicatechin (67%) as the main extender unit, with epigallocatechin making up the rest (30%). In *L. corniculatus* catechin (82%) was the most prevalent terminal unit, whereas in *L. pedunculatus* the terminal units were more heterogeneous.

The determination of the procyanidin (PC = catechin and epicatechin): prodelphinidin (PD = gallocatechin and epigallocatechin) ratio of the constituent flavan-3-ols of condensed tannins is a less detailed measurement, yet provides extremely useful information of the chemical structure of condensed tannins. Foo et al. (1982) reported this ratio (PC: PD for brevity) for the condensed tannins of 11 of the most common fodder legumes. Results ranged from 100: 0 (pure PC) for the condensed tannins extracted from the leaves of *Vicia hirsuta* and *V. sativa* to 0: 100 (pure PD) in those

extracted from the flowers of *Trifolium repens*. In earlier work, Foo and Porter (1980) had reported on the structures of 38 proanthocyanidin polymers from 14 widely distributed families of plants. Eight of the plant species examined contained condensed tannins composed of pure procyanidins, whereas the highest prodelphidinin content (96%) was observed in condensed tannins extracted from leaves of *Ribes nigrum*.

Instead of describing the oxidation pattern (PC: PD ratio) of the constituent flavan-3-ol units of condensed tannins, other workers prefer to describe their stereochemistry at C-3 relative to C-2. As a general rule, there is little pattern to both measurements (Foo et al. 1982). Surveys carried out so far have shown that the 2,3-*cis* configuration predominates in nature although great variation has been observed in this condensed tannin characteristic as well. For example, the 2,3-*cis*: 2,3-*trans* ratios of condensed tannins from five pine species varied from 65: 35 to 81: 19 (Eberhardt and Young, 1994). Much greater variation is observed among the condensed tannins extracted from different plant species. For example, Foo and Porter (1980) reported that this value ranged from 12: 88 in the leaves of *Ribes sanguineum* to 97: 3 in unripe fruits of *Aesculus hippocastanum*. Ayres et al. (1997) reported that CT from *Betula alleghaniensis* were 100% *cis*. Likewise, Porter and Woodruffe (1984) reported that the proanthocyanidin polymers of *Chaenomeles chinensis, Aesculus x carnea, Rhopalostylis sapida* and *Strelitzia reginae* were 100% *cis*, whereas those of *Crataegus oxyacantha* were 100% *trans*.

Results from research carried out so far indicate that there is great variation in the chemical structures of condensed tannins even among those coming from plant within the same genera or even more, among those extracted from different varieties within the same species. This variation extends to plant parts and it raises the question of whether the chemical structure of condensed tannins also varies in response to environmental factors. Because very few studies of the effects of condensed tannins have employed purified tannins and even fewer have characterised them, it is difficult at present to link particular tannin structures with particular tannin effects.

2.2.4.3 *Molecular weight of condensed tannins*

Average chain lengths of condensed tannins range from two flavan 3-ol units in barley seeds to 20 or 25 in *Lotus pedunculatus* and sainfoin leaves (Mueller-Harvey and McAllan, 1992). When examining the structures of 38 condensed tannin polymers, Foo

and Porter (1980) observed a range in the number-average molecular weight (M_n) between 810 (*Dicksonia squarossa*, frond) and 6000 (*L. pedunculatus*, roots), although almost all condensed tannins had M_n lying somewhere between 1750 and 3300. Likewise, the M_n observed by Foo et al. (1982) in condensed tannins from 11 forage legumes ranged between 2000 (*L. corniculatus*, leaves and roots) and 4000 (*L. pedunculatus*, roots). Similarly, the M_n of the peracetate derivatives of 32 samples varied widely, being in the range of 1600-5500 (Williams et al. 1983). Additionally, the latter authors observed that the molecular weight profiles of these condensed tannins varied greatly from those with narrow, smooth distributions, to those that were discontinuous

Condensed tannins are polydisperse, that is $D_p = M_w$ (*weight-average* molecular weight) /M_n (*number-average* molecular weight) is greater than one. For reference, if the the molecular weights possess a normal distribution then $M_n : M_w : M_z = 1 : 2 : 3$, with M_z being the *z- average* molecular weight. All the condensed tannins examined by Foo et al. (1982) had a D_p between 1.4 to 2.7. Those examined by Williams et al. (1983) in a larger survey had D_ps ranging between 1.14 and 44.0. Thus, on the basis of these data, the condensed tannin polymers and oligomers exist over an extremely wide molecular weight range, from dimers through the species with molecular weights of several hundred thousand, containing hundreds of flavan-3-ol units (Williams et al. 1983). However, the majority of tanniniferous species contain condensed tannin molecules of molecular weights mostly in the range 2000-20000 (Williams et al. 1983).

As it will be discussed in Section 2.4, such properties as astringency of condensed tannin are directly influenced by their chemical composition, their stereochemistry and their molecular weight. The more straightforward relationship is that between the molecular weight and astringency of condensed tannins, but still more research is needed to elucidate the nutritional impact of a given structural characteristic of condensed tannins. Another dimension is added to the problem when those nutritional effects are measured in ruminants, given the high complexity of their gastrointestinal tract, especially at the level of the rumen. The problem becomes even more complex if one considers the existence of several different types of tannins, such as the phlobatannins (Bonnet et al. 1996a; 1996b; 1996c; Saunders et al. 1996), the flavanol glycosydes (Orians, 1995; Orians and Fritz, 1995; Murray et al. 1996) and the flavanol gallates (Demello et al.

1996) and their co-existence within a given plant with hydrolysable and/or condensed tannins.

2.2.5 Function of tannins in plants

The function of tannins (of both the hydrolysable and condensed types) in plants is a much-discussed issue. A great deal of research with tannins has followed an approach that looks at biological relationships: taxonomy, phylogeny, biosynthesis, etc. Under this approach it has been considered that condensed tannins are present in the most primitive vascular plants, gallo- and ellagitannins appearing for the first time in the dicotyledons (Bate-Smith, 1977). Using the haemanalysis technique of Bate-Smith (1973a) gallotannins were found to be more astringent (more efficient as precipitant of proteins) than ellagitannins, which in turn, were found to be more astringent than the condensed tannins. These observations fitted nicely with those showing that ferns and gymnosperms have condensed tannins but not hydrolysable tannins, and that ellagitannins are more common in dicots than gallotannins. Thus, collectively, these observations appear to suggest a trend towards increasing biological activity in tannins associated with evolutionary advancement, as it was observed in the case of the toxicity of alkaloids in the angiosperms (Gottlieb, 1982). However, it was reported that vascalagin and castalagin predominate in oak leaves in spite of the fact that the astringency of the precursor of these two hydrolysable tannins, namely pento-O-galloyl-D-glucose (see Figure 2-1) is considerably higher (Spencer et al. 1988).

The widespread occurrence and high concentrations of tannins in many plants have been frequently interpreted as the result of selective pressures exerted by herbivores. Some research with mammals supports the hypothesis that tannins act as an antiherbivore defence by binding with dietary protein and digestive enzymes to limit assimilation in herbivores (Mitaru et al. 1988; Robbins et al. 1987a,b, 1991; Hagerman and Robbins, 1993). On the other hand, other studies have suggested that toxicity, rather than inhibition of digestive enzymes, is the mode of action (Lindroth and Batzli, 1984; Blytt et al. 1988; Mole et al. 1990b). Yet other studies with insects have suggested that tannins do not have any detectable impact on herbivores (Klocke and Chan, 1982, Manuwota and Scriber, 1986; Martin et al. 1987).

There may be considerable natural variation in tannin-herbivore interactions, due to structure specificity in the effects of tannins (Zucker, 1983; Clausen et al. 1990). Indeed, in a study of the effects of condensed tannins from 16 woody plant species on six herbivorous insect species, Ayres et al. (1997) observed that condensed tannins differed markedly in their antiherbivore effects and herbivores differed in their sensitivity towards condensed tannins. Thus, these authors doubted that selective pressures from herbivorous insects could be the main explanation for the diversion of so much carbon, in so many plant species, into the synthesis of condensed tannins. To the contrary, Feeny, (1976) wrote: "It is a precept of modern chemical ecology that energy is not likely to be "wasted" in the production of secondary metabolites unless there is some compensating adaptive advantage to the organism in question". He had observed that the period of highest insect attack on oak leaves correspond to the time when condensed tannins are virtually absent (Feeny, 1970). Gottlieb (1990) concluded that secondary metabolites are essential to plant life and that internal factors dominate the biosynthetic driving forces at all the metabolic ranks and those environmental factors, such as hervibory, constitute additional driving forces.

Throughout this discussion I have purposely ignored the fact that research with tannins has been plagued with methodological difficulties, as the methods used for the extraction, quantification and structure determination of tannins vary from one author to the other. Although methodological explanations might not be sufficient to explain the range of observed results (Ayres et al. 1997), it seems dangerous to underestimate their impact. Furthermore, the interpretation of a given set of results might be hampered if it is assumed beforehand that the results obtained in tests such as protein precipitation by tannins will be related to the nutritional effects of those tannins.

There are some observations with regard to the presence of tannins that deserve some attention. For example, within plant cells, tannins are found in the vacuole (Chafe and Durzan, 1973; Lees et al. 1993, 95) and this has been suggested to be a way of preventing inhibition of the cell metabolism by tannins (Haslam, 1974). One must wonder about the energetic costs and on the reasons for such a practice, especially when plants devote so much carbon to the production of tannins. Perhaps as suggested by Haslam (1986) "secondary metabolism serves to maintain primary metabolism in circumstances not propitious for growth".

2.3 EXTRACTION, ISOLATION AND ANALYSIS OF CONDENSED TANNINS

Research with tannins has been carried out with different interests and from different standpoints. Accordingly, the analytical techniques described in the literature for the extraction, quantification and identification of tannins are abundant. Most quantification methods involve colorimetric measurement of extracts derived from plant or animal tissues. More recently, highly specific techniques such as high performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) have been employed for the elucidation of the molecular structure of tannins.

2.3.1 Extraction and isolation of condensed tannins

Many properties, either positive or negative, have been attributed to condensed tannins. Elucidation of the true impact of plant tannins begins with their extraction and isolation for use in carefully designed experiments. As a general rule, there has been a subtle distinction (solvent-wise) between the procedures used for the extraction of condensed tannins when extracts are intended for quantification or when isolating condensed tannins for other, more specific measurements. Additionally, it is now generally accepted that tannins can be found in two forms in plant tissue. In one of these forms, condensed tannins are easily extractable from plant tissue by organic solvents (extractable condensed tannins), whereas another form is bound to cell protein and/or carbohydrate components of the cell wall (bound condensed tannins, Terrill et al. 1992b).

2.3.1.1 *Extractable condensed tannins*

Condensed tannins are usually extracted from plant tissue by the use of a polar solvent, such as 50% aqueous methanol or 70% aqueous acetone. However, there is a great deal of variation in the reported methods for tannin extraction. For example, Bate-Smith (1973a)'s procedure involved extracting samples three times with boiling 50% aqueous methanol with the extract concentrated *in vacuo*. Similarly, Hagerman and Robbins (1987) used 50% aqueous methanol with sonication for 15 minutes and then three times with boiling solvent for 8 minutes to extract tannins from various high-tannin plants. In another variation, Hagerman (1987) extracted tannins from sorghum using 50% methanol for 1 hour at room temperature. In turn, Telek (1989) used a 70% methanol solution containing 0.5% formic acid and 0.05% ascorbic acid to extract tannins from

several tropical legumes. Burns (1971) suggested the use of pure methanol for 20-28 hours to extract tannins from sorghum. As a final example, Bartolome et al. 1996 extracted proanthocyanidins from grape seeds using a synthetic wine solution containing tartaric acid (0.7 g/l), potassium bitartrate (1.11 g/l) in water-ethanol (80: 20, v/v).

Another variation that is commonly found in tannin extraction protocols involves pre-extracting the samples in order to eliminate lipids or pigments before the actual extraction with the chosen solvent is made. To accomplish this workers have used a variety of protocols and solvents. These include extracting with grinding in diethyl ether (Butler, 1982, Butler et al. 1982), with 10% acetic acid in ether (Distel and Provenza, 1991), three times with ethyl ether for 1 hour (Hagerman and Butler, 1978) and with 10 mM ascorbic acid in ethanol for 2 hours (Hagerman and Butler, 1980b). Interestingly, Strumeyer and Malin (1975) defatted sorghum seeds by shaking with peroxide-free ether (three times) and then extracted the tannins using 95% ethanol. A more complex protocol involved extracting sorghum seeds with petroleum ether, ethyl acetate and acetone (two times with each solvent) before extracting the tannins with ethanol (Strumeyer and Malin, 1975).

By far the most popular solvent for the extraction of tannins is 70% aqueous acetone containing 0.1% ascorbic acid. However, Koupai-Abyazani et al. (1992, 1993) preferred to use 75% aqueous acetone for the extraction of condensed tannins from sainfoin (*Onobrychis*). Ascorbic acid is usually added to the aqueous organic solvent to prevent tannin oxidation during extraction (Jones *et al*., 1976). Aqueous acetone solutions are powerful solvents for proanthocyanidin polymers extraction (Foo and Porter, 1980) and these solutions are generally recognised as the most efficient for extracting condensed tannins from forages (Terrill et al. 1990). A problem with the use of aqueous acetone to extract condensed tannins is that other compounds such as soluble lipids and non-tannin pigments are extracted as well, and it has been suggested that these cause interference with colour development (Walton et al. 1983). Hence, for most techniques of condensed tannin analysis, the use of 70% aqueous acetone for condensed tannin extraction necessarily involves the use of procedures to purify the crude extracts.

In order to remove contaminants from crude condensed tannin extracts, Jones et al. (1976) saturated the acetone extract with NaCl, which resulted in separation of acetone from the condensed tannin extract. Then, the condensed tannin extract was washed with

petrol to remove fats and pigments. In a later development, Broadhurst and Jones (1978), followed the NaCl saturation step with extraction of the condensed tannin extract with diethyl ether and then with ethyl acetate. Because saturation with NaCl is time consuming and might result in some condensed tannin loses, the extraction procedure recommended by Terrill et al. (1992b) used a mixture of acetone-water-diethyl ether (4.7: 2.0: 3.3) which "extracts condensed and washes out and non-tannin pigments in one step with no transfers". In another version of this protocol, Li et al. (1996) extracted condensed tannins from leaves by performing three 30-minutes and one overnight extraction with 70% aqueous acetone. After combining the supernatants, diethyl ether was added and the mixture was left to stand at –20C in the dark for separation of the two phases. The lower phase was used for assay of extractable condensed tannins.

2.3.1.2 *Bound condensed tannins*

The existence of bound condensed tannins was first reported by Bate-Smith (1973a, 1975) especially when he attempted to extract tannins from sainfoin (*Onobrychis viciifolia*). This author obtained a measure of the extractability of tannins by comparing the absorbance of the methanol extracts with those of the powdered dried leaf. Bound condensed tannins exist because at neutral pH, condensed tannins in forage plants form stable complexes with protein and carbohydrates, lowering their extractability (McLeod, 1974, Terrill et al. 1990). However, until the work of Terrill et al. (1992b) no method was available to measure bound condensed tannins. Under this protocol, samples are subjected to extraction with an aqueous 70% acetone solvent. After extraction of this fraction with diethyl ether to remove interfering pigments and lipids, free tannins are estimated using a colorimetric reaction with butanol-HCl. The residue from the acetone extraction is treated (2x) with sodium dodecyl sulfate (SDS) in a boiling water bath for 45 min, and the extract obtained is reacted with butanol-HCl to determine protein-bound tannins. The residue remaining after SDS extraction is also reacted with butanol-HCl to determine fibre-bound condensed tannins.

This technique has received some criticism because the buffered SDS solution contains HCl which may react with tannins, thus leading to a underestimation of tannins when subsequently reacted with butanol/HCl (Perez-Maldonado, 1994). Modifications to Terrill's method have been suggested by Perez-Maldonado (1994)

and by Li et al. (1996). It is difficult to say how specific the extraction with the SDS solution is and thus, whether it is appropriate to label the fraction obtained with this procedure as "protein" bound condensed tannins. It must also be established whether there is some merit, in terms of animal nutrition, in differentiating between tannins that are bound to "protein" and those that are bound to "fibre". It is logical to assume so, but up to now very scant work has been done to elucidate the role played by bound condensed tannins as a whole, let alone to account for the impact of these two fractions of bound condensed tannins in the overall nutritive quality of forages. This is definitely an area that deserves more attention. For the time being, some researchers have preferred to obviate the SDS extraction and to simply proceed with the determination of total bound condensed tannins.

2.3.1.3 *Isolation of condensed tannins*

After extraction of condensed tannin from plant tissue and elimination of lipids and fats, it is still necessary to use more specific procedures to purify even further the extracted tannins. As noted before, when the extraction of condensed tannins is intended for the isolation of purified tannins, there are some slight changes in the solvent systems used than when extracts are going to be used for such purposes as quantification of condensed tannins, or for some protein precipitation tests. Strumeyer and Malin (1975) proposed the use of lipophilic Sephadex (LH-20) for further purification of the condensed tannin extract. Their procedure involved equilibration, application and washing of the tannin extract using 95% aqueous ethanol. After the 95% ethanol eluate become colourless, condensed tannins were eluted as a discrete band from the Sephadex LH-20 column with 50% acetone-water. A variation of this method was reported by Hagerman and Butler (1980b), who used 80% ethanol for the washing of condensed tannins. In the other hand, Jones et al. (1976) used a combination of Sephadex G-50 and LH-20 for the purification of condensed tannins from six species of pasture legumes. They used 50% aqueous methanol to remove impurities from the tannins absorbed to the Sephadex LH-20 and used 70% aqueous acetone for the elution of condensed tannins from the column. This last combination of solvents in conjunction with Sephadex LH-20 has become the most popular with researchers. Other reported methods for the isolation of condensed tannins include the use of nylon beads to absorb condensed tannins (Telek, 1989) and of

trivalent yterbium to precipitate them, which reportedly requires less time and resources that the Sephadex LH-20 method (Giner Chavez et al. 1997b).

Throughout this section this discussion to become very detailed, and perhaps a little cumbersome as well, with the aim of demonstrating the great variability among the methods employed by researchers for the extraction of tannins. This might be of importance in the light of several observations. First, in most plants condensed tannins are found in several sizes, from dimers to polymers with a very high degree of polymerisation. Indeed, tannins are polydisperse molecules. Some extraction procedures have been criticised because their application might result in losses of some of those fractions, especially of those of low molecular weight. The impact of these losses upon quantification of condensed tannins or upon the determination of the biological activity of condensed tannins could be significant if these low molecular weight phenolics represent a high proportion of total phenolics. Second, there has been some debate on whether to use a given solvent system or another to carry out condensed tannin extraction from plant tissue. Whereas data obtained in the extractability of condensed tannins with a given organic solvent is useful in some fields of study, this same information might be useless when examined from the standpoint of animal nutrition. Tannins that are soluble upon a given extraction procedure might not be soluble at all in rumen liquid or in other physiological fluids. Finally, research has demonstrated that the way samples are collected and handled prior to and during tannin determination can have a significant effect on the values obtained (Chiquette et al. 1988; Ahn et al. 1989, 1997; Terrill et al. 1989; Cano et al. 1994; Makkar and Singh, 1995; Orians, 1995; Lindroth and Koss, 1996). Discussion of sample treatment was not included in this section but the procedures reported varied in the sample treatment methods used (fresh vs. dried, whole vs. ground, freeze-dried vs. oven-dried, etc.).

2.3.2 Methods for the determination of tannins and their astringency

Most methods for the determination of condensed tannins and their astringency can be classified as one of two types, chemical (colorimetric) and protein binding assays. Both types of methods convey information that is of extreme utility in condensed tannin research. Methods for the quantification of tannins of the hydrolysable type have also been reported and show great variability as well, however, in the next sections only the methods primarily related to the analysis of condensed tannins are discussed.

2.3.2.1 *Colorimetric methods*

As was the case for extraction of condensed tannins, several methods have been reported for the determination of tannins and related compounds. Some of the methods initially used include hide powder absorption and permanganate oxidation, which are non-specific for tannins (Mangan, 1988). Other non-specific methods include the colorimetric Folin-Dennis (Folin and Denis, 1915, Swain and Hillis, 1959, Burns, 1963), Prussian blue (Price and Butler, 1977) and Folin-Ciocalteau (Folin and Ciocalteu, 1927, Singleton and Rossi, 1965), and the gravimetric determination by precipitation with trivalent ytterbium (Reed et al. 1985). These methods are useful for the determination of total phenolics, a determination which includes both hydrolysable and condensed tannins. A major drawback of these methods is that they do not discriminate between tannin and non-tannin phenolics (Salunkhe et al. 1990). For example, the Folin-Denis and Folin-Ciocalteu reagents react with a range of substances which include phenol, phlorogucinol, L-tyrosine, salicylic acid, coumarin, catechol, pyrogallol, quercetin and some other readily oxidised substances such as ascorbic acid (Singleton and Rossi, 1965). Equating phenolic with tannins is a problem, because many are not tannins by strict definition (they do not precipitate protein), but some can be converted into tannins by oxidative polymerisation (Reed et al. 1985). However, phenolics that do not precipitate protein can have significant nutritional effects (Fahey and Jung, 1990)

A more specific method for condensed tannin determination is the use of acidified vanillin (Bate-Smith, 1954, Swain and Hillis, 1959, Burns, 1963, 1971, Broadhurst and Jones, 1978, Price et al. 1978, Butler et al. 1982). While a very sensitive method, the acidified vanillin reagent may also react with flavonoids that are not tannins such as chalcone derivatives and monomeric flavanols (Waterman et al. 1980, Mangan, 1988). However, the vanillin-HCl reagent has been effectively used to detect soluble monomeric and polymeric flavan-3-ols in leaf tissue (Bathe-Smith and Lerner, 1954, Bate-Smith and Metcalf, 1957, Burns, 1963) and in conjunction with gel electrophoresis in a survey of condensed tannins in pasture legumes (Jones et al. 1973). The vanillin-H_2SO_4 (Swain and Hillis, 1959) and the vanillin-HCl (Burns, 1963) have been criticised due to lack of reproducibility between samples, days and laboratories. Another major drawback of the vanillin-HCl procedure is that some protocols use catechin as a

standard, a flavan-3-ol that bears little relation to the form in which tannins are usually found in plant tissue.

In an attempt to correct problems observed with the vanillin assay, Broadhurst and Jones (1978) excluded light and controlled the temperature of the reaction mixture. In addition, these authors suggested the use of purified condensed tannins isolated from the species under investigation as standards. This resulted in an improved method, although there are still some problems with this assay. (i) Sarkar and Howarth (1976) reported that dihydrochalcones and anthocyanidins react with acidified vanillin to give similar product to tannins. An adequate condensed tannin extraction procedure should remove dihydrochalcones (Broadhurst and Jones, 1978) and anthocyanidins should be taken care of by the use of a proper blank (Jackson et al. 1996a). (ii) Li et al. (1996) suggested that the vanillin-HCl method is not sensitive enough to detect condensed tannins in low-tannin plants. (iii) The rate and extent of colour development in the vanillin assay were observed to be different depending on whether methanol or glacial acetic acid was used as solvent (Butler et al. 1982). (iv) Acetone reacted with acidified vanillin to produce a chromogen with λ_{max} at 548 nm that caused substantial error in the condensed tannin assay (Makkar and Becker, 1993). (v) The vanillin-HCl reagent is not appropriate for the determination of fibre bound tannins as there is no colour development at all with this fraction (Terrill et al. 1992b). (vi) The vanillin-HCl is specific for condensed tannins. (vii) When the reaction is carried out in methanol and catechin is used to standardise the assay, considerable overestimation of the tannin content is observed (Price et al. 1978, Gupta and Haslam, 1980) because more chromophore is produced per mg of tannin than per mg of catechin (Butler et al. 1982). In contrast, when the reaction is carried out in glacial acetic acid reaction with vanillin occurs only at the end groups of polymeric flavan-3-ols. Butler (1982) took advantage of this to calculate, in conjunction with absorbance of the butanol-HCl hydrolysis product, the relative degree of polymerisation of sorghum as the growth season progressed. (viii) When the reaction is conducted in methanol and catechin is used to standardise the assay, catechin and tannin react with vanillin with quite different kinetic patterns (Price et al. 1978, Gupta and Kaslam, 1980). (ix) The presence of methanol in the condensed tannin extract inhibits profoundly chromophore development in the vanillin-CT reaction (Butler et al. 1982).

The most popular colorimetric technique for determining condensed tannins is the butanol-HCl procedure. Bate Smith (1954, 1973a), Hillis and Swain (1959), Swain and Hillis (1959), Govindarajan and Mathew (1965), Gupta and Haslam (1980), Porter et al. (1986) and Terrill et al. (1992b) have proposed protocols for this technique. On heating with dilute acid and butanol, condensed tannins are partially converted to the strongly coloured anthocyanidins. The reaction is specific for cyanidin, delphinidin, pelargonidin and other anthocyanidins (Mangan, 1988). Porter et al. (1986) suggested this reaction was, at least partially, an autoxidation because of the dependence of the reaction on oxygen partial pressure and the ability of metal-ions to act as catalysts. They observed that the presence of the transition-metal ions Fe^{II}, Fe^{III}, Mn^{II} and CU^{II} enhances the colour reaction and increases the sensitivity of analysis for samples of low condensed tannin content. However, at high concentrations, Mn^{II} and CU^{II} lead to decomposition of anthocyanidins. These same authors also showed that in samples with water content above 6%, the yield of anthocyanidins would drop.

Porter et al. (1986) also observed that if a solution of condensed tannins was stored in butanol-HCl at room temperature for 48 hours the yield of anthocyanidin was the same as for the reaction if it had been heated. However if Fe^{III} was added there was no increase in anthocyanidin yield. All these observations allowed these authors to suggest that the final yield of anthocyanidin on the hydrolysis of condensed tannins by butanol-HCl depends on three types of reactions. (i) Autocatalytic: no oxygen or catalyst required. (ii) Autoxidation: oxygen required. (iii) Direct oxidation: Fe^{III} required.

A disadvantage of the butanol-HCl procedure is that at very low concentrations of condensed tannins, it is difficult to correct effectively for background absorption at 550 nm not due to anthocyanidins, especially for the SDS extracts in Terrill's protocol. Therefore, the procedure is more accurate for condensed tannin concentrations of 1% or higher (Terrill et al. 1992b). To take account of this, Carron et al. (1994) introduced the scanning of the hydrolysed samples between 450 and 650 nm. By doing this, it is observed that within this range, the absorbance of the anthocyanidin solution is never equal to zero. From a printout of the scan, it is possible to determine how much of the total absorbance is really due to anthocyanidin presence. This procedure also shows the wavelength of maximum absorbance is dependent upon the species studied ranging from

540 to 560 nm. By analogy, in most methods, total absorbance is measured exclusively at 550 nm.

Another problem with the butanol-HCl method is that underestimates condensed tannin content in digesta and faecal samples, probably as a result of changes in structure of condensed tannins during passage through the ruminant gastrointestinal tract, reducing conversion to anthocyanidin on acid hydrolysis (Terrill et al. 1994). Indeed, Bae et al. (1993a) observed that both water and liquid medium as compared to methanol reduced the chromophore yield of the butanol-HCl assay. Apparently, the addition of water or physiological fluids to the butanol-HCl assay increases the occurrence of competitive side reactions and decreases the yield of anthocyanidins. This might limit the use of the butanol-HCl method in studies a large number of liquid samples are produced (i.e. microbiological studies) or when lyophilisation of samples is not desirable.

Other colorimetric methods for the determination of condensed tannins have been reported in the literature. Bae et al. (1993a) reported a method that used 43% (v/v) H_2SO_4 in methanol that was suggested to be ideal for working with rumen bacteria in microbiological or in ruminant nutrition studies. Li et al. (1996) reported a method that used HCl-acidified 4-dimethylaminocinnamaldehyde (DMACA) which was shown to be five times more sensitive than the best vanillin-HCl method these authors tested.

2.3.2.2 *Protein precipitation assays*

A large number of protein precipitation assays have been reported in the literature. A summary of some of these methods is reported in Table 2-10. Given that tannins form insoluble complexes with proteins, one approach to measuring the amount of tannin in an extract has been to determine its capacity to precipitate proteins from solution. As an added bonus, since it is this precipitating capacity of tannins, especially of digestive enzymes or ingested food plant proteins, which is postulated to be responsible for the adverse effects of tannins, these assays appear to be particularly appropriate to study the significance of tannins in herbivory. Protein-precipitation methods reported in Table 2-10 are only a small portion of those reported in the literature. Earlier protein-precipitation methods include that of the Association of Agricultural Chemists (1965) for the determination of tannins in tea that is based on the precipitation of gelatine. Leather chemists also used protein solutions that include gelatine, calfskin or hide powder.

Table 2-10. *Some of the protein-precipitation methods reported in the literature.*

Method	*Basic procedure*
β-Glucosidase precipitation assay (Goldstein and Swain. 1965)	React tannin extract plus β–glucosidase solution for 15 minutes. Centrifugation (15 min at 12000 x *g*). Activity of the enzyme remaining in the supernatant determined. Results expressed as units of enzyme activity precipitated.
Haemanalysis: Relative astringency (Bathe-Smith, 1973a)	1.0 ml of tannin extract in H_2O plus 1.0 ml of haemolysed blood, mixture shaken. Centrifugation (5 min at 3000-7000 rpm). Absorptivity of supernatant read at 578 nm. Results expressed as tannic acid equivalents.
Colorimetric assay for protein-precipitable phenolics (Hagerman and Butler, 1978)	React 1.0 ml tannin extract in MeOH or H_2O plus 2 ml of bovine serum albumin (BSA) solution for 15 minutes. Centrifuge (15 min at 5000 x *g*). Add ferric chloride to the pellet, read at 510 nm. Results expressed as A_{510}/g of dry matter extracted.
Radio-labeled protein precipitation assay (Hagerman and Butler, 1980a)	React 2.0 ml of iodine-125-labeled-BSA for 15 minutes. Centrifuge (15 min 5000 x *g*). Count 1.0 ml aliquot of supernatant to determine protein precipitated which is expressed as mg of BSA precipitated.
Bovine serum albumin precipitation assay (Martin and Martin, 1982)	React tannin extract with a solution of BSA 15 minutes. Centrifuge (15 min at 12000 x *g*). Apply supernatant to Sephadex G-25. Add Coomasie Brilliant Blue G-250 dye to recovered proteins. Read at 595 nm. Precipitated BSA (mg) calculated by difference.
Dye-labeled protein precipitation assay (Asquith and Butler, 1985)	React tannin extract in MeOH with Remazol brilliant blue R-labelled BSA for 5 minutes. Centrifuge (bench top centrifuge). Pellet dissolved in SDS-triethanolamine-isopropanol solution. Read absorbance at 590 nm. Results expressed as mg of BSA precipitated.
Radial diffusion assay (Hagerman, 1987)	Add tannin extract to wells punched in Petri dishes containing a BSA-agar gel. Incubate at 30°C for 96-120 hours. Measure diameter of ring formed. Calculate tannin concentration in extract using diameter of ring and an appropriate calibration curve.
Rubisco precipitation assay (McNabb et al. 1998)	Incubate tannin extract in buffer with Rubisco-containing solution at 39°C for 90 minutes. Centrifugation (12000 x *g* for 10 min). Pellet dissolved in SDS-PAGE loading buffer. After SDS-PAGE, developed gels are quantified by imaging densitometry.

A very attractive procedure towards determining the astringency of tannins (i.e. their efficiency as precipitants of proteins; Bathe Smith, 1973a), involves the use of various enzymes. Goldstein and Swain (1965) suggested a method based on the ability of tannins to inhibit the enzymatic activity of β–glucosidase, but the results of the assay were difficult to interpret, because the relationship between enzymatic activity and the formation of insoluble complexes was not fully understood (Hagerman and Butler, 1978). There have been other attempts to estimate tannin concentration by its inhibition of various enzymes (Davis and Hoseney, 1979; Becker and Martin, 1982; Marks et al. 1987). However, they have had little success because the correlation between tannin concentration and the degree of inhibition has proven unsatisfactory (Daiber, 1975;

Gupta and Haslam, 1980; Earp et al. 1981; Bullard et al. 1981). Moreover, the effects of pH and solvent composition cannot be rigorously studied with enzyme inhibition methods

In another approach, Bate-Smith (1973a) developed the technique of precipitation of haemoglobin from haemolysed blood solutions by tannins into a general method for judging the relative astringency of tannin preparations. This author used with great effectiveness this assay in chemotaxonomic studies (Bathe-Smith 1973b, 1975, 1977, 1978, 1981). Unfortunately, in this assay tannin levels must exceed a rather high threshold value before the haemoglobin precipitates, possibly because other blood proteins precipitate first (Martin and Martin, 1982). In addition, many plant metabolites including saponins (Bathe-Smith, 1977) and pigments such as anthocyanidins (Asquith and Butler, 1985) also absorb at 578 nm interfering with the precipitation assay and causing high blank values. Subsequent studies of Schultz et al. (1981) have enhanced the value of this method in assaying for tannins, however in their studies these authors noted that this assay is absolutely dependant on a fresh supply of blood, since commercial preparations were unsatisfactory (Asquith and Butler, 1985).

Yet another approach has involved the use of proteins labelled with radioisotopes. Protocols involving this principle have been suggested by Hagerman and Butler (1980a) and Asquith et al. (1983). Because pigmented compounds do not interfere with the radiochemical determination, these methods are sensible and reliable. However, they are limited by their dependence upon the availability of the sophisticated radioisotope equipment. Additionally, the preparation of labelled protein may be difficult.

The colorimetric method of Martin and Martin (1982; 1983) is free from those difficulties. However, this assay is technically difficult and would be inappropriate for studies involving more than a few samples (Hagerman and Robbins, 1987). Moreover, this is an indirect assay since it uses the Bradford protein-dye test (Bradford, 1976) to measure non-precipitated protein, a feature that makes it inherently less accurate. Two simpler protein precipitation methods have been described: the dye-labelled protein precipitation method of Asquith and Butler (1985) and the colorimetric assay for protein-precipitable phenolics (Hagerman and Butler, 1978). Both methods use bovine serum albumin (BSA) as the test protein but do not give comparable results to each other (Hagerman and Robbins, 1987).

In most of the above reported assays, solvents such as acetone interfere with the precipitation and must be removed from the extract before analysis. Additionally, water-insoluble compounds frequently found in the tannin extract interfere with precipitation assays (Hagerman, 1987). All these observations led Hagerman (1987) to devise a protein-precipitation assay based on the diffusion of tannins through a protein-containing gel. The resulting radial diffusion method does not suffer from interference by non-tannin phenolics including catechin and gallic acid and solvent used. Additionally, with the use of an appropriate standard, this method was found to be adequate for the quantification of tannins in crude tannin extracts. Lareo et al. 1990 modified the original radial diffusion technique of Hagerman (1987) to develop a simple method for measuring the astringency of condensed tannins. The modified assay is still based on the diffusion of tannins through a protein (BSA) in a gel (pH 5) and the formation of a precipitate in the form of a ring. The diameter of the ring is proportional to the amount of precipitated protein (Lareo et al. 1990). As suggested by Hagerman (1987), another modification to the radial diffusion assay involved the use of alfalfa leaf protein instead of BSA (Giner Chavez et al. 1997a).

In terms of ruminant nutrition, especially of ruminants raised in grazing systems, the use of Rubisco in protein precipitation assays would be the most meaningful, given that Rubisco represents the principal dietary protein (Mangan and West, 1977). Consequently, several studies have been conducted to investigate the interaction between plant protein and tannins and to allow understanding of their implications upon the nutrition of ruminants (Jones and Mangan, 1977; Jackson et al. 1996b; McNabb et al. 1996). As a final development, McNabb et al. (1998) suggested a protein-precipitation method that used Rubisco as the test protein that should convey very useful information about protein-tannin interactions under rumen-like conditions. However, these same authors concluded that reactivity alone was unlikely to account for the differences in nutritive value that occur with forages containing condensed tannins.

Evidently, all existing protein-precipitation methods have some shortcomings and this is probably more eloquently demonstrated by the constant modification of protocols by researchers to include what they consider more satisfactory methods. Examples of these are the series of protocols reported by Hagerman and Butler (1978, 1980a, 1981), Asquith and Butler (1985) and Hagerman (1987) or Martin and Martin (1982, 1983) and

Martin et al. (1985). As it should be expected by differences in experimental protocols, the results yielded by these methods are not comparable. For example, after comparing three protein-precipitation methods that used BSA as the test protein, Hagerman and Robbins (1987) reported that results obtained with a given method were not comparable to the results obtained with the other two. Furthermore, the regions of linearity were different for each of the assays and for each of the leaf extracts tested.

Similarly, the protein-precipitating constituents in mature foliage of six oak species as determined by the methods of Goldstein and Swain (1965), Hagerman and Butler (1978) and Martin and Martin (1982) were not correlated to total phenolic and condensed tannin concentration (Martin and Martin, 1982). Thus, there are limitations to the conclusions that can be made from protein-precipitation assays. Protein precipitating capacity is a measure not simply of the quantity of tannins present, but rather of a property of the extract, which depends upon both the quantity and quality of protein-precipitating species present (Martin and Martin, 1982).

A final problem of the protein-precipitation assays was illustrated by the work of Hagerman and Robbins (1987) who observed that tannin-protein ratio in the reaction mixture influenced the solubility of the resulting tannin-protein complexes. In particular, when the protein to tannin ratio was larger than the optimum ratio, soluble protein complexes apparently formed instead of insoluble complexes. Additionally, Mole and Waterman (1987a) demonstrated that as the protein to tannin ratio increases, the amount of protein precipitated by a constant amount of tannins increases until a plateau is reached. Then, the tannin to protein ratio that the researcher is working with will influence the results obtained in most precipitation assays. Such behaviour really complicates the analysis of the data obtained in protein-precipitation assays.

Other techniques for the determination of tannins that do not fit in either the colorimetric and protein-precipitation categories have also been reported in the literature. For example, the Near Infrared Reflectance Spectroscopy technique has been used for the prediction of the condensed tannin concentrations in greater lotus (*Lotus uliginosus* Schkuhr; Smith and Kelman, 1997). Standard errors of the prediction equated to coefficients of variation in the order of 20% percent. The advantage of the proposed technique is that it will allow rapid evaluation and selection of genotypes. However, the prediction equations employed can only be as good as the chemical assay in which they

are based. In turn, Mathews et al. (1997) combined the depolymerisation of condensed tannins in the presence of phloroglucinol or benzyl mercaptan nucleophiles with either gas chromatography or high performance liquid chromatography for the quantitative determination of condensed tannins. In another approach, Silanikove et al. (1996) suggested to use the binding of polyethylene glycol to samples of forage plants as an essay of tannins and their negative effects in ruminal degradation. They reported that the amount of PEG bound to a sample was reproducible with a small coefficient of variation (0.5%) and it was a good indicator of the deleterious effects of tannins in ruminant nutrition.

2.3.3 Structural characterisation of condensed tannins

Methods for the detailed study of the structure and chemistry of tannins are varied. These include thin layer, low pressure, and high performance liquid chromatography, ultra-centrifugation, fast atom bombardment-mass spectrometry and nuclear magnetic resonance.

Paper (PC) and thin layer (TLC) chromatography afford an inexpensive, versatile and accessible technique for the qualitative analysis of tannins. For example, for the detection of condensed tannins in legumes, Jones et al. (1973) developed a very rapid screening method that involved crushing plant samples between two layers of 3MM chromatography paper and detecting condensed tannins by spraying with vanillin.

The choice of plates, solvents and spray reagents for PC and TLC available to researchers is great. Papers include Whatman No. 1 and 3MM and plates include those with silica gel G or H and cellulose. Solvent systems used include acetic acid-HCl-water (30:3:10; Forestal solvent; Harborne, 1967), benzene-methanol-acetic acid (45:8:4), chloroform-ethyl acetate-formic acid (5:4:1), acetone-water (9:1), formic acid-HCl-water (5:2:3) and 1-butanol-acetic acid-water (4:1:5; Strumeyer and Malin, 1975). For two dimensional TLC, the use of *tert*.-butanol-acetic acid-water (3:1:1) in the first direction and 6% acetic acid in the second direction has been commonly used (Brandon et al. 1982, Koupai-Abyazani et al. 1992, Foo et al. 1996). Other solvent combination reported used *n*-hexane-ether-acetic acid (80:20:1) in the first direction and acetone-water (9:1) in the second direction (Strumeyer and Malin, 1975). Detection reagents that have used include the vanillin reagent (Bate-Smith, 1954), the DMACA reagent (Li et al. 1996)

and 3% p-toluenesulphonic acid for condensed tannins; 5% phosphomolybdic acid, bisdiazotized benzidine and 2,6-dichlorobenzoquinone chloroimide (Haslam, 1966) for phenols and ultraviolet light followed by exposure to ammonia for flavonoids.

Low-pressure chromatography has been mainly used for the isolation of condensed tannins. However, fractionation of tannins involving the use of Sephadex LH-20 has been reported in the literature (Strumeyer and Malin, 1975; Kumar and Horigome, 1986, Foo et al. 1996; Foo et al. 1997). Fractionation of tannins using Sephadex LH-20 is accomplished by eluting absorbed tannins by the application of 70% acetone and monitoring the absorbance at 280-350 nm. Using this procedure, Kumar and Horigome (1986) observed five discrete peaks in the condensed tannins from black locust (*Robinia pseudo acacia*) which were composed by tannins of different molecular weights. Fractions can be further treated chromatographically by alternating between two different columns (Sephadex LH20 or G50) which allows chromatographicaly pure samples including monomers, dimers and trimers to be purified (Foo et al. 1996).

High-performance liquid chromatography (HPLC) has been proven to be very powerful, highly resolving, and versatile for the analysis of low molecular weight condensed tannins. Using HPLC and TLC, Mueller-Harvey et al. (1987) were able to fractionate, characterise and measure complex mixtures of condensed and hydrolysable tannins, flavanoids, catechin gallates and phenolic acids. Similarly, the use of reverse-phase HPLC allowed Brandon et al. (1982) to monitor the changes that occurred in the proanthocyanidin composition of barley ears as the growth season progressed. HPLC has been proven a very effective in resolving (based on their retention times) the acid-catalysed depolymerised products formed in the presence of α–toluenethiol (Shen et al. 1986). Similarly, Mathews et al. (1997) have proposed a method that uses either gas chromatography or HPLC to quantify the products resulting from the depolymerisation of condensed tannins in the presence of phloroglucinol or benzyl mercaptan nucleophiles. In another approach, Powell et al. (1995) suggested the use of HPLC for the analysis of the anthocyanidin pigments produced by the autoxidative depolymerisation of condensed tannins produced by heating in a solution of n-butanol-HCl and Fe^{III} salts (Porter et al. 1986).

When coupled to more versatile detectors such as the photodiode-array type, HPLC becomes even more powerful and resolving. Ultraviolet-visible spectra provide by these

detectors are used for identification of peaks by comparison with standards and to check peak purity. Parameters such as the wavelength of absorption maxima and convexity interval are very useful to distinguish between compounds that have similar retention times or to confirm their identity. Using these parameters, along with retention time and a newly defined parameter they labelled as the min-max distance (distance between the minimum and the maximum in the original spectrum), Bartolomé et al. (1996) could effectively identify 24 different peaks in the chromatogram of an extract from grape seeds (*Vitis vinifera*).

Photodiode detection coupled to a HPLC system fitted with a LiChrospher 100 RP-18 column was also effectively used by Koupai-Abyazani, (1992; 1993) to identify the constituent flavan-3-ol units (i.e. catechin, epicatechin, gallocatechin and epigallocatechin and their phloroglucinol adducts) in sainfoin condensed tannins after their degradation in the presence of phloroglucinol. All the peaks were well resolved except for (-)-epicatechin-4-phloroglucinol and (+)-catechin-4-phloroglucinol which form a critical pair. Because these compounds differ only in the stereochemistry at C-3, these authors suggested that using a chiral column could attain a better separation. As it can be observed in Table 2-11, compounds with three hydroxyl groups on the B-ring had shorter retention times than those of the corresponding compounds with two hydroxyl groups. Additionally, all phloroglucinol adducts eluted faster than their corresponding flavan-3-ols.

Table 2-11. *Retention times of some flavan-3-ols and their phloroglucinol adducts*

Compound	OH position	Absolute configuration	Retention time (min)
(+)-Catechin	3,5,7,3',4'	2*R*:3*S*	39.56
(+)-Gallocatechin	3,5,7,3',4',5'	2*R*:3*S*	23.06
(-)-Epicatechin	3,5,7,3',4'	2*R*:3*R*	44.68
(-)-Epigallocatechin	3,5,7,3',4',5'	2*R*:3*R*	38.49
(+)-Catechin-4-phloroglucinol	3,5,7,3',4'	2*R*:3*S*	26.47
(+)-Gallocatechin-4-phloroglucinol	3,5,7,3',4',5'	2*R*:3*S*	13.61
(-)-Epicatechin-4-phloroglucinol	3,5,7,3',4'	2*R*:3*R*	25.83
(-)-Epigallocatechin-4-phloroglucinol	3,5,7,3',4',5'	2*R*:3*R*	16.74

HPLC conditions: Column, LiChrospher 100 RP-18 (25 cm *x* 4 mm, I.D., 5 μm); flow rate, 1ml per minute; column temperature; ambient; detection, 280 nm.

Source: Koupai-Abyazani et al. (1992).

Mass spectrometry has also been used in conjunction with both gas chromatography (Galletti and Bocchini, 1995; Galletti et al. 1995) and HPLC (Self et al. 1986) to elucidate and characterise the structure of condensed tannins. When gas-liquid chromatography is used in conjunction with mass spectrometry, trimethylsilylation or permethylation is required to confer volatibility (Mangan, 1988). Recently, Galletti and Bochini (1995) reported on the behaviour of catechin and ellagic acid subjected to thermally assisted hydrolysis methylation in the presence of tetramethylammonium hydroxide (25% aqueous). The reaction was carried out at 600°C for 5 seconds using a platinum filament pyrolyzer which, in turn, was connected to a gas chromatograph-ion trap detector mass spectrometer. Likewise, Galletti et al. (1995) reported on the analysis of wine tannin that were subjected to either pyrolysis-gas chromatography-mass spectrometry or thermally assisted hydrolysis-methylation- gas chromatography-mass spectrometry. Self et al. (1986) have applied fast atom bombardment mass spectrometry to plant polyphenols. Although complex to interpret, analysis by HPLC-mass spectrometry is rapid and definitive (Mangan, 1988).

Another technique that has been very useful in the elucidation of the molecular structures of tannins is ^{13}C nuclear magnetic resonance (^{13}C NMR). A great number of researchers have used ^{13}C NMR in their study of tannins (Jacques et al. 1973; Czochanska et al. 1979; Brandon et al. 1982; Hemingway et al. 1983; Clausen et al. 1990; Roux, 1992; Eberhardt and Young, 1994; Ayres et al. 1997; McNabb et al. 1998). Assignments for the resonances for model compounds and isolated polymer preparations have been reported by Czochanska et al. (1980), Karchesy and Hemingway (1980), Porter et al. (1982), Newman et al. (1987), Foo et al (1996) and Foo et al. (1997). The use of ^{1}H NMR has also been reported in the literature for the study of the structure of tannins (Haslam, 1977, Porter and Foo, 1982, Hemingway et al. 1983)

The use of ^{13}C NMR provides information on the procyanidin:prodelphinidin ratios, the stereo chemistry of the heterocyclic ring of the monomer units, the nature of the chain-terminating unit and the number average molecular weight (estimated as average chain length x 288; Foo and Porter, 1980). The purity of the tannin preparation can also be determined by studying ^{13}C NMR spectra. For example, peaks at 56 ppm (1 ppm corresponds to a 22.6 Hz shift from TMS on a 22.6 MHz applied field strength) have been related to the presence of lignin (Newman and Porter, 1992). Likewise, peaks

between 160 and 170 ppm have been related to the presence of hydrolysable tannins. Other peak assignments are described in Figure 2-5.

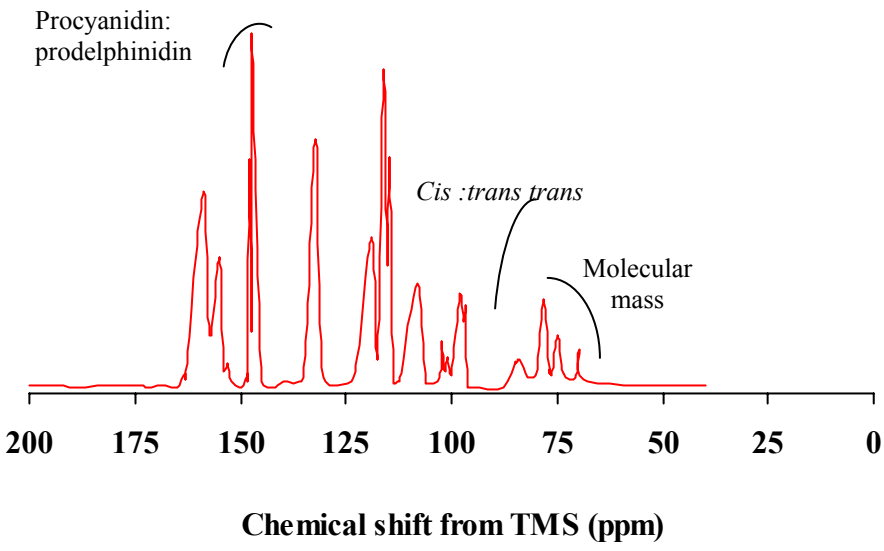


Figure 2-5. *An example of a ^{13}C NMR spectrum of condensed tannins. Peaks at 146 ppm represent C-3' and C-5' carbons of prodelphinidin unit, whereas peaks at 145 and 144 ppm represent C-3' and C-4' carbons of procyanidin unit. Peaks at 77 and 85 ppm represent the C-2 carbons of* cis *and* trans *units, respectively. The peak at 73 ppm the C-3 carbon of the extender units, whereas the peak at 69 ppm represent the C-3 of a terminal unit. Absorbances at 38 and 40 ppm correspond to C-4 (*cis *and* trans*, respectively) and allow confirmation of the C-2 interpretations. (Note: 1 ppm corresponds to a 22.6 Hz shift from TMS on a 22.6 MHz applied field strength.*

Adapted from Ayres et al. (1997).

Czochanska et al. (1980) used ^{13}C NMR to study seventeen species of plants including leaf, root, fruit and bark samples. Foo and Porter (1980) have used this technique to characterise samples in a broader survey and Foo et al. (1982) characterised the tannins of fodder legumes using ^{13}C NMR, vapour pressure osmometry and gel permeation chromatography. In successive reports and using mainly ^{13}C NMR, Foo and co-workers (1996, 1997) reported on the detailed study of condensed tannins from *Lotus corniculatus* and from *Lotus pedunculatus*. Tannins from these closely related species were found to differ greatly in their composition of extender and terminal flavan-3-ol units.

Work by Hemingway et al. (1996) has suggested that when analysing flavan-3-ols in their biologically significant phenolic forms or their methyl ether or methyl ether acetate derivatives, some of the assignments commonly reported for the H atoms of the A-ring are incorrect. However, recent work has suggested that two-dimensional NMR techniques for the study of tannins afford a powerful probe not only for distinction

between different proanthocyanidin dimers, but also allows full and unambiguous assignments, even for both major rotameric forms, without the need for derivatisation (Debruyne et al. 1996).

As was already stated, ^{13}C NMR has been successfully used for the determination of *number-average* molecular weight of tannins. Likewise, vapour pressure osmometry is a very reliable technique that has been used to estimate this same parameter (Foo et al. 1982). A problem with using these techniques for the determination of molecular weights is that two tannins with the same *number-average* molecular weight could have very different molecular mass profiles. This was demonstrated by the work of Williams et al. (1983). These authors used gel permeation chromatography for the determination of the molecular weights of peracetate derivatives of condensed tannins from 32 different samples, which yielded both the *number-average* MW and *weight-average* molecular weight of the compounds under study. Gel permeation chromatography profiles differed considerably in shape and the authors considered necessary to classify them into five types according to peak symmetry. As it is shown in Table 2-12, tannins with similar *number-average* MW can have completely different *weight-average* MW.

Table 2-12. *Molecular weights of some condensed tannins.*

Plant species: organ	Number-average	Weight-average	Polydispersity[1]
Trifolium repens, flower	3100	5000	1.61
Lotus tenuifolius, root	3100	10000	3.22
Phoenix canariensis, leaf	3400	4300	1.26
Musa sapientum, fruit skin	3400	16100	4.74
Astelia fragrans, inflorescence	3500	5500	1.57
Phormium cookianum, leaf	3500	154000	44.0
Feijoa sellowiana, fruit (unripe)	4200	7800	1.86
Podocarpus totara, leaf	4200	9400	2.24
Grevillea robusta, leaf	4500	79000	17.6
Lotus pedunculatus, root	4500	34000	7.39

[1]*Polydispersity = number-average MW/ weight-average MW.*

Source: Williams et al. (1983).

Other researchers have also used gel permeation chromatography for the determination of the molecular weight of tannins following derivatisation (Foo et al. 1982; Clausen et al. 1990). One disadvantage of this technique is that because tannins are too polar to be

separated on most gel permeation columns, they must be derivatised. This involves acetylation with pyridine-acetic anhydride (1:1) overnight and subsequent precipitation of the resulting peracetates by the addition of water. Besides the obvious inconveniences involved in the handling of pyridine and acetic anhydride, the main problem with this procedure is that it produces a form of tannins that is distant from the form tannins are found in nature. A further problem arises from the fact that the eluted tannin peracetates are detected with a UV monitor at 270 nm, although it is recognised that procyanidin and prodelphinidin have different extinction coefficients at this wavelength. Thus, for this technique it is assumed that the prodelphinidin: procyanidin: propelargonidin ratio remains constant throughout the molecular weight profile, a condition which seems very difficult to satisfy judging by the diversity in composition of most tannin extracts.

The further assumption that the resulting degree of acetylation is not variable (i.e. always complete) appears to be easier to satisfy, although to our knowledge this has not been fully tested over a range of condensed tannins. Hence, the molecular weight of a constituent peracetate unit is considered to be *ca* 500 Dalton, an estimation that would correspond to the penta-acetate of procyanidin units. However, although it is well established that procyanidin is the most abundant constituent of condensed tannins, it has also been observed that in some tannins, prodelphinidin is more prevalent. As shown in Table 2-13, the peracetates resulting from the acetylation of prodelphinidin units have molecular weights that are more than 11% higher than those of procyanidin peracetate units. It should be pointed out that the preceding discussion has not included the potentially enormous impact that glycosylation of tannins (Foo et al. 1997) could have on the molecular weight of the resulting peracetates.

Table 2-13. *Molecular weights of the peracetates of some flavan-3-ols.*

Flavan-3-ol peracetate	MW of flavan-3-ol	MW of peracetate
Triacetate of guibourtinidol	254	380
Tetra-acetate of afzelechin or fisetinidol	271	439
Penta-acetate of catechin, robinetinidol or prosopin	288	498
Hexa-acetate of gallocatechin	305	557

Ultra-centrifugation is another technique that has been used to estimate the molecular of condensed tannins (Jones et al. 1976). However, the ultracentrifuge yields *z-average* molecular weights (M_z), and the relationship between *number-average* molecular weight

and M_z is not straightforward (Foo and Porter, 1980). In turn, Porter (1984) reported the use of low-angle laser light scattering for the determination of the weight-average molecular weight of condensed tannins.

Indeed, a great number of techniques are now available to researchers for the elucidation and characterisation of tannins. Some are better than others are, but almost without exception, there are limitations to the information provided by these techniques. This was well illustrated by Ayres et al. (1997) who used ^{13}C NMR to determine the procyanidin: prodelphinidin ratio in a number of condensed tannins, including those from *Salix alaxensis*, which were observed to consist of a 50:50 mixture of procyanidins to prodelphinidins. These authors hypothesised that such a ratio could arise from (i) a blend of two tannins, one a pure procyanidin and the other a pure prodelphinidin; (ii) condensed tannins in which the units are randomly incorporated; or (iii) condensed tannins in which the units are assembled in an ordered manner. To the knowledge of these authors, none of these unresolved features of condensed tannins can be directly elucidated with ^{13}C NMR spectroscopy, and some are beyond any established analytical techniques.

Undoubtedly, however, great strides in our understanding of tannin structure and its relationship to tannin biological activity have been attained through the use of these techniques. This is certainly becoming more the case of the condensed tannins from temperate forage legumes. Unfortunately, research with tanniniferous tropical forage legumes, has not been blessed with this degree of sophistication and even today, the impact of the presence of tannins in tropical legumes on the nutritional status of ruminants is investigated with the aid of colorimetric methods at best. It is not unreasonable to expect that condensed tannins from tropical legumes will differ from those of temperature legumes both in molecular weights and constituent flavan-3-ol units. If anything, the concentration of condensed tannins in tropical legumes is far greater than in temperate legumes. It then becomes necessary to study the structure of condensed tannins from tropical legumes in far greater detail than it is being done at present.

2.4 INTERACTIONS OF CONDENSED TANNINS WITH OTHER MOLECULES

2.4.1 Mechanisms of the interaction

Although it has been shown that condensed tannins form complexes with proteins, polysaccharides, nucleic acids, steroids, alkaloids, and saponins (Haslam, 1986) and even minerals (Reed, 1995), the great trust of research has been directed towards understanding the mechanisms of tannin-protein interactions. However, studies of tannin-carbohydrate and tannin-caffeine interactions suggest that the basic mechanism of these interactions is essentially the same to that underlying tannin-protein complexation (Spencer et al. 1988, Mueller-Harvey and McAllan, 1992). In consequence, it seems appropriate to discuss these interactions as a whole rather than as separate mechanisms.

In principle, given the diversity in molecular structures among tannins, the complexation of tannins with other molecules may either occur by hydrogen bonding, ionic bonding, hydrophobic bonding or covalent bonding. Although charged groups are absent in the condensed tannins (Gustavson, 1956; Haslam, 1966), the possibility of ionic interactions in preparations of hydrolysable tannins (especially ellagitannins) cannot be entirely excluded since charged groups could arise through partial hydrolysis of the depside linkages (Oh et al. 1980). On the other hand, when discussion is limited to the condensed tannins at acidic to neutral conditions that possibility is very remote (Haslam, 1977). Condensed tannins become charged only at high pH by dissociation of the phenolic hydroxyl groups with formation of phenoxide ions (Oh et al. 1980).

Tannins can form covalent bonds with proteins through oxidative polymerisation reactions as a result of heating, exposure to ultra-violet radiation and the action of polyphenol oxidase (Reed, 1995). This represents an irreversible change that requires molecular oxygen and is favoured by high pH (Oh et al. 1980). On the hand, tannin-protein complexes formed via hydrophobic and hydrogen bonds are of a reversible nature (Loomis and Battaile, 1966: Oh et al. 1980).

It is now generally accepted that these last two types of attachment modes, hydrogen bonding and hydrophobic bonding, are the most important in the complexation of tannins with other molecules. The presence of hydrogen donors in the form of phenolic hydroxyl groups in the tannins and of hydrogen acceptors in the form of carbonyl

functions of the peptide linkages of the proteins would naturally lead to the formation of hydrogen bonds (Oh et al. 1980; Haslam, 1989). Likewise, both protein and tannins contain hydrophobic regions, the aromatic nuclei of the tannins and aliphatic side chains of the protein amino acids, which would seem equally feasible would participate in the interaction phenomena.

Many investigators have suggested that hydrogen bonding would be the primary mode of attachment employed by tannins to bind proteins (Goldstein and Swain, 1963; Loomis and Battaile, 1966; van Sumere et al. 1975). The emphasis of hydrogen bonding as a vehicle for association was derived from the work of K.H. Gustavson, who observed that large amounts of tannins were "fixed" by modified polyamides, such as nylons, which contain the peptide linkages as "the only reactive group of importance". In turn, other workers, notably Oh et al. (1980) have drawn attention to the fact that hydrophobic effects may dominate the complexation process.

Evidence has accumulated that is most reasonably interpreted in terms of hydrophobic bonding in the formation of tannin-protein complexes. For example, Goldstein and Swain (1965) observed that detergents could dissociate tannin-protein complexes. Gray (1978) observed that low molecular weight phenolic substances were strongly absorbed by polystyrene resins. In addition, Hagerman and Butler (1980a) observed that the interaction of tannins with bovine serum albumin was stimulated by the addition of simple alcohols (methanol or ethanol). Likewise, Asquith and Butler (1985) reported that purified tannins from sorghum and quebracho precipitated more protein when the solvent contained 20% alcohol than in 100% water; with methanol giving more precipitation than ethanol. Similarly, Calderon et al. (1968) noted that ethanol increased the amount of gelatine precipitated by quebracho tannin. On the other hand, Hagerman and Butler (1980), observed that the non-polar solvent dioxane inhibits bovine serum albumin precipitation. Finally, the work of Oh et al. (1980) showed that proteins absorbed in a column of Sepharose contained immobilised phenolics were effectively eluted by anionic and non-ionic detergents. A study of the interaction between condensed tannins and polyamino acids free in solution showed similarly that the extent of complexation was related to the number of methylene groups in the amino-acid side chain (Oh et al. 1980). All these observations imply that forces other than hydrogen bonding must be involved in the formation of tannin-protein complexes.

In view of the heterogeneous nature of potential attachment sites on the surface of proteins and of the diversity of structures in tannins, it could be reasonably assumed that the interaction between tannins and proteins would be one of mixed modes (Oh et al. 1980). In this regard these authors pointed that "both hydrogen bonding and hydrophobic interaction are solvent-dependent phenomena. Thus, hydrogen bonding is favoured to occur in hydrophobic solvents such as carbon tetrachloride, while hydrophobic bonding between solutes primarily occurs in hydrophilic or polar solvents. The two functional groups that interact in the formation of hydrogen bonds are in aqueous media surrounded with hydrogen-bonded water molecules before the reaction takes place. The overall reaction involves breaking these solvent bonds as well as establishing the new hydrogen bonds between the solutes and will proceed if the reaction is accompanied by a decrease in free energy. Available thermodynamic data suggests that this reaction is not favoured thermodynamically. On the other hand, hydrophobic interaction between solvated hydrophobic amino acid residues is strongly favoured and will take place if steric factors permit".

A characteristic feature of hydrophobic interaction is that its strength increases with ionic strength and temperature. On the other hand, hydrogen bonding depends more strongly than hydrophobic bonding on solution pH (Mueller-Harvey and McAllan, 1992). Many investigators have observed that precipitation of protein by tannins is greatest within one pH unit of the isoelectric point of the protein (Jones and Mangan, 1977; Hagerman and Butler, 1978; Martin and Martin, 1983). Interestingly, Oh et al. (1980) observed that increases in neither temperature nor ionic strength resulted in an increment in the precipitation of bovine serum albumin by tannins. On the other hand, increments in these two factors resulted in significant increments in the observed precipitation of gelatine and poly-L-proline. In turn, Hagerman and Butler (1978) observed that the precipitation of bovine serum albumin was not affected by ionic strength (0.05 to 5.0 M) or temperature (4 and 22 °C).

The above observations appear to support those of Haslam and Lilley (1986) that the complexation of bovine serum albumin by gallo- or ellagitannins is mostly governed by hydrogen bonding. In turn, Baxter et al. (1997) after performing NMR studies which involved titration of a series of polyphenols with a synthetic 19-residue proline-rich protein fragment, observed that the predominant mode of association is a hydrophobic

stacking of the polyphenol ring against the pro-S face of proline. These authors also observed that the first proline residue of a Pro-Pro sequence is a particularly favoured binding site. Hence, it appears that the relative importance of hydrogen bonding versus hydrophobic bonding depends on the nature of both the protein and the tannin (Mueller-Harvey and McAllan, 1992).

2.4.2 Factors affecting the formation of complexes between tannins and other molecules

As the preceding discussion suggests, the interaction between tannins and other molecules can be expected to be very complex and likely to be affected by a number of factors. Indeed, research on tannin-protein complexation has shown that characteristics of both tannins and proteins can affect complex formation (Spencer et al. 1988; Mueller-Harvey and McAllan, 1992), so that tannin-protein binding can be specific for both the protein (Hagerman and Butler, 1981) and the tannin (Asquith and Butler, 1986). As a consequence, there is no such thing as a generalised tannin-protein interaction (Mole and Waterman, 1987b).

2.4.2.1 *Characteristics of tannins that influence their complexation with other molecules*

One of the factors that can influence the astringency of tannins (i.e. their ability to precipitate proteins) is their molecular weight. It has been suggested that phenolic compounds of low molecular weight form unstable crosslinks with proteins, while those of high molecular weight are ineffective as tannin agents (Salunkhe et al. 1990). The molecular weight of condensed tannins varies widely according to factors such as forage species and plant tissue (Foo and Porter, 1980; Foo et al. 1982; Williams et al. 1983; Cano-Poloche, 1993; Cano et al. 1994), sample treatment (Cano et al. 1994) and throughout the growth season (Butler, 1982).

Bate-Smith (1973a) effectively used his haemanalysis technique to demonstrate that the ability of condensed tannins to precipitate the proteins of haemolysed blood increased as the molecular weight increased from 576 to beyond 1134 (see Table 2-14). In Bate-Smith's study of the relative astringency of condensed and hydrolysable tannins, tannic acid was found to be the most effective precipitating agent and was assigned a relative astringency of 1.0. However, after application of his technique to extracts containing

only condensed tannins or only ellagitannins, Bathe-Smith (1973a) failed to observe an extract that displayed a relative astringency similar to that of tannic acid. The only exception to this was an extract from leaves of *Shorea macrophylla,* which the author had found to contain tannins of both the hydrolysable and condensed types. Because genuinely pure condensed tannins were not available in Bate-Smith's study, Porter and Woddruffe (1984) carried out haemanalysis measurements on a number of condensed tannins of known molecular weights (see Table 2-14). Their results suggested that condensed tannins with a *number-average* molecular weight of 2400-2700, have a very similar relative astringency to tannic acid (1250 ± 60 *number-average* molecular weight).

Table 2-14. *Relative astringency (as described by Bate-Smith, 1973a) of some condensed tannins*.

Condensed tannin	Molecular weight, Dalton	Relative stringency
Mixed dimers[1]	576	~0.10
Mixed trimers[1]	864	~0.28
Tetramer[1]	1134	~0.38
Higher oligomers[1]	Beyond 1134	~0.50
Crataegus oxyacantha[2]	1200	0.73
Watsonia ardernei[2]	2100	0.83
Pinus radiata, phloem[2]	2100	0.89
Pinus radiata, leaf[2]	2700	1.00
Aesculus x carnea	3300	0.91
Rhopalostylis sapida[2]	3750	0.99

Adapted from [1]Bathe-Smith (1973a) and [2]Porter and Woodruffe (1984).

Perhaps contrary to the findings of Porter and Woodruffe (1984), Cano et al. (1994) observed that the astringency of condensed tannins from tropical legumes, as determined by radial diffusion, decreased when the *relative degree of polymerisation* (as described by Butler 1982) of tannins reached 8-9 monomer units (2400-2700 Dalton). A very interesting observation in the study of Cano and co-workers was that tannins extracted from lyophilised and oven-dried *Calliandra* sp. and *Phyllodium* sp. leaves, were shown to have a higher *relative degree of polymerisation* than tannins extracted from fresh leaf samples. Such an increase in molecular weight was accompanied by a marked increase (2x) in the protein precipitating capacity of *Calliandra* tannins. In turn, Jones et al. (1976) reported that tannins of high *z- average* molecular weight (17,000-28,000 Dalton)

from sainfoin (*Onobrychis vicifolia*) leaves were less effective in precipitating proteins than smaller tannins from other legumes including *Lotus* sp. (6000-7100 Dalton). Perhaps in agreement with these observations, Kumar and Vaithiyanathan (1990) suggested than when the molecular weight (*number-average*?) is rather large (>5000), the condensed tannins become rather insoluble in physiological solutions and lose their protein-precipitating capacity.

A final point with regard to the molecular weight of tannins involves the concept that tannins are polydisperse molecules (see Section 2.2.5.3), thus creating the possibility that different tannin fractions from the same sample will display different binding affinities for the same protein. This was elegantly shown by the work of Oh and Hoff (1979) who fractionated the tannins of grape (*Vitis vinifera* L.) using affinity chromatography in a Sephadex G-50 column. They reported that the gelatine precipitation of the resulting tannin fractions increased as their tentative molecular weight increased from 900 to 2,100 Dalton. Further increases in molecular weight resulted in a decline in gelatine-precipitating capacity. Likewise, Kumar and Horigome (1986) observed that the astringency of five condensed tannin fractions from black locust (*Robinia pseudo acacia* L.) leaves were related to their molecular weight (see Table 2-15).

Table 2-15. *Protein-precipitable phenolics (Hagerman and Butler, 1978) and ability to inhibit the activity of β–glucosidase (Goldstein and Swain, 1965) of five different fractions of the condensed tannins from black locust leaves.*

Parameter	Fraction				
	A	B	C	D	E
Degree of polymerisation	4.12	3.00	2.13	1.91	1.53
Protein-precipitable phenolics	0.99	0.96	0.94	0.80	0.74
% inhibition of β–glucosidase	51.9 ± 2.3	27.4 ± 2.6	22.7 ± 0.9	17.4 ± 1.3	11.0 ± 0.9

Source: Kumar and Horigome (1986).

From these trials, it is readily apparent that the molecular weight of condensed tannins plays a very significant role in determining their effectiveness as protein-precipitants. Nonetheless, from these studies it is difficult to predict with certainty at what molecular weight the condensed tannins from a given source will display the highest astringency. Factors contributing to the ambiguity include the use of different test proteins and assay

methods to determine tannin astringency and degree of polymerisation, but perhaps the reported results are also indicative of the specificity of tannin-protein interactions.

Another factor that can influence the observed astringency of tannins is their conformational flexibility. Tannins with highly flexible conformations interact more strongly with protein such as bovine serum albumin (Beart et al. 1985; Horigome et al. 1988). In fact, the "relatively lower astringency" of condensed tannins compared to other polyphenols may be explicable, in part, in terms of the conformational restraints imposed by restricted rotation about the repeating 4→8 and 4→6 interflavan bonds (Spencer et al. 1988). The loss of conformational freedom is reflected in a reduced capacity to bind to protein. Such is the case with the rigid, virtually inflexible, propeller-shape vescalagin and castalagin, which are analogous to β-1,2,3,4,6-penta-*O*-galloyl-D-glucose, but in a molar basis are bound less effectively to protein than its precursor (Spencer et al. 1988).

Table 2-16. *Inhibition of β-glucosidase activity by polyphenols of different molecular weights and conformational flexibility*

Polyphenol	Molecular weight	Inhibition of β-glucosidase, K_i (10^{-4} M) [1]
β-1,2,6-tri-*O*-galloyl-$_D$-glucose	636	10.80
β-1,2,3,6-tetra-*O*-galloyl-$_D$-glucose	788	2.50
β-1,2,3,4,6-penta-*O*-galloyl-$_D$-glucose	940	0.85
Casuarictin	936	1.57
Rugosin-D	1874	0.08
Sanguin H-6	1870	0.40

[1]Non-competitive inhibition, assuming Michaelis-Menten kinetics, K_M remains unaffected and K_i is a quantitative measure of the affinity of the polyphenol for the enzyme. Low values of K_i indicate a relatively strong affinity for the protein.

Source: Ozawa et al. (1987)

The impact of conformational flexibility on the astringency of tannins was elegantly demonstrated by Ozawa et al. (1987) who studied the impact of different hydrolysable tannins on the activity of β-glucosidase. As shown in Table 2-16, β-glucosidase inhibition was heavily influenced by the molecular weight of the tannin. However, polyphenols of similar molecular weight, for example β-1,2,3,4,6-penta-*O*-galloyl-$_D$-glucose and casuarictin, were observed to differ in their ability to inhibit β-glucosidase. Less inhibitory compounds were those that had less flexible configurations due to the

formation of digalloyl moieties, since casuarictin possess two of such biphenyl linkages whereas β-1,2,3,4,6-penta-*O*-galloyl-D-glucose has none. In turn, sanguin H-6 has three digalloyl complexes and rugosin-D just one.

Condensed tannin stereochemistry has also been reported as a factor that affects tannin astringency. Jones et al. (1976) observed that the astringency of condensed tannins from pasture legume species was approximately in order of their prodelphinidin content. The greater the amount of prodelphinidin, the more astringent the tannins. Likewise, Ayres et al. (1997) reported that high antiherbivore activity in condensed tannins was highly correlated with a high proportion of prodelphinidin. However, McNabb et al. (1998) observed that the contrasting prodelphinidin content in tannins from *Lotus corniculatus* (1900 Dalton) and *Lotus pedunculatus* (2200 Dalton) had little or no effect on their reactivity with Rubisco. Clausen et al. (1990) observed that purified tannins from bitterbrush (*Pursia tridentata*) were more astringent than those from blackbrush (*Coleogyne ramosissima*), despite being of similar average molecular weight. However, the stereochemistry at C-3 and C-4 was different, with catechin/epicatechin ratios of 55:45 and 20:80 for bitterbrush and blackbrush tannins, respectively. Interestingly, snowshoe hares fed basal diets supplemented with these purified tannins preferred tannins from bitterbrush over those from blackbrush.

From the standpoint of tannins, a final factor that is of importance in the complexation of tannins with proteins is the water solubility of the polyphenol. A broadly inverse relationship exists between the strength of the association with protein and the solubility of the polyphenol in water. Low solubility favours strong association (Spencer et al. 1988).

2.4.2.2 *Characteristics of proteins and other molecules that influence their complexation with tannins*

Given that tannin-protein binding can be quite specific for both the protein and the tannin (Butler, 1989), there are protein characteristics that dictate their affinity for tannins. Indeed, a protein with a high affinity for tannin may be selectively bound and precipitated out of a large excess of proteins with lesser affinity. Hagerman and Butler (1981) devised a competitive binding assay that has been extremely useful in studies of proteins to determine structure-activity relationships. The assay uses bovine serum

albumin and lysozyme labelled with iodine-125, somewhat in the fashion of a competitive immunoassay. Using this technique, several investigators have observed that the relative affinities of the various competitors varies, sometimes over more than four orders of magnitude (Hagerman and Butler, 1981; Mehansho et al. 1983; Asquith and Butler, 1985; see Table 2-17).

Table 2-17. *Relative affinity of different protein for sorghum tannin*

Protein	Relative affinity
Gelatine	14.0
Proline-rich salivary protein	6.8
Pepsin	1.1
Bovine serum albumin	1.0
Bovine haemoglobin	0.068
Ovalbumin	0.016
β-Lactoglobulin	0.0087
Lysozyme	0.0048
Soybean trypsin inhibitor	<0.0010

Source: Butler (1989)

Indeed, proteins differ greatly in their affinities for a particular tannin (Asano et al. 1982; Hagerman and Klucher, 1986). Those proteins that bind strongly to tannins have properties that include high molecular weight and open and flexible structures (Asquith and Butler, 1986; Mueller-Harvey and McAllan, 1992). The working principle behind those factors is exactly the same as in the case of tannins. Longer amino acid chains signify a greater number of attachment sites, while greater conformational freedom reduces the limitations caused by steric factors.

Without doubt, the role played by protein size and conformational flexibility in tannin-protein interactions is highly significant. However, there are some other factors that also influence the affinity of protein for tannins. One obvious factor to consider is amino acid composition. Indeed, Handley (1954) observed that free amino acids of basic type precipitated tannins from solution, an observation that was corroborated by Mole and Waterman (1987a) with the basic amino acids arginine and histidine. Likewise, Oh et al. (1980) observed that the basic poly-L-lysine displayed high affinity for tannins, whereas no reaction was observed when tannins were mixed with the acidic poly-L-aspartic acid

or poly-L-glutamic acid. Oh et al. (1980) also reported that among the polyamino acids tested, poly-L-proline and poly-L-hydroxyproline also exhibited high affinity for tannins.

Indeed, a common feature of protein and polypeptides with a high affinity for tannins is their richness in the imino acid proline. Hagerman and Butler (1980b) observed that proteins that remain associated with tannins during tannin purification have a very high proline content. Proline-rich (up to 45%; Mehansho et al. 1987b) salivary proteins with high affinity for tannins are produced in tannin-fed rats (Mehansho et al. 1983) and mice (Mehansho et al. 1985), in mule deer (Robbins et al. 1987b), deer (Austin et al. 1989), monkeys (Mehansho et al. 1987a), rabbits, hares and humans (Mole et al. 1990a).

Hagerman and Butler (1981) suggested that proline richness could increase protein affinity for tannins due to two major factors. The first is that proline rich polypeptides have much more open and flexible configurations. Second, proline rich proteins have an increased capacity to form strong hydrogen bonds due to increased accessibility of the peptide linkages. In this regard, Spencer et al (1988) observed that compact globular proteins have poor affinity for β–pentagalloyl-D-glucose, perhaps because they do not provide in aqueous media, sufficient sites for polyphenol complexation.

It has also been reported that the presence of oligosaccharides in glycoproteins enhances the affinity and selectivity of binding to tannins and increases the solubility of the resulting complexes (Asquith et al. 1987). Although the oligosaccharide itself had no affinity for the tannins, its removal apparently caused proteins to assume a more compact conformation. With regard to the effect of carbohydrate on the solubility of the resulting tannin-protein complex, it has been reported that bovine submaxillary mucin is not precipitated by tannins (Jones and Mangan 1977; Perez-Maldonado et al. 1995), perhaps suggesting that the resulting complex is of soluble nature. On the other hand, Strumeyer and Malin (1970) suggested that the resistance of a yeast invertase against denaturation by tannins was due to glycosylation.

Finally, with reference to tannin-polysaccharide complexation, research has shown that polysaccharides that can develop secondary structure containing hydrophobic cavities do associate strongly with tannins (Ozawa et al. 1987). In view of this, these authors suggested that the loss of astringency that occurs upon ripening of some fruits might well

be due to the production of water-soluble fragments of the pectin structure as the cellular structure softens.

2.4.2.3 *Additional factors that affect the complexation of tannins with other molecules*

Evidence has accumulated suggesting that the formation of insoluble protein complexes is dependent upon the relative concentration of tannin and protein. One of the first reports of this behaviour was provided by Van Buren and Robinson (1969), who observed redissolution of tannin protein complexes in the presence of an excess of either component. In a related observation, Robbins et al. (1987a) reported that the amount of bovine serum albumin precipitated by tannin depended upon the protein-precipitation assay used. In studying this anomaly, Hagerman and Robbins (1987) concluded that differences were related to the tannin-protein ratios used in the different protein-precipitation assays. When protein precipitation by a constant amount of tannin is plotted against the amount protein in the reaction mixture, a *quasi*-bell-shaped curve is obtained that could be divided into three regions. (i) When little protein is present, there is an excess of tannin in the reaction mixture, and little precipitate is formed. (ii) When a moderate amount of protein is present, the optimal ratio between tannin and protein, or equivalence point, is achieved, and precipitation is maximised. (iii) When a large excess of protein is added, a small precipitate is formed, which was composed only of protein.

Similar observations were reported by Mole and Waterman (1987a), who also observed that the shape of the protein precipitation curve was dependent upon the source of tannin used. Thus, with some tannins such as from quebracho, the equivalence point occurred over a very broad range of tannin-protein ratios. On the other hand, with other tannins (i.e. *Quercus* sp.) the equivalence point occurred over a very narrow range. Furthermore, when tannic acid was reacted with protein, the shape of the protein precipitation curve was not the same as that reported by Hagerman and Robbins (1987).

Another interesting occurrence in the study of Mole and Waterman (1987a) concerned the behaviour of the phenolics during the same precipitation assays. Determination of the amount of phenolics remaining in solution showed that the fall in supernatant phenolics did not exactly reciprocate the pattern seen in the curve for protein precipitation. There was no flat minimum, but rather a supernatant phenolic curve that began to rise again at

the higher concentrations of protein. Again, the shape of this second curve was dependent upon the tannin source used. A final observation from this work was that in most cases, maximum protein precipitation and minimum supernatant phenolics did not coincide, generally the latter occurring first. These observations indicate that at higher protein concentrations, protein in the precipitate is associated with relatively less and less tannins, until a point is reached where precipitates becomes less stable due to the smaller percentage of tannins incorporated within them (Van Buren and Robinson, 1969).

These observations are perhaps better understood in light of the concepts of Spencer et al. (1988): "The association of polyphenols with protein is principally a surface phenomenon. The efficacy of polyphenol binding to proteins derives from the fact that polyphenols are multidentate ligands able to bind simultaneously (via different phenolic groups) at more than one point at the protein surface. When polyphenols cause precipitation of proteins from solution two situations may be envisaged. At low protein concentrations the polyphenol associates at one or more sites at the protein surface, to give a mono-layer that is less hydrophilic than the protein itself. Aggregation and precipitation then ensue. Where the protein concentration is high the relatively hydrophobic surface is formed by complexation of the polyphenol onto the protein and by cross-linking of different protein molecules by the multidentate polyphenols. Precipitation then follows as above. This tendency to cross-link protein molecules explains the changing stoicheiometry of the aggregates with changing protein concentrations. More polyphenol is thus required to precipitate proteins from dilute solution than from concentrated solutions".

The above hypothesis is well supported by the observations of Jones and Mangan (1977) who studied the precipitation of Fraction 1 leaf protein in the presence of increasing concentrations of condensed tannins. Fraction 1 protein was precipitated by one-twelfth its weight of sainfoin tannin. This represents a molar ratio of Fraction 1 protein to tannin of *ca* 1:2. Further tannin, up to a maximum of 23 molecules of tannin per molecule of protein, could be absorbed provided there was a large excess of tannin in solution. It also explains why at Hagerman and Robbins's "equivalence point", even simple phenolics can precipitate proteins from solution (McManus et al. 1981), provided they can be maintained at concentrations sufficient to push the equilibrium in favour of the protein-phenol complex (Spencer et al. 1988).

As discussed earlier, under the proper conditions, other factors can also influence the complexation of tannins with other molecules. These include temperature (Jones and Mangan, 1977; Hagerman and Robbins, 1987), reaction time (Hagerman and Robbins, 1987), ionic strength (Goldstein and Swain, 1963, Oh et al. 1980), pH (Hagerman and Butler, 1978; McNabb et al. 1998) and solvent used (Loomis and Battaile, 1966; Hagerman and Butler, 1980a).

Conjugation of these and the above-discussed factors make the study of tannin complexation and tannin nutritional effects an extremely complex subject. And this without considering herbivore adaptive responses to tannin intake (see next section). Even in "simpler" case-scenarios, the behaviour of tannins is bewildering to say the least. Illustrations of this point abound in the literature. Take for example the work of Barahona et al (1996), who treated leaves from different tropical legumes with different amounts of polyethylene glycol 8000 (PEG) to bind soluble condensed tannins and then determined the astringency of the unbound tannins. As the amount of PEG added increased, the concentration of 70% aqueous methanol-extractable tannins decreased. At the same time, a corresponding increase in "fibre" bound tannins was observed, so that the total amount of tannins in the sample remained relatively constant. Interestingly, astringency (g of precipitated protein per g of unbound tannins) of those samples, determined by the radial diffusion assay of Hagerman (1987) with modifications by Lareo et al. (1990), had a quadratic relationship to the amount of PEG added.

To explain this behaviour it must be considered that responses in Hagerman's assay are due to the diffusion of soluble tannins through a protein-containing gel and that tannin-PEG complexes are very stable at pH 5.0 (Makkar et al. 1995b) and even withstand extraction with boiling detergents (Carulla, 1994). Thus, it appears that under the conditions of Barahona's study, the assayed tannin where comprised of at least three different fractions, that differed in their affinities for bovine serum albumin, the test protein. Fraction one consisted of tannins with very high affinity for PEG but with very low affinity for the test protein. Tannins in fraction two have a low affinity for PEG but high affinity for bovine serum albumin. Finally, tannins in fraction three have little or no affinity for both PEG and bovine serum albumin. Certainly, it is difficult to predict with any certainty if this would also be the situation *in vivo*.

2.4.3 Dissociation of tannin-protein complexes

Condensed tannins have traditionally been thought to reduce plant preference by digestion inhibition (Feeny, 1969, 1976; Rhoades and Cates, 1976; Swain, 1979) because they can complex and render inactive digestive enzymes (Swain, 1979) and precipitate dietary proteins (Feeny, 1969). Precipitated protein would presumably be less digestible than soluble protein and the herbivore would thus obtain inadequate dietary protein from tannin-rich plants. Under this presumption, disruption of the complexes between tannins and other molecules will counteract the potentially deleterious effects of tannins. At any rate, since most interactions involving tannins are reversible, it is always helpful to understand the mechanics of the dissociation of tannin complexes.

Goldstein and Swain (1965) observed that detergents could dissociate tannin-protein complexes. Similar results were reported by Oh et al. (1980) using non-ionic, ionic and cationic detergents. Hagerman and Butler (1978) observed that tannin-protein complexes formed *in vitro* by mixing up to 1 mg of tannin and 2 mg of protein, could be reversed by the addition of 4 ml of a 1.0% sodium dodecyl sulphate (SDS), 5% triethanolamine solution. By contrast, precipitation of protein by sorghum tannin was completely prevented by 0.1% SDS (Hagerman and Butler, 1980a).

Loomis and Battaile (1966) suggested that organic solvents might disrupt tannin-protein complexes. Addition of some solvents, including dioxane and dimethylformamide inhibited protein precipitation by tannins (Hagerman and Butler, 1980a), whereas phenol dissociated tannin-protein complexes, releasing up to 90% of precipitated protein (Hagerman and Butler, 1980b). Likewise, acetone was reported to inhibit the formation of tannin-protein complexes (Hagerman, 1987). Quite on the contrary, the presence of simple alcohols resulted in increased precipitation of proteins by tannins (Calderon et al. 1968; Hagerman and Butler, 1980a; Asquith and Butler, 1985).

In vitro, tannin precipitates protein from aqueous solutions at moderate pH (Goldstein and Swain, 1965; Hagerman and Butler, 1980a; McManus et al. 1981; Hagerman and Klucher, 1986). Accordingly, Mole and Waterman (1987a) observed that precipitation of free amino acids by tannins was inhibited at pH 8.0 or higher, whereas Loomis and Battaile (1966) suggested that precipitation of proteins by tannin was independent of pH at pH values less than 8.0, and decreased sharply at pH 8.0. In turn, Hagerman and

Butler (1978) and Martin and Martin (1983) observed that precipitation of protein was maximised within one pH point of the isoelectric point of the protein.

Various tannin-binding agents have been employed for various purposes such as quantification of tannins (Makkar et al. 1993), extraction of enzymes (Badran and Jones, 1965) and in alleviating the adverse effects of tanniniferous foods and feeds (Barry and Duncan, 1984; Barroga et al. 1985: Carulla, 1994; Barahona et al. 1997). These tannin-binding agents include polyethylene glycol, polyvinyl pyrrolidone and polyvinyl polypyrrolidone. With varying degrees of effectiveness (Makkar et al. 1995b), all these compounds have shown to bind vegetable tannins and Jones and Mangan (1977) have suggested that tannins bind preferentially to polyethylene glycol, which displaces protein from tannin-protein complexes.

Jones and Mangan (1977) showed that, in the presence of polyethylene glycol, tannin-protein complexes are insoluble and stable at pH 4 to 6.5, but 95% of the protein was released between pH 1 and 3 and 30 % protein was released by PEG at pH values between 8-8.5. From the view point of animal nutrition, one would then expect that after escaping the rumen (pH 5 to 7), protein in tannin-protein complexes would be degraded readily by the gastric (about pH 2.5) and pancreatic (about pH 8-9) secretions (Mangan, 1988). If this is true, tannins may be a practical way of protecting protein from rumen degradation and increasing the amount of available dietary protein that reaches the duodenum. However, McNabb et al. (1998) suggested that this relationship might not be strictly correct in the small intestine, considering the observations of Wang et al. (1996c), that pH of the digesta in the abomasum was about 3-4 and increased to about 5.5 at the beginning of the small intestine. Maximum digesta pH in the small intestine was 8.0 and furthermore, digesta would only be in acidic conditions in the abomasum for 20-35 minutes.

Some of these mechanisms of dissociation have not gone unnoticed by members of the herbivore community. In fact, diverse mechanisms have been proposed by which herbivores could mitigate tannin effects, by impeding the formation of tannin-protein complexes. In insects, these include high gut pH (Berenbaum, 1980; Felton and Duffy, 1991), surfactants (Martin and Martin, 1984; De Veau and Schultz, 1992; Tugwell and Branch, 1992), high concentration of cations (Martin et al. 1985) and gut redox potential (Appel and Martin, 1990). In mammals, the interaction of tannin and

protein could also be interrupted by surfactants (Freeland et al. 1985; Mole and Waterman, 1985). However, Miller et al. (1997) observed that although surfactants can affect digestion of the tanniniferous mulga (*Acacia aneura*) by sheep, using polyethylene glycol to precipitate condensed tannins is more effective in improving mulga digestion and animal production than the use of surfactants. It should be remembered, however, that Hagerman and Robbins (1987) observed that when protein is present in excess relative to tannins, soluble tannin-protein complexes apparently formed instead of insoluble complexes, with unknown metabolic effects.

2.5 NUTRITIONAL EFFECTS OF CONDENSED TANNINS

In terms of ruminant nutrition, claims for detrimental and beneficial effects of tannins abound in the literature. For example, because dietary protein is first available to the rumen microorganisms than to the ruminant, precipitation of dietary proteins by tannins might result in enhanced efficiency of protein utilisation. This is because with most high quality forages, protein usage by rumen microorganisms can be a rather wasteful process (Mangan, 1988). Indeed, problems associated with extensive proteolysis and (or) deamination in the rumen limit production in modern feeding systems (Beever et al. 1989). By preventing the access of protein to microorganisms or their enzymes, tannins could increase the flow of dietary protein to the abomasum and small intestine of ruminant fed tanniniferous forages. Other potential beneficial effects of tannins in ruminants include the prevention of bloat in cattle (Mangan, 1988) and the control of internal parasites in ruminants without the recourse to anthelmintics (Niezen et al. 1996). On the other hand, especially when present in large quantities, tannins might lead to reduced forage consumption, perhaps as the result of reduced forage palatability and digestibility and by inhibition of digestive enzymes.

Given the intricacies of the ruminant digestive tract, determination of the nutritional effects of tannins is not a straightforward matter. Additionally, it must be recognised that tannins are only one among the extensive array of secondary metabolites produced by plants. The likely interactions between different forage constituents make singling out specific effects a very difficult task. This was aptly illustrated by the work of Freeland et al. (1985). Mice fed on diets containing tannins or saponins experienced adverse effects on voluntary feed intake and weight gain. However, when mice were allowed to choose between tannin- and saponin-containing diets, they selected combinations that suppressed the ill effects of either metabolite alone.

Diet selection, animal protective responses and the interplay of ingested dietary constituents between themselves and with rumen microorganisms and post-rumen digestive enzymes, among other factors, will determine the extent of the nutritional impact of tannins. At the end, in what concerns to forage research, animal productive performance and well being should be among the most important criteria by which to judge the nutritional impact of the presence of tannin in forages.

Table 2-18. *Effects of tannins on the voluntary feed intake by sheep*

Source	Key comparison	Effect on voluntary feed intake	Reference
Tara tannin	Soybean meal offered as a protein supplement with and without tannins (0.89%[1])	No difference between treatments.	Driedger and Hatfield (1972)
Lotus pedunculatus	Forage offered had low (4.6%) and high (10.6%) tannin content	No difference in organic matter intake, but lower intake of digestible organic matter with the high tannin forage.	Barry and Duncan (1984)
Lotus pedunculatus	Forage offered with and without PEG[2] (2.4 g/g of tannin)	No difference in organic matter intake, but higher intake of digestible organic matter and metabolizable energy with the PEG-treated forage	Barry and Duncan (1984)
Lotus corniculatus	Forage offered contained 0.5% and 3.3% tannins	No difference between treatments.	Waghorn et al. (1987)
Lotus corniculatus	Forage (2.2% tannins) offered with and without PEG (50 g /day)	No difference between treatments.	Waghorn et al. (1987)
Mulga (*Acacia aneura*)	Forage offered with and without PEG (24 g/day)	Supplementation with PEG increased intake of forage by 50%	Pritchard et al. (1988)
Sericea lespedeza	Forage offered had high (18.1%) and lower (3.1-8.7%) tannin content	Sheep consumed more of the lower tannin forage	Terrill et al. (1989)
Mountain mahogany (*Cercocarpus montanus*)	Forage (4.1% tannins) offered as a protein supplement with and without PEG (2 g/g of tannin)	No difference between treatments.	Nuñez-Hernandez et al. (1991)
Sulla (*Hedysarum coronarium*)	Forage (4.5-5.0% tannins) offered with and without PEG (100 g/day)	PEG supplementation slightly depressed forage intake	Terrill et al. (1992a)
Lotus pedunculatus	Forage (5.0-5.5% tannins) offered with and without PEG (100 g/day)	No difference between treatments.	McNabb et al. (1993)
Desmodium ovalifolium	Forage (4.6% tannins) offered with and without PEG (5% of DM[3])	PEG supplementation increased forage intake by 21%	Carulla (1994)
Ceratonia siliqua	Forage offered with and without 25 g of PEG/ day	PEG supplementation increased forage intake by a two-fold	Silanoke et al. (1994)
Lotus pedunculatus	Forage (5.5% tannins) offered with and without PEG (100 g/ day)	PEG supplementation increased forage intake by about 9%	Waghorn et al. (1994a)
Lotus corniculatus	Forage (3.5% tannins) offered with and without PEG (100 g/ day)	No difference between treatments.	Wang et al. (1994)
Sesbania sesban and *S. goetzei*	Forage offered as a protein supplement differed greatly in tannin content	Intake of the basal diet decreased as the level of tannins in the supplement increased	Wiegand et al. (1995)

Table 2-18. *Effects of tannins on the voluntary feed intake by sheep (continued)*

Source	Key comparison	Effect on voluntary feed intake	Reference
Lotus corniculatus	Forage (4.55% tannins) offered with and without PEG	No difference between treatments.	Wang et al. (1996a)
Lotus corniculatus	Forage (3.4% tannins) offered with and without PEG	No difference between treatments.	Wang et al. (1996b)
Calliandra calothyrsus and *Gliricidia sepium*	Forage offered as protein supplements either fresh or oven-dried, the latter form containing less tannins	Oven-drying significantly increased the consumption of the basal diet	Ahn et al. (1997)
Desmodium ovalifolium, Flemingia macrophylla	Forage offered with and without PEG (3.5 % of DM)	PEG supplementation increased forage intake by an average of 10% over the control forages.	Barahona et al. (1997)
Acacia cyanophylla	Forage offered either fresh (5.1% tannins) or air-dried (4.3% tannins)	Air-drying increased dry matter intake by *ca* 8%.	Ben Salem et al. (1997a)
Sesbanea sesban	Six accessions differing in tannin content were offered as protein supplements	With increasing tannin levels there was increased intake of the basal diet	Kaitho et al. (1997b)
Calliandra calothyrsus	Fresh and dried forage was offered as a protein supplement with and without PEG	Both drying and the addition of PEG increased the intake of the basal diet	Norton and Ahn (1997)
Lotus corniculatus	Forage (2.8% tannins) offered with and without PEG	No difference between treatments.	Min et al. (1998)

[1]Tannin concentration expressed as a percent of forage (diet) dry matter.
[2]PEG = polyethylene glycol, to bind and inactivate free, soluble tannins.
[3]DM = dry matter.

