TANNINS IN LIVESTOCK AND HUMAN NUTRITION

ACIAR PROCEEDINGS No. 92

Priority Setting Discussion

Background

In northern Australia, and in many countries with similar tropical climates, the supply of quality feed to support a high daily liveweight gain of younger domestic livestock is not consistently available. The resulting poor nutritional status of the animals leads to constraints in reproductive performance and animal health, with associated affects on farmer livelihoods, especially in developing countries. The use of perennial, nitrogen fixing shrub legumes offers an attractive alternative to traditional pasture as a means of overcoming this shortfall in nutrient supply for livestock.

Protein supply to ruminants is the most common nutrient deficit encountered in tropical regions, but although shrub legumes are protein rich (up to 28% w/w protein), their digestibility is restricted by relatively high levels of endogenous tannins. Where tannin levels do not exceed 5% dry weight, this can be an advantage in preventing bloat and reducing rumen degradation of protein, thereby providing valuable rumen bypass protein for the animal. However, where tannin levels are as high as 10–15% dry weight, they have a significant anti-nutritional effect; overall digestibility is low, protein availability is greatly reduced, feed palatability is poor and digestive upsets in the animals may occur.

Previous work in Adelaide showed that feral goats were able to successfully browse tannin-containing *Acacia aneura* without detrimental effects, and in fact to thrive on a diet comprising mainly *Acacia*. This work also showed that transfer of crude rumen fluid from feral goats to domestic sheep was successful in allowing sheep to digest *Acacia* without detrimental effects, and to recover liveweight gains previously lost through under-nutrition. These results indicated that previously unknown micro-organisms from feral goats were capable of resisting the toxic effects of tannins.

ACIAR-funded project ASI/1993/018 was established to investigate this phenomenon further, with particular emphasis on developing the technology for the use of the shrub legume, *Calliandra calothrysus* as livestock feed in Indonesia. Outcomes from this project included the isolation and identification of 5 different bacterial species that were resistant to, or could degrade hydrolysable and condensed tannin, the development of procedures for analysing tannin composition in plant samples, the formulation of an HPLC profile for tannins from different shrub legumes, the discovery that plant treatment (drying) significantly effected tannin composition, the indication that tannins also inhibit lower digestive tract functions in ruminants, the demonstration that co-feeding with non tannin-containing plants could have positive effects, the impact of 'Browse-Plus' on nutrition in tannin-fed sheep, and confirmation of increased animal production following microbial transfers from tannin-adapted animals.

This project has therefore set the groundwork for the introduction of microbial transfer technology to farming systems in developing countries where tannin-containing plants may be utilised as livestock feed. Nevertheless, there are many gaps in our knowledge and these need to be addressed before the technology can be used most effectively. Some accessions of plants have high levels of tannins yet are highly digestible (in nylon bag trials); tannin levels and profiles in plants seem to be affected by environmental changes; tannins appear to accumulate differentially in specific regions of the plant; assay procedures for tannins are ambiguous and often very misleading in determining feed digestibility; tannins may have other as yet undescribed effects on digestive tract structure and function; optimum mixtures of beneficial micro-organisms need to be established; tannin-degrading genes need to be identified and transferred between microbial species to enhance the nutritive value of tanniniferous legume species.

To answer some of these questions and to establish research priorities for the future in this area, it was important to discuss these issues in a forum of experts who are familiar with animal production problems in developing countries, have expertise in tannin chemistry and analysis, are knowledgeable about microbial ecology in the rumen and understand plant structure and function. Such a diverse range of experts are drawn from the disciplines of microbiology, agronomy, animal production and wine chemistry. The aim of this workshop was to bring them together for 3 days to discuss the above issues, and to develop a set of priorities for future research on the use of tropical shrub legumes in animal production.

Objectives

1. Develop an understanding of the potential for tannin-containing plant resources to be used as livestock feed.
2. Identify whether any microbes are able to degrade tannins under anaerobic conditions.
3. Further an understanding of tannin biosynthesis in plants and degradation in other systems.
4. Progress knowledge of microbial degradation of tannins and tannin complexes in animal digestive systems.
5. Develop an understanding of the chemistry and methodologies for analysis of total tannins and tannin structure, so as to enhance knowledge of the biological activities of tannins.
6. Review the current state of knowledge and establish research priorities for future programs on the biological effects of tannins, with particular emphasis on animal production in developing countries.

Purpose of the planning session

1. To summarise major outcomes from scientific sessions.
2. To determine the gaps in current knowledge relating to tannins in:
   Livestock nutrition;
   Chemistry and analysis;
   Microbiology.
3. To establish what research programs are required to address these gaps.
4. To establish research priorities (H = high, M = medium, L = low) for:
   Developing countries;
   Scientific understanding.
5. To identify the constraints to achieving these research objectives.

Summary of Major Scientific Sessions

Tannins and their role in livestock nutrition

Tannins represent an extremely complex range of polyphenolic compounds that are poorly understood in a number of different areas. In particular, species and age-specific changes in tannin profiles occur, the role of environmental factors (e.g. heat, light, water, predation) on tannin synthesis is little understood as is the biological impact of tannins on livestock.

Understanding structure/activity relationships of tannins is particularly important, together with the development of new methods of quantitation that relate chemical structures to biological activity. Potential interactions of tannins with other primary and secondary plant compounds may be the key to understanding palatability and other biofunctions.