2.5.1 Voluntary feed intake and palatability

The presence of tannin in a forage has been assumed to affect voluntary feed intake (McLeod, 1974), perhaps because of reduced palatability or limited digestion of ingested feed (Reed, 1995). Kumar and Vaithiyanathan (1990) suggested that a high concentration of tannins could depress feed intake in three ways. (i) Tannins may slow down the digestion of dry matter in the rumen, react with the outer cellular layer of the gut wall (Mitjavila et al. 1977) and thus diminish the permeability of the gut wall. All of this would give signals of physical distension - an important feedback in the ruminant for controlling feed intake. (ii) Tannins may influence some hormone (cholecystokinin, bombesin) levels in the same manner the affect plasma growth hormone concentration in

sheep (Barry, 1984). (iii) The depression on intake could also be due to unpalatability (Burns and Cope, 1974).

As depicted in Table 2-18, reports on the effects of tannin intake on voluntary feed intake are varied, and this perhaps reflects differences in the concentrations and sources of tannins fed and the diverse methodology employed for tannin determination. Several studies have reported that tannins do not influence feed intake (Driedger and Hatfield 1972; Waghorn et al. 1987; Nuñez-Hernandez et al. 1991; McNabb et al. 1993; Wang et al. 1994, 1996a, 1996b). On the other hand, in other studies the presence of tannins has been associated with reduced feed intake (Barry and Duncan 1984; Pritchard et al. 1988; Terrill et al. 1989; Carulla, 1994; Wiegand et al. 1995; Barahona et al. 1997; etc.). Additionally, when used as protein supplements for low quality basal diets, legumes with high tannin contents are associated with low basal diet intake, whereas those containing moderate tannin levels are associated with higher basal diet intake (Woodward and Reed, 1989, Ahn et al. 1997, Norton and Ahn, 1997). These effects of tannins are very important when tanniniferous legumes are used as protein supplements for crop residues in developing countries (Reed 1995).

Closer analysis of the results portrayed in Table 2-18 provides two interesting observations. First, in six different experiments, contrasting concentrations of condensed tannins from *Lotus corniculatus* did not have any impact on the voluntary intake of this legume by sheep. On the other hand, with just one exception, the presence of increasing amounts of condensed tannins on *Lotus pedunculatus* resulted on diminished intake of this legume by sheep. These differences could be the result of the higher concentration of tannins in the latter legume, but could also be related to differences in the molecular structure of tannins in these legumes, such as those demonstrated by the work of Foo et al. (1996, 1997).

The other interesting observation that can be made from Table 2-18 is that, with only one exception, increasing concentrations of tannins in tropical forage legumes were associated with reductions in forage intake. This not necessarily means that tannins from tropical legumes are biologically more active or astringent that those in temperate legumes. Explanation to these differences might not only be related to tannins but also to other factors. McLeod (1974) suggested that coarseness of stem in *Lespedeza cuneata* or the presence of the glycoside coronillin in *Coronilla varia* might help explain the low

acceptability of these tanniniferous forages by ruminants. In turn, Waghorn et al. (1994a) suggested that decreased ruminal turnover and rate of digestion was more important than palatability in reducing intake of sheep fed pure diets of *L. pedunculatus* in comparison to sheep fed *L. pedunculatus* plus polyethylene glycol. A characteristic of the legumes in which tannins were shown to negatively affect intake is that *in vivo* dry matter digestibility in these legumes was very low. On the other hand, *in vivo* digestibility of *L. corniculatus* were at least 68% or better. It might be possible that in energy-limited ruminants the nutritional impact of tannins is more significant. Up to date, very little is understood about tannin-energy interactions in the ruminant digestive tract.

A factor that comes to mind regarding dry matter digestibility is fibre composition and degradability. Waghorn et al. (1994a) observed that sheep selected leaves of *L. pedunculatus* over stems, although leaves were higher (4x) in tannin content. Similarly, Barahona et al. (1997) observed that sheep preferred leaves of *Desmodium ovalifolium* over stems, although leaves contained about 2.5 times more tannins than stems. On the contrary, stems had 1.4 times more NDF and two times more indigestible ADF than leaves. Similar observations regarding leaf-stem selectivity by sheep were made by Nsahlai et al. (1998). Studies have shown than leaves are more readily consumed than stems because of the lower resistance of leaves to chewing (Minson 1990) and longer ruminal retention of the more fibrous stem particles (Thornton and Minson, 1972).

In summary, prediction of the impact of condensed tannins on the voluntary intake of forages with an acceptable degree of certainty is still not possible. To isolate tannin effects, studies where pure tannins have been added to a tannin-free basal diet or where tannin-binding agents are added to a tannin-containing diet seem to be preferable to studies where intake of different tannin containing legumes are compared. Results obtained using this last approach are prune to be confounded by many non-tannin factors. However, no approach is free from criticism. For example, it is obvious that animals do not consume pure chemicals and polyethylene glycol might bind and inactivate non-tannin phenolic compounds. In consideration of the fact that non-tannin phenolic compounds in forages are also likely to affect nutritional responses by ruminants, an approach that might be worth considering would be to include treatments where crude extracts are added to tannin-free diets.

Table 2-19. *Effect of tannins on the degradation, absorption and excretion of nitrogen*

Source	Key comparison	Effect	Reference
Tara tannin	*In vitro* ammonia production of soybean meal treated with graded levels of tannins	The presence of 10% tannin resulted in a 90% decrease in deamination	Driedger and Hatfield (1972)
Mung beans (*Vigna radiata*)	*In vitro* protein digestibility of boiled seeds measured with and without tannins	Addition of tannins reduced protein digestibility values by 3-4%	Barroga et al. (1985)
72 West African fodder trees	*In vitro* degradability of the protein from 72 fodder trees varying in tannin content	There was an inverse relationship between in vitro protein degradability and both soluble phenolics and insoluble tannin concentration	Rittner and Reed (1992)
Lotus corniculatus	*In vitro* degradable protein determined on 12 *L. corniculatus* clones differing in tannin content	As tannin content of the clones increased, *in vitro* protein degradability decreased	Miller and Elke (1994)
Cottonseed hulls	Potential degradability of two cottonseed proteins measured *in vitro* with and without hulls and PEG	Addition of hulls depressed degradation of the two proteins and the addition of PEG only partially removed that effect	Yu et al. (1996a)
Faba bean (*Vicia faba*)	*In vitro* degradability of protein in six pairs of near-isogenic lines, in each pair a tannin-free and a tannin-containing line	Seeds from the tannin-free lines had 10% higher *in vitro* protein digestibility than tannin-containing lines	Helsper et al. (1996)
Faba bean (*Vicia faba*)	Rumen degradability of protein of 12 faba bean cultivars determined *in vitro*	There was a strong correlation between tannin content and *in vitro* rumen protein degradability	Makkar et al (1997a)
12 tropical browse legumes	Nitrogen digestibility from tropical legumes with tannin content ranging from 0 to 2.8% determined *in sacco*	Tannin content (vanillin-HCl and butanol-HCL) was not a reliable indicator of the degradation of nitrogen *in sacco*	Ahn et al. (1989)
Temperate fodder legumes	*In situ* protein degradability determined in cultivars of fodder legumes containing between 0 and 2.8% tannic acid equivalents	Amount of undegraded protein after 12 hours of incubation was positively related to tannin content, with 38% and 86% remaining in non-tannin and tannin legumes, respectively	Messman et al. (1996)
Tara tannin	Soybean meal treated with and without 0.89% tannins fed to sheep	There were no differences in nitrogen digestion, but lambs receiving the tannin-treated diet had higher nitrogen retention due to reduced urinary nitrogen excretion	Driedger and Hatfield (1972)
Lotus pedunculatus	Forage with low (4.6%) and high (10.6%) tannin content fed to sheep	More nitrogen reached the duodenum, was gained across the rumen and was excreted in faeces in sheep fed the high tannin forage	Barry (1984); Barry and Manley (1984)
Lotus pedunculatus	Forage (9.5% tannins) was fed to sheep treated with zero, low and high rates of PEG	Less nitrogen reached the duodenum, was digested in the post-ruminal region and was excreted in faeces as PEG supplementation increased	Barry et al. (1986)

Table 2-19. *Effect of tannins on the degradation, absorption and excretion of nitrogen (continued)*

Source	Key comparison	Effect	Reference
Lotus corniculatus	Forage containing 0.5% and 3.5% tannins fed to sheep	Sheep fed the 3.5% tannin forage had higher non-ammonia nitrogen pool, lower rumen ammonia nitrogen and higher nitrogen retention	Waghorn et al. (1987)
Lotus corniculatus	Forage containing 2.2% tannins fed to sheep with and without PEG (50 g/day)	PEG treatment resulted in higher rumen ammonia, less nitrogen reaching the abomasum and ileum and excreted in faeces, less amino acids reaching the abomasum and being absorbed in the small intestine	Waghorn et al. (1987)
Lotus corniculatus	Two iso-synthetic strains of *L. corniculatus* containing 1 and 3% tannins fed to sheep	There was higher apparent nitrogen digestibility in the low (1%) tannin strain (76.6 versus 69.9%)	Chiquette et al. (1989)
Sericea lespedeza	High tannin (18.1%) and low tannin (3.1-8.7%) forage fed to sheep	Sheep fed the high tannin forage had higher faecal excretion and lower apparent digestibility, but similar nitrogen retention than those fed the lower tannin forage	Terrill et al. (1989)
Mountain mahogany (*Cercocarpus montanus*)	Forage (4.1% tannins) with and without PEG (2.3 g/g of tannins) fed to sheep and goats	Animals fed PEG-treated forage had lower faecal but higher urinary nitrogen excretion than those fed the control forage. Overall, there were no differences in nitrogen retention	Nuñez-Hernandez et al. (1991)
Mountain mahogany (*Cercocarpus montanus*)	Forage (3.8% tannins) with and without PEG (2.3 g/g of tannins) fed to sheep	Sheep fed PEG-treated forage had higher rumen ammonia concentrations than those fed the control forage	Nuñez-Hernandez et al. (1991)
Desmodium ovalifolium	Forage (6.8% tannins) with and without PEG (5% of DM) was fed to sheep	Sheep fed PEG-treated forage has higher nitrogen intake and rumen ammonia nitrogen, lower faecal nitrogen and higher nitrogen retention than sheep fed the control forage	Carulla et al. (1994)
Desmodium ovalifolium	Forage (6.1% tannins) treated with PEG (0, 3.5 and 7.0% of DM) was fed to sheep	At similar nitrogen intake, PEG addition was linearly associated with reduced nitrogen (microbial, non-microbial and total) flow to the duodenum, reduced faecal excretion and reduced nitrogen retention	Carulla et al. (1994)
Lotus pedunculatus	Forage (5.5% tannins) fed to sheep with and without an intra-ruminal infusion of PEG (100 g/ day)	More nitrogen in the rumen and reached the omasum, abomasum, ileum and faeces and less amino acids were degraded in the rumen in control sheep than in the PEG-infused sheep	Waghorn et al (1994b)
Lotus corniculatus	Forage (3.5% tannins) fed hourly to sheep with and without PEG (50 g/ day)	PEG supplementation increased both rumen ammonia and apparent digestion of nitrogen and reduced the utilization of plasma cystine for synthetic body reactions	Wang et al. (1994)

Table 2-19. *Effect of tannins on the degradation, absorption and excretion of nitrogen (continued)*

Source	Key comparison	Effect	Reference
Acacia saligna	Forage (8.3% tannins) fed to sheep and goats as a sole diet	Apparent digestibility of nitrogen was 17.5% and 0.9% for goats and sheep, respectively. Both species were in negative nitrogen balance	Degen et al. (1995)
Lotus pedunculatus	Lotus and ryegrass mixture (33% lotus; 1.8% tannins) fed to sheep with and without PEG	PEG increased rumen ammonia concentrations and increased apparent nitrogen digestion. However, there no differences in nitrogen retention	Waghorn and Shelton (1995)
Sesbania sesban	Three *S. sesban* accessions with varying levels of tannins fed to sheep	Faecal nitrogen was highest in rams fed the highest tannin forage, whereas urinary nitrogen excretion was highest in those fed the lowest tannin accession. Retention of nitrogen was maximised with the forage of intermediate tannin content	Wiegand et al. (1995)
Lotus pedunculatus	Forage (4.5-5.5% tannins) fed to sheep with and without intra-ruminal infusion of PEG	PEG addition increased Rubisco digestion in the rumen (72% to 96%) and decreased that in the small intestine (27% to 4%), but did not affect the solubilization of Rubisco. Thus tannins appear to slow protein degradation by microorganisms	McNabb et al. (1996)
Desmodium intortum and *Calliandra calothyrsus*	These tanniniferous and two tannin-free legumes were fed to sheep and goats	Higher proportions of dietary nitrogen reached the abomasum, more nitrogen was absorbed and was excreted in faeces when animals were fed the tanniniferous forages	Perez-Maldonado and Norton (1996b)
Sulla (*Hedysarum coronarium*)	Forage (7.2% tannins) fed to sheep with and without PEG	Animals receiving PEG had higher rumen ammonia concentration and higher (74%) apparent digestibility of nitrogen than control (no PEG) sheep	Stienezen et al. (1996)
Lotus corniculatus	Forage (3.0% tannins) was fed to sheep infused a S^{35}-labelled lotus homogenate with and without PEG	Tannins reduced the true digestibility of plant methionine and cysteine, but increased the total amounts of these plant amino acids absorbed in the small intestine	Wang et al. (1996c)
Calliandra calothyrsus and *Gliricidia sepium*	Fresh and oven-dried forage of *C. calothyrsus* (11.7 vs. 8.2% tannin) and *G. sepium* (2.0 vs. 0% tannin) fed to sheep	Sheep given *Gliricidia* had higher ammonia and protein degradability in the rumen than those fed *Calliandra*. In both legumes, drying increased faecal nitrogen and increased nitrogen retention by decreasing rumen protein degradation and increasing post-ruminal nitrogen absorption	Ahn et al. (1997)
Desmodium ovalifolium and *Flemingia macrophylla*	Forages (9.0% extractable tannins) fed to sheep with and without PEG (3.5% of DM)	Addition of PEG resulted in less ruminal escape protein, and less faecal nitrogen with both legumes, but apparent post-ruminal absorption of nitrogen was not affected	Barahona et al. (1997)

Table 2-19. *Effect of tannins on the degradation, absorption and excretion of nitrogen (continued)*

Source	Key comparison	Effect	Reference
Sesbania sesban	Six accessions with varying tannin levels were fed as a protein supplement to sheep receiving teff straw *ad libitum*	With increasing tannin levels there was an increase in estimated escape protein and faecal nitrogen excretion and a decrease in the rate of degradation and urinary excretion of nitrogen	Kaitho et al. (1997c)
Calliandra calothyrsus	Fresh-frozen (3.6% tannins) and dried (2.2% tannins) forage fed as a protein supplement to sheep with and without PEG	Both drying and PEG addition increased total nitrogen digestibility. PEG increased the proportion of nitrogen degraded in the rumen, rumen ammonia concentration and nitrogen absorption, but did not affect nitrogen retention	Norton and Ahn (1997)
Quebracho	Quebracho tannins added (5%) to a basal diet and treated with 0, 0.01 and 0.1% Browse Plus (PEG)	Addition of quebracho reduced *in vivo, in sacco* and *in vitro* digestibility of protein, with addition of PEG removing only partially these effects in the *in vivo* and *in sacco* studies	Salawu et al. (1997a)
Forty browse species	Degradability of browse species with varying tannin content measured in ruminally-fistulated sheep	Browse species with high tannin content had high rumen by-pass and low apparent nitrogen digestibility. Other browse legumes had high by-pass that was digested in the small intestine	Kaitho et al. (1998)
Lotus corniculatus	Forage fed to sheep with and without twice a day daily oral administration of PEG	PEG supplementation increased rumen ammonia, blood plasma urea and decreased plasma cysteine concentrations	Min et al. (1998)

Explanatory notes: The concentration of tannins always expressed as percent of forage dry matter. PEG = polyethylene glycol, added to bind and inactivate tannins. DM = dry matter.

2.5.2 Nitrogen metabolism

Excessive degradation of dietary protein in the rumen has long been recognised as undesirable. Soluble proteins such as casein and Fraction 1 leaf protein degrade very rapidly in the rumen with less than 10% of the casein consumed reaching the duodenum (Mangan, 1988). Ruminants have been shown to use protein more efficiently if it is protected against bacterial deamination in the rumen (McLeod, 1974). Precipitation of protein by the formation of insoluble tannin-protein complexes has been shown to protect dietary protein from ruminal fermentation (Barry and Duncan, 1984; Barry, 1989). This complexation has been suggested to occur during mastication, an idea supported by the findings of Burrit et al. (1987) and Terrill et al. (1992b), who reported a significant reduction of solvent-extractable condensed tannins in plant tissue after

chewing. This reduction is accompanied by an increase in protein-bound and fibre-bound tannins (Terrill et al. 1992b; Carulla, 1994; Perez-Maldonado, 1994).

Protection of protein in tannin-protein complexes against deamination by microbial enzymes is indeed a potentially beneficial feature of tannins. However, protein escaping ruminal degradation may not become available in the small intestine (Waghorn et al. 1994b). Indeed, it has been pointed out that the digestibility of protein in plants containing significant amounts of tannins is low and that the reduction in protein availability is proportional to the protein-precipitating capacity of tannins (Robbins et al. 1987a). Nonetheless, taking structure-activity relationships into account, there is a sense of futility in the making of broad generalisations regarding tannin effects.

2.5.2.1 *Effects of tannin on ruminal protein degradation*

Logically, any account of the effects of tannin on the degradation of nutrients in the rumen must essentially circumscribe to the effects of tannin on ruminal microorganisms and their enzymatic activity. This is an entire issue on its own and we have dedicated some brief comments to it in Sections 2.5.5 and 2.5.6.

It has been suggested that tannins inhibit microbial extracellular enzymes (Scalbert, 1991). Indeed, Jones et al. (1994) reported reductions of the activity of some of the most important ruminal proteolytic bacterial species in the presence of tannins from sainfoin (*Onobrychis viciifolia*). Likewise, *in vitro* experiments have shown that, with varying degrees of effectiveness, tannins are effective in protecting proteins against deamination by enzymes and ruminal fluid (Driedger and Hatfield 1972; Barroga et al. 1985; Miller and Elke, 1994; Helsper et al. 1996; see Table 2-19). Additionally, *in vitro* work by Rittner and Reed (1992) showed a negative correlation between protein degradability and plant concentration of insoluble condensed tannins, which was explained by the formation of insoluble tannin-protein complexes. On the other hand, Yu et al (1996a) showed that whereas addition of tannin-containing cottonseed hulls depressed the *in vitro* degradation of cottonseed proteins, the addition of the tannin-binding polyethylene glycol only partially removed that effect.

In vivo experiments have also suggested that one the principal effect of dietary tannins is to reduce protein degradability. Several authors have reported reduced ruminal ammonia concentrations in response to tannin consumption (Waghorn et al. 1987; Chiquette et al.

1989; Terrill et al. 1992a; McNabb et al. 1993; Carulla 1994, etc.; see Table 2-19). This has been attributed to lower solubility and reduced deamination of plant proteins when tannins were present. However, McNabb et al. (1996) observed that whereas tannins from *Lotus pedunculatus* reduced the digestion of Rubisco in the rumen, they did so without affecting its solubilization. This suggests that tannins in *L. pedunculatus* act by slowing protein degradation by rumen microorganisms.

This evident reduction in protein availability to rumen microorganisms is often accompanied by increased post-ruminal nitrogen flow (Barry et al. 1986; Waghorn et al. 1987, 1994b; Carulla, 1994; McNabb et al. 1996; Barahona et al. 1977; etc.; see Table 2-19). Barry and Manley (1984) observed that feeding fresh *Lotus pedunculatus* with low- and high-tannin concentrations (4.6 vs. 10.6% of DM) to sheep increased nitrogen gains (i.e. duodenal N flow - N intake) across the rumen (1.8 and 10.5 g/d). By comparison, prediction (McRae and Ulyatt, 1974) of these values in sheep fed similar but non-tannin containing fresh forages returns nitrogen losses of 3.7 and 2.1 g/day. Other workers have also reported nitrogen gains across the rumen in response to increased tannin intake (Barry et al. 1986; Waghorn et al. 1987; Carulla 1994; Perez-Maldonado 1994). Overall, the trend observed when feeding diets containing tannins has been increased duodenal non-ammonia nitrogen flow compared with similar but non- or low-tannin diets.

The potential benefits of reducing the extent of ruminal protein degradation can only be attained if this reduction is not associated with detrimental reductions in the digestibility coefficient of other dietary components. Evidently, a balance must exist between protein and energy availability in order to maximise feed utilisation. Barry et al. (1986) suggested that in *Lotus sp*. a tannin concentration of 3-4% of DM should be adequate to improve the efficiency of protein digestion without affecting carbohydrate digestion. On the other hand, Miller and Ehlke (1994) suggested that birdsfoot trefoil (*Lotus corniculatus* L.) with tannin concentrations in the range of 27 to 85 g catechin equivalents/ kg of DM can reduce ruminal protein degradation, with little or no corresponding reduction in dry matter digestibility. These differences could have a methodological basis, but could also be the reflection of structure-activity relationships. In simple systems, tannin reactivity is dependent upon tannin molecular structure, work is still required to determine what is the true impact of tannin structure *in vivo*.

2.5.2.2 *Effect of tannins on nitrogen absorption and amino acid supply to the small intestine*

In the previous Section it was demonstrated that ingested tannins increase the amount of dietary protein that reaches the duodenum of ruminants. However, the important question is whether or not this extra protein becomes available to the ruminant. In this regard, some studies that suggested that the efficiency of post-ruminal non-ammonia nitrogen absorption (g absorbed/g reaching the duodenum) is similar in forages with contrasting tannin concentrations. This was the case in sheep fed low- and high-tannin *Lotus pedunculatus* (Barry and Manley, 1984); in sheep fed *Desmodium ovalifolium* diets with and without addition of polyethylene glycol (Carulla, 1994); and in sheep and goats fed high tannin and non-tannin legumes (Perez-Maldonado, 1994). These findings suggest an increased efficiency in the absorption of nitrogen per g of nitrogen ingested in animals fed tannin diets compared to those fed diets of similar characteristics but non-tanniniferous. Nonetheless, Waghorn et al. (1987) observed no differences in apparent nitrogen absorption (g/d) in sheep fed two *Lotus corniculatus* cultivars of low- and medium-tannin concentration.

A very interesting effect of ingested tannins is that they appear to act selectively on the type of amino acids they protect from deamination in the rumen. Indeed, sheep fed dried sainfoin had a greater flow of essential amino acids to the duodenum compared to sheep fed dried alfalfa (Harrison et al. 1973). Similar results with both fresh and dried sainfoin have been reported by other researchers (Thomson et al. 1971; Beever and Siddons, 1986). Likewise, sheep fed *Lotus corniculatus* (2.2% tannins) had higher abomasal flow of essential (*ca* 50%) and non-essential amino acids (14%) compared to those fed the same diet but receiving an intraruminal infusion of polyethylene glycol. In addition, polyethylene glycol infusion was associated with a decrease of 62% and an increase of 9% in the absorption of essential and non-essential amino acids, respectively (Waghorn et al. 1987). On the contrary, later work by Waghorn et (1994b) showed that although a higher concentration of tannins in *Lotus pedunculatus* resulted in higher post-ruminal fluxes of essential and non-essential amino acids, this beneficial effect was negated by a corresponding reduction in amino acid absorption in the small intestine.

Research with non-ruminants has suggested that tannins decrease the absorption of essential amino acids, especially methionine (Butler et al. 1986). In cecectomized

cockerels, the digestibility of eight individual amino acids and the mean digestibility of essential and non-essential amino acids in various sorghum cultivars differing in tannin content was negatively related to tannin content (Elkin et al. 1996). In pigs, inclusion of hulls of faba beans (*Vicia faba*) of high tannin content (3.3% of DM) resulted in reductions of 4 to 29 units in the apparent ileal digestibility of amino acids compared to those receiving low tannin (0.1%) hulls (Jansman et al. 1995). In turn, Lizardo et al. (1995) observed that sorghum tannin levels exceeding 0.25% of DM affected the ileal apparent digestibility of energy and nitrogen by weaned piglets. In the growing rat, inclusion of tannin-containing cottonseed hulls to ten semi-purified diets depressed the apparent and true ileal digestibilities of nitrogen and amino acids (Yu et al. 1996b). However, this response was dependent on protein source. With casein-based diets, depressions caused by hull addition were completely reversed by the addition of polyethylene glycol. On the other hand, with cottonseed-kernel-based diets, addition of hulls had no effects in the digestibility of methionine and cystine and depressed that of other amino acids, with the addition of polyethylene glycol only reversing between 0% and 50% of that depression. In related research, Yu et al. (1996c) noted apparent digestibility of cottonseed amino acids was lower in the pig than in the rat and concluded that studies into the effects of cottonseed tannins should be carried out in the target animal species.

In what refers to sulphur amino acids, sheep fed *Lotus pedunculatus* (5.5% tannins) had higher (100 vs. 71%) protection of both methionine and cystine in the rumen compared to those fed the same diet but receiving an intraruminal infusion of polyethylene glycol (McNabb et al. 1993). This was accompanied by a 27% increase in apparent absorption of methionine (4.5% for cystine) in the small intestine of control sheep (i.e. the post-ruminal digestibility of methionine was not affected by tannins, although it was reduced for cystine). Additionally, there was a 52% increase in plasma cystine concentration and a 78% increase in cystine irreversible loss rate in control sheep as compared to those infused with polyethylene glycol. This resulted in a 110% increase in the cystine flux to productive processes and maintenance in control sheep. Similar increases in the amount of cystine leaving the plasma cystine pool to be used for body synthetic reactions were reported by Wang et al. (1994) when sheep were fed fresh *Lotus corniculatus* (2.7% CT) with and without an intraruminal polyethylene glycol infusion.

2.5.2.3 *Tannins and nitrogen retention*

As depicted in Table 2-19, consumption of a diet high in tannins has been commonly associated with increased faecal N excretion in ruminants. On the other hand, animals fed high tannin diets commonly show decreased urinary nitrogen excretion as well. In consequence, reports of reduced nitrogen retention due to CT intake have not been as common as one would initially expect. Quite on the contrary, several workers have reported increased nitrogen retention due to the presence of tannins in ruminant diets. For example, Driedger and Hatfield (1972) reported higher N retention in sheep fed a soybean meal supplement treated with 10% tara tannin, due to reduced urinary N excretion. Similarly, condensed tannins in sainfoin increased both nitrogen retention and the amount of metabolised nitrogen recycled to the digestive tract, increased nitrogen excretion in faeces, and decreased nitrogen excretion in the urine (Egan and Ulyatt, 1980). Likewise, Barry et al. (1986) observed that high tannin content in *Lotus pedunculatus* increased faecal nitrogen excretion and decreased urinary nitrogen excretion, leading to higher overall nitrogen retention by sheep. Increased nitrogen retention due to the presence of tannins on forages has also been reported by Waghorn et al. (1987), Waghorn et al. (1990) and by Perez-Maldonado and Norton (1996b).

On the other hand, other workers have reported that the nitrogen balance of ruminants has been unaffected by the presence of contrasting levels of tannins in forages. These include the studies of Terrill et al. (1989) with Sericea lespedeza, Nuñez-Hernandez et al. (1991) with mountain mahogany, Waghorn and Shelton (1995) with *Lotus pedunculatus*, Barahona et al. (1997) with *Desmodium ovalifolium* and *Flemingia macrophylla* and Norton and Ahn (1997) with *Calliandra calothyrsus*. Finally, in the studies of Carulla (1994) with fresh *D. ovalifolium* and of Ahn et al (1997) with *C. calothyrsus* and *Gliricidia sepium,* the presence of tannins was associated with reduced nitrogen in ruminants. However, after conducting more experiments with *D. ovalifolium*, Carulla (1994) concluded that although faecal nitrogen excretion is increased by consumption of tannins, this appears to be related to increased nitrogen flow to the duodenum while nitrogen digestion or absorption in the small intestine is not affected by tannins.

This diversity of results is probably a function of tannin molecular structure and tannin concentration in ingested dry matter. The effects of tannin concentration were neatly

demonstrated in the work of Wiegand et al. (1995) and Kaitho et al. (1997c) with *Sesbania sesban*. In both these studies, increasing tannin contents resulted in increased faecal nitrogen excretion with a concomitant reduction in urinary nitrogen. Additionally, Wiegand et al. (1995) observed that nitrogen retention was maximised at an intermediate tannin concentration.

While it is evident that the mechanism by which tannins increase faecal nitrogen excretion is based upon their ability to bind and precipitate protein, the mechanisms underlying reduced urinary output are not that clear. Waghorn et al. (1987) suggested that increased nitrogen retention with *Lotus corniculatus* appeared to be due to improved utilisation of absorbed nitrogen. Indeed, it has been suggested that tannins might increase the efficiency of urea recycled to the rumen (Reed, 1995) because they lower the rate of protein degradation and deamination in the rumen and therefore lower ruminal NH_3. This was demonstrated by the work of Waghorn et al (1994b), who observed low rumen ammonia concentration and associated rapid turnover of the plasma urea pool in sheep fed *Lotus pedunculatus* (5.5% tannins). In that study, the irreversible loss rate of urea was more than twice the urinary urea excretion, which was similar to a finding of Egan and Ulyatt (1980) with mature sainfoin, but greater than values for fresh ryegrasses or white clover. These authors concluded high degradation rate of urea-nitrogen was indicative of an extensive recycling both to the rumen and to the intestine. Both plasma urea concentrations and rumen ammonia concentrations affect this extensive recycling (Egan et al. 1986).

2.5.3 Tannins and the digestibility of carbohydrate and dry matter

It has been postulated that tannins may defend plants against herbivory by reducing cell wall digestion. This may depend on the type and level of tannins consumed and on the animal species studied (Robbins et al. 1987b). Using *in vitro* methods, Rittner and Reed (1992) found that soluble proanthocyanidin content of West African browse was negatively correlated to neutral detergent fibre degradability. Likewise, the *in vitro* dry matter disappearance for tree leaves was found to decline with an increase in tannin content (Waterman et al. 1980, Forwood and Owensby, 1985). Van Hoven (1984) reported that the *in vitro* dry matter digestibility of lucerne decreased almost linearly in response to increasing concentration of condensed tannins (tannin range: 0 to 35%, *in vitro* dry mater digestibility: 49.7 to 0.0%, respectively).

Table 2-20. *Effects of tannins on the digestibility of carbohydrate and dry matter*

Source	Key comparison	Effect	Reference
Sericea lespedeza	IVDMD determined for plants with low and high tannin content	Low tannins plants had higher IVDMD (71.8% vs. 63.4%) than high tannin plants	Donnelly and Anthony. (1969)
Lotus corniculatus	IVDMD determined for two isosynthetic strains differing in tannin content (1.3 and 5.4%)	Low tannin strain had higher IVDMD, gas pressure increase and VFA production than high tannin strain	Chiquette et al. (1988)
Twelve tropical browse legumes	*In sacco* DMD determined in freeze-dried or oven-dried samples varying in tannin content (0-11%)	Oven drying reduced *in sacco* DMD, and the presence of tannins decreased the rate on dry matter disappearance	Ahn et al. (1989)
72 West African fodder trees	*In vitro* degradability of NDF determined in these samples with contrasting tannin content	*In vitro* degradability of NDF was negatively correlated with soluble condensed tannin concentration in the samples	Rittner and Reed (1992)
Lotus corniculatus	IVDMD determined in 12 *L. corniculatus* clones differing in tannin content	At tannin concentrations between 0 and 8.5% catechin equivalents, there were little effects in IVDMD attributable to tannins	Miller and Elke (1994)
Eleven tropical forage legumes	Fermentation kinetics and non-starch polysaccharide loss determined in leaf samples of varying tannin content (0 to 17.5%)	Both extractable and bound condensed tannin concentrations in leaves were inversely related to gas accumulation and non-starch polysaccharide loss	Longland et al. (1995)
Quercus incana and *Dichostachys cinerea*	Rate of fermentation of hay was determined in vitro in the presence of different concentrations of condensed tannins	Rate of digestion affected to a greater extent than the potential extent of digestion; different tannins at the same level can have different degree of effect	Makkar et al. (1995c)
Twelve Bolivian fodder trees	*In vitro* gas production measured in these samples of varying phenolic, protein precipitating ability and tannin content	Total phenolics and, less accurately, protein precipitation ability, were useful indicators of the degree on inhibition of rumen microorganisms by phenolics. This was not the case with tannin content (butanol-HCl)	Wood and Plumb, 1995
Robinia pseudoacacia and other legumes	In vitro gas production of these forages with and without different tannins binding agents	Addition of tannin binding agsents increased gas accumulation, with polyethylene glycol being the most effective tannin-binding agent	Khazaal et al. (1996)
Legume foliage and straw samples	*In vitro* fermentation (gas accumulation) in samples containing 0.3 and 1.6% extractable tannins	At these low levels tannins did not appear to adversely affect the nutritive value of these samples, since maximum decrease in organic matter digestibility was 3%	Makkar et al. (1996)
Forty tropical browse species	Rumen degradability and *in vitro* gas production determined in these samples of varied tannin content	Condensed tannins were negatively related to potential and effective degradability and to asymptotic gas production; phenolics more related to DMD than to forage preference	Kaitho et al (1997a)

Table 2-20. *Effects of tannins on the digestibility of carbohydrate and dry matter (continued)*

Source	Key comparison	Effect	Reference
Seventeen Zimbabwean browse species	*In vitro* fermentation (gas production) measured in these samples which differed greatly in tannin content	Neither protein precipitating polyphenolics not insoluble tannins were related to gas production constants, indicating that their effect on gas production is complex	Ndlovu and Nherera (1997)
Calliandra calothyrsus	*In vitro* gas production determined in leaves and stems with and without PEG (at 3 levels)	Increasing levels of PEG only slightly improved the gas production from the leaves and progressively improved gas production from the stems	Salawu et al. (1997c)
Quebracho	*In sacco* determination with quebracho tannins added (5%) to a basal diet and treated with 0, 0.01 and 0.1% Browse Plus (PEG)	Addition of quebracho tannins had no effect on *in sacco* digestibility of *Calliandra calothyrsus* leaves, but reduced that of stems, with addition of PEG removing significantly those effects	Salawu et al. (1997b)
Four browse species	*In vitro* gas production of these samples determined with and without PEG addition	In terms of gas accumulation, the response to PEG treatment increased with increasing level of phenolic compounds in the browse plants	Tolera et al. (1997)
Tara tannin	Soybean meal treated with and without 0.89% tannins fed to sheep	There were no differences in the apparent dry matter digestion by sheep	Driedger and Hatfield (1972)
Lotus pedunculatus	Forage with low (4.6%) and high (10.6%) tannin content fed to sheep	The only effects attributable to tannins were a shift in the site of digestion of hemicellulose and readily fermentable carbohydrates, with less digestion of these constituents occurring in the rumen	Barry and Manley (1984)
Lotus pedunculatus	Forage (9.5% tannins) was fed to sheep treated with zero, low and high rates of PEG	Increasing PEG supplementation linearly increased apparent digestibility of organic matter and energy, but had no effect on that of cellulose, hemicellulose, pectin and soluble carbohydrates. However, tannins increased the rate at which undegraded particles left the rumen	Barry et al. (1986)
Lotus corniculatus	Forage containing 0.5% and 3.5% tannins fed to sheep	There no differences between forages in the proportions of OM and NDF digested in the rumen	Waghorn et al. (1987)
Lotus corniculatus	Forage containing 2.2% tannins fed to sheep with and without PEG (50 g/day)	There no differences in the apparent digestibility of DM and NDF, but PEG increased the proportion of DM digested in the rumen	Waghorn et al. (1987)
Lotus corniculatus	Two isosynthetic strains (1 and 3% tannins) fed to sheep	No differences in dry and organic matter digestibility, however, ADF digestibility was lower in the 3% tannin strain	Chiquette et al. (1989)

Table 2-20. *Effects of tannins on the digestibility of carbohydrate and dry matter (continued)*

Source	Key comparison	Effect	Reference
Sericea lespedeza	High (18.1%) and low (3.1-8.7%) tannin forage fed to sheep	Digestibility of DM, NDF, ADF and hemicellulose depressed in sheep fed the high tannin forage	Terrill et al. (1989)
Fodder trees and straw diets	Forages with varying content of phenolics fed to sheep	Forage with the highest level of phenolics and insoluble tannins had the lowest OM and NDF digestibility	Reed et al. (1990)
Mountain mahogany (*Cercocarpus montanus*)	Forage (4.1% tannins) fed to sheep with and without PEG (2.3 g/g of tannins)	There were no difference in OM and ADF digestibility, but PEG addition decreased that of NDF, however, there were no differences in the *in situ* extent of NDF digestion	Nuñez-Hernandez et al. (1991)
Sulla (*Hedysarum coronarium*)	Forage (4-5% tannins) fed to sheep with and without PEG (40 g/day)	PEG supplementation reduced molar proportions of *n*-butyrate and increased those of iso-butyrate and total valerate	Terrill et al. (1992a)
Desmodium ovalifolium	Forage (6.8% tannins) fed to sheep with and without PEG (5% of DM)	There were no differences in DM, OM, NDF and ADF digestibility between sheep fed both forages	Carulla (1994)
Lotus pedunculatus	Forage (5.5% tannins) fed to sheep with and without PEG (100 g/day)	Digestibility and site of digestion of DM, hemicellulose, cellulose and ash were not affected by tannins	Waghorn et al. (1994a)
Lotus corniculatus	Forage (3.5% tannins) fed hourly to sheep with and without PEG (50 g/day)	Digestibility of DM (0.766 vs. 0.786), OM (0.774 vs. 0.797) and hemicellulose (0.605 vs. 0.666) were lower in control than in PEG sheep	Wang et al. (1994)
Acacia saligna	Forage (8.3% tannins) fed to sheep and goats as the sole diet	Digestibility of DM, OM and energy were low in both species, but higher for goats; negative ADF and ADL digestibility were observed	Degen et al. (1995)
Lotus pedunculatus	Lotus and ryegrass mixture (33% lotus, 1.8% tannins) fed to sheep with and without PEG	Tannins decreased digestibility of DM by 3-7 percentage units and had no effects on major VFA but reduced proportions of iso-butyrate, valerate and iso-valerate	Waghorn and Shelton (1995)
Sesbania sesban and *S. goetzei*	Three accessions of *S. sesban* and one *S. goetzei* with varying tannin contents fed to sheep	High levels of tannins depressed NDF digestibility; the accession with the highest tannin content had negative ADL digestibility	Wiegand et al. (1995)
Desmodium intortum and *Calliandra calothyrsus*	These tanniniferous and two other tannin-free legumes fed to sheep and goats	Tanniniferous diets were digested less well in the rumen (63 vs. 73%); there as an apparent net gain of ADF across the digestive tracts of animals fed *Calliandra*	Perez-Maldonado and Norton (1996b)
Sulla (*Hedysarum coronarium*)	Forage (7.2% tannins) fed to sheep with and without PEG	Digestibility of DM was lower (70.5 vs. 72.5%) in control sheep than in PEG sheep	Stienezen et al. (1995)

Table 2-20. *Effects of tannins on the digestibility of carbohydrate and dry matter (continued)*

Source	Key comparison	Effect	Reference
Lotus corniculatus	Forage (4.5% tannins) fed to sheep with and without PEG	The concentrations of non-esterified fatty acid were similar for the two groups, however concentrations of iso-butyric, iso- and n-valeric acids were lower in the PEG minus group	Wang et al. (1996a)
Calliandra calothyrsus and *Gliricidia sepium*	Fresh and oven-dried forage of *C. calothyrsus* (11.7 vs. 8.2% tannin) and *G. sepium* (2.0 vs. 0% tannins) fed to sheep	For both species, drying increased dry matter digestibility	Ahn et al. (1997)
Desmodium ovalifolium and *Flemingia macrophylla*	Forages (9.0% extractable tannins) fed to sheep with and without PEG (3.5% of DM)	Total digestibility of OM, NDF and ADF were increased for both legumes with the addition of PEG	Barahona et al. (1997)
Acacia cyanophylla	Air-dried forage (4.5% tannins) fed as a supplement for sheep receiving a basal diet of lucerne hay	As the intake of acacia increased, the digestibility of OM, NDF and ADF were reduced	BenSalem et al. (1997b)
Sesbania sesban	Six accessions with varying tannin levels were fed as protein supplements to sheep receiving teff straw *ad libitum*	With increasing tannin levels there was a decrease in the rate of DM degradation, however the digestibility of DM did not differ between diets, although that of NDF decreased with supplementation	Kaitho et al (1997b)
Calliandra calothyrsus	Fresh-frozen (3.65 tannins) and dried (2.2% tannins) forage fed as a protein supplement to sheep with and without PEG	Digestibility of OM of the diets was increased by drying but not by the infusion of PEG	Norton and Ahn (1997)

Explanatory notes: The concentration of tannins is always expressed as percent of forage dry matter. PEG = polyethylene glycol, added to bind and inactivate tannins. DM = dry matter, OM = organic matter, NDF = neutral detergent fibre, ADF = Acid detergent fibre, ADL = acid detergent lignin, IVDMD = *in vitro* dry matter digestibility, DMD = dry matter digestibility, VFA = volatile fatty acids.

Other workers, using *in vitro* techniques, have also shown that the presence of tannin in forages in inversely related to dry mater and fibre digestibility (see Table 2-20). However, there have been some notable exceptions to that rule, which include the work of Wood and Plumb (1995) and of Ndlovu and Nherera (1997). In these works poor correlation existed between tannin content and *in vitro* gas accumulation.

The use of *in vitro* techniques for the evaluation of forages with tannins has received some criticism. This is because of the poor mimicking in the closed *in vitro* system of several aspects of ruminal digestion that are critical to understanding the potential digestive reduction due to allelochemicals (i.e. tannins; Robbins et al. 1987b; Lascano

and Carulla 1992). These aspects include (i) Absorption and differential flow of fibrous, volatile and soluble feed constituents from the rumen. (ii) Extensive dilution of allelochemicals in newly ingested food entering the relatively large rumen pool. (iii) Addition and continuous mixing of saliva with dry matter prior to and during fermentation. (iv) Potential inequality between solubility *in vitro* and absorbability *in vivo*. However, given the versatility of *in vitro* techniques, and with their adequate use, they should provide powerful tools with which to unravel some of the most important key mechanistic issues underlying the nutritional impact of tannins in forages.

Results from *in vivo* research have also pointed at variable responses on fibre and dry matter digestion due to tannin consumption. For example, Barry and Duncan (1984) reported that sheep fed high-tannin *Lotus pedunculatus* (6.3% tannins) had reductions of 5 and 8% in the apparent digestibility of cellulose and hemicellulose, respectively, as compared to those fed lotus plus polyethylene glycol. However, in a later report, Barry and Manley (1984) observed that even though high tannin (10.6%) *L. pedunculatus* had lower apparent digestibility of cellulose and hemicellulose than forage of lower tannin content, these effects were not attributable to tannins. On the other hand, tannins were found responsible for reducing the rumen degradability of hemicellulose and readily fermentable carbohydrate, effects that were compensated by greater post-ruminal digestibility of these constituents. Likewise, Barry et al. (1986) showed that levels of reactive tannins of 9.5, 4.5 and 1.4% of DM in *L. pedunculatus* (obtained by PEG addition) did not influence the apparent total tract digestibility of either cellulose or hemicellulose. However, increasing dietary CT concentration linearly decreased the ruminal digestion of hemicellulose but not that of cellulose. It was also observed that increasing the dietary reactive CT concentration had no effect on the rate at which CHO were degraded in the rumen per unit time, but instead increased the rate at which their undegraded residues left the rumen per unit time.

Several authors have reported decreased *in vivo* digestibility of fibre and/or dry matter due to the presence of contrasting levels of tannins in forages (see Table 2-20). These include the studies Terrill et al. (1989) with high tannin (18.1%) *Sericea lespedeza*; Wang et al. (1994) with *L. corniculatus* (3.5% tannins); Waghorn and Shelton (1995) with *L. pedunculatus*; Wiegand et al (1995) with *Sesbania sesban*, and seven other workers (see Table 2-20). Conversely, Table 2-20 reports eight different instances when

contrasting concentrations of tannins had no effect in either fiber or dry matter digestibility. For example, Waghorn et al. (1987) observed similar neutral detergent fiber digestibility at medium and low levels of tannins birdsfoot trefoil. Similarly, Chiquette et al. (1989) reported similar acid detergent fibre digestibility for two birdsfoot trefoil isosynthetic strains containing tannins at 3.2 and 1.2% of dry matter. More interestingly, Nuñez-Hernandez et a*l*. (1991) observed greater neutral detergent fibre digestibility in sheep and goats fed mountain mahogany containing 4.1% tannins compared with those fed diets with polyethylene glycol added.

Another observation from Table 2-20, is that in several instances the presence of tannins was associated with reduction of the molar proportion of the minor volatile fatty acids (iso-butyrate and iso- and n-valerate; Terrill et al. 1992a; Waghorn and Shelton, 1995; Wang et al. 1996a). Terrill et al. (1992a) explained these changes on their observation that high rumen protozoal numbers were associated with the intake of sulla and that shifted the end products of fermentation. The effect of tannin on rumen microbes will be discussed with greater detail in Section 2.5.6.

2.5.4 Tannin-lignin interactions

It has been proposed that both tannins and lignins are formed biosynthetically, either wholly or partially, from the products of the shikimic acid pathway and share many common intermediates (Van Soest, 1982). It is therefore no accident that plants that have high levels of tannins also tend to be highly lignified, and that the stresses that elevate tannin concentration, also tend to elevate that of lignin (Barry, 1989). In *Lotus* sp., increasing soil nutrient and climatic stresses caused large and similar increases in the concentration of total condensed tannins and lignin (Barry and Manley, 1986). Accordingly, a positive correlation of lignin with both soluble and insoluble condensed tannins was reported for East (Reed, 1986) and West (Rittner and Reed, 1992) African browse species.

Reports on the effects of tannins on the digestion of lignin have consistently pointed that the presence of tannins is related to a reduction in the apparent digestibility of lignin. Addition of polyethylene glycol to *Lotus pedunculatus* diets significantly improved lignin apparent digestibility and low tannin lotus had significantly higher lignin apparent digestibility than high tannin lotus (Barry and Duncan, 1984; Barry and Manley, 1984;

Barry et al. 1986). Additionally, ruminal digestion of lignin as a proportion of total digestion was decreased as tannin concentration increased in the diet (Barry et al. 1986).

Several workers have reported that in animals fed tanniniferous legumes there is a net gain in lignin across the digestive tract. For example, Wiegand et al. (1995) reported that accessions of *Sesbania sesban* with high content of tannins had negative digestion coefficients for acid detergent lignin. Similarly, Degen et al. (1995) reported that sheep and goats fed the highly tanniniferous *Acacia saligna* had negative digestibility for acid detergent lignin. As a further example, Perez-Maldonado and Norton (1996a) reported that in sheep and goats fed a mixture of *Digitaria decumbens* and *Desmodium intortum* there was an apparent net gain in lignin across the rumen and whole intestinal tract. Perhaps, a similar gain in lignin is what explains the net gain in acid detergent fibre (30%) across the digestive tract of sheep and goats fed *Calliandra calothyrsus* (Perez-Maldonado and Norton, 1996b). Indeed, it was observed that insoluble (bound) tannins increased the apparent content of cell wall fractions, including lignin, as determined by the detergent system of forage analysis (Rittner and Reed, 1992).

In a revision of the detergent extraction techniques, Makkar et al. (1995d) analysed samples by the standard approach (which uses a separate sample for each determination) and by the sequential approach in which NDF is subjected to acid detergent solution to obtain ADF. These authors observed that when *Acacia saligna* leaves were subjected to a sequential extraction, acid detergent fibre and lignin content were higher than when these constituents were determined directly. The reverse was true when analysing faecal samples of sheep and goats. In later work, Makkar et al. (1997b) investigated how the presence of tannins could interfere with the determination of *in vitro* rumen true digestibility. Neutral and acid detergent fibre obtained after removal, by centrifugation, of the liquid phase of the incubation medium was compared with those obtained directly. The neutral detergent fibre in the centrifuged set was significantly higher than in the direct set, whereas the reverse was true in the case of acid detergent fibre. This was due to the presence of higher amounts of nitrogen and tannin in the ADF of both sets than in the ADF of the sample incubated. The same was true for the NDF of the centrifuged set. These observations suggest that the detergent system of forage analysis is inadequate for the determination of *in vivo* and *in vitro* cell wall digestibility of tanniniferous forages.

The implications of these observations for the determination of lignin *in vivo* were demonstrated by the work of Goodchild and McMeniman (1993) in a trial designed to monitor the disappearance of sorghum stover polyphenols and condensed tannins from browse in the sheep gut. These authors observed that the disappearance of tannins at various sites of the gut was quantitatively similar to increases in artefact lignin. This artefact lignin was found to contain 74 mg N per g, more than three times the nitrogen concentration in lignin, but less than the nitrogen concentration in Maillard products (Van Soest, 1982); and thus, mobilised several times less nitrogen than tannin-protein complexes would have done. The authors suggested that conversion of tannins in or around the abomasum into an inert lignin-like artefact would explain why tannin modifies rumen degradation more than it inhibits the digestion of protein in the small intestine.

A fact that comes to mind in light of these observations is that, although no absorption of *L. pedunculatus* tannins occurred in the digestive tract of sheep, the butanol-HCl assay was not able to detect all the tannins in ruminal, abomasal, duodenal, ileal and faecal samples (Terrill et al. 1994). In one experiment, tannin recovery in faeces was only 15% of intake. One of the explanations offered to explain this was the occurrence of conformational changes in the tannin molecule such that it is not longer detectable by colorimetric methods. If this were the case, this would also explain the appearance of artefact lignin and fibre discussed above. This merits further investigation.

2.5.5 Microbial degradation of tannins

Among the tannins, those of the condensed type are structurally more complex (Vennat et al. 1986; Osawa et al. 1993b), which probably results in their biodegradation being more difficult to attain or requiring more specialised conditions, enzymes and/or microorganisms. On the other hand, there is sufficient evidence on the degradation of hydrolysable tannins by a number of microorganisms (Scalbert, 1991) including those from the rumen (Field and Lettinga, 1992; Murdiatti et al. 1992).

A tannase enzyme that catalyses the hydrolysis of ester bonds between a phenolic acid and an alcohol has been isolated from *Aspergillus spp*. (Scalbert, 1991). This enzyme is also produced by other fungi (*Aspergillus niger, A. oryzae, A. flavus, A. japonicus, Penicillium chrysogenum. P. notatum,* and *P. Isladicum*), yeast (*Candida spp., Pichia*

spp., Debaryomyces hansenii), and bacteria (*Achromobacter spp., Bacillus pumilis, B. polymyxa, Corynebacterium spp., Klebsiella planticola,* and *Pseudomonas solanacearum*; Aoki et al. 1976; Jean et al. 1981; Pourrat et al. 1987). In addition, a gallotannin degrading esterase that catalysed the efficient hydrolysis of galloylglucose and related compounds was isolated from the leaves of pedunculate oak (*Quercus robur* syn. *Q. pedunculata*; Niehaus and Gross, 1997).

In turn, a great deal of the existing information on the microbial degradation of condensed tannins comes from studies with non-rumen microorganisms grown in pure cultures. For example, Deschamps (1985) isolated forty-eight bacterial strains from decaying bark that were able to grow on a mineral medium with added quebracho and mimosa tannin extracts as the sole carbon source. It was observed that tannins from mimosa were more strongly degraded than those from quebracho and that depolymerisation was much more important than the attack of monomeric structures. Likewise, a strain of *Penicillium adametzi* Zalenski was also isolated from enrichment cultures where condensed tannins were used as the only carbon source (Grant, 1976). When *P. adametzi* was grown in media containing catechin, the procyanidins B-3 and C-2 and higher molecular weight tannins as the sole carbon substrates, although the fungus would grow and degrade these substrates, the vitality of fungal growth was negatively related to the degree of polymerisation of the tannins.

In addition, Vennat et al. (1986) reported on the depolymerisation of a condensed tannin extract from strawberry rhizomes (*Fragaria vesca* L., *Rosaceae*) using a strain of the yeast *Saccharomyces rouxii*. In recent work, Makkar et al. (1994) showed that the fungus *Sporotrichum pulvurulentum* effectively degrades condensed tannins in oak (*Quercus incana*) leaves. Prompted by these observations, Gamble et al. (1996) also demonstrated the biological degradation of condensed tannins in Sericea lespedeza by the white rot fungi *Ceriporiopsis subvermispora* and *Cyathus stercoreus*. These authors monitored relative levels of condensed tannins and other nutrients as a function of fungal treatment by solid-state cross-polarisation and magic angle spinning ^{13}C nuclear magnetic resonance spectroscopy. The strains tested removed between 56 and 63% of bound and soluble tannins with *C. subvermispora* being more effective in doing so than *C. stercoreus*.

Several workers have also reported the degradation of small molecular weight phenolics by non-rumen microorganisms. For example, Chandra et al. (1969) reported that the moulds *Aspergillus fumigatus, A. terreus, A. niger, Penicillium* sp. and a *Streptomyces* sp. isolated from soil samples and *Aspergillus flavus* (dicat) were able to degrade catechin. Phloroglucinol carboxylic acid and protocatechuic acid were identified as products of the degradation. The degradation of quercetin to protocatechuic acid and phloroglucinol carboxylic acid by *A. flavus* was also reported by Westlake et al. (1959). In later work, Winter et al. (1989) reported the isolation of *Clostridium* strains capable of cleaving the C-ring of quercetin, kaempferol and naringenin from the faecal flora of humans. However, none of the strains cleaved catechin. Cleavage of quercetin and kaempferol produced 3,4-didydrophenylacetic acid and 3-hydrophenylacetic acid, respectively. The expected phloroglucinol could not be recovered. Finally, Hopper and Mahadevan (1997) reported on the degradation of catechin by *Bradyrhizobium japonicum*. This rhizobium cleaved catechin through catechin oxygenase with phloroglucinol carboxylic acid and protocatechuic acid identified as the initial products of degradation. Phloroglucinol carboxylic acid was further decarboxylated to phloroglucinol, which was further decarboxylated to resorcinol. Resorcinol was hydroxylated to hydroxyquinol. Protocatechuic acid and hydroxyquinol underwent intradiol cleavage to yield β–carboxy *cis*, *cis*-muconic acid and maleylacetate, respectively.

With its abundance in microbial species, the rumen should be a good source for tannin degrading microorganisms. This appears to be more probable in animals that commonly encounter tannins in their diets. Recently, microbial studies on ruminants that naturally browse woody plants have shown that these animals possess ruminal microorganisms that are not found in domestic ruminants and are resistant to the toxic effects of tannins. One of these organisms, *Streptococcus caprinus,* was isolated from ruminant content of feral goats browsing tannin-rich *Acacia* species (Brooker et al. 1994). The bacterium was a Gram-positive, facultative anaerobe capable of clearing tannic acid-protein complexes and tannin-resistant, growing in media containing at least 2.5% tannic acid or condensed tannins. Additionally, Skene and Brooker (1995) reported the isolation of a strain of *Selenomonas ruminantiun* subspecies *ruminantium* from feral goats browsing tannin-rich *Acacia* sp. The isolate was capable of growing on tannic acid or condensed tannin and displayed tannin acylhydrolase (EC 3.1.1.20) activity. Enzyme

activity was demonstrated to increase 17-fold and 36-fold respectively when cells were grown in the presence of gallic acid methyl ester or tannic acid. Finally, Nelson et al. (1995) reported on the isolation of an anaerobic diplococcoid bacterium able to degrade hydrolysable tannins from the ruminal contents of a goat fed *Desmodium ovalifolium*. This strain grew under anaerobic conditions in the presence of up to 30 g of tannic acid per litter and tolerated a range of phenolic monomers, including gallic, ferulic and *p*-coumaric acids. The predominant fermentation product from tannic acid breakdown was pyrogallol.

The degradation of condensed tannins by rumen microorganisms, on the other hand, appears to be more difficult to accomplish. For example, Makkar et al (1995c) reported that under *in vitro* conditions, rumen microbes from cattle which had never consumed tannin-containing diets, were not able to degrade condensed tannins from *Quercus incana*. Likewise, Makkar et al (1995a) reported that rumen microbes with eight days of tannin exposure were not able to degrade tannins from quebracho and from *Dichostachys cinerea* under the conditions of the rumen simulation technique (RUSITEC). On the other hand, McAllister et al. (1994) reported that after 120 hours of incubation with the rumen anaerobic fungi *Neocallimastix frontalis, N. patriciarum* and *Pyromyces communis*, the recovery of condensed tannins from *Lotus corniculatus* was less than 86%.

On the other hand, reports suggest that the capacity of rumen microorganisms to degrade small phenolics is far greater than their observed capacity to degrade higher molecular weight tannins. Indeed, Simpson et al. (1969) reported that microflora of the rumen rapidly degraded bioflavonoids such as rutin, quercitrin, naringin, and hesperidin to water-soluble products. Protocatechuic acid was decarboxylated to yield catechol, and catechol was not further degraded. In a series of studies, Cheng et al. (1969, 1971) and Krishnamurty et al. (1970) demonstrated that *Butyrivibrio* sp. from ruminal fluid cleaved the C-ring of rutin and quercitrin, but not that of the aglycone quercetin. Bacteria isolated from the rumen that degrade phloroglucinol include *Streptococcus bovis* and *copprococcus* sp. (Tsai and Jones 1975). The major products of phloroglucinol catabolism were acetate and carbon dioxide. In addition, it was reported that *Coprococcus* sp. at least partially degraded the flavonols quercetin and rhamnetin (Tsai et al. 1976). Finally, Krumholz and Bryant (1986, 1988) and Krumholz et al. (1987)

reported on the isolation and characterisation of a gram-positive ruminal bacterium which they named *Eubacterium oxidoreducens*, requiring H_2 or formate to degrade gallate, pyrogallol, phloroglucinol or quercetin to acetate and butyrate. An unidentified ruminal bacterium able to produce resorcinol from gallic acid was also observed.

In a very interesting study, Miller et al. (1995) transferred tannin-resistant microorganisms from feral goats to domestic livestock (sheep and goats), and examined the effect of this transfer upon the digestion of the tannin-rich mulga (*Acacia aneura*). Both sheep and domestic goats had greater feed intake and nitrogen retention after receiving a ruminal inoculation of feral goat rumen fluid. Inoculation also reduced the rate of weight loss in both species. On the contrary, Miller et al. (1996) reported that inoculation of sheep with *Streptococcus caprinus* (Brooker et al. 1994, see above) failed to improve the digestion of mulga. As the authors suggested, these results indicate that interactions between several populations of microorganisms are involved in achieving improvements in protein digestion.

The benefits accrued to ruminants by the degradation of tannin-protein complexes are not as evident as one might think. If the degradation of complexes takes place in the rumen, it is probable that none of that increased protein availability would benefit the host animal, since rumen microorganisms would de the ones to have first access to released protein. Other benefits, such as increased microbial nitrogen flow to the duodenum might ensue, provided that rumen microorganisms use the newly released protein efficiently. On the other hand, this might also signify a concomitant decrease in rumen escape protein. As discussed earlier (Section 2.5.2.2), increased rumen escape protein does not necessarily mean increased protein availability. However, in a great number of cases, improved nitrogen absorption and retention has been associated to increased nitrogen flows to the duodenum in animals fed tanniniferous diets.

It is noteworthy to mention that the discovery of tannin-protein-complex-degrading bacteria was done in the koala (Osawa 1990; Osawa and Mitsuoaka 1990; Osawa, 1991; Osawa 1992; Osawa and Walsh 1993; Osawa et al. 1993b). In this arboreal marsupial, these bacteria were observed to colonise “strategic” regions of the caecal wall and the proximal colon. This might facilitate access, although it is likely to be indirect, for the host animal to the nitrogen moiety liberated from breakdown of tannin-protein complexes (Osawa et al. 1993a). Tannin-protein-complex-degrading bacteria have also

been reported in caecal samples from ringtail possums, deer and some omnivores (Osawa and Sly, 1992). Thus, it appears that these bacteria are related to the distal portions of the digestive tract, where its role in degrading tannin-protein complexes would appear to bring indirect benefits, if any, to the host animal in terms of nitrogen availability.

However, what would be the destiny of the tannin moiety in tannin-protein complexes after their degradation by bacteria? If bacteria acted by releasing protein from tannin-protein complexes without affecting them, then it could be expected that tannins would be free to interact with other molecules such as digestive enzymes, and create new complexes. It is difficult to envision beneficial effects of the degradation of tannin-protein complexes under this scenario. If, on the other hand, microorganisms were also to degrade the tannins, then we would have to be concerned with the end products generated. This is because small molecular weight compounds resulting from the degradation of tannins could be easily absorbed by the host animal. Jimenez-Ramsey et al. (1994) reported that polymeric ^{14}C-labeled condensed tannin fractions extracted from sorghum were not absorbed from the intestinal tract of chickens. On the other hand, non-tannin related sorghum phenolics, presumably of small molecular weight, were readily absorbed.

In the studies of Osawa et al. (1993b), the enterobacteria isolated from koalas were shown to degrade tannin-protein complexes involving tannic acid (hydrolysable tannin), but did not release protein from those involving tannins from quebracho (a condensed tannin). Subsequent studies showed that these bacterial strains metabolised gallic acid into pyrogallol. Although pyrogallol was not degraded any further, no pyrogallol was detected in the faeces of the koalas. It is difficult to ascertain whether this could be due to absorption of pyrogallol by the koalas and whether absorption of pyrogallol entails an energetic cost or have positive or negative effects to the koala. Further discussion on this topic is carried out in Section 2.5.7.

2.5.6 Effects of Tannins on Rumen Microbes

Besides the possible role of tannins in deterring herbivory, tannins may also deter microorganisms from predating plant tissue, either by increasing resistance against pathogens or by protecting essential tissues such as wood against decay (Scalbert, 1991).

Tannins have been found to be toxic for fungi, bacteria and yeast. For the ruminant animal, depending on the need for microorganisms to degrade fibre, this toxicity may carry considerable nutritional importance. Hence, an accurate assessment of the effects of leguminous tree and shrub tannins upon ruminal microbes is important.

An estimation of toxicity can be obtained by measuring biochemical parameters characteristic of the metabolism of certain microorganisms, such as cellulose degradation, glucan synthesis, and nitrate, methane, or ethanol production (Scalbert, 1991). As it was shown in Tables 2-19 and 2-20, in most *in vitro* studies, a negative relationship between plant tannin content and degradability of protein, fibre, and dry matter by ruminal microorganisms has been reported. As a corollary, Cano-Poloche (1993) reported negative *in vitro* digestibility of neutral detergent fibre in tropical shrub legumes high in tannins. She attributed this to the binding of soluble tannins to components of the cell wall, with the resulting complexes becoming indigestible or inaccessible to bacterial enzymes. Additionally, *in sacco* studies have suggested that tannins are negatively associated to dry matter degradation by ruminal microorganisms (Chiquette et al. 1988; Salawu et al. 1997b).

The antimicrobial activities of tannins, both of the condensed and of the hydrolysable type, were demonstrated by the work of Henis et al. (1964), who studied the effects of carob pods water extracts and tannin acid on the growth of several microorganisms. Carob pods extracts inhibited *Cellvibrio fulvus* and *Clostridium cellulosolvens* at 15 μg/ml, *Sporocytophaga myxococcoides* at 45 μg/ml and *Bacillus subtilis* at 75 μg/ml. The inhibiting concentrations of tannic acid were found to be 12, 10, 45 and 30 μg/ml, respectively. However, the concentrations of both types of tannins required to inhibit the growth of microorganisms such *as Shigella dysenteriae* and *Salmonella typhosa* were reported to be in excess of 600 μg/ml. In that same study, it was observed that gallic acid and catechol were less effective in inhibiting the growth of these microorganisms than the polymeric compounds. Likewise, Andebrhan et al. (1995) reported that the effectiveness of partially purified tannins from cocoa seeds for inhibiting basiodiospore germination of *Crinipellis perniciosa* (Stahel) Singer, was directly related to the molecular weight of the phenolics added. In turn, Eberhardt and Young (1994) reported that the condensed tannins extracted from conifer seed cones were found to inhibit the growth of fungal cultures of *Ceratocystis coerulescens* and *Schizophyllum commune* but

not of *Trametes versicolor*. From these results, it appears that the observed degree of microbial inhibition due to the presence of tannins is a function of the type and concentration of tannin present and of the susceptibility of the microorganisms involved.

Similar studies have shown that tannins can also negatively affect microorganisms of the rumen and/or inhibit or alter their enzymatic activities. For example, *in sacco* studies showed that the activities of urease, carboxymethylcellulase, glutamate dehydrogenase and alanine aminotransferase were significantly lower in the tannin-rich treatment (*Quercus incana* leaves) as compared to the low tannin treatment (Makkar et al. 1988). On the other hand, the activities of glutamate ammonia ligase and λ–glutamyltransferase were higher in the high-tannin treatment. In turn, Bae et al. (1993b) reported that tannins from *Lotus corniculatus* inhibited extracellular culture fluid endoglucanase activity of the ruminal cellulolytic bacterium *Fibrobacter succinogenes* S85 at concentrations as low as 25 μg per ml. On the contrary, cell-associated endoglucanase activity increased at concentrations of condensed tannins between 100 and 300 μg per ml.

In another study, condensed tannins from sainfoin leaves were found to inhibit growth and protease activity in *Butyrivibrio fibrisolvens* A38 and *Streptococcus bovis* 45S1 but had little effect on *Prevotella ruminicola* B_14, or *Ruminobacter amylophilus* WP225 (Jones et al. 1994). Likewise, when studying the interactions among purified tannins from three sources with five strains of ruminal bacteria, Nelson et al. (1997) observed variation among the bacteria in the amount of tannin required to inhibit their growth and among the tannins in their capacity to inhibit microbial growth. Finally, McAllister et al. (1994) reported that tannins from *Lotus corniculatus* inhibited endoglucanase activity of four rumen anaerobic fungi grown in pure culture. However, there were differences in the susceptibility to tannin among the fungi, with *Neocallimastyx frontalis* being the most impervious and *Orpinomyces joyonii* the most susceptible to the presence of tannins.

In vivo, the effects of tannins on growth of the ruminal microflora can be estimated from measurements of the ruminal degradation of protein and other nutrients, and in most cases, the presence of tannins has been associated with reduced availability of nutrients to rumen microorganisms (see Tables 2-19 and 2-20). Another useful indicator of microbial growth is the amount of microbial nitrogen that reaches the duodenum of ruminants fed tanniniferous diets. However, it must be recognised that this measurement

is limited by our ability to distinguish between microbial, endogenous and feed nitrogen in duodenal digesta (Reed 1995). Indeed, techniques commonly used for measuring microbial nitrogen such as those based on diaminopimelic acid or in purine: nitrogen ratios in bacteria (Zinn and Owens, 1986) are not error-free.

Analysis of microbial nitrogen flow to the duodenum in response to tannin consumption shows a variety of responses. For example, Waghorn et al. (1987) reported reduced duodenal microbial nitrogen flux (DMN) in sheep fed first cut *Lotus corniculatus* containing medium (3.3%) tannin concentration when compared to those fed forages of low (0.5%) tannin levels. However, at the end of the trial, no overall difference in DMN was found between animals fed low- and high-tannin forages. In turn, McNabb et al. (1993) observed lower rumen microbial non-ammonia nitrogen fractions in sheep fed *Lotus pedunculatus* alone (5.5% tannins) as compared to those receiving a polyethylene glycol infusion. Conversely, Carulla (1994) observed a reduction in DMN and microbial efficiencies with increasing levels of polyethylene addition (i.e., lower free tannins) to *Desmodium ovalifolium* diets in one sheep trial. However, in another trial of similar design, this author observed no differences in DMN between animals fed low- and high-tannin forages. Finally, Perez-Maldonado (1994) and Barahona et al. (1997) observed no significant changes in DMN in sheep fed high-tannin diets, although DMN and microbial efficiencies were numerically higher for the high-tannin diets. In view of these results, some authors have hypothesised that tannins might increase microbial yields, probably as a result of improved endogenous nitrogen recycling (Beever and Siddons, 1986; Reed et al. 1990; Reed, 1995).

Direct measurements in microbial population shifts also give an indication of the impact of tannins on rumen microflora. Some reports have suggested a positive correlation between tannins and ruminal protozoan numbers. Chiquette et al. (1989) observed that *Lotus corniculatus* diets with 3.2% tannin tended to have higher protozoan populations than lotus diets with 1.2 % tannins, with the differences becoming significant 3 hours after feeding. Likewise, supplementation of sulla (*Hedysarum coronarium*) diets with polyethylene glycol resulted in decreased protozoan counts in sheep (Terrill et al. 1992a). Increases in protozoan populations might result in a reduction of bacterial numbers. Unfortunately, no measurements of bacterial growth parameters were performed in these two studies. On the other hand, Wang et al. (1994) reported increased

protozoan numbers in animals fed *Lotus corniculatus* and polyethylene glycol as opposed to those fed the control diet.

As discussed above, this great variation in responses both in the *in vitro* and *in vivo* data is the function of many different factors, among which with no doubt the way tannins exert their antimicrobial effects must be a very important consideration. In an excellent review, Scalbert (1991) suggested that tannins could mediate their antimicrobial effects in four different ways: (i) by inhibition of extracellular microbial enzymes. (ii) By limiting substrate availability to microorganisms. (iii) By action on microbial membranes: (a) by inhibition of oxidative phosphorylation or (b) by affecting the integrity of membranes. (4) By inducing metal ion deprivation. Of these mechanisms, the inhibition of oxidative phosphorylation is not likely to occur in anaerobic rumen microorganisms.

Butler (1989) suggested that under *in vivo* conditions digestive enzymes might retain full activity in the presence of tannins, possibly due to the presence of detergents and unfavourable pH conditions, all of which would prevent tannins from binding protein. In the ruminant, a substantial reduction in extractability of tannins occurs after chewing, indicating that low levels of soluble tannins, capable of inhibiting microbial enzymes, reach the rumen (Burrit et al. 1987; Terrill et al. 1992b). Additionally, it has been demonstrated that some animal species have the ability to secrete proline-rich salivary proteins with high affinity for binding tannins, which may result in a further reduction of extractable CT concentrations in the rumen (see Section 2.5.7). Thus, it may appear that the inhibitory effect of tannins on microbial activity are mainly due to substrate-tannin complex formation (Carulla, 1994). Yet, an increased efficiency in nitrogen usage and recycling to the rumen in response to tannins might help microbes to overcome tannin-induced nitrogen deficiencies and foster their growth (Egan and Ulyatt, 1980).

A final observation is that many workers have reported that, in the presence of tannins, many bacteria undergo morphological changes (Henis et al. 1964; Chiquette et al. 1988; Bae et al. 1993; Jones et al. 1994; Nelson et al. 1995) and fungi (McAllister et al. 1994). Among those changes is the tendency to grow in chains, the formation of protoplasts, the presence of large amounts of surface material, the tendency to form multiple adherent microcolonies, the flocculation of bacterial cultures and the elongation of bacterial cells.

It is probable that bacteria growing in glycocalyx-enclosed microcolonies are limited in the secretion of bacterial exoenzymes, in their proliferation, and in their penetration of plant tissues (Chiquette et al. 1988). However, the work of Bae et al. (1993b) and McAllister et al. (1994) suggested that the presence of extracellular material could indeed help prevent the inactivation of microbial enzymes by tannins. Indeed, McAllister et al. (1994) suggested that the filamentous material excreted by fungi in the presence of tannins might create microenvironments between the fungal mat and the surface of the substrate. Within these microenvironments, enzymes could continue to be active. A similar mechanism was reported in the anthracnose fungus *Colletotrichum graminicola*, which produces spores in water-soluble mucilage (Nicholson et al. 1986). A glycoprotein fraction of the mucilage had an exceptionally high affinity for binding purified tannins and protected spores from inhibition of germination by a variety of phenolic compounds. The ability of some microorganisms to degrade and metabolize tannins (see previous section) can also act as a protective mechanism against the deleterious effects of tannins upon microbes. Other factors such as the secretion of tannin resistant enzymes, tannin oxidation, and the formation of siderophores to sequester metal ions have also been suggested (Scalbert, 1991).

As a final thought, it is clear that both the rumen microflora as a whole and individual microbial species of the rumen possess a great versatility that allow them to survive in the face of high concentrations of tannins. It is undeniable that the presence of tannin could lead to changes in composition of the rumen microflora. However, it is also possible that the tannin-resistant microorganisms would continue to contribute to a healthy ruminal activity, especially in the presence of adequate concentrations and types of tannins.

2.5.7 Protective response to dietary tannins

Animals that normally consume tannin-containing plant materials presumably should have developed at least partially effective defences against the potentially deleterious effects of tannins. Additionally, it would be logical to assume that the protective responses observed in a given animal would be related to the nature of the problem the animal faces. In this regard, a very important distinction has to be made regarding whether the nutritional affects of tannins are due to their complexation with digestive enzymes and feed components, to their absorption from the digestive tract or to a

mixture of these two mechanisms. This distinction is needed given the likelihood that the nutritional implications resulting from these two mechanisms would be different both in the extent of their effects and in the counteractive responses associated with them.

Studies carried out with ^{14}C-labelled condensed tannins indicate that they are not absorbed from the digestive tracts of chickens (sorghum tannins, Jimenez-Ramsey et al. 1994) and sheep (*Lotus pedunculatus* tannins, Terrill et al. 1994). Quite on the contrary, Perez-Maldonado and Norton (1996a) using butanol-HCl and ^{14}C-labelled condensed tannins detected a substantial disappearance of *Desmodium intortum* and *Calliandra calothyrsus* tannins (free, protein, and fibre-bound) during metabolism in the gastrointestinal tract in sheep and goats.

It is possible that the differences between these assays could be related to the source of condensed tannin used. It has been suggested that certain condensed tannins can be easily depolymerised under a variety of mild conditions not unlike those found in many digestive tracts (Butler et al. 1986; Clausen et al. 1990). For instance, Porter et al. (1985) reported on the lability on the interflavan bonds of certain tannins under acidic conditions is similar to that of the glycosidic bond of phenol glycosides. Both the rate of depolimerisation and the products formed are dependent on tannin structure. Thus, procyanidins can be depolymerised under mild acidic conditions (0.6 N HCl; 30°C; Fletcher et al. 1977), whereas proanthocyanidin-A2 tannins (Jacques and Haslam, 1974) are highly resistant to depolymerisation. For procyanidins, 2,3-*cis*-procyanidins undergo stereospecific depolymerisation more rapidly than their 2,3-*trans*-isomers (Hemingway and McGraw, 1983; Hemingway et al. 1983). It has been suggested that the kinetic control in these latter reactions is the result of steric considerations (Roux et al. 1980).

The perspective of condensed tannin depolymerisation has not been examined in depth in nutritional studies. Indeed, the great majority of nutritional work has been directed towards explaining animal responses in the light of the formation of complexes involving tannins and dietary entities. It must be recognised that polyphenols in plants are not only comprised of the condensed tannins, but also include low molecular weight non-tannin phenolics which could be more readily absorbed from the digestive tract, resulting in systemic effects elsewhere in the body. For example, the effect of absorbed phenolics on intake could be mediated in one of three ways: (i) altering physiological

systems, (ii) creating toxicity or (iii) creating an additional energy requirement if their elimination is an energy-demanding process (Robbins et al. 1987a).

Logically, one of the first lines of defence against the deleterious effects of tannins involves control of intake. Mueller-Harvey and McAllan (1992) suggested that animals would preferentially eat low-tannin diets if given the choice. This is definitely the case in the grass-preferring feeders such as cows and sheep. However, Semiadi et al. (1995) observed that when the intermediate feeders sambar and red deer were offered a free choice of seven different plant species, sambar selected a diet higher in condensed tannins and lignin, but lower in protein. This was probably linked with the greater ability of sambar to neutralise some plant secondary compounds and their more efficient rumination pattern compared with red deer.

It must be kept in mind that dietary selection is the result of the interplay of many different factors. For example, animals develop preferences for foods ingested early in life in the presence of social models such as the mother (Provenza and Balph, 1987, 1988). Work by Distel and Provenza (1991) showed that experience early in life could have profound and persistent effects on intake. These authors observed that young goats that had been exposed to the low quality and tanniniferous shrub blackbrush (*Coleogyne ramossisima*) consequently ate more of the shrub than goats that had never been exposed to it. This corroborates the results of many other workers that prior experience ingesting foods increases the preferences for those foods by animals (Arnold, 1964; Martin, 1978; Bartmann and Carpenter, 1982).

Such observations imply that several physiological and morphological factors are actively involved in these adaptive responses. This was illustrated by the study of Distel and Provenza (1991), where experienced and inexperienced goats were offered a choice of either older growth or current season growth of blackbrush, the latter containing 2.4 times more tannins that the former. Early in the experimental period, inexperienced goats ingested more current season growth than experienced goats. However, inexperienced goats quickly (24 hours) learned an aversion to blackbrush current season growth, presumably because condensed tannins stimulated the emetic system of the midbrain and brain stem (Provenza et al. 1990). After that, both groups rejected current season growth, but experienced goats had significantly greater intakes of older growth than inexperienced goats. This higher intake was related to increased rumen capacity and

probably to a greater ability to detoxify ingested phenols in experienced goats. Likewise, research has shown that the alimentary canal of browsers such as deer may enlarge when they ingest poor-quality forages, allowing them to ingest more forage (Milne et al. 1978; Baker and Hobbs, 1987).

As it would probably be the case with depolymerised tannins, it has been suggested that simple phenolics and the phenol component of hydrolysable tannin would be readily absorbed in the digestive tract of animals (McLeod, 1974). This could explain why Robbins et al (1987a) observed that deer consuming leaves and flowers high in phenolics produced an orange to blood-red urine that was negative for both haemoglobin and red blood cells. While some animals may have detoxification capabilities, they must still balance the rate of intake with the rate of detoxification if they are to consume successfully forages containing absorbable phenolics (Robbins et al. 1987a). A corollary of this adaptive behaviour was reported by these authors in the very slow, continuous ingestion of high-phenolic forages by mule deer (*Odocoileous hemionus*) in response to limited feed options. Likewise, sheep fed the highly tanniniferous *Acacia cyanophylla* appeared to regulate tannin consumption in function of tannin accumulation elsewhere in the body, with intake changing from day to day (Bensalem et al. 1997a). These observations provide support to the hypothesis of Freeland and Janzen (1974) that mammalian herbivores ingest toxins in amounts they can detoxify.

Another line of defence against ingested tannins is the secretion of salivary proline-rich proteins that have very high affinity for tannins (see Section 2.4.2.2). It has been proposed that these proline-rich tannin-binding salivary proteins constitute the first line of defence against tannins in the digestive tract (Butler et al. 1986; Mehansho et al. 1987a, 1987b). Increased synthesis of proline-rich salivary proteins due to tannin-rich savanna sorghum diets has been demonstrated in rats and mice (Mehansho et al. 1983, 1985). Quite on the contrary, hamsters in a 2% tannin diet did not respond like rats and mice in inducing proline-rich salivary protein synthesis (Mehansho et al. 1987a).

The benefits accrued to animals by the production of salivary tannin-binding proteins have been unambiguously demonstrated. Mehansho et al. (1983) reported that during the first three days of feeding high-tannin sorghum, a lost of body weight was observed in rats. Within three days, weight of parotid glands and production of proline-rich proteins reached their maximum and coincidentally, rats started to gain weight again. However,

when induction of these dietary proteins is blocked by beta--specific blockers, rats become as vulnerable to the effects of dietary tannins as hamsters are (Mehansho et al. 1987a; Butler, 1989).

Early work showed that in ruminants the parotid salivary glands are three times larger in browsers than in grazers (Hoffman, 1973), which could probably be best explained by the range of tannin intake among these two groups of feeders. However, the presence of tannin-binding proline-rich salivary proteins in ruminants was only reported for the first time by Robbins et al. (1987b). They reported that the saliva of the browser mule deer (*Odocoileus hemionus*) had a higher proline content and greater tannin-binding capacity than saliva from the grazer sheep and cows.

Results from the work of Robbins et al. (1987b) and Austin et al. (1989) suggested that browsers that usually ingest dietary tannins produce tannin-binding salivary proteins, while the grazers do not produce such proteins. The efficacy of this adaptation was illustrated by Robbins et al. (1991), who observed that the digestibility of fibre by mule deer (*Odocoileus hemionus*), which produces proline-rich proteins, was not affected by condensed tannins, but it was reduced in sheep which have saliva with low affinity for tannins. Austin et al. (1989) also observed that these tannin-binding salivary proteins form soluble complexes with tannins that are stable at the pH values encountered during transit of the entire digestive tract.

In recent studies it has been shown that tannin-binding proline-rich salivary proteins are not produced in goats (Distel and Provenza, 1991), but are present in the root vole (*Microtonus oeconomus* Pallas; Juntheikki et al. 1996) and in moose (*Alces alces*; Juntheikki, 1996). However, Provenza and Malechek (1984) suggested that salivary or plant protein consumed by domestic goats might bind as much as 50% of the dietary tannins during ingestion. A further observation by Juntheikki, (1996) was that both Scandinavian and North American moose produce a salivary tannin-binding protein which binds only condensed tannins common in their diet.

It is interesting to note that with the exception of rats and mice, production of tannin-binding salivary proteins appears to be constitutive in all other animals and in amounts which reflect their approximate intake of tannins and related polyphenols (Butler, 1989). A drawback of this comportment is that if tannin-binding proteins are poorly digested (as

it appears to be the case), their continuous production might result in reduced protein retention when animals consume low-tannin diets (Robbins et al. 1987b). However, after analysing the amino acid composition of mammalian proline-rich proteins, Mole et al. (1990a) observed that they contained very low levels of nutritionally essential amino acids, which should make them expendable at a low cost for the animal.

Proline-richness is not always a guarantee of a protein having high affinity for tannins. For example, Mole et al. (1990a) isolated salivary proteins from sheep and cows with very high proline content (46 and 57%, respectively) that had no affinity for tannins, On the other hand, the relatively lower proline-rich deer saliva bound tannins very strongly. Caution must be exercised on the interpretation of these results, given that tannin-protein interactions can be very specific (see section 2.4.2). Additionally, if the production of proline-rich proteins is primarily intended to protect animals against tannins, then it could be expected that these proteins would have greater binding affinity for those tannins animals commonly encounter in their diets. However, after studying the information available on proline-rich proteins, McArthur et al. (1995) proposed that the ancestral function of these proteins was maintaining oral homeostasis and that counteracting dietary tannins by binding with them was a derived function. A recent in this field, however, was the isolation of histatins (histidine-rich salivary proteins) from human saliva with high affinity for binding tannins (Yan and Bennick, 1995). Indeed, in comparative studies, histatins showed greater ability to precipitate tannins than proline-rich salivary proteins.

Other lines of defence employed by herbivores have as a working principle the prevention of the formation of insoluble tannin-protein complexes and were discussed in Section 2.4.3. These include high gut pH, surfactants, high concentration of cations and gut redox potential in insects and the use of surfactants in mammals. Additionally, other mechanisms that allow herbivores to deal with the effects of ingested tannins have been reported in the literature. For example, voles (Lindroth and Batzli, 1983) and snowshoe hares (Bryant et al. 1985) possess enzymes systems that detoxify phenolic compounds through conjugation with glucuronic acid. Likewise, the enzyme polyphenol oxidase has been shown to inactivate tannins (Nichols-Orions, 1991). Finally, in tree locusts absorbed phenolics are not toxic but are utilised in the synthesis of the cuticle (Bernays and Woodhead, 1982).

2.5.8 Tannin effects upon animal productivity

The mechanisms and effects of tannin-ruminant interactions are so varied that accurate predictions of animal performance (i.e. milk, meat or wool production) as influenced by tannin consumption cannot be easily made. Indeed, it is not uncommon to find reports of beneficial and deleterious effects of tannins on animal performance in the literature. Given the great variety of tannin-nutrients, tannin-microorganisms and tannin-animals interactions, it would seem adventurous to try to find an explanation for these contrasting occurrences.

A good number of reports that show positive effects of tannin in terms of animal productivity can be found in the literature. For example, Driedger and Hatfield (1972) observed increased daily weight gains and greater gain to feed ratios in lambs fed a corn-based diet supplemented with tannin-treated (0.9% of diet dry matter) soybean meal as compared to lambs receiving supplemental urea or soybean meal with no tannin added. In turn, Terrill *et al*. (1992a) reported that tannins (4.0-5.0% of dry matter) in sulla (*Hedysarum coronarium*) increased wool growth rate in sheep as compared with sheep grazing the same pastures but given a daily dose of polyethylene glycol. This effect was only evident during the active periods of wool growth (spring and early summer) and was not observed during the winter. No differences in body growth or voluntary feed intake were observed in that study. With six accessions of the tropical legume *Sesbania sesban*, it was observed that with increasing tannin levels there as an increase followed by a decrease in the liveweight gain of sheep (Kaitho et al. 1997b).

The tannins in *Lotus corniculatus* have also been associated with improved performance by sheep. In a grazing trial with lactating ewes rearing twin lambs, Wang et al. (1996a) compared the milk yield and composition of ewes grazing *L. corniculatus* (4.5% tannins) with and without twice a day daily oral supplementation with polyethylene glycol. Although at peak lactation there were no differences among both groups, as the lactation progressed, the decline in milk production and in the secretion rates of milk protein and lactose were less in the ewes not receiving polyethylene glycol supplementation. In mid and late lactation control ewes secreted more milk (21%), more protein (14%) and more lactose (12%) than polyethylene glycol-supplemented ewes. Voluntary feed intake and wool growth was similar in both groups. Wang and co-workers (1996b) reported another trial in which weaned lambs grazed *L. corniculatus* and lucerne (*Medicago sativa*) with

and without supplementation with polyethylene glycol. Compared to lambs grazing lucerne, lambs grazing *L. corniculatus* had slightly lower forage intake, and higher liveweight gain, carcass weight gain, carcass dressing-out percentage and wool growth. Polyethylene glycol supplementation had no effect upon these measurements in lambs grazing lucerne. However, in lambs grazing *L. corniculatus*, polyethylene glycol supplementation reduced wool growth and slightly reduced liveweight gain. From these two experiments, it can be concluded that tannins in *L. corniculatus* increase the productive efficiency of sheep. This beneficial effect of *L. corniculatus* forage appears to be also true for liveweight gains in heifers (Marten and Ehle, 1984) and fat-corrected milk yields in dairy cows (Larsen et al. 1974; as cited by Reed, 1995).

Decreased carcass fatness in growing lambs in response to tannin consumption has been reported by Purchas and Keogh (1984) and by Terrill et al. (1992a), presumably due to increasing protein deposition and reducing fat as a proportion of the carcass. This effect of tannins can be useful in helping the meat industry to keep pace with the present-day nutritional concerns of humans.

An area in which tannins appear to be of benefit for animal production, is their possible use in controlling internal parasites without the recourse to anthelmintics. Specialty crops, particularly those containing condensed tannins (*Hedysarum coronarium, Lotus pedunculatus* and *L. corniculatus*) hold special promise as a means of counteracting parasite-induced production losses and dagginess (Niezen et al. 1996). Niezen et al. (1995) conducted two grazing experiments to evaluate the effects of the tanniniferous sulla (*Hedysarum coronarium*) and the non-tanniniferous lucerne (*Medicago sativa*) on the performance of parasitised and non-parasitised lambs. In both experiments, parasitised lambs feeding on sulla higher average daily gain (129-206 vs. 3-50 g/ day) than parasitised lambs grazing lucerne. In addition, in the first trial parasitised lambs feeding on sulla had lower faecal egg counts per gram of faeces than parasitised lambs grazing on lucerne. In the second trial, parasitised lambs grazing sulla had higher rate of wool growth from mid-side patches and larger wool fibre diameter than parasitised lambs grazing lucerne. At slaughter, parasite-induced anorexia was evident in lambs grazing lucerne but not in those grazing sulla.

Another issue that has received much attention regarding tanniniferous forages is their use in the prevention of bloat in cattle. The grazing of lush pastures such as white clover

(*Trifolium repens* L.), red clover (*Trifolium pratense* L.), or alfalfa (*Medicago sativa*) has been commonly related to the occurrence of bloat in cattle. The soluble proteins present in these pastures have been implicated as the surfactants responsible for the persistent foams that develop in the rumen of animals suffering from bloat (Jones and Mangan, 1977). This foam entraps rumen fermentation gasses and the free gas space normally present in the dorsal sac of the rumen is replaced by a frothy mass of rumen digesta. Unless this condition is relieved, the animal suffers severe compression of heart and lungs, leading to anorexia and rapid death. Bloat-provoking legumes release large amounts of soluble protein into the rumen but, due to their content of condensed tannins, non-bloating species such as sainfoin and *Lotus corniculatus,* do not (Mangan, 1988). Tannins would decrease the solubility of the cytoplasmic protein released in large quantities into the rumen by these legumes, thus preventing the occurrence of the typical frothy bloat (Jones and Mangan, 1977; Mangan, 1988; Barry, 1989).

As pointed by Mangan (1988), the fact that the main component of soluble protein acting as foaming agent is Rubisco, the most abundant protein in green leaves and on earth (Barbeau and Kinsella, 1988), makes a direct connection between the problem of bloat and the protein nutrition of forage-fed animals.

Recently, Li et al. (1996) with the use of a very sensitive method for the detection of condensed tannin, re-examined 22 legumes species, most of which had been reported to be bloat-safe but free of foliar condensed tannins. After examination, the tested plants could be divided into four classes: (i) Bloat-safe species which had neither observable nor measurable condensed tannins. Bloat safety in these plants could be related to mechanically stronger leaves (Lees et al. 1981), which can lower the initial rate of digestion and thus enable bloat safety (Howarth et al. 1978). (ii) Species with a history of bloat, but where a few tannin-positive cells were found. (iii) Bloat-safe plant with observable tannin positive cells, but without measurable levels of tannins. (iv) Bloat-safe plants with observable tannin-positive cell and measurable levels of condensed tannins.

In the study of Li et al. (1996), all plants with a measurable level of total tannins greater than 1 g per kg of dry matter had been reported as bloat-safe. This was in close agreement with reports that tannin levels between 1.1 and 2.3 g per kg of diet dry matter were associated with bloat safety (Waghorn and Jones, 1989; Stockdale, 1994). Additionally, theoretical extrapolations suggested that a total condensed tannin level of

2-5 g of tannin per kg of dry matter would be sufficient to prevent bloat in lucerne and clover species. Li et al. (1996) made this calculations based on the following observations: (i) These bloat-provoking legumes contain 14-20% soluble protein (McArthur and Miltimore 1968). (ii) Up to 60% of soluble protein was released in the rumen (Mangan, 1972). (iii) A 50% reduction in soluble protein is required for bloat safety (Howarth et al. 1977). (iv) Tannin precipitate protein when tannin; soluble protein ratios were greater than either 1:12 (Jones and Mangan 1977) or 1:20 (Tanner et al. 1994). In summary, the threshold level of condensed tannins required for bloat safety was between 1 and 5 g of tannins per kg of forage (diet) dry matter. Therefore, these authors recommended 5 g per kg of dry matter as a breeding objective or bloat safety in forage legumes.

In other reports, consumption of *Lotus pedunculatus* with and without polyethylene glycol supplementation had no effect in the growth rates or wool production of sheep (Waghorn et al. 1994a; Waghorn and Shelton, 1995).

Conversely, deleterious effects of tannin consumption on animal performance have also been frequently reported. Barry (1985) observed that daily administration of polyethylene glycol to sheep grazing *Lotus pedunculatus* diets increased liveweight gain by 41-61 g/d and increased wool growth as well. Pritchard et al. (1988) reported increased wool growth up to 45% and liveweight gain up to 33 g/d in sheep fed fresh mulga and supplemented with minerals and polyethylene glycol as opposed to those not receiving supplementation. Reed *et al*. (1990) reported weight loss in sheep fed a teff (*Eragrotis teff*) straw diet supplemented with *Acacia cyanophylla,* a fodder tree high in tannins. However, weight gains of sheep supplemented with *A. siberiana* and *A. seyal* (also high in soluble phenolics) were not different than those of sheep supplemented with noug cake (an oil seed cake) or urea.

Other studies where tannins were negatively associated with animal performance include that of Silanoke et al. (1994) who reported that supplementation with 25 g of polyethylene glycol per day was associated with recovery of weight loss by sheep fed solely on carob (*Ceratonia siliqua*) leaves. Likewise, the high content of condensed tannins and related polyphenols in *Carissa edulis* and *Dichrostachys cinerea* resulted in low intake, digestibility and rate of gain (6 g per day) when fed with maize and high weight loss (-63 g per day) without maize (Wiegand et al. 1996). In turn, Miller et al.

(1997) reported that provision of polyethylene glycol at a rate of 12 g/day significantly improved clean wool weight (0.809 vs. 0.745 mg per cm2) and liveweight gain (44 vs. 22 g per day) compared with unsupplemented sheep. Finally, Prasad et al. (1997) reported that sheep fed khejri (*Prosopis cineraria*) and supplemented with polyethylene glycol had higher wool yields (587 vs. 444 g) that unsupplemented sheep.

Barry (1984) hypothesised that the reduction of wool growth by tannins could obey two reasons: 1) decreased protein absorption, limiting the amount of sulphur amino acids available for wool production, and 2) an elevation of plasma growth hormone concentration which would divert amino acid away from wool synthesis. However, later work by McNabb et al. (1993) and Wang et al. (1994) appeared to suggest that tannins do increase the availability of sulphur for body synthetic reactions. Likewise, other workers have failed to record changes in the levels of growth hormone (Waghorn et al. 1994b; Wang et al. 1996a). Undoubtedly, a greater understanding of tannin effects upon ruminants is needed to allow man to make adequate use of the properties of tannins in improving animal nutrition.

2.6 CONCLUDING REMARKS

Despite a few problems, tropical legumes constitute a plausible alternative for improving pasture-based tropical animal agriculture. Already confined to low-fertility soils, Third World animal agriculture is under increased pressure due to the ongoing expansion of human settlements into agricultural land. As animal agriculture moves into areas of decreasing soil fertility and increasing soil acidity, the need for the selection and collection of leguminous fodder shrubs and trees adapted to these conditions attains greater importance. Although a good number of leguminous species that perform agronomically well under these conditions have been identified, their value as forages appears to be limited by several factors, among which, the presence of high levels of tannins seems to play a very important part.

Consumption of condensed tannins by ruminants has been associated with both positive and negative effects. Positive effects include protein protection from excessive fermentation in the rumen, potentially increasing protein absorption and preventing bloat development. Negative effects might include reduced voluntary feed intake and reduced fibre and dry matter digestibility due to impaired rumen function. Which of these effects is observed in a given situation appears to be a function of factors such as the structure of tannins and their concentration in plant tissue. Determination of the role played by these factors in determining the impact of tannins on ruminant nutrition, is essential to provide a framework for future germplasm evaluations.

It is undeniable that great strides have been given in our knowledge of tannin chemistry and in our understanding of the mechanisms underlying the interactions between tannins and other molecules under controlled conditions. A great deal of information has also been gathered regarding the effects of tannin consumption on the nutritional status of ruminants. However, it is also evident that we are far from completely comprehending the role of tannins with regard to both plant fitness and animal performance. Indeed, in almost all of the areas of research discussed, the field remains open and ripe for further exploitation and inquiry.

However, our capacity to pursue scientific interests is today greater than never before. A good corollary of this is our capacity to manipulate the nutrient content in plants. Tannin biosynthesis in forage legumes can be chemically (Morris and Robbins, 1992; Robbins

et al. 1992; Robbins et al. 1996), nutritionally and environmentally manipulated (Morris and Robbins, 1997). Genetic manipulation to either reduce (Carron et al. 1994; Robbins et al. 1994; Bavage et al. 1995) or increase (Crea et al. 1994; Bavage et al. 1995) tannin content in tanniniferous legumes has also been reported. Manipulation of tannin biosynthesis in tannin-free forage legumes is another issue that has received some attention (Morris and Robbins, 1997). This ability, together with the possibility to genetically alter tannin structure, provide extremely powerful tools to improve our understanding of plant processes and metabolite function.

Research in structure-activity relationships, however, can also be undertaken without the involvement of genetic manipulation, given that a great variability in tannin structure already exists in nature. Such was the approach followed to complete the work reported in this thesis, in which the effects of tannins from tropical legumes, differing in structural characteristics on enzymatic and microbiological degradation of substrates, among other aspects, were studied.

3. IN VITRO DEGRADABILITY OF MATURE AND IMMATURE LEAVES OF SEVEN TROPICAL FORAGE LEGUMES DIFFERING IN CONDENSED TANNIN AND NON-STARCH POLYSACCHARIDE CONTENT AND COMPOSITION

3.1 INTRODUCTION

3.1.1 The problem

A significant proportion of the ruminant production in tropical and sub-tropical South America is forage-based and relies on the grazing of native and introduced grasses growing in acid, low-fertility soils. Under these conditions, and especially during the dry season, the low quality and availability of forage limits animal production. The introduction of forage legumes into these grazing systems is intended to alleviate this problem. However, many of the tropical forage legumes which have good agronomic performance when planted on low-fertility, acidic soils (i.e. *Desmodium ovalifolium, Calliandra calothyrsus, Flemingia macrophylla*) have limited feeding value and are poorly accepted by livestock. This could be due, in part, to the high concentration of secondary metabolites such as condensed tannins in these plants. Condensed tannins are polyphenols with the ability to bind and precipitate proteins, carbohydrates and other molecules (Haslam, 1986), thereby making them less available for productive purposes in the ruminant animal.

High (≥ 4.0 % of forage dry matter) levels of condensed tannins in temperate forage legumes such as *Lotus pedunculatus* have been shown to depress intake and interfere with protein and cell wall digestion in ruminants (Barry and Duncan, 1984; Pritchard et al., 1988). Similar observations have been made with tropical forage legumes. For example, reduction of methanol-extractable condensed tannin content in leaves of *D. ovalifolium* (Carulla, 1994) by the addition of polyethylene glycol, resulted in increased intake and nitrogen retention by sheep. However, in a similar trial, intake and digestibility of *D. ovalifolium* and *F. macrophylla* was not only influenced by condensed tannin concentration, but also by cell wall carbohydrate content and probably composition as well (Barahona et al., 1997).

Delimitation of the role played by condensed tannins in determining forage quality is of primary importance to the ongoing efforts of identifying legume germplasm adapted to acid soils and with high forage quality. Recent reports have suggested that tannin content, as determined by the butanol-HCl or vanillin-HCl methods, is not a good indicator of potential nutrient degradability when comparisons are made across different legume species. For example, tannin content was not a reliable indicator of *in sacco* nitrogen degradability in twelve tropical legumes (Ahn et al. 1989) and of the *in vitro* degree of inhibition of rumen micro-organisms by phenolics in twelve Bolivian fodder trees (Wood and Plumb, 1995). Additionally, neither protein-precipitating phenolics nor insoluble proanthocyanidins were related to gas production in seventeen Zimbabwean browse species (Ndlovu and Nherera, 1997). Furthermore, Ndlovu and Nherera (1997) suggested that the fibre fraction of browse might be more important than tannins in limiting fermentation *in vitro*.

In consequence, a study was designed to examine the relationships between protein, fibre and condensed tannin content and cell wall carbohydrate content and composition with the overall nutritional quality of seven tropical legumes as determined *in vitro*. Both the two-stage *in vitro* dry matter digestibility technique of Tilley and Terry (1963) and the pressure transducer technique of Theodorou et al. (1994) were used for the determination of nutritional quality.

3.1.2 A brief description of the tropical legumes chosen for this study

The tropical legumes chosen for this study comprised *Desmodium ovalifolium, Flemingia macrophylla, Leucaena leucocephala, Leucaena pallida, Leucaena macrophylla, Calliandra calothyrsus* and *Clitoria fairchildiana*.

The genus *Leucaena* is native to the New World and extends from Southern United States through middle South America (Argel et al., 1998). Among the *Leucaena*, *L. leucocephala* is probably the species that has received the most attention. This tropical legume is an extremely useful, versatile and adaptable tree, that can provide several products and services which include firewood and poles, livestock fodder and green manure, food for human consumption and soil conservation benefits (Hughes, 1998). Presently, three subspecies of *L. leucocephala* have been recognised. These subspecies are: *leucocephala*, *glabrata* (Zarate, 1987) and *ixtahuacana* (Hughes,

1997). The value of *L. leucocephala* lies in its combination of multiple products, fast growth, ease of propagation and management by farmers, and high quality products (Hughes, 1998). It is the premier forage species in both extensive grazing systems and cut and carry systems for smallholders (Shelton, 1998), often described as the "alfalfa of the tropics" and was the first species used for the production of green manure in alley cropping systems (Hughes, 1998). However, the wider use of *L. leucocephala* has been limited by lack of cold and drought tolerance, poor growth on acid soils, heavy pod production and low wood durability. The most serious limitation of *L. leucocephala* is the susceptibility to the psyllid defoliator *Heteropsylla cubana*, which damage *Leucaena* trees by feeding from the phloem of developing shoots and young foliage (Hughes, 1998). Quantification of a commonly used psyllid damage rating scale in terms of loss of yield showed that, at even moderate damage ratings, yield loss of susceptible accessions can be greater than 50% (Mullen et al., 1998).

Leucaena leucocephala is one of the most nutritious tropical forages fed to ruminants (McNeill et al., 1998), consequently giving cattle high liveweight gains (Jones, 1994). However, previous research has shown that foliage of *L. leucocephala* has a considerable content of condensed tannins (1.8-7.30%, McNeill et al., 1998, Dalzell et al., 1998), which can restrict the utilisation of *Leucaena* forage. Additionally, foliage of *L. leucocephala* has been shown to contain the toxic amino acid mimosine (Brewbaker and Kaye, 1981, Jones and Jones, 1984).

Another member of the *Leucaena* that has received attention by researchers is *L. pallida*. Because it has moderate to high psyllid resistance, *L. pallida* has been promoted as a substitute for *L. leucocephala* for forage production in areas where high psyllid pressure limits yields from *L. leucocephala* (Hughes, 1998). *L. pallida* and its hybrid with *L. leucocephala* (KX2) show excellent psyllid resistance (Mullen et al., 1998), high forage yields (Mullen and Shelton, 1998), cool tolerance and have spreading branching habit, which make this accession ideal for forage production (Hughes et al. 1998). However, *L. pallida* may have lower nutritive value than *L. leucocephala* (Norton et al., 1995). Indeed, the high concentration of condensed tannins in *L. pallida* was associated with a reduction in the availability of nitrogen to sheep (McNeill et al. 1998). In turn, Jones et al (1998) observed that *L. pallida* was considerably less palatable than *L. leucocephala* and *L. collinsii*.

By comparison with *L. leucocephala* and *L. pallida*, *L. macrophylla* has little been used. As indicated by its name, this species has the largest leaflets (3-7 cm) of any *Leucaena* (Hughes, 1998). It occurs as subspecies *istmensis*, which is fast growing and outperforms subspecies *macrophylla* (Stewart et al., 1991). Work by Mullen et al. (1998) showed that *L. macrophylla*, subsp. *macrophylla* was moderately susceptible to psyllid attack. Additionally, during an evaluation of the *Leucaena* foundation collection in Subtropical Australia, Mullen and Shelton (1998) observed that *L. macrophylla* accessions yielded relatively well in the warm seasons, but were slow to establish. In turn, Middleton and Clem (1998), reported that among 27 different *Leucaena* accessions, *L. macrophylla* had one of lowest tolerances to frost. On the other hand, Argel and Pérez (1998) reported that the foliar retention of *L. macrophylla* and plant height after 9.8 months after planting was among the highest when 18 different *Leucaena* species were planted in Costa Rica, Central America. Another positive observation with regard to *L. macrophylla* is its low condensed tannin content (0.6 – 1.7% of dry matter; Dalzell et al., 1998).

Calliandra calothyrsus is a tropical mimosiod legume native to an extensive area from Mexico to Panama (National Academy of Science, 1983). It is a very fast growing tree, with leaves suitable for forage and out-yields other legumes, especially when planted in acid soils (Perdomo, 1991; Lascano and Carulla, 1992, Lascano et al., 1995). Foliage from *C. calothyrsus* contains high protein content (<22.0%, National Academy of Science, 1983, Lascano et al., 1995). However, the *in sacco* (Ahn et al., 1989) and *in vitro* (Perdomo, 1991, Salawu et al., 1997c) degradability of this forage is low, which might be related to its high content of condensed tannins (Lascano, 1995). Indeed, Jackson et al. (1996a) reported that condensed tannin content in *C. calothyrsus* leaves was 155 and 64 g kg^{-1} of dry matter when plants were grown in Colombia, South America and Northern Australia, respectively. In turn, Kang'ara et al. (1998) observed that the digestibility of both dry matter and protein increased greatly when *C. calothyrsus* was treated with polyethylene glycol, which binds condensed tannins in preference to protein and other dietary molecules. It should be pointed that work by Palmer and Schlink (1992) showed that, if *C. calothyrsus* is fed fresh, its forage value is high. Indeed, intake of fresh material by sheep was considerable higher than that of wilted material.

Flemingia macrophylla is a shrub legume from South East Asia and China with excellent adaptation to acid soils (Perdomo, 1991, Maass, 1996). It is, however, a high tannin legume (Lascano and Carulla, Jackson et al. 1996a). The presence of high concentrations of condensed tannins in this legume has been associated with its poor degradability. For example, Lascano and Carulla (1992) reported *in vitro* dry matter digestibility values of only 18.4% for this shrub legume. Likewise, Longland et al (1995) reported that loss of non-starch polysaccharide (NSP) after incubation of this legume with rumen microorganisms in batch culture was only 174 mg g^{-1} of NSP, whereas that of *L. leucocephala* was 784. Similarly, gas accumulation profiles from leaves of this legume were also very low. The low digestibility of this legume has also been reported in *in vivo* experiments. For example, Barahona et al. (1997) reported that the total tract digestibility of organic matter of this legume by sheep was only 45%. This value, however, was not improved by the addition of polyethylene glycol. Polyethylene glycol supplementation, however, was associated with an increase in organic matter intake of this legume by sheep. Similarly, when a mixture of *F. macrophylla* and *Cratylia argentea* was offered to sheep, a decline in fibre digestibility was observed as the level of *F. macrophylla* in the diet increased (Fässler and Lascano, 1995). The high content of condensed tannins in this legume has been also related to the low rate at which litter from this legume decomposes, although this characteristic might be advantageous for soil conservation purposes (Budelman, 1988).

Desmodium ovalifolium Wallep [synonymous *D. heterocarpum* (l.) DC subspecies *ovalifolium* (Prain.) Ohashi (Ohashi (1991)] is a herbaceous legume native from Southeast Asia. Its prostrate, stoloniferous habit of growth allows this legume to provide effective protection against soil erosion and tolerate heavy grazing, and to form persistent associations with very aggressive grasses of similar growth habit (i.e. *Brachiaria spp*.). Additionally, *D. ovalifolium* is drought and shade-tolerant (Suarez, 1985) and performs very well when planted in acid, low-fertility soils (Grof, 1982). Given its shade-tolerance and its growth habit, *D. ovalifolium* has been used for soil protection purposes in rubber tree and oil palm plantations in Southeast Asia. However, the adoption of *D. ovalifolium* as a forage legume by farmers in tropical America has been poor, given the apparent low palatability and acceptability of this

legume by livestock. This has been attributed to the to presence of high levels of condensed tannins in this legume (Lascano and Salinas, 1982). Indeed, reduction of extractable condensed tannin content in *D. ovalifolium* by the addition of polyethylene glycol resulted in higher intake of this legume by sheep (Barahona et al., 1997). This was probably associated to increased ruminal digestibility of fibre and organic matter in sheep fed the polyethylene-treated forage. On the other hand, the addition of polyethylene glycol resulted in decreased ruminal escape and flow of dietary nitrogen to the duodenum, although it also resulted in reduced faecal output of nitrogen. Conversely, Pérez (1997) observed that steers grazing on the association of *D. ovalifolium* with either *Brachiaria decumbens* or *B. humidicola* had greater liveweight gains than those grazing on the grasses alone. Differences between these studies could have been related to the existence of genotype-environment interactions in this legume, a subject that was studied in Chapters 5.1 and 5.2.

Among the tropical legumes included in this study, *Clitoria fairchildiana* is the one that has received less attention by researchers. The forage of this legume is known to be highly tanniniferous (C. E. Lascano, personal communication).

3.2 MATERIALS AND METHODS

3.2.1 Plant biomass

At two separate occasions, at an interval of four months, 1.5 kg samples of mature and immature (terminal) forage from seven tropical legumes, grown at the Centro Internacional de Agricultura Tropical (CIAT) in Cali, Colombia, were harvested. Stems were removed by hand, and leaves were frozen until freeze-dried and then ground to pass a 1-mm dry mesh screen. Legumes were chosen to provide an adequate range in forage quality (i.e. *in vitro* dry matter digestibility) and comprised *Desmodium ovalifolium, Flemingia macrophylla, Leucaena leucocephala, Leucaena pallida, Leucaena macrophylla, Calliandra calothyrsus* and *Clitoria fairchildiana*.

3.2.2 In vitro dry matter digestibility and chemical analyses

The *in vitro* dry matter digestibility (IVDMD) of all legumes was estimated on 0.5 g dry weigh of tissue using the two-stage Tilley and Terry technique (1963) with rumen liquor from a fistulated Brahman steer grazing star grass (*Cynodon dactylon)*.

Additionally, samples (0.2 g dry weight) were analysed for nitrogen content using the method from the Association of Analytical Official Chemists (AOAC, 1975). The amino acid composition of all samples was determined by ion exchange chromatography following the method of Mason et al. (1980). Acid hydrolysis with performic acid oxidation (to allow the determination of methionine and cysteine) was carried out. The amino acids in the resulting hydrolysate were separated by ion exchange chromatography in a Hilger Chromspeck amino acid analyser. Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were determined on separate 0.5 g of tissue as outlined by Van Soest et al. (1991). Condensed tannins extractable in 70 % aqueous acetone and total condensed tannins were determined using a modification of the method proposed by Terrill et al. (1992b). Triplicate 10-mg samples were extracted with a mixture of 2.0 ml of 70% aqueous acetone containing 0.05% ascorbic acid and 2.5 ml of diethyl ether. Tubes were shaken for two min at 2000 rpm and then centrifuged at 2500 rpm for five min. The upper phase of the two-phase separation containing acetone and diethyl ether was discarded and the extraction was repeated one more time. Traces of solvent in the aqueous phase were removed at 40°C on a hot plate under dry air. After centrifugation, the aqueous phase was separated from the pellet and the pellet was dried at 40°C on a heating block. After bringing the volume of the aqueous extract to 2.5 ml with distilled water, 0.5-ml aliquots were mixed with 3.5 ml of butanol-HCl (95:5, v/v) and the mixture was heated for one h at 95°C. Tannins still bound to the residue were determined by adding 4.0 ml of butanol-HCl to the dry pellet and hydrolysing the tannins by heating at 95°C for one h. In both cases, samples were allowed to cool before reading their absorption between 450 and 650 nm in a Philips PU 8720 UV/VIS scanning spectrophotometer. Absorbance due to the formation of anthocyanidins was estimated on a plot of the scan by subtracting background absorbance from total absorbance. Standard curves were obtained using tannins extracted from immature and mature leaves of all tanniniferous legumes using a 70% acetone, 0.05% ascorbic acid aqueous solution and then purified on a column packed with Sephadex LH-20. Known amounts of the purified tannin standards were reacted in triplicate with 4 ml of butanol- HCl.

3.2.3 In vitro gas production measurements

In each of two experiments, 1.0-g samples of all forage legumes were fermented in three 165-ml capacity serum bottles according to the pressure transducer technique of Theodorou *et al*. (1994). Grab samples of rumen-digesta were taken at 8.00 h before the morning feeding from fistulated wethers fed grass hay, and transported to the laboratory in a pre-warmed (39°C) vacuum flask. The microbial inoculum and culture media were prepared as described by Theodorou *et al.* (1994). Each serum bottle received 10 ml of microbial inoculum, 85 ml of buffer and 4 ml of reducing agent.

At the end of the incubation period, the contents of each serum bottle were filtered through pre-weighed sintered glass funnels and freeze dried to constant weight. Dry matter loss was calculated as the difference between the dry weight of the sample pre- and post-incubation. Additionally, the concentration of volatile fatty acids (VFA) in the liquid fraction of the culture media at the end of the 144-h incubation period was determined by gas chromatography. A Chrompack CP 9000 chromatograph fitted with an automatic sampler (Chrompack 911) and a flame-ionisation detector, linked to a Dell PC with A1-450 integration software, was used for VFA quantification.

3.2.4 Non-starch polysaccharide content and loss after fermentation

The content of non-starch polysaccharides (NSP) was measured in the second gas production run in all samples both pre- and post-incubation. Neutral sugars in NSP were measured by the method of Englyst and Cummings (1984), whereby alditol acetate derivatives of carbohydrate monomers derived from acid hydrolysates of washed, polymeric, de-starched samples were quantified by gas chromatography. A Varian 3400 chromatograph fitted with an automatic sampler (Varian 8000) and a flame ionisation detector, linked to a Dell PC with Dionex A1-450 integration software, was used for quantification of sugars. Uronic acids in the hydrolysates were determined by the colorimetric method of Scott (1979).

3.2.5 Statistical analyses

Gas production data were fitted to the model of France et al. (1993) using the MLP (Ross, 1987) package. The equation is in the form, $Y = A\{1 - e^{[-b(t-T) - c(\sqrt{t}-\sqrt{T})]}\}$ where Y is the cumulative gas production (ml), A is the asymptote (i.e. gas pool), T is lag time,

and b (h^{-1}) and c ($h^{-0.5}$) are decay rate constants. A combined fractional rate (h^{-1}) of gas production (μ) was calculated as, $\mu = b + c/2\sqrt{t}$, where t is the incubation time (h).

The relationships between gas accumulation at 12, 24, 48 and 144 h post-inoculation and nutrient content of the mature and immature leaf samples from the seven tropical legumes were investigated by simple linear regressions. Data obtained in the gas production experiments, including concentration and composition of VFA and dry matter disappearance, were analysed as a completely randomised design with tannin source and maturity as the main factors using the analysis of variance (ANOVA) procedure of SAS (1990). Duncan's Multiple Range Test at alpha level of 0.05 was used to compare means.

3.3 RESULTS

3.3.1 Chemical composition of leaves including condensed tannins and NSP

Nitrogen content ranged between 19.9 g kg^{-1} in mature *D. ovalifolium* and 46.0 g kg^{-1} in immature *L. leucocephala* and tended to decrease with maturity in all the legumes, with the exception of *D. ovalifolium* (Table 3-1). Fibre content, as determined by the detergent system of analysis, varied greatly among species. Content of NDF (g kg^{-1}) ranged from 204 in immature *C. calothyrsus* to *ca* 520 in *L. macrophylla*, whereas that of ADF ranged from 117 in immature *C. calothyrsus* to 350 in immature *D. ovalifolium* (Table 3-1). Estimates of hemicellulose content (NDF-ADF; g kg^{-1}) ranged from 10 in *D. ovalifolium* to *ca* 200 in *L. macrophylla*. In three legumes (*F. macrophylla, L. pallida* and *C. calothyrsus*), fibre content was higher in mature than in immature leaves.

Table 3-1. *Content of nitrogen, neutral and acid detergent fibre and* in vitro *dry matter digestibility estimations using the two-stage Tilley and Terry and the pressure transducer gas techniques of mature (non-terminal) and immature (terminal) leaves of seven tropical forage legumes*

		Chemical composition and *in vitro* digestibility, g kg^{-1} DM[1]				
Legume species	Maturity	Nitrogen	NDF[2]	ADF[3]	IVDMD[4]	PTDMD[5]
D. ovalifolium	Immature	20.0	377.7	352.0	345.4	434.7 [ef]
	Mature	19.9	336.9	326.7	423.6	461.3 [de]
F. macrophylla	Immature	26.1	441.8	313.0	243.5	254.4 [h]
	Mature	22.5	452.1	304.2	231.2	237.3 [h]
L. leucocephala	Immature	46.0	244.8	133.6	624.5	633.5[a]
	Mature	35.6	283.3	182.1	614.7	563.2 [b]
L. pallida	Immature	34.8	269.3	209.9	423.6	523.7 [bc]
	Mature	31.2	295.5	237.9	364.8	408.2 [f]
L. macrophylla	Immature	40.1	519.2	319.1	490.7	500.3 [cd]
	Mature	31.1	517.5	320.6	459.8	441.1 [ef]
C. calothyrsus	Immature	33.7	203.9	117.2	534.6	472.2 [de]
	Mature	29.9	232.6	182.7	397.0	366.0 [g]
C. fairchildiana	Immature	26.7	471.6	340.6	388.0	435.1 [ef]
	Mature	25.2	501.6	336.6	420.4	430.7 [ef]

[1]For nitrogen, NDF, ADF and IVDMD measurements were carried in triplicate. If the variation between replicate determinations was higher than 5%, samples were re-analysed. PTDMD values are the mean of six determinations (two gas production runs, each sample assayed in triplicate).

[2]NDF = Neutral detergent fibre.

[3]ADF = Acid detergent fibre.

[4]IVDMD = Two-stage Tilley and Terry (1963) *in vitro* dry matter digestibility.

[5]PTDMD = Pressure transducer technique in vitro dry matter digestibility after 144 h of incubation with rumen microorganisms. Values presented are the means of two independent runs.

[a,b,c,d,e,f,g,h] Means within a column with different superscripts are different ($P < 0.05$).

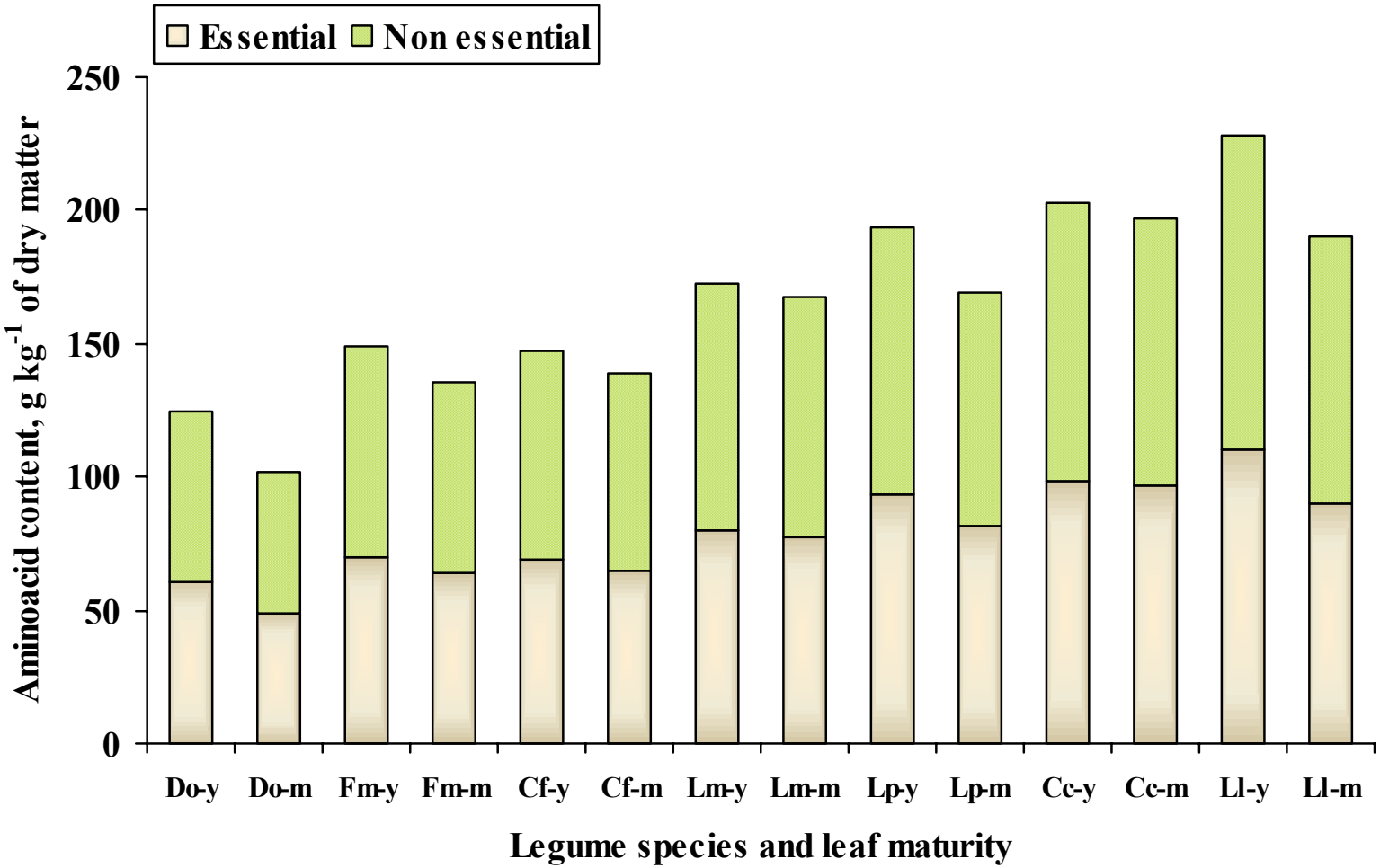


Figure 3-1. The *essential and non-essential amino acid content in mature (non-terminal) and immature (terminal) leaves of seven tropical legumes. ; Do* = Desmodium ovalifolium*; Fm* = Flemingia macrophylla*; Cf* = Clitoria fairchildiana*; Lm* = Leucaena macrophylla; *Lp* = Leucaena pallida*; Cc* = Calliandra calothyrsus*; Ll* = Leucaena leucocephala *y= immature forage; m = mature forage. Essential amino acids included arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine*

Estimations of dry matter digestibility by the technique of Tilley and Terry (1963) ranged from 231 in mature *F. macrophylla* to 625 in immature *L. leucocephala*, whereas the corresponding estimates from the pressure transducer technique were 237 and 634 g kg^{-1}, respectively (Table 3-1). Indeed, both measurements were highly correlated ($R^2 \geq 0.83$, $P < 0.001$). In four legumes (*C. calothyrsus* and the three *Leucaena*), dry matter disappearance in the pressure transducer technique was highest ($P < 0.5$) in immature than in mature leaves (see Table 3-1).

Great variation was observed in the amino acid composition of leaves from all legumes (see Figures 3-1, 3-3a and 3-3b). The total amino acid content (g kg^{-1} of dry matter) ranged from 101 in mature *D. ovalifolium* leaves to 227 in immature leaves of *L. leucocephala* (Figure 3.1). Similarly, the content of essential amino acids (i.e. arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine) was lowest in mature *D. ovalifolium* and highest in immature *L. leucocephala* (53 and 117 g kg^{-1} of dry matter, respectively). It was interesting to observe that the ratio of essential – non-essential amino acids was very similar for all leaf samples (range: 0.862:1 to 0.962:1).

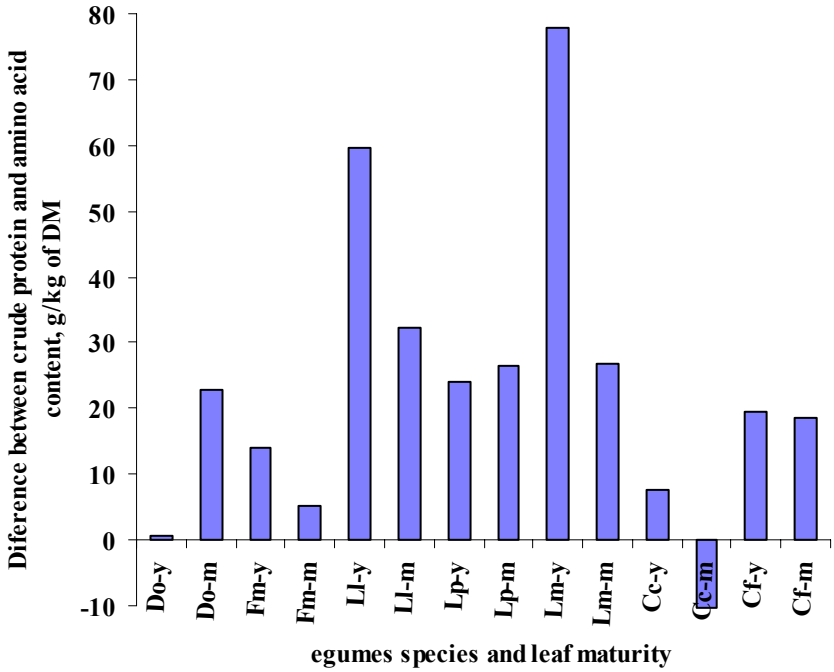


Figure 3-2. *ontent of amino acids* *ed:* Do = Desmodium ovalifolium, *Fm* = Flemingia macrophylla, *Ll* = Leucaena leucocephala, *Lp* = L. pallida, *Lm* = L. macrophylla, *Cc* = Calliandra calothyrsus, *Cf* = Clitoria fairchildiana, *y= immature (terminal) leaves, m = mature leaves.*

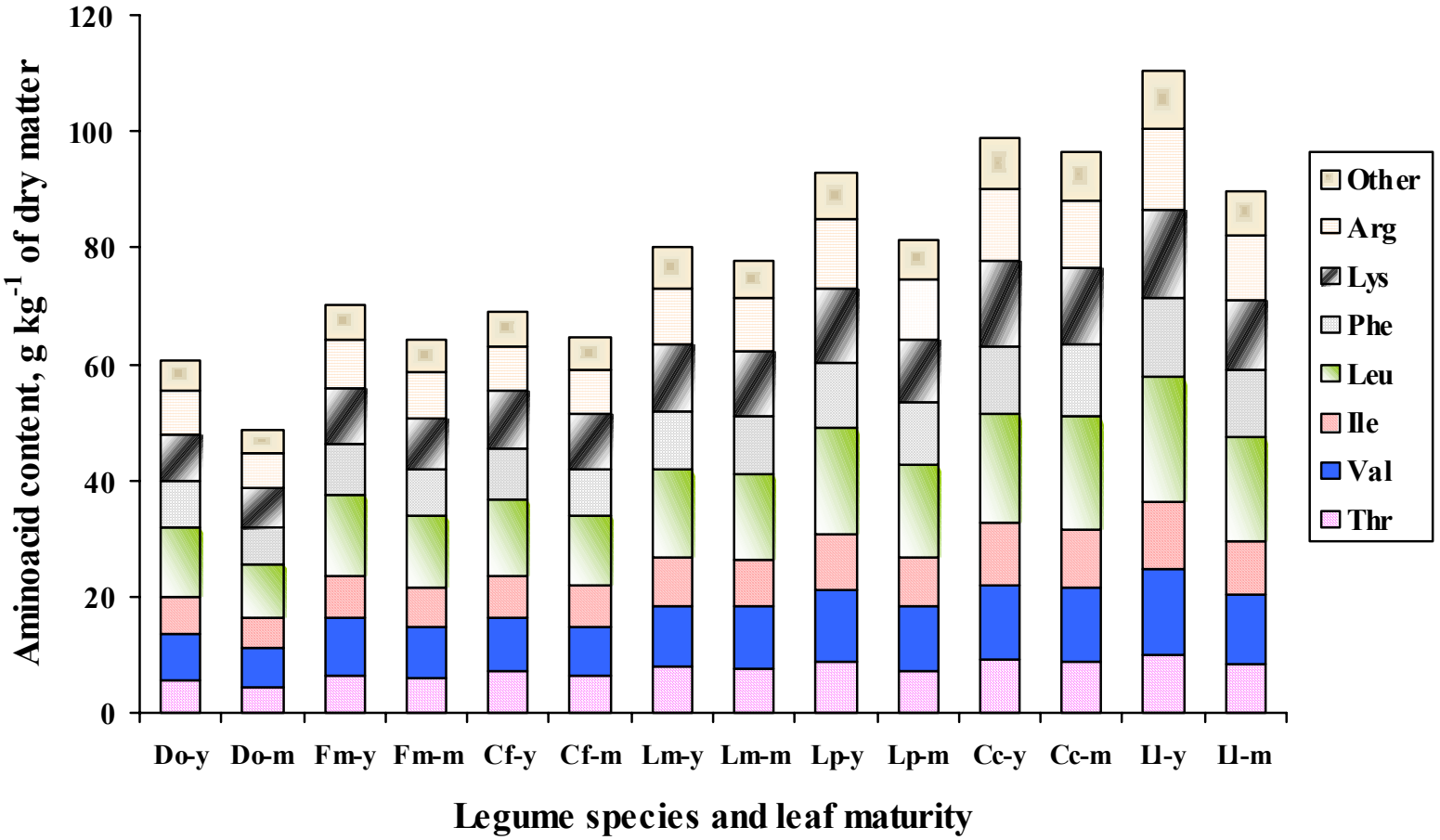


Figure 3-3a. The content of *essential amino acids in mature (non-terminal) and immature (terminal) leaves of seven tropical legumes. ; Do* = Desmodium ovalifolium*; Fm* = Flemingia macrophylla*; Cf* = Clitoria fairchildiana*; Lm* = Leucaena macrophylla; *Lp* = Leucaena pallida*; Cc* = Calliandra calothyrsus*; Ll* = Leucaena leucocephala *y= immature forage; m = mature forage. Key to amino acids: Thr = threonine, Val = valine, Ile = isoleucine, Leu = leucine, Phe = phenylalanine, Lys = lysine, Arg = arginine, others = histidine, methionine and tryptophan.*

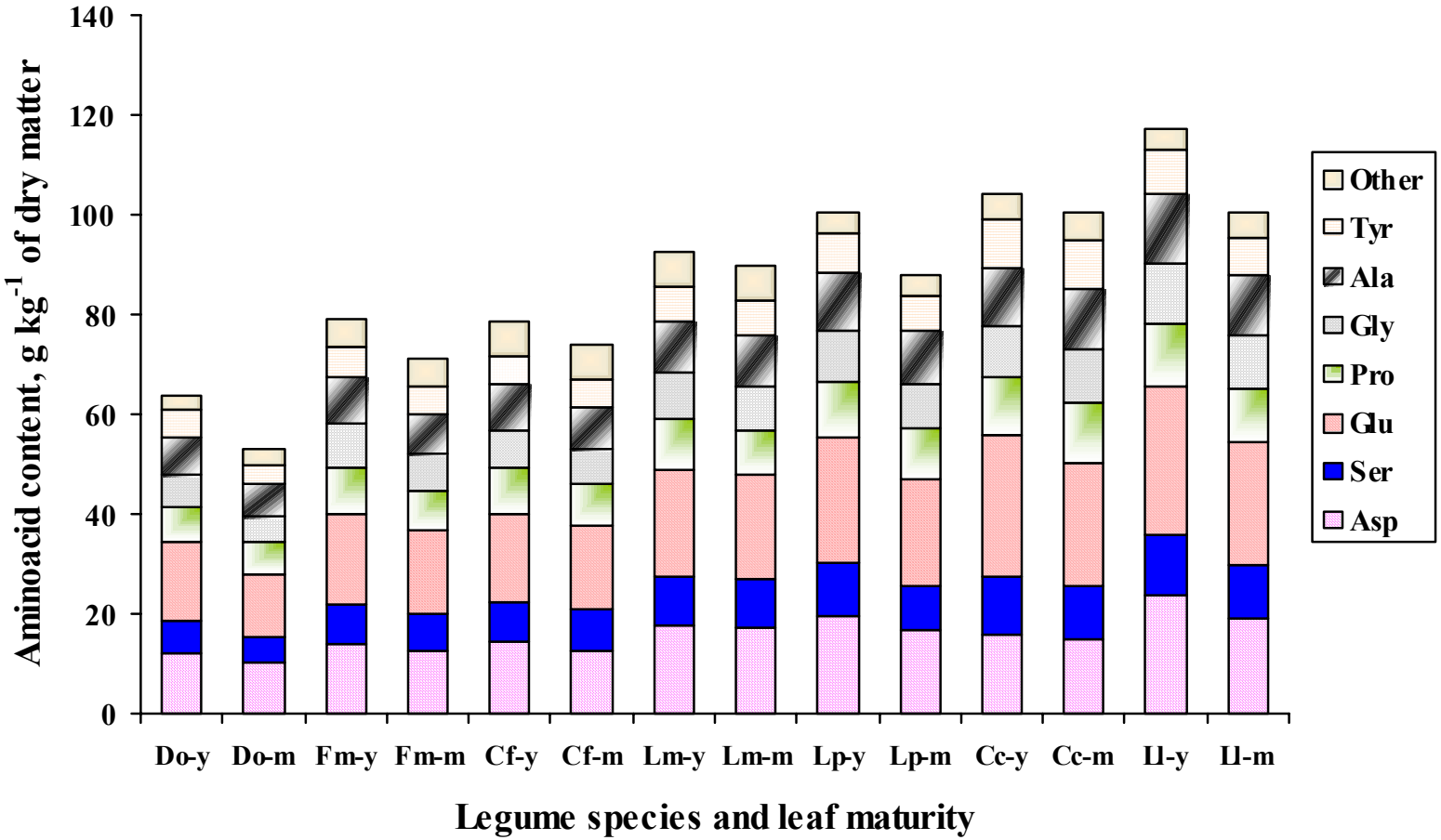


Figure 3-3b. The content of non-*essential amino acids in mature (non-terminal) and immature (terminal) leaves of seven tropical legumes. ; Do* = Desmodium ovalifolium*; Fm* = Flemingia macrophylla*; Cf* = Clitoria fairchildiana*; Lm* = Leucaena macrophylla; *Lp* = Leucaena pallida*; Cc* = Calliandra calothyrsus*; Ll* = Leucaena leucocephala *y= immature forage; m = mature forage. Key to amino acids: Asp =aspartate, Ser = serine, Glu = glutamate, Pro = proline, Gly = Glycine, Ala = Alanine, Tyr = Tyrosine, others = Cysteine, glutamine.*

The difference between the content of crude protein (nitrogen X 6.25) and the content of amino acids was low for samples from *D. ovalifolium*, *C. calothyrsus* and *F. macrophylla*, intermediate for *C. fairchildiana* and high for the *Leucaena* (Figure 3.2). In agreement with this, three unidentified peaks appearing between the methionine and phenylalanine peaks were observed in all the *Leucaena* samples (data not shown). Compared with the corresponding mature samples, the difference between the estimates of crude protein and amino content was higher in immature leaves from *L. macrophylla* and *L. leucocephala*.

Analysis of the content of essential amino acids in all legumes, showed that the essential amino acid present in the highest proportion was leucine (range 9.3 to 22 g kg^{-1} of dry matter), which was closely followed by lysine and valine (see Figure 3.3a). In turn, the content of histidine and methionine in leaves of the legumes assayed was never higher than 5.7 g kg^{-1} of dry matter. Furthermore, tryptophan was not detected in any of the samples assayed.

Among non-essential amino acids, glutamate was present in the highest concentration (range: 12.7 to 29.5 g/kg of dry matter), closely followed by aspartate (range: 10.0 to 23.6 g/kg of dry matter, see Figure 3-3b). In turn, the concentration of cysteine only ranged between 2.8 and 7.2 g/kg of dry matter. It must be recognised, however, that there were indications that the performic oxidation did not result in the oxidation of all the cysteine to cysteic acid. However, the observed cysteine peak (which in ideal conditions would have not been present) was very small in all cases. Neither asparagine nor glutamine was detected in any of the samples assayed.

Great differences were observed in the standard curves obtained for the quantification of the different condensed tannins by the technique of Terrill et al. (1992b, data not shown). The change in the absorbance at 550 nm per mg of tannin hydrolysed (i.e. slope) ranged from 0.6 in tannins extracted from mature *C. calothyrsus* to 4.66 in those from immature *D. ovalifolium* and appeared to be species-dependent. With the exception of tannins from *D. ovalifolium*, the slope of the obtained standard curve was higher for tannins extracted from immature leaves than for those extracted from mature leaves.

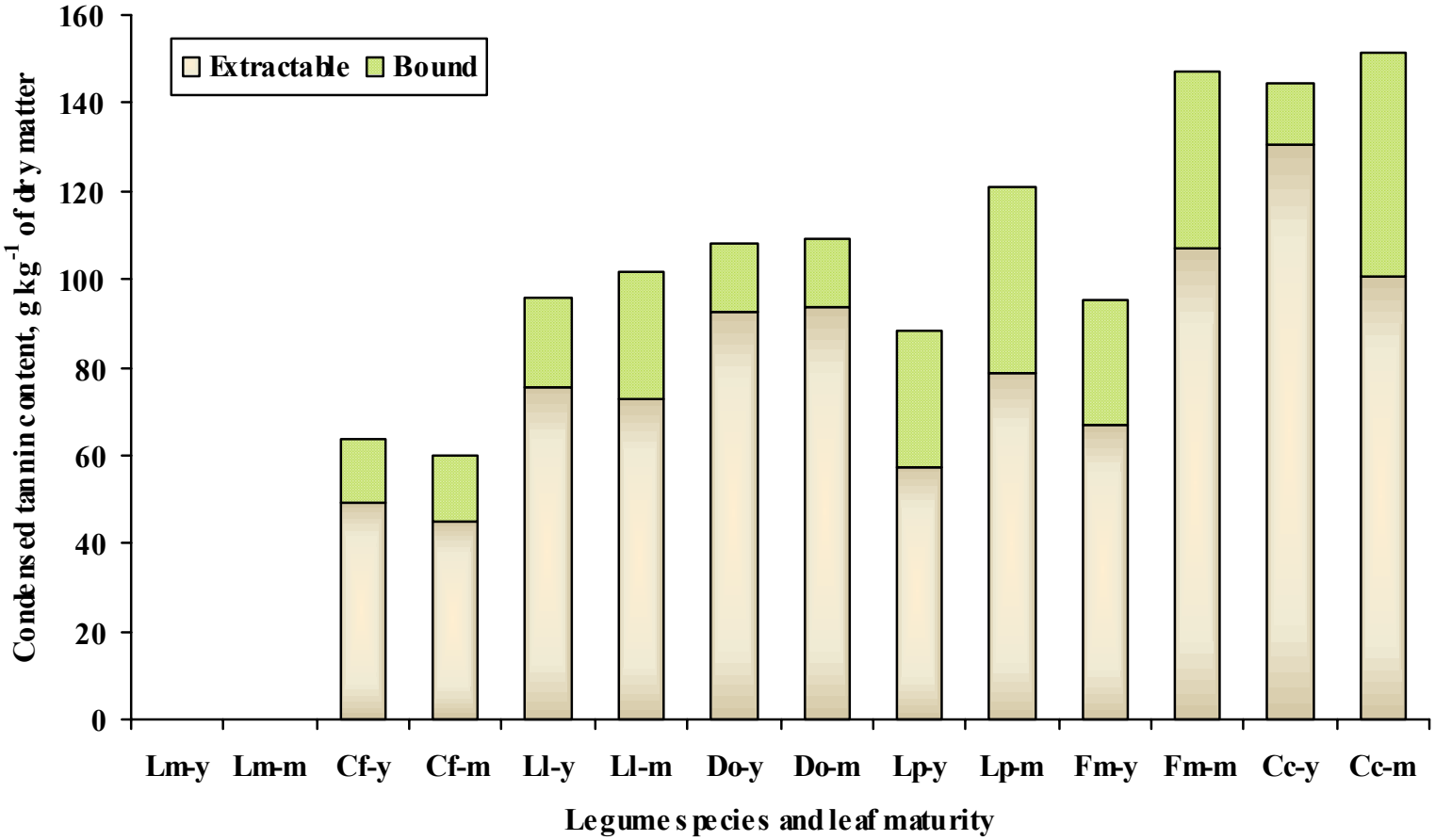


Figure 3-4. *Concentration of acetone-bound and acetone-extractable condensed tannins in mature (non-terminal) and immature (terminal) leaves of seven tropical legumes. Explanatory notes: Lm* = L. macrophylla (non-tanniniferous)*; Cf* = Clitoria fairchildiana*; Ll* = Leucaena leucocephala*; Do* = Desmodium ovalifolium*; Lp* = L. pallida*; Fm* = Flemingia macrophylla*; Cc* = Calliandra calothyrsus*; y= immature forage; m = mature forage.*

Table 3-2. *Non-starch polysaccharide (NSP) content and composition of mature (non-terminal) and immature (terminal) leaves from seven tropical legumes (g kg^{-1} of dry matter).*

Legume species	Maturity	NSP components							Total
		Rhamnose	Arabinose	Xylose	Mannose	Galactose	Glucose	UAC	NSP
D. ovalifolium	Immature	0.0	7.0	26.2	14.6	7.7	174.2	39.3	268.9
	Mature	0.0	6.9	25.3	11.6	6.5	154.0	33.7	238.1
F. macrophylla	Immature	0.0	9.9	24.4	5.7	9.8	123.4	34.3	207.5
	Mature	0.0	9.8	26.4	6.2	9.4	122.2	32.9	206.9
L. leucocephala	Immature	6.1	10.4	6.4	3.5	11.4	40.0	45.5	123.3
	Mature	6.5	10.3	5.5	2.8	11.6	37.8	45.6	120.1
L. pallida	Immature	1.2	10.2	3.6	2.8	11.5	35.8	44.1	109.1
	Mature	3.9	9.5	4.6	2.9	11.2	45.9	47.5	125.5
L. macrophylla	Immature	0.0	17.3	29.4	4.2	10.8	98.7	40.4	200.7
	Mature	0.0	17.2	29.2	3.4	10.6	86.9	35.7	183.0
C. calothyrsus	Immature	3.6	7.5	4.4	4.6	9.8	55.0	34.7	119.6
	Mature	5.5	5.4	4.7	2.9	7.3	45.5	37.5	108.7
C. fairchildiana	Immature	0.0	15.0	29.1	4.9	13.8	126.1	28.5	217.2
	Mature	0.0	14.3	27.3	4.3	10.0	122.1	35.1	213.1

NSP components (neutral sugars) were determined by gas chromatography as described by Englyst and Cummings (1984). Uronic acids (UAC) were determined by the colorimetric method of Scott (1979). Total NSP values were obtained by summing neutral sugars and uronic acids. Standard error for these determinations lied between 1 and 5.5% of the values for all samples.

The condensed tannin content (g kg^{-1} of dry matter) ranged between 0 in young and mature *L. macrophylla* to 151 in *C. calothyrsus* (see Figure 3-4). In these tanniniferous legumes, most of the tannins were found to be acetone-extractable. However, there were differences in the relative extractability of tannins, with bound tannins representing only 9.5 % of total tannins in immature *C. calothyrsus* and 35.0 % of the total tannins in *L. pallida*. Except for *C. fairchildiana,* the concentration (g kg^{-1} of dry matter) of condensed tannins tended to increase with leaf maturity in all legumes. Nonetheless, acetone-bound tannins as a proportion of total tannins (g kg^{-1} of total tannins) did not change with maturity in these samples, except in *C. calothyrsus* and to a lesser extent in *L. leucocephala*, where mature samples were found to have a greater proportion of bound tannins than immature samples.

The content of NSP (g kg^{-1}) of forage legumes varied greatly, ranging from 109 in mature *C. calothyrsus* to 269 in immature *D. ovalifolium* (see Table 3-2). Composition of the NSP varied between legume species, although glucose and uronic acids were the most abundant cell wall carbohydrate constituents in all samples, in combination accounting for ca. 67-79 % of the NSP. Content and composition of the NSP was similar between *L. leucocephala, L. pallida* and *C. calothyrsus*. In contrast, content and composition of NSP from *L. macrophylla* differed from that of the other two *Leucaena* species, especially in their glucose, xylose and arabinose content. Leaf maturity had no effect on the content and composition of NSP in *F. macrophylla, L. leucocephala* and *C. fairchildiana*. On the other hand, total NSP differed by at least 9% between mature and immature leaves in the case of the remaining legumes.

In general, contents of NDF and ADF were higher than the corresponding contents of NSP (Tables 3-1 and 3-2), although both estimations were significantly related to NSP content (NDF, $r = 0.70$, $p = 0.005$; ADF, $r = 0.90$, $p < 0.001$). Assuming that in these samples ADF is equal to cellulose plus lignin, an estimate of lignin content was then obtained by subtracting glucose in NSP from ADF. The values of this estimate (g kg^{-1}) ranged from 62 in immature *C. calothyrsus* to 215 in *C. fairchildiana*. Since some of the glucose in NSP will be derived from hemicellulose, it appears certain that this estimation will underestimate the lignin content in these samples. However, differences among samples were great and might not be affected by this imprecision.

Table 3-3. *Gas production of mature and immature (terminal) leaf samples from seven tropical legumes after incubation with rumen microorganisms.*

Legume	Maturity	Gas accumulated, ml[1]			
		12 hours	24 hours	48 hours	144 hours
D. ovalifolium	Immature	9.54 ^{g}	19.78 ^{g}	38.20 ^{g}	83.86 ^{fg}
	Mature	11.61 ^{fg}	25.92 ^{fg}	51.95 ^{efg}	99.97 ^{cdef}
F. macrophylla	Immature	24.43 ^{de}	35.67 ^{ef}	48.65 ^{fg}	68.64 ^{g}
	Mature	24.35 ^{de}	34.74 ^{ef}	46.49 ^{g}	64.40 ^{g}
L. leucocephala	Immature	58.78 ^{b}	67.98 ^{b}	103.97 ^{ab}	139.38 ^{a}
	Mature	45.52 ^{b}	74.79 ^{ab}	110.11 ^{a}	141.72 ^{a}
L. pallida	Immature	28.07 ^{cd}	53.28 ^{cd}	88.74 ^{c}	128.18 ^{ab}
	Mature	26.72 ^{cd}	43.19 ^{cde}	65.55 ^{de}	101.77 ^{cdef}
L. macrophylla	Immature	45.07 ^{b}	68.97 ^{b}	92.04 ^{bc}	122.42 ^{abc}
	Mature	58.99 ^{a}	84.61 ^{a}	106.84 ^{a}	108.89 ^{bcde}
C. calothyrsus	Immature	14.39 ^{efg}	26.79 ^{fg}	47.48 ^{g}	89.63 ^{ef}
	Mature	21.40 ^{def}	37.76 ^{ef}	62.33 ^{ef}	92.78 ^{def}
C. fairchildiana	Immature	27.26 ^{cd}	41.31 ^{de}	63.02 ^{ef}	115.58 ^{bc}
	Mature	36.06 ^{bc}	54.53 ^{c}	78.29 ^{cd}	113.69 ^{bcd}

[1] Predicted using the parameters from the model of France et al. (1993). Values reported are the means of two independent gas runs.
^{a,b,c,d,e,f,g,h} Means within a column with different superscripts are different ($P < 0.05$).

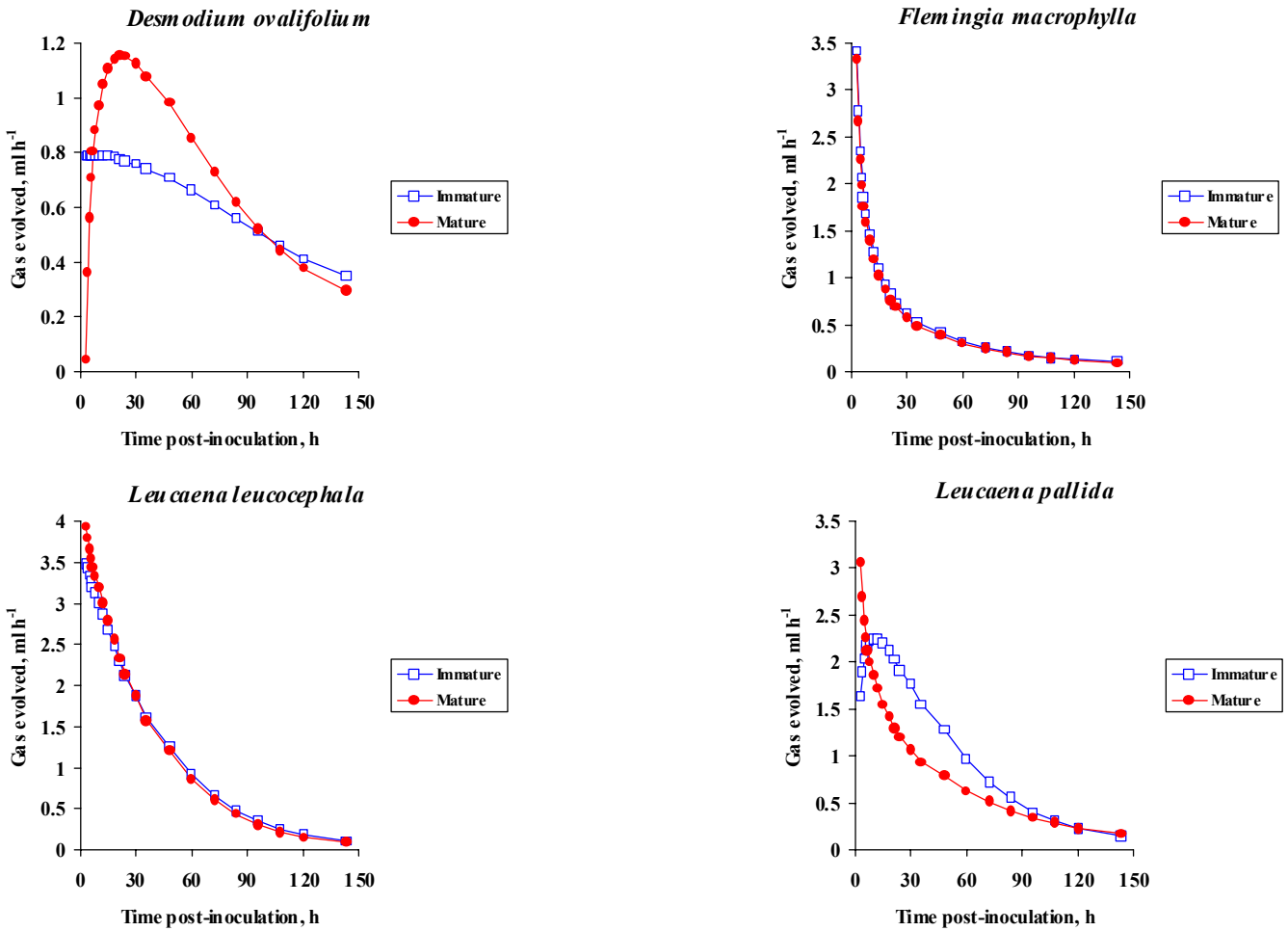


Figure 3-5a. *Rate of gas production (ml h^{-1}) at different times during the incubation of mature and immature (terminal) leaves from tropical legumes. Values depicted were estimated using predicted gas production parameters (France et al., 1992).*

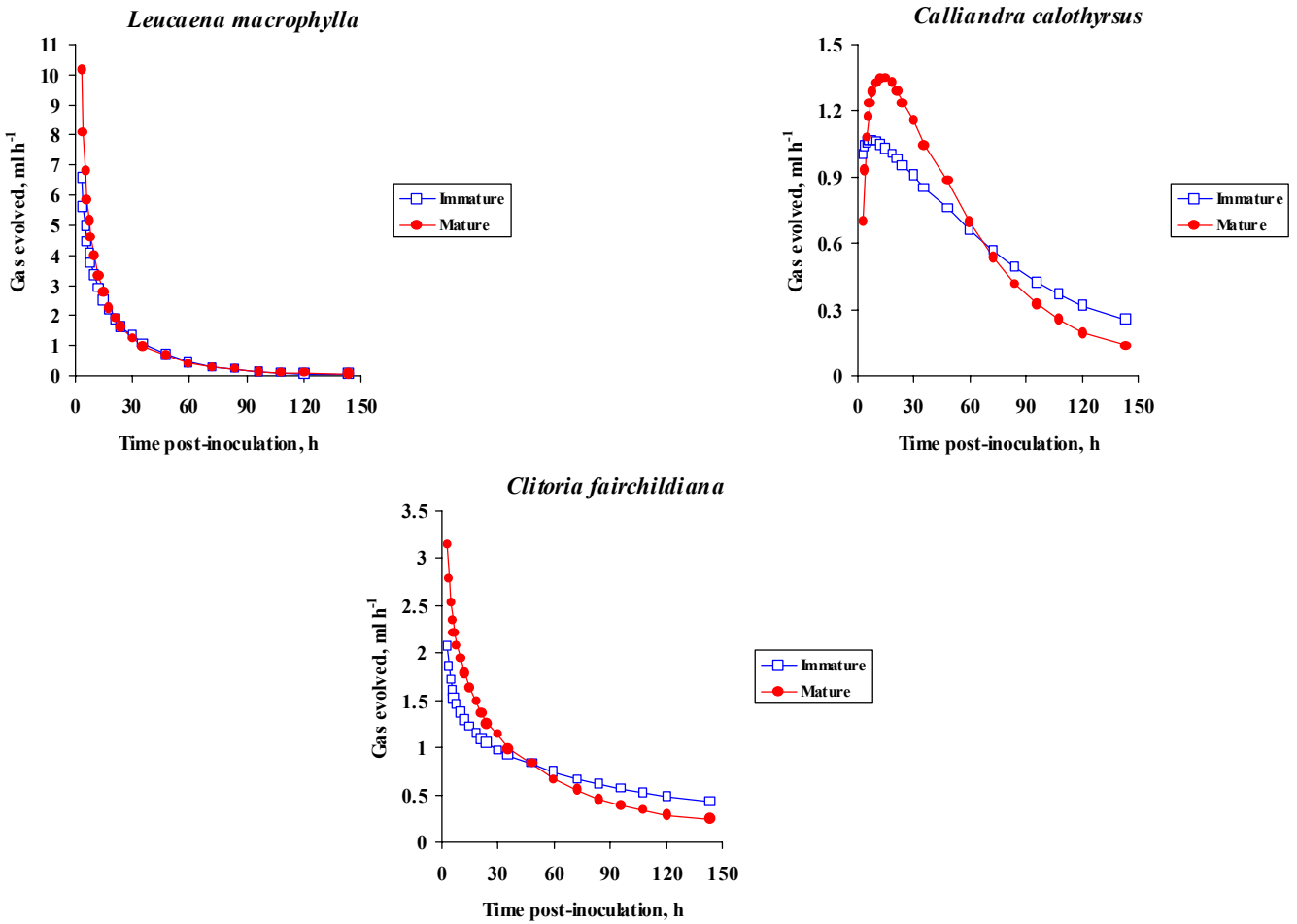


Figure 3-5b. *Rate of gas production (ml h^{-1}) at different times during the incubation of mature and immature (terminal) leaves from tropical legumes. Values depicted were estimated using predicted gas production parameters (France et al., 1992).*

3.3.2 Gas accumulation and loss of NSP

There were considerable differences in gas accumulation profiles (Table 3-3). At the end of the 144-h incubation period, gas pools in samples of *L. leucocephala* were about 140 ml, whereas those of *F. macrophylla* leaves were only 66 ml on average ($P < 0.05$). However, it is noteworthy that during the first 24 h of incubation, gas accumulation was highest in mature leaves of *L. macrophylla* (84 ml) and lowest in immature leaves of *D. ovalifolium* (20 ml, $P < 0.05$). Gas accumulation was not rapid during the initial fermentation period. In fact, in only three legumes *(F. macrophylla, L. leucocephala* and *L. macrophylla*), ≥ 50% of the total gas accumulation was produced within the first 24 h of fermentation.

Table 3-3 shows the influence of leaf maturity on gas accumulation, which varied with time of fermentation. Early in the fermentation (12 and 24 h) there were no differences in gas accumulation between mature and immature leaf samples in five of the seven legumes tested. Conversely, in the same period, mature leaves from *L. macrophylla* and *Clitoria fairchildiana* had significantly greater ($P< 0.05$) gas accumulation than immature leaves. After 48 h of incubation, mature leaves from *L. macrophylla, C. calothyrsus* and *C. fairchildiana* had significantly greater gas accumulation than immature leaves ($P < 0.05$). Conversely, after 48 h of incubation, immature leaves had significantly higher gas accumulation than mature leaves in the case of *L. pallida* ($P < 0.05$). Finally, Table 3-3 shows that, except in the case of *L. pallida* where gas accumulation continued to be greater in immature samples ($P < 0.05$), leaf maturity had no significant effect on the final gas pool size.

The model of France et al. (1993) was used to determine the gas pool size at discrete time intervals of 1, 2, 3, 6 or 12 h along the course of the fermentation period. These values were then divided by the number of hours in each interval. The resultant rates of gas evolution (ml of gas h^{-1}) were then plotted for the mature and immature samples of each legume species and can be seen in Figures 3-5a and 3-5b. These plots illustrate with greater clarity the variability in gas accumulation among legumes species and between the mature and immature samples of each legume.

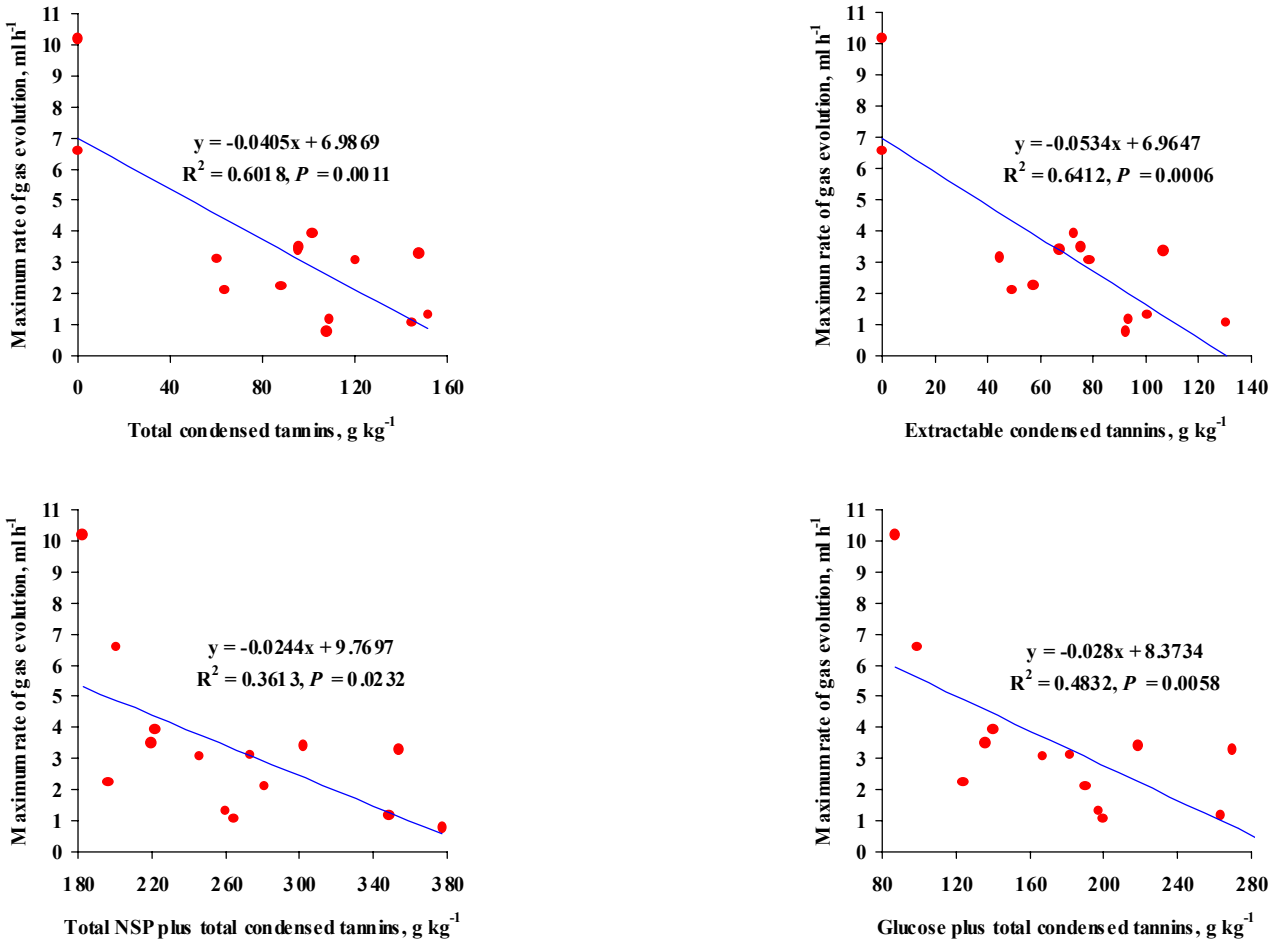


Figure 3-6. *Relationships between the maximum rate of gas production (ml h^{-1}) observed during the incubation of mature and immature leaves from seven tropical legumes and their condensed tannin and fibre-condensed tannin content.*

Table 3-4. *Concentration of volatile fatty acids (VFA) in the liquid fraction of the culture media at the end of the incubation period of mature (non-terminal) and immature (terminal) leaf samples from seven tropical legumes with rumen micro-organisms*[1].

Legume	Maturity	Mmoles litre^{-1}						
		Total VFA	Straight chain VFA				Branched chain VFA	
			Acetate	Propionate	Butyrate	Valerate	Iso-butyrate	Iso-valerate
D. ovalifolium	Immature	22.7 ^{f}	16.4 ^{e}	5.4 ^{e}	0.94 ^{ef}	0.13 ^{fg}	-0.018 ^{d}	-0.144 ^{e}
	Mature	24.6 ^{f}	17.3 ^{de}	6.1 ^{d}	1.30 ^{def}	0.03 ^{g}	-0.002 ^{cd}	-0.156 ^{e}
F. macrophylla	Immature	22.6 ^{f}	15.8 ^{e}	5.4 ^{e}	1.03 ^{ef}	0.23 ^{efg}	0.077 ^{cd}	0.005 ^{de}
	Mature	22.3 ^{f}	15.7 ^{e}	5.3 ^{e}	1.24 ^{def}	0.11 ^{fg}	0.003 ^{cd}	-0.078 ^{de}
L. leucocephala	Immature	46.8 ^{a}	30.7 ^{a}	10.0 ^{a}	3.22 ^{a}	1.30 ^{a}	0.597 ^{ab}	0.992 ^{ab}
	Mature	43.5 ^{a}	28.2 ^{a}	10.5 ^{a}	2.47 ^{b}	1.04 ^{b}	0.436 ^{b}	0.890 ^{b}
L. pallida	Immature	33.1 ^{bc}	22.8 ^{b}	8.2 ^{c}	1.28 ^{def}	0.47 ^{de}	0.181 ^{cd}	0.157 ^{cde}
	Mature	28.3 ^{e}	19.4 ^{cd}	8.1 ^{c}	0.82 ^{f}	0.22 ^{efg}	0.007 ^{cd}	-0.144 ^{e}
L. macrophylla	Immature	35.4 ^{bc}	21.9 ^{bc}	8.6 ^{bc}	2.14 ^{bc}	0.82 ^{bc}	0.661 ^{a}	1.335 ^{a}
	Mature	37.7 ^{b}	23.3 ^{b}	9.8 ^{ab}	2.32 ^{bc}	0.62 ^{cd}	0.568 ^{ab}	1.082 ^{ab}
C. calothyrsus	Immature	29.2 ^{de}	22.7 ^{b}	5.4 ^{e}	0.92 ^{ef}	0.37 ^{def}	0.029 ^{cd}	-0.108 ^{de}
	Mature	32.2 ^{cd}	23.3 ^{b}	6.2 ^{d}	1.64 ^{cde}	0.45 ^{de}	0.194 ^{c}	0.386 ^{c}
C. fairchildiana	Immature	33.4 ^{bc}	22.2 ^{bc}	8.2 ^{c}	1.88 ^{bcd}	0.38 ^{def}	0.287 ^{bc}	0.341 ^{cd}
	Mature	32.8 ^{cd}	20.6 ^{bc}	7.7 ^{c}	2.40 ^{b}	0.60 ^{cd}	0.501 ^{ab}	0.998 ^{ab}

[1]VFA concentrations are the average of the VFA concentrations recorded at the end of two separate gas production experiments.
^{a,b,c,d,e,f,g,h} Means within a column with different superscripts are different ($P < 0.05$).

According to their shape, these plots were classified into three groups. In the legumes of group one (*F. macrophylla, L. leucocephala* and *L. macrophylla*), rate of gas production was highest at the start of the fermentation period and showed a sharp decline as the incubation progressed. This rapid decline in the rate of gas production with time was particularly noticeable in leaf samples of the non-tanniniferous *L. macrophylla.* Incidentally, legumes in group one were the only ones in which 50% of the total gas production accumulated within the first 24 h of fermentation. In group two (*C. fairchildiana* and mature *L. pallida*), rate of gas production was also highest during the initial incubation period, but the decline was less rapid as compared to the first group. Finally, in group three (*C. calothyrsus, D. ovalifolium* and immature *L. pallida*), rates of gas production were initially low and reached maximum values 7–21 h post-inoculation. In all legumes considered, the rates of gas production in the last group declined more slowly with time, being the most persistent.

Determination of the maximum rates of gas production showed that there were differences both in the absolute value (i.e. ml h^{-1}) of this parameter and in the time required to achieve it (h). Linear regression analysis showed that the maximum rate of gas production was negatively related to the contents of total ($P = 0.0011$) and extractable ($P < 0.001$) condensed tannins (see Figure 3-6). Maximum rate of gas production was also negatively related to the aggregates of total condensed tannins with both total NSP and glucose content (Figure 3-6). Conversely, arabinose content was positively related to maximum rate of gas production ($P < 0.001$, data not shown). No other significant relationships were found between time to maximum rate of gas production and leaf chemical composition.

The concentration of VFA in the culture media at the end of the incubation period is shown in Table 3-4. Total VFA, acetate and propionate in cultures containing leaves from *F. macrophylla* and *D. ovalifolium* were only 50% of the values observed in cultures of *L. leucocephala* ($P < 0.05$). The highest levels of branched-chain VFA in cultures were observed in the non-tanniniferous *L. macrophylla* and in *L. leucocephala*, whereas negative (i.e. lower than those found in the blank) concentrations of branched chain fatty acids were observed with *D. ovalifolium* ($P < 0.05$).

Table 3-5. *Loss of non-starch polysaccharide (NSP_L) components from mature (non-terminal) and immature (terminal) leaves of seven tropical legumes (g kg^{-1} NSP or NSP constituents).*

Sample	Maturity	NSP component loss							Total
		Rhamnose	Arabinose	Xylose	Mannose	Galactose	Glucose	UAC	NSP_L
D. ovalifolium	Immature		604	476	585	591	597	512	572
	Mature		615	536	477	560	563	506	549
F. macrophylla	Immature		8	163	-102	34	246	422	234
	Mature		-118	71	-99	-97	131	439	143
L. leucocephala	Immature	958	644	412	650	676	673	933	767
	Mature	774	578	206	488	564	610	944	717
L. pallida	Immature	1000	777	534	558	656	650	689	676
	Mature	794	578	509	410	530	690	678	653
L. macrophylla	Immature		248	86	185	238	335	649	343
	Mature		52	49	-71	180	305	666	297
C. calothyrsus	Immature	-172	501	-281	71	234	350	478	337
	Mature	-79	519	37	298	159	564	467	439
C. fairchildiana	Immature		435	279	272	522	476	533	453
	Mature		258	299	133	210	302	522	327

NSP components (neutral sugars) were determined in samples pre- and post-incubation with rumen micro-organisms by gas chromatography as described by Englyst and Cummings (1984) and uronic acids (UAC) were determined by the colorimetric method of Scott (1979). Total NSP losses and NSP constituent losses were determined by subtracting the amounts remaining in the residue after 144 h of incubation from those initially present prior to incubation

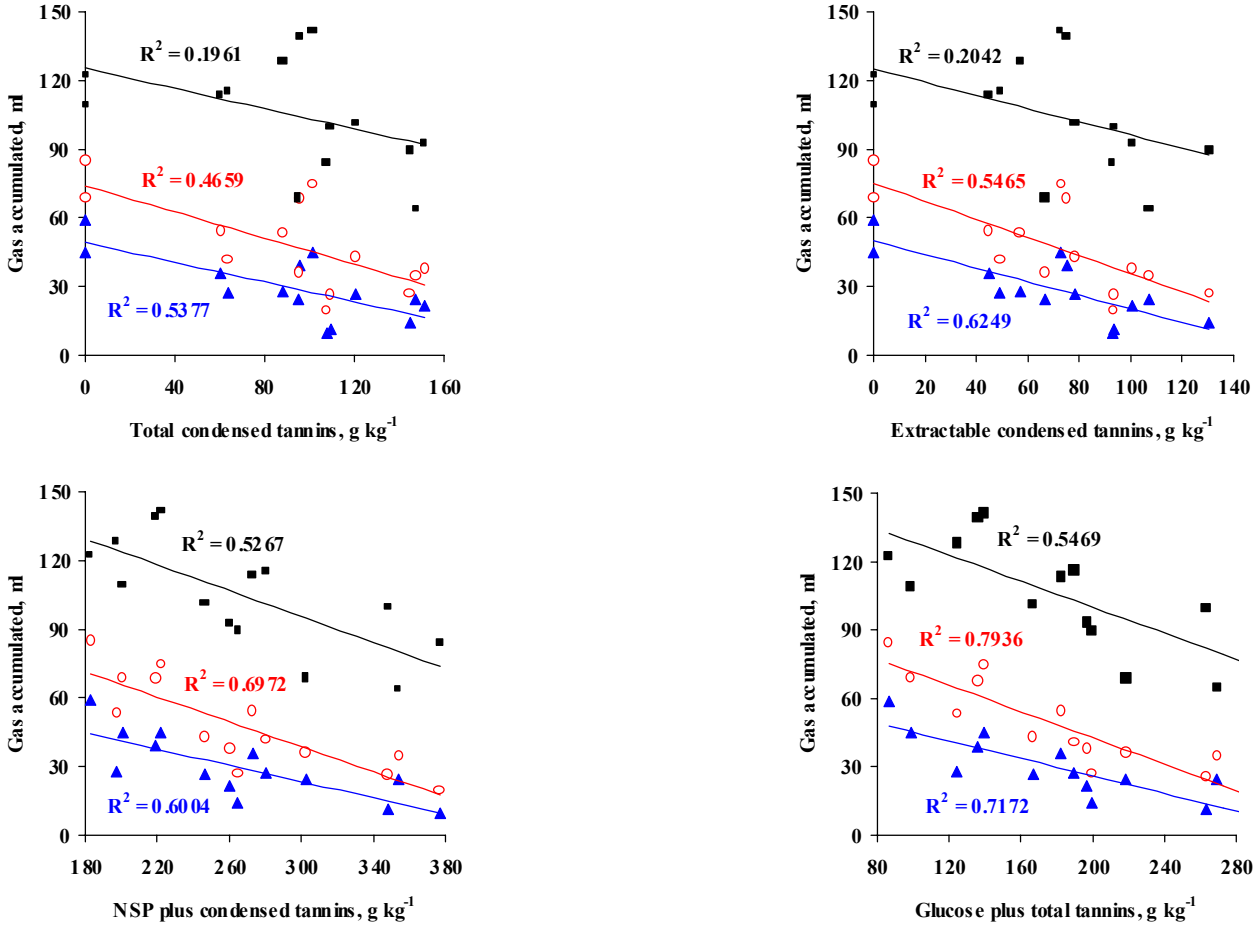


Figure 3-7. *Relationships between volume of gas accumulation (ml) at different times during the incubation of mature and immature leaves from seven tropical legumes and their condensed tannin and fibre-condensed tannin content.* ▲ *= gas at 12 h;* ○ *= gas at 24 h;* ■ *= gas at 144 h. With the exception of the relationship between gas at 144 h and both total and extractable tannins, all other relationships are significant (P ≤ 0.007).*

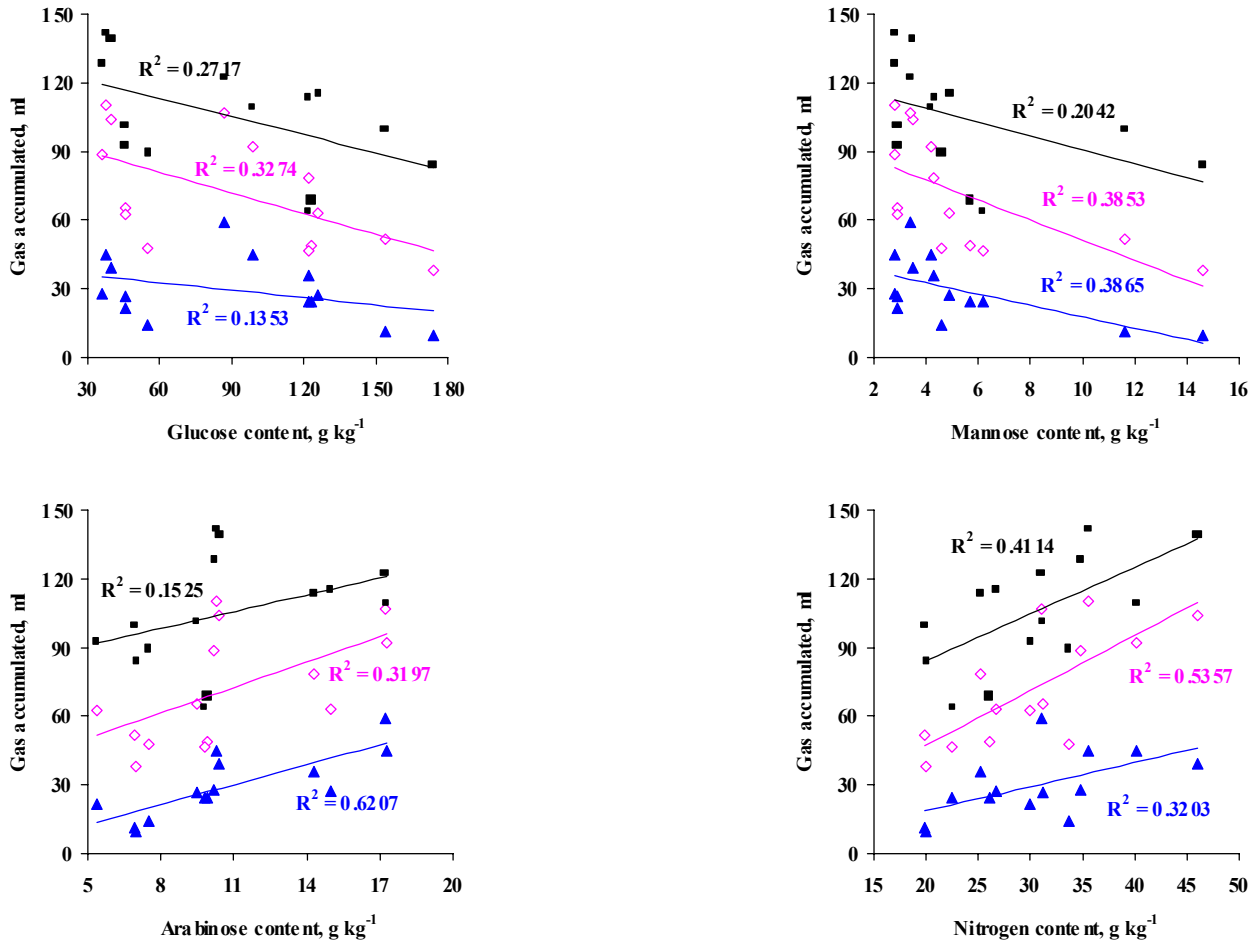


Figure 3-8. *Relationships between volume of gas accumulation (ml) at different times during the incubation of mature and immature leaves from seven tropical legumes and their content of some NSP constituents and of nitrogen.* ▲ *= gas at 12 h;* ◊ *= gas at 48 h;* ■ *= gas at 144 h. Please note: At* $R^2 = 0.283$, $P = 0.05$.

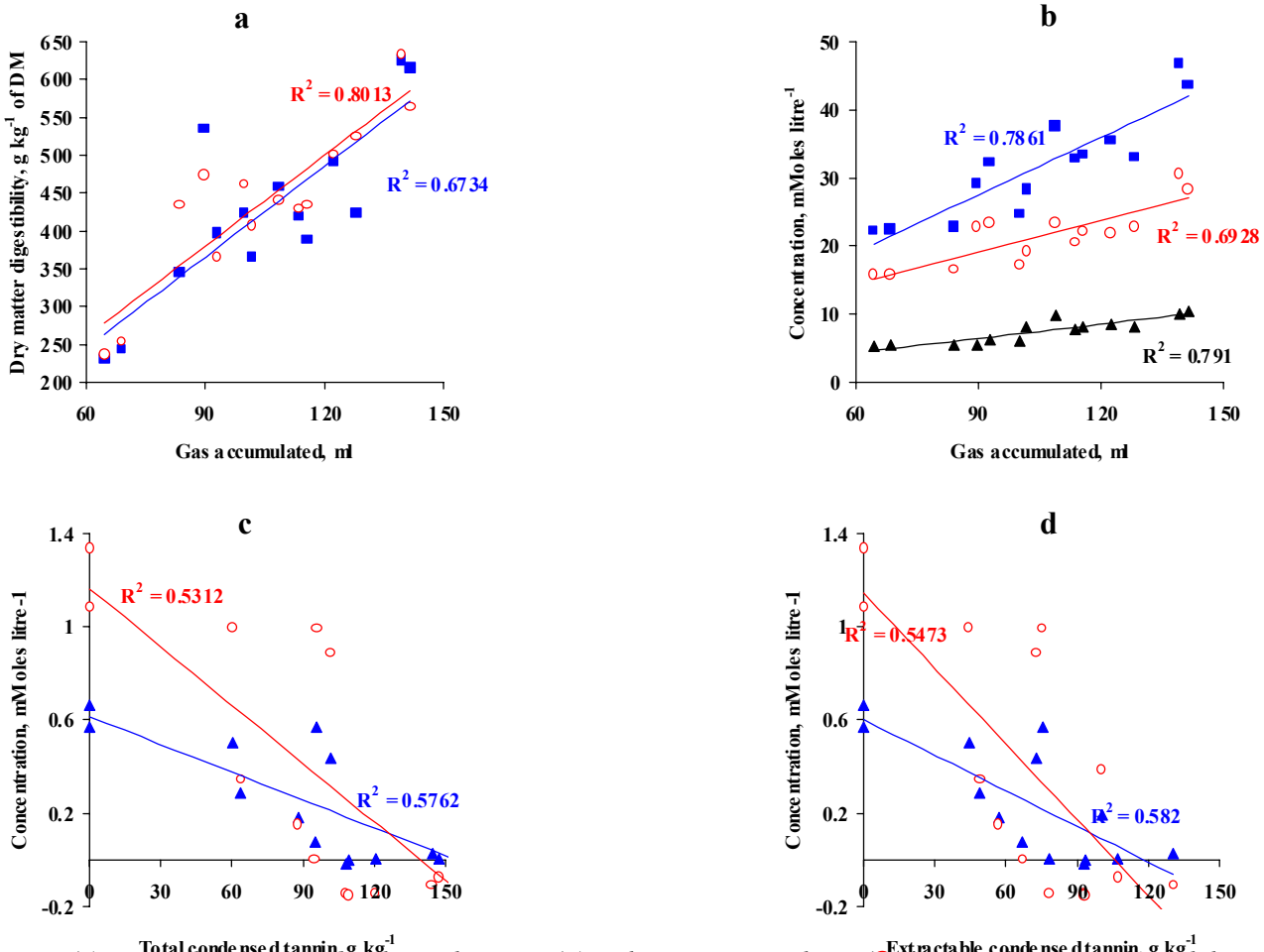


Figure 3-9. *Relationships between (***a***) gas pool at 144 h and Tilley and Terry's (■) and pressure transducer (○) in vitro dry matter digestibility; (***b***) gas pool at 144 h and propionate (▲), acetate (○) and total volatile fatty acids (■); (***c***) total condensed tannin and iso-butyrate (▲) and iso-valerate (○) and (***d***) extractable condensed tannin and iso-butyrate (▲) and iso-valerate (○). In all cases, P ≤ 0.003*

The loss of NSP (NSP_L) after incubation with rumen micro-organisms varied between legumes, ranging from 14.3 % in mature *F. macrophylla* to 76.7 % in immature *L. leucocephala* (Table 3-5). Losses of NSP from *L. leucocephala* and *L. pallida* did not differ greatly. Conversely, NSP_L from *L. macrophylla* was much lower than that of the other *Leucaena* species. Losses of uronic acids tended to be the highest among those of the other NSP constituents. Xylose was, generally, the least digestible NSP fraction. However, great variability in the digestibility of NSP constituents was observed between legumes. For example, NSP constituent losses were much greater in *L. leucocephala* and *L. pallida* than in *C. calothyrsus*, despite similar NSP content and composition among these legumes. Comparison of the NSP_L on the basis of leaf maturity, showed that immature samples tended to have greater NPS_L than mature samples in *F. macrophylla, L. leucocephala* and *L. fairchildiana*. The opposite was observed with *L. macrophylla* and *C. calothyrsus* (Table 3-5).

3.3.3 Relationships between leaf degradability and leaf chemical composition

Linear regression relationships between gas accumulation at different times during the incubation period and the nutrient content of the different leaves are shown in Figures 3-7 and 3-8. Early in the fermentation, gas accumulation was found to be inversely related to the content of extractable and total condensed tannins ($P < 0.05$; Figure 3-7). However, by the end of the fermentation period, this effect was no longer significant. On the other hand, the content of bound condensed tannins was not significantly related to gas accumulation at any point during the fermentation of these samples (data not shown). Gas accumulation was also inversely related to the concentration of other leaf components such as NSP, cell wall glucose (Figure 3-8), cell wall mannose (Figure 3-8), cell wall xylose, NDF and ADF. However, most of these relationships were weak and not significant ($P > 0.05$). An interesting point, however, was that most of these relationships became stronger as the incubation progressed. Notably, the relationship between glucose and gas accumulation became statistically significant ($P < 0.05$) by 48 h of incubation. Another point of interest is that higher correlation with gas evolution was obtained by the use of composite variables such as of NSP plus condensed tannins or cell wall glucose plus condensed tannins (see Figure 3-7).

Positive relationships were observed between gas accumulation and the content of arabinose (Figure 3-8), nitrogen (Figure 3-8) and uronic acids (data not shown). Notably, the relationships involving nitrogen and uronic acids were stronger by 48 h of incubation ($P \leq 0.06$). There were also significant positive relationships between gas pool size and the determinations of *in vitro* dry matter digestibility made by the techniques of Tilley and Terry (1963) and by the pressure transducer technique of Theodorou et al. (1994, see Figure 3-9a). Gas pool size was also significantly related to the concentration of acetate, propionate and total VFA observed at the end of the fermentation period (Figure 3-9b). Weaker positive relationships were observed between the concentrations of branched-chain VFA and gas accumulation by 144 h ($r \geq 0.67$, $P < 0.05$, data not shown).

A inverse relationship worthy of comment was that observed between the concentration of branched-chain VFA iso-butyrate, iso-valerate and the concentration of total condensed tannins (see Figure 3-9c) and extractable condensed tannins (see Figure 3-9d).

3.4 DISCUSSION

Previous research has suggested that at low levels, dietary tannins can be of nutritional benefit for ruminants. For example, Barry et al. (1986) suggested that at concentrations of 30-40 g kg^{-1} of dry matter, condensed tannins in *Lotus* sp. act by protecting dietary protein from excessive degradation in the rumen, without affecting forage intake or fibre digestion. The total condensed tannin content in the tanniniferous legumes examined in the present study was never lower than 60 g kg^{-1} of dry matter. However, the relationships between *in vitro* gas accumulation and both extractable and total condensed tannin content were only statistically significant during the initial stages of the incubation, with the effect declining as the incubation progressed. Conversely, the concentration of bound condensed tannins was not strongly related to gas accumulation. These observations suggest that tannins acted by decreasing the initial rate, but not the extent, of dry matter degradation. Similarly, Makkar et al. (1995c) reported that tannins from *Quercus incana* and *Dychostachys cineraria* affected the rate of digestion of a hay-based diet to a greater extent than they affected the potential extent of digestion. In turn, McNabb et al. (1996) observed

that whereas tannin from *Lotus pedunculatus* reduced the digestion of Rubisco in the rumen, they did so without affecting its solubilization, thus suggesting that tannins acted by slowing protein degradation by rumen micro-organisms. There is also evidence that tannins in forages increase the rate at which undegraded fibre molecules leave the rumen per unit time, with less digestion of these constituents taking place in the rumen (Barry and Manley, 1984; Barry et al. 1986). These observations, together with those of this thesis, illustrate how sampling and fermentation time can affect the results obtained in *in vitro* gas experiments. In agreement with the observations of Ndlovu and Nherera (1997), our results suggest that tannin effects on *in vitro* gas production experiments are complex and their interpretation should take into account *in vivo* responses.

In previous research, Longland et al (1995) found significant inverse relationships between gas accumulation at different times post-inoculation and the extractable, bound and total tannin contents of eleven tropical forage legumes. This could have been confounded by the fact that they dealt with two sets of tropical legumes obtained in two different locations (Colombia and Ethiopia). In their study, legumes harvested in Colombia which had the lower *in vitro* fermentation, not only had higher condensed tannin contents, but also had higher levels of phenolics and NSP than the Ethiopian samples. As an additional point, research in this thesis has demonstrated that condensed tannins from different legumes are not a uniform entity, but differ in characteristics such as molecular weight and monomeric composition (see Chapter 4.1), factors which in turn, affect their biological impact (see Chapters 4.2, 4.3 and 5.2). Indeed, some of the results from the present study can probably be best explained in terms of tannin structure-activity relationships. For example, immature *L. leucocephala* and immature *L. pallida* differed greatly in gas accumulation at 12, 24 and 48 h and *in vitro* DMD, despite similar NSP content and composition and condensed tannin content. As an additional example, a higher percentage of condensed tannins in *L. pallida* were bound to cell wall than in *L. leucocephala*. Additionally, there was greater VFA production with *L. leucocephala* than with *L. pallida*. These results are suggestive of differences in the biological effects of condensed tannins from both legumes.

Branched-chain fatty acids come from the fermentation of amino acids (valine, leucine) and have been known for some time to be growth promoting factors for many rumen bacteria (Hungate, 1966). The highest concentrations of branched fatty acids were found in *L. macrophylla* (zero tannin content) and in *L. leucocephala*. Conversely, with legumes such as *D. ovalifolium* and *F. macrophylla*, the concentration of branched-chain VFA was low (lower than that found in the control serum bottles containing only rumen inoculum and buffer). In a subsequent experiment, it was observed that among all legumes tested in this study, condensed tannins from *L. leucocephala* were the least efficient in inhibiting the fermentation of substrates by rumen micro-organisms (see Chapter 4.2). The present results thus suggest that in the presence of certain condensed tannins there is either decreased degradation of protein (hence less free amino acids and less conversion of these amino acid to branched-chain VFAs), or increased utilisation of branched-chain VFAs for protein synthesis by rumen micro-organisms. The most likely scenario within the rumen is the former, as the presence of condensed tannins in the diet has been consistently associated with reduction of protein fermentation by rumen micro-organisms (Barry and Manley, 1984; Waghorn et al., 1987; McNabb et al., 1993; Carulla, 1994). As some of the most important fibre-degrading bacteria have a requirement for branched-chain fatty acids for healthy growth and fibrolytic activity (Hungate, 1966), the observed deficit of branched-carbon chains in the presence of condensed tannins might lead to decreased bacterial protein synthesis and decreased fibre degradation.

Only a weak negative relationship was observed when comparing total NSP and glucose in NSP. This relationship beacame more significant when the aggregates of NSP + total condensed tannins and glucose + total condensed tannins were compared with gas production. This might be because condensed tannins bind to protein and/or carbohydrate components of the cell wall (Bate-Smith and Lerner, 1954) and rendering them less available for fermentation by rumen micro-organisms. Evidence of this binding is provided by comparison of the hemicellulose content as determined by the detergent system of forage analysis (NDF minus ADF) with that obtained by summation of rhamnose, arabinose, xylose, mannose and galactose content.

Traditional estimations of hemicellulose content were generally higher (up to three times) than those derived by the measurement of the individual NSP constituents.

It has been suggested that gravimetric methods for determining dietary fibre such as NDF and ADF can result in variable losses of one or more of the NSP components (Low, 1985) and lead to underestimation of the dietary fibre present in a given feed. On the other hand, techniques that measure the individual constituent monomers of the NSP fraction, do so without losses of those constituent groups (Longland and Low, 1989). Indeed, observations by Makkar et al. (1995d, 1997b) have suggested that the detergent system of forage analysis is inadequate for the determination of *in vivo* and *in vitro* cell wall digestibility of tanniniferous forages. Part of the difference may be due to the binding of condensed tannins and other phenolics to cell wall components with the formation of complexes that are insoluble in the detergents used for NDF and ADF determination, and which as suggested, also lead to substrate (fibre) deprivation to rumen micro-organisms.

The monosaccharide composition of the cell walls of *F. macrophylla*, *L. leucocephala* and *C. calothyrsus* reported herein was similar to the one reported by Longland et al. (1995). However, NSP_L from *F. macrophylla* and *C. calothyrsus* reported by Longland et al. (1995) were lower than the ones we report here. Greater variability on the loss of NSP and its constituents was observed among legume species than could be explained by variability in NSP composition. For example, losses of NSP and NSP constituents (mg g^{-1}) from *C. calothyrsus* were much lower than the corresponding losses from either *L. leucocephala* or *L. pallida*, despite the great similarity in NSP content and composition between these three legumes. Similar conclusions can be drawn when examining NSP_L from *F. macrophylla* and *C. fairchildiana.* These differences could be related to differences in factors such as side-chain substitution and binding of condensed tannins to cell wall components. Substitution of xylans with phenolics (Chesson et al., 1983) and arabinose (Brice and Morrison, 1982) was directly related to their degradability. Binding of condensed tannins to cell walls was found to have an inverse relationship to NSP_L in tropical legumes (Longland et al., 1995). However, in the current study, a strong negative relationship between content of bound condensed tannins and NSP_L was not observed. These findings appear to support the view that the biological activity of condensed tannins varies from legume

to legume. It should be noted as well that the absence of condensed tannins is not a guarantee for the superior quality (i.e. digestibility) of cell wall, as demonstrated by the case of *L. macrophylla*.