A more thorough understanding of these factors may then lead to the design of specific reagents that may inactivate or reduce the inhibitory effects of tannins. These include effects on microbial populations, digestive processes and the development of pathological changes in the intestinal tract of ruminants and monogastric livestock.

Livestock studies

In livestock, the extent of the decline in apparent nitrogen (N) digestibility and the rise in faecal N are reliable indicators of the extent of condensed tannin (CT) activity. However, in animals fed high N, high CT feeds, a depression in digestible crude protein (DCP) does not readily explain N retention depression, and a lack of DCP response shifts the focus of research towards post-ruminal effects of CTs. These questions point to a need to have a greater understanding of the effect of tannins on both ruminal and post-ruminal processes. Tannins in tropical forages are therefore a major problem, particularly in developing countries, with significant potential for improvement. At present this is not being realised.

Recent research also suggests that N release in the rumen may be mediated predominantly by plant-derived rather than microbial proteases. The fact that tannins will be more closely associated with plant rather than microbial protein points to a need to have a greater understanding of protein N release in the rumen and the role of plant versus microbial enzymes in mediating this reaction. Since a significant impact of CT is on the metabolism of protein N, there is a pressing need for assay procedures that relate CT levels to biological effects on N release and absorption. Assays may include in vitro N digestibility or polyethylene glycol (PEG) binding. In dairy cows, low levels of CTs improves milk output in late lactation. The mechanism is unclear but may be due to extra metabolisable protein or altered amino acid absorption. Data on the effect of CTs on the degradation of non-starch polysaccharides are unknown. In poultry diets in China, sorghum containing CT of up to 0.6% is used but DMD is not a major problem. At this level of supplementation, the focus is more on the role of tannins in reducing leg and beak colour. However, at a level greater than 1.5% DM, CTs fed to ducks causes endogenous protein wastage and inhibition of gastrointestinal enzyme activity. In contrast, CTs may have more positive effects on livestock production through bloat protection, potential anthelmintic properties, or they may increase the animals resistance to nematode infection.

Tannin biosynthesis and analysis

Quantitation of CTs in terms of rumen function is an interesting and important parameter to measure. Tannin bioassays in vitro can be based on gas production as a link with biological effects such as short chain fatty acid synthesis and microbial protein synthesis, but other biological assays are needed. Detergent systems of fibre analysis should be used with caution when characterising tannin-rich feeds. Enhancement of feeding value through feed storage in the presence of urea and the use of slow release PEG may be useful procedures, although the full biological effects of PEG are not yet known. The distinction between free and bound tannins in legumes is also important, and the protein binding assay is a useful link with the potential nutritive value of forage.

Analysis of grape seed and skin tannins has been carried out by mass spectrometry. Modern methods used in the ionisation stage such as electrospray and matrix associated light desorption ionisation (MALDI) have enabled the elucidation of polymorphic procyanidins in excess of eight monomers in size. However, other methods based on the cleavage of interflavanic bonds (e.g. thiolysis) would be useful in determining CT composition of forages. The formation and development of pigments in red wine has shed light on the mechanisms of tannin polymerisation although the intricate details are still not clear. Information derived from these studies will have a significant impact on future research of tannins in livestock forages.

The biosynthesis of proanthocyanidins in plants is an area of active research, particularly genetic control mechanisms. Questions arising from this work relate to the synthesis of epi-catechin, enzymatic control of condensation reactions and genetic manipulation of proanthocyanidin structures. Little is known of the control of condensation reactions, what directs plants to make specific types of tannins and how environmental influences are communicated to the plant biosynthetic machinery. Reducing CT levels in plants by genetic manipulation may be a strategy for the future.

Polyphenols in human health

Although there are links between flavonoid intake and protection against cardiovascular disease and cancer, the evidence is not strong. There is little information on the bio-availability and metabolism of tannins in humans, and more research is required. Tannins are also likely to have effects on gut bacteria and the environment of the bowel, which may be linked to short chain fatty acid synthesis and protection against cancer.

Tannins and rumen micro-organisms

Rumen micro-organisms can be selected for growth on tannins, and can degrade hydrolysable tannins, although little evidence is available to show degradation of the phenol ring in condensed tannins. These are found in a wide range of animals that naturally browse on tannin-containing feeds and it is likely that they contain a number of organisms that express some degree of resistance to tannins. Organisms can be transferred between animals and can be used to enhance tannin resistance in browsing livestock. However, it is not clear whether micro-organisms can cleave the aromatic ring or transform OH groups.

To answer these questions and to select for specific metabolic capabilities, defined model compounds need to be developed. Micro-organisms also produce extracellular polysaccharides in response to tannins but it is not clear whether this is a protective mechanism or a non-specific response to the environment. Some organisms produce esterases that degrade hydrolysable tannins, but it is not known whether other mechanisms such as enzyme glycosylation exist. More information on microbial interactions in the presence of tannins is necessary to understand and develop the potential for microbial alleviation of tannins in tropical forages. Other effects of tannins on micro-organisms include sequestering of trace elements and potential inhibition of microbial functions.