Sample maturity was found to have little effect on the content and composition of NSP, and on the relative distribution of condensed tannins. However, total tannin content tended to be greater and nitrogen content tended to be lower in the mature samples studied here. These tendencies, however, did not necessarily relate to the lower nutritional quality of the mature samples. For example, in only three of the seven legumes was DMD and NSP_L of mature leaves lower than that of immature leaves. Additionally, depressions in gas pool volume at 144 h due to leaf maturity were only observed in *L. pallida*. On the contrary, in three of the seven legumes, mature samples had a greater proportion of the gas produced within the first 24 h of incubation than immature samples. Other researchers have observed that up to a point, increases in molecular weight of condensed tannins were associated to increased astringency (Oh and Hoff, 1979; Kumar and Horigome, 1986). However, Jones et al. (1976) reported that tannins of high molecular weight from sainfoin were less effective in precipitating protein than the smaller *Lotus sp*. tannins. Similarly, Butler (1982) suggested that increases in the degree of polymerisation of sorghum tannins during seed development and maturation were associated with lower bird-resistance even in high-tannin sorghums. Whether the responses observed in this experiment are due to increases in polymerisation of condensed tannins with maturity is a subject that merits further investigation (see Chapters 4.1 and 4.2).

In vitro dry matter digestibility estimated by the technique of Tilley and Terry (1963) and by the pressure transducer technique of Theodorou et al. (1994) were highly correlated ($R \geq 0.91$, $P < 0.001$), despite differences such as species and diet of the donor animal and site (CIAT vs. IGER) of assessment. *In vitro* DMD was also highly correlated to the final volume of gas accumulated by 144 hours. These results demonstrate the robustness of the two *in vitro* techniques and show that the pressure transducer technique can be used with tropical legumes instead of the Tilley and Terry technique with the added advantage of providing information about the rates of fermentation as well as the extent of fermentation.

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4. STRUCTURE-ACTIVITY RELATIONSHIPS IN CONDENSED TANNINS FROM TROPICAL FORAGE LEGUMES

4.1 MOLECULAR WEIGHT AND PROCYANIDIN-PRODELPHINIDIN-PROPELARGONIDIN RATIO IN CONDENSED TANNINS FROM TROPICAL FORAGE LEGUMES

4.1.1 INTRODUCTION

The ability of tannins to form complexes with protein, carbohydrate and other molecules (Haslam, 1986) has traditionally been regarded as the means by which tannins inhibit digestion and reduce plant preference (Feeny, 1969, Rhoades and Cates, 1976, Swain, 1979). Tannins can complex with and inactivate digestive enzymes (Swain, 1979) and precipitate dietary protein (Feeny, 1969). In turn, the formation of complexes between dietary constituents such as fibre and tannins might result in nutrients becoming unavailable to rumen micro-organisms and/or to their hydrolytic enzymes. This could be mediated by the masking of potential binding sites (Martin and Martin, 1983).

Studies of tannin-protein interactions have highlighted the role played by tannin structure on the formation of tannin-protein complexes. Indeed, tannin characteristics such as molecular weight, conformational flexibility and water solubility can strongly influence the ability of tannins to precipitate proteins (Spencer et al. 1988). Likewise, the size, conformational flexibility and amino acid content of proteins strongly affect their affinities for a particular tannin (Asquith and Butler, 1986; Mehansho et al. 1983; Mole et al. 1990a). As a result, tannin-protein binding can be quite specific for both the protein and the tannin (Butler, 1989). This also signifies that nutritional studies must be concerned with determining not only tannin concentration, but also their structural characteristics.

The molecular structure of condensed tannins has been reported to be considerably diverse. Factors such as molecular weight, stereochemistry (*cis-trans* ratio) and monomeric composition contribute to this diversity (Foo and Porter, 1980; Foo et al. 1982; Williams et al. 1983; Eberhardt and Young, 1994). As in the case of tannin molecular weight, there is evidence that both stereochemistry and monomeric

composition of tannins can also play a role in determining the nutritional impact of condensed tannins (Jones et al. 1976; Clausen et al. 1990; Ayres et al. 1997). However, it is still not possible to delineate the role played by particular tannin structures on the observed effects of tannins.

In the present study, condensed tannins extracted from different tropical legumes were purified for their use in several *in vitro* experiments. In this chapter we report on (a) the use of gel permeation and high performance liquid chromatography to determine structural features of these tannins such as molecular weight and monomeric composition; (b) the effect of purified tannins on *in vitro* gas production from different substrates and (c) their effect on the activity of a number of cell wall degrading enzymes from rumen micro-organisms and discuss these effects in terms of structure-activity relationships.

4.1.2 MATERIALS AND METHODS

4.1.2.1 Purification of condensed tannins

Condensed tannins were isolated from mature and immature foliar samples of the tanniniferous tropical legumes *Desmodium ovalifolium, Flemingia macrophylla, Leucaena leucocephala, Leucaena pallida, Calliandra calothyrsus* and *Clitoria fairchildiana*. A sample of *ca* 30 g of lyophilized ground leaves was extracted with 200 ml a 70% acetone plus 0.05% (w/v) ascorbic acid aqueous solution for 1 h under continuous stirring at room temperature. After extraction was complete, acetone was recovered by filtration through four layers of cheesecloth and then through Whatman micro-fibre filter paper. Condensed tannins in the liquid phase were purified according to the method of Terrill et al. (1992b). Acetone was evaporated from the extract by rotary evaporation under reduced pressure and the resulting aqueous solution was extracted three times with diethyl ether followed by ethyl acetate (3x) extraction, in both instances keeping the aqueous phase and discarding the organic phase. After evaporation of remaining solvent, the aqueous solution was lyophilized and the obtained solid was re-dissolved in 50% aqueous methanol. Tannins were purified on a column packed with 60 ml of Sephadex LH-20 in 50% methanol slurry. Additional 50% aqueous methanol was run through the column until the liquid eluting from the column was colourless. Tannins were then recovered from the column by

elution with 70% aqueous acetone. After evaporation of the acetone, the aqueous solution was lyophilised to dryness. Purified condensed tannins were stored at -70 °C in the dark until used in these experiments.

4.1.2.2 Determination of the procyanidin-prodelphinidin-propelargonidin ratio in sequentially extracted and in purified condensed tannins

Duplicate (250-450 mg) ground-leaf samples of young and mature *Desmodium ovalifolium, Flemingia macrophylla, Leucaena leucocephala, L. pallida, L. macrophylla, Calliandra calothyrsus* and *Clitoria fairchildiana* were weighed into 20-ml screw cap tubes. Weighed samples were extracted with 10 ml of a 70% aqueous methanol solution for one hour under continuous shaking (1800 rpm) at room temperature. The extraction was repeated two times (another 1-h extraction and one overnight extraction) with the extracts being collected after centrifugation. All three methanol extracts were combined and the residue was subjected to a similar extraction but this time using a 70% aqueous acetone solution. After this was complete, the acetone extracts were combined and the residue dried at 40 °C in an air-convection oven and then weighed to determine total residual dry matter. Methanol and acetone were evaporated from their respective extracts by means of a sample concentrator set up at 40 °C. Interfering pigments in the acetone extract were eliminated by extraction with diethyl ether (3X). The total volume of all extracts was taken to 10 ml by the addition of distilled water. Then, the tannin content in 0.5-ml aliquots of all extracts and in 10-mg sub-samples of the residue fraction was determined by the addition of butanol-HCl (95:5, Terrill et al. 1992b) as described in Section 4.2.2. The standard curves obtained previously (see Section 4.2.2) were used to calculate the tannin content of the different samples.

For the determination of the procyanidin-prodelphinidin-propelargonidin ratio, condensed tannins in aliquots of all extracts, of solutions containing purified tannins and of the solid residue were hydrolysed by heating them at 95°C for 1-h in butanol-HCl. After hydrolysis was complete, the butanol-HCl in a 1-ml aliquot of the hydrolysed tannin solution was evaporated to dryness. Dried anthocyanidins were quickly re-dissolved in 50 µl of a solution containing 1% HCl in pure methanol. The procyanidin-prodelphinidin-propelargonidin ratio was determined by injecting 20 µl into a high-performance liquid chromatography system fitted with a 8 X 10 cm Nova

Pack C18 column and a photodiode array detector (Waters 990). Two solvents were used: A = 5% aqueous acetic acid; B = methanol and samples were eluted following a linear gradient of 30-100% B in 20 min. The photodiode array detector was set to acquire the spectra between 480-580 nm and to monitor the chromatograms at 525 nm. Data analysis was performed on a PC equipped with Millennium software. Using this software, chromatograms were extracted at 525 nm and peaks were identified by comparison of their retention time and wavelength of maximum absorption with those of pure anthocyanidin standards. These standards included cyanidin chloride, delphinidin chloride, pelargonidin chloride, fisetinidin chloride, robinetinidin chloride, luteolinidin chloride and apigeninidin chloride all purchased from Apin Chemicals, UK. Because all these anthocyanidins have different wavelength of absorption maximum (λ maximum), but similar extinction coefficient at their λ maximum, correction factors were used to correct for the difference in peak area at 525 nm and that at the wavelength of maximum absorption. These factors were 1.000 for procyanidin (λ maximum = 525 nm), 1.044 for prodelphinidin (λ maximum = 535 nm), 1.076 for propelargonidin (λ maximum = 517 nm) and 1.326 for profisetinidin (λ maximum = 507 nm).

4.1.2.3 Determination of the molecular weight of peracetate derivatives of condensed tannins

An initial estimation of tannin molecular weight was carried out on peracetate derivatives of condensed tannins according to the method of Williams et al. (1983). For the preparation of the peracetates, duplicate 10 mg of condensed tannins was mixed with 2.5 ml of a 1:1 (v/v) solution of pyridine: acetic anhydride and the resulting mixture was left to stand overnight at room temperature. Five ml of distilled water were slowly added and the mixture allowed to cool before centrifuging at 2500 rpm for 10 min. The resulting supernatant was discarded and the pellet (condensed tannin peracetates) was washed twice with a further 5 ml of water followed by centrifugation and recovery of the pellet. The recovered peracetates were then dried under a stream of compressed air. Prior to injection, peracetates were dissolved in 2 ml of tetrahydrofuran and 25 µl of the resulting solution was injected into a Shimadzu LC4A HPLC system fitted with two PLGel 5µm 500Å (300x7.5 mm, Polymer Laboratories, UK) columns in series. Samples were eluted from the column using

100% tetrahydrofuran at a rate of 1 ml min^{-1}. Under these conditions the system operating pressure was of 88 kg per cm^2. An UV detector (Shimadzu SPD2A) set up at 260 nm was used for detection of the peracetates derivatives. The GPC calibration was performed with polystyrene standards of molecular weights ranging from 162 to 22000 Dalton (Polymer Laboratories, UK). Data was acquired and handled via a PC equipped with GPC6000 chromatography data system software (version PR2; Jones Chromatography, UK).

4.1.2.4 Determination of the molecular weight of underivatised condensed tannins

Determination of the molecular weight was also carried out in samples of underivatised condensed tannins. For this measurement, duplicate 10 mg of purified condensed tannins was dissolved in 5 ml of a solution of 0.1% lithium bromide in dimethylformamide. An aliquot of 100 µl of the resulting solution was injected into a Shimadzu LC4A HPLC system fitted with two PLGel 5µm 500Å (300x7.5 mm) columns in series. Samples were eluted from the column using 100% dimethylformamide containing 0.1% (w/v) lithium bromide at a rate of 0.5 ml min^{-1} at ambient (25°C) temperature. Under these conditions the system operating pressure was of 92 kg per cm^2. A refractive index detector (BioRad) was used for detection of the condensed tannins. The GPC calibration was performed with polyethylene glycol standards (Polymer Laboratories, UK) of molecular weights ranging from 440 to 22800 Dalton. Data was acquired and handled via a PC equipped with GPC6000 chromatography data system software (version PR2; Jones Chromatography, UK).

4.1.3 RESULTS

4.1.3.1 Condensed tannin purification

Most freeze-dried purified condensed tannins were in the form of a fluffy, light brown powder. However, recovered tannins showed differences both in colour, from brown-yellow to brown-orange, and in form, with tannins from *C. calothyrsus* being almost crystalline. In general, yields were about 30% of the theoretical yields, with an average of one g of condensed tannins being obtained in each extraction. More than 60% of the losses occurred during purification in the Sephadex LH-20 column.

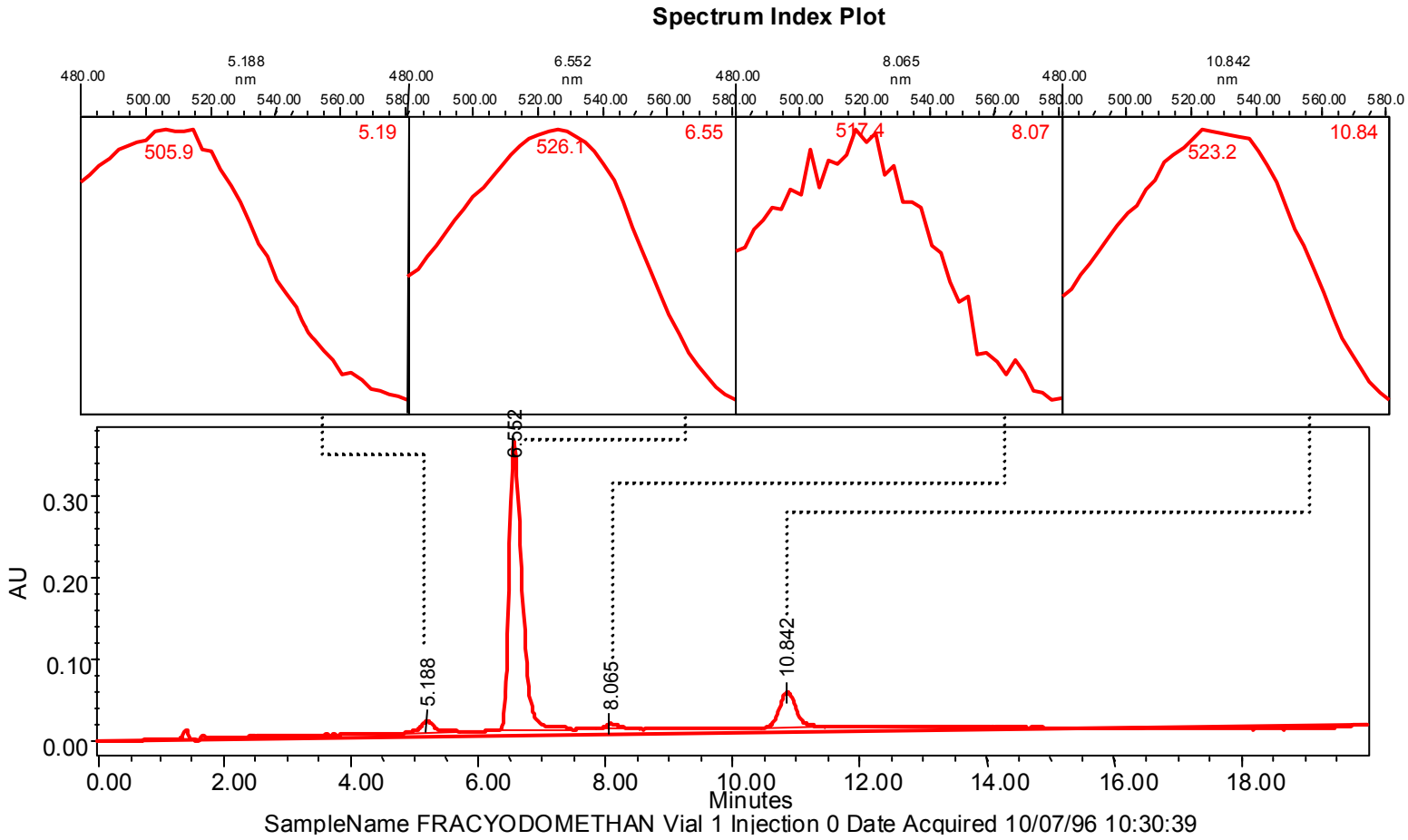

Figure 4.1-1a *Determination of the procyanidin-prodelphinidin-propelargonidin ratio in condensed tannins extracted from young* Desmodium ovalifolium *using 70% aqueous methanol*

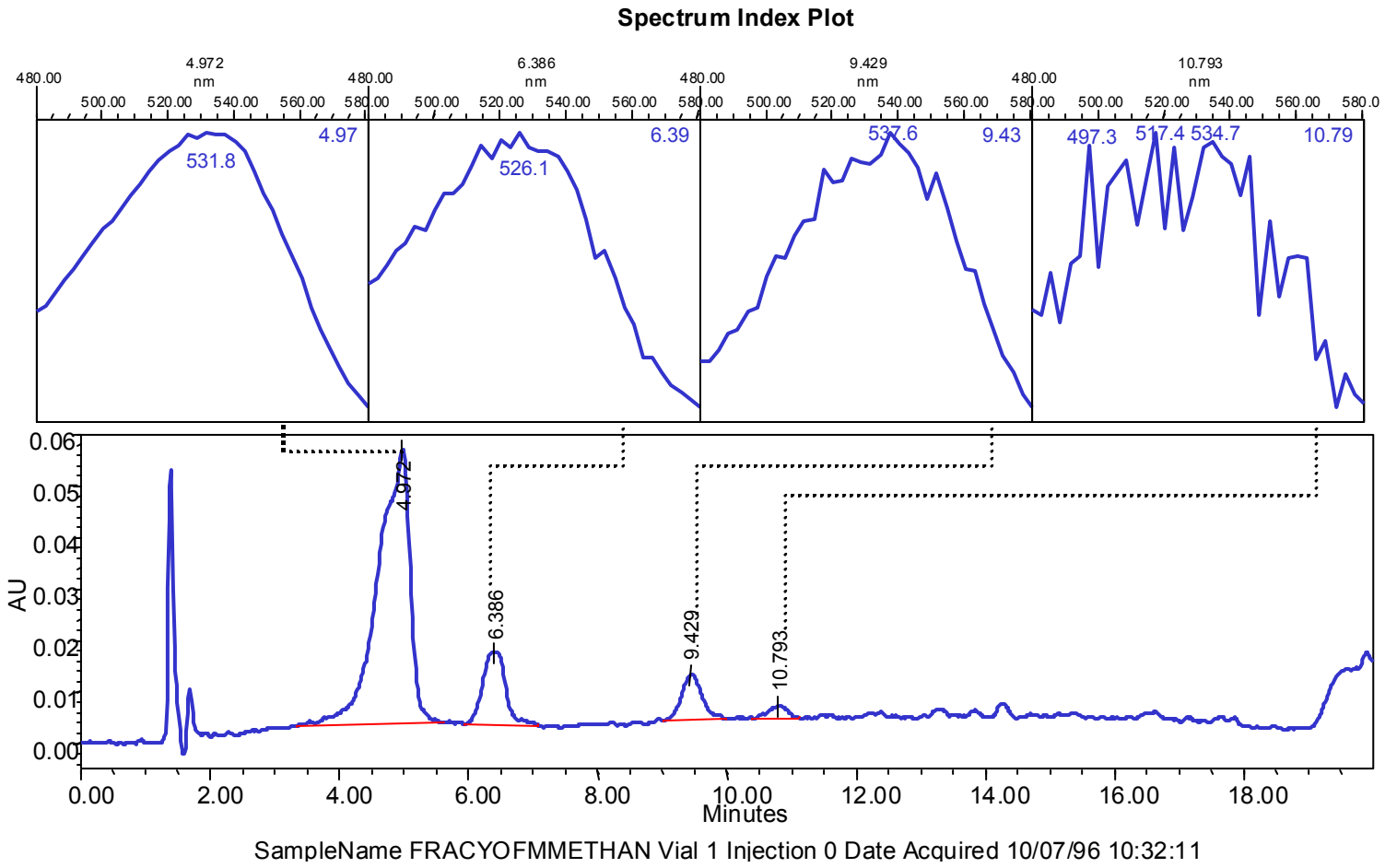

Figure 4.1-1b *Determination of the procyanidin-prodelphinidin-propelargonidin ratio in condensed tannins extracted from young* Flemingia macrophylla *using 70% aqueous methanol*

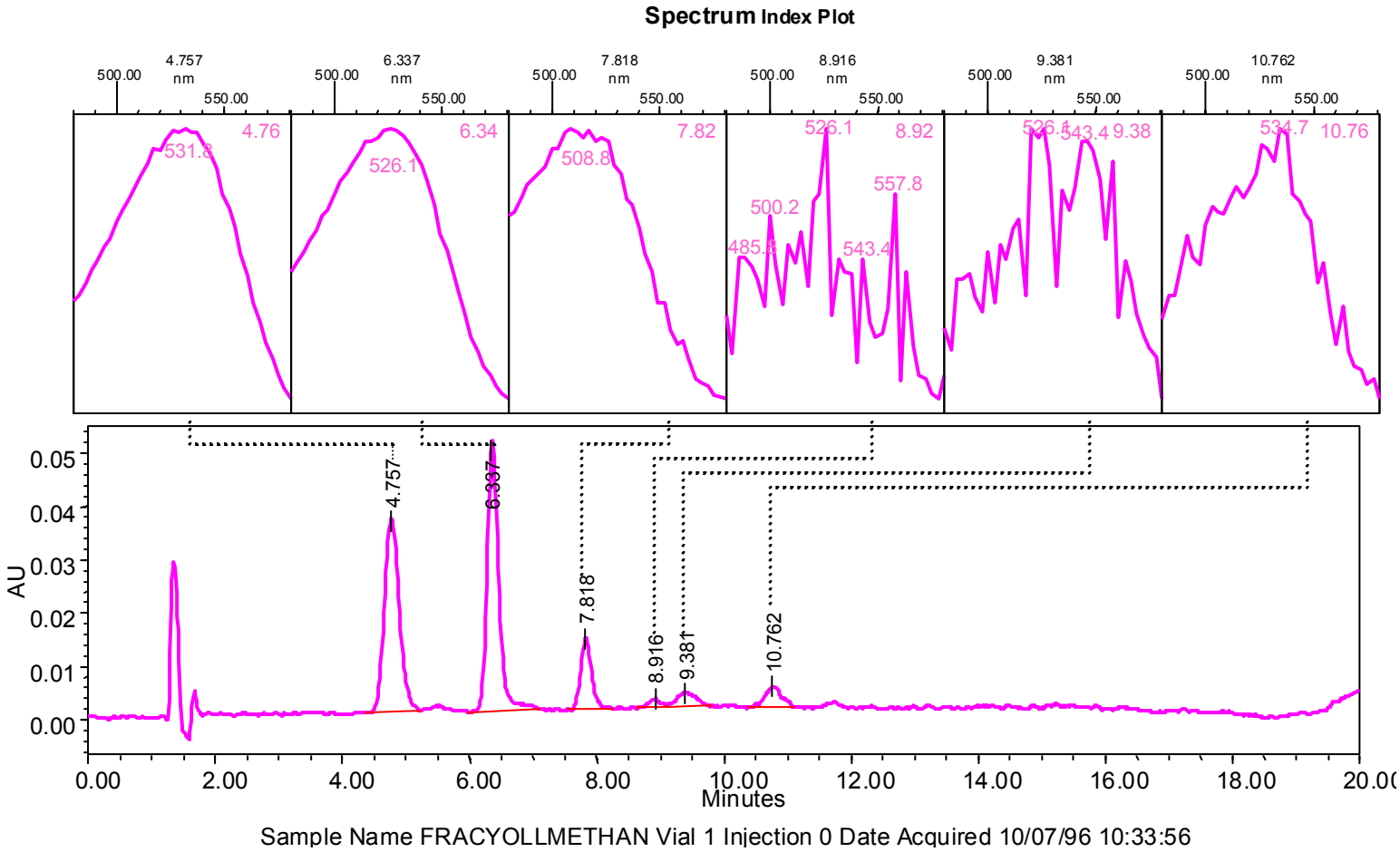

Figure 4.1-1c *Determination of the procyanidin-prodelphinidin-propelargonidin ratio in condensed tannins extracted from young* Leucaena leucocephala *using 70% aqueous methanol*

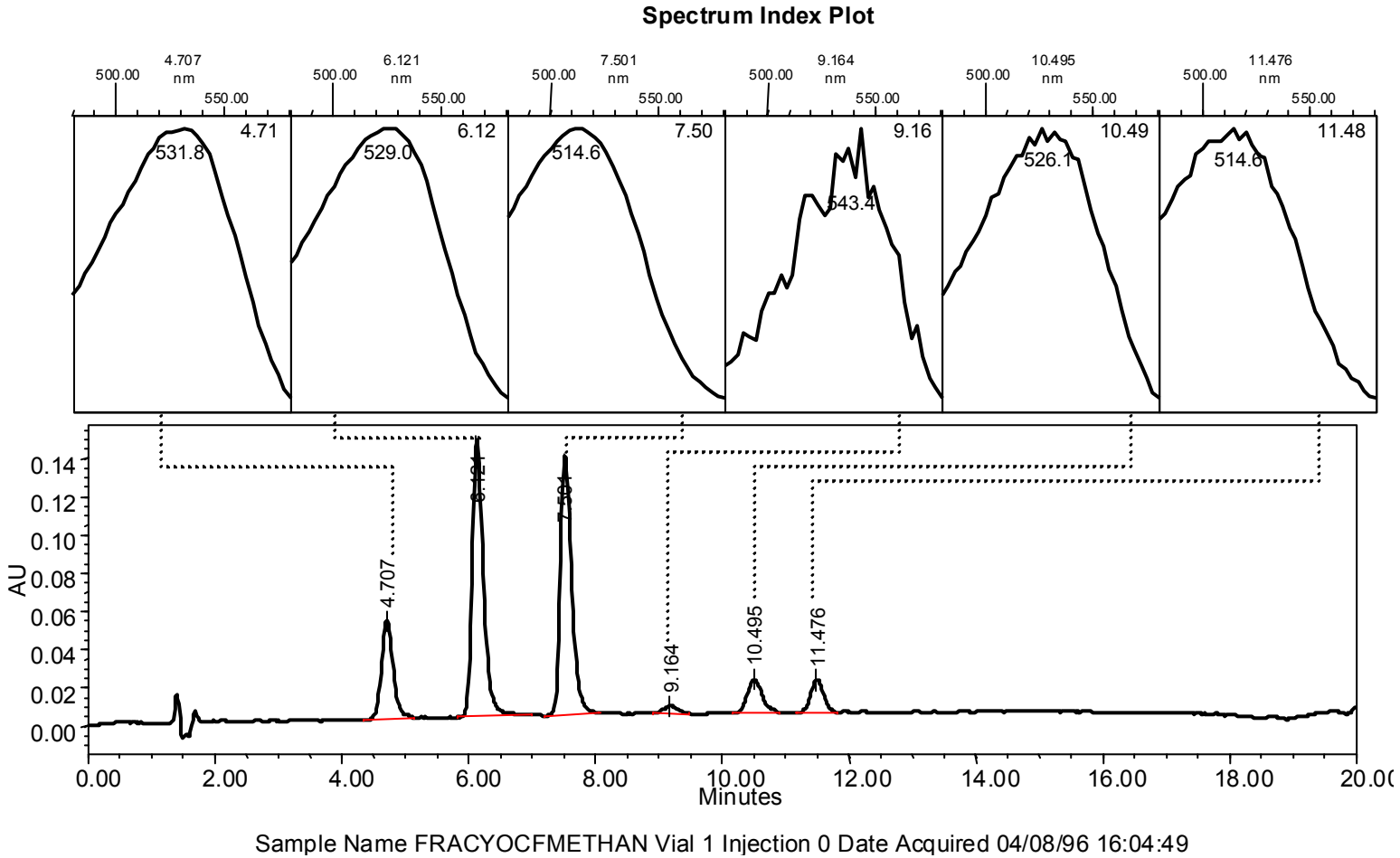

Figure 4.1-1d *Determination of the procyanidin-prodelphinidin-propelargonidin ratio in condensed tannins extracted from young* Clitoria fairchildiana *using 70% aqueous methanol*

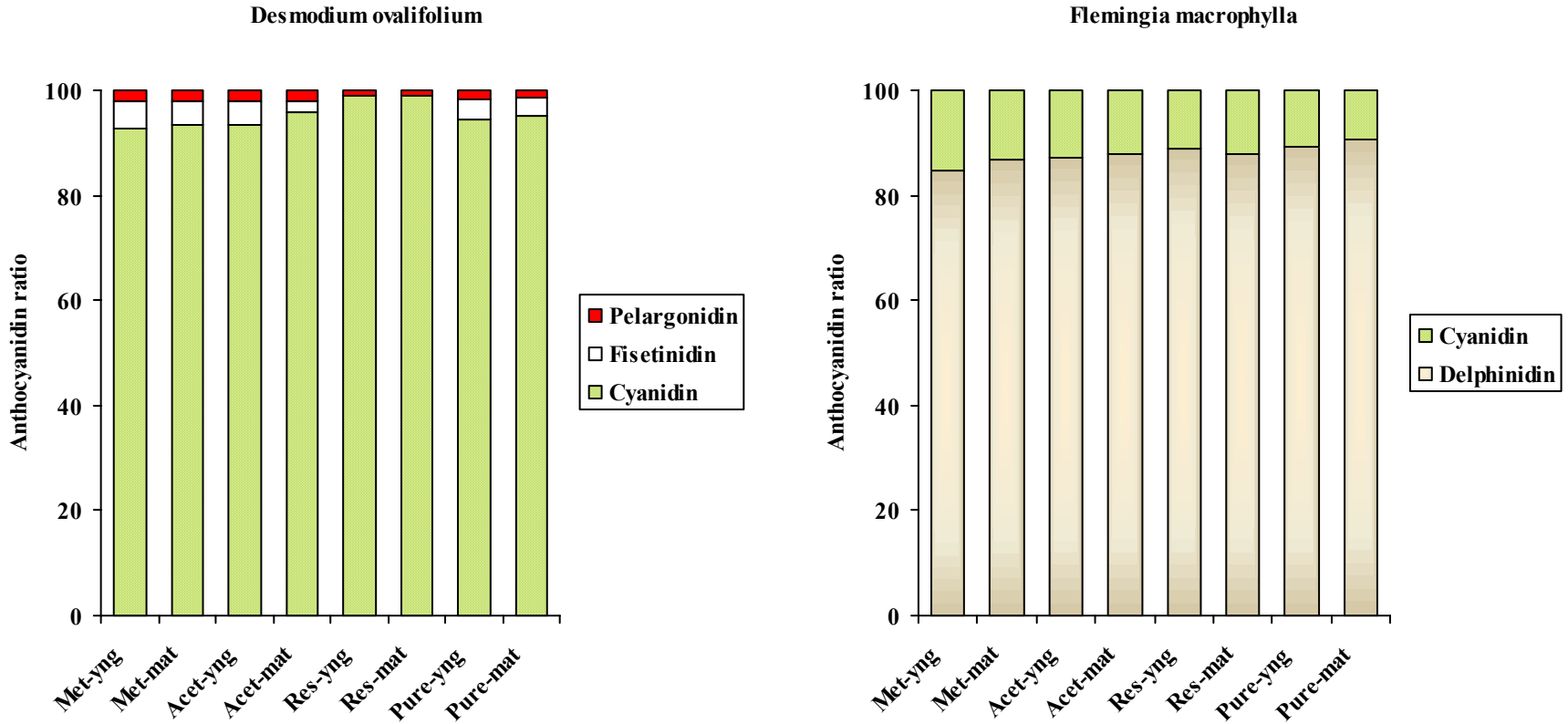

Figure 4.1-2a. *The procyanidin-prodelphinidin-propelargonidin ratio in sequentially extracted and in purified samples of condensed tannins. Met = methanol-extracted tannins; acet = acetone-extracted tannins; res = non-extractable tannins; pure = purified tannins; yng = immature leaves; mat = mature leaves*

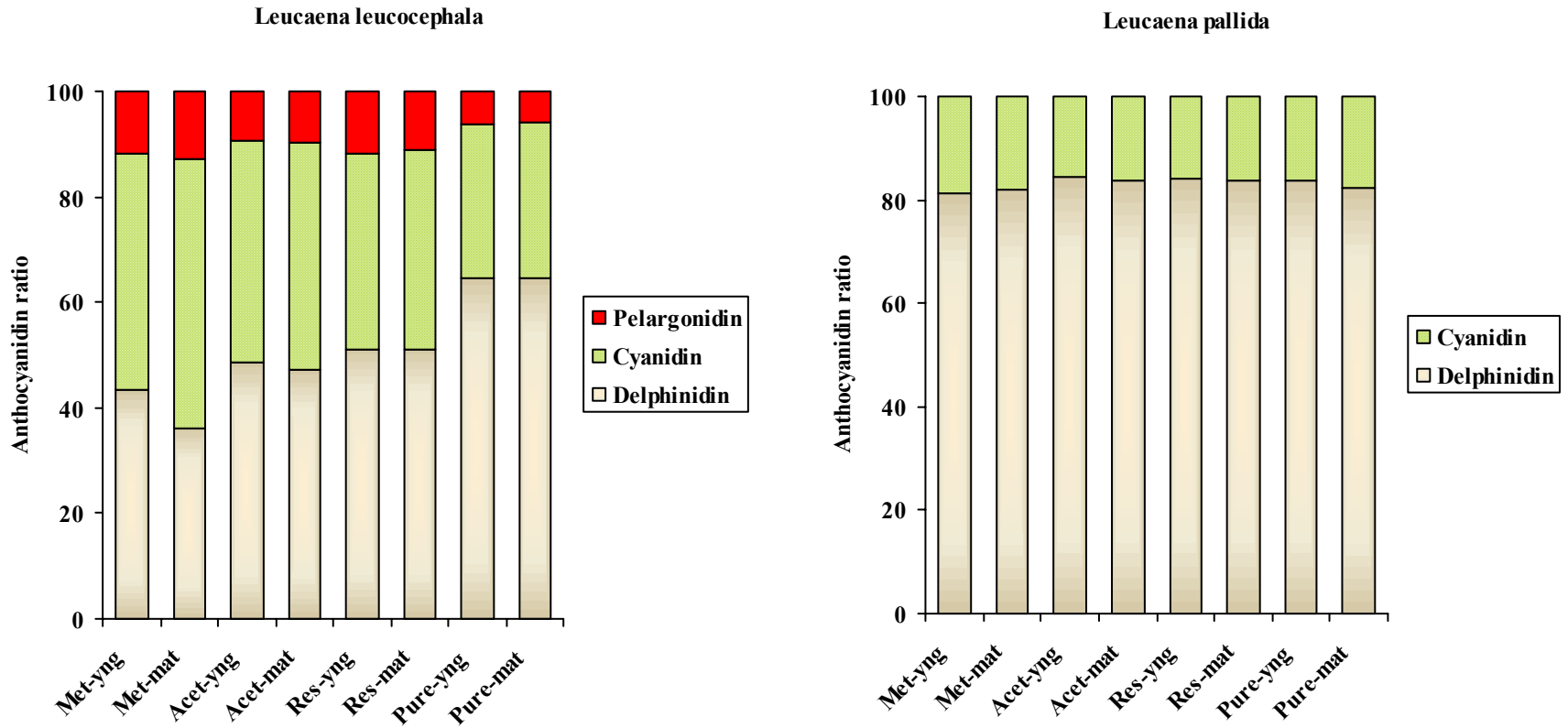

Figure 4.1-2b. *The procyanidin-prodelphinidin-propelargonidin ratio in sequentially extracted and in purified samples of condensed tannins. Met = methanol-extracted tannins; acet = acetone-extracted tannins; res = non-extractable tannins; pure = purified tannins; yng = immature leaves; mat = mature leaves*

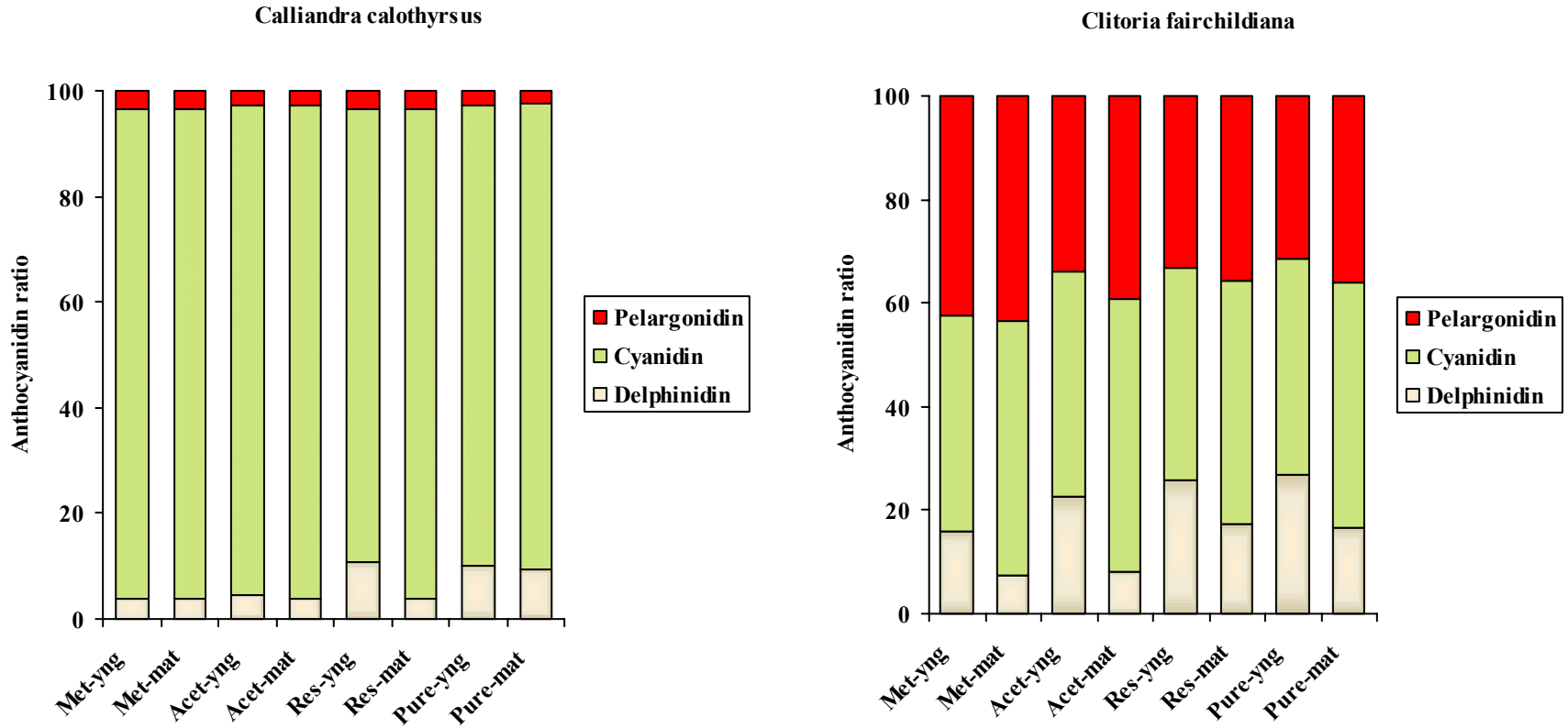

Figure 4.1-2c. *The procyanidin-prodelphinidin-propelargonidin ratio in sequentially extracted and in purified samples of condensed tannins. Met = methanol-extracted tannins; acet = acetone-extracted tannins; res = non-extractable tannins; pure = purified tannins; yng = immature leaves; mat = mature leaves*

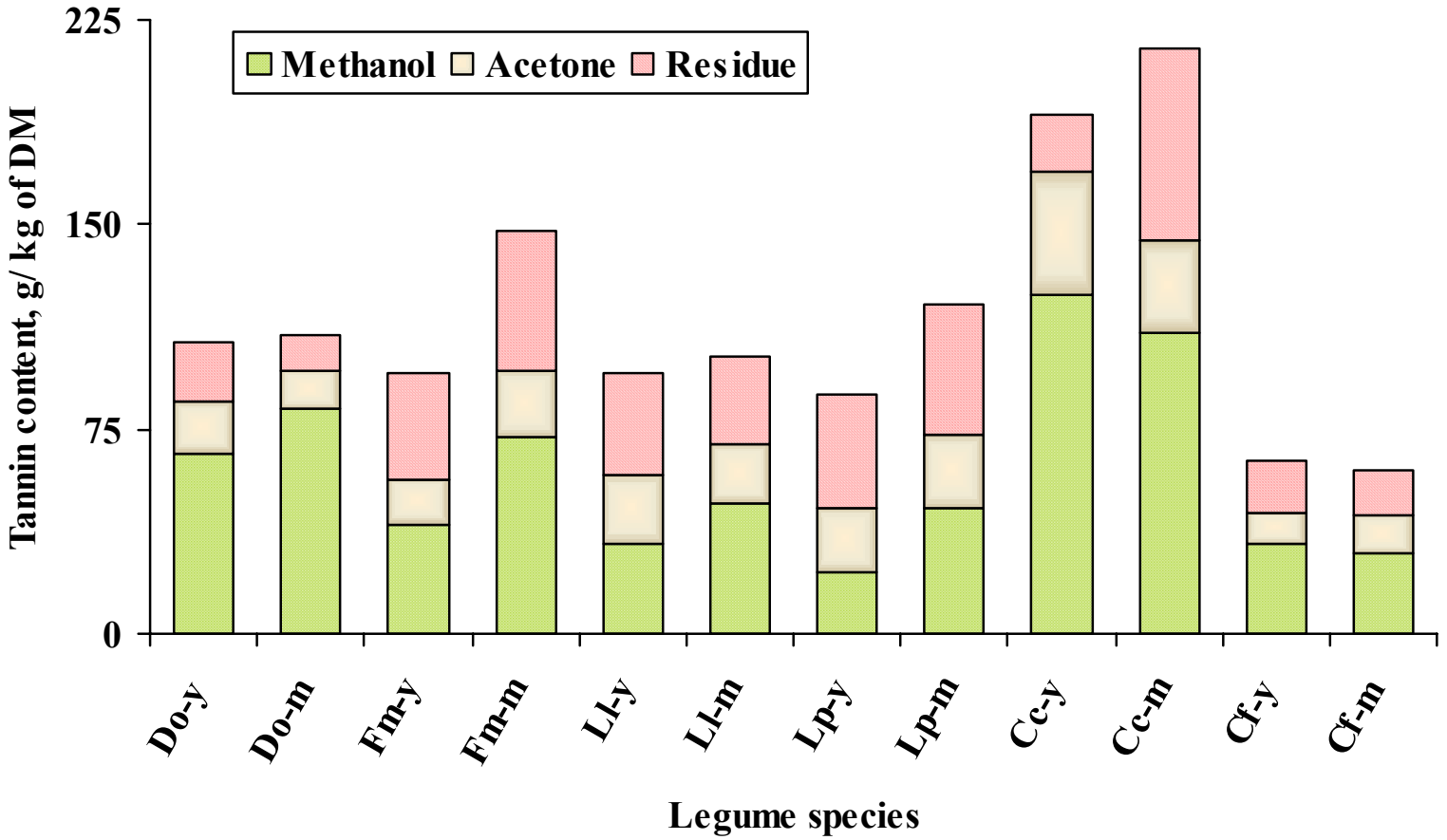

Figure 4.1-3. *The condensed tannin extracted by methanol, acetone and still attached to the residue after extraction in mature and immature leaf samples of six tropical legumes. Do* = Desmodium ovalifolium*; Fm* = Flemingia macrophylla*; Ll* = Leucaena leucocephala*; Lp* = Leucaena pallida*; Cc* = Calliandra calothyrsus*; Cf* = Clitoria fairchildiana*; y = young leaves; m = mature leaves.*

4.1.3.2 Procyanidin-prodelphinidin-propelargonidin ratio in sequentially extracted and in purified condensed tannins

The monomer composition [procyanidin-prodelphinidin-propelargonidin (PC-PD-PP) ratio] of purified condensed tannins is shown in Table 4.1-1. These data was calculated taking into account the area of all peaks, including those that could not be recognised. There was great variation in the anthocyanidin content of the tannins assayed. For example, tannins from *D. ovalifolium* (see Figure 4.1-1a) and *C. calothyrsus* consisted mostly of procyanidin. On the other hand, tannins purified from *F. macrophylla* (Figure 4.1-1b) and *L. pallida* were mostly composed of prodelphinidin. In turn, in purified condensed tannins from *L. leucocephala* (Figure 4.1-1c), procyanidin content was about 50% of prodelphinidin content. Finally, purified tannins from *C. fairchildiana* (Figure 4.1-1d) had an average PC-PD-PP ratio of 4.5 – 2.2 – 3.4. Overall, there were no differences in PC-PD-PP ratio among tannins due to leaf maturity.

Table 4.1-1. *Anthocyanidin content of purified condensed tannins extracted from immature and mature leaves of six tropical forage legumes*

Legume	Maturity	Anthocyanidin content, percent of total peak area				
		Procyan[1]	Prodelp[1]	Propela[1]	Profise[1]	Unknown
D. ovalifolium	Immature	85.2	-------	1.6	3.4	9.8
	Mature	86.1	-------	1.3	2.9	9.6
F. macrophylla	Immature	10.5	87.0	-------	-------	2.5
	Mature	9.0	86.7	-------	-------	4.3
L. leucocephala	Immature	28.2	62.	2.9	-------	3.7
	Mature	27.6	60.6	5.6	-------	6.2
L. pallida	Immature	14.3	74.4	-------	-------	11.3
	Mature	15.7	73.8	-------	-------	10.5
C. calothyrsus	Immature	82.0	9.5	2.8	-------	5.7
	Mature	82.6	8.7	2.2	-------	6.5
C. fairchildiana	Immature	39.0	25.1	29.6	-------	6.2
	Mature	43.0	15.0	34.5	-------	9.5

[1]Procya = procyanidin, prodelp = prodelphinidin, propela = propelargonidin, profise = profisetinidin.

The results obtained in the determination of the PC-PD-PP ratio in tannins sequentially extracted from leaf tissue are presented in Figure 4.1-2 (a, b and c). To allow for comparisons, data for the PC-PD-PP ratio of purified tannins is also included. It should be noticed that in this case, PC-PD-PP ratios in Figure 4.1-2 were calculated taking into account only the area of known peaks. In general, with the

exception of tannins from *L. pallida* (see Figure 4.1-2b) there were differences in the PC-PD-PP ratio of tannins in the different fractions. In the case of *D. ovalifolium*, for example, non-extractable tannins (those in the residue) did not contain profisetinidin, although tannins in the three other fractions examined (methanol and acetone extracts and purified tannins) did contain this anthocyanidin (see Figure 4.1-2a). A more striking example of this variation in PC-PD-PP ratio was observed in the tannin fractions from *L. leucocephala*. In this legume, examination of the PC-PD-PP ratio in the methanol, acetone, residue and purified tannin samples showed an increase in prodelphinidin and a decrease in procyanidin content (see Figure 4.1-2b). In most legumes, there were no differences in PC-PD-PP ratio between tannins extracted from immature and mature leaf samples. The only exception occurred with tannins from *C. fairchildiana*, where tannins from immature samples had greater prodelphinidin and lower procyanidin content than those of mature leaves (see Figure 4.1-2c).

Data on the content of condensed tannins in the methanol and acetone extracts and on the content of non-extractable (still attached to the residue after extraction) condensed tannins are presented in Figure 4.1-3. In general, the total tannin content measured in these samples was similar to that reported in Chapter 3 (Section 3.3.1). However, in the present study, observed condensed tannin content in leaves from *C. calothyrsus* was much higher than that reported earlier in Section 3.3.1. On average, 49.5 % of the total tannins were extractable in methanol, 22.4 % extractable in acetone and 28.1 % were non-extractable. As it was also observed in Chapter 3, there was great variability in the content and in the extractability of condensed tannins in the legumes studied here. Total tannin content (g kg^{-1} of plant tissue) ranged from 60 in *C. fairchildiana* to 200 in *C. calothyrsus*. There was a tendency for condensed tannin content to increase with leaf maturity, with most of that increase being due to increases in solvent-extractable tannins.

4.1.3.3 Molecular weight of the peracetate derivatives of condensed tannins

Under the conditions used for this determination, analyses were complete within 25 min. However, analyses were run for 30 min for reassurance. Figure 4.1-4 displays the results obtained for the calibration runs using polystyrene standards. The best fit to these values was obtained using a cubic equation whereby the minimum error was 0.51%, the maximum error was 0.56% and the standard deviation was 0.0140.

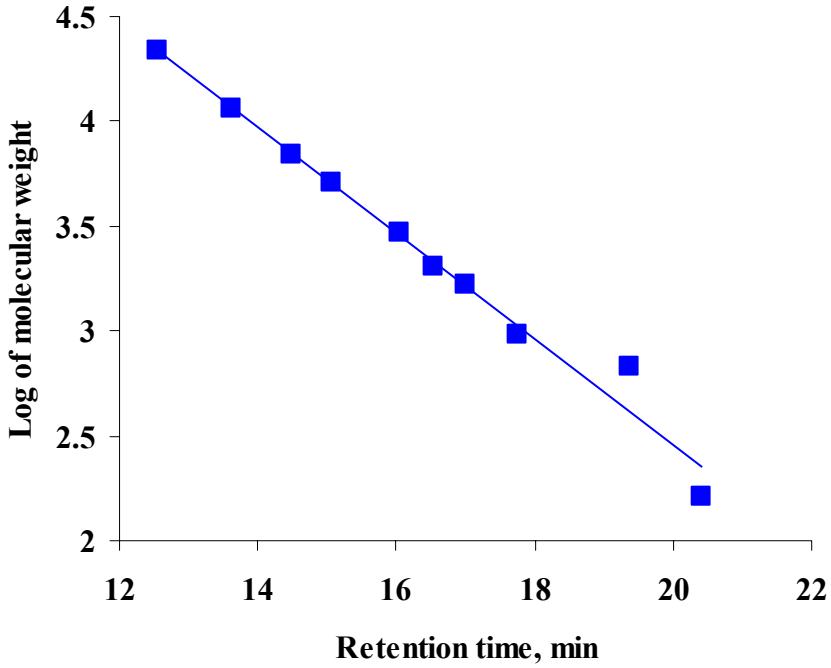

Figure 4.1-4 *Calibration curve for polystyrene standards of molecular weights ranging from 160 to 22000 Dalton. Solvent: Tetrahydrofuran; flow rate: 1 ml* min^{-1}*; detector: 260nm; column: 2 PLGel 5µm 500Å (300x7.5 mm) in series.*

Table 4.1-2. *Observed molecular weights of the peracetate derivatives of condensed tannins from tropical legumes*

Tannin source	Retention time	Molecular weight, Dalton			D_p
		Number average	*Weight* average	*Z* average	
Young *Desmodium ovalifolium*	15.18	5570	9680	17700	1.74
Mature *Desmodium ovalifolium*	15.18	5360	9560	17870	1.78
Young *Flemingia macrophylla*	15.07	5410	7850	11550	1.45
Mature *Flemingia macrophylla*	15.44	5270	9100	17040	1.72
Young *Leucaena leucocephala*	16.75	2300	2610	3000	1.13
Mature *Leucaena leucocephala*	16.46	2380	2450	2820	1.02
Young *Leucaena pallida*	16.15	2980	3550	5210	1.19
Mature *Leucaena pallida*	15.95	4190	6720	11340	1.60
Young *Calliandra calothyrsus*	15.87	3450	4820	7480	1.40
Mature *Calliandra calothyrsus*	15.82	3930	5790	9530	1.47
Young *Clitoria fairchildiana*	15.22	6440	11060	18430	1.72
Mature *Clitoria fairchildiana*	15.27	6130	10880	17880	1.78
Tadehagi sp.	16.00	3280	3720	4350	1.13

For reference, if the molecular weight (MW) possess a normal distribution, then *number average* MW **:** *weight average* MW **:** *z average* MW = 1 **:** 2 **:** 3. D_p = *number average* MW/*weight average* MW.

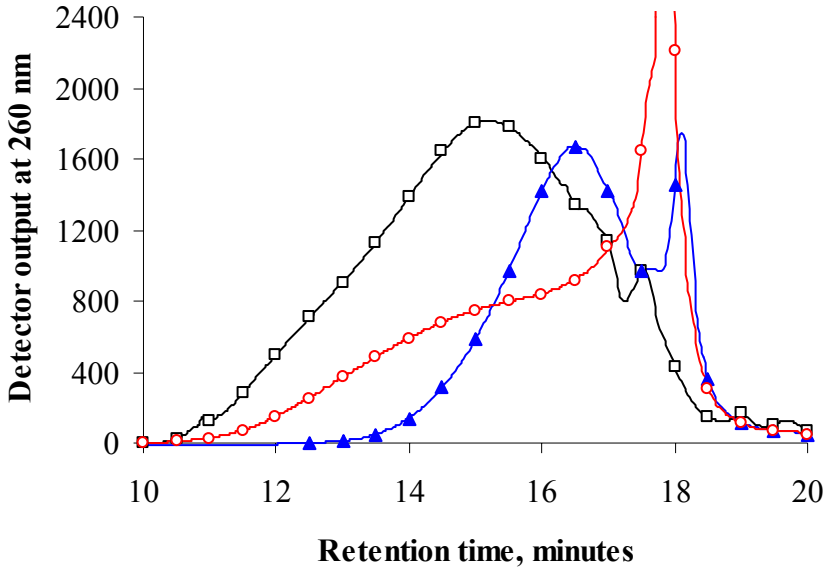

Figure 4.1-5a. *Determination of the molecular weight of peracetates derivatives of condensed tannins from mature leaves of* Desmodium ovalifolium *(□),* Leucaena leucocephala *(▲) and* Clitoria fairchildiana *(○). Solvent: tetrahydrofuran; flow rate: 1 ml min^{-1}; detector: UV 260 nm; columns: two PLGel 5 µm 500Å (300 x 7.5 mm).*

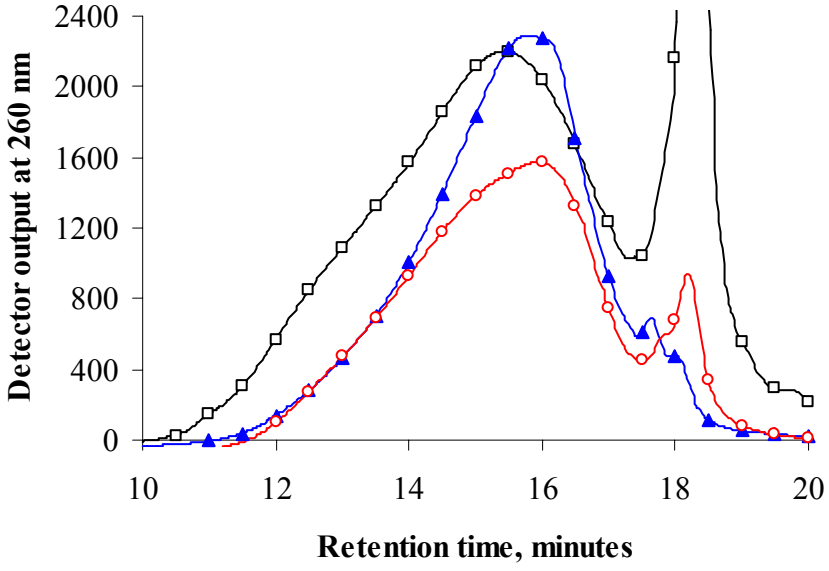

Figure 4.1-5b. *Determination of the molecular weight of peracetates derivatives of condensed tannins from mature leaves of* Flemingia macrophylla *(□),* Leucaena pallida *(○) and* Calliandra calothyrsus *(▲). Solvent: tetrahydrofuran; flow rate: 1 ml min^{-1}; detector: UV 260 nm; columns: two PLGel 5 µm 500Å (300 x 7.5 mm).*

The molecular weights of the peracetate derivatives of condensed tannins are reported in Table 4.1-2. Also reported in Table 4.1-2 is the data on the molecular weigh of the peracetates derivatives of condensed tannins purified from *Tadehagi sp*. There was considerable variation in the molecular weights of condensed tannins from different legumes. For example, the *number-average* molecular weight of tannins from *C. fairchildiana, F. macrophylla* and *D. ovalifolium* ranged between 5200 and 6400 Dalton. On the other hand, tannins from *L. leucocephala* had *number-average* molecular weights of only 2300 Dalton. There were no clear-cut effects of sample maturity on the observed molecular weight of condensed tannins. As Table 4.1-2 shows, all condensed tannins analysed are polydisperse, i.e. D_p = *weight-average* molecular weight/*number-average* molecular weight is greater than one. Estimations of D_p showed that this value ranged from 1.03 in tannins from mature *L. leucocephala* leaves to 1.78 in tannins from mature *D. ovalifolium* leaves (Table 4.1-2).

The difference in polydispersity among tannins analysed in this study is also shown in Figures 4.1-5a and 4.1-5b. As an example, Figure 4.1-5a shows that the distribution of molecular weights in peracetates of tannins from *L. leucocephala* was very narrow. Conversely, the distribution of molecular weights in tannins from *D. ovalifolium* (Figure 4.1-5a) and *F. macrophylla* (Figure 4.1-5b) was at least two times broader. These figures also show that, in varying proportions, the condensed tannin samples assayed in this study were composed of small molecular weight phenolics. This is made evident by the peaks appearing between 17.5 to 18.2 min, which would correspond to compounds of molecular weights of 1200 or 700 Dalton, respectively. Judging by peak area, in such tannins as those from *L. leucocephala*, *C. fairchildiana* and *F. macrophylla,* these small molecular weight compounds represent a significant proportion of the tannin sample.

4.1.3.4 Molecular weight of underivatised condensed tannins

Under the conditions of this assay, analyses were complete by 40 minutes. However, analyses were run for 50 minutes, in order to ensure that all tannins were eluted from the column. The relationship between the retention times and the molecular weight of the polyethylene glycol standards is displayed in Figure 4.1-6. The best description of this relationship was obtained using a cubic equation, with the minimum and maximum errors and a standard deviation of 0.69%, 0.96% and 0.0198, respectively.

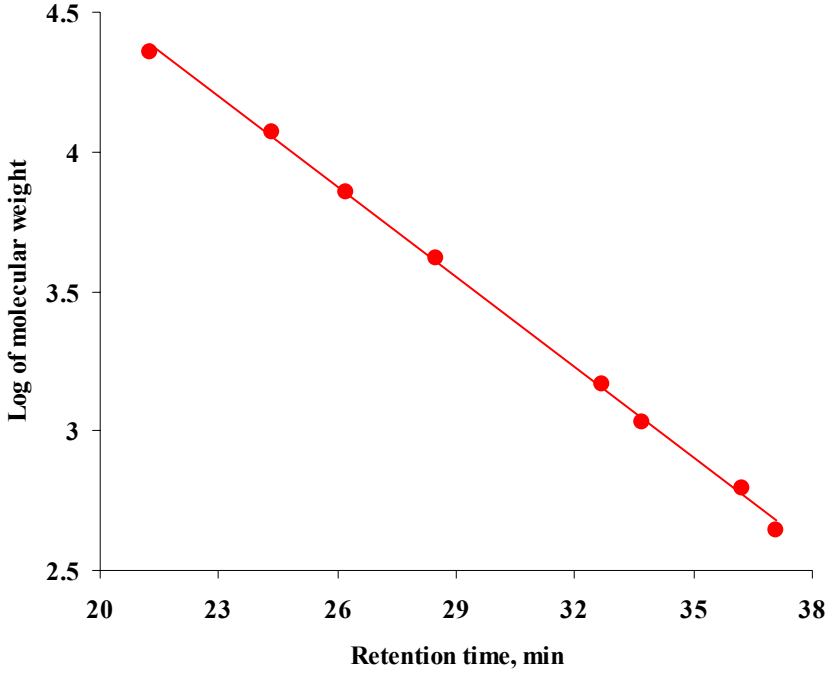

Figure 4.1-6. *The calibration curve for polyethylene glycol standards of molecular weight ranging from 440 to 22800 Dalton. Solvent: dimethylformamide containing 0.1% lithium bromide; flow rate: 0.5 ml min^{-1}; detector: refractive index; column: two PLGel 5µm 500Å (300x7.5 mm) in series.*

Table 4.1-3. *Observed molecular weights of underivatised condensed tannins from tropical legumes. Solvent: dimethylformamide containing 0.1% lithium bromide; flow rate: 0.5 ml min^{-1}; detector: refractive index; column: two PLGel 5µm 500Å (300x7.5 mm) in series.*

Tannin source	Retention time	Molecular weight, Dalton			
		Number average	*Weight* average	*Z* average	D_p
Young *Desmodium ovalifolium*	28.09	4880	7590	12010	1.55
Mature *Desmodium ovalifolium*	28.32	3850	6450	10600	1.68
Young *Flemingia macrophylla*	27.33	4320	6970	10910	1.61
Mature *Flemingia macrophylla*	28.45	4070	7300	12040	1.79
Young *Leucaena leucocephala*	31.10	2380	3060	4330	1.28
Mature *Leucaena leucocephala*	31.08	2360	2980	3990	1.26
Young *Leucaena pallida*	29.58	3250	4820	7510	1.48
Mature *Leucaena pallida*	29.29	3740	6050	9690	1.62
Young *Calliandra calothyrsus*	29.93	3320	5450	10440	1.64
Mature *Calliandra calothyrsus*	29.65	3540	5620	9450	1.59
Young *Clitoria fairchildiana*	27.01	4840	8310	12910	1.72
Mature *Clitoria fairchildiana*	28.36	4290	7060	11410	1.65
Tadehagi sp.	29.37	3110	4300	6150	1.38

Observed molecular weights of underivatised condensed tannins obtained using this technique are reported in Table 4.1-3. As for the peracetate derivatives, there was a great variation in the molecular weights of underivatised condensed tannins. For example, tannins from *C. fairchildiana, D. ovalifolium* and *F. macrophylla* were found to have *number-average* molecular weights with values ranging from 3850 to 4880 Dalton. Conversely, tannins from *L. leucocephala* had *number-average* molecular weights of only 2350 Dalton. The degree of polydispersity ranged from 1.26 in mature *L. leucocephala* to 1.79 in mature *F. macrophylla*. There was no clear distinction between the tannin molecular weights from mature and immature leaf samples.

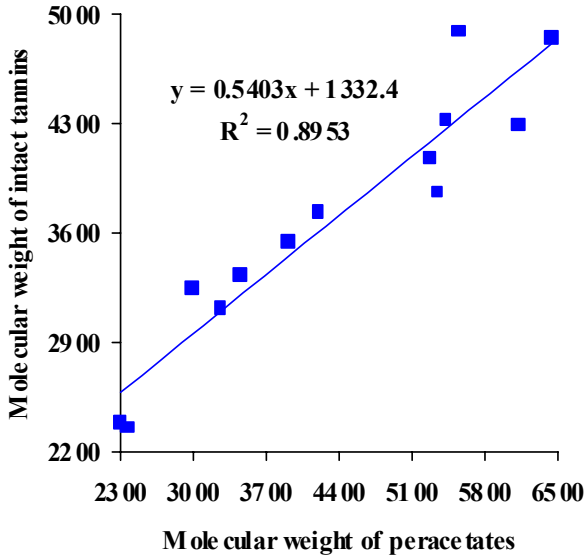

Figure 4.1-7. *Comparison of the estimation of* number-average *molecular of condensed tannin peracetates and of underivatised condensed tannins*

Values for the molecular weight of underivatised condensed tannins were lower than those for the corresponding condensed tannin peracetates. Indeed, mean *number-average, weight-average* and *z-average* molecular weight estimations of intact condensed tannins were only 84.5, 86.6 and 84.2%, respectively of the values for condensed tannin peracetates. However, as depicted in Figure 4.1-7, there was a high correlation between *number-average* molecular weight estimations obtained by both techniques. This was also the case for the estimates of *weight-average* and *z-average* molecular weight.

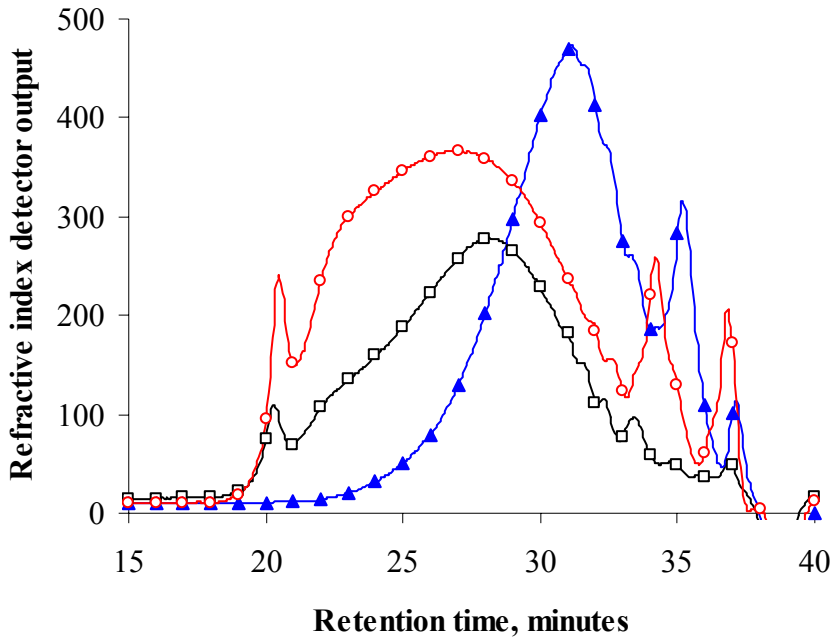

Figure 4.1-8a. *Determination of the molecular weight in underivatised condensed tannins from immature leaves of* Desmodium ovalifolium *(□),* Leucaena leucocephala *(▲) and* Clitoria fairchildiana *(○). Solvent: dimethylformamide; flow rate: 0.5 ml min^{-1}; detector: refractive index; columns: two PLGel 5 m 500 (300 x 7.5 mm) in series.*

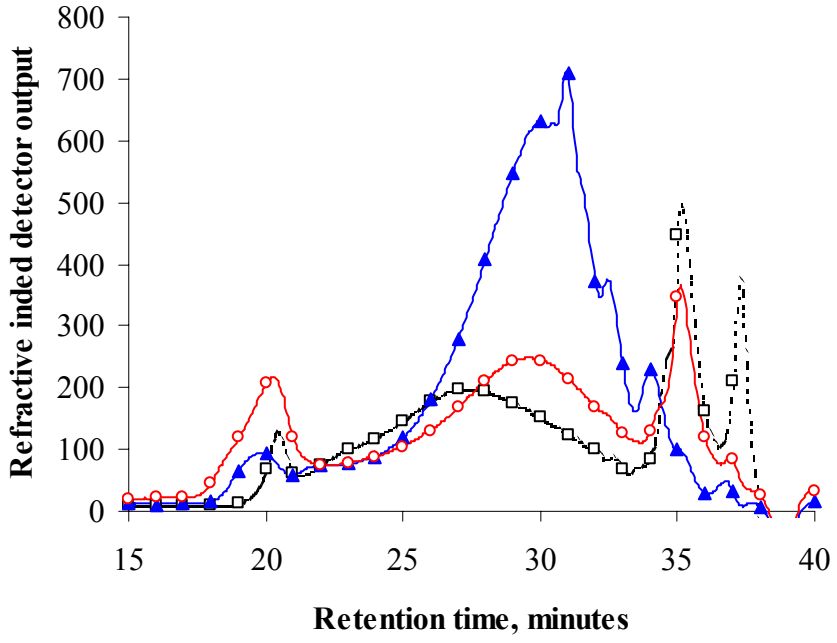

Figure 4.1-8b. *Determination of the molecular weight in underivatised condensed tannins from immature leaves of* Flemingia macrophylla *(□),* Leucaena pallida *(○) and* Calliandra calothyrsus *(▲). Solvent: dimethylformamide; flow rate: 0.5 ml min^{-1}; detector: refractive index; columns: two PLGel 5 m 500 (300 x 7.5 mm) in series.*

Figures 4.1-8a and 4.1-8b depict the chromatograms obtained during the determination of the molecular weight of underivatised condensed tannins. As for the peracetate derivatives, these chromatograms illustrate the considerable diversity in the distribution of molecular weight among the studied tannins. However, a greater number of tannin fractions (differing in molecular weight) were observed in the chromatograms of intact condensed tannins (Figures 4.1-8a and 4.1-8b) as compared with those from their peracetate derivatives (Figures 4.1-5a and 4.1-5b). Indeed, with the exception of tannins extracted from *L. leucocephala* and *Tadehagi sp*., discrete peaks with retention times of around 20.4 min (more than 30000 Dalton) were evident in tannins from all legumes (see Figures 4.1-8a and 4.1-8b) which were not evident in the chromatograms of the peracetates.

4.1.4 DISCUSSION

Considerable variation was observed in the structural features of the condensed tannins analysed in this study. Indeed, *number-average* molecular weight ranged from 2300 to 6400 and PC-PD-PP ratio from 94: 0: 2 to 9: 91: 0 Other researchers, primarily dealing with tannins extracted from temperate sources, have also reported great variation among tannins from different plant species. For example, using ^{13}C NMR, Foo and Porter (1980) reported that 38 proanthocyanidin polymers from 14 widely distributed plant families differed greatly in procyanidin-prodelphinidin ratio (100: 0 – 10: 90), *cis*: *trans* ratio (97: 3 - 12: 88) and in *number-average* molecular weight (810 to > 6000). Likewise, tannins extracted from 11 fodder legumes showed great variation in *number-average* molecular weight (2000-4000), D_p values (1.4-2.7), procyanidin-prodelphinidin ratio (0: 100 - 100: 0) and *cis*: *trans* ratio (55: 45 - 100: 0; Foo et al. 1982). In turn, Williams et al. (1983) reported that the *number-average* molecular weight of the peracetate derivatives of condensed tannins from 32 samples ranged from 1620 to 5500 and that D_p values ranged from 1.14 to 44.0.

Although the condensed tannins examined in the present study contrasted greatly both in molecular weight and in PC-PD-PP ratio, the variation observed for this latter trait was, by far, the most extensive. However, it is evident that the potential variability in composition of condensed tannins is even much greater. For example, in this study the definition of procyanidin includes both catechin and epicatechin, whereas that of prodelphinidin includes both gallocatechin and epigallocatechin. Thus, with the use of

other HPLC-based techniques such as the one proposed by Koupai-Abzayani (1992) and Foo et al (1997), based on the analysis of phloroglucinol addition products, far more detailed information of the proportion of constituent flavan-3-ols could be obtained. Nonetheless, ii must be also acknowledged that the protein-precipitating capacity of tannins is likely to be influenced not only by the proportion of their constituent flavan-3-ols, but also by also by the spatial arrangement of these constituents within the tannin molecule and the configuration of the tannin molecule in aqueous solutions. This illustrates the difficulties researchers face when trying to delineate the impact of anthocyanidin composition on tannin activity. In some studies, the prodelphinidin content of condensed tannins has been associated with tannin astringency (Jones et al. 1976) and tannin antiherbivore activity (Ayres et al. 1997). On the other hand, differences in prodelphinidin content in tannins from *Lotus pedunculatus* and *Lotus corniculatus* had little or no effect on their binding to Rubisco (McNabb et al. 1998).

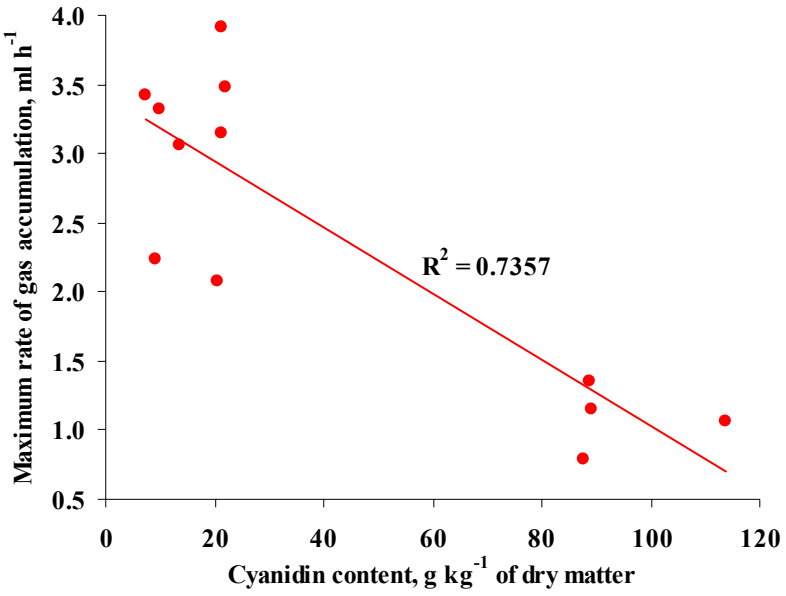

Figure 4.1-9. *The relationship between total content of cyanidin (g kg-1 of dry matter) and the maximum rate of gas production observed during the fermentation of mature and mature leaf samples of six tanniniferous tropical legumes (Chapter 3).*

In an attempt to assess the role played by anthocyanidin composition on the nutritional quality of tanniniferous legumes, the content of extractable, bound and total anthocyanidins was calculated for all legumes and related by linear regression to the forage quality data obtained in Chapter 3. Results showed that total and

extractable procyanidin content (g kg^{-1} of dry matter) was negatively related to gas accumulation at 12 and 24 hours ($P < 0.05$) and to the maximum rate of gas production ($P < 0.001$, see Figure 4.1-9). In turn, the maximum rate of gas production was positively related with extractable, bound and total prodelphinidin content ($P < 0.02$). Finally, content of propelargonidin (extractable, bound and total) was positively related to the concentration of iso-butyrate and iso-valerate observed at the end of the gas production experiments described in Chapter Three ($P < 0.05$). From these observations, is evident that the influence of flavan-3-ol composition on the nutritional effects of condensed tannins can be very complex. Thus, caution must be exercised in interpreting these results since it is evident that a greater number of data points are needed before broad generalisations can be made.

Another observation that deserves some consideration is that, in most legumes, tannin fractions differing in solvent-extractability (methanol and acetone extractable and non-extractable tannins, Section 4.1.2.2) also differed in PC-PD-PP ratios. Remarkably, in all prodelphinidin-containing legumes, examination of the data in Figures 4.1-2 (a-c) shows that the proportion of prodelphinidin in the PC-PD-PP ratio was lowest in tannins in the methanol extracts, intermediate in those in the acetone extracts and highest in the non-extractable tannins. The question then arises as to whether observed differences in extractability of condensed tannins were due to differences in anthocyanidin composition. Since differences in flavan-3-ol composition could determine the strength of the interactions between tannins and other molecules (Clausen et al. 1990), this appears to be a very strong possibility. Another possibility (not explored in this study) is for tannins in these three fractions to also differ in thei molecular weight, as observed in the study of Oh and Hoff (1979). This appears to be a certainty in the case of the extracts, as the majority of the small phenolic compounds should be extracted by the 70% methanol treatment. From our data it remains unclear how different combinations of PC-PD-PP ratios and molecular weights can influence the extractability of tannins into a given solvent.

The estimates of tannin molecular weight obtained in Sections 4.1.2.3 and 4.1.2.4 were also related via linear regression equations to the forage quality data obtained in Chapter Three. In general, increases in all estimates of molecular weight were negatively related to the concentration of acetate, n-valerate and total VFA, to the

accumulation of gas, especially at 24 and 48 hours and *in vitro* dry matter digestibility estimates obtained by the Tilley and Terry (1963) and pressure transducer techniques. However, stronger relationships were observed for the molecular weight estimates of underivatised tannins than for those of the peracetate derivatives. In turn, among different molecular weight estimates, the strongest relationships were observed for the *z-average* molecular weight. Then, *z-average* molecular weight of underivatised tannins was negatively related to NSP digestibility, to acetate, propionate, n-valerate and to total VFA concentration, to gas accumulation at 24, 48 and 144 hours and to *in vitro* dry matter digestibility (R^2 = 0.51 - 0.75; P ≤ 0.01, see Figure 4.1-10). Surprisingly, estimates of tannin molecular weight were not significantly related to maximum rate of gas production. On the other hand, both procyanidin and prodelphinidin content were significantly related to this parameter. Once again, this illustrates the complexity of the relationships between tannin structure and tannin activity. More research is needed to confirm and to extend the scope of these results, in order to elucidate the relative impact of particular structural features on the nutritional effects of condensed tannins.

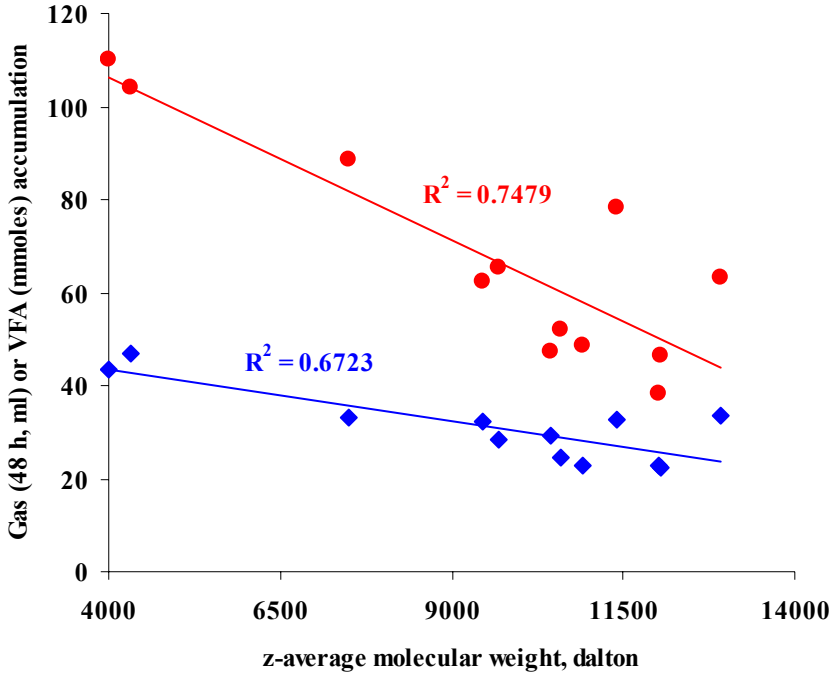

Figure 4.1-10. *The relationship between the z-average molecular weight of condensed tannin (dalton) with volume of gas production at 48 h (●) and total volatile fatty acid (VFA) accumulation (◆) during the fermentation of mature and mature leaf samples of six tanniniferous tropical legumes (Chapter 3).*

Finally, comment is needed to compare the two techniques employed in this study for the determination of the molecular weight of condensed tannins. As was discussed earlier, there are problems associated with the determination of molecular weight in peracetate derivatives of condensed tannins. These include the handling of pyridine and acetic anhydride during the derivatisation and the facts that the resulting peracetates are distant from the form tannins are found in nature. Because of differences in the degree of hydroxylation among the different flavan-3-ols, a further problem arises in that the molecular weights of the corresponding peracetates varies widely, ranging from 380 to 557 Dalton (see Table 2-13). However, it is a common practice to consider that the molecular weight of a constituent peracetate unit is 500 Dalton. Given the differences observed in the PC-PD-PP ratios on condensed tannins (such as the one observed in this study), this could lead to under and over-estimation of the average number of units constituting the tannin molecule. Development in GPC column techniques now make it possible to estimate tannin molecular weight in underivatised condensed tannins by the use of GPC. This is the first example of this.

Data on the molecular weight of the peracetate derivatives reported in Table 4-2 was not corrected by the extra weight added by the acetate residues, although such calculations were possible given the knowledge of the PC-PD-PP ratio is these samples. As a result, estimations of molecular weight in the underivatised samples were only 84% of those obtained for the peracetate derivatives, although it was also observed the determination made by both techniques were highly correlated. If differences between techniques were only due to the presence of added acetates, then estimations of molecular weight in underivatised tannins should be around 55% of those obtained for the peracetates.

It is difficult to establish were these differences reside, but when both techniques are compared, the determination of molecular weight in underivatised tannins appears to be more attractive. Firstly, there is no sample preparation beyond that of tannin purification. As a consequence, samples are analysed in the conformation and in the form that one would expect to find them in nature. Secondly, the data obtained does not have to be corrected for the presence of non-tannin moieties, which makes this measurement independent of other determinations (i.e. PC-PD-PP ratio). Thirdly, far greater detail on the distribution of molecular weights is obtained by the use of the

dimethylformamide technique. Additionally, although estimations of molecular weight by both methods were found very useful in explaining differences in forage quality, the molecular weight of underivatised tannins was a better predictor of forage quality.

One advantage of the peracetate technique over the dimethylformamide technique is its shorter run time. This difficulty arises due to the high viscosity of dimethylformamide and it could be overcome by the use of a column oven. This would allow to run analyses at 70°C, reducing system operating pressure and allowing higher flow rates, effectively decreasing run time.

4.2 EFFECT OF CONDENSED TANNINS FROM TROPICAL LEGUMES ON THE FERMENTATION OF *FESTUCA ARUNDINACEA* PLANT CELL WALLS: THE RELATIVE NUTRITIONAL IMPLICATIONS OF SUBSTRATE-ASSOCIATED AND SOLUBLE CONDENSED TANNINS

1.0.0 INTRODUCTION

Condensed tannins are widely distributed in higher plants and occur at high levels in various feeds and foods. The most widely recognised property of condensed tannins is their ability to bind strongly and selectively to proteins and other macromolecules. Since the work of Terrill et al. (1992b), condensed tannins in plant tissue have been classified as extractable (those that are easily extracted from plant tissue by aqueous organic solvents) or bound (those that remain bound to cell protein and/or carbohydrate components of the cell wall after such extraction). Clearly, tannins in these fractions differ in solubility as a result of their interaction with other plant tissue components and could also differ in their localisation within plant tissue. Hence, although based on an arbitrary criterion, the classification of tannins as extractable or bound leads us to speculate about the functional meaning of this categorisation. For example, it could be expected that condensed tannins in solution would mediate their effects by acting directly on rumen micro-organisms and their enzymes whereas those bound to plant tissue should exert their effects by affecting substrate availability.

Research carried out on tanniniferous tropical legumes has pointed to large differences on the extractability of condensed tannins in 70% aqueous acetone (Jackson et al., 1996a; Chapter three this thesis). Relatively little is known on how these differences in extractability could relate to the nutritional quality of tanniniferous forage legumes. However, it is generally believed that most of the anti-quality effects of tannins in forages are mediated via tannins that are in solution. Hence, a common practice in nutritional studies involving condensed tannins is to use polyethylene glycol to reduce the concentration of acetone-extractable condensed tannins (Barry and Duncan, 1984; Waghorn et al., 1987; Pritchard et al., 1988; McNabb et al., 1993, Carulla, 1994, Makkar et al., 1995b; Barahona et al., 1997). Improved understanding of how different levels of extractable condensed tannins affect ruminant nutrition has been gained through this research. Unfortunately, the

experimental approach used in these experiments has not permitted an assessment of the effect of extractable and bound condensed tannins on the nutritive value of tanniniferous feeds.

The pressure transducer technique of Theodorou et al. (1994) is an ideal system in which to investigate the relationships between the rumen microbial environment and forage components such as condensed tannins. In the present study, this technique was used to establish whether substrate-associated and condensed tannins in solution at different concentrations and from various sources differ in their relative impact on the degradation of substrates.

4.2.1 MATERIALS AND METHODS

4.2.1.1 Isolation of condensed tannins

The condensed tannins used in the experiments reported in this chapter were isolated from mature and immature foliar leaf samples of *Desmodium ovalifolium, Flemingia macrophylla, Leucaena leucocephala, Leucaena pallida, Calliandra calothyrsus* and *Clitoria fairchildiana* as described in Section 4.1.2.1.

4.2.1.2 Growth of *Festuca arundinacea* plant cells

For use in these experiments, non-embryogenic lignin-free cell suspension cultures from *Festuca arundinacea* were grown in cell culture as described by Dalton (1993). In short, cell cultures of *F. arundinacea* (tall fescue, suspension line 93BNI) were grown in M133 medium containing Murashige and Skoog basal plant medium (4.71 g l^{-1}), glucose (30.0 g l^{-1}) and 2,4-D (3 mg l^{-1}) in 2-l flasks containing one l of medium. Cell lines were maintained by sampling at a dilution rate of 1:5 in 250-ml flasks containing 100 ml of medium and grown under the same conditions. Flasks were kept at 25 ºC and shaken at 150 rpm for 28 days. Cells were harvested by filtration through a double layer of Mira cloth and then freeze-dried. To eliminate water-soluble components and starch, freeze-dried plant cells were re-suspended in water for 2 h with continuous stirring, re-filtered through Mira cloth and then freeze-dried to constant weight. Recovered cells, which will be referred to as plant cell walls for the remainder of this thesis, were stored in the dark at room temperature until used.

4.2.1.3 Determination of the binding affinity of condensed tannins for *Festuca arundinacea* plant cell walls

The binding affinity of condensed tannins from all legumes for *F. arundinacea* plant cell walls was determined by treating 25 mg of cell walls (previously re-hydrated with 1 ml of water) with 0, 0.25, 0.5, 1, 1.75 and 2.5 mg of condensed tannins dissolved in 0.95 ml of 50% aqueous methanol. After shaking for 2 h, 4.05 ml of distilled water was added and samples were allowed to further react overnight at 4°C. The next morning, samples were centrifuged (2000 rpm for 5 minutes) and aliquots (1-ml) of the supernatant were obtained. The remaining sample was then filtered under vacuum through one layer of Mira cloth with generous water washings. The condensed tannin-plant cell wall complexes were then freeze-dried to constant weight. The amount of condensed tannins present both in the complexes and in the supernatant was determined by the method of Terrill et al. (1992b). Substrate-associated tannins were determined by boiling (95°C for 1 h) duplicate 10-mg samples of the complexes suspended in 4 ml of butanol-HCl (95:5 v/v). Condensed tannins in the supernatant were determined by mixing duplicate 0.5-ml aliquots of the supernatant with 3.5 ml of butanol-HCl (after centrifugation of the supernatant at 10,000 rpm) and hydrolysing at 95°C for 1 h. Standards curves (see Section 3.2.2) were used to calculate the tannin content of the different samples.

4.2.1.4 Effect of different concentrations of condensed tannins from *Desmodium ovalifolium* on the fermentation of D-glucose and *Festuca arundinacea* cell walls

Experiments reported in this Section were made to establish whether condensed tannins free in solution behave in a significantly different manner to those attached (hence immobile) to the grass cell wall substrate. To this end, three different gas production experiments were conducted using the pressure transducer technique of Theodorou et al. (1994). In these pressure transducer experiments, samples were analysed in triplicate using 75-ml capacity serum bottles, each receiving 42.5 of digestion medium, 2 ml of reducing solution and 5 ml of rumen fluid inoculum. The purified condensed tannins used in these pressure transducer experiments were extracted from the mature leaves of *D. ovalifolium.*

Experiment One comprised of two independent gas production assays and was conducted with the aim of determining the effect of the presence of different concentrations of condensed tannins in solution on the fermentation of D-glucose by rumen micro-organisms. In both gas production assays, the main carbon source present in the digestion medium was D-glucose. Media were prepared so that, upon mixing of all constituent solutions, the final concentration of D-glucose was 0.25 g l^{-1} of medium. In the first assay, 0.5 ml of stock solutions containing condensed tannins dissolved in 50% aqueous methanol were added to serum bottles so as to obtain final concentrations of 0, 100, 200, 400, 800 and 2000 g of condensed tannins kg^{-1} of D-glucose (i.e. 0, 25, 50, 100, 200 and 500 mg of tannin l^{-1} of growth media). Samples were then incubated at 39°C for 94 h with gas readings taken at 0.5, 1.5, 3, 7, 7, 9, 23, 30, 50 and 94 h post inoculation. For the second assay, different concentrations (0, 200, 400, 800, 1400 and 2000 g kg^{-1} of D-glucose) of condensed tannins in solution were incubated for 30 h and gas readings were taken at 3, 6, 9, 12, 23 and 30 h post inoculation.

A second experiment was conducted to determine the effect of different concentrations of substrate-associated tannins on the fermentation of plant cell walls of *F. arundinacea*. For this experiment, condensed tannin-cell wall complexes of varying tannin content were prepared as described in Section 4.2.2.3. In short, triplicate 250-mg samples of *F. arundinacea* cell walls were treated with 0, 1.6, 3.1, 6.2, 12.4, 18.6 and 31 mg of condensed tannins from *D. ovalifolium*. The level of binding obtained ranged between 0 to 60.0 g of substrate-associated condensed tannins kg^{-1} of plant cells (dry matter basis). For the gas production assay, samples of varying weight (100-200 mg) of the condensed tannin-cell wall complexes were incubated at 39°C for a period of 120 h. Gas measurements were taken at 3, 6, 9, 12, 24, 36, 48, 72 and 120 h post-inoculation.

In a third experiment, the effect of different concentrations of condensed tannins in solution on the fermentation of plant cell walls from *F. arundinacea* was investigated. To this end, 0.5 ml of stock solutions containing condensed tannins from *D. ovalifolium* dissolved in 50% aqueous methanol were added to serum bottles containing 200 mg of freeze-dried plant cell walls. The concentrations of condensed tannins in solution were 0, 6.3, 12.5, 25, 50, 87.5 and 125 g kg-1 of cell wall dry

matter. In this gas production assay, samples were incubated for a period of 144 h with gas readings taken at 3, 6, 9, 12, 24, 36, 48, 72, 96 and 144 h post-inoculation.

4.2.1.5 Comparison of the effects of condensed tannins from different tropical legumes on the fermentation of D-glucose and of *Festuca arundinacea* cell walls

An additional set of experiments was conducted with the aim of establishing structure-activity relationships for the purified condensed tannins from the six legumes listed in Section 4.2.2.1. These experiments used the pressure transducer technique of Theodorou et al. (1994) and were concerned with substrate-associated condensed tannins and those in solution and their impact on the fermentation of different substrates by rumen micro-organisms. Relevant information regarding the structures of the condensed tannins was obtained from the experiments reported in Chapter 5.1.

Experiment four consisted of two separate gas production assays, whereby 0.5 ml of solutions containing purified condensed tannins from the six species listed above dissolved in 50% aqueous methanol were added to 75-ml serum bottles containing D-glucose as the main carbon source. The final concentration obtained for all condensed tannins was 1400 g of tannins in solution kg^{-1} of D-glucose (350 mg/l of fermentation media). In both assays, the incubation period was of 48 h and gas measurements were taken at 3, 6, 9, 12, 24, 36 and 48 h post-inoculation. The final concentration of D-glucose was 0.25 g l^{-1} of culture media.

In experiment five, condensed tannin-cell wall complexes were prepared as previously described (see Section 4.2.2.3). Samples (1.5 g) of *F. arundinacea* plant cell walls were treated with the appropriate amounts of condensed tannins from all legumes, so that the resulting complexes contained *ca* 30 g of condensed tannin kg^{-1} of plant cell wall dry matter. The actual level of binding ranged from 28.6 to 32.3 g of condensed tannin kg^{-1} of plant cell dry matter. In two separate gas production assays, 200-mg samples of the resulting condensed tannin-cell wall complexes were incubated for a period of 48 h with gas readings taken at 3, 6, 9, 12, 24, 36 and 48 h post-inoculation.

4.2.1.6 Additional analyses

For all gas production assays with the exception of the first assay in experiment one, the concentration of volatile fatty acids (VFA) in the supernatant was determined by gas chromatography. A Chrompack CP 9000 chromatograph fitted with an automatic sampler (Chrompack 911) and a flame-ionization detector, linked to a Dell PC with Dionex A1-450 integration software, was used for VFA quantification. Additionally, the disappearance of dry matter after fermentation was measured for gas production assays that comprised experiments two, three and five. At the end of the incubation period in these experiments, the contents of each serum bottle were filtered through pre-weighed sintered glass funnels and the residue freeze-dried to constant weight. Dry matter loss was calculated as the difference between the dry matter weight of the sample pre- and post-fermentation.

4.2.1.7 Statistical analyses

Gas production data were fitted to the model of France *et al*. (1993) using the MLP (Ross, 1987) package. The equation is in the form, $Y = A\{1 - e^{[-b(t-T) - c(\sqrt{t}-\sqrt{T})]}\}$ where Y is the cumulative gas production (ml), A is the asymptote (i.e. gas pool), T is lag time, and b (h^{-1}) and c ($h^{-0.5}$) are decay rate constants. A combined fractional rate (h^{-1}) of gas production (μ) was calculated as, $\mu = b + c/2\sqrt{t}$, where t is the incubation time (h).

Simple linear regressions were employed to investigate the relationships between concentration of tannins and both gas accumulation and end-point concentration of VFA in experiments one, two and three. The relationships between amount of substrate-associated tannins and dry matter recovery (experiment two) and between tannin structural features (molecular weight, anthocyanidin ratio) and gas and VFA accumulation (experiments four and five) were also investigated by simple linear regressions. Data obtained in experiments four and five was analysed as a completely randomised design with tannin source and maturity as the main factors using the analysis of variance (ANOVA) procedure of SAS (1989). Duncan's Multiple Range Test at alpha level of 0.05 was used to compare means.