Microbial ecology and phylogeny studies show that diverse populations of tannin tolerant bacteria can be isolated from feral livestock and wildlife. However very little is known of these organisms, their relationship with other rumen bacteria or their mechanisms of tannin resistance. A greater understanding of this area may lead to the development of appropriate inoculation strategies to improve livestock productivity on tannin-containing forages.

The manipulation of rumen microflora appears to be a promising approach if exotic organisms can be isolated and do persist in the rumen. In the worldwide search for micro-organisms capable of degrading tannins, rumen liquor from exotic sources are being evaluated for their ability to digest tannin-containing feeds. Samples can be digested both with and without PEG addition to measure the microbial tolerance to tannin as well as the affect of tannins on irreversibly complexing components of the feed such as protein. The estimation of digestibility could be under estimated because of PEG absorbed onto the residue. The response to PEG addition can be used both to identify regions/animal species where micro-organisms can be sought and also target areas where suitable micro-organisms could be used. Where the response is low, micro-organisms that are tolerant to or can degrade tannins could be present. Where the response is high a need is identified for introducing exotic micro-organisms.

Research Planning and Priority Setting

Animal nutrition

Gaps in current knowledge

1. Appropriate methods for relating tannin concentrations to biological responses in livestock.

Existing methods for the chemical analysis of CTs yield values that do not correlate with in vitro measurements of fibre digestibility. Rapid assay procedures that reflect the

biological impact of tannins on livestock production are essential for the evaluation of potential browse feeds, particularly in tropical environments.

2. Animal responses, including ruminal and post-ruminal effects to tannins in short-term and long-term feeding trials including directly grazed and cut-and-carry forage.

The assumption has always been that tannins inhibit microbial action in the rumen However, recent evidence suggests that this explanation is too restrictive and that tannins effect post-ruminal digestive functions as well. It is also not known whether long-term feeding of CT-containing forages will have detrimental effects on overall digestive tract functions. This information is essential if tannin-containing forages are to be recommended as alternate livestock feeds.

3. The development of integrated production systems using tannin containing forages.

Despite the potential benefits of tropical shrub legumes on livestock production, information on appropriate plant species or accessions is patchy. This information is required for each climatic region under consideration. It is important to incorporate such feeding strategies into an integrated production system, taking into account climatic, environmental and social factors.

4. Approaches towards amelioration of excessive tannins in forages by appropriate browsing or supplemental feeding (co-feeding) strategies.

High levels of CTs are detrimental to livestock production. However, mixed grazing/browsing has the potential to reduce total CT intake yet still retain the benefits of tannins on protein flow to the small intestine. Optimum browsing and supplemental feeding (co-feeding) strategies which involve high CT supplements mixed with low quality roughage may achieve this. More information on interactions between high and low CT forages in the rumen is essential.

Research programs to address these gaps

Programs	Priorities	
	Developing countries	Scientific understanding
The development of integrated biologically relevant assays of tannin activity in the total diet. This would include PEG-binding, protein-binding, chicken bioassays or HPLC assays. Appropriate standards to calibrate the assays need to be developed for each type of forage being investigated.	H	H
Investigation of the extent to which ruminal and post ruminal effects of tannins explains observed reductions in N balance for animals fed high CT diets.	L	M
In vivo evaluation of the potential nutritive value of tannin-containing forages.	L (tropics) H (temperate)	L L
Evaluation of economically feasible means of ameliorating high tannin contents of existing tannin-containing forage stands by co-feeding strategies.	H (tropics) L (temperate)	L L

Constraints to achieving research objectives.

1. The lack of a strong knowledge base in tannin structural chemistry and analytical methods.

2. Difficulty and cost in establishing and maintaining tannin-containing tropical forage resources for animal feeding trials in Australia and developing countries.

Tannin chemistry and analysis

Gaps in current knowledge

1. Methods for the quantitative and qualitative analysis of condensed tannins in forages. In particular, analyses need to be based on protein complexing reactions.

Analytical procedures that describe or measure the biological effect of tannins in forages are not available. Current procedures give misleading values or yield poorly correlating data.

2. The development of appropriate condensed tannin standards for each forage type and the design of model compounds that can be used in microbial selection systems and to assay for specific degradative enzyme activities.

Using current techniques, when condensed tannin is purified, it may not be truly representative of the condensed tannin in the forage, and may only comprise a small fraction of the extracted material.

Assays of tannins in forages need appropriate standards to define the concentration range of the assay. These are not available commercially, but are necessary to compare results across groups and between different accessions. Extraction procedures and assays need to take into account the fact that tannin structures may change as a consequence of feed processing.

3. Structural features of complex tannins and structure/function relationships in biological systems.

Although tannin-protein interactions have been well described, the chemical basis of these interactions are not clear. To evaluate the browse potential of forages, it will be necessary to define the binding potential of endogenous tannins with feed, microbial and animal protein, and to understand the variation in tannin structures across different plant species, and in the same species under different environmental conditions. Definition of tannin profiles in browse species will help distinguish between potentially beneficial and detrimental forages.

Research programs to address these gaps

Programs	Priorities	
	Developing countries	Scientific understanding
Establish reliability of a raft of tests against tannins isolated from different sources.	M	H
Develop a range of standards to be used in assays for different forage types and model compounds for use in microbial selection and enzyme screening.	H	H
Dissect tannin structures, particularly complex condensed tannins, and determine structure/function relationships.	L	H

Constraints to achieving research objectives

1. The lack of standardised methods for the isolation of condensed tannins from various forages.

2. Lack of modern research infrastructure in developing countries for the analysis of tannins extracted from indigenous forages.