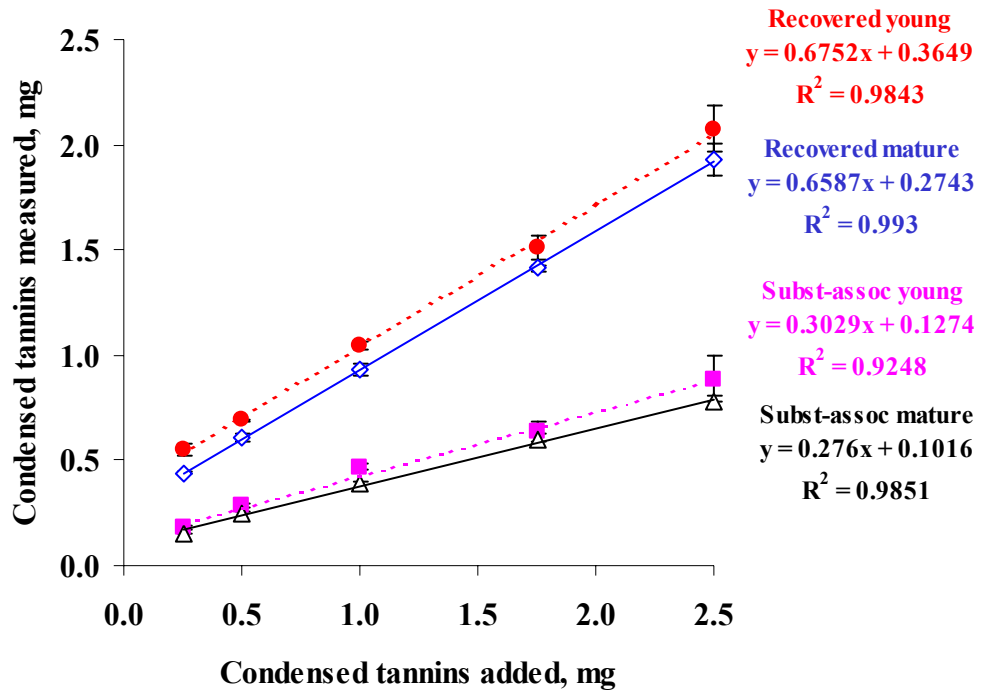

Figure 4.2-1a. *Binding of purified condensed tannins from* Desmodium ovalifolium *to plant cell walls from* Festuca arundinacea*. Upper lines depict the recovery (i.e. tannins in the supernatant plus those attached to the cell walls) of condensed tannins extracted from young (●) and mature (◊) leaves. Lower lines show the amount of substrate-associated tannins from young (■) and mature (Δ) leaves.*

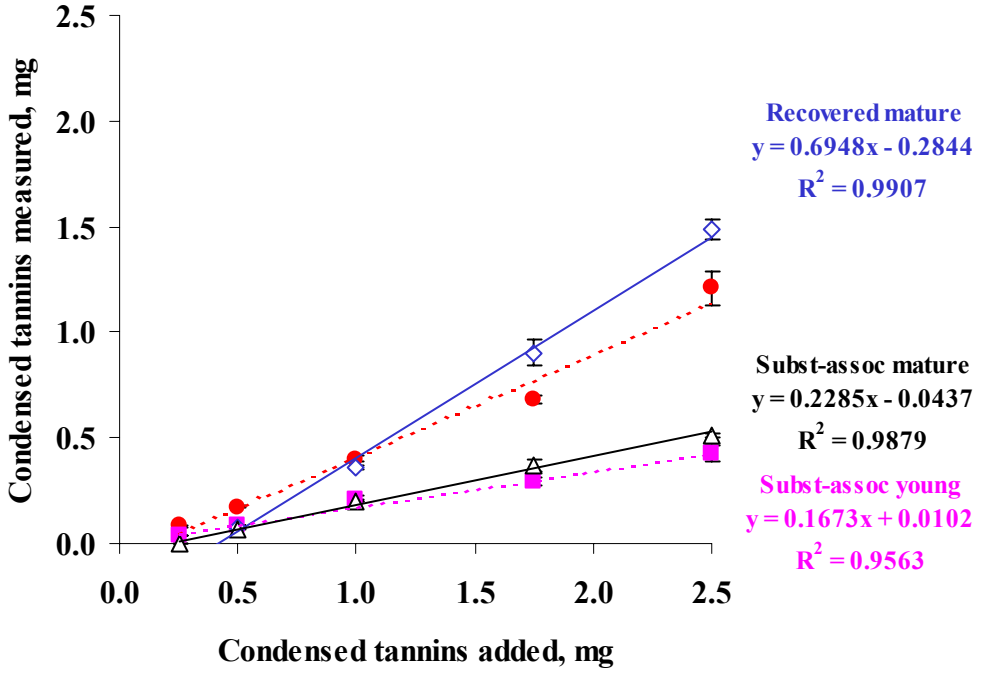

Figure 4.2-1b. *Binding of purified condensed tannins from* Flemingia macrophylla *to plant cell walls from* Festuca arundinacea*. Upper lines depict the recovery (i.e. tannins in the supernatant plus those attached to the cell walls) of condensed tannins extracted from young (●) and mature (◊) leaves. Lower lines show the amount of substrate-associated tannins from young (■) and mature (Δ) leaves.*

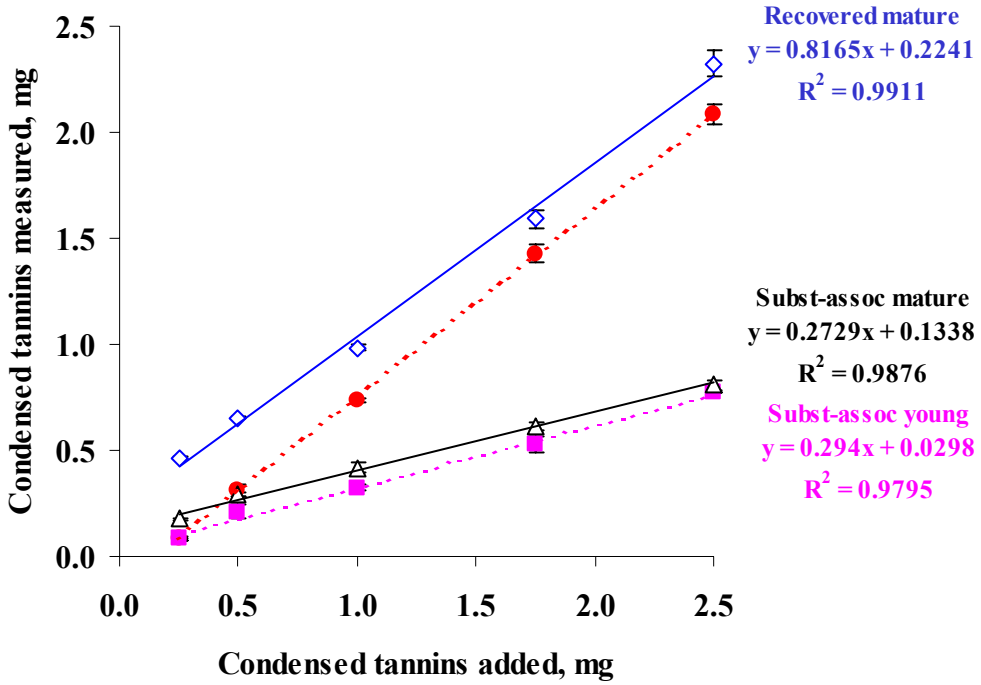

Figure 4.2-1c. *Binding of purified condensed tannins from* Leucaena leucocephala *to plant cell walls from* Festuca arundinacea*. Upper lines depict the recovery (i.e. tannins in the supernatant plus those attached to the cell walls) of condensed tannins extracted from young (●) and mature (◊) leaves. Lower lines show the amount of substrate-associated tannins from young (■) and mature (Δ) leaves.*

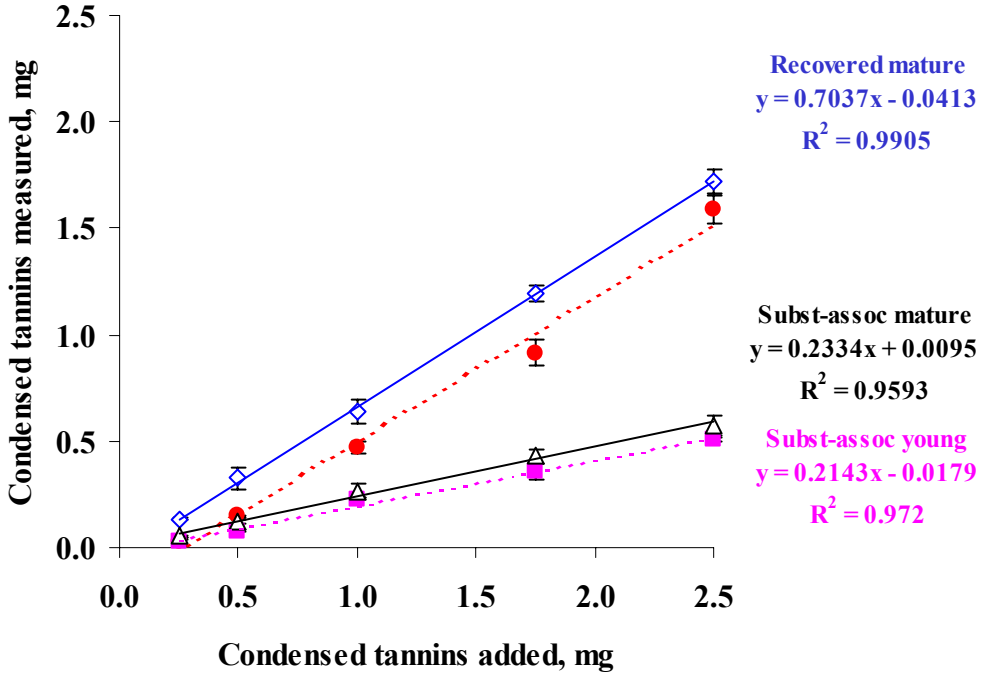

Figure 4.2-1d. *Binding of purified condensed tannins from* Leucaena pallida *to plant cell walls from* Festuca arundinacea*. Upper lines depict the recovery (i.e. tannins in the supernatant plus those attached to the cell walls) of condensed tannins extracted from young (●) and mature (◊) leaves. Lower lines show the amount of substrate-associated tannins from young (■) and mature (Δ) leaves.*

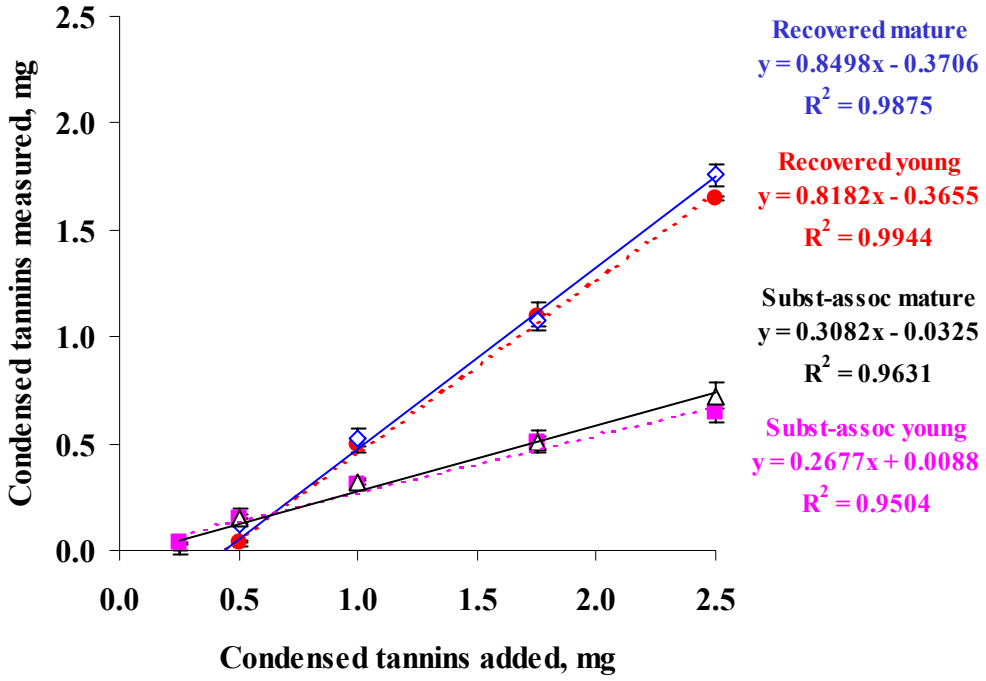

Figure 4.2-1e. *Binding of purified condensed tannins from* Calliandra calothyrsus *to plant cell walls from* Festuca arundinacea. *Upper lines depict the recovery (i.e. tannins in the supernatant plus those attached to the cell walls) of condensed tannins extracted from young (●) and mature (◊) leaves. Lower lines show the amount of substrate-associated tannins from young (■) and mature (Δ) leaves.*

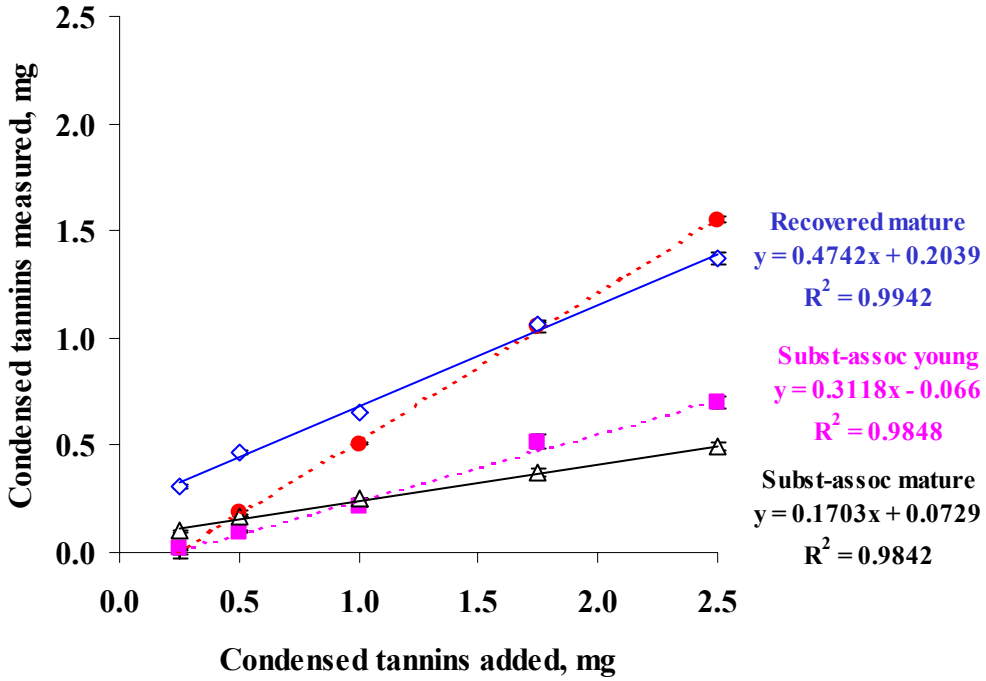

Figure 4.2-1f. *Binding of purified condensed tannins from* Clitoria fairchildiana *to plant cell walls from* Festuca arundinacea. *Upper lines depict the recovery (i.e. tannins in the supernatant plus those attached to the cell walls) of condensed tannins extracted from young (●) and mature (◊) leaves. Lower lines show the amount of substrate-associated tannins from young (■) and mature (Δ) leaves.*

4.2.2 RESULTS

4.2.2.1 Binding of condensed tannins to *Festuca arundinacea* cell walls

The binding of condensed tannins extracted from mature and immature leaves of tropical legumes is shown in Figures 4.2-1 (a-f). As these figures demonstrate, there were considerable differences in the binding of condensed tannins to the cell walls among the legumes tested in this study. For example, when 2.5 mg of condensed tannins were added to the cell walls, the highest level of binding was observed with tannins from *D. ovalifolium* (0.83 mg; Figure 4.2-1a) and *L. leucocephala* (0.79 mg; Figure 4.2-1c), whereas the lowest occurred with tannins from *F. macrophylla* (0.47 mg; Figure 4.2-1b). In general, the binding of condensed tannins to cell walls appeared not to be affected by the maturity of the leaves from which the condensed tannins were extracted. Furthermore, results from this experiment were not significantly related to the molecular weight and anthocyanidin ratio of the condensed tannins studied.

Figure 4.2-1 (a-f) also shows values on the recovery of condensed tannins in all binding experiments. The recovery of condensed tannins was estimated as the summation of the amount of tannins in the centrifuged supernatant and of those associated with the cell walls. As an example, when 2.5 mg of condensed tannins were added, average recovery values ranged from 1.35 mg with condensed tannins from *F. macrophylla* and 2.2 with those from *L. leucocephala*. On the other hand, when 0.25 mg of tannins were added, recovery ranged from –0.20 mg with tannins from *C. calothyrsus* to 0.49 mg with tannins from *D. ovalifolium*.

4.2.2.2 Gas accumulation as affected by different concentrations of soluble and substrate-associated condensed tannins from *Desmodium ovalifolium*

With comparisons made on the basis of tannin to substrate ratio, substrate-associated condensed tannins were more effective at inhibiting substrate fermentation by rumen micro-organisms than condensed tannins that were added in soluble form (see Figure 4.2-2). Indeed, concentrations of 60 g of substrate-associated tannins kg^{-1} of cell walls caused a of 34 % reduction in gas accumulation at 48 h of fermentation. By contrast, 1000 g of condensed tannins in solution kg^{-1} of D-glucose was required to observe approximately that same reduction in gas accumulation. Moreover, in experiment

three, the addition of condensed tannins in solution at concentrations up to 125 g per kg of cell walls resulted in an increase in gas accumulation.

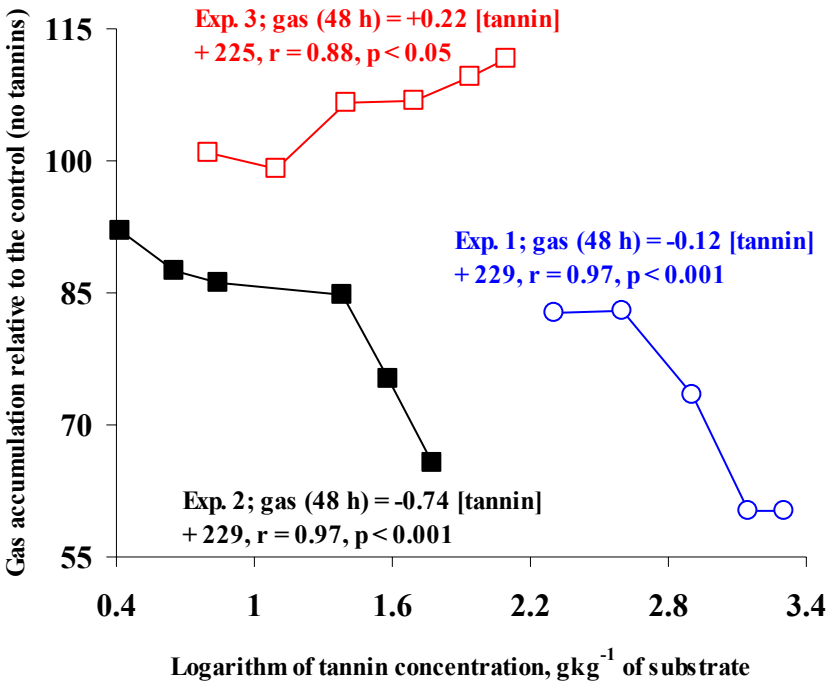

Figure 4.2-2. *Gas accumulation after 48 h during the fermentation of D-glucose and cell walls from* Festuca arundinacea *in the presence of purified condensed tannins from* Desmodium ovalifolium*. Exp. 1 (O) = Experiment one: tannins in solution and glucose as substrate; Exp. 2 (■) = Experiment two: substrate-associated tannins and plant cell walls as substrate; Exp. 3 (□) = Experiment three: tannins in solution and plants cell walls as substrate.*

In experiment one, accumulation of gas production after 48 h of incubation was inversely related to the concentration of condensed tannins in solution, both in the first ($r = 0.86$; $P < 0.001$; data not shown) and the second ($r = 0.97$; $P < 0.001$; Figure 4.2-2) gas production assays. Indeed, the gas pool at 48 h (average of the two gas production assays) was 527 ml for the control treatment (zero tannin) and only 286 ml for the highest tannin treatment. Similarly, in experiment two, an inverse relationship was observed between the concentration of substrate-associated condensed tannins and gas accumulation ($r = 0.96$; $P < 0.001$; Figure 4.2-2). In this experiment, gas pool at 48 h was 237 ml for the control (zero tannin) and 186 ml for the highest tannin treatment. On the other hand, in experiment three, a positive relationship ($r = 0.88$; $P < 0.05$) was observed between the concentration of condensed tannins in solution and gas accumulation. The gas pool after 48 h of incubation was 225 ml for the control treatment and 256 ml for the highest tannin treatment.

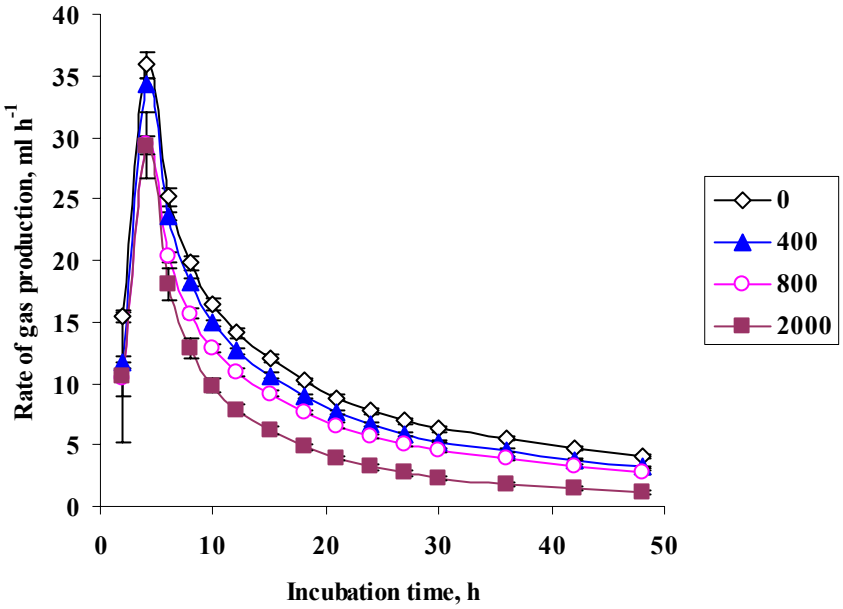

Figure 4.2-3a. *Rate of gas production (ml h^{-1}) during the fermentation of D- glucose by rumen micro-organisms in the presence of contrasting concentrations of condensed tannins in solution from* Desmodium ovalifolium*. For the sake of clarity, curves obtained by the addition of 200 and 1400 g of condensed tannins in solution kg^{-1} of D-glucose were excluded from this figure.*

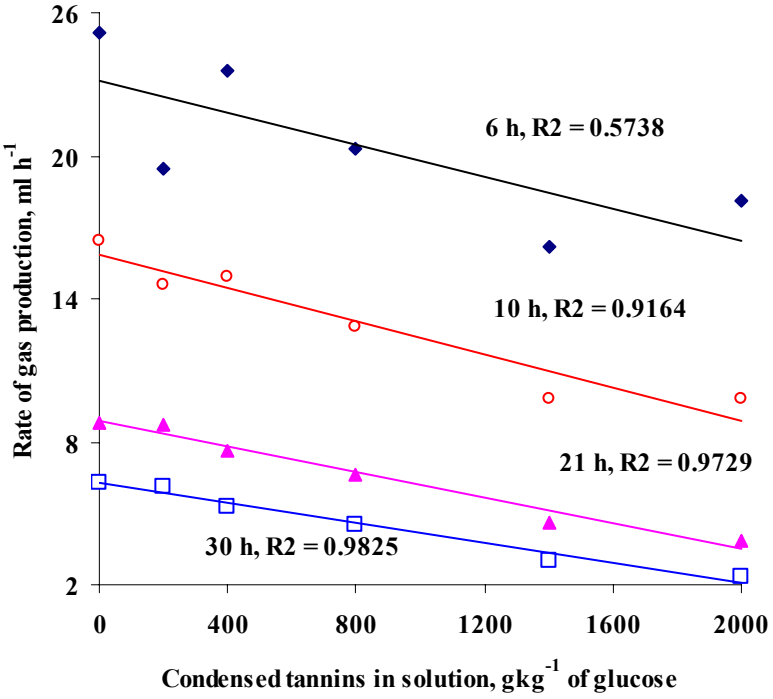

Figure 4.2-3b. *The relationship between the rate of gas production (ml h^{-1}) and the concentration of condensed tannins in solution from* Desmodium ovalifolium *at different times during the fermentation of D- glucose by rumen micro-organisms.*

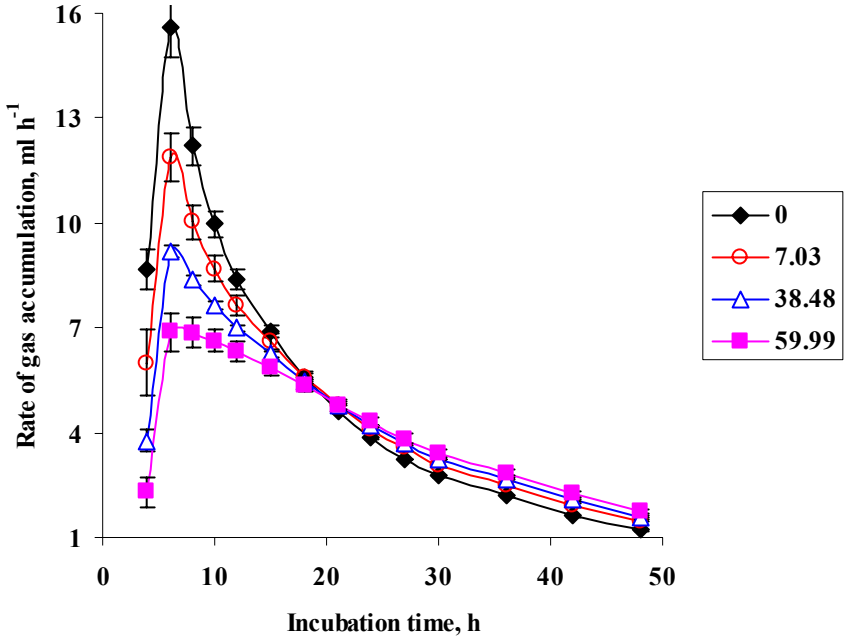

Figure 4.2-4a. *Rate of gas production (ml h^{-1}) during the fermentation of* Festuca arundinacea *cell walls by rumen micro-organisms in the presence of contrasting concentrations of substrate-associated condensed tannins from* Desmodium ovalifolium. *For the sake of clarity, curves obtained by the addition of 2.64, 4.51 and 24.01 g of substrate-associated condensed tannins kg^{-1} of plant cell walls were excluded from this figure.*

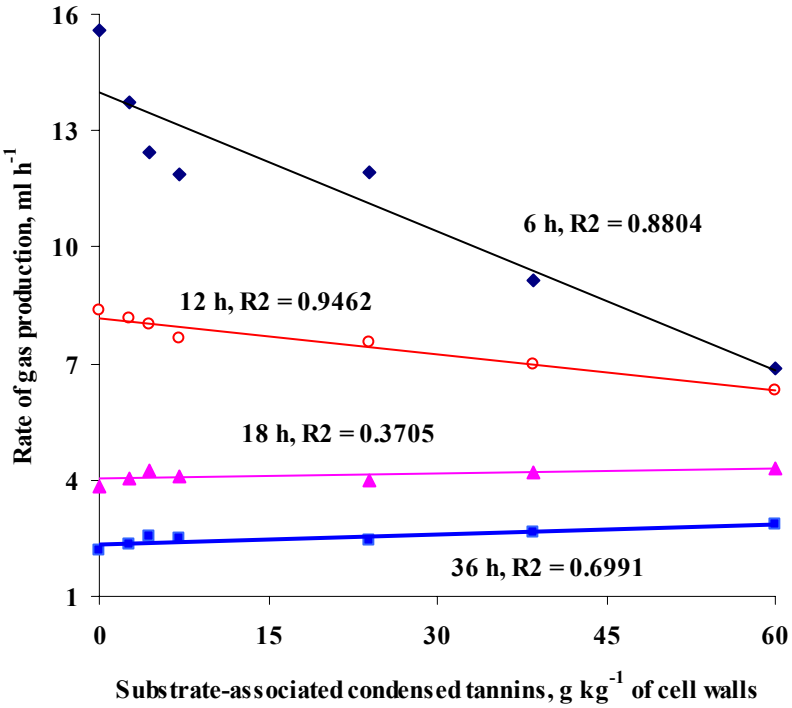

Figure 4.2-4b. *The relationship between the rate of gas production (ml h^{-1}) and the concentration of substrate-associated condensed tannins from* Desmodium ovalifolium *at different times during the fermentation of* Festuca arundinacea *cell walls by rumen microorganisms.*

Table 4.2-1. *Effect of the presence of varying concentrations of condensed tannins in solution from* Desmodium ovalifolium *on the concentration of volatile fatty acid (VFA) derived from the fermentation of D-glucose by rumen micro-organisms (Experiment One, second run)*

Added tannins, g kg^{-1} of D-glucose	MMoles per litre[1]						
	Total VFA	Straight chain VFA				Branched chain VFA	
		Acetate	Propionate	Butyrate	Valerate	Iso-butyrate	Iso-valerate
0 (control)	473.2	315.6	97.6	37.2	0.0	7.4	15.2
200	417.6	287.2	88.8	31.6	0.0	7.0	12.0
400	427.2	292.0	91.2	32.4	0.0	6.0	10.2
800	377.6	258.4	80.8	28.4	0.0	5.3	8.4
1400	418.4	288.4	89.6	31.2	0.0	0.0	8.0
2000	379.2	264.4	85.6	29.2	0.0	0.0	0.0
Linear regression relationships between volatile fatty acid components (Y) and concentration of condensed tannins in solution (SCT)							
	Total VFA	*Acetate*	*Propionate*	*Butyrate*	*Valerate*	*Iso-butyrate*	*Iso-valerate*
Slope	-0.032SCT	-0.017SCT	-0.004SCT	-0.003SCT		-0.004SCT	-0.006SCT
Intercept	441.1	298.3	92.0	33.7		7.7	14.0
R^2	0.48	0.42	0.27	0.42		0.92	0.89
P value	0.065	0.090	0.171	0.078		0.024	0.031

[1]After 30 h of incubation, values corrected for blank (containing only fermentation media and rumen fluid).

Table 4.2-2. *Effect of the presence of varying concentrations of substrate-associated condensed tannins from* Desmodium ovalifolium *on the concentration of volatile fatty acid (VFA) derived from the fermentation of* Festuca arundinacea *cell walls by rumen micro-organisms (Experiment Two).*

Added tannins, g kg^{-1} of Cell walls	MMoles per litre[1]						
	Total VFA	Straight chain VFA				Branched chain VFA	
		Acetate	Propionate	Butyrate	Valerate	Iso-butyrate	Iso-valerate
0 (control)	154.9	95.4	36.5	15.5	2.7	1.8	3.1
2.6	138.1	82.7	33.5	15.1	2.3	1.7	2.8
4.5	135.7	81.4	33.3	14.7	2.2	1.5	2.7
7.0	128.1	73.9	35.2	13.2	1.5	1.7	2.5
24.0	129.6	75.7	32.9	11.8	1.7	1.9	2.8
34.9	112.0	65.6	29.9	11.2	1.4	1.5	2.3
60.0	128.2	76.3	34.0	12.0	1.7	1.5	2.7
Linear regression relationships between volatile fatty acid components (Y) and substrate-associated condensed tannin added (SACT)							
	Total VFA	*Acetate*	*Propionate*	*Butyrate*	*Valerate*	*Iso-butyrate*	*Iso-valerate*
Slope	-0.354SACT	-0.234SACT	-0.037SACT	-0.062SACT	-0.013SACT	-0.002SACT	-0.004SACT
Intercept	138.7	83.2	43.3	14.5	2.2	1.7	2.8
R^2	0.36	0.31	0.17	0.63	0.40	0.10	0.15
P value	0.021	0.026	0.189	0.002	0.010	0.458	0.124

[1]After 120 h of incubation, values corrected for blank (containing only fermentation media and rumen fluid).

Table 4.2-3. *Effect of the presence of varying concentrations of condensed tannins in solution from* Desmodium ovalifolium *on the concentration of volatile fatty acid (VFA) derived from the fermentation of* Festuca arundinacea *cell walls by rumen micro-organisms (Experiment Three).*

Added tannins, g kg^{-1} of Cell walls	MMoles per litre[1]						
	Total VFA	Straight chain VFA				Branched chain VFA	
		Acetate	Propionate	Butyrate	Valerate	Iso-butyrate	Iso-valerate
0 (control)	255.7	160.2	59.5	22.2	4.2	3.5	6.1
6.25	247.8	155.6	57.7	21.6	4.1	3.1	5.8
12.50	269.5	169.4	62.5	23.0	4.3	3.7	6.6
25.00	197.0	122.8	46.9	17.3	2.9	2.8	4.4
50.00	250.9	157.4	59.2	21.8	3.8	3.3	5.4
87.50	265.9	169.2	62.1	22.0	3.9	3.3	5.5
125.00	231.4	149.3	53.5	17.9	3.1	2.9	4.6
Linear regression relationships between volatile fatty acid components (Y) and condensed tannins in solution added (SCT)							
	Total VFA	*Acetate*	*Propionate*	*Butyrate*	*Valerate*	*Iso-butyrate*	*Iso-valerate*
Slope	-0.045SCT	0.005SCT	-0.013SCT	-0.021SCT	-0.006SCT	-0.003SCT	-0.009SCT
Intercept	247.4	154.6	57.9	21.7	4.0	3.3	5.9
R^2	0.007	0.0003	0.011	0.183	0.252	0.147	0.30
P value	0.711	0.854	0.705	0.304	0.173	0.344	0.147

[1]After 144 h of incubation, values corrected for blank (containing only fermentation media and rumen fluid).

As the concentration of condensed tannins in solution increased, there was a corresponding decrease in the rate (ml h^{-1}) of gas accumulation in Experiment One (see Figure 4.2-3a). Notably, the maximum rate of gas production, which in all cases occurred four h post-inoculation, was not significantly related to tannin concentration. On the other hand, the predicted rate of gas production between 6 and 48 h post-inoculation was highly correlated to the concentration of tannins in solution ($P \leq 0.0016$). As demonstrated in Figure 4.2-3b, the presence of condensed tannins in solution reduced the rate of gas accumulation, and this effect remained relatively constant throughout the first 48 h of fermentation.

In turn, the relationship between the concentration of substrate-associated condensed tannins and the rate of gas accumulation during the fermentation of cell walls in experiment two was one of mixed modes. As shown in Figure 4.2-4a, during the first 18 h of fermentation, increased levels of substrate-associated condensed tannins were negatively related to rate of gas production ($P \leq 0.05$). On the other hand, between 24 and 48 h post-inoculation, this relationship became positive ($P \leq 0.05$), with the R^2 of the relationship increasing steadily as the incubation time increased. This behaviour is also illustrated in Figure 4.2-4b, where the rate of gas production is plotted against the concentration of substrate-associated condensed tannins at different points during the fermentation of the cell walls. As can be noted, the value of the slope for this relationship gradually changed from one that was steeply negative during the early stages of fermentation to one that was mildly positive later in the fermentation.

In the second gas production assay of experiment one, the mean concentrations of total and individual VFA observed at the end of the 30-h incubation period were inversely related to the amount of condensed tannins in solution present (Table 4.2-1). Notably, this inverse relationship was only significant for the branched chain VFA (iso-valerate: $r = 0.94$, $P < 0.05$ and iso-butyrate: $r = 0.96$, $P < 0.05$). Similarly, in experiment two an inverse relationship was observed between the concentration of substrate-associated condensed tannins and VFA accumulation by the end of the 144-h fermentation period (Table 4.2-2). On this occasion, however, the relationship between tannin concentration and branched VFA was weak ($P > 0.05$). On the other hand, butyrate, valerate, acetate and total VFA concentrations were significantly reduced by the presence of substrate-associate condensed tannins ($P < 0.05$). In

experiment three, although the relationships between the concentration of condensed tannins in solution and the concentration of VFA were negative, they were non-significant (Table 4.2-3). Remarkably, in all three experiments, the concentration of propionate was not affected by the presence of condensed tannins in any form.

In experiment two, the amount of dry matter recovered at the end of the 120-h incubation period increased as the concentration of substrate-associated condensed tannins increased ($R = 0.91$; $P < 0.001$). A negative relationship was also observed for dry matter recovery and gas accumulation in experiment two ($R = 0.94$; $P < 0.001$). By contrast, in experiment three, the recovery of dry matter was unresponsive to the concentration of soluble condensed tannins.

4.2.2.3 Effect of condensed tannins in solution and substrate-associated condensed tannins from different tropical legumes on the fermentation of D-glucose and *Festuca arundinacea* cell walls by rumen micro-organisms.

In experiment four, after 24 h of fermentation, condensed tannins in solution from the different legumes reduced gas accumulation by at least 16% (20% by 48 h) when compared to the gas accumulation observed for the control treatment (see Figure 4.2-5 a and b). At 48 h, the maximum reduction in gas accumulation (up to 37% relative to the control treatment) was observed with condensed tannins extracted from the immature leaves of *C. fairchildiana*, *L. pallida* and *C. calothyrsus* ($P < 0.05$). On the other hand, addition of condensed tannins from mature and immature of *L. leucocephala* resulted in the smallest (*ca* 20%, $P < 0.05$) reduction of gas accumulation during the fermentation of D-glucose by rumen micro-organisms. Condensed tannins in solution from immature leaves of *C. fairchildiana*, *F. macrophylla* and *L. pallida*, were more effective in preventing the accumulation of gas derived from the fermentation of D-glucose by rumen micro-organisms than those extracted from mature leaves ($P < 0.05$). Regression analysis showed that neither molecular weight or anthocyanidin composition of condensed tannins was significantly related to gas accumulation observed at 12, 24 and 48 hours post-inoculation in this experiment.

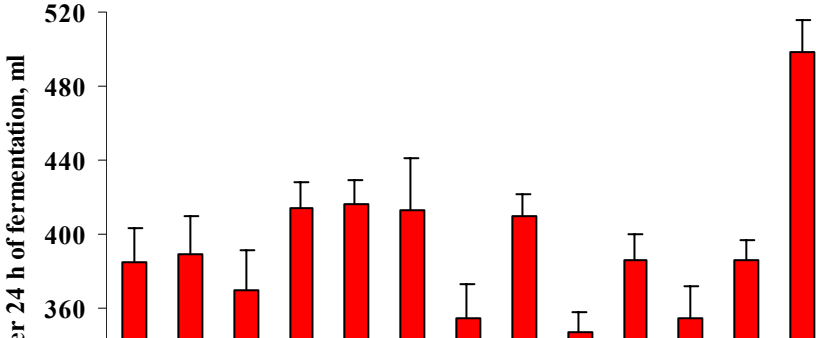

Figure 4.2-5a. *Accumulation of gas (ml) after 24 h during the fermentation of D- glucose by rumen microorganisms in the presence condensed tannins in solution from six tropical legumes. Do =* Desmodium ovalifolium, *Fm* = Flemingia macrophylla, *Ll* = Leucaena leucocephala, *Lp* = L. pallida, *Cc* = Calliandra calothyrsus, *Cf* = Clitoria fairchildiana, *y = tannins extracted from immature leaves, m = tannins extracted from mature leaves.*

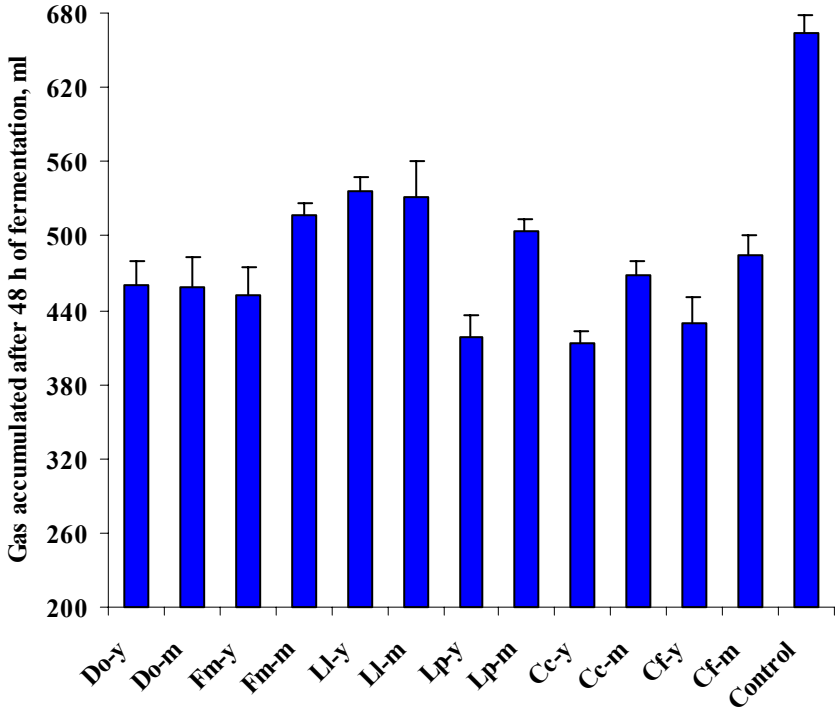

Figure 4.2-5a. *Accumulation of gas (ml) after 48 h during the fermentation of D- glucose by rumen microorganisms in the presence condensed tannins in solution from six tropical legumes. Do =* Desmodium ovalifolium, *Fm* = Flemingia macrophylla, *Ll* = Leucaena leucocephala, *Lp* = L. pallida, *Cc* = Calliandra calothyrsus, *Cf* = Clitoria fairchildiana, *yng = tannins extracted from immature leaves, mat = tannins extracted from mature leaves.*

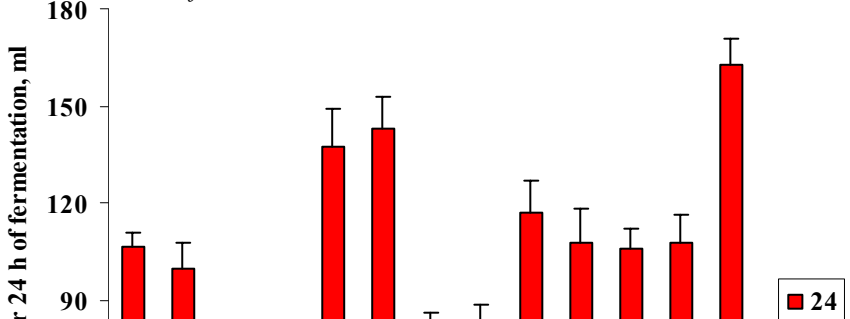

Figure 4.2-6a. *Accumulation of gas (ml) after 24 h during the fermentation of* Festuca arundinacea *cell walls by rumen micro-organisms in the presence substrate-associated condensed tannins from six tropical legumes. Do* = Desmodium ovalifolium, *Fm* = Flemingia macrophylla, *Ll* = Leucaena leucocephala, *Lp* = L. pallida, *Cc* = Calliandra calothyrsus, *Cf* = Clitoria fairchildiana, *y* = *tannins extracted from immature leaves, m = tannins extracted from mature leaves.*

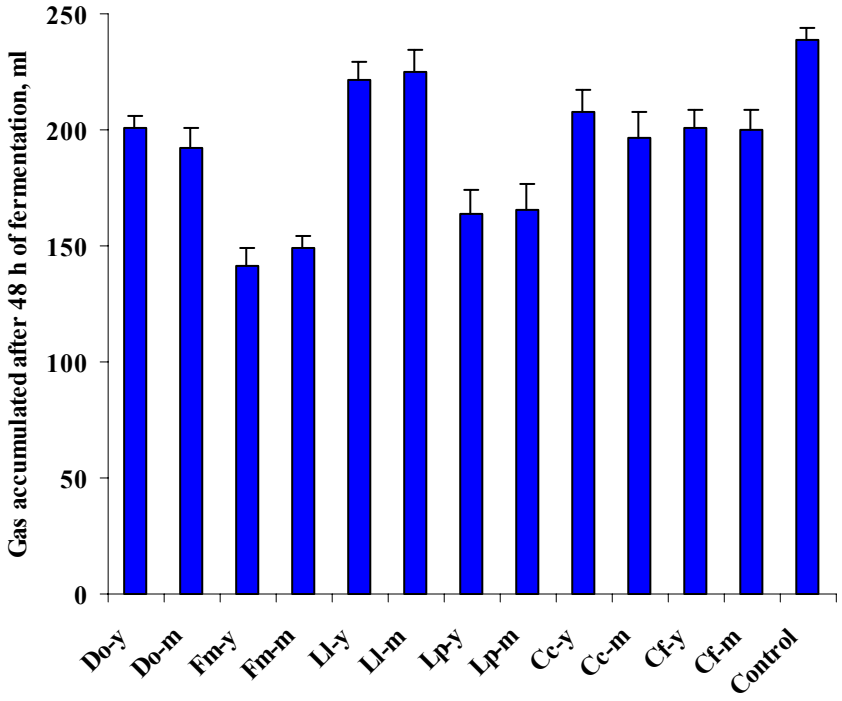

Figure 4.2-6b. *Accumulation of gas (ml) after 48 h during the fermentation of* Festuca arundinacea *cell walls by rumen micro-organisms in the presence substrate-associated condensed tannins from six tropical legumes. Do* = Desmodium ovalifolium, *Fm* = Flemingia macrophylla, *Ll* = Leucaena leucocephala, *Lp* = L. pallida, *Cc* = Calliandra calothyrsus, *Cf* = Clitoria fairchildiana, *y* = *tannins extracted from immature leaves, m = tannins extracted from mature leaves.*

Figure 4.2-7. *Rate of gas accumulation of gas (ml h^{-1}) at different times during the fermentation of D-glucose by rumen microorganisms in the presence of condensed tannins in solution (1400 g kg^{-1} of D-glucose). Cc* = Calliandra calothyrsus, *Ll* = Leucaena leucocephala, *y = tannins extracted from immature leaves, m = tannins extracted from mature leaves*

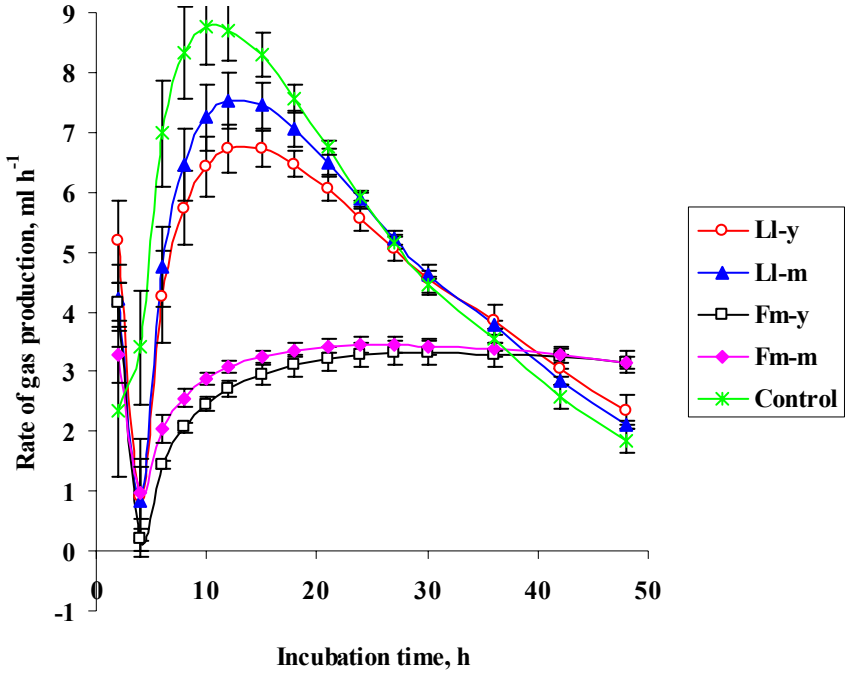

Figure 4.2-8. *Rate of gas accumulation of gas (ml h^{-1}) at different times during the fermentation of* Festuca arundinacea *cell walls by rumen microorganisms in the presence substrate-associated condensed tannins (30 g tannin kg^{-1} of cell walls). Ll* = Leucaena leucocephala, *Fm* = Flemingia macrophylla, *y = tannins extracted from immature leaves, m = tannins extracted from mature leaves.*

In experiment five, after 24 h of fermentation, the presence of substrate-associated condensed tannins reduced gas accumulation by at least 12 % (6% after 48 h) relative to the control treatment (see Figure 4.2-6 a and b). After 48 h, great variability was observed in the magnitude of this reduction, which ranged between 6 and 40%. The highest reduction was observed with condensed tannins from *F. macrophylla* ($P < 0.05$), whereas the lowest reduction continued to be observed with those from *L. leucocephala* ($P < 0.05$). Contrary to what was observed with condensed tannins in solution, substrate-associated tannins extracted from mature and immature leaves did not differ in their ability to inhibit the fermentation of plant cell walls. The only exception occurred with tannins extracted from *F. macrophylla,* where tannins from immature leaves were more efficient in preventing the fermentation of plant cells than tannins from mature leaves ($P < 0.05$). Regression analysis showed that the *number-average, weight-average* and *z-average* molecular weight of underivatised condensed tannins (see Section 4.1.3.4) was negatively related to gas accumulation at 8, 10, 12 and 24 hours post-inoculation in experiment five ($P < 0.05$). In turn, the anthocyanidin composition of condensed tannins was not significantly related to gas accumulation.

In experiment four, condensed tannins in solution from all legumes reduced the rate of gas production (ml h^{-1}) derived from the fermentation of D-glucose by rumen micro-organisms to varied extents, as illustrated in Figure 4.2-7 with tannins from mature and immature leaves of *L. leucocephala* and *C. calothyrsus*. As in experiment one, the maximum rate of gas production occurred very early in the fermentation. Additionally, the time required to attain the maximum rate of gas production was not affected by the presence of condensed tannins in solution. The maximum rate and the rate of gas production observed by 12, 24 and 48 h of incubation were not significantly related to molecular weight or to anthocyanidin composition of condensed tannins.

In experiment five, substrate-associated condensed tannins from all legumes also reduced the rate of gas production (ml h^{-1}) from the fermentation of cell walls by rumen micro-organisms. This effect and the range in the observed reduction are illustrated in Figure 4.2-8 with tannins from mature and immature leaves of *F. macrophylla* and *L. leucocephala*. As can be appreciated from this figure, substrate-

associated tannins not only reduced the maximum rate of gas production but also increased the time required to achieve it. Indeed, the maximum rate of gas production for the control treatment was 8.75 ml h^{-1} and occurred at 10 h after inoculation. In the presence of immature and mature tannins from *L. leucocephala*, maximum rate of gas production was 6.72 and 7.52 ml h^{-1} and occurred at 15 and 12 h after inoculation, respectively. More strikingly, in the presence of immature and mature tannins from *F. macrophylla*, the maximum rate of gas production was 3.31 and 3.45 ml h^{-1} and occurred at 30 and 24 h post-inoculation, respectively (see Figure 4.2-8). The *number-average, weight-average* and *z-average* molecular weight of underivatised condensed tannins (see Section 4.1.3.4) were negatively related to the rate of gas accumulation observed at 6, 8 (Figure 4.2-9), 10 and 12 h post-inoculation ($P < 0.05$). On the other hand, these three molecular weight estimates had a strong positive relationship with the rate of gas production observed after 48 h of incubation ($P < 0.01$, Figure 4.2-9). In turn, the rate of gas production after 24 h of incubation had a positive relationship with the procyanidin content of the condensed tannins ($P< 0.05$). The reverse was true in the case of prodelphinidin ($P < 0.05$).

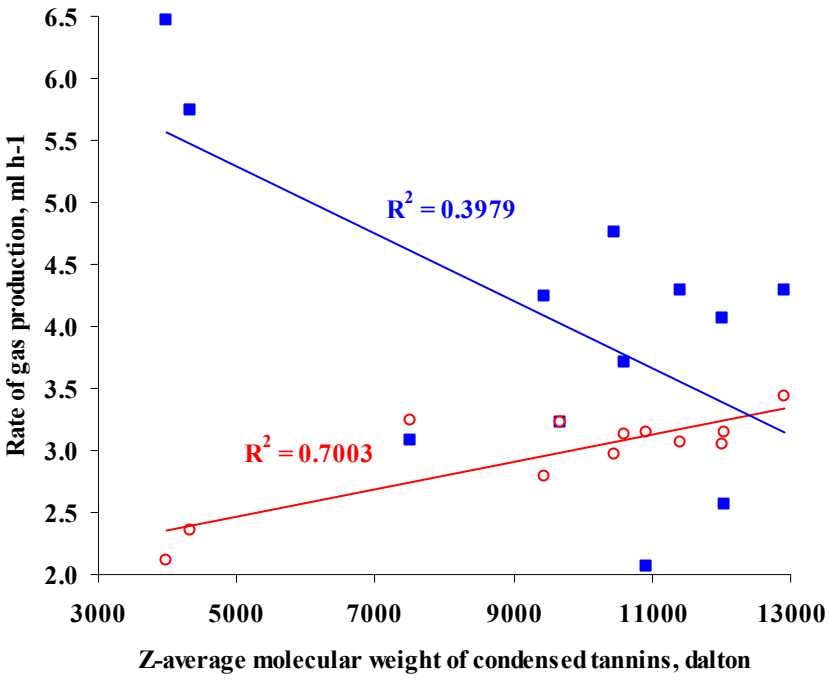

Figure 4.2-9. *The relationship between the molecular of substrate associated condensed tannins from mature and mature leaves of six tropical legumes and the rate of gas accumulation (ml h^{-1}) after 8 (■) and 48 (◊) h of fermentation of* Festuca arundinacea *cell walls by rumen micro-organisms. Condensed tannins were present at a concentration of 30 g tannin kg^{-1} of plant cell walls.*

Table 4.2-4. *Volatile fatty acid (VFA) concentration after fermentation of D-glucose by rumen micro-organisms as affected by the presence of condensed tannins in solution (1400 g per kg of D-glucose) extracted from mature and immature leaves of six tropic*al legumes *(Experiment Four)*

CT source	mMoles per litre[1]						
	Total VFA	Straight chain VFA				Branched chain VFA	
		Acetate	Propionate	Butyrate	Valerate	Iso-butyrate	Iso-valerate
Immature *D. ovalifolium*	466.4 ^{c}	329.6 ^{d}	87.6 ^{b}	37.0 ^{abc}	1.6 ^{ab}	1.6 ^{d}	8.6 ^{bc}
Mature *D. ovalifolium*	528.4 ^{bc}	376.0 ^{abc}	100.8 ^{b}	37.0 ^{abc}	0.0 ^{b}	6.8 ^{ab}	8.0 ^{bc}
Immature *F. macrophylla*	486.0 ^{bc}	341.6 ^{bcd}	93.6 ^{b}	36.0 ^{abc}	2.5 ^{ab}	3.6 ^{bcd}	8.8 ^{bc}
Mature *F. macrophylla*	518.0 ^{bc}	368.8 ^{bcd}	100.8 ^{b}	36.3 ^{abc}	0.0 ^{b}	3.0 ^{cd}	9.4 ^{bc}
Immature *L. leucocephala*	539.6 ^{b}	384.0 ^{ab}	104.0 ^{b}	38.4 ^{ab}	0.0 ^{b}	3.6 ^{bcd}	9.8 ^{bc}
Mature *L. leucocephala*	527.2 ^{bc}	366.0 ^{bcd}	102.4 ^{b}	37.0 ^{abc}	5.3 ^{a}	5.7 ^{abc}	11.2 ^{b}
Immature *L. pallida*	486.8 ^{bc}	346.8 ^{bcd}	96.8 ^{b}	34.4 ^{bc}	1.4 ^{ab}	1.1 ^{d}	6.2 ^{bc}
Mature *L. pallida*	524.8 ^{bc}	376.8 ^{abc}	100.4 ^{b}	35.7 ^{bc}	0.8 ^{ab}	4.0 ^{bcd}	7.4 ^{bc}
Immature *C. calothyrsus*	475.6 ^{bc}	342.0 ^{bcd}	98.0 ^{b}	27.6 ^{c}	0.0 ^{b}	2.6 ^{cd}	4.9 ^{c}
Mature *C. calothyrsus*	472.0 ^{bc}	332.2 ^{cd}	94.0 ^{b}	34.6 ^{bc}	0.0 ^{b}	2.6 ^{cd}	7.7 ^{bc}
Immature *C. fairchildiana*	482.0 ^{bc}	344.0 ^{bcd}	94.4 ^{b}	34.9 ^{bc}	1.0 ^{ab}	0.8 ^{d}	7.1 ^{bc}
Mature *C. fairchildiana*	520.8 ^{bc}	365.6 ^{bcd}	103.2 ^{b}	38.8 ^{ab}	0.0 ^{b}	4.0 ^{bcd}	9.3 ^{bc}
Control	604.0 ^{a}	412.0^{a}	119.6^{a}	45.9 ^{a}	0.9 ^{ab}	8.4 ^{a}	17.0 ^{a}

[1]After 48 h of incubation, values corrected for blank (containing only fermentation media and rumen fluid).

^{a,b,c,d} Values within a column without common superscript letters differ ($P< 0.05$). Values reported for each experiment are the means of those observed for each treatment in two gas production experiments.

Table 4.2-5. *Volatile fatty acid (VFA) concentration after fermentation of plant cells from* Festuca arundinacea *by rumen micro-organisms as affected by the presence of substrate-associated condensed tannins (30 g per kg of plant cells) extracted from mature and immature leaves of six tropical legumes (Experiment Five)*

CT source	mMoles per litre[1]						
	Total VFA	Straight chain VFA				Branched chain VFA	
		Acetate	Propionate	Butyrate	Valerate	Iso-butyrate	Iso-valerate
Immature *D. ovalifolium*	80.8 ^{abc}	39.3 ^{ab}	28.3 ^{ab}	10.3 ^{abc}	1.32	0.0	1.6 ^{b}
Mature *D. ovalifolium*	71.4 ^{bc}	36.5 ^{ab}	25.4 ^{ab}	8.9 ^{abc}	0.00	0.0	0.6 ^{b}
Immature *F. macrophylla*	59.5 ^{c}	33.1 ^{b}	21.0 ^{b}	5.3 ^{c}	0.00	0.0	0.1 ^{b}
Mature *F. macrophylla*	83.7 ^{abc}	45.5 ^{ab}	27.8 ^{ab}	9.1 ^{abc}	0.00	0.0	1.3 ^{b}
Immature *L. leucocephala*	128.9 ^{ab}	56.5 ^{ab}	38.9 ^{a}	13.8 ^{ab}	0.41	0.0	1.9 ^{b}
Mature *L. leucocephala*	109.2 ^{abc}	59.4 ^{ab}	35.4 ^{ab}	12.7 ^{ab}	0.35	0.0	1.4 ^{b}
Immature *L. pallida*	83.2 ^{abc}	47.3 ^{ab}	26.2 ^{ab}	8.5 ^{bc}	0.00	0.0	1.2 ^{b}
Mature *L. pallida*	77.5 ^{abc}	43.2 ^{ab}	25.4 ^{ab}	7.8 ^{bc}	0.00	0.0	1.1 ^{b}
Immature *C. calothyrsus*	117.7 ^{abc}	68.1 ^{ab}	34.1 ^{ab}	11.7 ^{ab}	0.46	0.0	1.7 ^{b}
Mature *C. calothyrsus*	105.8 ^{abc}	59.1 ^{ab}	32.9 ^{ab}	11.9 ^{ab}	0.00	0.0	1.5 ^{b}
Immature *C. fairchildiana*	83.3 ^{abc}	46.1 ^{ab}	27.1 ^{ab}	8.9 ^{abc}	0.00	0.0	1.2 ^{b}
Mature *C. fairchildiana*	78.6 ^{abc}	41.8 ^{ab}	26.3 ^{ab}	8.8 ^{abc}	0.39	0.0	1.3 ^{b}
Control	134.6 ^{a}	71.0 ^{a}	42.1 ^{a}	14.6 ^{a}	1.4	0.0	3.9 ^{a}

[1]After 48 h of incubation, values corrected for blank (containing only fermentation media and rumen fluid).

^{a,b,c} Values within a column without common superscript letters differ ($P< 0.05$). Values reported for each experiment are the means of those observed for each treatment in two gas production experiments.

In experiment four and with the exception of valerate, the concentration of VFA at the end of the incubation period was closely related to gas accumulation at 48 h ($r \geq 0.78$; $P < 0.01$). In this experiment, reductions in VFA concentrations ranging from 11 to 23 % relative to that of the control treatment were associated with the presence of condensed tannins in solution (see Table 4.2-4). Although there were no statistically significant differences between tannin treatments, it should be noted that the highest concentration of total VFA among the soluble condensed tannin treatments was observed with tannins from *L. leucocephala*. Likewise, in four of the six legumes, higher depressions in VFA concentrations were recorded in the presence of tannins from immature leaves as compared to those from mature leaves, although these differences were not statistically significant. Examination of the concentration of individual VFA, showed that iso-butyrate and iso-valerate were reduced the most, and acetate and propionate were reduced the least by the presence of soluble condensed tannins. Regression analysis showed that neither molecular weight or anthocyanidin composition of condensed tannins was significantly related to the concentration of VFA observed at the end of incubation period in this experiment.

In experiment five, the mean concentrations of total VFA, acetate, propionate and butyrate were positively related to gas accumulation ($r \geq 0.89$, $P < 0.01$). The presence of substrate-associated condensed tannins was associated with reductions in total VFA that ranged from 5 to 55 % relative to the control treatment (see Table 4.2-5). The highest reductions in concentration of total VFA were observed with tannins from *F. macrophylla* ($P < 0.05$), whereas the lowest were recorded with tannins from *L. leucocephala*. Maturity of leaves from which condensed were extracted from had no effect on the concentrations of VFA observed in this experiment. Regression analysis showed that the *number-average*, *weight-average* and *z-average* molecular weight of condensed tannins were negatively related to the concentration of acetate, propionate, n-butyrate and total VFA observed at the end of Experiment Five ($P < 0.05$). Conversely, the anthocyanidin composition of condensed tannin was not significantly related to VFA accumulation.

Recovery of dry matter was highly correlated with gas pool at 48 h in experiment five ($r \geq 0.87$; $P < 0.01$; data not shown). Dry matter recovery was on average 57.5% for the *F. macrophylla* treatment and 22.5% for the control treatment.

4.2.3 DISCUSSION

Throughout this chapter, I have purposely chosen to refer to the tannins used in the reported gas production experiments as "in solution" and "substrate-associated". This was a required distinction to avoid confusions with the terms "extractable" and "bound" that I have used to refer to condensed tannins in earlier chapters of this thesis. The definition of extractable and bound tannins refers solely to the fact that upon application of an organic solvent to a tanniniferous sample, two tannin fractions are generated: tannins that go into solution (extractable) and tannins that remain attached to the sample (bound). On the other hand, the terms "in solution" and "substrate-associated" carry a connotation of functionality, or mode of action. In terms of ruminant nutrition for example, condensed tannins "in solution" are tannins which could affect the fermentation of forages through interaction with micro-organisms or formation of complexes with microbial enzymes or with the substrate itself. Although there is some common ground between both definitions, acetone-extractable tannins cannot be equated to condensed tannins in solution.

Results obtained in this study suggest that, at similar concentrations, substrate-associated condensed tannins are most effective at inhibiting the fermentation of substrates than soluble condensed tannins. Indeed, much lower substrate-associated condensed tannins to substrate ratios were required in experiments two and five to obtain comparable levels of inhibition than observed in experiments one and four, where tannins in solution to substrate ratios were very high. This conclusion was corroborated by the results obtained in experiment three, where addition of "low" soluble tannin to substrate ratios did not inhibit the fermentation of the plant cell walls.

It might be argued that the observed differences in the ability of both forms of condensed tannins to inhibit the fermentation of substrates were inflated by comparing results on the basis of tannin-substrate ratio. This would appear especially so in the experiments were D-glucose was used as substrate, given the low (0.25 g l^{-1}) D-glucose concentration used. Nonetheless, comparison of the results on the basis of tannin concentration in the fermentation medium also suggests that substrate-associated tannins possess a greater ability to inhibit substrate fermentation. Indeed,

maximum tannin concentration used (mg $l^{-1}$of fermentation media) was 500, 240 and 500 for experiments one (in solution, D-glucose as substrate), two (associated, plant cell walls) and three (in solution, plant cell walls), respectively. Furthermore, the impact of tannin form was efficiently illustrated in the contrasting results observed in experiments two and three, where the only difference was the form in which tannins were present.

In the process of unravelling the mechanistic factors underlying these results, one could speculate that the differences observed are due to a dilution effect. That is, the probabilities that tannins in solution could exert any effect are directly related to the probability that they encounter a dietary entity, be it substrate, microbial digestive enzymes or the micro-organisms themselves, with which to interact. In these experiments, tannins in solution were diluted in a great volume of fermentation media thus lowering the possibilities of these random encounters. On the other hand, substrate-associated tannins would appear to be in the ideal position to interact with microorganisms and their enzymes. Assumptions under this scenario are in agreement with earlier suggestions that condensed tannins in solution would mainly act by interacting directly with the rumen microbial population and their extra-cellular enzymes (McLeod, 1974). In turn, substrate-associated condensed tannins might primarily affect substrate availability by masking potential binding sites for microorganisms or their enzymes (Martin and Martin, 1983).

However, it must be considered if this explanation is in agreement with other experimental observations. For example, soluble and substrate-associated condensed tannins had specific effects on the concentration of individual VFA observed at the end of the incubation period. In experiments one and four, the VFA fractions most affected by the presence of condensed tannins in solution were the branched-chain VFA iso-butyrate and iso-valerate. On the other hand, the greatest reductions in VFA concentrations due to the presence of substrate-associated tannins were observed for straight-chain fatty acids (acetate, butyrate and valerate). Since branched-chain fatty acids come from the fermentation of amino acids (valine, leucine; Hungate, 1966), our results would support the hypothesis that extractable (meaning free-soluble) tannins have their largest effects on protein degradability due to the formation of indigestible complexes with protein (Robbins et al., 1987a; Rittner and Reed. 1992).

Decreased degradation of protein would mean less free amino acids and less conversion of these amino acids to branched-chain fatty acids. Certainly, the presence of acetone-extractable tannins in ruminant diets has been consistently associated with reduction of protein fermentation by rumen microorganisms (Barry and Manley, 1984; Waghorn et al., 1987; McNabb et al., 1993; Carulla, 1994). On the other hand, data on VFA concentration in gas experiments two and five suggests that the effects of substrate-associated condensed tannin are more related to fibre degradability. It is no clear, however, whether these observations are the result of specific interactions between tannins and the substrate used in these experiments.

There was another clear distinction between the effects of condensed tannin in solution and those associated to the substrate. Indeed, the impact on rate of gas production attributable to the presence of tannins in solution was relatively stable throughout the first 48 h of fermentation. On the other hand, the effect of substrate-associated tannins on the rate of gas production was mixed: negative during the initial stages of fermentation and positive after the first 24 hours post-inoculation. This could either mean that micro-organisms gradually adapted to the presence of substrate-associated tannins, that tannins were degraded by the action of microbial enzymes, or that substrate-associated tannins became progressively soluble in the culture media as the incubation time increased. Gentle methods were used to bind condensed tannins to plant cell walls in the experiments reported here, which lends support to the latter supposition. This would provide further corroboration that tannins in solution are less effective at inhibiting substrate fermentation than tannins that are associated with the substrate. However, it is not possible to say which of these factors (or if some other factor) mediated this response.

Previous research has shown that among tanniniferous tropical legumes, there is great variation in the extractability of tannins in 70% aqueous acetone (Longland et al. 1995; Jackson et al., 1996a; Chapter Four this thesis). For example, Jackson et al (1996a) observed that the content of bound condensed tannins, as a proportion of total tannin content, ranged from 0% in *Sena velutina* to 99.8% in *Gliricidia sepium*. Moreover, the extractability of tannins from tropical legumes varies not only between legume species, but also in response to factors such as sample maturity (Chapter 4 this

thesis), genotype x environment interactions (A. Schmidt, personal communication) and post-harvest treatment of forages (Cano et al., 1994, Barahona et al., 1996).

However, little is known about the impact of this variation on the nutritive quality of forages. This is related to the fact that the criterion used for distinguishing between bound and extractable condensed tannins is somewhat arbitrary. Tannins, especially those of high molecular weights, which are extractable in 70% aqueous acetone, might not be solution in rumen liquor during the fermentation of tanniniferous feeds simply because of the lower solvating power of rumen liquor. This was demonstrated in chapter 4.1, where it was observed that the proportion of the total tannin in forages that could be labelled as extractable was dependent upon the type of solvent used. In those experiments, distinctions were made between acetone-extractable and methanol-extractable condensed tannins. As a further point, condensed tannins in intact plant leaves are contained in cell vacuoles. It is then possible that tannin extractability in acetone might only reflect this compartmentalisation and not be related to the actual binding ability/status of tannins. Yet another difficulty is that during ingestion and fermentation of tanniniferous feeds, tannins bind to substrates in what has been shown to be a very dynamic process. For example, previous research has shown that the concentration of solvent-extractable tannin in plant tissue decreases substantially after chewing (Burrit et al., 1987; Terrill et al., 1992a). This reduction is accompanied by an increase in the concentration of non-extractable tannins (Terrill et al., 1992a). Thus, it is difficult to ascertain what proportion of the total condensed tannin will effectively act as "in solution" or as "substrate-associated" within the rumen during fermentation of tanniniferous forages. Further definition of the true behaviour of condensed tannins within the rumen will certainly be required to aid in processes such as screening and evaluation of tanniniferous forage germplasm.

Great differences were observed in the ability of condensed tannins from different tropical legumes to inhibit the fermentation of D-glucose (experiment four) and plant cells (experiment five). In both experiments, the lowest inhibition of fermentation was observed with tannins from *L. leucocephala*. On the other hand, the highest inhibition in the fermentation of substrates was observed in the presence of tannins in solution from immature *C. fairchildiana* and *L. pallida* and substrate-associated tannins from *F. macrophylla* and *L. pallida*. It should be noted that in experiment five, the

concentration of substrate-associated tannins was highest for the *F. macrophylla* treatment and this could have influenced the obtained results.

Previous research has shown that many factors can affect the biological effects (astringency) of condensed tannins. Among them, molecular weight has received great attention (Jones et al., 1976; Oh and Hoff, 1979; Porter and Woodruffe, 1984; Kumar and Horigome, 1986; Cano et al., 1994). Phenolic compounds of low molecular weight are thought to form unstable cross-links with proteins, whereas those with high molecular weight are said to be ineffective as tannin agents (Salunkhe et al., 1990). As observed in chapter 4.1, tannins from *L. leucocephala* have the lowest *number-average* molecular weight of all the tannins assayed for in this study (*ca* 2400 Dalton). All other tannins were found to have *number-average* molecular weights greater than 3200 Dalton.

It is interesting to note that in this study, the impact of tannin molecular weight on fermentation was only significant in the case of substrate-associated tannins. Indeed, in experiment five, molecular weight of condensed tannins had significant inverse relationships with gas accumulation at 8, 10, 12 and 24 h, rate of gas production at 6, 8, 10 and 12 h and acetate, propionate, butyrate and total VFA concentrations. No such relationships were evident in experiment four with condensed tannins in solution. From the data gathered in these experiments, it is not possible to obtain a plausible explanation for this divergence. One source of divergence between soluble and substrate-associated tannins (besides form) is that the process used in the production of tannin-cell wall complexes fermented in experiment five also resulted in some sort of tannin fractionation. For tannins from *D. ovalifolium* and *L. leucocephala*, binding of tannins to cell walls was greater than 62% of the tannins added. On the other hand, this value was not greater that 37% for tannins from *C. fairchildiana*, *L. pallida* and *F. macrophylla*. Judging from previous results, there is a distinct probability that tannins that bound to cell walls differed in structural features to those that did not bind. The other difference between experiment four and five is, of course, the substrate fermented. Different substrates generally mean different sets of enzymes produced for their fermentation and this could lead to different results given that the interaction between tannin and protein can be quite specific for both the tannin and the protein (Hagerman and Butler, 1981; Asquith and Butler, 1986).

Tannins from different legumes differed greatly in their ability to bind to plant cells of *F. arundinacea* (section 4.2.3.1). The highest binding was observed with tannins from *D. ovalifolium* and *L. leucocephala*, whereas the lowest binding was observed with tannins from *F. macrophylla*. In previous research it was also found that tannins from *D. ovalifolium* have a greater ability to precipitate bovine serum albumin than tannins from *F. macrophylla* (Barahona et al., 1996; 1997). The binding of condensed tannins to cell walls observed in the present study was not related to the levels of bound condensed tannins observed in lyophilised leaves. In chapter three, for example, higher concentration of bound condensed tannins (mg g^{-1} of DM) was observed in leaves from *F. macrophylla* (up to 40.5) than in those of *D. ovalifolium* (15.9). These results provide further support to the idea that extractability of tannins in 70% aqueous acetone does not reflect the actual solubility of condensed tannins in other, more biological solvents.

Recovery of tannins in these binding experiments was generally no higher than 80% of added tannins. It is evident that at the lowest level of tannin addition, estimates of tannin binding and tannin recovery in these experiments are not reliable. This is because the butanol-HCl technique is not very sensitive at low tannin concentrations (Terrill et al. 1992b). However, this is not a feasible explanation to explain the low recovery when 2.5 mg of tannin were added to the cell walls. Explanation to this low recovery might reside in the experimental protocol followed for tannin determination. During these binding experiments, a fraction of the plant cells became soluble, which then could have led to the formation of soluble tannin-substrate complexes of the kind first reported by Hagerman and Robbins (1987). After freezing, thawing and centrifugation of the supernatant samples, these complexes could have become insoluble, hence the observed results. Further study of this is needed since the formation of soluble tannin-protein complexes in the gut may be relevant to the digestibility of nutrients such as protein (Mole and Waterman, 1985).

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Table 1. Binding of condensed tannins (CT) extracted from immature (terminal) and mature leaves of tropical legumes to plant cells from *Festuca arundinacea* as determined by directly measuring the amount of CT bound to plant cells.

Tannin source+	Maximum total binding (mg)	Linear regressions between total binding (mg) and added tannins (CT, mg)
Immature *D. ovalifolium*	0.89	Binding = 0.3029CT + 0.1274 R^2 = 0.9928
Mature *D. ovalifolium*	0.78	Binding = 0.2760CT + 0.1015 R^2 = 0.9964
Immature *F. macrophylla*	0.42	Binding = 0.1673CT + 0.0102 R^2 = 0.9856
Mature *F. macrophylla*	0.51	Binding = 0.2285CT - 0.0437 R^2 = 0.9948
Immature *L. leucocephala*	0.77	Binding = 0.2940CT + 0.0298 R^2 = 0.9933
Mature *L. leucocephala*	0.81	Binding = 0.2729CT + 0.1338 R^2 = 0.9955
Immature *L. pallida*	0.51	Binding = 0.2136CT - 0.0168 R^2 = 0.9907
Mature *L. pallida*	0.58	Binding = 0.2334CT + 0.0095 R^2 = 0.994
Immature *C. calothyrsus*	0.64	Binding = 0.2676CT + 0.0088 R^2 = 0.9788
Mature *C. calothyrsus*	0.72	Binding = 0.3082CT - 0.0325 R^2 = 0.9845
Immature *C. fairchildiana*	0.70	Binding = 0.3118CT - 0.066 R^2 = 0.9919
Mature *C. fairchildiana*	0.49	Binding = 0.1703CT + 0.0729 R^2 = 0.9944

Table 2. Effect of different concentrations of condensed tannins (CT) from *Desmodium ovalifolium* on the fermentation of glucose and *Festuca arundinacea* plant cells by rumen micro-organisms in three different gas production experiments.

Experiment One[1]			Experiment Two			Experiment Three		
Added CT, g per kg of D-glucose	Rate of gas production (μ_{24}, h^{-1})[2]	Gas accumulated at 48 h, ml	Added CT, g per kg of plant cells	Rate of gas production (μ_{24}), h^{-1}	Gas accumulated at 48 h, ml	Added CT, g per kg of plant cells	Rate of gas production (μ_{24}), h^{-1}	Gas accumulated at 48 h, ml
0	0.0133	494.6	0.0	0.0479	237.9	0.0	0.018	225.0
200	0.0258	449.3	2.6	0.0477	226.6	6.25	0.020	228.3
400	0.0158	434.6	4.5	0.0459	223.2	12.5	0.017	221.0
800	0.0157	375.5	7.0	0.0438	219.0	25.0	0.019	237.4
1400	0.0153	283.2	24.0	0.0450	214.3	50.0	0.021	237.6
2000	0.0253	273.1	34.9	0.0434	200.4	87.5	0.021	236.4
			60.0	0.0415	186.1	125.0	0.022	256.0

Linear regression relationships between gas accumulation at 48 h (GP) and condensed tannins (CT)

Exp. One: GP = -0.115CT + 477; r = 0.97; P < 0.001

Exp. Two: GP = -0.752CT + 229; r = 0.96; P < 0.001

Exp. Three: GP = 0.271CT + 225; r = 0.88; P < 0.05

[1]Gas production experiments were carried out using tannins purified from *D. ovalifolium* as follows: Experiment One = Soluble CT with D-glucose as energy source, Experiment 2 = CT bound to plant cells from *Festuca arundinacea*; Experiment 3 = soluble CT with plant cells from *F. arundinacea* as substrate.

[2]Gas production parameters estimated using the model of France et al. (1993). Rate of gas production (μ_{24}) was calculated at t = 24 h and gas pool was estimated at 48 h post inoculation

Table 6. Effect of different types of condensed tannins (CT) extracted from immature and mature leaves of tropical legumes on the fermentation of glucose and *Festuca arundinacea* plant cells by rumen micro-organisms in two different gas production experiments

CT source	Experiment 4: Extractable CT and glucose[1]		Experiment 5: Bound CT and plant cells[2]	
	Gas accumulated at 48 h, ml	Rate of gas production (μ^{24}), h^{-1}	Gas accumulated at 48 h, ml	Rate of gas production (μ^{24}), h^{-1}
Immature *D. ovalifolium*	460.5 ^{defg}	0.0338 ^{b}	200.9 ^{d}	0.0295
Mature *D. ovalifolium*	458.3 ^{defg}	0.0605 ^{a}	192.3 ^{d}	0.0255
Immature *F. macrophylla*	452.8 ^{efg}	0.0223 ^{b}	142.7 ^{f}	0.0074
Mature *F. macrophylla*	516.3 ^{bc}	0.0188 ^{b}	150.5 ^{e}	0.0085
Immature *L. leucocephala*	536.7 ^{b}	0.0147 ^{b}	219.3 ^{bc}	0.0430
Mature *L. leucocephala*	531.7 ^{bc}	0.0145 ^{b}	225.8 ^{ab}	0.0487
Immature *L. pallida*	418.3 ^{g}	0.0357 ^{b}	164.6 ^{e}	0.0123
Mature *L. pallida*	504.2 ^{bcd}	0.0178 ^{b}	165.8 ^{e}	0.0120
Immature *C. calothyrsus*	413.4 ^{fg}	0.0215 ^{b}	208.2 ^{cd}	0.0288
Mature *C. calothyrsus*	468.6 ^{def}	0.0193 ^{b}	196.9 ^{d}	0.0319
Immature *C. fairchildiana*	429.3 ^{g}	0.0190 ^{b}	200.7 ^{d}	0.0176
Mature *C. fairchildiana*	485.0 ^{cde}	0.0153 ^{b}	200.0 ^{d}	0.0272
Control	677.1 ^{a}	0.0087 ^{b}	240.3 ^{a}	0.0558

[1]Experiment 4 studied the effect of extractable CT from different sources on the fermentation of D-glucose by rumen micro-organisms. Extractable CT were added in concentrations of 1400 g of CT per kg of D-glucose.

[2]Experiment 5 studied the effect of bound CT from different sources on the fermentation of *Festuca arundinacea* plant cells by rumen micro-organisms. Bound CT were added in concentrations of 30 g of CT per kg of plant cells.

^{a,b,c,d,e,f,g}Values within a column without common superscript letters differ ($P< 0.05$). Values reported for each experiment are the means of those observed for each treatment in two gas production experiments.

4.3 EFFECT OF CONDENSED TANNINS FROM SIX TROPICAL LEGUMES ON THE ACTIVITY OF FUNGAL FIBROLYTIC ENZYMES

4.3.1 INTRODUCTION

Forage-fed ruminants meet their energy requirements through the degradation of dietary fibre in the rumen, which principally occurs through the action of fungal and bacterial extracellular cellulases hemicellulases, pectinases and esterases. It has been suggested that anaerobic fungi participate in the primary colonisation of plant biomass in the rumen (Bauchop, 1979). They penetrate and preferentially colonise tissues traditionally regarded as resistant to degradation (Akin et al. 1990), are capable of degrading fibrous particles of dissimilar size at similar rates and to a similar extent (Davies, 1991, France et al., 1993) and possess the most potent cellulases yet discovered (Wood et al., 1986).

Several factors can limit the degradation of fibre in the rumen. These include condensed tannins, polyphenols with the ability to bind and precipitate protein, carbohydrate and other molecules (Haslam, 1986). Condensed tannins occur in high concentrations in a great number of tropical forage legumes, especially in those adapted to acid, low-fertility soils. High levels of condensed tannins in temperate (Barry and Duncan, 1984, Pritchard et al., 1988) and tropical (Carulla, 1994, Barahona et al. 1997) forage legumes have been associated with depressed intake and reduced digestion of protein and dietary fibre in ruminants. These effects are probably mediated via the inactivation of microbial enzymes or by an increase in the resistance of substrates to degradation by micro-organisms and/or their enzymes.

Substantial effort has been directed towards the identification of high-quality tropical forage legumes adapted to acid soils. During this process, the need to fully comprehend how the presence of condensed tannins in tropical forage legumes affects their overall quality has become evident. This task becomes difficult to accomplish, as condensed tannins are not a uniform chemical entity. For example, condensed tannins extracted from six tropical legumes differed greatly in molecular structure (i.e. molecular weight and monomer composition, chapter 4.1) and in their ability to inhibit the fermentation of substrates (chapter 4.2). Likewise, previous research has

demonstrated that the protein-precipitating capacity of condensed tannins is greatly affected by their molecular weight (Jones et al., 1976; Oh and Hoff, 1979; Kumar and Horigome, 1986; Cano et al., 1994). It has been suggested that phenolic compounds of low molecular weight form unstable cross-linkages with proteins, whereas those with high molecular weight are ineffective as tannin agents (Salunkhe et al., 1990). Moreover, condensed tannins that are bound to the substrate appear to be more effective in inhibiting microbial degradation of feeds than tannins that are present in the soluble form (chapter 4.2.).

The objective of this work was to clarify how the presence of condensed tannins from tropical legumes affects the activity of the gut fungal enzymes responsible for cellulose and xylan degradation in the rumen. This was accomplished by using crude extracts of culture filtrates of the anaerobic fungi *Neocallimastix hurleyensis* and different concentrations of condensed tannins extracted from six different tropical legumes, which differed in molecular weight and monomer composition. Additionally, the activity of a recombinant esterase in the presence of different concentrations of the condensed tannins including those from *Lotus sp.* was also examined.

4.3.2 MATERIALS AND METHODS

4.3.2.1 Condensed tannin source

The condensed tannins were isolated from mature stem-free leaf samples of *Desmodium ovalifolium, Flemingia macrophylla, Leucaena leucocephala, Leucaena pallida, Calliandra calothyrsus* and *Clitoria fairchildiana* as described in Section 4.1.2.1. Purified condensed tannins from *Lotus corniculatus* var. Leo were generously provided by Dr. Phil Morris (Cell Manipulation Group, IGER). With the exception of the ferulic acid esterase assay, stock solutions of condensed tannins were prepared using 5 mg of condensed tannin ml^{-1} of citrate phosphate buffer (pH 6.5), which contained 5% methanol in order to dissolve the tannins completely. These solutions were kept at 4°C in the dark until required and prepared fresh to be used within a one-week period.

4.3.2.2 Enzyme source

Dr. Wei-Yun Zhu and Mr. Emin Oskodze (Microbiology Group, IGER) generously provided the culture filtrates containing the *Neocallimastix hurleyensis* enzymes used for this work. Carboxymethylcellulase (CMCase) and β-D–glucosidase were obtained by growing the monocentric fungus *Neocallimastix hurleyensis* in modified medium C (Davies et al., 1993) on milled wheat straw at 39°C without agitation in gas-tight serum bottles. Xylanase and β-D–xylosidase were obtained by growing the fungus on xylan (Zhu et al. 1996). Culture media solutions containing the enzymes were centrifuged for 5 minutes at 14000 rpm at room temperature in a Microcentaur micro-centrifuge. The supernatant containing the enzymes was recovered and kept at -70°C until used in these experiments. In all experiments reported herein, a mixture of the appropriate enzyme solution diluted with citrate phosphate buffer (1:1, v/v) was used as the enzyme source. A recombinant ferulic acid esterase enzyme was provided by Genencor International.

4.3.2.3 Activity of different enzymes from *Neocallimastix hurleyensis* in the presence of different concentrations of condensed tannins extracted from six tropical legumes

4.3.2.3.1 *CMCase*

CMCase activity in the presence of condensed tannins was determined by measuring reducing sugars released from carboxymethylcellulose (CMC) using a modification of the method reported by Zhu et al. (1996). This research consisted of two independent experimental runs, whereby duplicate determinations were carried out in 10-ml cell culture tubes. In turn, each determination consisted of two different assay sets. Samples in the first assay set received 100 µl of the appropriate condensed tannin solution, 100 µl of the enzyme solution and 800 µl of substrate [1.5% (w/v) of CMC sodium salt, medium viscosity, (Sigma) in citrate-phosphate buffer, pH 6.5]. Samples in the second assay set received 800 µl of citrate-phosphate buffer, pH 6.5 in place of the substrate solution. Tannin solutions were prepared by dissolving purified condensed tannins (Section 4.1.2.1) in a solution containing 5% methanol in citrate buffer and provided 0, 12.5, 25, 37.5 and 50 µg of condensed tannin ml^{-1} of reaction mixture. The reaction mixture was incubated for 30 minutes at 50°C. At the end of the incubation, released reducing sugar was measured according to the method of

Somogyi (1953) using a FP-901 Chemistry Analyser (LabSystems) to read the absorbance of the copper-sugar-arsenomolybdate complex at 540 nm. Standard curves for released reducing sugars were obtained by reacting 100 µl of the appropriate standard solution, 100 µl of 5% methanol in citrate buffer and 800 µl of either CMC (first set) or buffer (second set). The standard solutions contained 0, 0.25, 0.5, 1 and 2 mg of glucose ml^{-1} of distilled water. The net released glucose, due to the degradation of CMC by CMCases, was defined as the amount of glucose in first set samples minus glucose present in second set samples.

4.3.2.3.2 *Xylanase*

Xylanase activity in the presence of condensed tannins was determined by measuring the release of reducing sugars from soluble xylan using a modification of the method used in Section 4.3.2.3.1 for the determination of CMCase activity. The substrate solution used in this experiment contained 6.25 mg ml^{-1} of soluble xylan (Sigma; Ghangas et al., 1989) in citrate-phosphate buffer, pH 6.5. For the preparation of the standard curve, solutions containing 0, 0.5, 1, 1.5, 2 and 3 mg of xylose ml^{-1} of distilled water were prepared. Working solutions of all purified condensed tannins were prepared in each of the two experimental runs for this assay, providing 0, 3.125, 6.25, 12.5 and 25 µg of condensed tannin ml^{-1} of final reaction mixture.

4.3.2.3.3 *β-D-glucosidase*

The procedure used in the determination of β-D-glucosidase activity was modified from that of Garcia-Campayo and Wood (1993). In each of two experimental runs, determinations were carried out in triplicate in 1.5 ml Eppendorf tubes. Samples received 400 µl of the citrate-phosphate buffer (pH 6.5), 50 µl of enzyme, 25 µl of substrate (25 mM of *p*-nitrophenyl-β-$_D$-glycopiranoside, Sigma) and 25 µl of the appropriate condensed tannin solution. Working solutions from all purified condensed tannin provided 0, 12.5, 25, 50 and 100 µg of tannin ml^{-1} of reaction mixture. Enzyme blanks (400 µl of the assay buffer, 25 µl of substrate and 75 µl of water) and substrate blanks (400 µl of the assay buffer, 50 µl of enzyme and 50 µl of water) were also included in each run. All samples were incubated for 1 h at 39°C and the enzymatic reaction was stopped by the addition of 500 µl of NaOH-glycine buffer, pH 10.6. Aliquots (200 µl) of the resulting solutions were placed in 96-well micro-plates and

their absorbance read at 405nm in a BIO Kinetics Reader, (Biotek Instruments, Luminar Technology Ltd). Standard curves for *p*-nitrophenol were prepared by mixing 400 µl of the assay buffer, 50 µl of water and 50 µl of the appropriate standard solution. The standard solutions contained 0, 50, 100, 150, 200 and 300 mg of *p*-nitrophenol ml^{-1} of distilled water.

4.3.2.3.4 *β-D-xylosidase*

The method used to determine the activity of β-D-glucosidases was also used, with some modifications, for the determination of β-D-xylosidase activity. As for the β-D glucosidase, determinations were carried in triplicate in each of two experimental runs. The substrate solution contained 25 mM of *p*-nitrophenyl-β-$_D$-xylopiranoside (Sigma). The working tannin solutions used in these assays provided 0, 6.25, 12.5, 25 and 50 µg of tannin ml^{-1} of reaction solution.

4.3.2.3.5 *Determination of the activity of fibrolytic fungal enzymes in the presence of condensed tannins when using plant cell walls from* **Festuca arundinacea** *as substrate*

This work was designed to compare the effect of soluble and substrate-associated condensed tannins on the enzymatic degradation of substrates. The general procedure used in these experiments for measuring fibrolytic activity was modified from that used in Section 4.3.2.3.1 for the determination of CMCase activity in the presence of tannins. In these experiments, however, *Festuca arundinacea* cell walls grown in culture (and therefore lignin-free; Dalton, 1993) were used as the primary substrate. Prior to their use in these experiments, plant cell walls were washed with distilled water and citrate buffer to remove any excess of sugar and other soluble components. The culture filtrate used in these assays was a 1:1 (v/v) mixture of that containing the CMCases and β-$_D$-glucosidases and the one containing the xylanases and β-$_D$-xylosidases. All other reagents were the same as those used in the CMCase assay. For the evaluation of the effect of condensed tannins in solution on the enzymatic degradation of plant cells, quadruplicate samples per treatment received 10 mg of plant cells, 800µl of buffer, 100µl of the appropriate tannin solution and 100µl of enzyme (first set) or water (second set). Tannins were added to the enzyme-substrate complex just at the beginning of the assay. In turn, the effect of substrate-associated

condensed tannins on the enzymatic degradation of plant cells was evaluated in plant cell walls that had condensed tannins previously bound to them. Quadruplicate samples received 10 mg of cell wall with substrate-associated tannins, 800µl of buffer and 100µl of enzyme (first set) or water (second set). The level of tannin addition, either in solution or substrate associated form was at 3.0 % of plant cell dry matter. In both experiments, samples were placed in 1.5 ml Eppendorf tubes and incubated in a water bath for 1 h at 50°C. The reaction mixture was then centrifuged and a 0.5-ml aliquot removed from each tube and mixed with 0.5 ml of copper reagent. The resulting solution was then boiled for 15 minutes. After cooling, 1 ml of arsenomolybdate was added and the resulting solution left to react at room temperature for a further 30 min. The absorbance of the final solution was read at 540 nm in a FP-901 Chemistry Analyser (LabSystems). An empty cuvette was used as a blank.

4.3.2.4 Determination of the activity of a recombinant ferulic acid esterase in the presence of different concentrations of condensed tannins

An existing assay was modified to measure the kinetics of ferulate breakdown by a recombinant ferulic acid esterase (FAE) in the presence of different concentrations of condensed tannins. Two experiments were carried out to evaluate the effect of different types and concentrations of condensed tannins on the activity of FAE. All determinations were carried out in 1-ml disposable cuvettes. The aim of the first experiment was to evaluate the effect of reaction time between tannins and FAE on the activity of this enzyme. For this experiment, samples received 965 µl of sodium phosphate buffer (pH 5.0), 10 µl of the enzyme solution and 10 µl of a solution containing 6 mg of condensed tannins ml^{-1} from *D. ovalifolium* dissolved in 95% ethanol. Triplicate samples prepared this in way were left to react at room temperature for 0, 1, 2, 4, 6 and 10 minutes after which, 25 µl of the substrate solution (5 mMolar ethyl-4-hydroxy-3-methoxy-cinnamate in 95% ethanol) was added. After mixing thoroughly, the change in absorbance at 334 nm was read in a Philips PU 8720 UV/VIS scanning spectrophotometer for two minutes.

The second experiment was designed to compare the effects of different concentration and types of condensed tannins on the activity of FAE. Quadruplicate samples

received 965 µl of sodium phosphate buffer, pH 5.0, 10 µl of the enzyme solution and then 25 µl of the substrate solution. After mixing thoroughly, the change in absorbance at 334 nm was read for 45 seconds. An aliquot (10 µl) of the appropriate tannin solution was added and after vigorous mixing, the change in absorbance at 334 nm was recorded for a further 60 seconds. Condensed tannins employed for this experiment included those extracted from *Lotus sp.* and those obtained from the six tropical legumes mentioned in Section 5.3.2.1. At least five different condensed tannin concentrations were used for each tannin; all condensed tannin solutions were prepared in 95% ethanol. There was considerable variation in the ability of condensed tannins to inhibit the activity of this enzyme and thus, different concentrations of condensed tannin were used in each case. Results from this experiment are reported by expressing the rate of ferulate breakdown after the addition of condensed tannins as a percentage of the rate observed in the first 45 seconds.

4.3.2.5 Statistical Analyses

For all experiments, except for the degradation of *F. arundinacea* cell walls, relationships between observed enzyme activity and concentration of the individual condensed tannins were analysed using second degree polynomial regressions. Data obtained in the experiments with CMCases, xylanases, β-D-glucosidases, β-D-xylosidases and the one measuring the degradation of *F. arundinacea* cell walls were analysed as a completely randomized design using the analysis of variance (ANOVA) procedure of SAS (1989) with concentration and/or source of condensed tannins as terms in the model. Duncan's Multiple Range Test at alpha level of 0.05 was used to compare means. Linear regression equations were used to investigate the relationships between tannin molecular weight and anthocyanidin composition with observed enzymatic activity.

4.3.3 RESULTS

4.3.3.1 CMCase activity in the presence of condensed tannins

As shown in Figure 4.3-1, addition of condensed tannins from the different tropical legumes resulted in inhibition of CMCase activity. However there was considerable variation in ability of condensed tannins to so (Figure 4.3-1 and Table 4.3-1). All comparisons are made relative to the enzyme activity observed for the control (no tannins) treatment. The highest degree of enzymatic inhibition was observed with condensed tannins from *D. ovalifolium*; 12.5 μg of the tannin ml^{-1} of reaction mixture resulted in a decline of 58% in the activity of CMCase ($P < 0.05$). The lowest degree of inhibition of CMCase activity was observed in the presence of condensed tannins from *C. calothyrsus* and *L. leucocephala*. At 12.5 μg ml^{-1} of condensed tannins from these legumes, CMCase activity was reduced by 24 and 20% respectively ($P > 0.05$). Linear regression analysis showed that neither molecular weight nor anthocyanidin composition of the condensed tannins was significantly related to the reductions in CMCase activity (data not shown). However, as the concentration of the condensed tannins increased in the reaction solution, an inverse relationship between molecular weight of the peracetate derivatives of condensed tannins and CMCase activity became stronger. At 50 μg ml^{-1} of tannins, this relationship became a trend (R^2 = 0.549-0.633, $P \leq 0.1$, see Figure 4.3-2).

The concentration of condensed tannins (μg ml^{-1} of reaction solution) required to inhibit the activity of CMCase by 50% was estimated using second degree polynomial equations fitted to the experimental data points. The fit between the experimental and predicted data points was very high (R^2 =0.968 to 0.999). The predicted value was estimated to be 14 for *D. ovalifolium* and circa 38 and 40 for *L. leucocephala* and *C. calothyrsus* condensed tannins, respectively (Table 4.3-1). Condensed tannins from *F. macrophylla, L. pallida* and *C. fairchildiana* showed an intermediate and somewhat comparable ability to inhibit CMCases, with 22, 25 and 29 μg ml^{-1} of tannins from these legumes, respectively, required to achieve 50% reduction in CMCase activity (Table 4.3-1).

Table 4.3-1. *Amount (μg ml^{-1}) of condensed tannins from mature leaves of different tropical legumes required to inhibit the activity of different* Neocallimastix hurleyensis *enzymes and a recombinant ferulic acid esterase (FAE) by 50% of the corresponding control (no tannins) treatment.*

Tannin source	*Neocallimastix hurleyensis* enzymes				
	CMCase	Xylanase	β-$_D$-Glucosidase	β-$_D$-Xylosidase	FAE
D. ovalifolium	13.7	2.8	53.2	21.3	47.6
F. macrophylla	21.9	2.7	96.8	Not observed	29.4
L. leucocephala	37.9	5.1	Not observed	Not observed	170.8
L. pallida	25.0	3.2	80.7	Not observed	10.3
C. calothyrsus	40.3	5.5	Not observed	Not observed	17.8
C. fairchildiana	29.5	3.4	Not observed	29.8	85.1

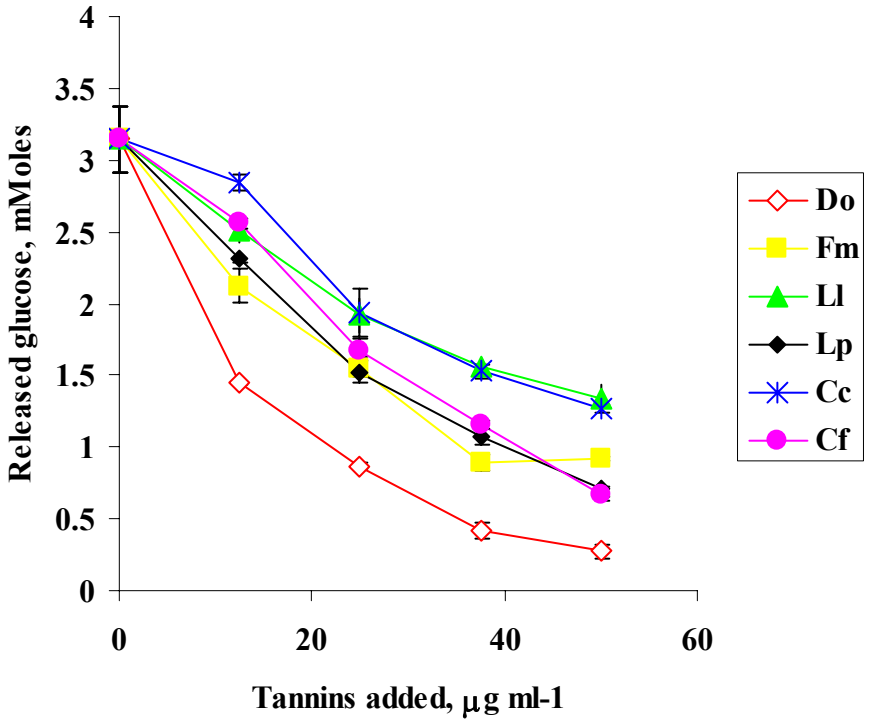

Figure 4.3-1. *Activity of* Neocallimastix hurleyensis *CMCase in the presence of different concentrations of condensed tannin extracted from mature leaves of six tropical legumes. Do =* Desmodium ovalifolium*, Fm =* Flemingia macrophylla*, Ll =* Leucaena leucocephala*, Lp =* Leucaena pallida*, Cc =* Calliandra calothyrsus *and Cf =* Clitoria fairchildiana*. Portrayed curves are the average of two independent assays. Error bars represent SEM in each figure where n= 4. Where error bars are not shown, they are smaller than the size of the symbols.*

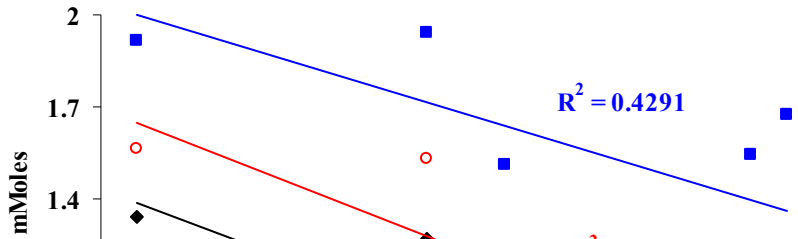

Figure 4.3-2. *The relationship between the molecular weight of peracetate derivatives of condensed tannins from mature leaves of six tropical legumes* and the a*ctivity of* Neocallimastix hurleyensis *CMCase in the presence of different concentrations [25 (■), 37.5 (O) and 50 (◆) mg ml^{-1}] of these condensed tannins.*

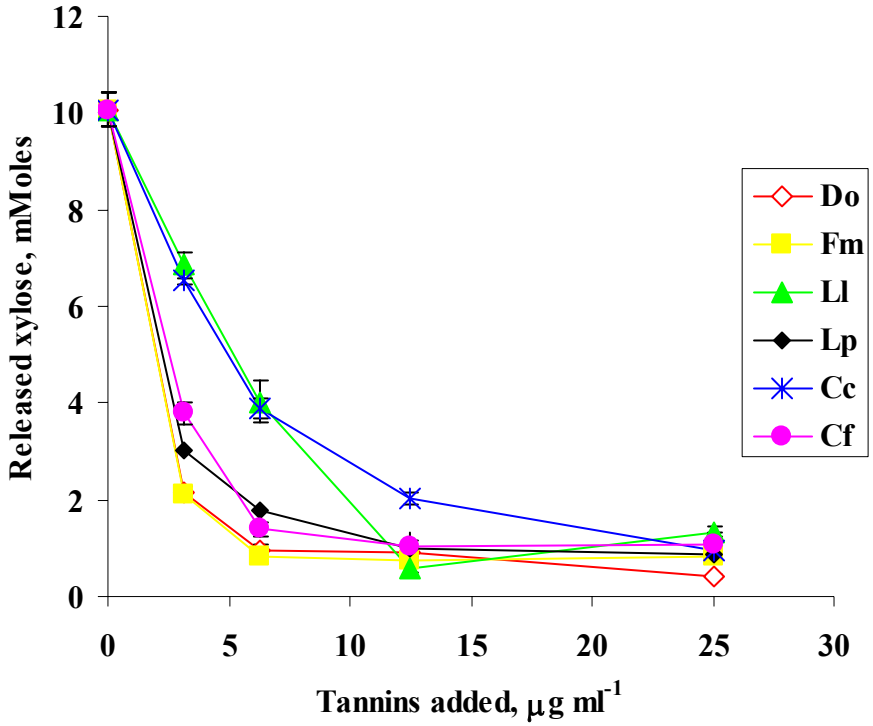

Figure 4.3-3. *Activity of* Neocallimastix hurleyensis *xylanase in the presence of different concentrations of condensed tannin extracted from mature leaves of six tropical legumes. Do =* Desmodium ovalifolium*, Fm =* Flemingia macrophylla*, Ll =* Leucaena leucocephala*, Lp =* Leucaena pallida*, Cc =* Calliandra calothyrsus *and Cf =* Clitoria fairchildiana*. Portrayed curves are the average of two independent assays. Error bars represent SEM in each figure where n= 4. Where error bars are not shown, they are smaller than the size of the symbols.*

4.3.3.2 Xylanase activity in the presence of condensed tannins

Contrary to the effect on CMCase activity, lower concentrations of added condensed tannins resulted in greater inhibition of the activity of xylanases (Figure 4.3-3, and Table 4.3-1). In fact, in the presence of all combinations of types and concentrations of condensed tannins assayed in this experiment, the activity of xylanase was always significantly lower ($P < 0.05$) than that observed for the control (no tannin) treatment. It should be noted that the highest concentration of condensed tannins used in this assay was only half of that used in the CMCase assay (25 vs. 50 μg ml^{-1}). Nonetheless, at 25 μg ml^{-1} the reduction in xylanase activity relative to the control treatment ranged between 87 and 96 %. On the other hand, at 25 μg ml^{-1}, the corresponding reduction in CMCase activity ranged between 50 and 87 %.

As shown in Figure 4.3-3, differences between condensed tannin types were more pronounced when they were added at low concentrations. For example, at 3.125 μg ml^{-1}, condensed tannins from *F. macrophylla* and *D. ovalifolium* reduced xylanase activity by about 79 %. On the other hand, at this same concentration, condensed tannins from *C. calothyrsus* and *L. leucocephala* reduced the activity of xylanases by only 32 and 35%, respectively ($P < 0.05$). The higher concentrations of condensed tannins used in this experiment were equally effective at inhibiting xylanase activity regardless of their origin (Figure 4.3-3). Estimation (using the second degree polynomial equations, with R^2 values ranging from 0.755 to 0.999) of the concentration of condensed tannins (μg ml^{-1}) required to reduce the activity of xylanase by 50%, showed that this value ranged from 2.7 (*F. macrophylla*) to 5.5 (*C. calothyrsus*; see Table 4.3-1).

At low tannin concentrations (3.125 and 6.25 μg ml^{-1} of reaction solution), the molecular weight of condensed tannins was inversely related to xylanase activity ($P \leq 0.1$). At 6.25 μg ml^{-1}, the relationship involving the estimates of *number-average*, *weight-average* and *z-average* molecular weight of the condensed tannin peracetates was particularly strong (R^2: 0.707-0.820, $P \leq 0.05$, Figure 4.3-4). In turn, monomer composition of condensed tannins was not significantly related to the activity observed for this enzyme in the presence of condensed tannins.

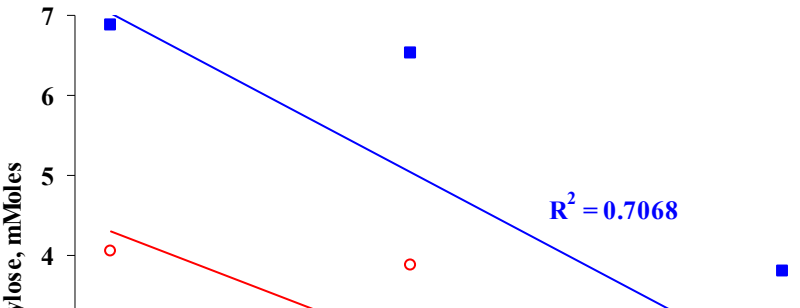

Figure 4.3-4. *The relationship between the molecular weight of peracetate derivatives of condensed tannins from mature leaves of six tropical legumes* and the a*ctivity of* Neocallimastix hurleyensis xylana*se in the presence of different concentrations [3.125 (■), and 6.25 (O) mg ml*$^{-1}$*] of these condensed tannins.*

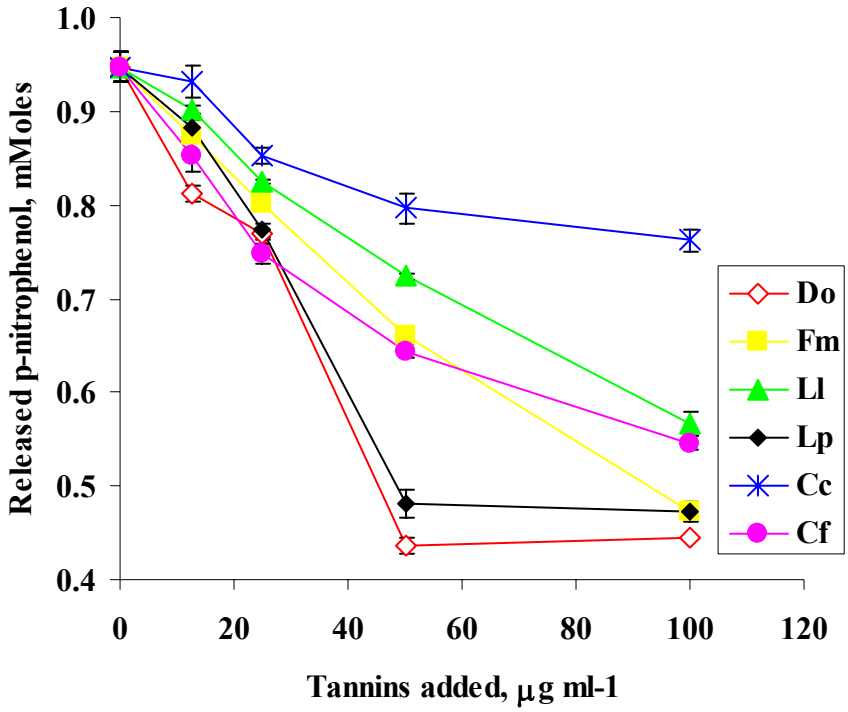

Figure 4.3-5. *Activity of* Neocallimastix hurleyensis *β-D-glucosidase in the presence of different concentrations of condensed tannin extracted from mature leaves of six tropical legumes. Do* = Desmodium ovalifolium, *Fm* = Flemingia macrophylla, *Ll* = Leucaena leucocephala, *Lp* = Leucaena pallida, *Cc* = Calliandra calothyrsus *and Cf* = Clitoria fairchildiana. *Portrayed curves are the average of two independent assays. Error bars represent SEM in each figure where n= 6. Where error bars are not shown, they are smaller than the size of the symbols.*

4.3.3.3 β-D-glucosidase activity in the presence of condensed tannins

The ability of condensed tannins from different legumes to reduce the activity of β-D-glucosidase from *N. hurleyensis* was variable (Figure 4.3-5 and Table 4.3-1). As for the CMCase and the xylanase assays, the addition of condensed tannins from *D. ovalifolium* resulted in the highest inhibition of β-D-glucosidase activity. At 50 μg ml^{-1} of condensed tannins from this legume, the activity of this enzyme was reduced to only 46% ($P < 0.05$). Conversely, as was observed in the CMCase assay, the lowest inhibition in the activity of β-D-glucosidase was observed in the presence of condensed tannins from *C. calothyrsus*. At 100 μg ml^{-1}, condensed tannins from this legume reduced enzymatic activity by only 20% ($P > 0.05$).

The estimated concentration of condensed tannins required to produce a decline of 50% in β-D-glucosidase activity was 53, 81 and 97 μg ml^{-1} for *D. ovalifolium*, *L. pallida* and *F. macrophylla*, respectively (Table 4.3-1). Despite the use of high concentrations of condensed tannins (up to 100 μg ml^{-1}), condensed tannins from *C. calothyrsus*, *L. leucocephala* and *C. fairchildiana* failed to reduce the activity of this enzyme by more than 30%.

Linear regression analysis showed that neither molecular weight nor monomer composition of condensed tannins was significantly related to the activity of β-D-glucosidase. The strongest relationship occurred at condensed tannin concentrations of 12.5 and 25 μg ml^{-1}, with *z-average* molecular weight (estimated on peracetate derivatives of condensed tannins) being inversely related to enzymatic activity ($R^2 = 0.533$, $P < 0.1$, see Figure 4.3-6).

4.3.3.4 β-D-xylosidase activity in the presence of condensed tannins

The highest concentration of condensed tannins employed in the β-D-xylosidase experiments was only 50% of the maximum tannin concentration used in the experiment with β-D-glucosidases. As was observed in the previous assays, results from the experiment with β-$_D$-xylosidases showed that condensed tannins from different legumes differed greatly in their ability to inhibit the activity of this enzyme (Figure 4.3-7, Table 4.3-1).

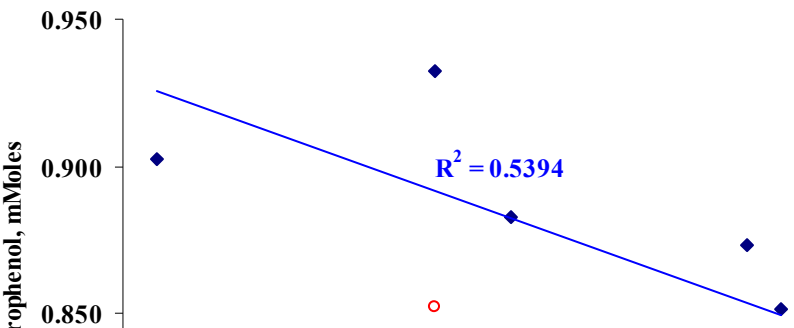

Figure 4.3-6. *The relationship between the molecular weight of peracetate derivatives of condensed tannins from mature leaves of six tropical legumes* and the a*ctivity of* Neocallimastix hurleyensis *β-D-glucosidase in the presence of different concentrations [3.125 (■), and 6.25 (O) mg ml^{-1}] of these condensed tannins.*

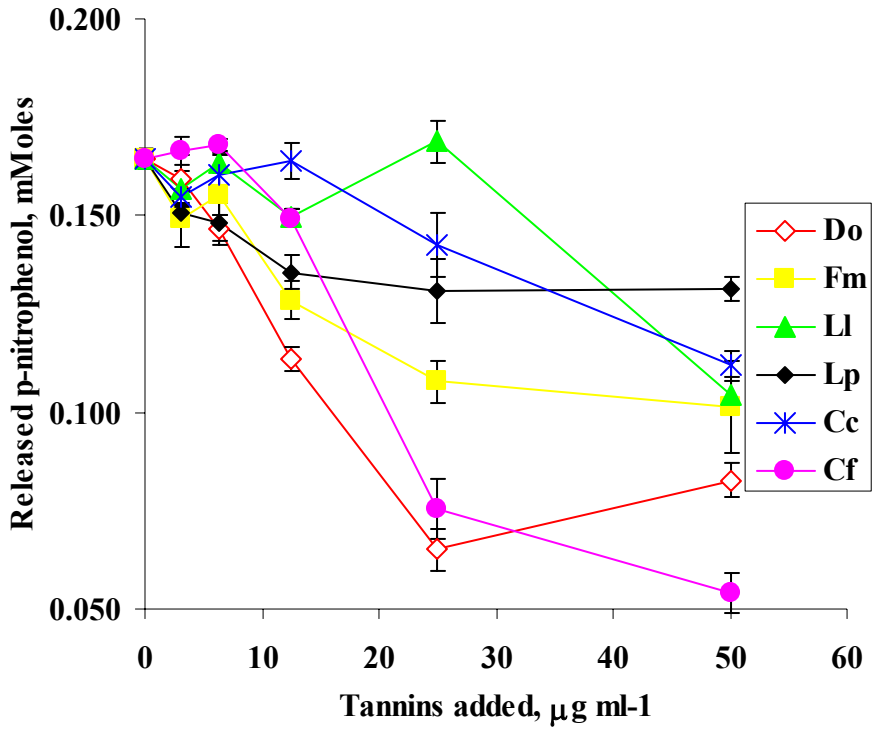

Figure 4.3-7. *Activity of* Neocallimastix hurleyensis *β-$_D$-xylosidase in the presence of different concentrations of condensed tannin extracted from mature leaves of six tropical legumes. Do =* Desmodium ovalifolium*, Fm =* Flemingia macrophylla*, Ll =* Leucaena leucocephala*, Lp =* Leucaena pallida*, Cc =* Calliandra calothyrsus *and Cf =* Clitoria fairchildiana*. Portrayed curves are the average of two independent assays. Error bars represent SEM in each figure where n= 6. Where error bars are not shown, they are smaller than the size of the symbols.*

In the β-D-xylosidase experiment, condensed tannins from *D. ovalifolium* and *C. fairchildiana* were the most effective in inhibiting this enzyme, with 21 and 30 µg ml^{-}

[1] of tannins resulting a reduction of 50% in the activity of β-D-xylosidase ($P < 0.05$). Condensed tannins from all other legumes failed to reduce the activity of β-D-xylosidase by more than 40% with respect to the control (no tannins) treatment.

There were no significant relationships between molecular weight and monomer composition of condensed tannin and the activity of β-D-xylosidase at 6.25 and 12.5 μg ml^{-1} of condensed tannins. On the other hand, at 25 μg ml^{-1} of condensed tannins, *number-average*, *weight-average* and *z-average* molecular weights (estimated in tannin peracetates) were negatively related to β-$_D$-xylosidase activity ($R^2 \geq 0.861$, $P \leq 0.0054$ for the linear regression). However, as shown in Figure 4.3-8 the shape of the relationship between condensed tannin molecular weight and β-D-xylosidase activity was better described by the use of second-degree polynomial equations. In turn, at 50 μg $ml^{-1}$of tannins, propelargonidin content of condensed tannins was inversely related to β-$_D$-xylosidase activity ($R^2 = 0.66$, $P = 0.049$, see Figure 4.3-9).

4.3.3.5 Degradation of plant cell walls from *Festuca arundinacea* by fibrolytic enzymes from *Neocallimastix hurleyensis* in the presence of condensed tannins from tropical legumes

With the exception of condensed tannins from *L. pallida*, substrate-associated tannins were more effective ($P < 0.05$) as inhibitors of fibrolytic enzymes from *N. hurleyensis* than the corresponding soluble condensed tannins (see Figure 4.3-10). Moreover, when condensed tannins from different legumes were ranked according to their ability to inhibit cell wall degrading activity, the ranking obtained for soluble condensed tannins was not the same as the one obtained for substrate-associated tannins. For example, the highest release (i.e. net absorbance of the copper-arseno-molybdate complex) of reducing sugars in the presence of soluble tannins was observed with condensed tannins from *C. calothyrsus* (0.265), whereas the lowest was observed with tannins from *L. pallida* (0.189). On the other hand, the highest and lowest release of reducing sugars in the presence of substrate-associated tannins occurred with condensed tannins from *L. pallida* (0.198) and *F. macrophylla* (0.071), respectively.

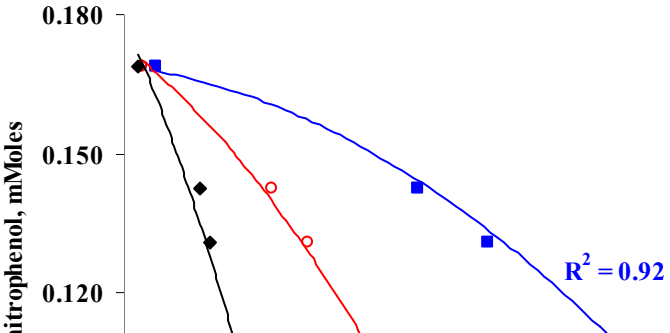

Figure 4.3-8. *The relationship between of molecular weight of peracetate derivatives of condensed tannins from mature leaves of six tropical legumes* and the a*ctivity of* Neocallimastix hurleyensis *β-D-xylosidase in the presence of 25 mg ml*$^{-1}$ *of these condensed tannins. Symbols represent the relationship involving* number-average *(◆)*, weight average *(O) and* z-average *(■) molecular weight.*

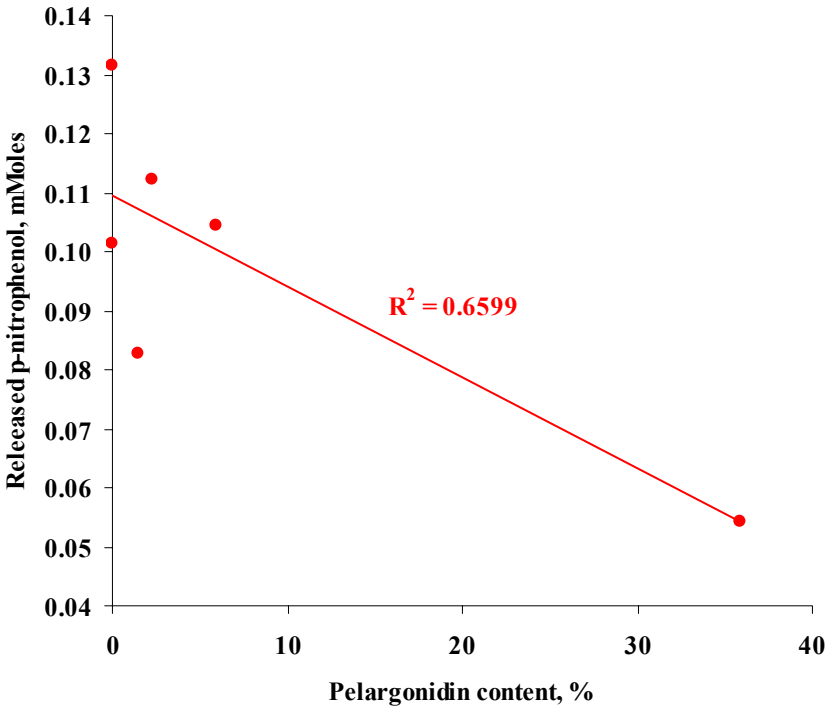

Figure 4.3-9. *The relationship between the propelargonidin content of condensed tannins from mature leaves of six tropical legumes* and the a*ctivity of* Neocallimastix hurleyensis *β-D-xylosidase in the presence of 50 mg ml*$^{-1}$ *of these condensed tannins.*

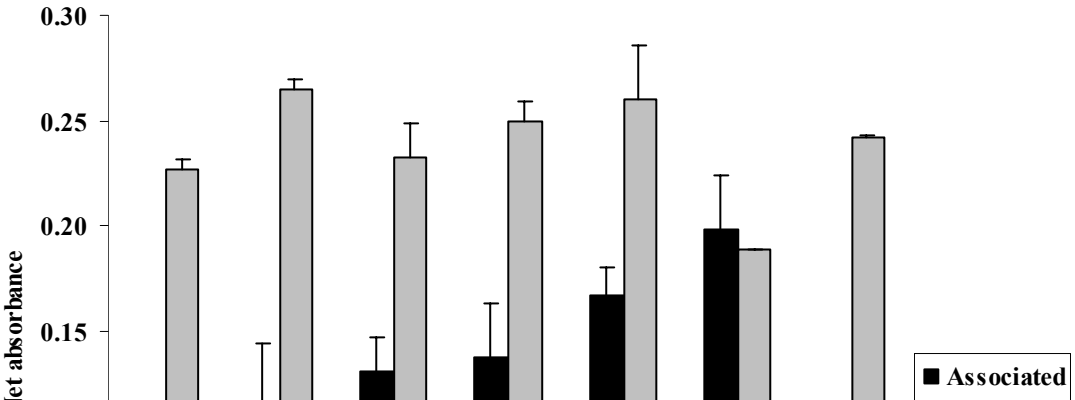

Figure 4.3-10. *Net release of reducing sugars from cell walls of* Festuca arundinacea *by the action of fibrolytic enzymes from* Neocallimastix hurleyensis *in the presence of soluble and substrate-associated condensed tannins (30 g/kg of cell walls). Fm* = Flemingia macrophylla*, Cc* = Calliandra calothyrsus, *Ll* = Leucaena leucocephala*, Cf* = Clitoria fairchildiana, *Do* = Desmodium ovalifolium *and Lp* = Leucaena pallida*. Error bars represent SEM in each figure where n= 4*

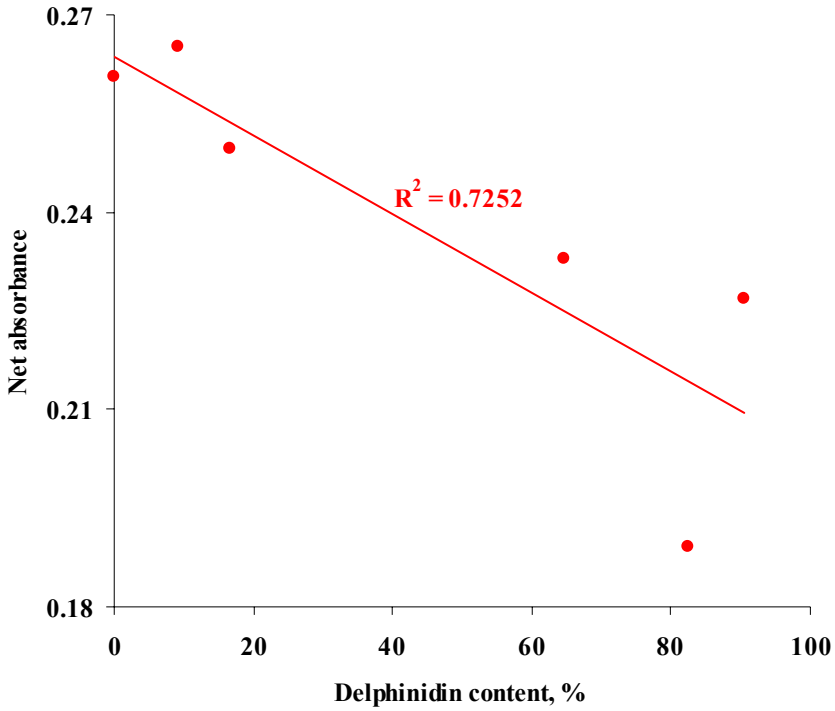

Figure 4.3-11. *The relationship between the propelargonidin content of condensed tannins from mature leaves of six tropical legumes* and the a*ctivity of* Neocallimastix hurleyensis *β-D-xylosidase in the presence of 50 mg ml^{-1} of these condensed tannins.*

These contrasting results explain the great variability observed when calculating the ratio between the release of reducing sugars in the presence of substrate-associated tannins to the release of reducing sugars released in the presence of soluble tannins.

Such ratios ranged from 0.313 in the case of *F. macrophylla* to 1.049 in the case of *L. pallida,* although they were never higher than 0.624 in all other legumes.

Linear regression analysis showed that the activity of fungal fibrolytic enzymes in the presence of soluble and substrate-associated condensed tannins was not significantly related to tannin molecular weight. Likewise, monomer composition of condensed tannins was not significantly related to cell wall degradation in the case of substrate-associated tannins. For soluble tannins, on the other hand, procyanidin content of condensed tannins was positively related ($R^2 = 0.667$, $P < 0.05$) to release of reducing sugars from the cell walls. In turn, prodelphinidin composition was inversely related to cell wall degradation ($R^2 = 0.725$, $P < 0.05$, see Figure 4.3-11).

4.3.3.6 Activity of a recombinant ferulic acid esterase (FAE) in the presence of different concentrations of condensed tannins from different tropical legumes and from *Lotus sp.*

Results obtained in the first FAE experiment are reported in Figure 4.3-12. In this experiment, the activity of FAE was measured in the presence of condensed tannins from *D. ovalifolium* after allowing different times of reaction between the condensed tannins and the enzyme. Control treatments consisted of enzyme samples diluted in buffer which were also allowed to stand at room temperature for different periods of time before adding the substrate and determining the enzyme activity. The activity of FAE in the presence of condensed tannins was expressed as a percentage of activity observed for the control samples. A quadratic relationship was observed between the time allowed for reaction between the condensed tannins and FAE and FAE's activity ($R^2 = 0.706$). Thus, when the reaction time was 0 and 10 minutes, the observed reduction in the activity of FAE was 51.6 and 54.1 % of the rate observed for the corresponding control treatment, respectively. On the other hand, when the reaction time was 4 minutes, the reduction in FAE activity was 63.0% in relationship to the activity observed for the corresponding control (no tannins) treatment.

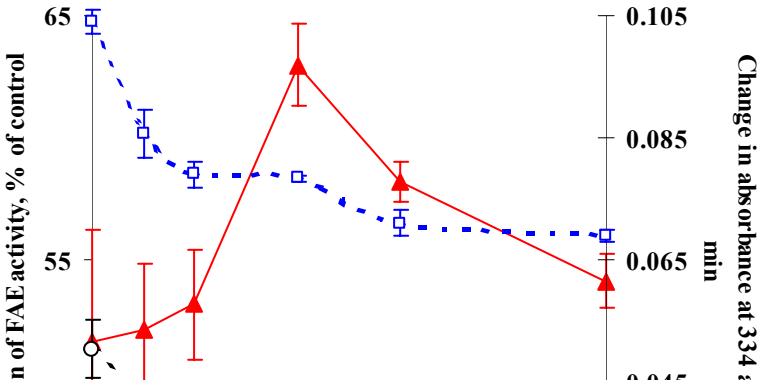

Figure 4.3-12. *Changes in the activity of a recombinant ferulic acid esterase after reaction with condensed tannins from mature leaves of* Desmodium ovalifolium *for discrete periods of time. The tannin to protein ratio used in all determinations was 12.5 (w/w). The curve with the solid line (▲) represent the inhibition in enzyme activity expressed as a percentage of the corresponding control samples, whereas curves with broken lines represent the change in absorbance after 2 min for the control ( ) and tannin treatments (*O*). Error bars represent SEM in each figure where n= 3.*

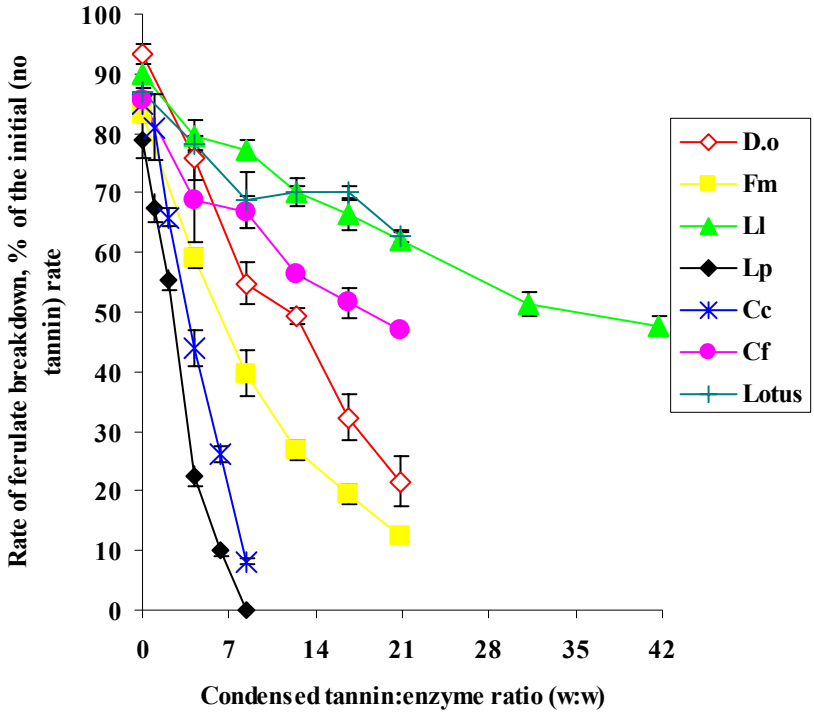

Figure 4.3-13. *Activity of a recombinant ferulic acid esterase in the presence of different concentrations of condensed tannin extracted from mature leaves of six tropical legumes. Sources of tannins were the tropical legumes: Do =* Desmodium ovalifolium*, Fm =* Flemingia macrophylla*, Ll =* Leucaena leucocephala*, Lp =* Leucaena pallida*, Cc =* Calliandra calothyrsus *and Cf =* Clitoria fairchildiana *and the temperate legume* Lotus corniculatus*. Error bars represent SEM in each figure where n= 4. Where error bars are not shown, they are smaller than the size of the symbols.*

By far, the greatest variation in the ability of the condensed tannins to inhibit enzymatic activity was observed in the assay with FAE (Figure 4.3-13 and Table 4.3-1). The condensed tannins that were most effective in reducing the activity of this

enzyme came from *L. pallida* and from *C. calothyrsus*, with 10.3 and 17.8 μg ml^{-1} reducing the activity of FAE by 50% (Table 4.3-1). Conversely, 170.8 μg ml^{-1} of condensed tannins from *L. leucocephala* reduced the activity of this enzyme to the same extent (Table 4.3-1). It was interesting to note that condensed tannins extracted from *Lotus sp.* proved to be similarly effective at inhibiting this recombinant enzyme as those from *L. leucocephala*, with additions of 80 and 100 μg of *Lotus* tannin causing reductions of only 30 and 37% in the activity of FAE, respectively. No significant linear regression relationships were observed between molecular weight or monomer composition of condensed tannins and inhibition of FAE activity. The strongest relationships occurred at tannin to protein ratios (w/w) of 16.7 and 20.8 between z-average molecular weight (estimated on the peracetates) and FAE activity ($R^2 = 0.533$, $P < 0.1$).

4.3.4 DISCUSSION

The anaerobic fungi play a very important role in the degradation of fibre in ruminants that are fed highly fibrous diets, as it is the case of most ruminants raised in tropical production systems. The scientific evidence that supports this idea was summarised by Theodorou et al (1996) in a recent review of the rumen anaerobic fungi as follows:

- In fibrous diets, a substantial proportion of the plant fragments in the rumen is rapidly and extensively colonised by anaerobic fungi. Based on these observations, Bauchop (1979) suggested that the role of anaerobic fungi in the rumen would be that of initial colonisers in ligno-cellulose digestion.
- The larger populations of anaerobic fungi are found in ruminants fed highly fibrous diets, as opposed to ruminants fed predominantly concentrates or soft leafy tissue.
- Some anaerobic fungi possess remarkable cellulases. For example, Wood et al. (1986) observed that the cellulase from a coculture of *N. frontalis* and a rumen methanogen was able to solubilize the highly ordered cotton cellulose to the extent of 98% in 72 hours, considerably better that the most active cellulase previously known.

- Anaerobic fungi have been shown to penetrate and preferentially colonise tissues traditionally regarded as resistant to degradation (Akin et al. 1990) and are able to degrade fibrous particles of dissimilar size at similar rates and to a similar extent (Davies, 1991).

The anaerobic fungi that was studied in the present work (*N. hurleyensis*) possesses very potent cellulases (Theodorou et al., 1989; Akin et al., 1990). However, it was observed that the addition of 40 μg ml^{-1} or less of soluble condensed tannins from six tropical legumes resulted in reductions of at least 50% in the activity of *N. hurleyensis* CMCases. Moreover, lower concentrations of soluble condensed tannins were required to inhibit xylanase activity by 50% (5.5 μg ml^{-1} or less, Table 4.3-1). This is in agreement with the observations of Salawu et al. (1998) that xylanase activity from a cell-free preparation of the rumen fungus *N. frontalis* was more affected by a *C. calothyrsus* leaf extract than the corresponding CMCase activity.

Contrary to the CMCases and xylanases data, β-D-glucosidases and β-D-xylosidases were less sensitive to the presence of soluble tannins. Indeed, in only a few cases did the presence of condensed tannins result in reductions of 50% in the activity of these enzymes, even at concentrations of 100 μg ml^{-1}. An observation that is not evident from the figures presented in this chapter is that, at similar concentrations of soluble condensed tannins, β-D-glucosidases were less inhibited than the β-D-xylosidases. For example, at 12.5, 25 and 50 μg $ml^{-1}$of soluble tannins, average inhibition was 8, 16 and 34 % for the β-D-glucosidases and 14, 26 and 40 % for the β-D-xylosidases, respectively.

These observations suggest that digestive enzymes differ in their susceptibility to inhibition by condensed tannins. The interactions involved are complex and given the simplicity of the experimental design used in this study, it is difficult to point at specific factor(s) to explain these differences. For example, because culture filtrates of *N. hurleyensis* were used, it is not possible to comment on the results as they relate to differences in enzyme concentration. The use of the values of total protein content in these culture filtrates to aid in these comparisons would not be meaningful, because only a variable fraction of that protein would be acting in the degradation of a given substrate. On the other hand, because during fibre degradation there is synergy among

the different cellulases and xylanases (Gilbert and Hazlewood, 1993), it would appear that the use of culture filtrates would provide more realistic estimates of fibre degradation in the face of tannins than the use of purified enzymes.

It is possible that the variation in the susceptibility of these fungal enzymes to inhibition by tannins could be due differences inherent to the enzymes themselves. Possible factors include differences in amino acid composition and secondary and tertiary structure and degree of glycosylation among these enzymes. For example, Hagerman and Butler (1980b) observed that proteins associated to sorghum tannins during tannin purification have a high proline content and are highly hydrophobic. These features might enhance the binding of tannins to the proteins. Likewise, Mehansho et al. (1983) observed that tannin-fed rats and mice produce highly hydrophobic, proline-rich salivary glycoproteins for which tannins exhibit high affinities and that protect these animals against the deleterious effects of condensed tannins. Although I lack knowledge on the amino acid composition of the enzymes assayed for in this study, there is little doubt that they differ greatly in these characteristics. It is possible that as observed in many other endoglucanases and xylanases (Gylkes et al., 1991), the CMCases and xylanases of *N. hurleyensis* contain proline-rich sequences that could probably make these enzymes susceptible to the inhibitory actions of condensed tannins (McAllister et al., 1994).

The role played by the degree of glycosylation in determining the binding affinity of tannins for glycoproteins is still unclear. Strumeyer and Malin (1970) observed that a yeast invertase (a glycoprotein) was resistant to inhibition by tannins and suggested that the carbohydrates of glycosylated enzymes may protect these glycoproteins against inhibition by tannins. On the other hand, Asquith et al (1987) observed that in salivary proline-rich glycoproteins, oligosaccharides enhance the affinity and selectivity of binding to tannins. Early reports on the composition of β-$_D$-glucosidases mentioned the existence of a carbohydrate moiety (3 to 6%) in purified preparations of this enzyme from *Stachybotrys atra* and *Aspergillus niger* (Gascoigne and Gascoigne, 1960). Likewise, an extracellular β-glucosidase from the anaerobic fungus *Orpinomyces* sp. was shown to be a monomeric glycoprotein containing 8.55% (w/v) carbohydrate (Chen et al., 1994). On the other hand, the β-$_D$-xylosidase purified from

the anaerobic fungus *N. frontalis* by Garcia-Campayo and Wood (1993) was shown to be a dimer of two polypeptide sub-units.

Other protein characteristics such as their isoelectric point and conformational flexibility can also influence their binding affinities for condensed tannins. Precipitation of many proteins by condensed tannins is greatest within one pH unit of the isoelectric point of the protein (Hagerman and Butler, 1978). In turn, proteins with loose, open conformations have greater affinity for tannins (Asquith et al, 1987). However, besides the question of which mechanistic factors are responsible for our observations, another relevant issue is whether in ruminants fed tanniniferous forages the degradation of xylan continues to be more compromised than that of cellulose. Previous work *in vitro* has consistently shown that among the non-starch polysaccharides from tanniniferous tropical legumes, xylose is the least degradable constituent (Longland et al., 1995; Chapter 3 this thesis). If this is also the case *in vivo*, future work with tanniniferous forage legumes must pay closer attention to the interaction between condensed tannin content and fibre content and composition.

Given that condensed tannins bind not only to proteins but also to other macro-molecules, it is possible that the observed variation in the inhibition of fungal enzymes by tannins could also be related to factors inherent to the substrate hydrolysed by these enzymes. This is not a novel idea. When investigating the proteolysis of bovine serum albumin by trypsin, Mole and Waterman (1987b) concluded that tannic acid deprived trypsin of substrate rather than acting directly on the enzyme. All substrates utilised in the assays reported herein were in soluble form. However, those employed in the β-D-glucosidase and β-D-xylosidase assays are of relatively low molecular weights (301.3 and 271.7, respectively). On the contrary, CMC and xylan are substrates of much higher molecular weights, with backbones that vary in their degree of substitution. These factors could increase their affinity for binding to CMC and xylan by condensed tannins in at least two different ways. First, the residues attached to CMC and xylan could lead to stronger tannin-polysaccharide interactions. Second, larger, yet soluble polysaccharides like CMC and xylan used in these assays, could allow for the formation of hydrophobic cavities, a condition which leads to stronger associations with condensed tannins (Ozawa et al., 1987).

Substrate-associated tannins were more effective in preventing the enzymatic degradation of *F. arundinacea* plant cells than tannins in free, soluble form. These results are in agreement with our earlier observations that substrate-associated tannins prevented the *in vitro* degradation of substrates by a mixed population of rumen microorganisms to a greater extent than soluble tannins (see chapter 4.2). The implications of these observations for ruminants fed tanniniferous forages are very important for at least two reasons:

- It has been shown that among tanniniferous tropical legumes there is great variation in the extractability of condensed tannins in aqueous solvents (Jackson et al 1996a, Chapter Four this thesis). Moreover, tannin extractability has been shown to vary with factors as sample maturity (Chapter Four this thesis), genotype x environment interactions (A. Schmidt, pers. comm.) and post-harvest treatment of forages (Cano et al., 1994, Barahona et al., 1996).
- A great proportion of the extractable tannins becomes attached to feed constituents during the mastication of feeds (Burrit et al., 1987; Terrill et al., 1992a) and this is often accompanied by an increase in non-extractable tannins (Terrill et al., 1992a).

Results in this thesis therefore point to the need to determine the true form in which condensed from different tropical legumes occur in the rumen. As discussed in chapter 4.2, the use of tannin extractability in an organic solvent results too simple a measurement to allow accurate description of the nutritional effects of condensed tannins in ruminants.

There were considerable differences in the ability of condensed tannins from different tropical legumes to reduce the activity of the fungal enzymes assayed for in this study. In the majority of the cases, the greatest reductions on the activities of these enzymes were observed with tannins from *D. ovalifolium* and *F. macrophylla*. On the other hand, the lowest reductions were mostly observed with tannins from *L. leucocephala*. This is in agreement with our previous observations that among the condensed tannins tested in this study, those from *L. leucocephala* were the least effective in reducing the fermentation of substrates by a mixed population of rumen microorganisms (Chapter 4.2 this thesis).

In the present study, the molecular weight of the condensed tannins assayed was found to be more related to the capacity of tannins to inhibit these fungal enzymes than the monomeric composition of condensed tannins. Determination of the molecular weight of tannins extracted from mature leaves of *L. leucocephala* showed them to be of only 2360 Dalton. All other tannins were found to have number-average molecular weights greater than 3500 Dalton (see chapter 4.1 this thesis). Previous research has suggested that the biological activity of condensed tannins is strongly related to their molecular weight (Jones et al., 1976; Oh and Hoff, 1979; Kumar and Horigome, 1986; Cano et al., 1994). Given the potential variation in monomeric composition and the small number of tannin samples assayed in this study, our data does not necessarily proves that the impact of this variable towards the biological activity of tannin is unimportant. A greater survey of tannin sources is required to elucidate the impact of this aspect of tannin biochemistry.

It was interesting to note that in all assays, the impact of molecular weight was influenced by tannin concentration. In most assays, except for the one for xylanase activity, the relationship between tannin molecular weight and enzymatic activity in the presence of tannins was maximized at the higher tannin concentrations used. This appears to suggest the existence of a threshold for tannin concentration, below which the ability of tannins to inhibit these digestive enzymes cannot be predicted in terms of tannin molecular structure. On the other hand, inhibition of xylanase activity was dependent upon tannin structure at the lowest tannin concentration used in this assay. This is probably the result of the high susceptibility to inhibition by tannins that was demonstrated for this enzyme. As the concentration of tannin increased, it was no longer possible to differentiate between the effects of the different tannins used in the xylanase assay. This is not necessarily in contradiction of the observation of a threshold value for tannin concentration in all other enzymatic assays. It is simply the case that this value was too low in the case of the xylanases. Moreover, this observation further suggests that beyond that threshold value, the impact of tannin structure upon their ability to inhibit digestive enzymes becomes negligible.

Tannins from the temperate legume *Lotus* sp. proved to be about as efficient at inhibiting the activity of FAE as condensed tannins from *L. leucocephala*. This was interesting to note given that condensed tannins from *Lotus* are considered to be good

in terms of animal nutrition (Waghorn et al., 1987, McNabb et al., 1998) and among all the condensed tannins from tropical legumes tested here, those from *L. leucocephala* have been consistently shown to be the more benign. Barry (1989) suggested that for *Lotus sp.*, 20-40g of condensed tannins kg^{-1} of dry matter is the concentration of forage condensed tannins that will improve the efficiency of protein utilization without depressing rumen fiber digestion and voluntary intake. In view of the results presented here, this value could be even lower for the condensed tannins of most tropical legumes.

Even though tannins characteristics explain a great deal of the observed differences in the ability of condensed tannins to inhibit fungal enzymes, our data also points to the fact that tannin-enzyme interactions are very specific and are probably related to characteristics of the three components (tannin, enzyme and substrate) involved. To illustrate, condensed tannins from *L. pallida* were among the most effective inhibitors of β-D-glucosidases and of the recombinant FAE. However, these condensed tannins were also the least effective against the β-D-xylosidases. Similar shifts in the specificity of inhibition toward the different enzymes were observed for condensed tannins from *D. ovalifolium* and *C. calothyrsus* (all other enzymes versus FAE). A further illustration of this was provided in the assay where plant cells were used as substrate. These results aptly illustrate the need to further investigate the role of condensed in tannin-fed ruminants under the scope of the structure-activity relationships. This would also include the interactions between content and type of condensed tannins and composition of the fibre. This might be of vital importance in understanding the effects of the supplementation of grass-fed ruminants with tanniniferous legumes or when studying the co-feeding of non-tanniniferous and tanniniferous forages.

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5. GENOTYPE X ENVIRONMENT INTERACTIONS IN THE TROPICAL LEGUME DESMODIUM OVALIFOLIUM

5.1 EFFECT OF GENOTYPE AND ENVIRONMENT ON FORAGE QUALITY AS DETERMINED BY GAS PRODUCTION

5.1.1 INTRODUCTION

Condensed tannins are very important in a range of forage legumes as their presence can have positive or negative impact on ruminant nutrition. Research reported in the previous chapters of this thesis has suggested that the nutritional impact of condensed tannins is a function of both the tannin concentration in plant tissue and of tannin structural chemistry. The concentration of condensed tannins in plant tissue has been shown to vary with many factors. These include plant species (Jackson et al., 1996a; Chapter 3 this thesis), plant part (Foo et al., 1982, Barahona et al., 1997), plant maturity (Lees et al., 1995), growing season (Clarke et al., 1939; Donnelly, 1959, Feeny, 1970), soil fertility (Barry and Forss, 1983), growth temperature (Fales, 1984, Carter et al., 199), CO_2 concentration and drought stress (Carter et al. 1999). For example, Anuraga et al. (1993) observed that a combination of moisture stress and high temperature resulted in an increase in condensed tannin concentrations in *Lotus pedunculatus*. In turn, Barry and Forss (1983) observed that the condensed tannin concentration was 2.5 times higher when *L. pedunculatus* was grown in low-fertility, acid soils than when it as grown in high fertility soils.

With regard to structural chemistry, it has been shown that both the molecular weight and the monomer composition of condensed tannins vary greatly according to factors such as species and plant organ (Foo and Porter, 1980; Foo et al., 1982; Williams et al., 1983) and sample treatment (Cano et al., 1994). Additionally, it was observed that the molecular weight of condensed tannins from sorghum increased as the growth season progressed (Butler, 1982). However, little or no work has been conducted to examine the changes in condensed tannin structure in tanniniferous plants growing under different environmental conditions.

Desmodium ovalifolium Wallep [synonymous *D. heterocarpum* (l.) DC subspecies *ovalifolium* (Prain.) Ohashi (Ohashi (1991)] is a tropical legume native to Southeast

Asia. It has a prostrate, stoloniferous growth habit, which not only allows this legume to provide effective protection against soil erosion and tolerate heavy grazing, but also to form persistent associations with very aggressive grasses of similar growth habit (i.e. *Brachiaria spp*.). Additionally, *D. ovalifolium* is drought and shade-tolerant (Suarez, 1985) and performs very well when planted in low-fertility acid soils (Grof, 1982). However, the adoption of *D. ovalifolium* as a forage legume by farmers in tropical America has been poor largely due to the apparent low palatability and acceptability of this legume by livestock. This has been attributed to the to presence of high levels of condensed tannins in this legume (Lascano and Salinas, 1982). Nonetheless, there is anecdotal evidence that in certain regions, the presence of *D. ovalifolium*, alone or in association with grasses, leads to improved animal production. It has certainly been observed that in the Colombian Orinoquia, animals grazing on mixtures of *Brachiaria* and *D. ovalifolium* had greater liveweight gains than those grazing on the *Brachiaria* alone (Perez, 1997). Likewise, it has been shown that *D. ovalifolium* responds well to fertilizer, and the application of sulphur improves the nutritional quality of the legume (Lascano and Salinas, 1982; Perez, 1997). Perhaps, as observed with *L. pedunculatus,* this increase in forage quality in *D. ovalifolium* corresponds to a decrease in condensed tannin concentration.

The above observations prompted investigators from the University of Hohenheim, Stuggart, Germany and from the Centro Internacional de Agricultura Tropical (CIAT) in Cali, Colombia to undertake an investigation into the genotype-environment interactions in *D. ovalifolium*. In this study, financed by the German Agency for Technical Co-operation (GtZ) and conducted by Axel Schmidt (PhD student, University of Hohenheim), a selection of 18 accessions of *D. ovalifolium* was planted at two fertilizer levels in six sites in four contrasting environments in Colombia. Through a special agreement, samples from five of the 18 accessions were made available for this study in order to investigate the changes in nutritional quality brought about by changes in condensed tannin concentration and structure. This chapter reports the changes in forage quality as determined by the pressure transducer technique of Theodorou et al. (1994) and also the preliminary attempts to use near infrared reflectance spectroscopy (NIRS) to predict gas production in these samples.

5.1.2 MATERIALS AND METHODS

5.1.2.1 General experimental design of the agronomic experiment

A collection of 18 accessions of *D. ovalifolium* were selected from the World collection (approximately 160 accessions) and used for this study, The criteria for their selection is depicted in Table 5.1-1.

Table 5.1-1. *Collection of accessions used for the study of the genotype-environment interactions in the tropical legume* Desmodium ovalifolium

Main criteria for selection	Origin[1]	Selected accessions (CIAT numbers)
Origin	IDN	23195, 23665, 23672
	THA	13655, 13086, 33058
	LAO	23618
Controls	?	350 [commercial accession]
	THA	13089 [widely evaluated]
Early flowering	THA	3788
High seed production	THA	13647
Rapid establishment	MYS, THA	13105, 13125
High crude protein content	MYS	13305
High soluble nitrogen content	THA	3793
Low condensed tannin content	MYS	13110
High digestibility	THA	13030, 13651
High relative acceptability	MYS	13105, 13305

1 IDN = Indonesia, MYS = Malaysia, THA = Thailand, LAO = Laos

Adapted from Schultze-Kraft (1997)

The eighteen accessions were established in six sites in four contrasting environments in Colombia. In the *well-drained savanna* of the Colombian Llanos there were two sites: *Alcancia* and *Maquenque*, both located in Carimagua, a research station shared by the Corporacion Colombiana de Investigacion Agricola (CORPOICA, Colombian Institute for Agricultural Investigation) and the Centro Internacional de Agricultura Tropical (CIAT, International Centre for Tropical Agriculture). In the *humid tropic* there were two other sites: *Macagual* located in CORPOICA's Macagual Research Station and *La Rueda*, a farm owned by Agroganadera del Valle, a commercial firm. The other two sites were *Cauca*, representative of a *dry hillsides* environment, located in El Melcho, Cauca and *Chinchina*, typical of the *wet hillsides* environment, located

at La Romelia Research Station of the Centro Nacional de Investigaciones de Café (CENICAFE, National Centre for Coffee Research), Chinchiná, Caldas. Climatic characteristics and soil chemistry on the chosen sites are depicted in Table 5.1-2.

Table 5.1-2. *Characteristics of the experimental sites chosen for the study of the genotype-environment interactions in the tropical legume* Desmodium ovalifolium

Characteristic	*Hillsides*		*Humid tropic*		*Savanna*	
	Cauca	Chinc[1]	Macag[1]	Rueda	Alcan[1]	Maque[1]
Altitude (m.a.s.l.[2])	1555	1360	150	150	190	180
Precipitation (ml per year)	1800	2600	2300	2300	3500	3500
Minimum temperature (°C)	14.2	16.7	21.2	21.2	22.3	22.3
Max. temperature (°C)	24.1	26.4	31.1	31.1	30.4	30.4
Mean temperature (°C)	18.4	21.0	26.1	26.1	26.3	26.3
Soil characteristics (0-20 cm)[3]						
Organic matter (%)	19.50	14.14	3.15	5.75	4.20	1.75
PH	5.05	4.95	4.50	3.95	3.50	4.05
Phosphorus (ppm)	1.15	1.90	3.00	3.65	2.35	1.70
Aluminium (mE/100 g)	0.25	0.60	1.04	16.49	3.10	1.30
Calcium (mEq/100 g)	1.68	2.78	1.34	1.39	0.25	0.13
Magnesium (mEq/100 g)	0.50	0.86	0.55	1.16	0.11	0.04
Potassium (mEq/100 g)	0.30	0.20	0.22	0.38	0.09	0.04
Sulphur (ppm)	219.80	37.00	18.95	16.40	22.40	24.30
Boron (ppm)	0.19	0.45	0.26	0.63	0.19	0.18
Zinc (ppm)	2.30	1.95	1.56	2.31	1.05	0.56
Manganese (ppm)	8.08	11.68	101.16	84.59	3.28	0.63
Copper (ppm)	0.12	0.17	0.88	1.31	0.54	0.17
Iron (ppm)	5.14	5.83	27.39	49.83	45.45	33.62
Aluminium saturation (%)	9.48	13.42	33.15	84.92	88.10	86.82
Sand (%)	48.28	61.09	34.49	15.61	8.16	45.22
Limous (%)	34.68	25.26	29.55	14.59	49.96	30.36
Clay (%)	17.04	13.65	35.96	69.80	42.38	24.42

[1]Chinc = *Chinchina*, Macag = *Macagual*, Alcan = *Alcancia*, Maque = *Maquenque*

[2]m.a.s.l. = meters above sea level

[3]Values portrayed are the average of determinations made on soils samples taken during the dry and rainy seasons and receiving the low fertilizer treatment

After the preparation of the plots (6 x 5 m), sexual seeds were planted by hand, leaving 50 cm between rows and plants. There were a total of 108 plots per site (18 accessions x 2 fertilizer levels x 3 replicates per treatment combination). In each plot

there were 80 planting sites, with each planting site receiving four seeds. After plants emerged and to differentiate between the effects related to temperature and those related to soil acidity and fertility, two fertilizer treatments (high and low) were included in all sites. The low fertilizer treatment represented a minimal level of nutrients, adjusted for all sites, to ensure that plants would grow and produce sufficient dry matter to carry the necessary quality tests. The high fertilizer treatment, also adjusted for the conditions in each site, was designed to completely eliminate any stress related to soil fertility and thus to promote growth at near maximum levels. The fertilizer treatments per site are presented in Table 5.1-3.

Table 5.1-3. *Fertilizer treatments applied to each site*

Environment	Fertilizer[1]	Phosphor[2]	Potassium	Calcium	Sulphur	Micro-mineral
		Kilograms hectare^{-1}				
Dry hillsides	Low	10	20	150	5	-------
	High	50	50	500	20	2.5 Zn, 0.3 B
Wet hillsides	Low	------	------	------	------	-------
	High	50	50	500	20	2.5 Zn, 0.3 B
Humid tropic	Low	------	------	------	------	-------
	High	50	50	500	20	2.5 Zn, 0.3 B
Savanna	Low	10	20	150	5	-------
	High	50	50	500	20	2.5 Zn, 0.3 B

[1]Fertlizer applied after first weeding (i.e. approximately four weeks after seeding). Sulphur was applied every year following the standardisation cut.

[2]Phosphor = phosphorus, Zn = zinc, B = boron.

For the study reported in this thesis, samples used came from accessions 350, 3788, 13125, 23618 and 33058. The main criteria for their selection were the differences in condensed tannin content observed in a preliminary evaluation of samples harvested from the *Chinchiná* site under the high fertilizer treatment. Harvest of these materials took place following a standardisation cut, and included re-growth samples of six (rainy season) and eight (dry season) weeks of age. Stem-free leaf samples were immediately frozen (-20°C), freeze-dried to dryness and then ground to pass a 1-mm dry mess screen. In order to reduce the number of samples, replicates within each treatment were combined, thus generating 120 different samples (five accessions x six sites x two fertilizer levels x two harvesting seasons).

5.1.2.2 *In vitro* dry matter digestibility and chemical analysis of the samples

The *in vitro* dry matter digestibility (IVDMD) of all samples was determined at the Forage Quality Laboratory, CIAT according to the two-stage technique of Tilley and Terry (1963). Rumen fluid was obtained from two Brahman steers grazing on a star grass (*Cynodon dactylon*) pasture and supplemented daily with a soy-based concentrate. Samples were also analysed for crude protein content at the Analytical Services laboratory of CIAT using an Skalar auto-analyser. Neutral detergent and acid detergent fibre was determined on separate samples at the Forage Quality laboratory of CIAT following the procedures outlined by Van Soest et al. (1991). Nitrogen bound to the residue of acid detergent extraction was also determined using the auto-analyser technique. The amount of condensed tannins extractable and non-extractable in 70 % aqueous acetone was determined according to a modification of the method of Terrill et al (1992b) as outlined in Section 3.2.2 of this thesis. These determinations, with the exception of the protein analysis, were performed in triplicate samples.

5.1.2.3 *In vitro* gas production experiments

Gas production experiments were performed at CIAT's Forage Quality Laboratory according to the method of Theodorou et al. (1994) as described in Section 3.2.3. Rumen liquor was obtained from the same donor animals that provided the inoculum for the IVDMD determination. Given the high number of samples, analyses were performed in singular (1-g sub-samples), with measurements for the same sample being repeated in a separate gas production run. In total, five different gas production runs were required to include the duplicate determinations of all 120 samples. At least four blanks were included in each gas production run. In all runs, the fermentation was allowed to proceed for 144 h with gas measurements taken at 3, 6, 9, 12, 24, 33, 48, 60, 72, 96 and 144 h post-inoculation.

5.1.2.4 Preliminary evaluation of the potential of near infrared reflectance spectroscopy (NIRS) to predict gas accumulation

A NIRSystem 6500 (NIRSystems, Silver Spring, MD) monochromator was used to collect near infrared spectra from 1100 to 2498 nm as log 1/*R*, where *R* = reflectance. Ninety of the 120 samples were used to develop predictive equations with the

remaining 30 samples being used to validate the resulting equations. Modified partial least squares with cross validation was used to develop predictive equations for gas accumulation at 6, 12, 24, 33, 48, 72 and 144 h post-inoculation. Standard Normal Variate transformation and the Detrend option of the ISI software was used for scatter correction of the near infrared reflectance spectra. The prediction equations obtained will be used to estimate gas accumulation profiles in all 1226 samples collected in the agronomic experiment described in Section 5.1.2. Further work is needed for the reliable application of this technique, and thus, these results will be reported later.

5.1.2.5 Statistical analysis

Gas production data were fitted to the model of France et al. (1993) as described in Section 3.2.5. The relationships between tannin and nutrient content with gas accumulation were investigated using linear regressions.

5.1.3 RESULTS

5.1.3.1 Chemical composition of the samples

The chemical composition of the five accessions of *D. ovalifolium* varied in response to the different variables (site, soil fertility and harvest season). The results are depicted pictorially as this allows for easier illustration of the influence of the growth variables on nutrient content. Given the high number of samples (120) it was necessary to graph the changes observed for each measured constituent.

Protein: The mean crude protein content of the five accessions in each combination of planting site and fertilizer treatments is shown in Figures 5.1-1a and 5.1-1b. During the dry season, all samples considered, the protein content (g kg^{-1} of dry matter) ranged from 105 to 220. In turn, protein content (average of the five accessions) was high in plants grown under high fertilizer in the well-drained savanna (*Maquenque* 174, *Alcancia* 192). On the other hand, plants grown in *La Rueda* had a mean protein content of only 127 g kg^{-1} of dry matter (see Figure 5.1-1a). Besides the marked increment observed for the samples originating from *Alcancia* and *Maquenque*, where mean increases in protein content were in the order of 50 and 31 g kg^{-1} of dry matter, improved soil fertility had little effect on protein content in samples from the remaining four sites.

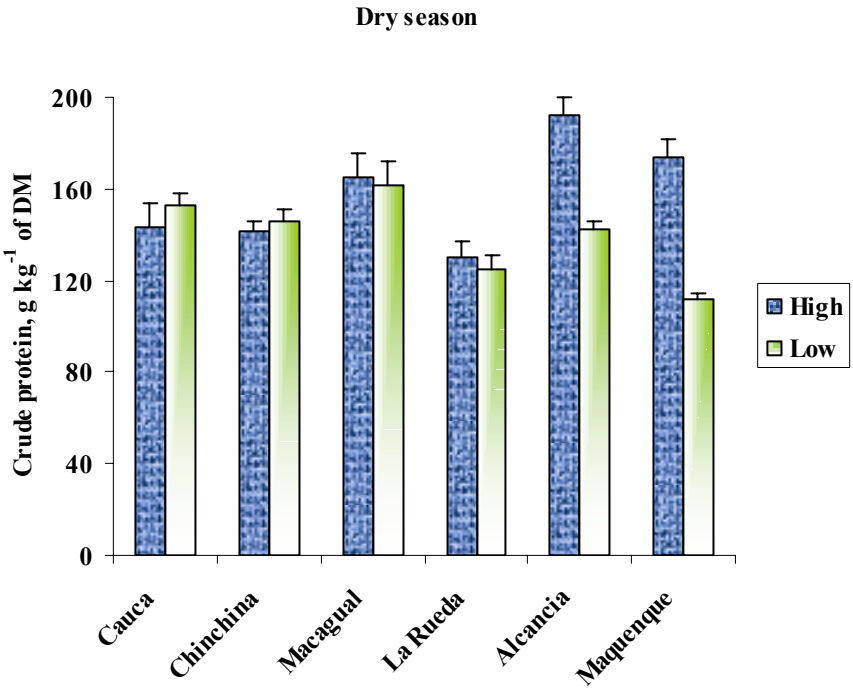

Figure 5.1-1a. *Environment-related variation in the crude protein content of five* Desmodium ovalifolium *accessions planted at two fertilizer levels in four environments in Colombia and harvested during the dry season (eight weeks re-growth).* Cauca = *dry hillsides,* Chinchiná = *wet hillsides;* Macagual *and* La Rueda = *humid tropics and* Alcancia *and* Maquenque = *well-drained savanna. Bars represent SEM where n= 5.*

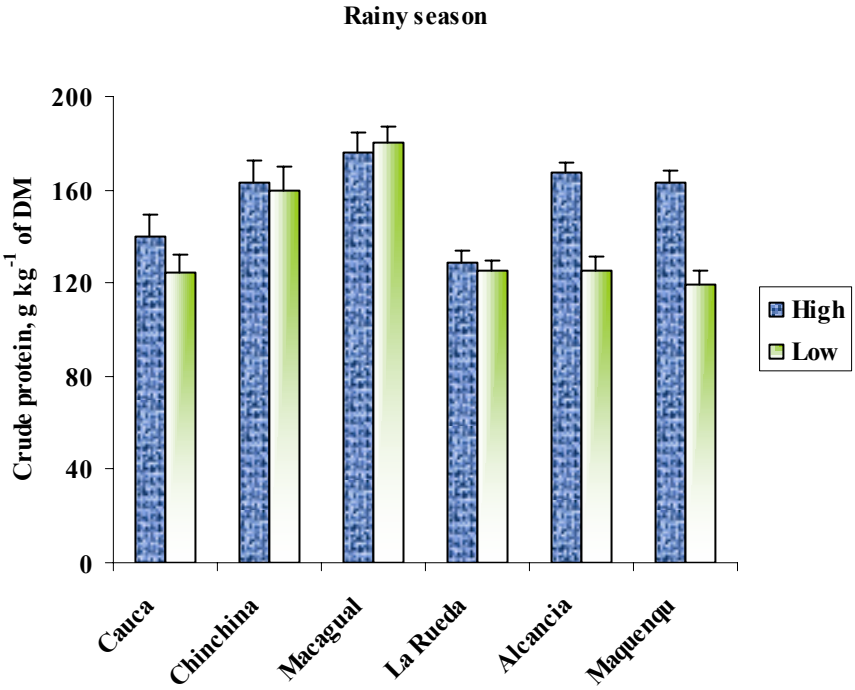

Figure 5.1-1b. *Environment-related variation in the crude protein content of five* Desmodium ovalifolium *accessions planted at two fertilizer levels in four environments in Colombia and harvested during the rainy season (six week re-growth).* Cauca = *dry hillsides,* Chinchiná = *wet hillsides;* Macagual *and* La Rueda = *humid tropics and* Alcancia *and* Maquenque = *well-drained savanna. Bars represent SEM where n= 5.*

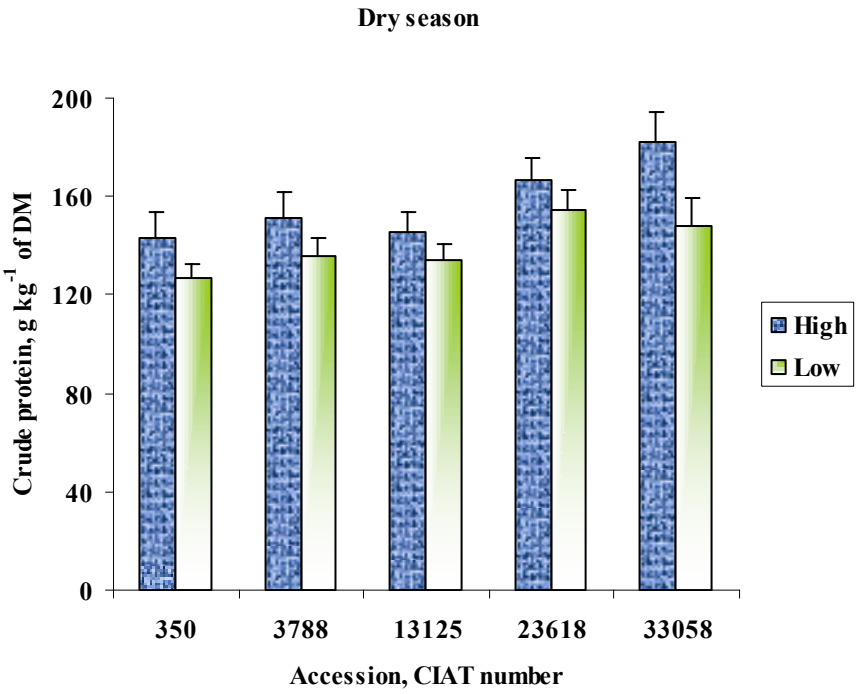

Figure 5.1-1c. *Genotype-related variation in the crude protein content of five* Desmodium ovalifolium *accessions planted at two fertilizer levels in six sites (four environments) in Colombia and harvested during the dry season (eight weeks re-growth). Bars represent SEM where n= 6.*

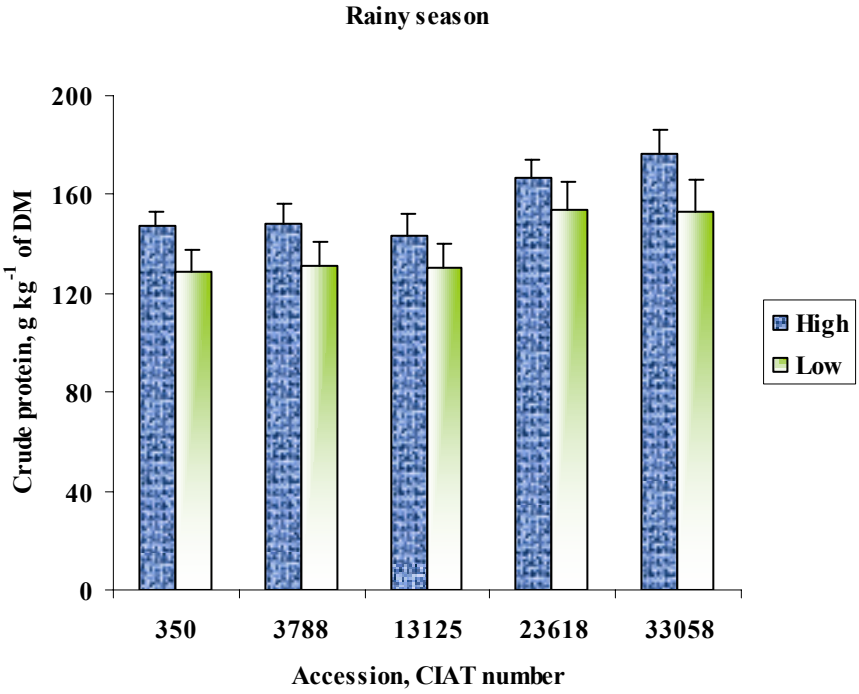

Figure 5.1-1d. *Genotype-related variation in the crude protein content of five* Desmodium ovalifolium *accessions planted at two fertilizer levels in six sites (four environments) in Colombia and harvested during the rainy season (six weeks re-growth). Bars represent SEM where n= 6.*

During the rainy season, all samples considered, the protein content ranged from 107 to 207 g kg^{-1} of dry matter. In turn, the mean protein contents (averaged by accession and fertilizer for each site) were highest in plants grown in *Macagual* (178) and lowest in those grown in *La Rueda* (127; see Figure 5.1-1b). As observed in the samples harvested in the dry season, high fertilizer was associated with a increase of 42 g kg^{-1} of dry matter in the protein content of the accessions grown in *Alcancia* and *Maquenque* (well-drained savanna). Differences in protein content attributable to fertilizer were negligible in plants grown in the other four sites.

The genotype-related variation in protein content (averaged across planting sites) is shown in Figure 5.1-1c for dry season samples and 5.1-1d for rainy season samples. In both sets of samples, mean protein content was higher in accession CIAT 33058 and lower in accessions CIAT 350 and CIAT 13125. In all accessions, mean protein content was higher in plants grown under high fertilizer as compared to those grown under low fertilizer.

Neutral detergent fibre: In plants harvested during the dry season, the content of neutral detergent fibre (g kg^{-1} of dry matter) ranged from 410 to 562. Mean neutral detergent fibre, averaged by accessions and fertilizer for each site, was highest in samples from *La Rueda* and lowest in those from *Chinchiná* (531 and 439 g kg^{-1} of dry matter, respectively; see Figure 5.1-2a). fertilizer had no effect on the mean content of neutral detergent fibre content observed in plants harvested during the dry season.

In plants harvested during the rainy season, the content of neutral detergent fibre ranged from 375 to 551 g kg^{-1} of dry matter. Mean neutral detergent fibre, averaged by accession and fertilizer for each site, was highest in samples from *La Rueda* and lowest in samples from *Chinchiná* (498 and 407, respectively; Figure 5.1-2b). Mean content of neutral detergent fibre in plants grown in *Cauca*, *Chinchiná* and *La Rueda* was not affected by the fertilizer treatments applied. On the other hand, mean increases in the content of neutral detergent fibre of 33, 35 and 53 g kg^{-1} of dry matter were associated with the high fertilizer treatment in samples from *Macagual*, *Maquenque* and *Alcancia*, respectively.

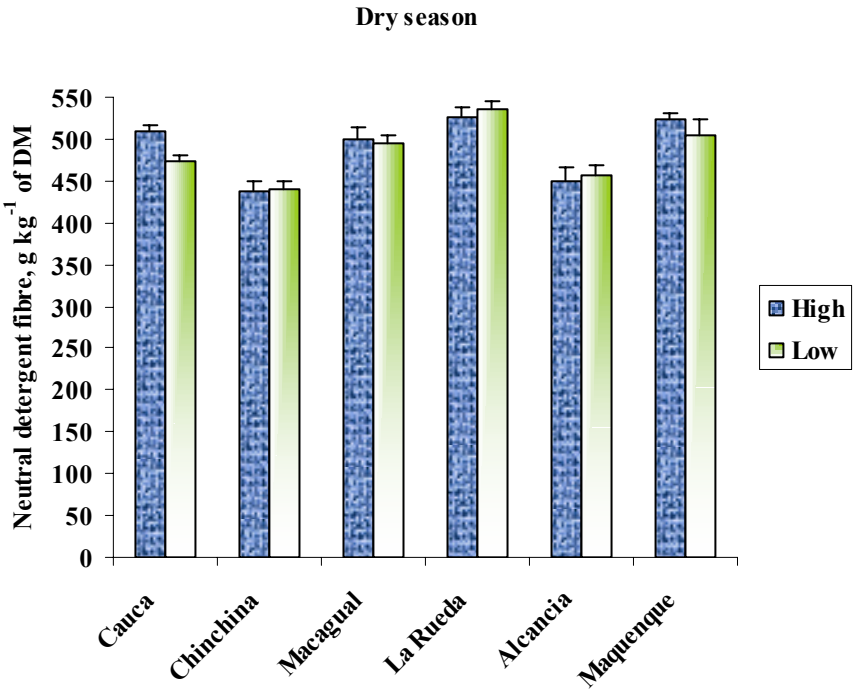

Figure 5.1-2a. *Environment-related variation in the mean neutral detergent fibre content of five* Desmodium ovalifolium *accessions planted at two fertilizer levels in four environments in Colombia and harvested during the dry season (eight weeks re-growth).* Cauca = *dry hillsides,* Chinchiná = *wet hillsides;* Macagual *and* La Rueda = *humid tropics and* Alcancia *and* Maquenque = *well-drained savanna. Bars represent SEM where n= 5.*

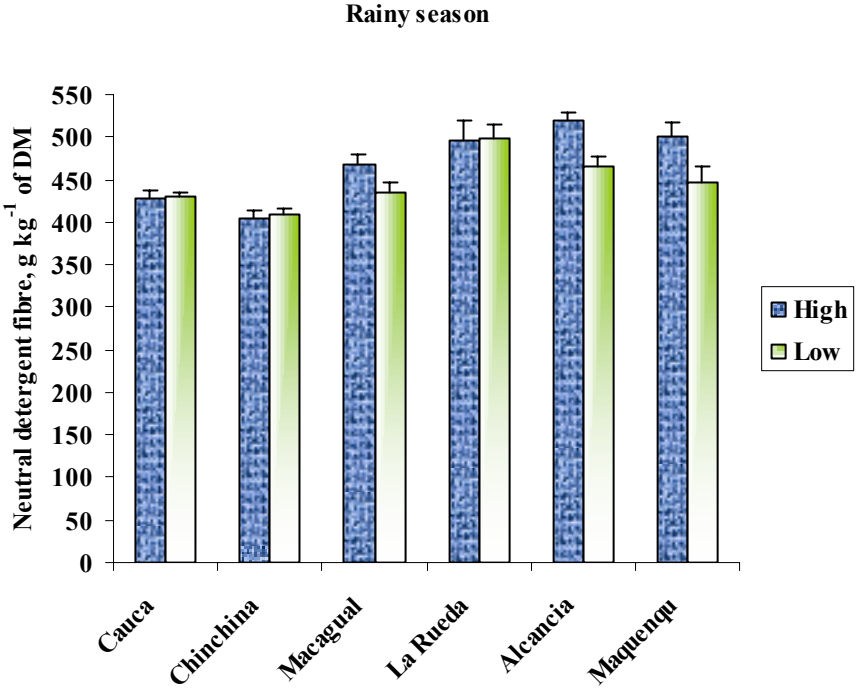

Figure 5.1-2b. *Environment-related variation in the mean neutral detergent fibre content of five* Desmodium ovalifolium *accessions planted at two fertilizer levels in four environments in Colombia and harvested during the rainy season (six weeks re-growth).* Cauca = *dry hillsides,* Chinchiná = *wet hillsides;* Macagual *and* La Rueda = *humid tropics and* Alcancia *and* Maquenque = *well-drained savanna. Bars represent SEM where n= 5.*

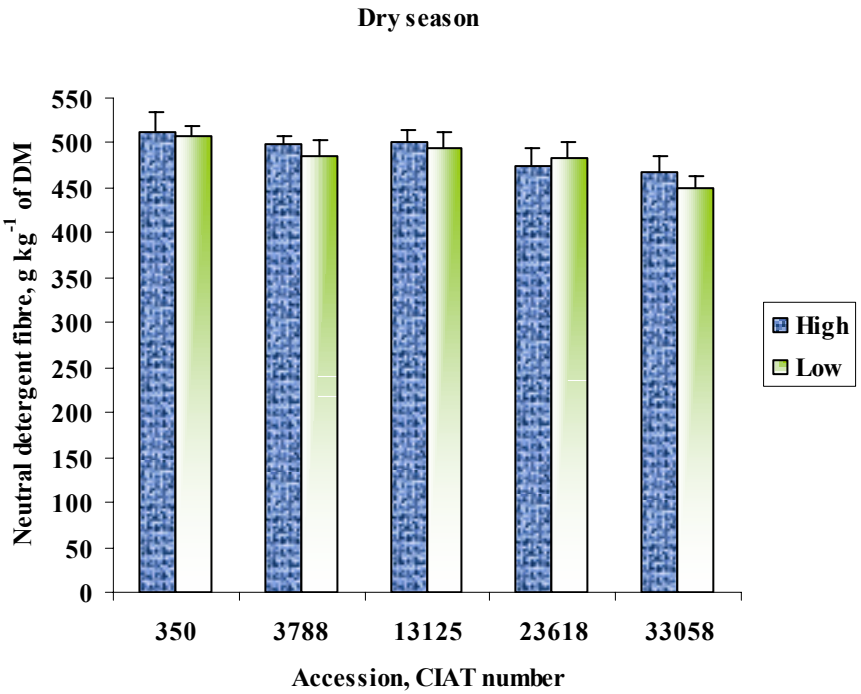

Figure 5.1-2c. *Genotype-related variation in the neutral detergent fibre content of five* Desmodium ovalifolium *accessions planted at two fertilizer levels in six sites (four environments) in Colombia and harvested during the dry season (eight weeks re-growth). Bars represent SEM where n= 6.*

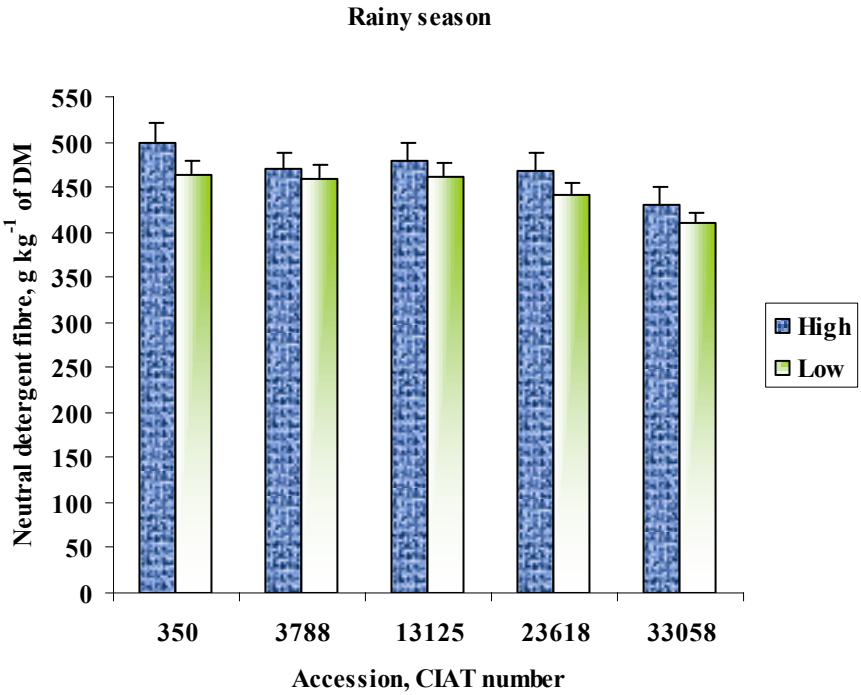

Figure 5.1-2d. *Genotype-related variation in the neutral detergent fibre content of five* Desmodium ovalifolium *accessions planted at two fertilizer levels in six sites (four environments) in Colombia and harvested during the rainy season (six weeks re-growth). Bars represent SEM where n= 6.*

Regardless of harvesting season, the mean content of neutral detergent fibre (averaged by site and fertilizer) was highest in accession CIAT 350 and lowest in accession CIAT 33058. Indeed, for plants harvested during the dry season, mean content of neutral detergent fibre (g kg^{-1} of dry matter) was 512 for accession CIAT 350 and 459 for accession CIAT 33058 (Figure 5.1-2c). For rainy season plants, mean values were 481 and 420, respectively (see Figure 5.1-2d). When grouped by harvesting season, dry-season plants had higher content of neutral detergent fibre than rainy-season plants (488 vs. 458 g kg^{-1} of dry matter, respectively).

Acid detergent fibre: The content of acid detergent fibre in plants harvested during the dry season ranged from 238 to 452 g kg^{-1} of dry matter. When averaged by accession and fertilizer, plants grown in *La Rueda* were found to have the highest contents of acid detergent fibre (417 g kg^{-1} of dry matter). Conversely, plants grown in *Cauca* had mean contents of acid detergent fibre of only 299 (see Figure 5.1-3a). In *Alcancia* and *Maquenque,* and compared to those receiving the low fertilizer treatment, plants receiving the high fertilizer treatment had mean decreases of 35 and 40 g kg^{-1} of dry matter in their content of acid detergent fibre. In the rest of the sites, there were no differences in the content of acid detergent fibre attributable to the fertilizer treatment applied

The content of acid detergent fibre in rainy season samples ranged from 237 to 384 g kg^{-1} of dry matter. In turn, mean content of acid detergent fibre (g kg^{-1} of dry matter and averaged by accession and fertilizer) was 296 in plants grown in *Cauca* and 353 in plants grown in *La Rueda* (see Figure 5.1-3b). Differences in content of acid detergent fibre between plants grown under low and high fertilizer in *Alcancia* and *Maquenque* were only 19 and 28 g kg^{-1} of dry matter, respectively. In all other sites, fertilizer had no effect on the content of acid detergent fibre.

In dry-season samples, mean content of acid detergent fibre (averaged across sites and fertilizer regime) was highest for accession CIAT 13125 and lowest for accession CIAT 33058 (362 and 308 g kg^{-1} of dry matter, respectively; Figure 5.1-3c). Similarly, for plants harvested during the rainy season, accessions CIAT 13125 and CIAT 33058 continued to have the highest and the lowest (352 and 285 g kg^{-1} of dry matter, respectively; Figure 5.1-3d) content of acid detergent fibre.

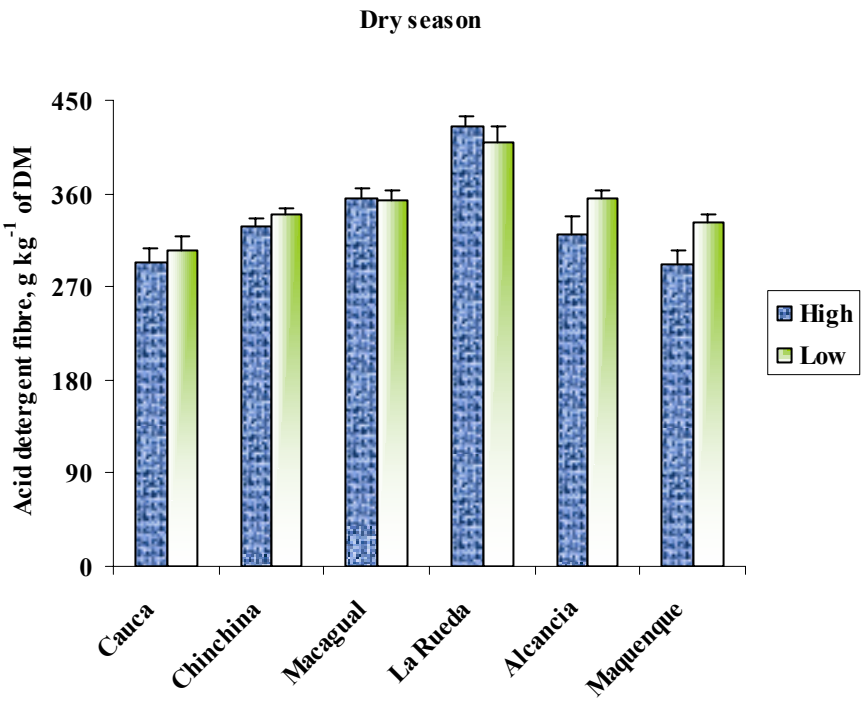

Figure 5.1-3a. *Environment-related variation in the mean acid detergent fibre content of five* Desmodium ovalifolium *accessions planted at two fertilizer levels in four environments in Colombia and harvested during the dry season (eight weeks re-growth).* Cauca = *dry hillsides,* Chinchiná = *wet hillsides;* Macagual *and* La Rueda = *humid tropics and* Alcancia *and* Maquenque = *well-drained savanna. Bars represent SEM where n= 5.*

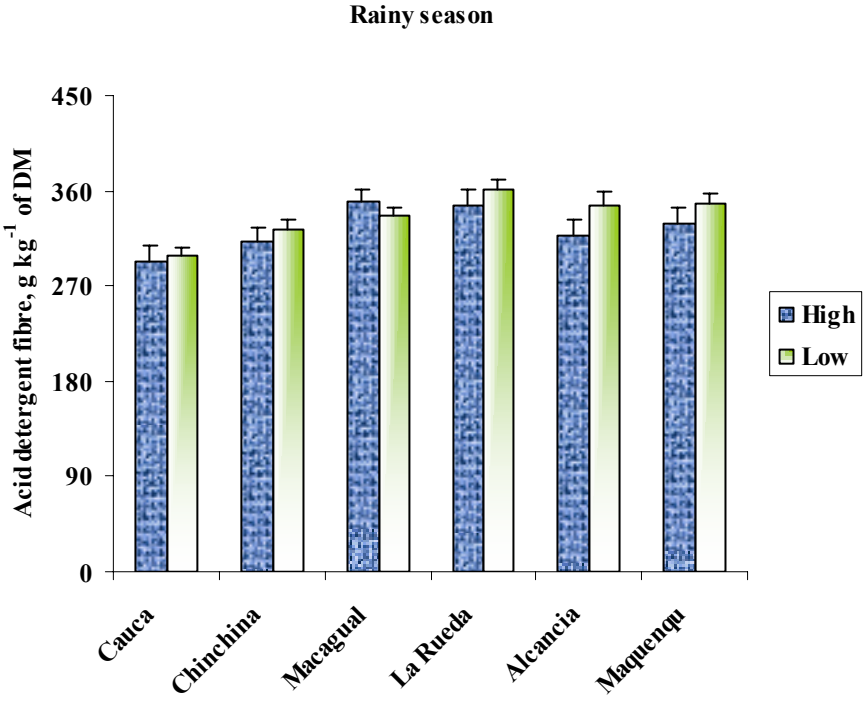

Figure 5.1-3b. *Environment-related variation in the mean acid detergent fibre content of five* Desmodium ovalifolium *accessions planted at two fertilizer levels in four environments in Colombia and harvested during the rainy season (six weeks re-growth).* Cauca = *dry hillsides,* Chinchiná = *wet hillsides;* Macagual *and* La Rueda = *humid tropics and* Alcancia *and* Maquenque = *well-drained savanna. Bars represent SEM where n= 5.*

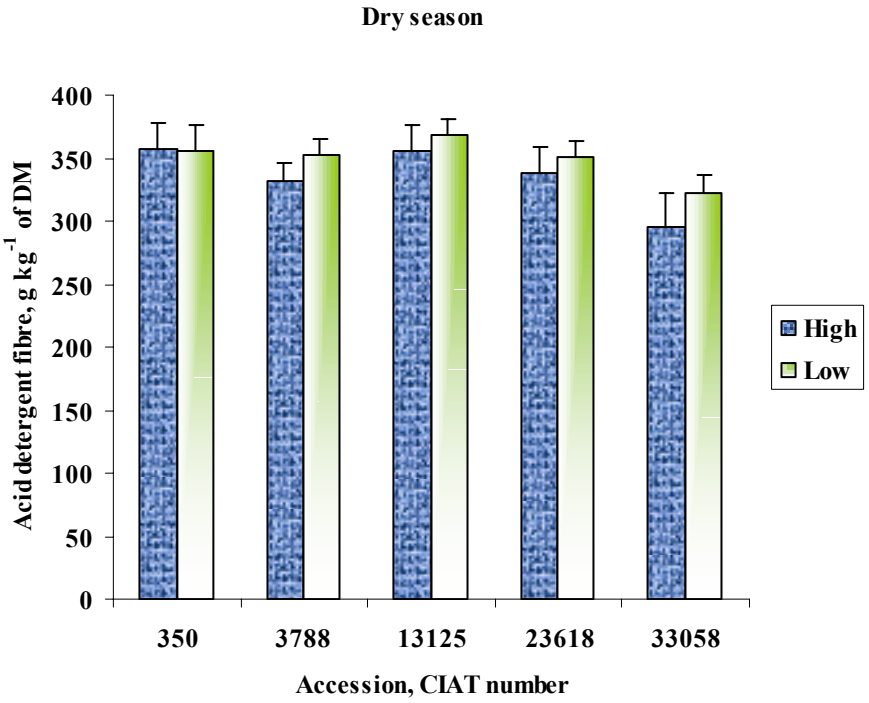

Figure 5.1-3c. *Genotype-related variation in the acid detergent fibre content of five* Desmodium ovalifolium *accessions planted at two fertilizer levels in six sites (four environments) in Colombia and harvested during the dry season (eight weeks re-growth). Bars represent SEM where n= 6.*

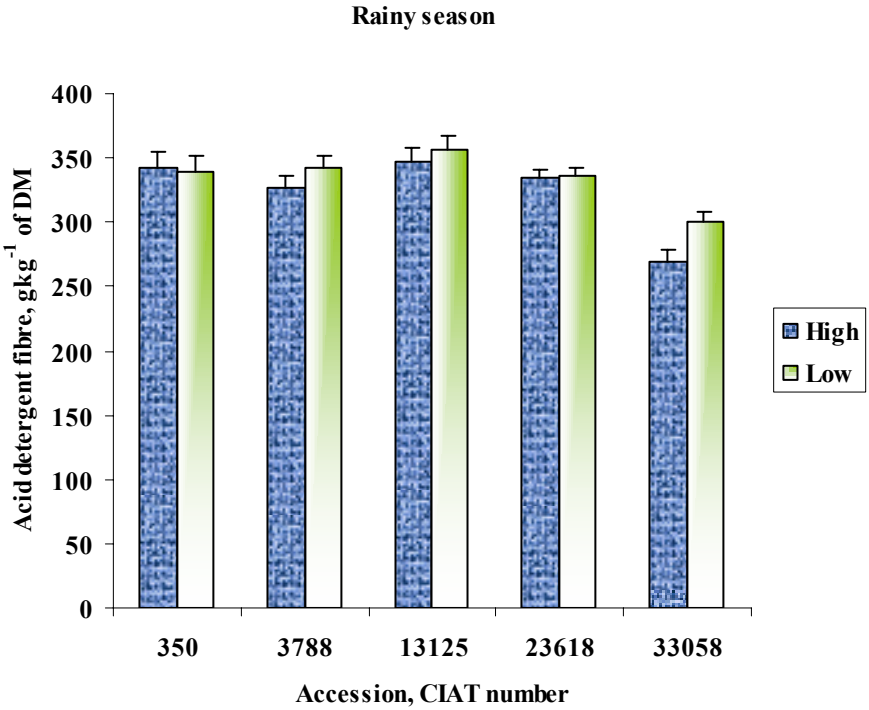

Figure 5.1-3d. *Genotype-related variation in the acid detergent fibre content of five* Desmodium ovalifolium *accessions planted at two fertilizer levels in six sites (four environments) in Colombia and harvested during the rainy season (six weeks re-growth). Bars represent SEM where n= 6.*

During the rainy season, the high fertilizer treatment resulted in a mean decrease of 18 and 28 g kg^{-1} of dry matter in the content of acid detergent fibre of accessions CIAT 3788 and 33058. In the other three accessions, the concentration of acid detergent fibre was unresponsive to fertilizer regime.

Extractable condensed tannins: In plants harvested during the dry season, the content of condensed tannins extractable in 70% aqueous acetone ranged from 42 to 98 g kg^{-1} of dry matter. In turn, mean content of extractable condensed tannins, averaged by accession and fertilizer ranged from 59 in samples from *Maquenque* and *Macagual* to 74 in samples from *Cauca* (see Figure 5.1-4a). The mean concentration of acetone-extractable condensed tannins was largely unresponsive to fertilizer in plants grown in *Cauca*, *Chinchiná* and *Macagual*. On the other hand, high fertilizer was associated with decreases of 12, 25 and 26 g kg^{-1} of dry matter in the concentration of extractable condensed tannin in plants grown in *La Rueda*, *Maquenque* and *Alcancia*.

The content of extractable condensed tannins ranged from 49 to 105 g kg^{-1} of dry matter in samples harvested during the rainy season. Plants grown in *Macagual* had a mean extractable condensed tannin content of 58 g kg^{-1} of dry matter. Conversely, plants grown in *Alcancia* and *Maquenque* had a mean content of extractable condensed tannins of 85 g kg^{-1} of dry matter. In plants grown in *Macagual* under high fertilizer there were increases of nearly 11 g of extractable condensed tannins kg^{-1} of dry matter as compared to those grown under low fertilizer (see Figure 5.1-4b). Conversely, high fertilizer treatment was associated with reductions of 20 and 29 g kg^{-1} of dry matter in the content of extractable condensed tannins in plants grown in *Alcancia* and *Maquenque*, respectively.