3. Lack of a generally accepted model system to test tannin structure/function relationships in livestock.

Digestive microbiology

Gaps in current knowledge

1. The microbial biodiversity in the rumen, phylogenetic relationships between tannin tolerant bacteria and culture methods for studying new microbial species.

A range of tannin tolerant bacteria have been isolated from various animals browsing tannin-containing forages but it is not clear what impact these bacteria have on rumen function, what interactions occur between them and what organisms are missed through lack of appropriate culture methods. More organisms that degrade condensed tannins are needed. To evaluate the true potential for microbial alleviation of tannins, we need to understand these issues.

2. The availability of model substrates and assay methods to measure anaerobic degradation of CTs by bacteria.

Current bacterial selection procedures suffer from variable tannin sources and poorly defined descriptions of tannin composition. Model compounds that mimic tannins and which can serve as substrates for microbial degradation will greatly enhance our understanding of microbial reactions that mediate tannin tolerance in micro-organisms.

3. The potential for tannin modification (conjugation, glycosylation) by microbial systems, interactions with secondary plant compounds and the effect of PEG on microbial function.

The complexity of tannins is exacerbated by possible modifications or interactions with other plant compounds. An understanding of these interactions will be necessary in order to design appropriate control systems.

4. The significance of tannin tolerant bacteria to rumen function in animals browsing tannin-containing forages.

Despite the fact that several tannin tolerant and some tannin degrading bacteria have been isolated from animals browsing tannin-rich forages, there is no evidence that these bacteria contribute to the animals ability to utilise these feeds. More information is needed on the role of these bacteria, their populations in the rumen and possible interactions between them. Only then will microbial inoculations to overcome tannins be a viable option.

5. Tannin interactions with gastrointestinal functions.

Recent evidence points to post-ruminal effects of tannins, including inhibition of nutrient degradation and absorption. To alleviate CT effects or to select for appropriate plant browse species, it is essential to understand how tannins effect gastrointestinal tract function and to correlate tannin structures with inhibition of gut function.

Research programs to address these gaps

Programs	Priorities	
	Developing countries	Scientific understanding
Microbial biodiversity and phylogeny to understand interactions between tannin tolerant bacteria in the alleviation of tannins in the diet.	M	H
The development of functional assays for the isolation and identification of beneficial organisms.	H/M	H
Interactions of tannins with micro-organisms and mechanisms of tannin resistance.	L	H
The effect of tannins on gastrointestinal functions.	M/L	H
The effect of microbial inoculants on productivity in animals grazing tannin-containing forages.	L	L

Constraints to achieving research objectives

1. Knowledge of tannin structure/function relationships and the development of appropriate assay methodologies and standards.

2. Reproducible sources of tannins and model compounds for selection and enzyme assays.

3. Environmental impact studies and biological diversity agreements between partner countries.

J.D. Brooker
Animal Science,
University of Adelaide

Concluding Comments

Research on condensed tannins (CT) and animal nutrition has progressed markedly in the past 20 years. In devising priorities for developing countries, it is useful to look first at the conclusions of research in this area in temperate countries.

Research in New Zealand (NZ) has shown that the conclusions obtained with temperate forages depend on the structure or type of CT as well as on the concentration. The CT in *Lotus corniculatus* have given the best effects in animal nutrition, increasing the absorption of essential amino acids from the small intestine and increasing wool growth, lactation performance and reproductive performance in grazing sheep. In contrast, the CT in sulla appear to have the greatest action against gut nematodes. Legumes such as *Lotus corniculatus* and sulla have persistency problems in mixed pastures and have to be grazed as pure species on a small area of the farm. The key to success with temperate forages is increasing the CT content of forages such as perennial ryegrass, white clover, red clover and lucerne, which have widespread agricultural applications, from approximately 1 g CT/kg DM to 5 g CT/kg DM or greater, mainly to control bloat. Molecular techniques thus have great potential.

In contrastto temperate forages, the CT content of most tropical legumes (60–150 g/kg DM) is far too high for optimum animal nutrition and needs to be diluted by mixing these forages with greater quantities of non CT-containing feeds. The key here will be getting effective transfer of CT from one feed to another (co-feeding), such that the efficiency of protein digestion in the non CT-containing forage is improved in the same way as shown for *L. corniculatus* in temperate forages.

At this workshop, we have heard about the exciting work of neutralising the effects of high CT concentrations by transferring rumen fluid from 'adapted' animals to 'non-adapted' animals. This must have considerable potential for practical exploitation, in the same manner as the excellent work of Jones et al. (1986) has found application throughout the tropics in transferring an inoculum of rumen micro-organisms to counteract the toxic effects of the amino acid mimosine in Leucaena.

Some comment on the funding of CT related animal research is also required. Most research in temperate countries has been done in NZ and in southern Australia, where the range of CT-containing plants is small, highly skilled scientific groups have been developed, and adequate funding mechanisms are available. In contrast, a much greater range of CT-containing plants are available in the tropical developing countries, the scientific skill base is less and funding of research is either difficult or impossible. To adequately solve problems of CT in animal nutrition in developing countries will therefore require inputs of finance and scientific skills from developed countries, and the development of joint programs involving scientists from both developed and developing countries.

A comment in relation to human health: Using standard epidemiological studies, Roger King showed that there was an established beneficial link between moderate alcohol consumption and the incidence of coronary heart disease (CHD), but a weak link between flavonoid intake and protection against CHD. One of the problems here is

that alcohol only occurs in beverages, at relatively high concentrations, and the consumption must therefore be easy to measure. In contrast, flavonoids occur in low concentrations across a range of foods, including vegetables, fruit skins and red wine in relatively low concentration. Against this background, it is perhaps not surprising that beneficial effects of consuming flavonoids in red wine are difficult to quantify using epidemiological studies. Perhaps longer term controlled nutritional studies are needed, where the effects of the alcohol consumption in red wine can be separated from the effects of flavonoids. Such studies may be difficult to design, but would be most enjoyable for the people participating.

Finally, a comment about the continued need for basic scientific research, aimed at generating new knowledge. One of the aims of this Workshop is the solving of problems of applied animal nutrition in developing countries. I have always believed that progress in applied research will be as good as the basic research that underpins it. In this case, basic research means developing a knowledge of the structure of a range of CTs and a knowledge of their reversible reactivity with proteins, especially in both the rumen and small intestine. Progress in the CT field is going to depend on continued funding of basic science, as well as of the more applied nutritional work.

T.N. Barry
Institute of Food, Nutrition and Human Health
Massey University
New Zealand

The Tannins — An Overview

P.G. Waterman[1]

CONFRONTED with the task of presenting an overview of the current state of our knowledge of tannins and their importance, both commercial and ecological, it is difficult to know quite where to start. For example, when we refer to something as 'a tannin' are we all meaning the same thing? In my old laboratory, we always worked to a rather simple 'operational' definition (Mole and Waterman 1987) which labelled them as 'water-soluble phenolic natural products that can precipitate proteins from aqueous solution'. Note the definition states 'can' and does not imply that such a precipitation 'must' occur and many examples are now present in the literature that demonstrate how protein precipitation can be prevented or reversed.

To a leather chemist, it is the binding of tannin to protein that assumes critical importance, while, to a viniculturist, it is their astringency and the taste perception they impart that is the focus. As pointed out by Haslam (1989), astringency can be produced by small phenolics incapable of binding with proteins in a manner that will lead to precipitation, and which is therefore completely at odds with the view of the leather chemist. The animal nutrition scientist will be primarily concerned with the anti-nutritional properties, how their presence in the diet impacts on growth and well-being and how to minimise deleterious effects through diet selection or pre-treatment of foodstuffs. For the agronomist, the beneficial growth rates and yield that can be gained from selecting for low tannin varieties have to be balanced against a higher susceptibility to pests and pathogens. In the case of food and beverage plants, account has to be taken of the impact that tannin concentration and structure have on the taste and visual perception of the product. Those interested in human nutrition and health have to weigh up their potential anti-oxidant activities against possible harmful effects. For the environmental scientist, an understanding of the distribution of tannins and the levels of production in different plant communities can have implications for the sustainable population levels of herbivores and fresh-water habitats.

Whatever the specific interest, however, the problem of understanding the impact of tannins will generally come back to a consideration, from one viewpoint or another, of their interaction with proteins and polysaccharides and in particular with the potentially irreversible binding between tannin and protein and how it can be circumvented. In the remainder of this overview, I am going to deal with some of the areas which seem to me to be critical for an understanding of the role of tannins and where further work is needed.

The chemistry and biosynthesis of tannins

It might well seem that we know a great deal about the chemistry of tannins and, indeed, in terms of the structures of the major types, this is true. We are well aware that condensed tannins are flaven-3-ol oligomers carrying varying degrees of oxidation on the A and C rings of each monomer and that the chirality of C-4, where linkage of monomers occurs, and C-3 open up the possibilities for considerable structural variability. Likewise, we know that the hydrolysable tannins are based on a hexose

[1] Centre for Phytochemistry, Southern Cross University, PO Box 157, Lismore, NSW 2480, Australia

KEYWORDS: Tannins, Proteins, Tannin protein interaction, Phlorotannins, Complexation

(usually glucose) linked to a number of gallic acid or modified gallic acid units. Less well known are the phlorotannins, which are restricted in distribution to some algae, notably marine.

What we still lack is sufficient knowledge, particularly where condensed tannins are concerned, about the complement of tannins produced by a species. Does a species always produce oligomers with the same stereochemistry based on monomers with the same substitution pattern? We still lack the techniques to identify these rapidly, and insufficient natural examples have been isolated and purified to allow structure-activity relationship studies to define optimum chain length for biological effects (different effects may be optimised at different molecular weights or stereochemistry).