The content of extractable condensed tannins was similar among the five accessions studied. In plants harvested during the dry season, content of extractable condensed tannins (g kg^{-1} of dry matter, averaged across sites and fertilizer regime) ranged from 63.9 for accession CIAT 350 and 70.3 for accession CIAT 13125 (Figure 5.1-4c). In all accessions, high fertilizer was associated with a reduction in content of extractable condensed tannins. This reduction averaged 11.3 g kg^{-1} of dry matter and varied from 6.7 (accession CIAT 23618) to 14.7 (accession CIAT 3788).

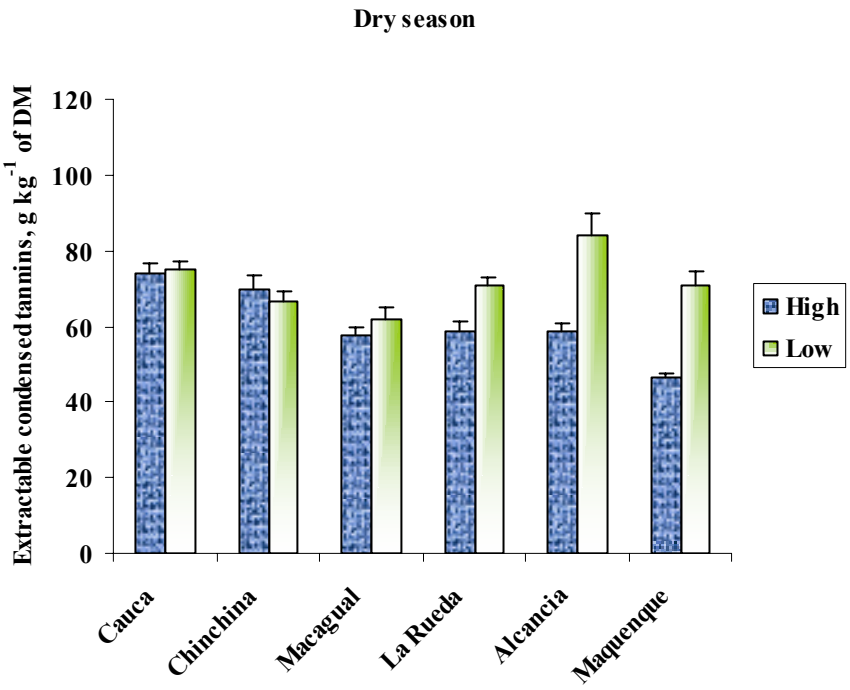

Figure 5.1-4a. *Environment-related variation in the extractable condensed tannin content of five* Desmodium ovalifolium *accessions planted at two fertilizer levels in four environments in Colombia and harvested during the dry season (eight weeks re-growth).* Cauca = *dry hillsides,* Chinchiná = *wet hillsides;* Macagual *and* La Rueda = *humid tropics and* Alcancia *and* Maquenque = *well-drained savanna. Bars represent SEM where n= 5.*

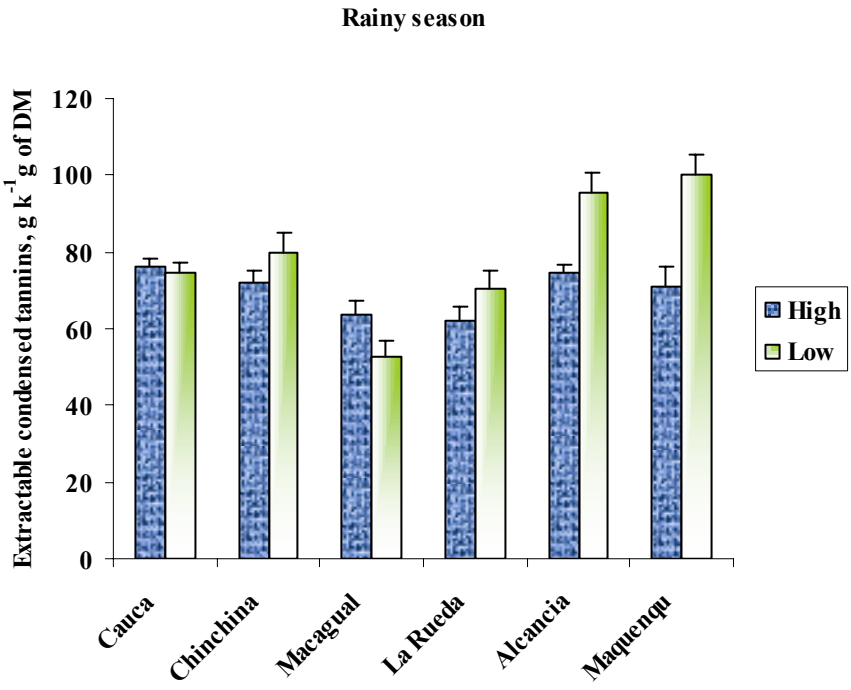

Figure 5.1-4b. *Environment-related variation in the extractable condensed tannin content of five* Desmodium ovalifolium *accessions planted at two fertilizer levels in four environments in Colombia and harvested during the rainy season (six weeks re-growth).* Cauca = *dry hillsides,* Chinchiná = *wet hillsides;* Macagual *and* La Rueda = *humid tropics and* Alcancia *and* Maquenque = *well-drained savanna. Bars represent SEM where n= 5.*

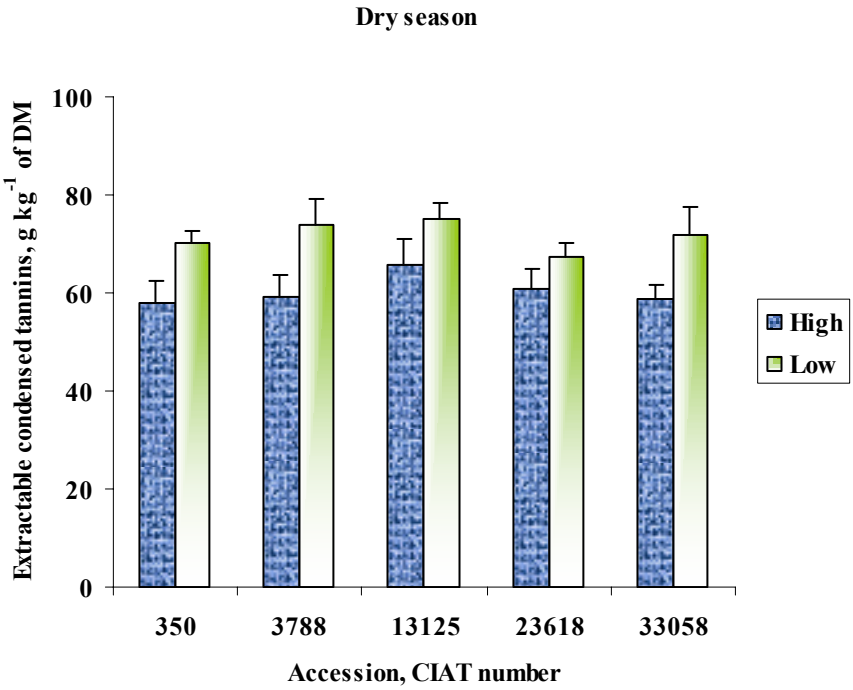

Figure 5.1-4c. *Genotype-related variation in the extractable condensed tannin content of five* Desmodium ovalifolium *accessions planted at two fertilizer levels in six sites (four environments) in Colombia and harvested during the dry season (eight weeks re-growth). Bars represent SEM where n = 6.*

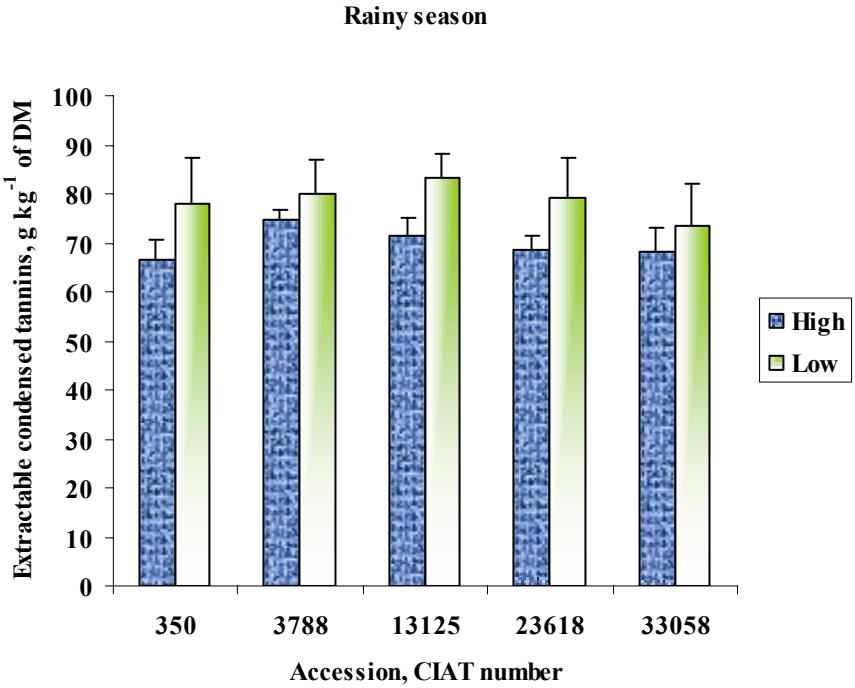

Figure 5.1-4d. *Genotype-related variation in the extractable condensed tannin content of five* Desmodium ovalifolium *accessions planted at two fertilizer levels in six sites (four environments) in Colombia and harvested during the rainy season (six weeks re-growth). Bars represent SEM where n= 6.*

In plants harvested during the rainy season, the content of extractable condensed tannins (g kg^{-1}) ranged from 70.7 in accession CIAT 33058 to 77.5 in accession CIAT 13125. Plants grown in all sites under high fertilizer regime tended to lower extractable condensed tannin concentration than those grown under low fertilizer. This reduction (g of extractable condensed tannins kg^{-1} of dry matter) averaged 8.8 and ranged from 5.1 for accession CIAT 3788 to 11.7 for accession CIAT 13125 (see Figure 5.1-4d). As an additional observation, plants harvested during the rainy season had a higher content of extractable condensed tannins than those harvested during the dry season (overall mean: 74.3 and 66.1 g kg^{-1} of dry matter, respectively).

Bound condensed tannins: The content of bound condensed tannins ranged from 7.3 to 21.6 g/kg of dry matter in plants harvested during the dry season. In turn, the mean content of bound condensed tannins ranged from 9.0 to 18.8 g kg^{-1} of dry matter in plants grown in *Chinchiná* and *Cauca*, respectively. Mean concentration of bound condensed tannins was unresponsive to fertilizer treatment in plants from *Macagual* and *Cauca*. In turn, in plants grown in *La Rueda*, high fertilizer was associated with an increase of 2.32 g of bound condensed tannins kg^{-1} of dry matter. Conversely, high fertilizer application was associated with reductions of 0.73, 2.51 and 3.13 g kg^{-1} of dry matter in the content of bound condensed in plants from *Chinchiná*, *Alcancia* and *Maquenque*, respectively (Figure 5.1-5a).

In plants harvested during the rainy season, the concentration of bound condensed tannins ranged from 8.3 to 20.0 g kg^{-1} of dry matter. Mean concentration of bound condensed tannins (g kg^{-1} of dry matter) ranged from 13.8 to 17.4 in plants grown in *Maquenque* and *Alcancia*, respectively No differences in content of bound condensed tannins were associated with fertilizer regime in plants from *Macagual* and *Maquenque*. In turn, high-fertilizer plants from *Chinchiná* had higher content of bound condensed tannins than low-fertilizer plants (16.5 and 14.5 g kg^{-1} of dry matter, respectively). Conversely, reductions of 0.80, 1.05 and 1.25 g of bound condensed tannins kg^{-1} of dry matter were observed for high-fertilizer plants from *La Rueda*, *Cauca* and *Alcancia*, respectively (see Figure 5.1-5b).

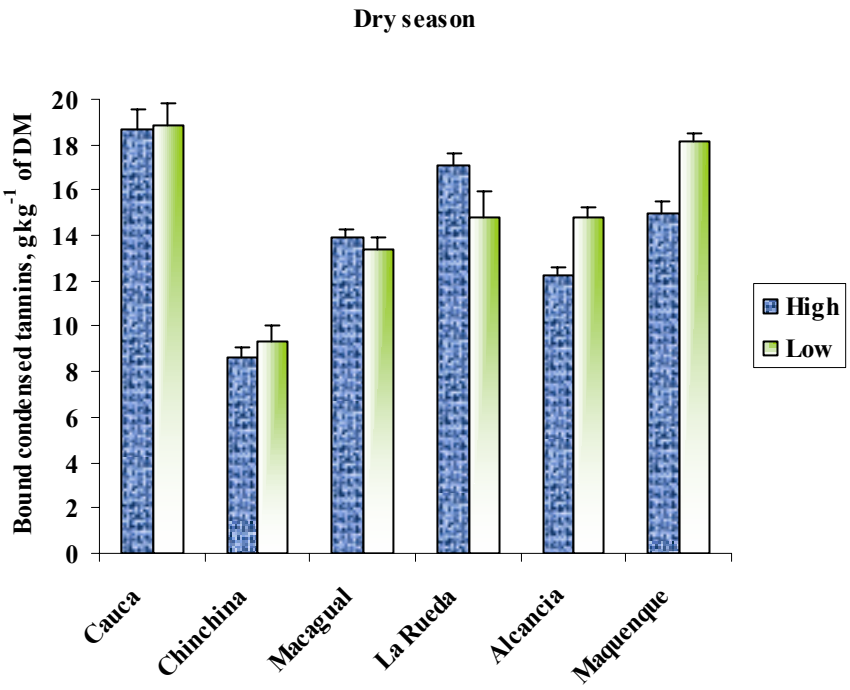

Figure 5.1-5a. *Environment-related variation in the content of bound condensed tannins of five* Desmodium ovalifolium *accessions planted at two fertilizer levels in four environments in Colombia and harvested during the dry season (eight weeks re-growth).* Cauca = *dry hillsides,* Chinchiná = *wet hillsides;* Macagual *and* La Rueda = *humid tropics and* Alcancia *and* Maquenque = *well-drained savanna. Bars represent SEM where n= 5.*

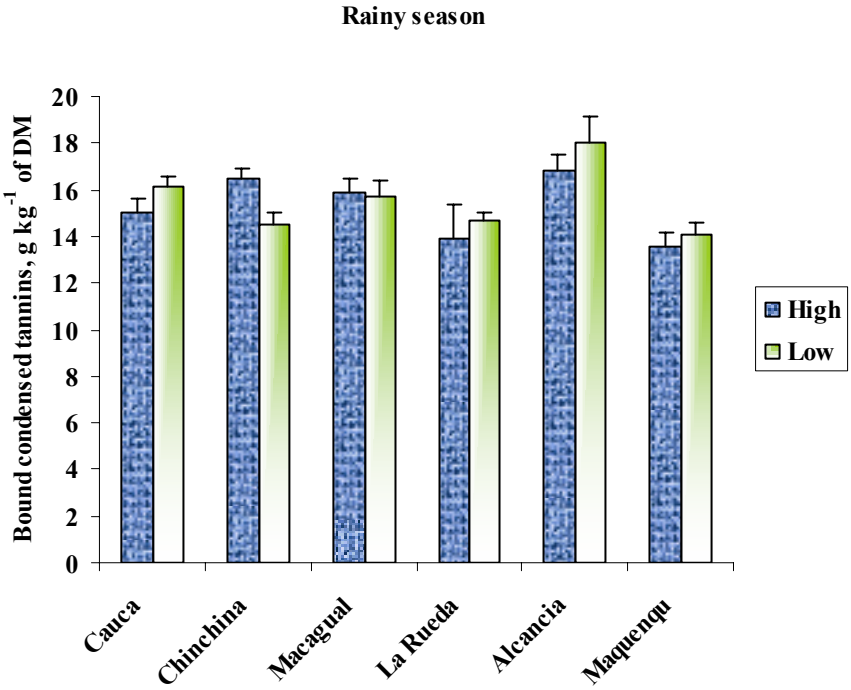

Figure 5.1-5b. *Environment-related variation in the content of bound condensed tannins of five* Desmodium ovalifolium *accessions planted at two fertilizer levels in four environments in Colombia and harvested during the rainy season (six weeks re-growth).* Cauca = *dry hillsides,* Chinchiná = *wet hillsides;* Macagual *and* La Rueda = *humid tropics and* Alcancia *and* Maquenque = *well-drained savanna. Bars represent SEM where n= 5.*

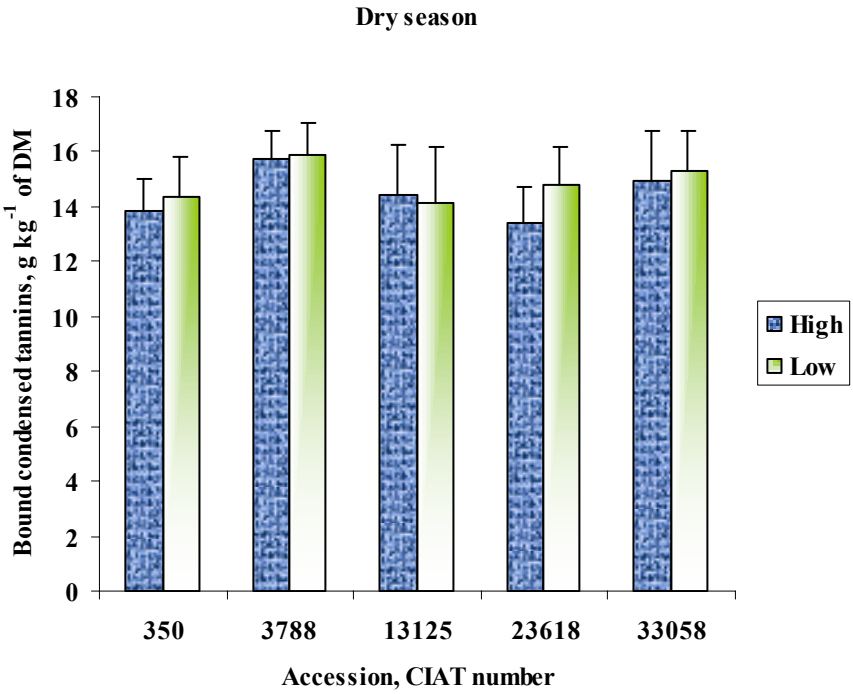

Figure 5.1-5c. *Genotype-related variation in the content of bound condensed tannins of five* Desmodium ovalifolium *accessions planted at two fertilizer levels in six sites (four environments) in Colombia and harvested during the dry season (eight weeks re-growth). Bars represent SEM where n= 6.*

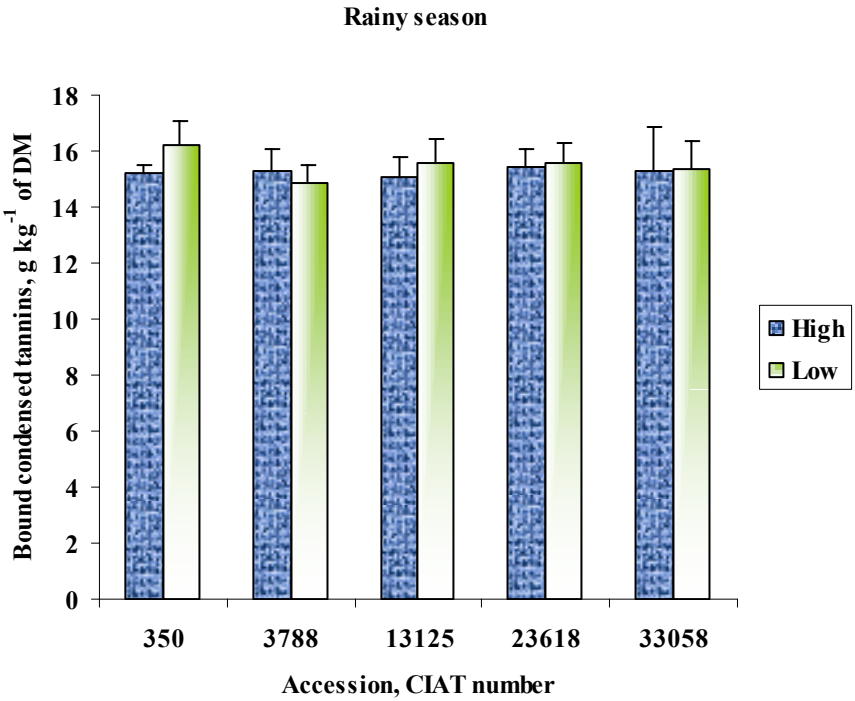

Figure 5.1-5d. *Genotype-related variation in the content of bound condensed tannins of five* Desmodium ovalifolium *accessions planted at two fertilizer levels in six sites (four environments) in Colombia and harvested during the rainy season (six weeks re-growth). Bars represent SEM where n= 6.*

In dry-season plants, the mean content of bound condensed tannins (averaged across sites and fertilizer regime) was lowest in accession CIAT 350 and highest in accession CIAT 3788 (14.1 and 15.8 g kg^{-1} of dry matter, respectively). The highest difference in content of bound condensed tannins related to fertilizer, was a reduction of 9 % observed in accession CIAT 23618 in response to high fertilizer application. In all other accessions, bound condensed tannin concentration was similar among plants grown under high and low fertilizer regime (see Figure 5.1-5c).

Mean content of bound condensed tannins ranged from 15.1 (accession CIAT 3788) to 15.7 (accession CIAT 350) for samples harvested during the rainy season. In these plants, there were no differences associated with fertilizer treatments (Figure 5.1-5d).

5.1.3.2 In vitro determinations of forage quality

In vitro *dry matter disappearance*: In samples harvested during the dry season, the disappearance of dry matter measured *in vitro* by the technique of Tilley and Terry (1963) ranged from 302 to 587 g kg^{-1} of dry matter. Mean *in vitro* dry matter disappearance (averaged by accession and fertilizer) was lowest in plants grown in *La Rueda* and highest in those grown in *Maquenque* (343 and 482 g kg^{-1} of dry matter, respectively). fertilizer had no effect on the *in vitro* dry matter disappearance of plants grown in *Cauca* and *Chinchiná*. On the other hand, high fertilizer was associated with increments of 33, 40, 114 and 183 g kg^{-1} of dry matter in the *in vitro* dry matter disappearance of plants grown *La Rueda*, *Macagual*, *Alcancia* and *Maquenque*, respectively (Figure 5.1-6a).

In plants harvested during the rainy season, *in vitro* dry matter disappearance ranged from 181 to 560 g kg^{-1} of dry matter. In turn, mean *in vitro* dry matter disappearance (g kg^{-1} of dry matter and averaged by accession and fertilizer) ranged from 367 to 499 in samples from *Maquenque* and *Chinchiná*, respectively. High fertilizer was associated with increases in the *in vitro* dry matter disappearance of samples harvested from all from all sites during the rainy season (see Figure 5.1-6b). Such increments (g kg^{-1} of dry matter) averaged 82 across all sites, and were lowest in samples from *Macagual, Chinchiná, Cauca* and *La Rueda* (28-36) and highest in samples from *Alcancia* (137) and *Maquenque* (235).

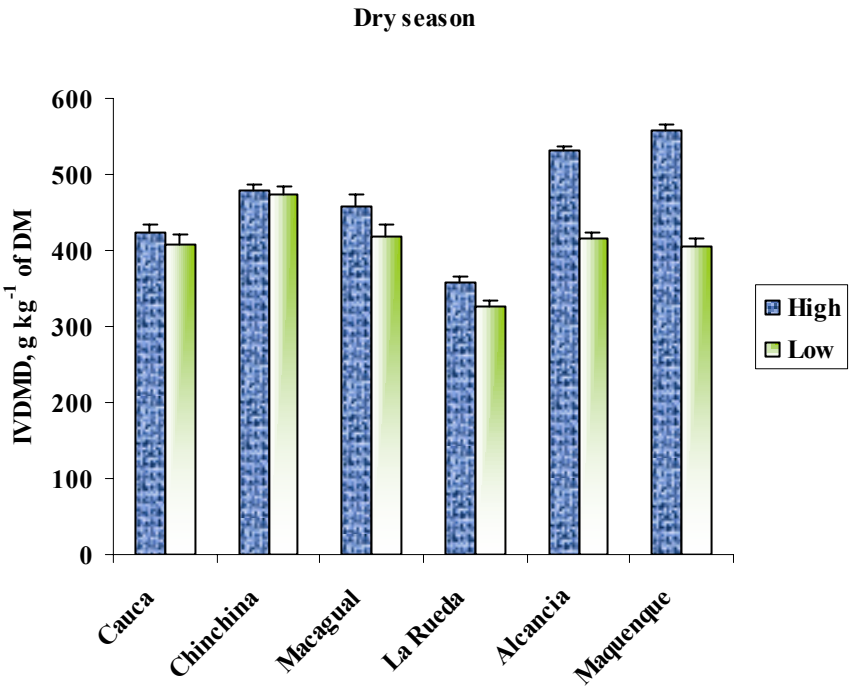

Figure 5.1-6a. *Environment-related variation in the* in vitro *dry matter disappearance of five* Desmodium ovalifolium *accessions planted at two fertilizer levels in four environments in Colombia and harvested during the dry season (eight weeks re-growth).* Cauca = *dry hillsides,* Chinchiná = *wet hillsides;* Macagual *and* La Rueda = *humid tropics and* Alcancia *and* Maquenque = *well-drained savanna. Bars represent SEM where n= 5.*

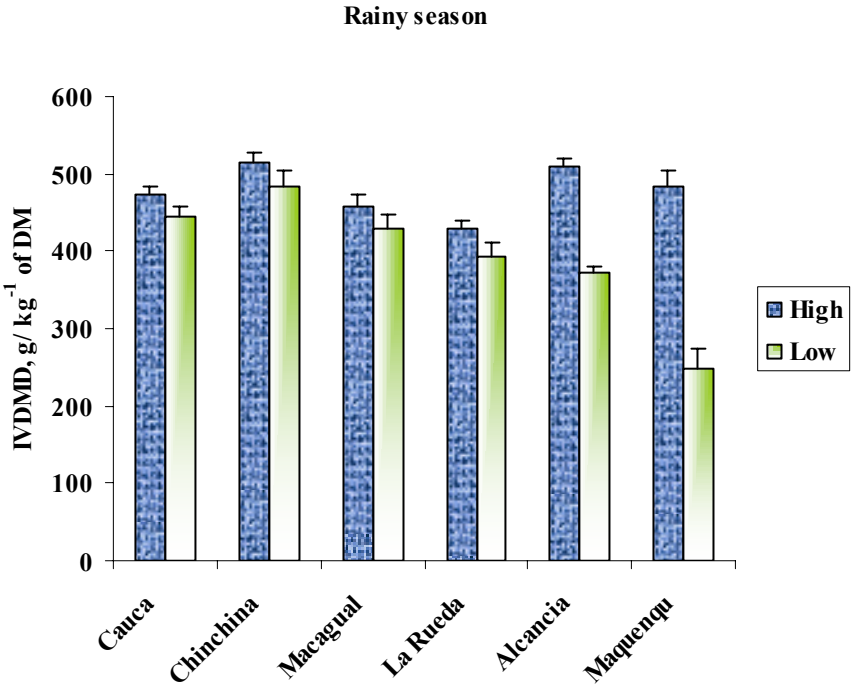

Figure 5.1-6b. *Environment-related variation in the* in vitro *dry matter disappearance of five* Desmodium ovalifolium *accessions planted at two fertilizer levels in four environments in Colombia and harvested during the rainy season (six weeks re-growth).* Cauca = *dry hillsides,* Chinchina = *wet hillsides;* Macagual *and* La Rueda = *humid tropics and* Alcancia *and* Maquenque = *well-drained savanna. Bars represent SEM where n= 5.*

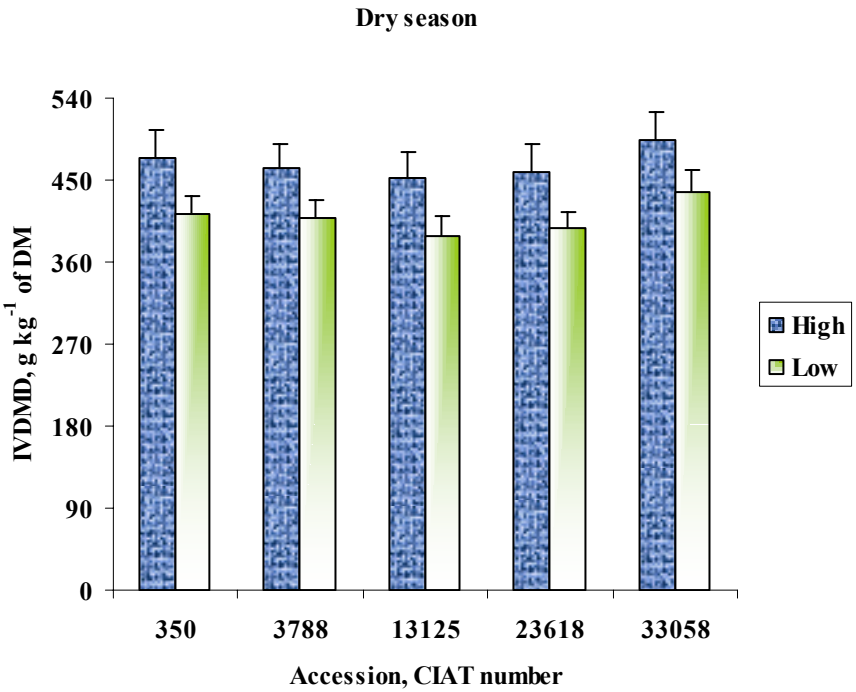

Figure 5.1-6c. *Genotype-related variation in the* in vitro *dry matter disappearance of five* Desmodium ovalifolium *accessions planted at two fertilizer levels in six sites (four environments) in Colombia and harvested during the dry season (eight weeks re-growth). Bars represent SEM where n= 6.*

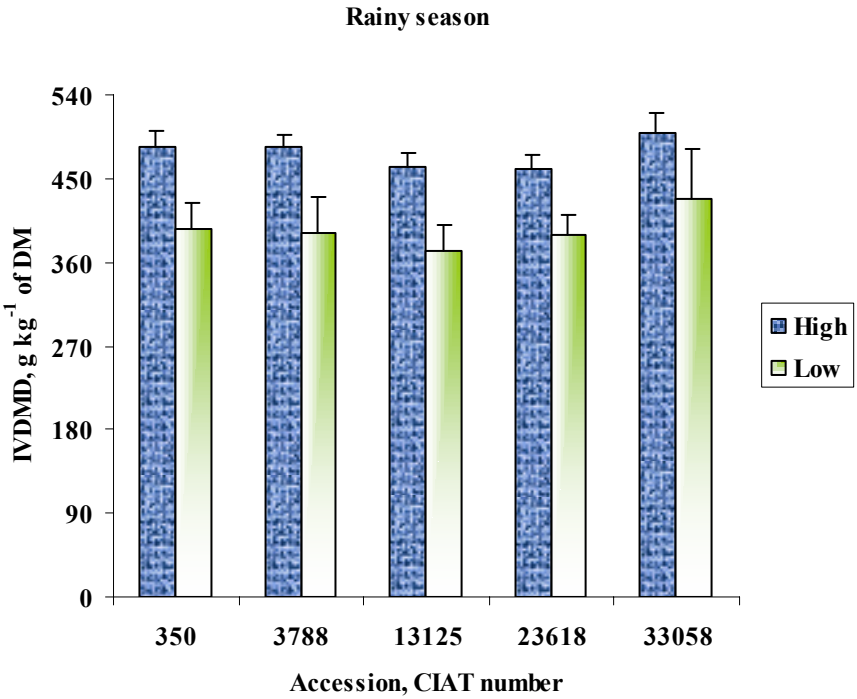

Figure 5.1-6d. *Genotype-related variation in the* in vitro *dry matter disappearance of five* Desmodium ovalifolium *accessions planted at two fertilizer levels in six sites (four environments) in Colombia and harvested during the rainy season (six weeks re-growth). Bars represent SEM where n= 6.*

In plants harvested during the dry season, mean *in vitro* dry matter disappearance (averaged across site and fertilizer treatments) was lowest for accession CIAT 13125 and highest for accession CIAT 33058 (420 and 464 g kg^{-1} of dry matter, respectively). All five accessions considered, high fertilizer application resulted in an average increase of *circa* 59 g kg^{-1} of dry matter in *in vitro* dry matter disappearance in samples harvested during the dry season (Figure 5.1-6c).

Mean *in vitro* dry matter disappearance was also lowest for accession CIAT 13125 and highest for accession CIAT 33058 (418 and 463 g kg^{-1} of dry matter, respectively) in samples harvested during the rainy season. Across all five accessions, high fertilizer application resulted in an average increase in *in vitro* dry matter disappearance of 82 g kg^{-1} of dry matter (see Figure 5.1-6d). This increase was lowest in accessions CIAT 23618 and 33058 and highest in accession CIAT 3788 (70, 70 and 94 g kg^{-1} of dry matter, respectively).

Gas accumulation experiments: To illustrate differences observed in the gas production experiments, the rate of gas accumulation (ml h^{-1}, as defined in Chapter 3) were plotted against incubation time for the first 48 h of incubation. The first of such figures (5.1-7a and 5.1-7b) depict the mean rate of gas accumulation (averaged by accession) during the incubation of samples harvested during the dry season. When compared across sites, samples from *La Rueda* were shown to have the lowest rate of gas evolution. Indeed, the maximum rate of gas (ml h^{-1}) observed for *La Rueda* samples ranged from 0.97 to 1.6 ml h^{-1}. In contrast, the maximum rate of gas production in the high-fertilizer samples from *Maquenque* and *Alcancia* ranged from 4.4 to 7.2 ml h^{-1}. Another difference between samples from the different sites was related to the amount of time (h) that was needed to achieve the maximum rate of gas evolution. For example, in samples from *Chinchiná* and *La Rueda*, the maximum rate of gas evolution was achieved after 21 and 24 h of inoculation, respectively. On the other hand, the maximum rate of gas accumulation during the fermentation of high-fertilizer samples from *Maquenque* and *Alcancia* was observed after only 6 and 4 h of incubation, respectively. As a final comparison, the rate of gas evolution after 48 h of incubation corresponded to 86 % and 37 % of the maximum rate of gas production in samples from *La Rueda* and *Maquenque*, respectively.

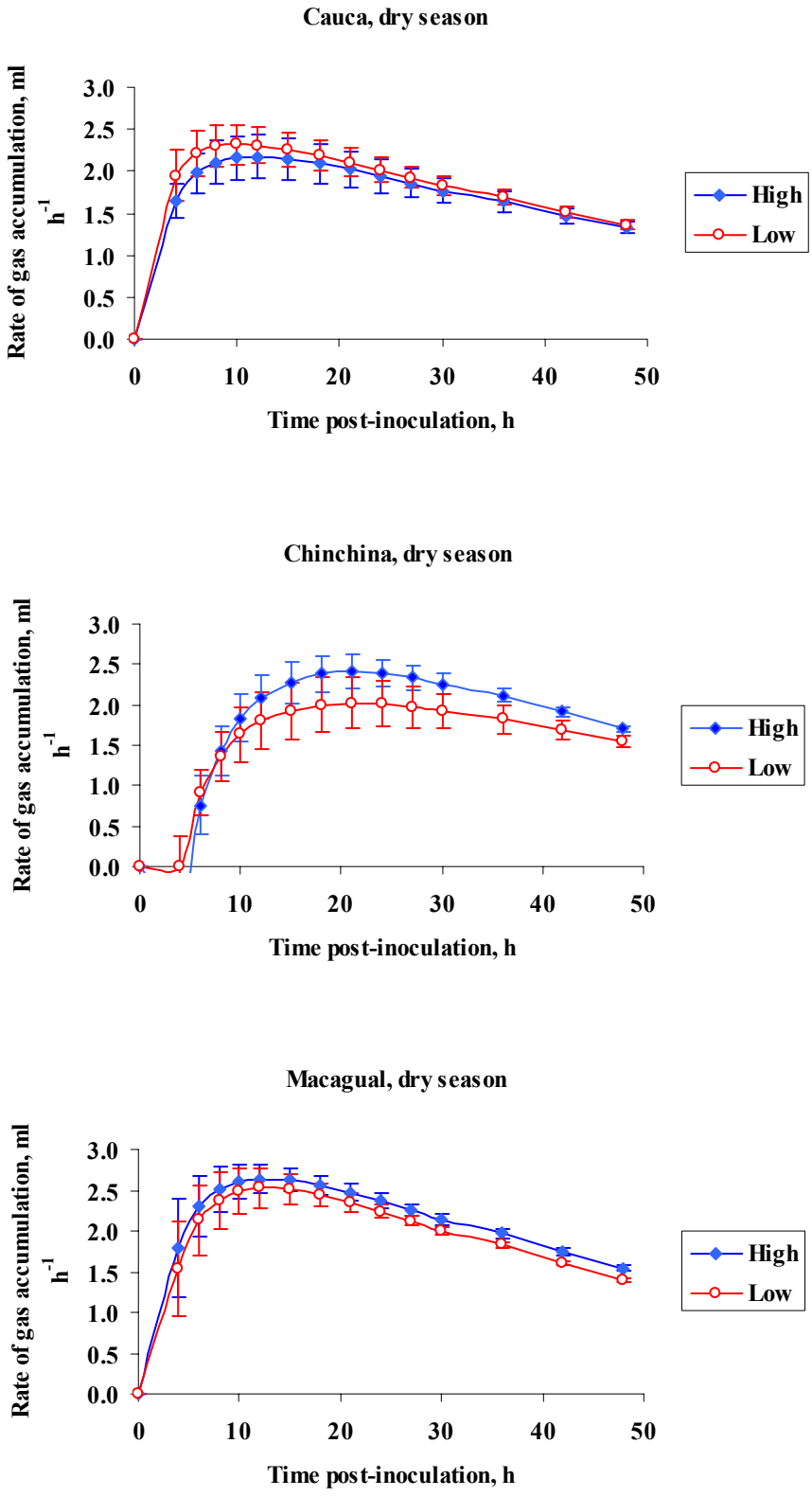

Figure 5.1-7a. *Environment-related variation in the rate of gas accumulation of five* Desmodium ovalifolium *accessions planted at two fertilizer levels in contrasting sites in Colombia and harvested during the dry season (eight weeks re-growth). Samples plotted in this figure came from three environments: hillsides* = Cauca *(dry) and* Chinchiná *(wet) and humid tropic* = Macagual. *Bars represent SEM where n= 5.*

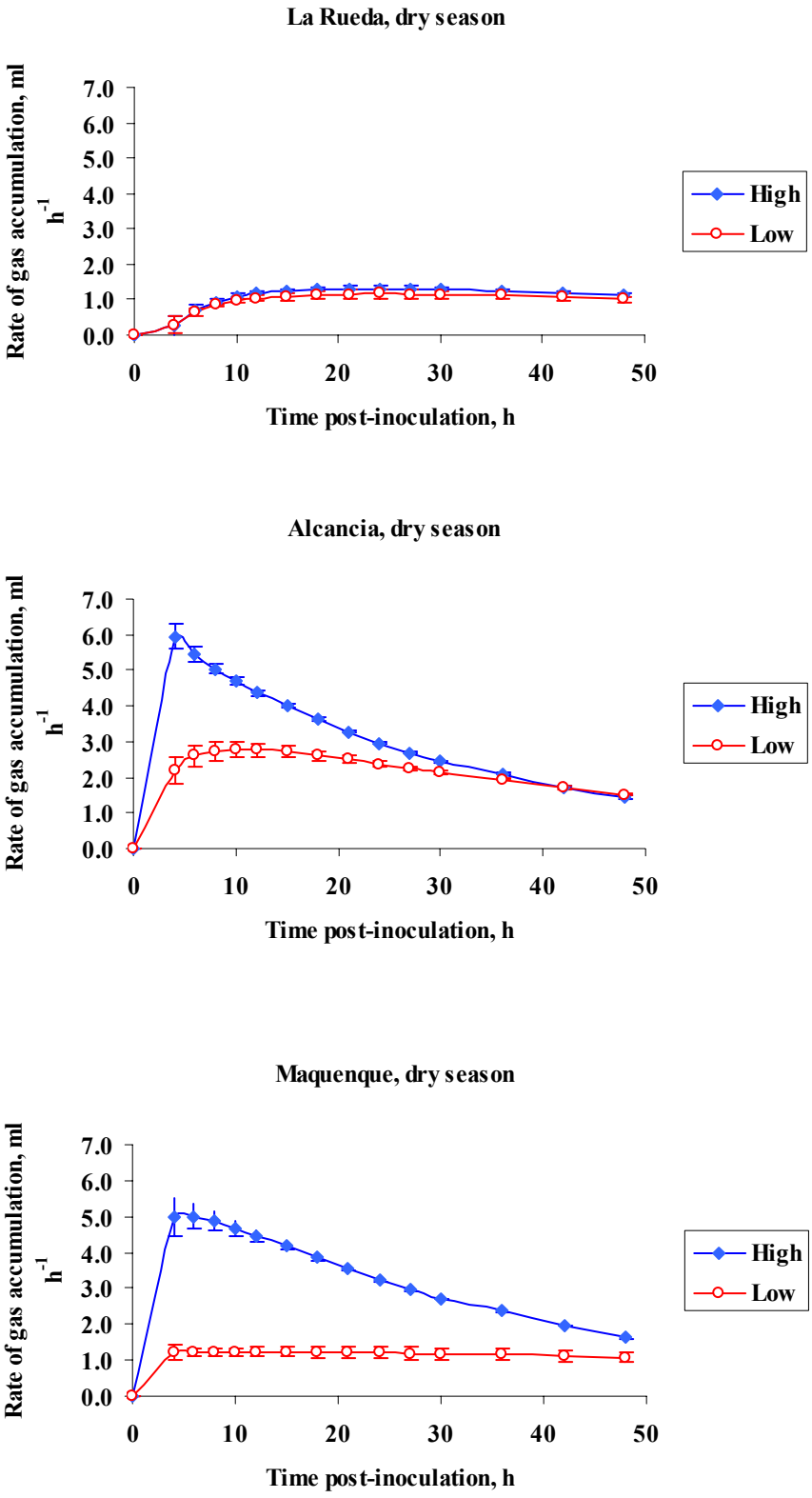

Figure 5.1-7b. *Environment-related variation in the rate of gas accumulation of five* Desmodium ovalifolium *accessions planted at two fertilizer levels in contrasting sites in Colombia and harvested during the dry season (eight weeks re-growth). Samples plotted in this figure came from two environments: humid tropic* = La Rueda *and well-drained savanna* = Alcancia *and* Maquenque. *Bars represent SEM where n= 5.*

Fertilizer had a dramatic impact in the rate of fermentation in samples from the *well-drained savanna*. Indeed, while the maximum mean rate of gas production was 5.9 and 5.0 ml h^{-1} for the high-fertilizer samples from *Alcancia* and *Maquenque*, respectively, that of the corresponding low-fertilizer samples was only 2.8 and 1.2 ml h^{-1} (see Figure 5.1-7b). Additionally, during the fermentation of high-fertilizer samples from *Alcancia* and *Maquenque*, the maximum rate of gas evolution was achieved very early during the fermentation (4 and 6 h post-inoculation) and thereafter, the amount of gas produced per hour tended to decline very rapidly as the fermentation progressed. On the other hand, the maximum rate of gas evolution was only achieved after 10 and 12 h of fermentation in the corresponding low-fertilizer samples, and gas evolution remained relatively constant during the next 38-36 h of incubation (see Figure 5.1-7b). In the remaining sites, fertilizer did not elicit the same response observed in samples from the *Alcancia* and *Maquenque* sites, a feature that was particularly true in samples from *Cauca* (see Figure 5.1-7a).

When compared across accessions, little differences in gas accumulation were evident in samples harvested during the dry season. The rate of gas accumulation was numerically greater for accession CIAT 33058 than for the other four accessions studied. However, the SEM associated with the means portrayed in Figures 5.1-8a (high fertilizer) and 5.1-8b (low fertilizer) were very high, which is an indication of the great variability in rate of gas evolution observed across plants grown at the different sites. In turn, the results depicted in figures 5.1-8a and 5.1-8b are suggestive of little differences in fermentation kinetics among the five *D. ovalifolium* accessions studied. Overall, the observed rate of gas evolution increased in response to high fertilizer in all the accessions in these experiments.

There was also considerable variability in gas accumulation during the fermentation of samples harvested during the rainy season. At maximum rate of gas evolution (27 h post-inoculation), samples from *Cauca* produced an overall average of 1.76 ml h^{-1} (range = 1.4 to 3.9 ml h^{-1}). By contrast, after only eight h of incubation, samples from *Chinchiná* had an overall maximum rate of gas production of 3.64 ml h^{-1} (range: = 2.5 to 7.7 ml h^{-1}; see Figure 5.1-9a).

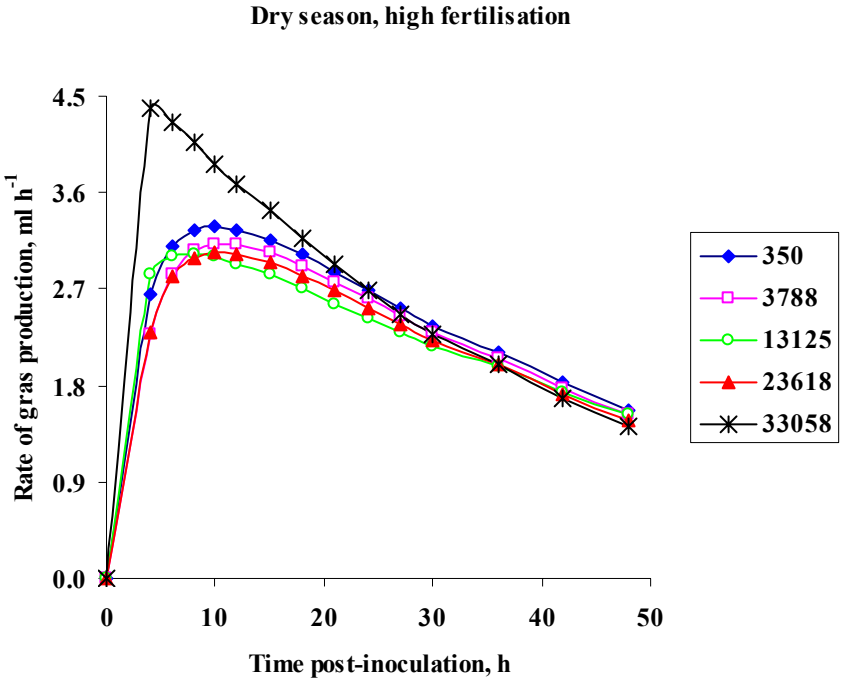

Figure 5.1-8a. *Genotype-related variation in the rate of gas accumulation (averaged across planting site) of five* Desmodium ovalifolium *accessions planted at under high fertilizer in six sites (four environments) in Colombia and harvested during the dry season (eight weeks re-growth). For clarity and due of its high values, SEM was omitted from this figure.*

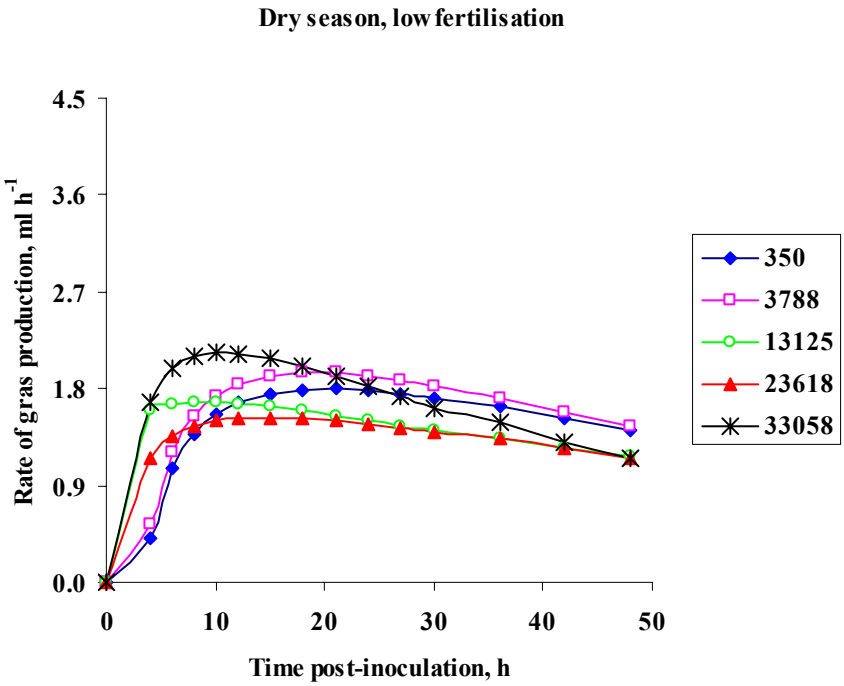

Figure 5.1-8b. *Genotype-related variation in the rate of gas accumulation (averaged across planting site) of five* Desmodium ovalifolium *accessions planted at under low fertilizer in six sites (four environments) in Colombia and harvested during the dry season (eight weeks re-growth). For clarity and due of its high values, SEM was omitted from this figure.*

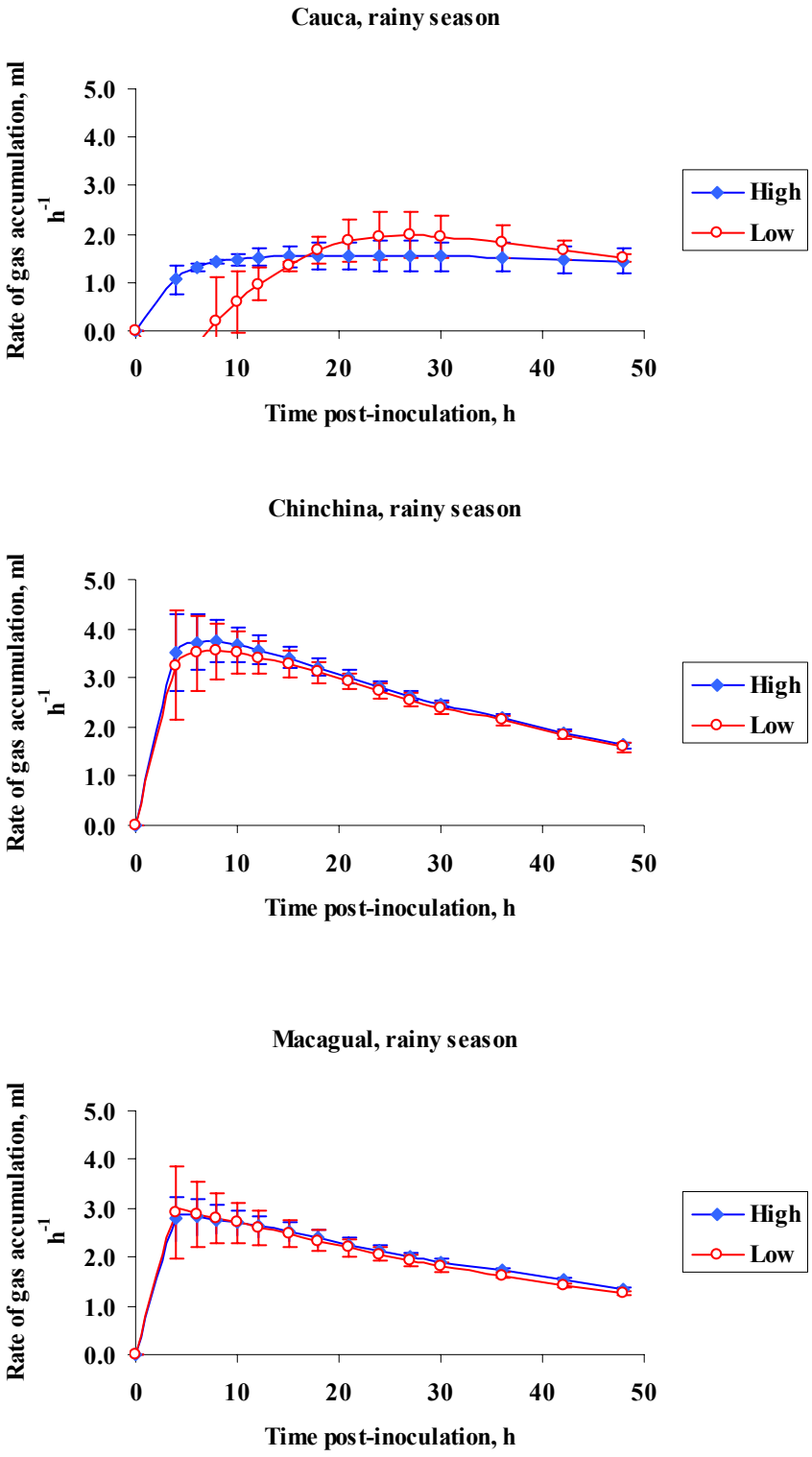

Figure 5.1-9a. *Environment-related variation in the rate of gas accumulation of five* Desmodium ovalifolium *accessions planted at two fertilizer levels in contrasting sites in Colombia and harvested during the rainy season (six weeks re-growth). Samples represented in this figure came from three environments: hillsides* = Cauca *(dry) and* Chinchiná *(wet) and humid tropic* = Macagual. *Bars represent SEM where n= 5.*

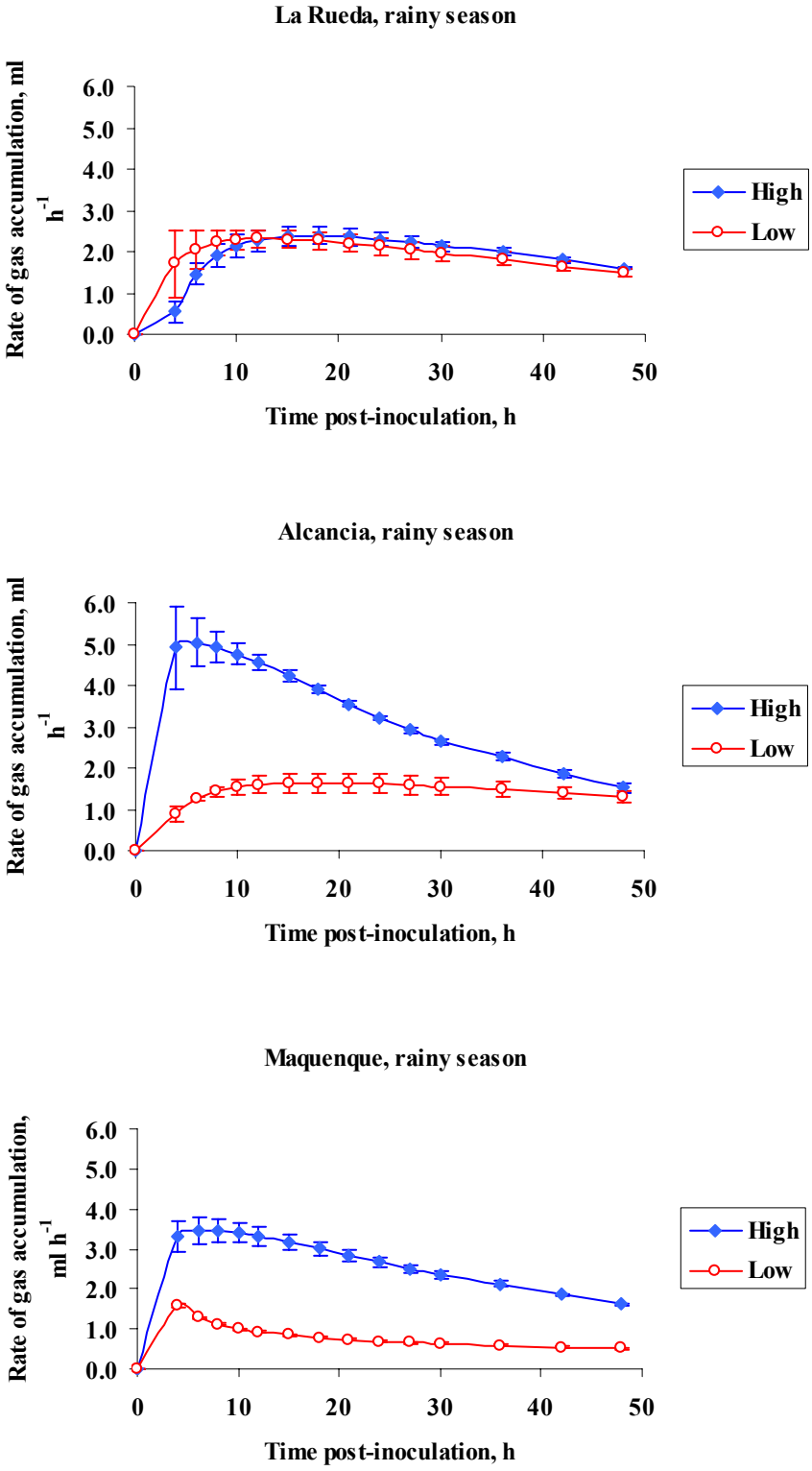

Figure 5.1-9b. *Environment-related variation in the rate of gas accumulation of five* Desmodium ovalifolium *accessions planted at two fertilizer levels in contrasting sites in Colombia and harvested during the rainy season (six weeks re-growth). Samples represented in this figure came from two environments: humid tropic* = La Rueda *and well-drained savanna* = Alcancia *and* Maquenque. *Bars represent SEM where n= 5.*

As was observed in the dry season samples, high fertilizer was associated with a dramatic increase in the rate of gas evolution in samples harvested from the *Alcancia* and *Maquenque* sites during the rainy season (see Figure 5.1-9b). Indeed, plants grown under high fertilizer in *Alcancia* and *Maquenque* had an average maximum rate of gas evolution of 5.04 and 3.46 ml h^{-1}, respectively. On the other hand, the corresponding low-fertilizer plants had only 1.65 and 1.60 ml h^{-1}. Conversely, in samples harvested during the rainy season from all other sites, the fertilizer treatments were not associated with changes in the rate of gas evolution, an observation that had also been made in plants harvested during the dry season.

Comparison of gas accumulation among the five accessions studied showed little difference between accessions, although gas production during the initial stages of fermentation was numerically greater for accession CIAT 33058 (Figures 5.1-10a and 5.1-10b). This effect was more evident in plants grown under low fertilizer (see Figure 5.1-10b). Overall, plants grown under high fertilizer had the greatest rate of gas evolution than those grown under low fertilizer. Indeed, the maximum rate of gas evolution was in average 3.38 ml h^{-1} in high-fertilizer samples and 2.04 ml h^{-1} in low-fertilizer plants. These values were observed after 6 and 4 h of incubation, respectively. After 48 h of incubation, the overall rate of gas evolution was 1.55 and 1.11 ml h^{-1} for the high and low fertilizer samples, respectively.

5.1.3.3 Relationships between chemical composition and gas production

In an attempt to determine the relative impact of chemical composition on *in vitro* gas accumulation, their relationships were examined by means of linear regression analyses. Given the apparent differences between these two sets of samples, these relationships were investigated separately for samples harvested during the dry and rainy season.

Neutral detergent fibre: The concentration of neutral detergent fibre was not significantly related to the maximum rate of gas accumulation observed during the fermentation of samples harvested during the dry and rainy season (data not shown). Furthermore, in rainy-season plants the content of neutral detergent fibre was not significantly related to the rate of gas evolution observed at discrete time points during the first 48 h of incubation of these samples.

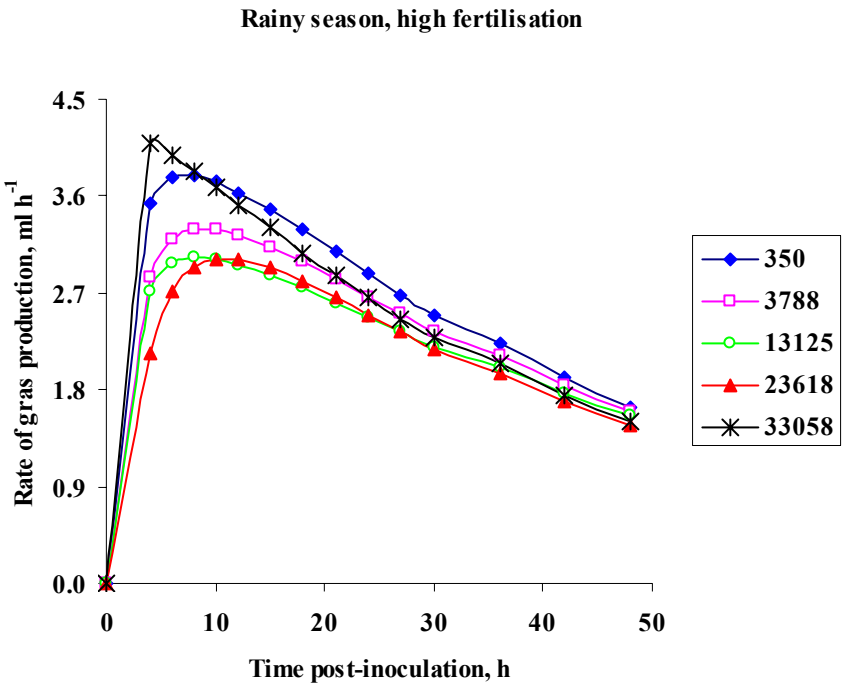

Figure 5.1-10a. *Genotype-related variation in the rate of gas accumulation (averaged across planting site) of five* Desmodium ovalifolium *accessions grown under high fertilizer in six sites (four environments) in Colombia and harvested during the rainy season (six weeks re-growth). For clarity and due of its high values, SEM was omitted from this figure.*

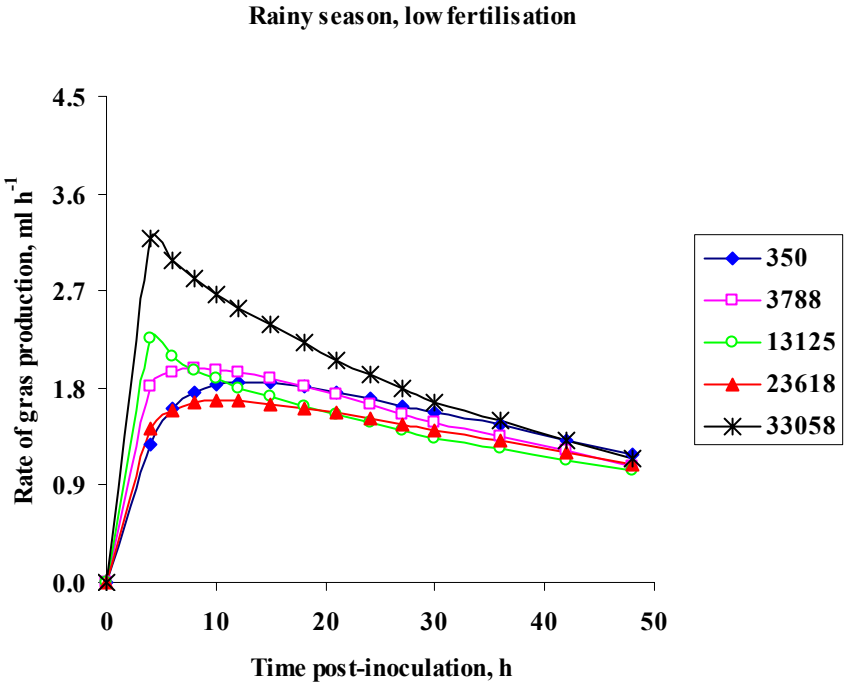

Figure 5.1-10b. *Genotype-related variation in the rate of gas accumulation (averaged across planting site) of five* Desmodium ovalifolium *accessions grown under low fertilizer in six sites (four environments) in Colombia and harvested during the rainy season (six weeks re-growth). For clarity and due of its high values, SEM was omitted from this figure.*

In plants harvested during the dry season, there was a negative relationship between neutral detergent fibre and rate of gas evolution. The r^2 of this relationship increased as the incubation time progressed, attaining statistical significance after 18 h of incubation (r^2 = 0.068, P < 0.05). In turn, the strongest r^2 for this relationship was observed after 42 h of incubation (r^2 = 0.109, P < 0.01).

Acid detergent fibre: The content of acid detergent fibre was negatively related to the rate of gas evolution in samples harvested during the dry and rainy seasons. In dry-season plants, the overall relationship between the maximum rate of gas evolution (y) and the content of acid detergent fibre (x) was described by the equation y = -0.0173x + 8.67, r^2 = 0.285, P < 0.001 (see Figure 5.1-11a). Closer examination of this relationship revealed that the impact (denoted by the slope of the linear regression relationship) of acid detergent fibre content on the maximum rate of gas evolution varied greatly according to site. For example, in *La Rueda* increases in acid detergent fibre were associated with very small increases in the observed maximum rate of gas evolution (slope = 0.0048, r^2 = 0.45, P = 0.035). Conversely, at all other sites, the slope was negative and ranged from –0.014 (r^2 = 0.57, P = 0.012) in *Cauca* to –0.050 (r^2 = 0.595, P = 0.009) in *Maquenque*. One interesting observation from Figure 5.1-11a is the clustering of the data points in samples from *La Rueda* and the formation of two groups in plants from *Alcancia* and *Maquenque*, in response to the fertilizer treatment applied. In these sites, high-fertilizer samples were the ones with the lower content of acid detergent fibre and the higher rate of gas production.

In samples harvested during the rainy season, the overall relationship between the content of acid detergent fibre and the maximum rate of gas evolution was also negative (see Figure 5.1-11b). The linear regression obtained was as follows: *Maximum rate of gas evolution* = -0.0197(*acid detergent fibre*) + 9.90, (r^2 = 0.198, P = 0.001). In samples from *Cauca*, *La Rueda* and *Maquenque*, the relationship between the content of acid detergent fibre and the maximum rate of gas production was weak and failed to reach significance. Conversely, the relationship between acid detergent fibre and the maximum rate of gas accumulation in plants grown in *Alcancia*, *Macagual* and *Chinchiná* was negative and highly significant (r^2 = 0.57 - 0.66, P = 0.011 – 0.005). In turn, the corresponding slope of the linear regression was –0.040, -0.049 and –0.055 for samples from *Alcancia, Macagual* and *Chinchiná*, respectively.

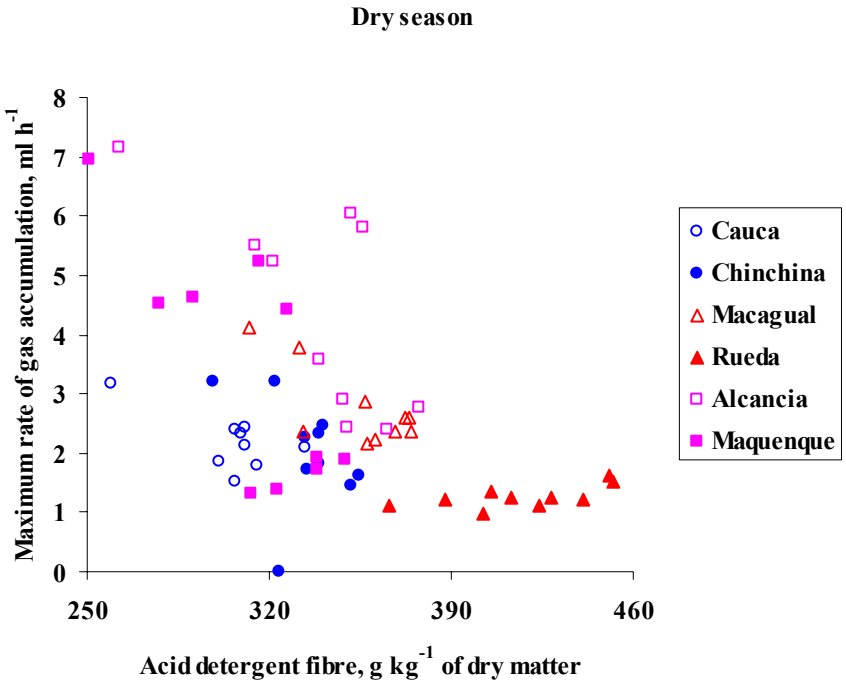

Figure 5.1-11a. *The relationship between the maximum rate of gas accumulation and acid detergent fibre content of five* Desmodium ovalifolium *accessions planted in six sites (four environments: hillsides* = Cauca *(dry) and* Chinchiná *(wet), humid tropic* = Macagual *and* la Rueda *and well-drained savanna* = Alcancia *and* Maquenque*) in Colombia. Dry season samples (eight weeks re-growth).*

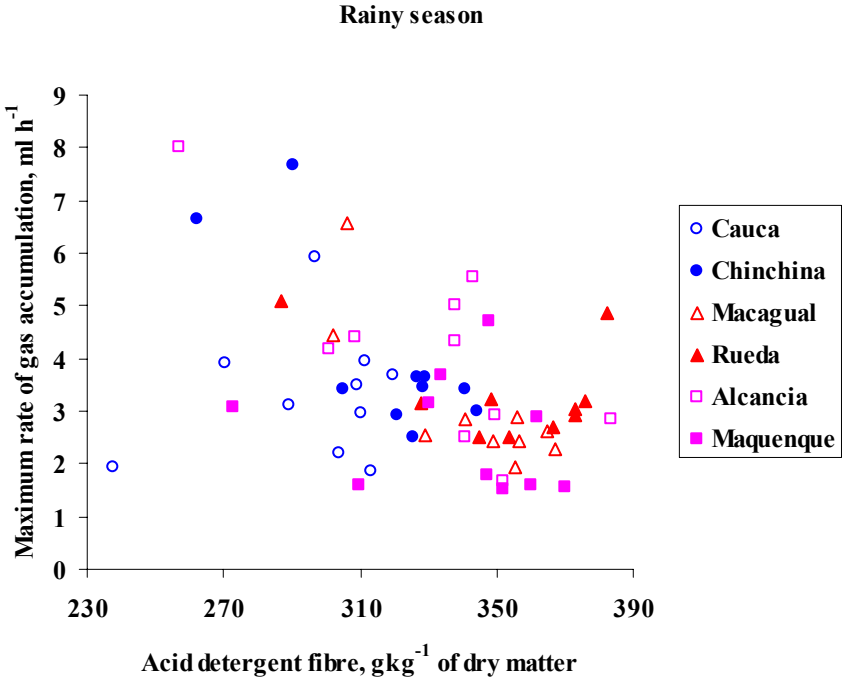

Figure 5.1-11b. *The relationship between the maximum rate of gas accumulation and acid detergent fibre content of five* Desmodium ovalifolium *accessions planted in six sites (four environments: hillsides* = Cauca *(dry) and* Chinchiná *(wet), humid tropic* = Macagual *and* la Rueda *and well-drained savanna* = Alcancia *and* Maquenque*) in Colombia. Rainy season samples (six weeks re-growth).*

Figures 5.1-11a and 5.1-11b show the relationship between the content of acid detergent fibre and the maximum rate of gas accumulation, this latter value occurring at different times post-inoculation depending on the sample fermented. We also examined the relationship between the content of acid detergent fibre and the rate of gas accumulation at discrete time points during the first 48 h of the incubation period. For dry season samples, this analysis allowed us to confirm that during the first 48 h of incubation, the content of acid detergent fibre was negatively related to the rate of gas production. The r^2 of this relationship increased as the fermentation progressed, reaching its maximum at 15 h post-inoculation ($r^2 = 0.314$, $P < 0.001$) and then gradually decreased as the fermentation continued. In turn, for rainy season samples, the maximum r^2 of the relationship between content of acid detergent fibre and rate of gas evolution was observed after 36 h of incubation ($r^2 = 0.064$, $P = 0.0507$).

Condensed tannins: In samples harvested during the dry and rainy season the content of acetone-extractable and total condensed tannins was negatively related to the maximum rate of gas production from the fermentation of these samples. Such relationship was not evident in the case of bound condensed tannins. In consequence and because in these experiments, the impact of acetone-extractable and total condensed tannins on fermentation kinetics was practically the same, it was decided to show only those relationships involving total condensed tannins

In plants harvested during the dry season, the overall relationship between total condensed tannins (x) and the maximum rate of gas production (y) was described by the equation $y = -0.0593x + 7.41$, $r^2 = 0.23$, $P < 0.001$ (see Figure 5.1-12a). Within each site, however, there was great variation in the impact of total condensed tannins upon the maximum rate of gas accumulation derived from the fermentation of these samples. Although this relationship was negative at all sites, this only achieved statistical significance in samples from *Alcancia* and *Maquenque* ($y = -0.077x + 10.8$, $r^2 = 0.56$, $P = 0.012$ and $y = -0.113x + 11.7$, $r^2 = 0.77$, $P = 0.0008$, respectively). Significantly, it was among samples from these two sites that the greatest range in content of total condensed tannins was observed (see Figure 5.1-12a). It was also interesting to observe that the maximum rate of gas accumulation in samples from *La Rueda* was lower than it could be predicted given their total tannin content.

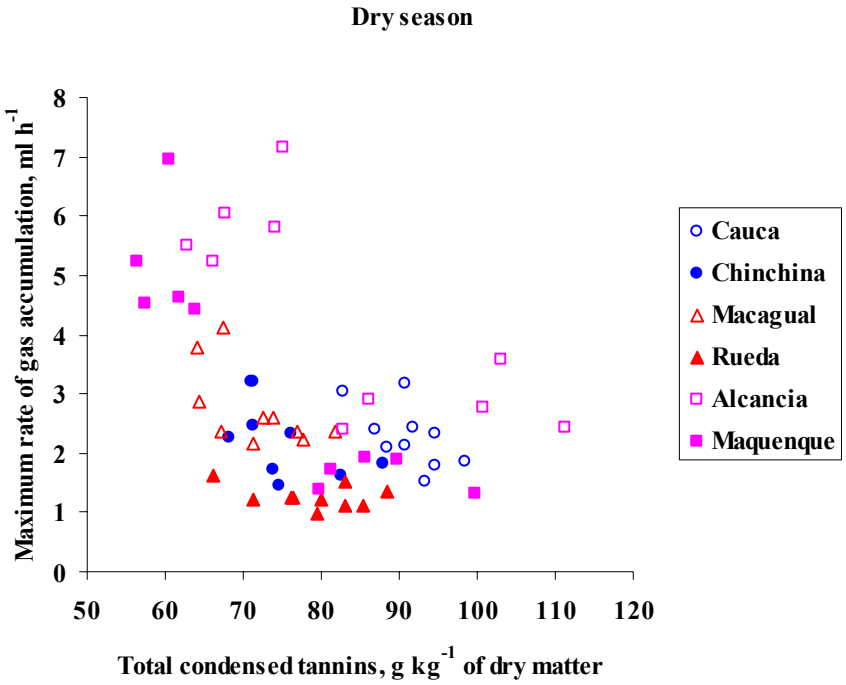

Figure 5.1-12a. *The relationship between the maximum rate of gas accumulation and total condensed tannin content of five* Desmodium ovalifolium *accessions planted in six sites (four environments: hillsides* = Cauca *(dry) and* Chinchiná *(wet), humid tropic* = Macagual *and* la Rueda *and well-drained savanna* = Alcancia *and* Maquenque*) in Colombia. Dry season samples (eight weeks re-growth).*

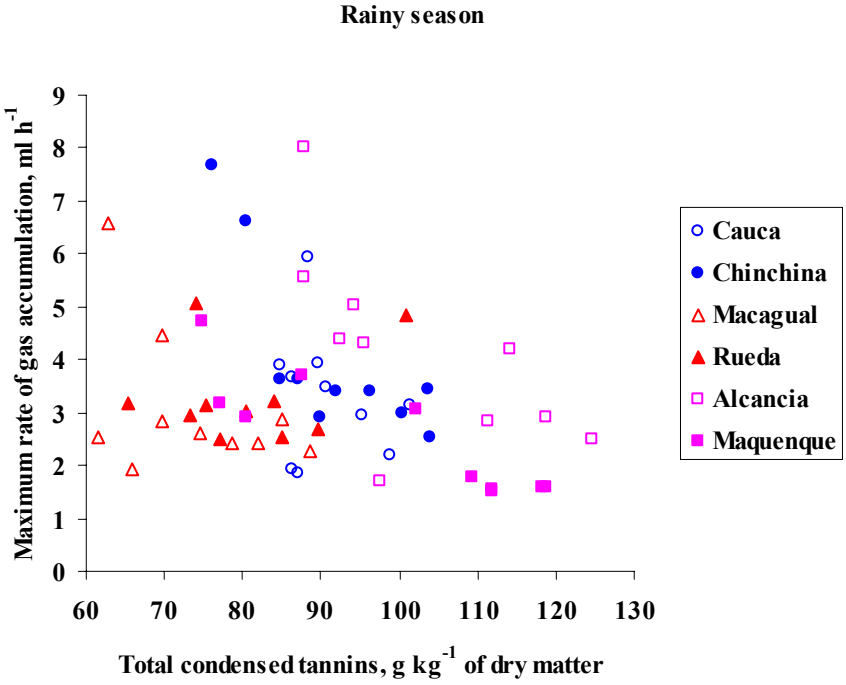

Figure 5.1-12b. *The relationship between the maximum rate of gas accumulation and total condensed tannin content of five* Desmodium ovalifolium *accessions planted in six sites (four environments: hillsides* = Cauca *(dry) and* Chinchiná *(wet), humid tropic* = Macagual *and* la Rueda *and well-drained savanna* = Alcancia *and* Maquenque*) in Colombia. Rainy season samples (six weeks re-growth).*

In rainy-season samples, the maximum rate of gas accumulation (y) was also negatively related to total condensed tannin content (x, see Figure 5.1-12b). This relationship, described by the equation: $y = -0.029x + 6.0$, $r^2 = 0.09$, $P < 0.05$, was weaker than the one observed for dry season samples. When regression analysis was performed within each site, this relationship failed to achieve statistical significance in samples from *Cauca*, *Macagual* and *La Rueda*. On the other hand, there was a significant negative relationship between total condensed tannins and maximum rate of gas accumulation in samples from *Alcancia*, *Maquenque* and *Chinchiná* ($P < 0.05$).

Regression analysis was also performed between total condensed tannins and the rate of gas evolution observed at different time points during the fermentation of dry and rainy season samples. For dry season samples, the r^2 of this relationship was strongest after 24 h of incubation ($r^2 = 0.249$, $P < 0.001$, data not shown) and steadily decreased afterwards. In turn, in rainy season samples, the r^2 of this relationship was highest after 36 h of incubation ($r^2 = 0.177$, $P < 0.001$, data not shown).

Protein: In dry-season samples, the content of protein was strongly and positively related to the maximum rate of gas accumulation. The overall relationship, depicted in Figure 5.1-13a, obeyed the equation $y = 0.046x - 4.1$, $r^2 = 0.63$, $P < 0.001$. As with the other chemical components, this relationship varied greatly according to site. In samples from *La Rueda*, for example, this relationship was negative ($y = -0.008x + 2.3$, $r^2 = 0.38$, $P = 0.057$). In all other sites, the relationship was positive, although it was not statistically significant for *Chinchiná* samples. The relationship was particularly strong in samples from the well-drained savanna, being $y = 0.055x - 4.7$, $r^2 = 0834$, $P < 0.001$ and $y = 0.055x - 4.4$, $r^2 = 094$, $P < 0.001$ in samples from *Alcancia* and *Maquenque*, respectively.

In rainy-season samples, the relationship between the maximum rate of gas production and the crude protein content was also positive, but not as strong as observed for the dry season samples ($y = 0.023x + 0.13$, $r^2 = 0.172$, $P < 0.001$, Figure 5.1-13b). Regression analysis performed within each site showed that this relationship varied greatly from site to site. In samples from *Cauca*, *Macagual* and *La Rueda* the relationship was weak and non-significant. On the contrary, in samples from *Chinchiná*, *Alcancia* and *Maquenque*, the r^2 of this positive relationship ranged from 0.55 to 0.71 ($P = 0.013$ to $P = 0.002$)

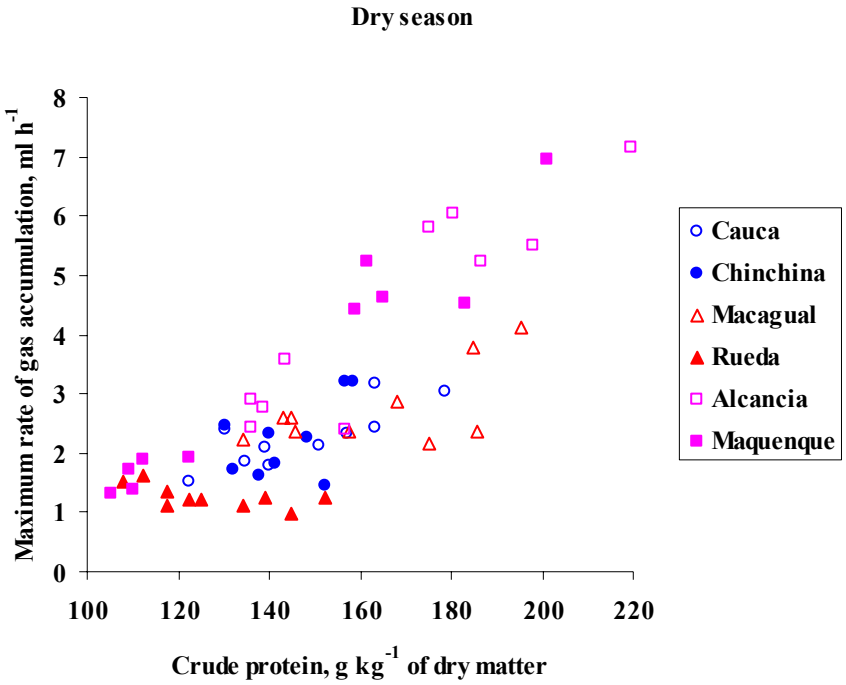

Figure 5.1-13a. *The relationship between the maximum rate of gas accumulation and crude protein content of five* Desmodium ovalifolium *accessions planted in six sites (four environments: hillsides* = Cauca *(dry) and* Chinchiná *(wet), humid tropic* = Macagual *and* la Rueda *and well-drained savanna* = Alcancia *and* Maquenque*) in Colombia. Dry season samples (eight weeks re-growth).*

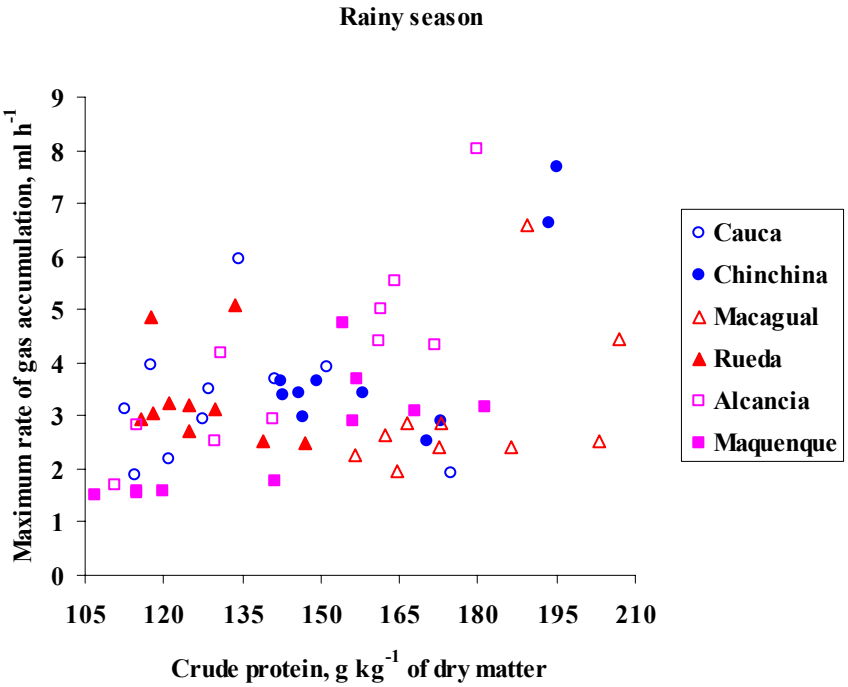

Figure 5.1-13b. *The relationship between the maximum rate of gas accumulation and crude protein content of five* Desmodium ovalifolium *accessions planted in six sites (four environments: hillsides* = Cauca *(dry) and* Chinchiná *(wet), humid tropic* = Macagual *and* la Rueda *and well-drained savanna* = Alcancia *and* Maquenque*) in Colombia. Rainy season samples (six weeks re-growth).*

Other relationships: There was a strong relationship between *in vitro* dry matter digestibility (Tilley and Terry, 1963) and the maximum rate of gas accumulation in both dry and rainy season samples. Such relationship is plotted in Figure 5.1-14 for dry season samples and could mathematically be described as $y = 0.0185x - 5.33$, $r^2 = 0.696$, $P < 0.001$. In turn, for rainy season samples the relationship was $y = 0.012x - 1.84$, $r^2 = 0.417$, $p < 0.001$.

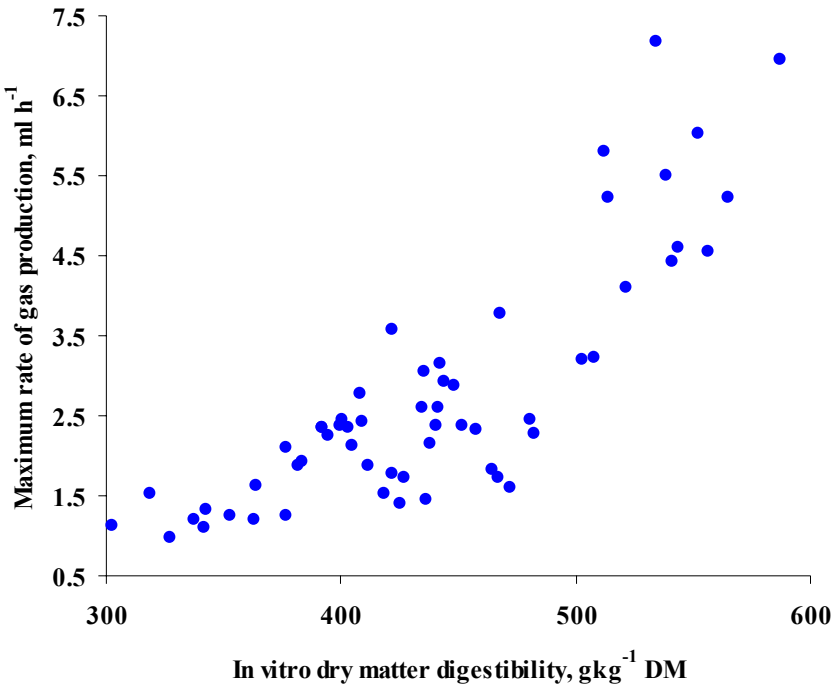

Figure 5.1-14. *The relationship between the maximum rate of gas accumulation and the* in vitro *dry matter digestibility of five* Desmodium ovalifolium *accessions planted in six sites (four environments: hillsides* = Cauca *(dry) and* Chinchiná *(wet), humid tropic* = Macagual *and* la Rueda *and well-drained savanna* = Alcancia *and* Maquenque*) in Colombia. The relationship shown corresponds to samples harvested during the dry season (eight weeks re-growth).*

5.1.3.4 Evaluation of the use of NIRS to predict gas accumulation

Near infrared reflectance spectroscopy showed promise to predict gas accumulation for the 1226 samples generated for the entire agronomic experiment conducted by Axel Schmidt (70 samples were lost out of a possible total of 1296). Most of those missing samples (62 of the 70) were lost in *Cauca* due to the extremely stressful conditions that prevailed during plant establishment and resulted in the lost of 31 of the 108 plots planted in that site. The remaining missing samples were lost in the *well-drained savanna* sites (6 in *Maquenque* and 2 in *Alcancia*).

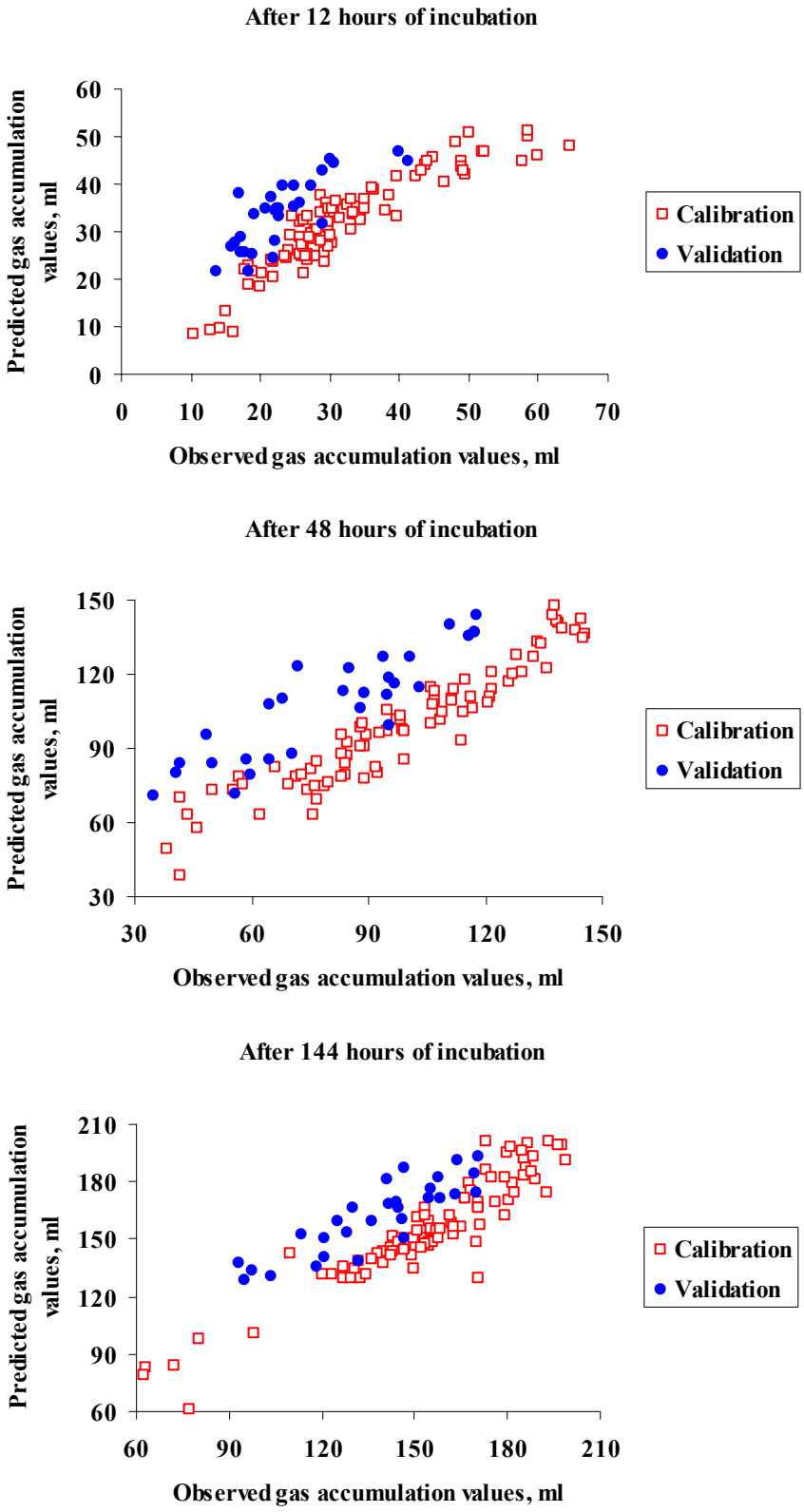

Figure 5.1-15. *The relationship between predicted (via near infrared reflectance spectroscopy) and observed gas accumulation at 12, 48 and 144 hours during the fermentation of five* Desmodium ovalifolium *accessions planted at two fertilizer levels in six contrasting sites in Colombia and harvested during the dry and rainy seasons. Calibration = 90 samples, validation = 30 samples.*

The typical fit obtained between the predicted and observed gas accumulation values for both the calibration and validation sets is shown in Figure 5.1-15, which shows the relationships for gas accumulation after 12, 48 and 144 h of fermentation. Overall, the r^2 between predicted and observed values increased as the fermentation progressed and was highest for the values observed after 48 h of incubation. Thus, after 12 h of incubation. the relationship between predicted and observed gas accumulation obeyed the equation $y = 0.784x + 6.513$, $r^2 = 0.843$, $P < 0.001$. In turn, after 48 h of incubation the relationship was $y = 0.903\ x + 9.805$, $r^2 = 0.905$, $P < 0.001$. Finally, after 144 h of incubation the relationship became $y = 0.896\ x + 16.69$, $r^2 = 0.868$, $P < 0.001$.

Also included in Figure 5.1-15 is the relationship between observed and predicted gas accumulation values for the 30 samples in the validation set. Although the r^2 for that relationship was high (0.63, 0.80 and 0.76, at 12, 48 and 144 h of incubation, respectively, $P < 0.001$), it is evident from Figure 5.1-15 that predicted values significantly overestimate observed gas accumulation in those particular sample sets. It must be recognised that the resulting ranking of the samples was unaffected by such bias. Additionally, for the purpose of establishing differences in quality among samples, absolute values are not necessary. However, it is evident that further work is needed to allow for the reliable use of this technique in the prediction of gas accumulation in these samples. That work will be carried out and presented in a later report.

5.1.4 DISCUSSION

Great emphasis was placed on the description of the results obtained in this study of the genotype-environments interactions in *D. ovalifolium*. This approach was necessary given the high number of samples at hand and the great variability in responses obtained, especially due to environmental variables (site, fertilizer and growth season). It is evident that, by describing results from this experiment on the basis of means rather than of individual values, we effectively smoothed out a great deal of that variability. However, all available data was included when performing linear regression analyses, which in a way, complements for the shortcomings of our chosen approach.

It must be emphasised that the thrust of this research is towards explaining differences in forage quality by virtue of differences in forage chemical composition, of which condensed tannins constitute a focal point. However, this objective must reconcile with the fact that, in more than a way, this part of our research is dependent on and is limited by the objectives of the main agronomic experiment conducted by Axel Schmidt. Hence, probably the greatest difficulty in writing this chapter is related to the need to minimise potential conflicts originated from similarities (or differences) in the use of experimental data by the main researcher in this experiment (A. Schmidt) and in this thesis. To illustrate, it is beyond the scope of this thesis to determine which agronomic factors are the underlying forces resulting in changes in the chemical composition of *D. ovalifolium*. Similarly, it is not our primary aim to identify the ideal agronomic conditions and the characteristics of the site that would optimise the use of *D. ovalifolium* as a forage legume. This is also true for the related objective of identifying superior or inferior *D. ovalifolium* accessions. On the other hand, it must be recognised that in the analysis and interpretation of our results great benefits would be gained by the inclusion of these considerations.

In the present study, changes in the chemical composition and nutritional quality of the five *D. ovalifolium* accessions were more related to environmental factors than to genotype-related aspects. Indeed, there was greater variability when results were plotted on a per-site basis than when presented using a per-accession approach (i.e. differences between means were lower and the standard error of the mean was higher in the latter than in the former). In agreement with these observations, isozyme characterisation failed to show the existence of genetic variability among the *D. ovalifolium* accessions studied in the main agronomic experiment (Bettina Klein and Axel Schmidt, personal communication).