Consequently, I would highlight the need to determine condensed tannin oligomer profiles within target species and, assuming a mixture, to obtain sufficient of each to explore their biological activity. The same issues are relevant for hydrolysable tannins and for phlorotannins but because of their greater ubiquity in food and livestock feed plants condensed tannins should get priority. Coupled with the need to further define the structure of the metabolites produced is the need to better understand their biosynthesis. Here again the most pressing questions surround the condensed tannins and I would identify two particular issues as I think it fair to assume we know the mode of production of the monomer. The first of these is the origin of the precursor cinnamate unit. While it certainly arises from the shikimate pathway, there has been considerable speculation to the effect that some condensed tannin formation reflects a metabolic shunt for the elimination of excess carbon in metabolic pools (Coley et al. 1985). Irrespective of whether the interpretation of why it happens is correct, it is very clear that extrinsic effects do impact on tannin production. The possibility that there is production through both a basal (controlled) pathway and a separate overload pathway (Waterman and Mole 1989) is still in need of exploration. The second issue is the degree to which the chirality of the linkage and the extent of polymerisation of the monomers is controlled. If this is defined and the gene(s) responsible located, then the opportunities arise for selecting for particular condensed tannin sizes and shapes, and the ramifications of being able to do this are considerable.

The tannin protein interaction

The complexation between tannin and protein is central to our interest in tannins. The very obvious precipitation that occurred when tannins and proteins were mixed and the critical nature of the formation of stable complexes resistant to microbial degradation in the leather industry has lead to a view that complexation is irreversible. While this is true under some circumstances, it is far from being always the case. In recent years, there has been ample evidence that the interaction of tannin and protein is an event with a very variable outcome and that complexation without precipitation or with reversible precipitation is not infrequent. For excellent short reviews of this, see Haslam (1989, 1998).

A particular tannin is now known to exhibit different affinities for different proteins and the extent of that variation is considerable. Proteins with an open structure and those rich in the amino acid proline appear to have a particularly high complexation coefficient while glycoproteins, globular proteins and those of low molecular weight have low affinities. The high proline content of the salivary proteins of some mammalian herbivores has attracted attention as a possible pre-digestion process for the elimination of anti-nutritive tannins (see, for example, Austin et al. 1989).

There is abundant evidence to confirm that the tannins produced by different species or by the same species in different parts or at different times vary in their capability to precipitate tannins. However, such experiments have generally been performed on crude tannin mixtures and evidence as to the relative potency of pure tannins is less easy to come by. What does exist suggests that different hydrolysable tannins do exhibit structure-related protein precipitation profiles while, in condensed tannins, molecular weight is important.

The conditions under which complexation takes place has been shown to cause considerable variation in the strength of that interaction. The solubility of the tannin in water is a prime biological consideration that has rarely been taken into account. Relative proportions of tannin and protein and tannin can lead to very different outcomes as in many cases excess levels of protein will solubilise a precipitate and render it insoluble again although it seems this may not be true for the proline-rich salivary proteins (Luck et al. 1994). The pH of the system and the presence of solubilising agents such as bile acids are able to modify the interaction between tannin and protein to a considerable degree (Mole and Waterman 1985).

Our understanding in all of the above areas remains inadequate.

Environmental Effects on Tannin Production Within and Between Individuals

However much we improve our knowledge of the formation and function of tannins in plants we are going to be left with the problem that production seems to depend to a considerable extent on extrinsic factors, most notably soil conditions and light intensity. The impact of light can be quite extraordinary at the intraplant level so that the foliage in different parts of a shrub or tree can vary by several percentage points in its tannin content (for examples see Waterman and Mole 1989). The underlying mechanisms by which extrinsic factors, notably light, influence tannin levels has been speculated upon but remains in need of hard experimental data performed under conditions where as many as possible of the potential variables are controlled.

Analysing tannins in vitro

The in vitro analysis of tannins, both to obtain quantitative data on the level of compounds present and qualitative or quantitative data on their capacity to interact with proteins and other substances remains highly problematical. Some five years ago (Waterman and Mole 1994), we examined all available chemical methods of analysis and concluded they were inadequate for telling us about either the levels present or their protein precipitating capacity. While biochemical methods, based on some measure of the actual protein precipitation ability of the tannins in an extract, were more revealing, here again artificial test-tube procedures were considerably divorced from reality and still had many variables to be controlled.

I know of no developments in the past five years that reverse the opinion that we held then. The methods available to us are, to put in bluntly, not really up to the job. I fear this will continue to be the case but efforts to improve on methods for the rapid assessment of biological activity of tannins in vitro remain an urgent requirement.

In vivo studies

In vivo studies have been carried out primarily with domestic livestock and insects. Many of these studies have revealed that tannins have a net negative impact on performance and well being but this has not uniformly been the case. Perhaps the most clear-cut evidence has come from studies using poultry feeding on tannin containing pulses. Studies on sheep and cattle have also generally shown a negative correlation between performance and tannin intake but there are exceptions. Wild mammalian herbivores have been far more variable in their responses to food selection experiments, perhaps reflecting the potential value of having proline rich saliva. However, where the comparisons have been made, fibre and lignification seem to be more important variables in many cases. Likewise in insects conclusions vary from species to species and there is evidence of adaptation to a high tannin diet in some insect and vertebrate herbivores (Waterman and Mole 1989).

Certainly one of the most revealing and exciting areas for tannin research in the next few years will be to explore in vivo activity using a chemically more defined starting material.

References

Austin, P.J., Suchar, L.A., Robbins, C.T. and Hagerman, A.E. 1989. Tannin-binding proteins in saliva of deer and their absence in saliva of sheep and cattle. Journal of Chemical Ecology, 15: 1335–1347.

Coley, P.D., Bryant, J.P. and Chapin, S. 1985. Resource availability and plant antiherbivore defences. Science, 230: 895–899.

Haslam, E. 1989. Plant Polyphenols: Vegetable tannins revisited. Cambridge University Press, Cambridge, 230 p.

Haslam, E. 1998. Practical Polyphenolics: from Structure to Molecular Recognition and Physiological Action. Cambridge University Press, UK, 422 p.

Luck, G., Liao, H., Murray, N.J., Grimes, H.R., Warminski, E.E., Williamson, M.P., Lilley, T.H. and Haslam, E. 1994. Polyphenol, astringency and proline-rich proteins. Phytochemistry, 37: 357–371.

Mole, S. and Waterman, P.G. 1985. Stimulatory hydrolysis of proteins. Ecological implications. Journal of Chemical Ecology, 11: 1323–1332.

Mole, S. and Waterman, P.G. 1987. Tannins as antifeedants to mammalian herbivores: still an open question? In: Waller, G.R. ed. Allelochemicals: Role in Agriculture and Forestry, American Chemical Society, Washington DC, 572–587.

Waterman, P.G. and Mole, S. 1989. Extrinsic factors influencing production of secondary metabolites in plants; In: Bernays, E.A. ed. Focus on Plant-Insect Interactions, CRC Press, Boca Raton, 107–134.

Waterman, P.G. and Mole, S. 1994. Analysis of Phenolic Plant Metabolites, Blackwell Scientific Publications, London, 238 p.

The Significance of Tannins in Tropical Animal Production

B.W. Norton[1]

Abstract

Legume forages and fodder trees have a significant role in maintaining the quality and continuity of supply of feed for grazing animals in the tropics. A major limitation to feed quality is the presence of secondary plant compounds, such as hydrolysable (HT) and condensed (CT) tannins, which can depress feed intake and utilisation by animals in these areas. The following review briefly describes the nature and occurrence of these compounds in tropical forage legumes and fodder trees and outlines the beneficial and detrimental effects that tannins have on animal metabolism and nutrition. Research on tannins and their action has resulted in techniques that might be applied to overcome the toxic and other effects of tannins. These techniques are also briefly described, and some recommendations made about future needs for research on tannins in tropical feeds. It was concluded that little further progress could be made without a standardised method for CT analysis and more detailed descriptions of the relationships between CT structure, chemistry and biological activity. There is also a need to develop simple techniques to overcome the detrimental effects of tannins. These may include the addition of supplements that inactivate tannins, or microbial inoculates which render tannins inactive in the rumen.

ANIMAL production systems in the tropics and sub-tropics utilise a wide range of feedstuffs, varying from grains, crop and industrial by-products to the extensive use by grazing animals of available grasses, legumes, shrubs and trees.

The level of animal production achieved in any one environment is generally related the quantity, quality and continuity of supply of feed available throughout the year, which, in turn, is related to rainfall, temperature, soil type and fertility. In any one environment, farmers face the challenge of matching available feed supplies with the animals' needs within a framework of a sustainable farming system.

New technologies are being continually devised to assist the farmers with these decisions, and new sources of forages are being presented as one avenue of development. New high-producing grasses are replacing slow growing traditional varieties, and exotic forage legumes, shrubs and fodder trees are being promoted in some areas as more productive sources of feed than indigenous varieties and species.

The maintenance of feed continuity depends on both the quantity and quality of feed produced, and in the tropics, forage legumes improve diet quality by supplementing grasses with protein during the growing season, while fodder trees provide protein and energy supplements during the dry season. However, many tropical legumes contain secondary plant compounds (SPCs) which may diminish their potential value as high quality feeds, and there is an increasing awareness that the effects of these compounds on feed quality and animal production need greater study.

The following paper broadly reviews the relationship between SPC content, particularly tannins, of tropical forage legumes and fodder trees, nutritive value and effects on animal production systems in the tropics and sub-tropics. This information is intended to set the scene for this workshop on a more detailed discussion of the value of tannins in animal and human nutrition.

Plant secondary compounds and tannins

The occurrence and significance of SPCs in plants has been the subject of a number of recent reviews (Norton 1994; Kumar and D'Mello 1995; Lowry et al. 1996; Foley et al. 1999). Phenolic compounds are the largest single group of SPCs, and total phenolics in plants can reach up to 40% dry matter (Reed

[1] School of Land and Food, The University of Queensland, Brisbane Qld 4072 Australia

KEYWORDS: Legume forages, Fodder trees, Hydrolysable tannins, Condensed tannins, Animal nutrition

1986; Tanner et al. 1990). In grasses, the major phenolic is lignin that is bound to all plant cell walls, and is a significant limiting factor in their digestion in the rumen (Minson 1990). Lignin is also a limiting factor in the digestion of legumes, but is bound largely to the vascular tissue (Wilson 1993), with often high concentrations of other free and bound phenolic compounds (phenolic acids, coumarins and flavonoids) in floral, leaf and seed tissue (McLeod 1974).