This is not to say that there were no differences in forage quality among the five accessions studied in sections 5.1.3.1 to 5.1.3.3 of this thesis. On the contrary, accession CIAT 33058 was found to have the highest protein content and the lowest fibre (neutral and acid detergent fibre) content. This combination, together with an intermediate content of condensed tannins, explains why this accession had *in vitro* dry matter digestibility values and initial rates of gas accumulation that were greater than those of the other four accessions studied. On the other hand, accession CIAT

13125 had the lowest protein content and the highest content of acid detergent fibre and extractable condensed tannins, a combination that resulted in this accession having the lowest *in vitro* dry matter digestibility among the five accessions studied.

Whereas there was a measurable impact of genotype on forage quality in *D. ovalifolium*, the corresponding impact of the environmental factors was truly remarkable in comparison. For example, plants grown under improved soil fertility in the *well-drained savanna* sites (*Alcancia* and *Maquenque*) had 1.4 times more protein (174 vs. 124 g kg^{-1} of dry matter) than those grown under low soil fertility conditions. In turn, *D. ovalifolium* grown under low fertility conditions in these two sites had 1.35 times more condensed tannins (103.9 and 77.0 g kg^{-1} of dry matter) than when grown under conditions of improved soil fertility. Changes in acid detergent fibre in samples from these sites were more modest, with contents in low-fertility plants being 1.1 times higher than in the high-fertility plants.

The impact of environmental variables on chemical composition of *D. ovalifolium* was also apparent in the differences in chemical composition of plants grown in *Macagual* and *La Rueda.* Indeed, these plants differed greatly in neutral and acid detergent fibre and in protein content, despite the similarity in climatic characteristics at these two sites. Although differences in mineral composition of soils in these sites might account for these changes in plant chemical composition, differences in their physical characteristics (drainage, aeration, see Table 5.1-2 for differences in clay content) could have also played an important role in producing these responses.

Growth season also influenced the chemical composition of *D. ovalifolium*. For example, in samples from *La Rueda,* those harvested during the dry season had more fibre content (1.1 times more neutral detergent fibre and 1.18 times more acid detergent fibre) than those harvested during the rainy season. In turn, plants harvested from *Maquenque* during the rainy season had 1.32 times more total condensed tannins than those harvested during the dry season

Overall, the content of condensed tannins (g kg^{-1} of dry matter, average of the values observed for the five accessions within each site-fertilizer-season combination) ranged from 61 (*Maquenque*, dry season, and high fertilizer) to 114 (*Maquenque*, rainy season, and low fertilizer). Similar variations in condensed tannin concentration,

in response to different environmental factors, have been reported in the literature. For example, Barry and Forss (1983) reported that the condensed tannin concentration (g kg^{-1} of dry matter) in *Lotus pedunculatus* was 80-110 when grown in acid soils without fertiliser application and 20-30 when grown in high fertility soils. In turn, high growth temperature was associated with increased condensed tannin concentration in a normal and a low-tannin cultivar of sericea lespedeza (*Lespedeza cuneata*, Fales, 1984). This response was very dramatic in the leaves of the normal cultivar, where tannin concentration increased from 145 to 249 g kg^{-1} of dry matter. Likewise, *Lotus pedunculatus* grown under moisture stress (20% of field capacity) had a tannin content of 68 g kg^{-1} of dry matter when the temperature regimen was high (26/22 °C) and less that 20 when the growth temperature was low (10/14 °C, Anuraga et al., 1993). Conversely, Carter et al. (1999) observed that although the concentration of condensed tannins in leaves and stems of *Lotus corniculatus* CV Leo (S41) was increased by doubling the CO2 concentration, increasing the growth temperature reduced it. These authors also observed that drought stress caused a significant reduction in tannin concentration in S33 and S41 leaves. It could be that condensed tannin accumulation in response to a given environmental factor is species dependent.

In turn, it was interesting to observe that, with the exception of samples harvested from *Chinchiná* during the dry season, the concentration of bound (non-extractable in 70% aqueous acetone) condensed tannins was relatively stable across sites and accessions. In fact, there were no significant linear regression relationships between the content of bound condensed tannins and that of all other chemical constituents measured in this experiment. This was unexpected, given that the corresponding concentration of extractable condensed tannins and of protein and fibre (chemical constituents upon which bound tannins attach, Terrill et al., 1992b) did vary significantly. Logically, it could be expected that the concentration of bound condensed tannins would be related to the availability of attachment sites, a reasoning that receives support from the fact that in low-tannin plants, most of the tannins are non-extractable (Phil Morris, personal communication). On the other hand, it has been contended that true bound tannins do not exist within the living plant (Axel Schmidt, personal communication) as they are confined to the vacuole (Chafe and Durzan, 1973; Jones et al. 1973; Lees et al., 1993; 1995). Such compartmentalisation would be

needed to avoid tannins interfering with the plant own enzymatic processes. Under this perspective, bound tannins would come into existence during plant death, as the vacuole ruptures and tannins become free to interact with other chemical constituents of the plant. However, the content of bound tannins should also increase as the availability of binding sites increases. Of course, this discussion is based under the assumption that the specificity of the interaction between tannins and other molecules *from the same plant* remained unchanged throughout this experiment, although this is an area where little research has been advanced. Likewise, by harvesting samples of the same re-growth age and giving them the same post-harvesting treatment, most non-treatment sources of variation affecting the concentration of bound condensed tannins in *D. ovalifolium* should have been eliminated. Nonetheless, none of the observations from the current experiment helped to improve our understanding of the great variation previously observed for the concentration of bound condensed tannins among different tanniniferous legumes (Jackson et al. 1996a, Chapter 3 this thesis).

The observation that, among the 120 samples examined in the current study, condensed tannin content was never lower than 58 g kg^{-1} of dry matter warrants further discussion for two reasons. First, if as in many other plants, high condensed tannin concentration in *D. ovalifolium* is related to severe environmental conditions, then our failure to observe low tannin concentrations appears to suggest that the environments tested in this experiment failed to provide the optimum conditions for the growth of this legume. The alternative explanation would be that, at least partially, condensed tannin production is constitutively high in *D. ovalifolium*. If this was the case, then a series of other questions regarding the evolutionary significance of this behaviour and its energetic cost to the plant might ensue. Obviously, on the basis of our experimental data, we could only speculate about these issues.

The high content of condensed tannins in *D. ovalifolium* observed in the current study has also to be examined from the ruminant nutrition perspective. Traditionally, the presence of high concentrations of condensed tannins in *D. ovalifolium* has been associated with the low palatability and acceptability of this legume by livestock (Lascano and Salinas, 1982). Likewise, in *Lotus pedunculatus*, a concentration of condensed tannins higher than 40 g kg^{-1} of dry matter has deleterious effects on dry matter consumption and in nutrient digestibility and availability to ruminants (Barry

et al., 1986, Barry, 1989). In turn, *in vitro* studies suggested that, in *Lotus corniculatus*, condensed tannin concentrations in the range of 27 to 85 g of catechin equivalents kg^{-1} of dry matter would reduce ruminal protein degradation with little or no corresponding reduction in dry matter digestibility (Miller and Ehlke, 1994). Earlier findings reported in this thesis confirmed that the nutritional impact of condensed tannins is dependent upon their concentration in plant tissue, but also demonstrated the important role that tannin structure plays in this regard (see chapter 4). Viewed from this perspective, it seems feasible that the contrasting results in the studies of Barry and co-workers and those of Miller and Ehlke (1994) are a reflection of structure-activity relationships.

In reference to the impact of condensed tannins on the nutritive quality of *D. ovalifolium*, it is important to note that the nutritional quality observed in the current study for some *D. ovalifolium* samples was comparable to that reported in Chapter 3 for *Leucaena leucocephala*. Indeed, *in vitro* dry matter digestibility (g kg^{-1} of dry matter, all determinations made at CIAT, Colombia) was 620 in leaves of *L. leucocephala* and 560 (range 540 to 590) in *D. ovalifolium* grown during the dry season and under high fertilizer in *Maquenque*. As a further comparison, rate of gas accumulation in a great number of *D. ovalifolium* samples was found to be similar or even greater than that observed during the degradation of the non-tanniniferous *L. macrophylla*. This is a very important comparison, since it must be remembered that among all legumes studied in Chapter 3, the highest rate of gas accumulation during the first 12 h of incubation was observed with leaves from *L. macrophylla*. It must be clarified that the comparison we are referring to involved gas production experiments that were carried out at CIAT under the exact same conditions, a condition without which such comparison would not be possible. Before any other comparisons take place, it must emphasised that this "high" (with reference to other tropical legumes grown in acid soils) quality of *D. ovalifolium* occurred concomitantly with a high content of condensed tannins.

In the present study, linear regression analysis showed that condensed tannin concentration in *D. ovalifolium* was inversely related to *in vitro* degradation. However, when plants from all sites were included in this comparison, the resulting relationship, although statistically significant, was not very strong. This is explained

in the great variation in chemical composition that was observed among plants grown in the different sites. Indeed, the concentration of nutrients such as fibre and protein varied greatly in samples grown in the different sites. In addition, each of these components had its own significant contribution to the degradation of *D. ovalifolium,* with that involving protein being quite strong and positive. These observations suggest that we must re-evaluate the role of condensed tannins from the standpoint of forage (or diet) chemical composition and possible interaction between different chemical constituents. Likewise, structure-activity considerations must be included in this evaluation, and the following section of this thesis follows that approach (see Chapter 5.2).

When regression analyses were performed on a per site basis, there was a strong inverse relationship between the condensed tannin content and the rate of gas evolution in samples grown in the *well-drained savanna*. There are at least two reasons that account for this. First, variability in chemical composition was generally at its lowest when samples were compared on a per site basis. Additionally, in samples grown in *Alcancia* and *Maquenque*, fertilizer had a tremendous effect on chemical composition, effectively creating two clusters of samples, which not only differed in condensed tannin content, but also in their entire chemical composition. Thus, samples with a high tannin content also had a high content of acid detergent fibre and a low concentration of crude protein. Second, the greatest range in content of condensed tannins was observed among plants grown in these two sites under high and low fertilizer. This accentuated the effect of condensed tannin concentration upon the *in vitro* degradation of *D. ovalifolium*.

From this last observation, it would appear that a stronger relationship between tannin content and *in vitro* degradation of *D. ovalifolium* may have been observed had samples with truly low tannin levels been available for this comparison. This is not only because the greatest range in tannin concentration would make tannin effects more discernible, but also because there was an inverse relationship between the concentration of protein (x) and extractable condensed tannin (y) in samples harvested during the dry and rainy seasons. This relationship was $y = -0.267x + 95.44$, $r^2 = 0.227$, $P < 0.001$ for dry season samples and $y = -0.291x + 117.42$, $r^2 = 0.266$, $P < 0.001$ for rainy season samples. Such broadly inverse relationships between the content of crude

protein and condensed tannins have also been reported in sericea lespedeza (Cope, 1962, Donnelly and Anthony, 1969, Fales, 1984, Petersen et al., 1989). In the current experiment, the content of crude protein was also negatively related to that of acid detergent fibre.

Near infrared reflectance spectroscopy was shown to be a very useful technique for the prediction of gas accumulation in *D. ovalifolium*. Probably the greatest advantage of this non-destructive technique is the relatively little time and effort (especially when compared with the alternative of running gas accumulation experiments for all samples) that is required in the development of the predictive equation and its use to estimate gas accumulation. One potential problem with the use of this technique is that different equations (which could be based in different wavelengths) are used to predict gas accumulated at different time points during sample fermentation. In consequence, values for predicted gas accumulation at time x can be higher than those predicted at time $x+ y$, a situation that could never take place during a normal incubation. Another problem that was observed with the use of this technique was that the prediction of gas accumulation during the early stages of sample fermentation (6 h post-inoculation) was not as accurate as the prediction of gas accumulation for the later stages of fermentation (12 hours of incubation or more). Although it was not attempted to create prediction equations for the rate of gas accumulation, it is probable that estimates of the maximum rate of gas accumulation would be equally compromised, since this value tended to occur very early during the fermentation in a great number of samples. This is a potentially limiting problem given that the concentration of condensed tannins was proven to be strongly associated with the observed maximum rate of gas evolution (see chapter 3). Likewise, in the current work, there was a strong correlation between the maximum rate of gas accumulation and *in vitro* dry matter digestibility (Figure 5.1-14).

Preliminary predictions gas accumulation (data not shown) confirmed earlier observations that greater changes in nutritional quality were observed among these different accessions in response to environmental variables than to genotype-related factors. It was very interesting to note that in samples harvested during the rainy season, variation between sites was only evident in plants receiving the low-fertilizer treatment. This suggests that during the rainy season, the observed differences in

forage quality when *D. ovalifolium* was grown in different sites were more related to soil fertility that to either temperature or precipitation. On the other hand, notable differences were observed in the gas accumulation profiles of plants grown under high fertilizer and harvested during the dry season. From this it can be concluded that moisture stress and probably growth temperature do play a role in affecting quality of *D. ovalifolium* grown during the dry season.

5.2 MOLECULAR WEIGHT AND PROANTHOCYANIDIN MONOMER COMPOSITION IN PURIFIED CONDENSED TANNINS AND THEIR IMPACT ON FORAGE QUALITY IN DESMODIUM OVALIFOLIUM

5.2.1 INTRODUCTION

In the previous section, the chemical composition and forage quality of *Desmodium ovalifolium* was shown to vary greatly in response to environmental and genotypical factors. In particular, the concentration of condensed tannins varied greatly among the 120 samples (5 accessions, 6 sites, 2 fertilisation levels and 2 harvesting seasons) of *D. ovalifolium* that comprised the previously reported experiment. Observations in Chapter 5.1 confirmed earlier observations that the effects of tannins upon forage quality are a function of their concentration in plant tissue (Chapters 4.2 and 4.3). In turn, results reported in this thesis corroborated the results obtained by other researchers that the concentration of condensed tannins in plant tissue varies greatly in response to different environmental factors (Clarke et al. 1939; Donnelly, 1959, Feeny, 1970; Barry and Forss, 1983; Fales, 1984; Anuraga et al. 1993, Carter et al., 1999).

However, as observed earlier in this thesis (Chapter 4.1), the nutritional effects of condensed tannins are also influenced by their structural features, among which proanthocyanidin composition and molecular weight have received attention in earlier chapters of this thesis. These structural features have been shown to vary according to factors such as species and plant organ (Foo and Porter, 1980; Foo et al., 1982; Williams et al, 1983), sample treatment (Cano et al., 1994) and maturity (Butler, 1982). However, there is little knowledge on the changes in condensed tannin structure that could potentially occur in tanniniferous plants grown under different environmental conditions. Indeed, it seems possible that different environmental conditions could alter the expression of the enzymes controlling the biosynthesis of condensed tannins and, in consequence, alter the structural features of the resulting products. For example, changes in the relative expression of dihydroflavanol reductase and flavan-3,4-*cis*-diol reductase could effectively alter the ratios of flavan monomers and condensed tannin chain length (Morris and Robbins, 1997).

In the present study, we determined the structure of condensed tannins purified from five accessions of *Desmodium ovalifolium* grown under different conditions of

fertility and climate. Implications of the observed results are then discussed from the standpoint of forage nutritional quality.

1.0.0 MATERIALS AND METHODS

5.2.1.1 Purification of condensed tannins

Due to time constraints, condensed tannins analysed in the present study were purified from 30 out of the 120 *D. ovalifolium* samples available (i.e. those analysed in Chapter 5.1). Hence, samples from accessions CIAT 350, 3788, 13125, 23618 and 33058 that had been grown under high and low fertilisation in *Cauca*, *Macagual* and *Alcancia* and harvested during the rainy season were chosen for condensed tannin purification. The procedure for tannin purification was modified slightly, to increase speed and efficiency. From each of the available samples, a 2.0-g sub-sample was extracted with 20 ml of a 70% aqueous acetone solution containing 0.05% (w/v) ascorbic acid. After shaking for one h, samples were filtered through Whatman No. 1 filter paper, discarding the solid residue. The recovered filtrate was then extracted with 10 ml of diethyl ether (3x) in order to remove the chlorophyll that had been co-extracted with the condensed tannins. After this extraction, residual ether in the aqueous phase was evaporated by heating samples in a forced-draught oven for two h at 40°C. In the meantime, to each of ten 50-ml centrifuge tubes, 20 ml of a Sephadex LH-20 slurry in 50% aqueous methanol were added. An additional 15 ml of 50% methanol were added, tubes were mixed thoroughly by shaking in a vortex for 30 seconds and were then centrifuged at 3000 rpm for 10 min. The resulting supernatant was decanted, and the methanol wash was repeated two more times with the purpose of eliminating fine particles and allowing full equilibration of the Sephadex LH-20. Once ether had been evaporated from the samples, the aqueous solution containing the extracted condensed tannins (4-5 ml) was dissolved in an equal volume of 100% methanol and samples were added to the Sephadex tubes. An additional 10 ml of 50% methanol was used to ensure that most of the extract was added to the Sephadex tube. After thorough mixing of the samples, tubes were centrifuged at 3000 rpm for 10 min, discarding the supernatant. Then, the tubes were washed with 20-ml fractions of 50% methanol until the obtained supernatant was colourless (3-4 washes). Condensed tannins were then separated from the Sephadex by addition of 20 ml of a 70% aqueous acetone solution containing 0.05% (w/v) ascorbic acid. To eliminate possible

contamination by Sephadex, the resulting supernatant was filtrated through Whatman No. 1 filter paper. Additional acetone washes were performed until the obtained supernatant was colourless. Finally, after evaporation of the acetone at 40°C under a stream of dry air, samples were freeze dried and the purified tannins were stored at 20°C until their use in these experiments.

5.2.1.2 Structural characterisation of condensed tannins

The monomer composition (procyanidin-prodelphinidin-propelargonidin ratio) was determined in duplicate of all purified condensed tannins following the protocol that was described in Section 4.1.2.2. This time, however, the high-performance liquid chromatography system was fitted with a Waters 996-photodiode array detector.

In turn, the molecular weight of underivatised, purified condensed tannins was determined following the procedure described in Section 4.1.2.4, using dimethylformamide plus 0.1% (w/v) lithium bromide as the solvent and polyethylene glycol standards (440 to 22800 dalton) for the calibration curve. Determinations were made in duplicate, with samples dissolved in dimethylformamide immediately prior to their injection into the chromatography system.

5.2.1.3 Data analysis

The relationships between condensed tannin structure (molecular weight, monomer composition) and variables such as chemical composition and rate of gas evolution (that reported in Chapter 5.1) were investigated using linear regression analysis. For comparisons involving proanthocyanidins, their concentration in the original sample (g kg^{-1} of dry matter) was calculated by multiplying the total content of condensed tannins times the proanthocyanidin ratio (as a percentage, unknown peaks included) in the corresponding purified condensed tannins. Such calculation was not possible in the case of molecular weight estimates. Thus, regression analyses were performed between the molecular weight estimates and all other variables. To account for the variability due to differences in tannin concentration, linear regression relationships were plotted taking care to group samples according to their total condensed tannin content.

5.2.2 RESULTS

5.2.2.1 Condensed tannin structure

There was great variation in the procyanidin-prodelphinidin-propelargonidin ratio of purified tannins extracted from *D. ovalifolium* grown under different environmental conditions (see Figure 5.2-1). For example, the procyanidin content (mean of all 10 samples in each site and unidentified peaks included) comprised 57.5, 58.0 and 75.8% of the condensed tannins from *Alcancia, Macagual* and *Cauca*, respectively. In turn, the corresponding pelargonidin content in samples from those sites was 0.55, 2.42 and 1.09%, and the fisetinidin content was 0.27, 2.27 and 0%, respectively. No delphinidin was detected in any of the samples studied. Unidentified peaks comprised 41.7 and 37.3% of the condensed tannins from *Macagual* and *Alcancia* and 23.1% of those extracted from plants grown in the *Cauca* site.

Examination of the results from the perspective of fertilisation showed that condensed tannins from plants grown under low fertilisation in *Alcancia* tended to have higher cyanidin and pelargonidin content than their high-fertilisation counterparts (see Figure 5.2-1). In consequence, condensed tannins from plants grown under low fertilisation in *Alcancia* had a lower proportion of unidentified peaks than those extracted from plants grown under high fertilisation. On the other hand, condensed tannins purified from plants grown under low fertilisation in *Cauca* had lower contents of cyanidin and pelargonidin and a higher proportion of unidentified peaks (Figure 5.2-1). Finally, there were no changes in the proanthocyanidin composition of condensed tannins extracted from plants grown in *Macagual* under low and high fertilisation.

Differences in the monomer composition of condensed tannins were also apparent when results from different genotypes were examined (see Figure 5.2-2). For example, the cyanidin content (averaged across sites and fertilisation, $n = 6$) ranged from 57.5% in condensed tannins from accession 23618 to 68.7% in those from accession 350. In turn, the content of pelargonidin ranged from 0.38% (accession 23618) to 2.58% (accession 33058) and that of fisetinidin from 0.61% (accession 3788) to 1.04% (accession 350). Finally, the content of unidentified components ranged from 29.5% (accession 350) to 41.0% (accession 23618).

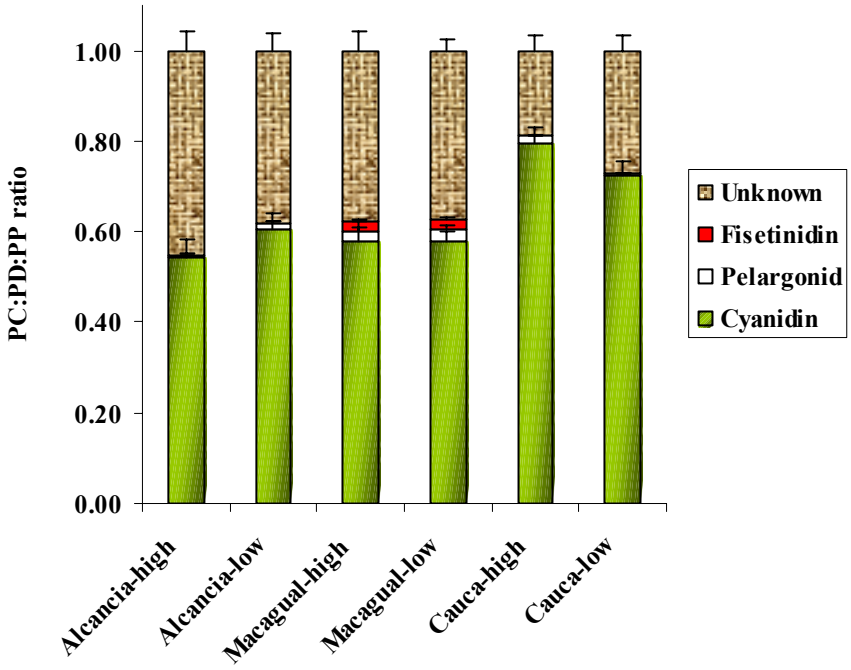

Figure 5.2-1. *Environment-related variation in the procyanidin-prodelphinidin-propelargonidin ratio of purified condensed tannins from five* Desmodium ovalifolium *accessions planted at two fertilisation levels (high and low) in three contrasting sites at Colombia and harvested during the rainy season. Experimental sites comprised* Cauca *(dry hillsides),* Macagual *(humid tropics) and* Alcancia *(well-drained savanna). Bars represent SEM where n= 5.*

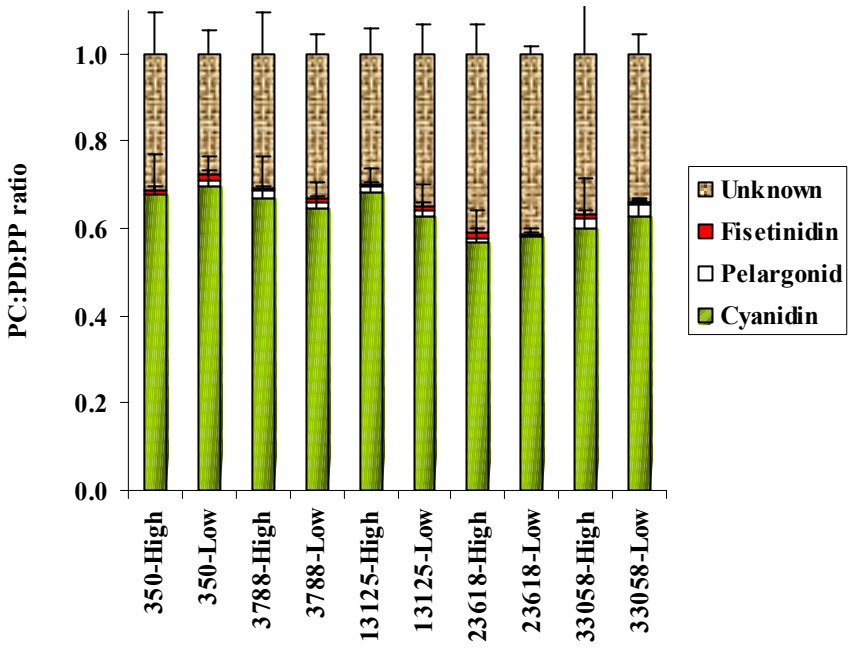

Figure 5.2-2. *Genotype-related variation in the procyanidin-prodelphinidin-propelargonidin ratio of purified condensed tannins from five* Desmodium ovalifolium *accessions planted at two fertilisation levels (high and low) in three contrasting sites at Colombia and harvested during the rainy season. Experimental sites comprised* Cauca *(dry hillsides),* Macagual *(humid tropics) and* Alcancia *(well-drained savanna). Bars represent SEM where n= 3.*

In general, viewed from the standpoint of the different accessions studied in this experiment, fertilisation was not associated with great changes in the procyanidin content of condensed tannins (see Figure 5.2-2). Indeed, the greatest change in cyanidin content in response to fertilisation was observed in samples from accession 13125, where this value (averaged across sites) was 68.1 and 62.9% in samples from plants grown under high and low fertilisation, respectively. On the other hand, the greatest changes in pelargonidin content in response to high fertilisation was the reduction (from 1.14 to 0.33 %) and the increment (from 0 to 0.77 0%) observed for accessions 350 and 23618, respectively.

As with the case of the proanthocyanidin content of condensed tannins, there was great variation in the molecular weight of condensed tannins extracted from plants grown in the different experimental sites. This variation can be easily appreciated in Figures 5.2-3 and 5.2-4, that shows typical chromatograms for condensed tannins extracted from accessions 3788 and 13125 grown in the three experimental sites under high and low fertilisation. From these figures, it is evident that not only did the average estimations of condensed tannin molecular weight change across these three sites, but also the mass distribution profiles were radically different. For example, in accessions 3788 and 13125, condensed tannins from plants grown in *Alcancia* had a distinctive peak at about 20 minutes (> 25000 dalton, see Figures 5.2-3 and 5.2-4). This peak was also present, although less prominently, in tannins from *Macagual,* but was absent in those from *Cauca*. Likewise, the distribution of molecular weight in the main peak (that observed between 21 and 30 minutes) in samples from *Macagual* and *Cauca* was very symmetrical, closely resembling the normal distribution curve, but not in tannins from *Alcancia*. As a final example, some low molecular weight peaks could be observed between 32 and 37 min (1500 - 500 dalton) in condensed tannins from *Macagual* and *Alcancia*, which were not present in tannins from *Cauca*.

For the purpose of this investigation, changes in molecular weight in response to environmental and genotypical factors were investigated by monitoring the changes in the molecular weight of tannins eluted between 21 and 30 minutes (i.e. “main” peak). Using these estimations, great variation in the molecular weight of condensed tannin was observed among samples from the different experimental sites (see Figure 5.2-5), with estimations of molecular weight being lowest in condensed tannins from *Cauca*,

intermediate in those from *Macagual* and highest in those from *Alcancia*. Estimations of *number-*, *weight-* and *z-average* molecular weight were 4960, 7590 and 11710 for condensed tannins extracted from plants grown in *Cauca* and 8185, 11640 and 15570 in those from *Alcancia*.

Examination of the combined effect of fertilisation and planting site showed that, in *Macagual*, condensed tannins from plants grown under high-fertilisation had greater molecular weight estimates than those grown under low soil fertility (see Figure 5.2-5). On the other hand, condensed tannins extracted from plants grown in *Cauca* under low fertilisation had greater molecular weight estimates then those grown under conditions of improved soil fertility. In turn, there were no differences in the molecular weight of condensed tannins extracted from plants grown under high and low fertilisation in *Alcancia*.

When compared across accessions, differences in molecular weight were not as pronounced. Indeed, the lowest molecular weight estimates (*number-*, *weight-* and *z-average*, averaged across site and fertilisation treatments, $n = 6$), were 6220, 9095 and 12860 for tannins from accession CIAT 23618, whereas the highest were 6840, 9985 and 13650 for tannins from accession CIAT 13125. In turn, the molecular weights of the different accessions (averaged across sites) varied to some extent in response to the fertilisation treatments (see Figure 5.2-6). For example, plants from accession CIAT 23618 had tannins of higher number average molecular weight when grown under low-fertility soils than when grown under improved soil fertility. The opposite behaviour was observed for accessions CIAT 350, 3788 and 33058.

5.2.2.2 The impact of condensed tannin structure on in vitro gas production

In the current experiment, all estimates of condensed tannin molecular weight were positively related to the rate of gas production during the early stages of the fermentation (see Figure 5.2-7). For example, the *number average* molecular weight (x) was positively related to the rate of gas accumulation observed between 4 and 24 h post-inoculation ($P < 0.05$), but it had its strongest relationship with rate of gas production after 12 h of fermentation ($rate_{12} = 0.0049x - 0.88$, $r^2 = 0.303$, $P = 0.002$). In turn, the strongest relationships involving *weight-* and *z-* average molecular weights occurred with rates of gas accumulation after 15 and 18 h post-incubation ($P \leq 0.05$).

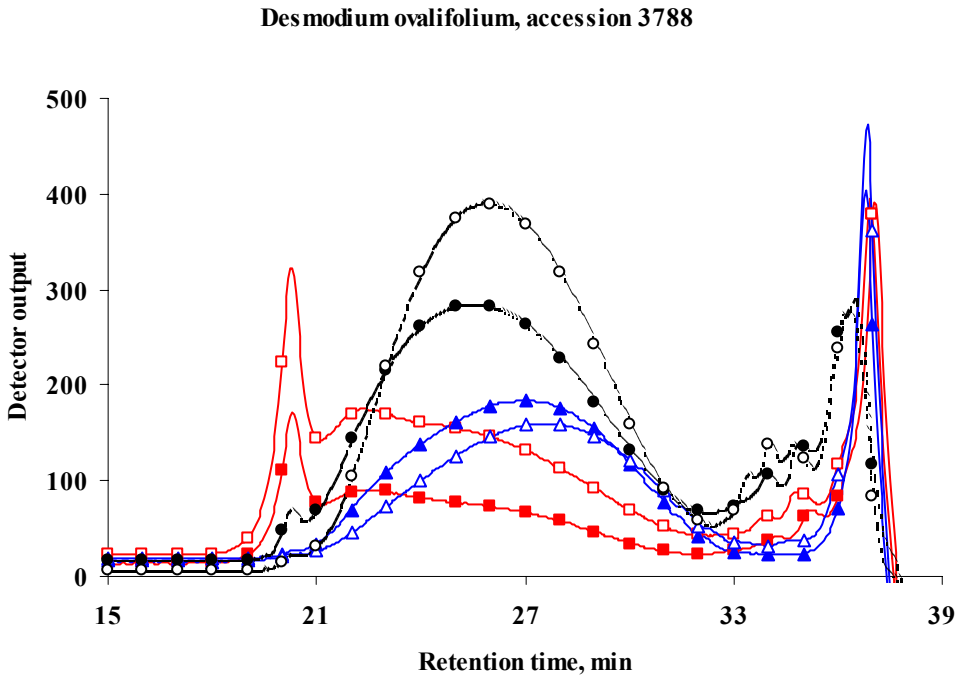

Figure 5.2-3. *Variation in the molecular weight of condensed tannins from* Desmodium ovalifolium *accession CIAT 3788 grown under two fertilisation levels (high and low) in three contrasting sites at Colombia and harvested during the rainy season. Experimental sites comprised* Cauca *(dry hillsides, high = ▲, low = Δ),* Macagual *(humid tropics, high = ●, low =* O*) and* Alcancia *(well-drained savanna, high = ■, low =). Bars represent SEM where n= 5.*

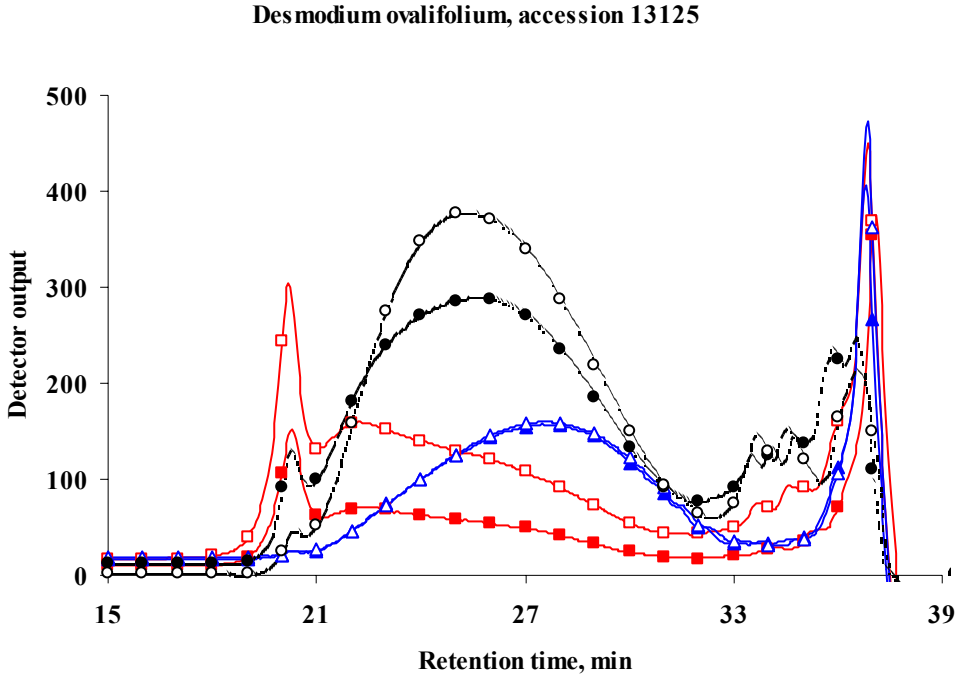

Figure 5.2-4. *Variation in the molecular weight of condensed tannins from* Desmodium ovalifolium *accession CIAT 13125 grown under two fertilisation levels (high and low) in three contrasting sites at Colombia and harvested during the rainy season. Experimental sites comprised* Cauca *(dry hillsides, high = ▲, low = Δ),* Macagual *(humid tropics, high = ●, low =* O*) and* Alcancia *(well-drained savanna, high = ■, low =). Bars represent SEM where n= 5.*

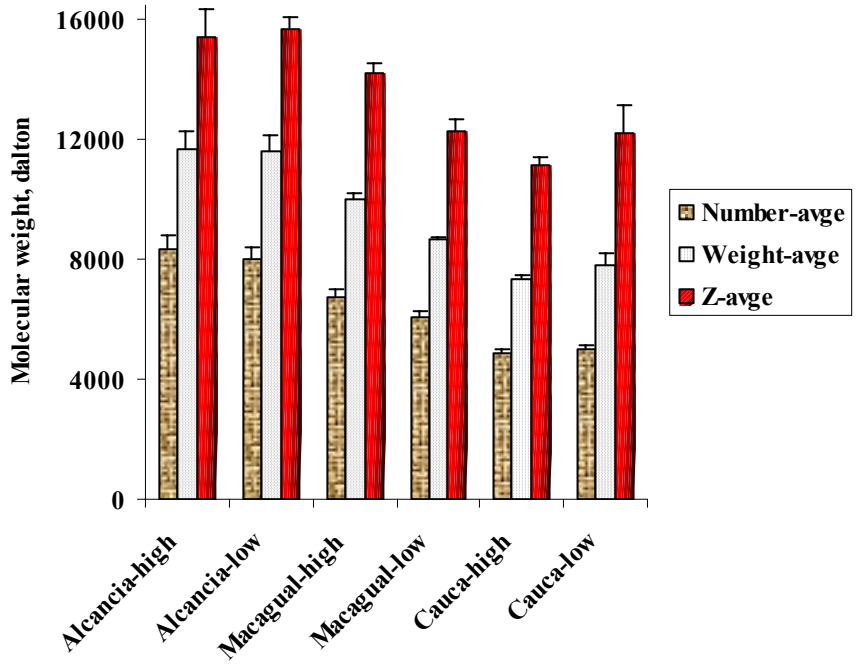

Figure 5.2-5. *Environment-related variation in the molecular weight of purified condensed tannins from five* Desmodium ovalifolium *accessions planted at two fertilisation levels (high and low) in three contrasting sites at Colombia and harvested during the rainy season. Experimental sites comprised* Cauca *(dry hillsides),* Macagual *(humid tropics) and* Alcancia *(well-drained savanna). Bars represent SEM where n= 5.*

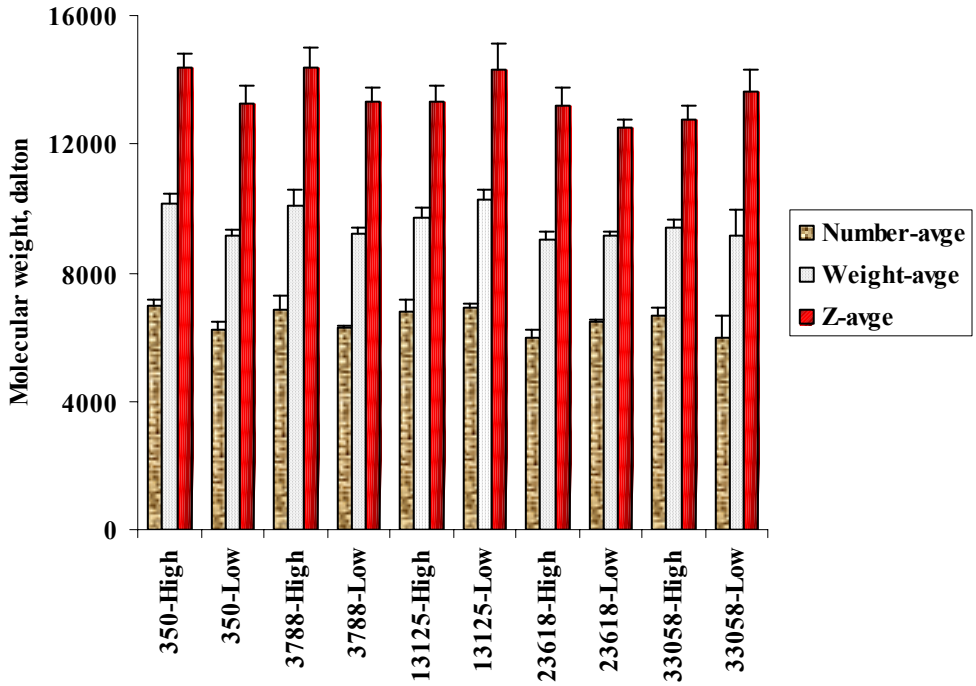

Figure 5.2-6. *Genotype-related variation in the molecular weight of purified condensed tannins from five* Desmodium ovalifolium *accessions planted at two fertilisation levels (high and low) in three contrasting sites at Colombia and harvested during the rainy season. Experimental sites comprised* Cauca *(dry hillsides),* Macagual *(humid tropics) and* Alcancia *(well-drained savanna). Bars represent SEM where n= 3.*

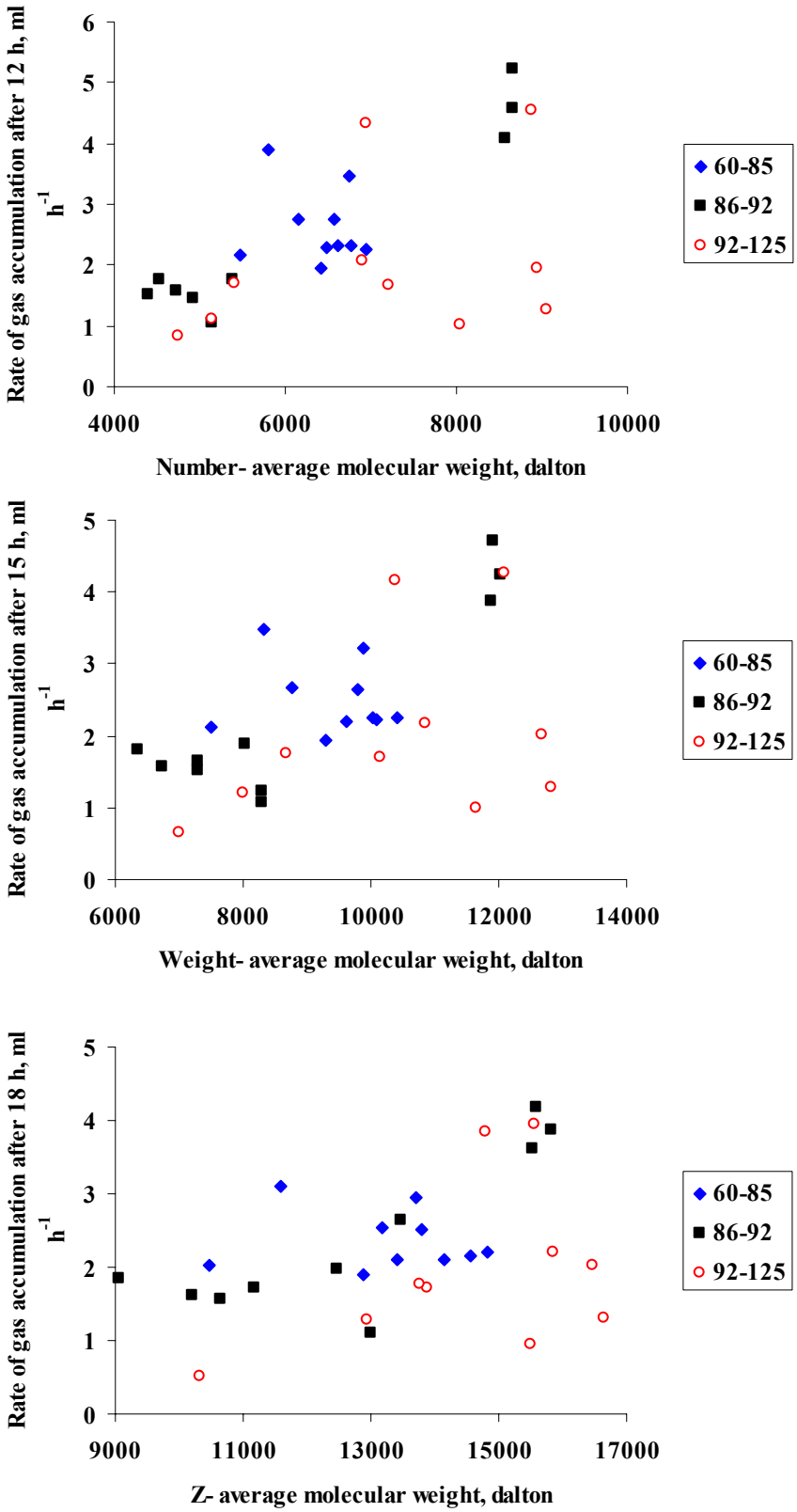

Figure 5.2-7. *The relationship between condensed tannin molecular weight and the rate of gas accumulation observed during the fermentation of five* Desmodium ovalifolium *accessions planted at two fertilisation levels in three contrasting sites in Colombia and harvested during the rainy season. Numbers in the legend represent the range in condensed tannin concentration (g kg^{-1} of dry matter) observed in these samples.*

It should noted that in all the relationships plotted in Figure 5.2-7, there were four samples that, did not follow the general trend, as they had lower rates of gas production than could be predicted in terms of condensed tannin concentration and molecular weight. All these samples came from plants grown under low fertilisation in the *Alcancia* site.

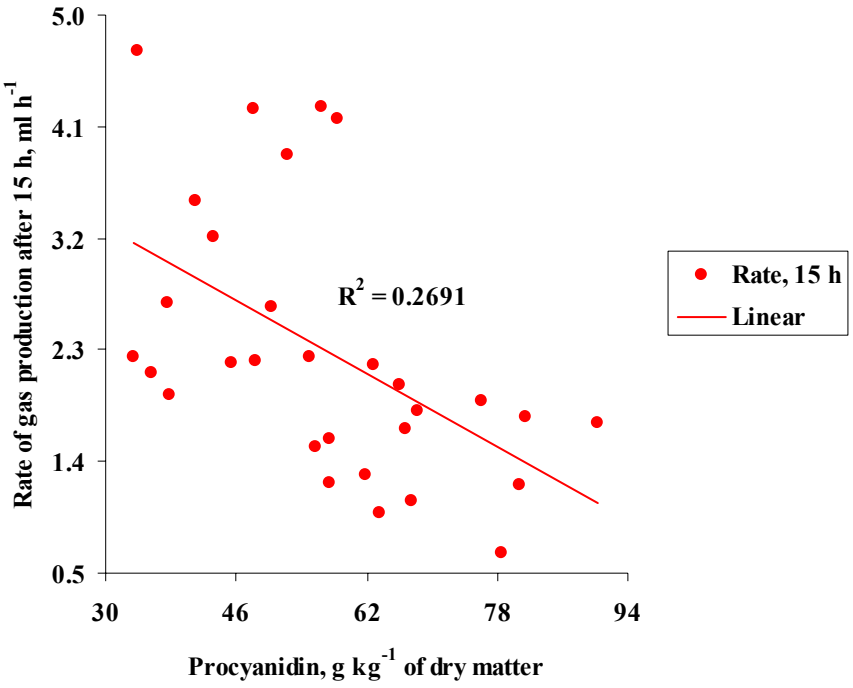

Figure 5.2-8. *The relationship between cyanidin content (g kg^{-1} of dry matter) and the rate of gas accumulation observed during the fermentation of five* Desmodium ovalifolium *accessions planted at two fertilisation levels in three contrasting sites in Colombia and harvested during the rainy season. Experimental sites comprised* Cauca *(dry hillsides),* Macagual *(humid tropics) and* Alcancia *(well-drained savanna).*

The procyanidin content (g kg^{-1} of dry matter) was negatively related to the rate of gas accumulation (ml h^{-1}) observed between 4 and 27 h post-inoculation ($P < 0.05$). This relationship reached its strongest point after 15 h of fermentation and was described by the equation: $Rate_{15\ hours} = -0.0369(cyanidin) + 4.4$, $r^2 = 0.269$, $P < 0.01$ (see Figure 5.2-8 above).

5.2.2.3 Other relationships involving condensed tannin structure

Linear regression analysis showed that the content of neutral detergent fibre was positively related to the molecular weight of condensed tannins (see Figure 5.2-9). For example, the relationship with *number-average* molecular weight (*y*) followed the equation y = 28.6 (neutral detergent fibre) + 6563, $r^2 = 0.588$, $P < 0.001$.

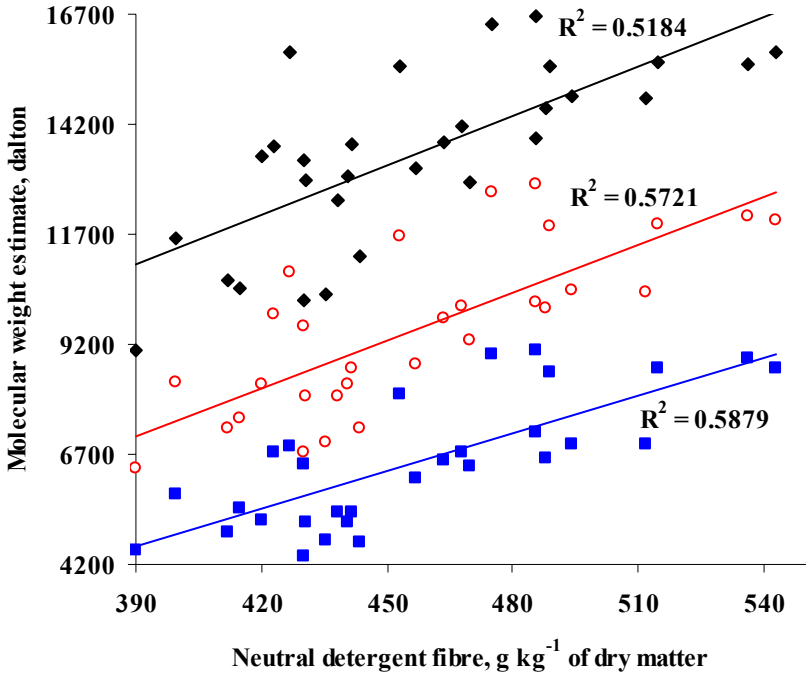

Figure 5.2-9. *The relationship between neutral detergent fibre (g kg^{-1} of dry matter) and the* number- (■), weight- (O) *and* z- (◆) *average molecular weight of condensed tannins from five* Desmodium ovalifolium *accessions planted at two fertilisation levels in three contrasting sites in Colombia and harvested during the rainy season. Experimental sites comprised* Cauca *(dry hillsides),* Macagual *(humid tropics) and* Alcancia *(well-drained savanna).*

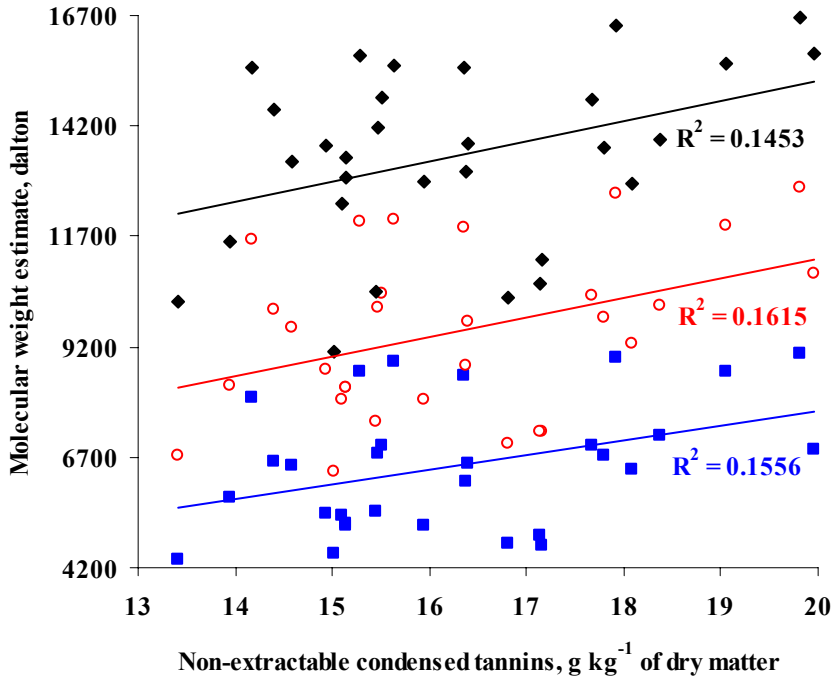

Figure 5.2-10. *The relationship between non-extractable condensed tannins (g kg^{-1} of dry matter) and the* number- (■), weight- (O) *and* z- (◆) *average molecular weight of condensed tannins from five* Desmodium ovalifolium *accessions planted at two fertilisation levels in three contrasting sites in Colombia and harvested during the rainy season. Experimental sites comprised* Cauca *(dry hillsides),* Macagual *(humid tropics) and* Alcancia *(well-drained savanna).*

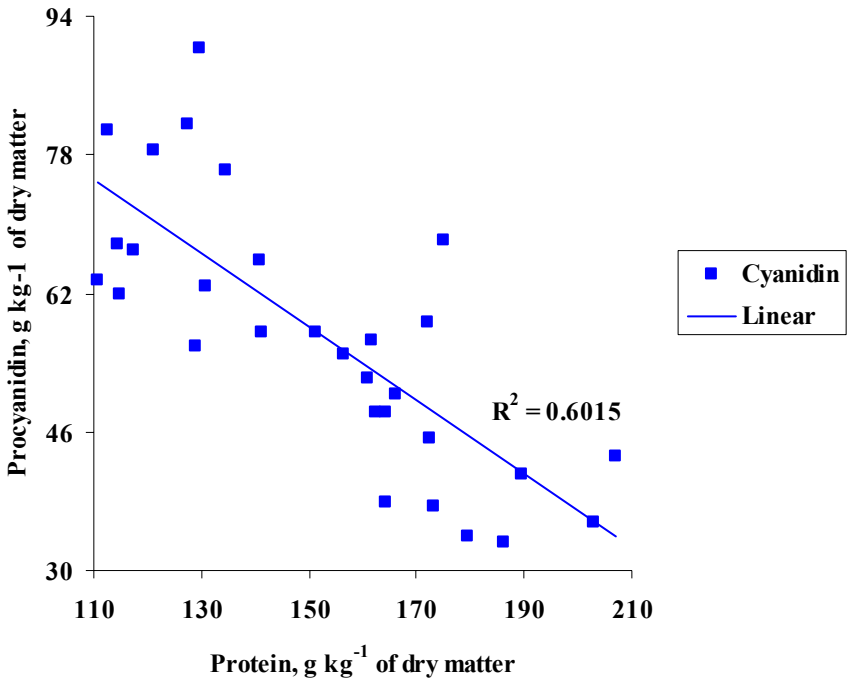

Figure 5.2-11. *The relationship between the contents of protein and procyanidin (g/ kg of dry matter) in five* Desmodium ovalifolium *accessions planted at two fertilisation levels in three contrasting sites in Colombia and harvested during the rainy season. Experimental sites comprised* Cauca *(dry hillsides),* Macagual *(humid tropics) and* Alcancia *(well-drained savanna).*

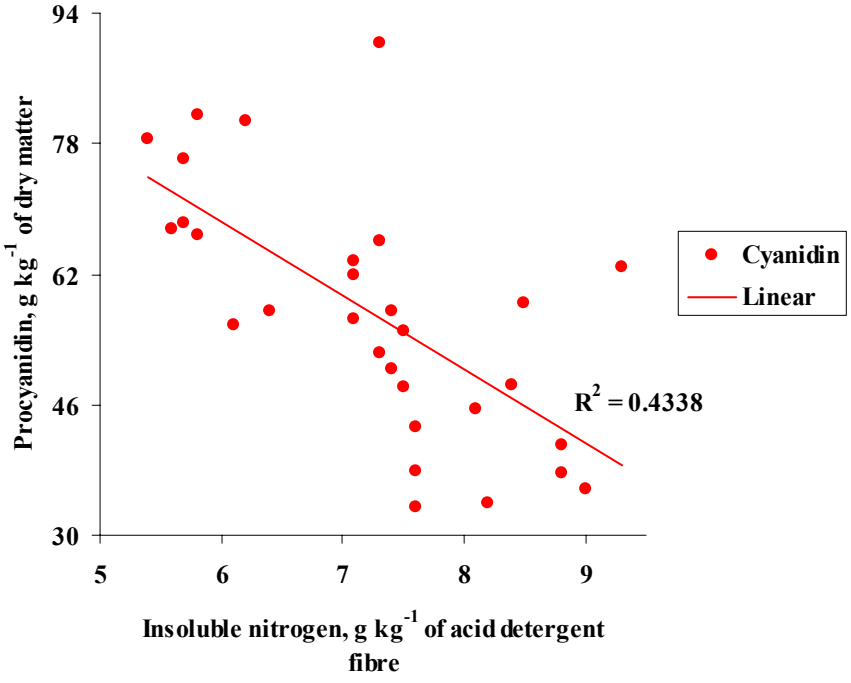

Figure 5.2-12. *The relationship between the contents of insoluble nitrogen (g kg^{-1} of acid detergent fibre) and procyanidin (g kg^{-1} of dry matter) in five* Desmodium ovalifolium *accessions planted at two fertilisation levels in three contrasting sites in Colombia and harvested during the rainy season. Experimental sites comprised* Cauca *(dry hillsides),* Macagual *(humid tropics) and* Alcancia *(well-drained savanna).*

In turn, the content of insoluble condensed tannins (g kg^{-1} of dry matter) was also positively related to the estimates of tannin molecular weight, although these relationships were weaker than those observed for neutral detergent fibre (see Figure 5.2-10). The r^2 of the linear regression relationship ranged 0.145 (*z-average* molecular weight) and 0.162 (*weight-average* molecular weight; $P < 0.05$). In addition to these relationships, all estimates of condensed tannin molecular weight were positively related to the content of acid detergent fibre ($P \leq 0.05$), and to the content of insoluble nitrogen in the acid detergent fibre residue ($P \leq 0.05$).

With reference to the proanthocyanidins, the content of procyanidin (g kg^{-1} of dry matter) was found to have a very strong and inverse relationship with protein content (see Figure 5.2-11). This inverse relationship followed the equation: *procyanidin* = -0.426 (*protein*) + 121.9, $r^2 = 0.602$, $P < 0.001$. The content of procyanidin was also inversely related to the concentration of insoluble nitrogen in acid detergent fibre and this relationship could be described as: *procyanidin* = -9.072 (*protein*) + 118.3, $r^2 = 0.434$, $P < 0.001$ (see Figure 5.2-12). Additionally, as expected, the procyanidin content (g kg^{-1} of dry matter) was strongly and positively related to acetone-extractable and total condensed tannins content ($r^2 \geq 0.533$, $P < 0.001$). Of the other proanthocyanidins, only profisetinidin was found to have significant relationships with other chemical constituents of *D. ovalifolium*. Indeed, fisetinidin content (g kg^{-1} of dry matter) was positively related to the contents of acid detergent fibre ($P < 0.05$), acid detergent insoluble nitrogen ($P < 0.01$) and protein ($P < 0.001$). It should be noted that neither the content of fisetinidin nor that of pelargonidin was significantly related to rate of gas accumulation. Additionally, no measure of condensed tannin structure was significantly related to *in vitro* dry matter digestibility as determined by the technique of Tilley and Terry (1963).

5.2.3 DISCUSSION

The effects of environmental factors upon condensed tannin concentration have been well documented (Feeny and Bostock, 1968; Feeny, 1970, Barry and Forss, 1983; Fales, 1984; Anuraga et al., 1993; Lees et al., 1995, Carter et al., 1999). Conversely, the effects of environmental factors upon condensed tannin chemistry have received less attention. Although some studies have dealt with changes in tannin chemistry throughout the growth season of fruits (Goldstein and Swain, 1963) and cereals

(Bullard et al., 1981; Brandon et al., 1982; Butler, 1982), little is known about the effect of variables such as growth temperature and soil fertility upon tannin structure. The present results have demonstrated that the impact of environmental factors are not only restricted condensed tannin concentration in plant tissue, but can also affect the condensed tannin structure. To my knowledge, the current study is the first to document changes in tannin structure in response to changes in environmental variables.

In the current experiment, the impact of environmental factors upon condensed tannin structure was more pronounced than that of the genotype variables. This observation is in agreement with those made in the previous chapter with regard to the chemical composition of *D. ovalifolium*. Changes in condensed tannin structure observed in the present experiment seem plausible, given our present knowledge of condensed tannin biosynthesis. Indeed, as discussed earlier in this chapter, changes in the relative expression of key enzymes in the biosynthetic pathway of condensed tannins, could effectively alter the structure of the resulting product (Morris and Robbins, 1997). Several examples of this can be found in the literature. For example, in auxin-treated root cultures of *Lotus corniculatus*, not only was tannin accumulation inhibited, but also there was some evidence for increased procyanidin content in the condensed tannins produced (Morris and Robbins, 1992). Likewise, after the genetic modification of roots of *L. corniculatus*, Carron et al. (1994) observed reductions in condensed tannin accumulation and an increase in the levels of procyanidin from 80% up to 100%. In turn, introduction of a full length *Antirrhinum majus* gene in sense in *L. corniculatus*, resulted in increased incorporation of propelargonidin in the condensed tannin produced (Bavage et al., 1995, 1997).

In the present study, the mechanisms eliciting these structural changes in condensed tannins from *D. ovalifolium* are not clear. On the other hand, despite the fact that the conditions of the present experiment were not ideal to test such hypothesis, these results suggest that these changes in condensed tannin structure would be of importance in determining the nutritive value of this tropical forage legume. In this experiment, discussion on structure-activity relationships of condensed tannins must acknowledge the fact that the tannins under study did not contain prodelphinidin and that their molecular weight was very high. In fact, in the current study estimates of *number-average* molecular weight in underivatised condensed tannins ranged from

4400 to 9000 dalton. By comparison, estimates of *number-average* molecular weights reported in Chapter 4.1 for underivatised condensed tannins from six tanniniferous tropical forage legumes, ranged from 2360 to 4900 dalton.

The observation that the molecular weight of condensed tannins from *D. ovalifolium* was positively related to the rate of gas production, supports the idea that as the size of tannins exceeds the optimum value, tannins lose their ability to form insoluble complexes with other molecules (Salunkhe et al., 1990). In early research, Jones et al (1976) reported that condensed tannins isolated from sainfoin were less effective in precipitating protein than tannins from *Lotus* sp. and hypothesised that this difference was due to the higher (17,000-28,0000 vs. 6000-7100 dalton) *z-average* molecular weight of the sainfoin tannins. Similarly, Kumar and Vaithiyanathan (1990) suggested than when their molecular weight is rather large, condensed tannins become rather insoluble in physiological conditions and lose their protein-precipitating ability.

One interesting aspect of the change in molecular weight of condensed tannins in response to different environmental factors lies on the implications of this response, if any, in terms of plant fitness. Likewise, questions regarding the mechanisms involved in this response need to be raised. For example, how is this change in molecular weight mediated? Or what are the specific environmental factors eliciting such a response? The data available in the present study showed that soil fertility was not a major factor in this response in condensed tannins purified from plants grown under low and high fertilisation in *Alcancia*. On the other hand, there were fertilisation-associated changes in the molecular weight of condensed tannins from plants grown at the other two sites. It is also probable that growth temperature, precipitation and soil pH (all of which were different across the three sites, see Table 5.1-2, chapter 5.1) also played a role in producing these changes in tannin molecular weight. It is evident that further investigation is needed to isolate the specific effects of these changes in tannin size and the mechanisms underlying them.

In the present experiment, the procyanidin content was found to have a negative relationship to the rate of gas accumulation observed during the early stages of the fermentation of samples from *D. ovalifolium*. Likewise, in the gas experiments reported in chapter 3, the content of procyanidin in condensed tannin from six tropical legumes was negatively related to the maximum rate of gas production and to gas accumulation at 12 and 24 hours post-inoculation. On the other hand, in the present

experiment, the presence of the proanthocyanidins propelargonidin and profisetinidin in tannins from *D. ovalifolium* had no significant relationship with gas accumulation. It is uncertain whether this was due to significantly lower proportion in which these proanthocyanidins were found in tannins from *D. ovalifolium* or to actual differences in hydroxylation patterns between these two proanthocyanidins and procyanidin. On the other hand, we must remember that although proanthocyanidin ratio is important in determining the nutritional impact of condensed tannins, the spatial distribution of these proanthocyanidins within the tannin molecule is also likely to have an effect in this regard. Hence, this data does not allow to completely dismiss the role played by these minor proanthocyanidins in the biological impact of these condensed tannins

Since procyanidin was the major component of the tannins from *D. ovalifolium*, there was a very strong relationship between procyanidin content and condensed tannin content. On the other hand, the contents of procyanidin and protein were inversely related to each other. Thus, this observation raises questions of a similar nature to those generated by the observed changes in molecular weight of condensed tannins (see above). An additional question generated by the determination of proanthocyanidin ratio in tannins from *D. ovalifolium* should concern the nature of the compounds that we labelled as unknown. It was evident that these compounds were related to the condensed tannins assayed, given that all of them had wavelengths of maximum absorption ranging from 500 to 540 nm. However, most of then appeared very late in the chromatograms, indicating differences in their level of hydroxylation, possibly relating to substitution with moieties such as methyl and/or other groups or other such modifications. The probability of such compounds being an artefact of the analytical technique employed appear to be minimised in the light of the observation that the presence of this unidentified compounds was very consistent across samples grown at the different sites.

Finally, although the present observations have confirmed the importance of structure-activity relationships in condensed tannins, they also have demonstrated that forage quality is the result of the interplay of many different factors. This was very effectively illustrated when plotting the relationship between condensed tannin molecular weight and rate of gas production in Figure 5.2-7. In these plots, it becomes obvious that the rate of gas accumulation in the low-fertilisation samples from *Alcancia*, although influenced by condensed tannin molecular weight, were also

influenced by some other factors. In chapter 5.1 we identified protein and fibre content as some of these factors. Thus, further research aimed at determining the relative impact of tannins upon the quality of tanniniferous forage legumes should be concerned with determining not only condensed tannin content, but also their corresponding structure and their interaction with other dietary constituents.

6. GENERAL CONCLUSIONS AND RECOMMENDATIONS FOR FURTHER WORK

6.1 GENERAL CONCLUSIONS

Several conclusions can be drawn from the experimentation reported in this thesis:

i) The presence of condensed tannins in tropical forage legumes has an overall negative relationship with their *in vitro* degradability. This proposition is thoroughly supported by the results obtained in the *in vitro* gas production experiments reported in Chapters 3, 4.2 and 5.1 and in the enzymatic activity experiments reported in Chapter 4.3. It must be understood, however, that this does not necessarily traduce to a negative impact on ruminant production. A reduction of ruminal protein degradation in response to the presence of condensed tannins, for example, is deemed as beneficial (Mangan, 1988), provided that the rumen-undegraded protein is later made available to the ruminant. Likewise, there have been reports of decreased carcass fatness in animals consuming tannin-containing diets (Purchas and Keogh, 1984; Terrill et al., 1992a). These observations could be associated with the changes in the molar proportion of volatile fatty acid produced during the fermentation of tanniniferous diets (such as those observed in the gas accumulation experiments reported in Chapter 4.2).

ii) Results from the gas production experiments reported in Chapters 3, 4.2 and 5.1 of this thesis suggest that condensed tannins in tropical forage legumes affect the rate of forage degradation to a greater extent than they affect the overall extent of forage degradation. Furthermore, in the majority of the cases, the impact of condensed tannins upon the rate of forage degradation (as indicated by the r^2 of the appropriate linear regression relationship) reached its maximum during the early stages of fermentation (usually within the first 20 hours post-inoculation). Although it is evident that the present results must be corroborated *in vivo*, these observations are in agreement with earlier reports that condensed tannins in *Lotus pedunculatus* reduced the rumen degradability of dry matter in sheep (Barry and Manley, 1984, Barry et al., 1986). In those experiments, however, the total tract apparent digestibility was unaffected by the presence of condensed tannins.

iii) The overall impact of the presence of condensed tannins upon forage degradability is a function of condensed tannin concentration (Chapter 3, 4.2, 4.3 and 5.1), form (soluble vs. substrate-associated, Chapter 4.2) and structure (Chapter 4.1, 4.2, 4.3 and 5.2). For the most part, in the gas production experiments reported in Chapter 4.2, there was a linear relationship between increasing concentrations of condensed tannins from *D. ovalifolium* and the resulting reductions in gas production. However, in the experiments where D-glucose was used as substrate, there was evidence that at a high tannin-substrate ratio, a point was reached where further increases in this ratio were not associated with more reductions in gas accumulation. Similarly, when the activity of different fibrolytic enzymes in the presence of condensed tannins was determined (Chapter 4.3), the observed relationship between increasing condensed tannin concentration and resulting enzyme activity was curvilinear and best described by polynomial equations. This is in agreement with earlier observations that the correlation between tannin concentration and the degree of enzymatic inhibition has proven unsatisfactory (Daiber, 1975; Gupta and Haslam, 1980, Bullard et al., 1981). Likewise, previous reports showed that not only the formation of insoluble tannin-protein complexes (Van Buren and Robinson, 1969; Hagerman and Robbins, 1987) but also the stoicheiometry of the resulting precipitates (Mole and Waterman, 1987a) is dependent upon the relative concentration of tannin and protein.

When comparisons were made on the basis of tannin form, substrate-associated tannins were consistently shown to be more effective in preventing the degradation of forages by rumen microorganisms (Chapter 4.2) and in inhibiting the activity of fungal fibrolytic enzymes (Chapter 4.3) than soluble tannins. In contrast, in Chapters 3 and 5.1, the concentration of condensed tannins non-extractable in 70% aqueous acetone was not related to forage degradability. The lack of correlation between the concentration of non-extractable condensed tannin and forage degradability might be partly explained on the basis of structure-activity relationships. However, it is also evident that the extractability of condensed tannins in organic solvents bears no relationship to the form in which condensed tannins will be found in the digestive tract after the ingestion of tanniniferous diets by ruminants. An observation that can be interpreted as providing support for this idea, was the fact that the concentration of non-extractable condensed tannins did not vary to a great extent when *D. ovalifolium* was grown under different environmental conditions (Chapter 5.1). On the other hand,

great variation in the content of protein, fibre and even condensed tannins was observed in this experiment.

The impact of condensed tannin structure (i.e. molecular weight and monomer composition) upon forage degradability was demonstrated by the results reported in all chapters of this thesis. A combination of the results obtained in the experiments reported in Chapters 3 and 4.1 with those reported in Chapters 5.1 and 5.2 leads to the conclusion that the negative impact of condensed tannins upon forage degradability was maximised when their number-average molecular was somewhere around 4900 dalton (see Figure 6-1). Similarly, Kumar and Vaithiyanathan (1990) and Salunkhe et al. (1990) suggested that there is an optimum molecular weight below and above which there is a reduction in the ability of condensed tannins to precipitate proteins and other molecules.

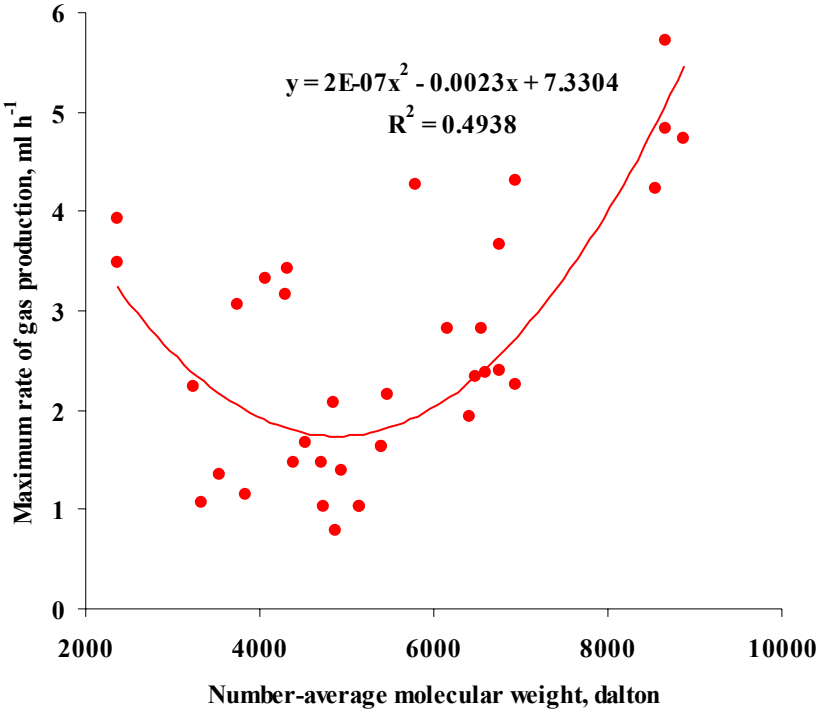

Figure 6-1. *The combined relationship between the maximum rate of gas production and the number average molecular weight of condensed tannins extracted from mature and immature leaves from six tropical legumes (Chapters 3 and 4.1) and those extracted from five* Desmodium ovalifolium *accessions grown under different conditions (Chapters 5.1 and 5.2).*

In turn, although in a less consistent fashion than observed for molecular weight, results from these experiments provided illustration of the influence that monomer composition of condensed tannins can have upon forage quality of tropical legumes.

For example, in the gas production experiments reported in Chapters 3 and 5.1, the rate of gas accumulation was found to have an inverse relationship with the content of procyanidin as determined in Chapters 4.1 and 5.2, respectively. On the other hand, other researchers have reported that increases in prodelphinidin content were associated with increases in astringency (Jones et al., 1976) and antiherbivore activity (Ayres et al., 1997) of condensed tannins. Indeed, great care must be taken when interpreting these results. First of all, as evidenced in the present experiments, the potential variability in monomer composition in condensed tannins from different sources is likely to be much greater than encountered in the experiments reported in this thesis. A greater survey of tanniniferous legumes than that evaluated in the current study is needed to unequivocally establish the impact of monomer composition of condensed tannins upon the nutritional value of these legumes. Likewise, because the influence of tannin molecular weight appears to be so dominant (Bathe-Smith, 1973a; Porter and Woodruffe, 1984; Oh and Hoff, 1979; Kumar and Horigome, 1986), it would be ideal to study the role played by different levels of hydroxylation in condensed tannins of similar molecular weight. Secondly, as suggested before, there is a very strong likelihood that the nutritional impact of condensed tannins would be not only a reflection of their monomer composition, but also of the sequential arrangement of these monomers within the tannin molecule.

iv) Condensed tannins are not a uniform chemical entity. Indeed, great variation was observed both in the molecular weight and the monomeric composition of condensed tannins extracted from the six tanniniferous tropical legumes studied in this thesis (Chapter 4.1). Other researchers, have also reported great variation in the structure of condensed tannins from different temperate species (Foo and Porter, 1980; Foo et al., 1982; Williams et al., 1983). In addition, in the present work, there was great variation in the molecular weight and monomer composition of condensed tannins from different *Desmodium ovalifolium* accessions grown under different environmental conditions (Chapter 5.2). As a further observation, the use of gel permeation chromatography to determine condensed tannin molecular weight clearly showed that condensed tannins are polydisperse molecules. The coexistence of tannin molecules of different molecular weight within the same plant creates the possibility of having specialised tannins. This is because as their molecular weight changes, the ability of these tannins to bind and precipitate other molecules will also change (Oh

and Hoff, 1979; Porter and Woodruffe, 1984; Kumar and Horigome, 1986). Finally, there were also indications that the monomer composition of acetone-extractable tannins would differ from that of non-extractable tannins (Chapter 4.1). Because all these changes in structure will result in changes in condensed tannin activity, it is not possible to continue to study the nutritional impact of condensed tannins on the sole basis of their concentration in plant tissue.

v) Among the condensed tannins studied for this thesis, those from *L. leucocephala* were consistently found to have the lower anti-nutritional impact. By comparison, condensed tannins from the closely related *L. pallida* were among the ones with the most deleterious impact on tissue digestibility and enzyme activity. Similarly, in a recent study, McNeill et al. (1998) observed that condensed tannins from *L. leucocephala* were exceptionally weak binders of protein, in comparison to those from *L. pallida*, *L. trichandra, Lotus pedunculatus* and *Acacia aneura* (mulga). Concomitantly, as reported in Chapter 3, the digestibility of *L. leucocephala* was the highest among the tropical legumes studied in this thesis. As discussed below, it is evident that this high quality is not only related to the structural features of its condensed tannins, but also to factors such as protein and fibre content and composition. It is difficult, then, to predict at which concentration in plant tissue does the presence of condensed tannins from different species becomes deleterious in terms of animal nutrition. However, the present results suggest that in tanniniferous forage legumes, the presence of low (*ca* 2000 dalton) molecular weight condensed tannins is advantageous in terms of forage quality. Proanthocyanidin composition of the condensed tannins from *L. leucocephala* was peculiar in that they contained ca 30% prodelphinidin and 3-6% propelargonidin. This balance could also be responsible for the desirable characteristics of these condensed tannins, although this needs to be corroborated in further experimentation.

vi) At a given tannin-enzyme ratio, the inhibition of enzymatic activity observed *in vitro* is a function of the characteristics of both the protein and the condensed tannin. For example, by virtue of their low molecular weight, condensed tannins from *Leucaena leucocephala* were consistently shown to be the least effective in inhibiting the activity of enzymes from the anaerobic fungus *Neocallimastix hurleyensis* (Chapter 4.3). In sharp contrast, the larger condensed tannins from *D. ovalifolium* and

Flemingia macrophylla were generally shown to be the most effective in inhibiting these enzymes. In turn, the xylanases were shown to be more susceptible to inhibition by condensed tannins that the CMCases (Chapter 4.3), which is in agreement with the observations of Salawu et al. (1998) who measured the activity of these enzymes in the presence of an extract from *Calliandra calothyrsus*. Interestingly, the glucosidases and xylosidases appeared to be even less susceptible to inhibition than the CMCases. It is also possible for these differences in susceptibility to inhibition by condensed tannins to be related to characteristics of the substrate hydrolysed by these enzymes.

vii) Although the present results showed that condensed tannins negatively affect the degradation of dry matter, they also demonstrated that *in vitro* degradability of the tropical legumes studied was a function of their entire chemical composition. In particular, the content of neutral and acid detergent fibre was found to have a negative impact on forage degradability (Chapter 5.1). Furthermore, it was also demonstrated that fibre degradability is a function of fibre composition, as non-starch polysaccharide constituents such as xylose were found to be highly undegradable, whereas the uronic acids were highly degradable (Chapter 3). Similar findings were reported by Longland et al., (1995). Protein, on the other hand, was positively related to the *in vitro* degradability in the legumes studied (Chapter 3 and 5.1) which is in agreement with the findings of Sileshi et al. (1996). Interestingly, in *D. ovalifolium* the contents of condensed tannins and fibre were positively related whereas a negative relationship was observed between protein and condensed tannin content. Such relationships make it difficult to elucidate the role played by a particular factor in determining the nutritional quality of a given forage legume.

6.2 RECOMMENDATIONS FOR FURTHER WORK

In the elucidation of the impact of condensed tannins upon forage quality, a clear distinction should be made between the nutritional, microbial and enzymatic effects of condensed tannins. Likewise, it must be recognised that whereas *in vitro* experimentation allows for the examination of the effects of individual variables, this simplicity also acts as a limitation. This is because, at any of these levels, the responses to the presence of condensed tannins are influenced by many factors, which are likely to differ from one level to the other. In consequence, extrapolation of the *in vivo* effects of condensed tannins based on observations made *in vitro* is not

advisable. Rather, all observed responses must be analysed following an integral approach, in which the interpretation of tannin effects observed *in vitro* takes into account all probable *in vivo* responses. *In vitro* experiments are, however, invaluable as probes for the detailed examination of specific aspects of a scientific problem and in making predictions for testing *in vivo*.

As suggested earlier, future research on the nutritional impact of condensed tannins must not only be based upon the concentration of condensed tannins in plant tissue, but must also include structure-activity considerations. Unfortunately, the techniques required for the structural study of condensed tannins involve some degree of sophistication, especially in respect to instrumentation (i.e. chromatography systems, specialised detectors, nuclear magnetic resonance). However, if this obstacle is removed, any laboratory can easily adopt the techniques used in the current study to measure molecular weight and monomer composition in condensed tannins. Both techniques are simple and reliable and do not require extensive sample preparation. The technique used to measure monomer composition has the advantage of having a short run time (20 min). Depending on the chromatography system, the run time for this technique can even be shortened to 15 min. Likewise, by the use of a column oven, the run time of the technique used to measure condensed tannin molecular weight can also be significantly reduced.

Over the last decade, much investigative effort has been dedicated to study the impact of condensed tannins upon the forage quality of tropical tanniniferous legumes As a result, our understanding of forage utilisation in the face of condensed tannins has been improved. However, it is evident that this is an area where research opportunities still exist in abundance. For example, little attention has been given to the possible interaction between energy availability (both rate and extent) and the nutritional impact of condensed tannins. In one end of this proposition, research reported in this thesis suggested that the presence of condensed tannins could result in reduced energy availability to ruminants, especially during the early stages of fermentation. In turn, when forage-fed ruminants are considered, energy availability is highly dependent upon fibre degradability, which relates to fibre composition (see Chapter 3). Since the rate and extent of energy availability to ruminants is affected by factors other than the presence of condensed tannins, it becomes possible for energy availability to become

a factor in determining the nutritional impact of tannins. This is the converse to the situation normally envisaged, where energy availability is a function of the presence of condensed tannins. Concepts relevant to this question include the relationships between voluntary intake and retention time of feeds in the rumen, the synchrony between the release of protein and energy and microbial growth efficiency and the specific inhibition of fibrolytic enzymes in the presence of tannins (Chapter 4.3) and fibre composition.

Research presented in this thesis suggested that substrate-associated tannins are more efficient in inhibiting the fermentation of substrates by rumen microbes and substrate degradation by microbial enzymes than soluble condensed tannins (Chapters 4.2 and 4.3). Although we could speculate about the mechanisms underlying this response, we are still far from understanding the basis of this phenomenon. However, our current knowledge suggests that in nutritional studies emphasis must be placed in establishing whether during transit through the gastrointestinal tract, condensed tannins act as substrate-associated or as soluble tannins. It seems apparent that this distinction should take precedence over that of determining the extractability of tannins into an organic solvent. The observation that the extractability of condensed tannins in 70% aqueous acetone was related to differences in tannin structure (see Chapter 4.1), does not necessarily contradict the present conclusions. Additionally, when appropriate (as determined by the actual use of the forage legume) studies attempting to understand the nutritional impact of condensed tannins must be carried using fresh forage, instead of freeze- or oven-dried material.

As observed in the experiments with fungal enzymes (Chapter 4.3), the interaction between proteins and condensed tannins can be quite specific. Indeed, both tannin and protein characteristics can influence the strength of the interaction (see above). An implication from this observation relates to the idea of co-feeding tanniniferous and non-tanniniferous forages, which is conducted with the purpose of protecting dietary protein (from the non-tanniniferous component) from excessive degradation in the rumen. For this idea to be successful, the specificity of binding between tannins and the protein to be protected must be very strong. Otherwise, condensed tannins might end up binding with other molecules and not providing the intended protection. In a related idea, it would also be interesting to determine what is the specificity of

binding between condensed tannins and protein produced by the same plant. These determinations would have practical application to other processes such as protein preservation during the production of silage from tanniniferous species. Additionally, these observations might shed some light on the subject of condensed tannin function in plants and their evolutionary significance.

Research summarised in this thesis demonstrated the influence of condensed tannin structure upon the nutritional effects of condensed tannins. For example, results reported in this thesis allowed clear delimitation of the influence of tannin molecular weight upon *in vitro* degradation of dry matter (see Figure 6.1). On the other hand, from these observations it would still be tentative to conclude about the concomitant role of monomer composition of condensed tannins. This is probably an indication that a greater number of observations than used in the present experiments are needed to clarify the nutritional impact of monomer composition (hence hydroxylation level) of condensed tannins. Furthermore, as stated before, there is a strong likelihood that the spatial arrangement of those monomers within the tannin molecule would also be likely to have a significant role in determining the nutritional impact of condensed tannins. A distant goal of condensed tannin research must then be the determination of the sequential order of monomers in the tannin molecule and their concomitant impact on forage nutritional value.

Experiments reported in this thesis demonstrated that the structure of condensed tannins from *Desmodium ovalifolium* changed dramatically when this tropical legume was grown under different environments. The mechanisms and environmental factors bringing about these changes still remain unknown and future research should be devoted to their identification. Furthermore, identification of the nutritional impact of these changes is also a worthwhile enterprise. Another aspect of these observations that deserves some attention is the determination of the evolutionary advantage gained by plants as a result of the production of condensed tannins of different structure. If anything, these observations suggest the existence of molecular mechanisms that allow the alteration of condensed tannin structure and with that, the possible amelioration or increment of their nutritional effects.

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Appendix One

Description of the data (.xls) files contained in the compact disk attached to the back of this thesis.

Filename	Contents	Associated Chapter (folder)
Aminoacid composition	Determination of the amino acid content in immature and mature leaves of seven tropical legumes	Chapter 3 (Chapter 3)
Analisis Gerardo Gas 1	Gas and VFA accumulation data during gas production experiment with immature and mature leaves of seven tropical legumes, MLP parameters, related ANOVA and linear regression analyses	Chapter 3 (Chapter 3)
NSP data	Determination of the NSP content before and after the fermentation of immature and mature leaves of seven tropical legumes, associated linear regression analyses	Chapter 3 (Chapter 3)
Rolly	Determination of the standard curves for amount of condensed tannins hydrolysed with butanol/HCl and absorbance due to anthocyanidin presence for tannins from mature and immature *Leucaena pallida*, *Calliandra calothyrsus* and *Clitoria fairchildiana*	Chapter 3 (Chapter 3)
Stdcurves	Determination of the standard curves for amount of condensed tannins hydrolysed with butanol/HCl and absorbance due to anthocyanidin presence for tannins from mature and immature *Desmodium ovalifolium*, *Flemingia macrophylla* and *Leucaena leucocephala*	Chapter 3 (Chapter 3)
TROPLEG	Entire data obtained during the first run of the gas production experiment with immature and mature leaves from seven tropical legumes, MLP parameters and DMD data	Chapter 3 (Chapter 3)
Tropleg2	Entire data obtained during the second run of the gas production experiment with immature and mature leaves from seven tropical legumes, MLP parameters and DMD data	Chapter 3 (Chapter 3)
VFA1	Determination of the VFA content after the two gas production experiments with immature and mature leaves from seven tropical legumes	Chapter 3 (Chapter 3)
COMPARIS	Determination of the proanthocyanidin composition in condensed tannins from mature and mature leaves from seven tropical legumes	Chapter 4.1 (Chapter 3)
Fraction	Determination of the condensed tannin content in tropical legumes following a sequential extraction procedure, determination of the proanthocyanidin composition in the resulting fractions	Chapter 4.1 (Chapter 4.1)

Description of the data (.xls) files contained in the compact disk attached to the back of this thesis (continued…).

Filename	Contents	Associated Chapter (folder)
Graph DMF one	Graphical representation of the chromatogram resulting from the determination of the molecular weight of the underivatised condensed tannins from immature leaves of *Desmodium ovalifolium*, *Leucaena leucocephala* and *Clitoria fairchildiana*	Chapter 4.1 (Chapter 4.1)
Graph DMF two	Graphical representation of the chromatogram resulting from the determination of the molecular weight of the underivatised condensed tannins from immature leaves of *Flemingia macrophylla*, *Leucaena pallida* and *Calliandra calothyrsus*	Chapter 4.1 (Chapter 4.1)
Graph peracetates one	Graphical representation of the chromatogram resulting from the determination of the molecular weight of the peracetate derivatives of condensed tannins from mature leaves of *Desmodium ovalifolium*, *Leucaena leucocephala* and *Clitoria fairchildiana*	Chapter 4.1 (Chapter 4.1)
Graph peracetates two	Graphical representation of the chromatogram resulting from the determination of the molecular weight of the peracetate derivatives of condensed tannins from mature leaves of *Flemingia macrophylla*, *Leucaena pallida* and *Calliandra calothyrsus*	Chapter 4.1 (Chapter 4.1)
Tannin-structure activity relationships	Linear regression analysis of the relationship between condensed tannin structure and gas and VFA accumulation in the gas production experiments reported in Chapter 3 of this thesis	Chapter 4.1 (Chapter 4.1)
Tannin-structure activity relationships	Linear regression analysis of the relationship between condensed tannin structure and gas and VFA accumulation in the gas production experiments reported in Chapter 4.2 of this thesis	Chapter 4.2 (Chapter 4.1)
Analisis Gerardo gas 2	Data on gas and VFA accumulation during the fermentation of D-glucose in the presence of soluble tannins from *Desmodium ovalifolium*, MLP parameters and linear regression analyses	Chapter 4.2 (Chapter 4.2)
Analisis Gerardo gas 3	Data on gas and VFA accumulation during the fermentation of cell walls in the presence of substrate-associated tannins from *Desmodium ovalifolium*, MLP parameters and linear regression analyses	Chapter 4.2 (Chapter 4.2)
Analisis Gerardo gas 4	Data on gas and VFA accumulation during the fermentation of cell walls in the presence of soluble tannins from *Desmodium ovalifolium* and associated MLP parameters	Chapter 4.2 (Chapter 4.2)
Analisis Gerardo gas 5	Data on gas and VFA accumulation during the fermentation of glucose in the presence of soluble tannins all tanniniferous tropical legumes and associated MLP parameters	Chapter 4.2 (Chapter 4.2)

Description of the data (.xls) files contained in the compact disk attached to the back of this thesis (continued…).

Filename	Contents	Associated Chapter (folder)
Analisis Gerardo gas 6	Data on gas and VFA accumulation during the fermentation of cell walls in the presence of substrate-associated tannins from all tanniniferous legumes and associated MLP parameters	Chapter 4.2 (Chapter 4.2)
Binding kinetics	Determination of the binding of condensed tannin extracted from immature and mature leaves of six tropical legumes to cell walls from *Festuca arundinacea*	Chapter 4.2 (Chapter 4.2)
Boundtangp	Raw data (gas and VFA) obtained during the fermentation of *Festuca arundinacea* cell walls containing different concentration of substrate-associated condensed tannins from *Desmodium ovalifolium*	Chapter 4.2 (Chapter 4.2)
Gas all bound CT	Raw data (gas and VFA) obtained during the fermentation of *Festuca arundinacea* cell walls containing different concentration of substrate-associated condensed tannins from all tanniniferous tropical legumes	Chapter 4.2 (Chapter 4.2)
Gasdossl	Raw data (gas) obtained during the fermentation of D- glucose in the presence of different concentrations of soluble condensed tannins from *Desmodium ovalifolium* (small and large serum bottles)	Chapter 4.2 (Chapter 4.2)
Predicted rate Chapter 5b	Predicted rate of gas accumulation in the gas production experiments reported in Chapter 4.2 of this thesis	Chapter 4.2 (Chapter 4.2)
SMALLDO	Raw data (gas) obtained during the fermentation of D- glucose in the presence of different concentrations of soluble condensed tannins from *Desmodium ovalifolium* (small serum bottles)	Chapter 4.2 (Chapter 4.2)
Soltangp	Raw data (gas and VFA) obtained during the fermentation of D-glucose in the presence of soluble condensed tannins extracted from mature and mature leaves of six tropical legumes	Chapter 4.2 (Chapter 4.2)
Soluble CT Festuca	Raw data (gas and VFA) obtained during the fermentation of *Festuca arundinacea* cell walls in the presence of different concentration of soluble condensed tannins from *Desmodium ovalifolium*	Chapter 4.2 (Chapter 4.2)
VFA2	Determination of the accumulation of VFA during the fermentation of D-glucose in the presence of different concentrations of soluble condensed tannins from *Desmodium ovalifolium* (see file SMALLDO)	Chapter 4.2 (Chapter 4.2)

Description of the data (.xls) files contained in the compact disk attached to the back of this thesis (continued…).

Filename	Contents	Associated Chapter (folder)
Anova 3rd paper	ANOVA of the experiments where the activity of different enzymes in the presence of different concentration of condensed tannins from mature leaves of six tropical legumes was determined	Chapter 4.3 (Chapter 4.1)
B-xylopiranosidase	Raw data on the experiments carried out to determine the impact of different concentrations of condensed tannins from mature leaves of six tropical legumes on the activity of b-xylopirosidase from *Neocallimastix hurleyensis*	Chapter 4.3 (Chapter 4.3)
CMCases	Raw data on the experiments carried out to determine the impact of different concentrations of condensed tannins from mature leaves of six tropical legumes on the activity of CMCases from *Neocallimastix hurleyensis*	Chapter 4.3 (Chapter 4.3)
FAE assay	Raw data on the experiments carried out to determine the impact of different concentrations of condensed tannins from mature leaves of six tropical legumes on the activity of a recombinant ferulic acid esterase (FAE)	Chapter 4.3 (Chapter 4.3)
Graphs 3rd paper	Graphical representation of the activity of the different fungal enzymes in the presence of different concentration of condensed tannins from six tropical forage legumes and related regression analyses	Chapter 4.3 (Chapter 4.3)
Rep B-glucosidase 98	Raw data on the experiments carried out to determine the impact of different concentrations of condensed tannins from mature leaves of six tropical legumes on the activity of B-glucosidases from *Neocallimastix hurleyensis*	Chapter 4.3 (Chapter 4.3)
Solange xylanase	Raw data on the experiments carried out to determine the impact of different concentrations of condensed tannins from mature leaves of six tropical legumes on the activity of xylanases from *Neocallimastix hurleyensis*	Chapter 4.3 (Chapter 4.3)
Wall enzyme activity	Raw data on the experiments carried out to determine the impact of different concentrations of condensed tannins from mature leaves of six tropical legumes on the ability of different enzymes from *Neocallimastix hurleyensis* to degrade cell walls from *Festuca arundinacea*	Chapter 4.3 (Chapter 4.3)
Averages comp quim D.o. axel	Mean protein, fibre and tannin content and in vitro dry matter digestibility in the 120 *Desmodium ovalifolium* samples examined in the genotype-environment study	Chapter 5.1 (Chapter 5.1)
Chem CT Do (Axle)	Raw data on the determination of the condensed tannin content in the 120 *Desmodium ovalifolium* samples	Chapter 5.1 (Chapter 5.1)

Description of the data (.xls) files contained in the compact disk attached to the back of this thesis (continued…).

Filename	Contents	Associated Chapter (folder)
Clima y suelo axel	Climatic and soil chemistry data related to the six sites where the agronomic experiment took place	Chapter 5.1 (Chapter 5.1)
Fertil	Data on the fertilizer treatments applied per site	Chapter 5.1 (Chapter 5.1)
Gasaxl1-out	MLP parameters associated with the gas production experiments conducted with the 120 *Desmodium ovalifolium* samples, their use to predict rate of gas evolution and associated linear regression analyses	Chapter 5.1 (Chapter 5.1)
GASROLANDO	Raw data (gas and dry matter disappearance) with 90 *Desmodium ovalifolium* samples	Chapter 5.1 (Chapter 5.1)
GASSIETE	Raw data (gas and dry matter disappearance) with 40 *Desmodium ovalifolium* samples	Chapter 5.1 (Chapter 5.1)
Predicted Gas Axel	Prediction of the gas accumulation data for all 1200 plus samples obtained in the main agronomic experiment	Chapter 5.1 (Chapter 5.1)
Chemistry of Do (Axel)	Determination of the molecular weight and he proanthocyanidin composition in condensed tannins purified from 30 of the 120 *Desmodium ovalifolium* samples, associated linear regression analyses	Chapter 5.2 (Chapter 5.2)
Graph Mol wt Do 13125	Chromatogram of changes of molecular weight of tannins in *Desmodium ovalifolium* 13125 in response to changes in environment	Chapter 5.2 (Chapter 5.2)
Graph Mol wt Do 23618	Chromatogram of changes of molecular weight of tannins in *Desmodium ovalifolium* 23618 in response to changes in environment	Chapter 5.2 (Chapter 5.2)
Graph Mol wt Do 33058	Chromatogram of changes of molecular weight of tannins in *Desmodium ovalifolium* 33058 in response to changes in environment	Chapter 5.2 (Chapter 5.2)
Graph Mol wt Do 350	Chromatogram of changes of molecular weight of tannins in *Desmodium ovalifolium* 350 in response to changes in environment	Chapter 5.2 (Chapter 5.2)
Graph Mol wt Do 3788	Chromatogram of changes of molecular weight of tannins in *Desmodium ovalifolium* 3788 in response to changes in environment	Chapter 5.2 (Chapter 5.2)

Note: Also included in this CD are the WORD files Chapter Four Thesis, Chapter 5a Thesis, Chapter 5b Thesis, Chapter 5c Thesis, Chapter 6 thesis, Chapter 6-a Thesis (correspondingly Chapters 3, 4.1, 4.2, 4.3, 5.1 and 5.2), Literature Review PhD Thesis, References PhD Thesis and Conclusions, Recommendations.