Plant tannins are a distinctive group of polyphenolic polymers of relatively high molecular weight (MW = 1000–20 000) which have the capacity to form complexes with carbohydrates and proteins. These tannins may be further categorised as hydrolysable tannins (HTs) or condensed tannins (CTs) on the basis of their structure and reactivity. HTs are relatively rare in nature, are of low MW (500–3000) and are cleaved under enzymatic or acid conditions to a monosaccharide and either gallic acid (gallotannins) or ellagic acid (ellagitannins). CTs have no carbohydrate core and are polymers of flavanoid units (polyhydroxyflavan-3-ol units) of varying composition and MW (1900–28 000) (Jones et al. 1976, Foo et al. 1982). Hydrolytic cleavage of CT yields anthocyanidins, and for this reason are now commonly described as proanthocyanidins (PAC) or more broadly as polyflavonoids. Although procyanidin and prodelphinidin are commonly found as the major repeating PAC units of condensed tannins, a further eight compounds may be found in tannins from different plant sources. Variation in both PAC type and polymer chain length is responsible for differences in biological activity and reactivity.

In the living plant cells, both HT and CT molecules are isolated within the cell in vaculoles, and believed to be only released into the cytoplasm when cell damage or death occurs. It is now recognised that CT may occur in either a ‘free (soluble)’ or a bound form to either protein or cell-wall carbohydrate, and that only the soluble CT depresses in vitro protein and fibre digestibility (Rittner and Reed 1992).

Research on the nutritional and metabolic significance of CT has been limited by a poor understanding of the relationship between biological reactivity and CT chemical structure and composition. This situation is partly due to the wide variety and complex nature of these molecules, but is further confused by the range of extraction methods and different qualitative and quantitative colorimetric techniques based on a number of different standards and precipitation and gravimetric methods by which CT concentrations are measured in plant tissues. For example, while it is now recognised that the Folin-Ciocalteau reagent is more specific for phenolic compounds than is the Folin-Denis reagent, it does not discriminate between free phenols and HT, and does not react with phenols in CT.

Similarly, the vanillin/HCl method developed by Broadhurst and Jones (1978) is specific for CT, but absorbance intensifies as molecular size decreases, and when monomeric catechin or tannic acid is used as a standard, this method will overestimate CT content. The method of Hagerman and Butler (1989) adapted from the Butanol/HCl method of Bate-Smith (1954), and using CT standards purified from the plant under test, is now the preferred method of analysis.

There is an urgent need for some standardisation of techniques across laboratories and for further research into the chemical character and biological activities of the molecular species that aggregate to form a plant CT.

Factors causing variation in plant tannin content

Secondary plant compounds are thought to be produced as defence mechanisms against tissue invasion by micro-organisms (bacteria, fungi) and destruction by herbivory (insects, birds, animals). The tannin content of plants is affected by plant species, genotype and stage of growth, and may vary with plant part (leaf, stem, inflorescence, seed), season of growth and other specific environmental factors such as temperature, rainfall, cutting and defoliation by grazing herbivores including insects. Although a detailed discussion of these factors is outside the scope of this review, an understanding of these influences on tannin content is essential for the manipulation of tannins to maximise nutritive value for animals.

Further variation is caused by not only the different analytical techniques used, but also by the methods of tissue preparation for analysis. For example, drying calliandra leaf by heat decreased both extractable and total CT content by 27% and 21% respectively, but increased the proportion of tannin bound to protein and the cell wall from 2.4% to 10.6% (Perez-Maldonado and Norton 1996a). Similar observations have also been made with other fodder trees, and for gliricidia, drying actually reducing extractable CT to levels undetectable by the vanillin-HCl and butanol-HCl tests (Ahn et al. 1989). Although not measured in these studies, it is presumed that drying binds gliricidia tannins to cell components, rendering it unavailable for reaction. As will be discussed later, the binding of CT to plant protein and cell walls can be used to manipulate the effects that these tannins have on animal metabolism.

Tannins in tropical forage legumes

While the tannins of some temperate forage legumes, such as *Medicago* spp., *Lotus* spp., *Lespedeza cuneata* and *Onobrychis viciifolia* (sainfoin), have been studied in some detail, less is known about tropical forage legumes. Table 1 shows values for the concentrations of protein, total phenolics and tannin (where present) in the dry matter (DM) of some tropical legumes. While entries from the Desmodium genus are well represented in these data, there appears to be no information available on phenolics or tannin contents of many other common tropical legumes such *Arachis* spp., *Lablab purpureus*, *Lotononis bainseii*, *Macroptilium atropurpureum* (siratro), *Neonotonia wightii* (Tineroo glycine), *Stylosanthes* spp. or *Trifolium semi-pilosum* (Kenya white clover). Skerman et al. (1988) have listed the following tropical legumes as having compounds other than tannins which have deleterious effects on animals: *Aeschynomene indica* (unknown factor(s)), *Canavalia ensiformis* (Canavanine), *Macroptilium lathyroides* (alkaloids) and *Indigofera spicata* (indospicine).

Tropical legumes have been used as companion plantings with tropical grasses in many tropical areas, and are included to improve both the quality of the diet provided to grazing animals in these areas, and as a source of nitrogen for grass growth. It is therefore surprising that tannins have not received more attention as part of the quality evaluation of these legumes as forages for grazing animals. However, it is worth noting that with the exception of two genera of minor importance (clitoria, mimosa), the tannin contents of the forage legumes surveyed were generally low when compared with that found in fodder tree leaves (see later), and less than that considered as inhibitory in temperate legume species (Waghorn et al. 1990).

Tannins in tropical fodder trees

Fodder trees and shrubs are probably the most important source of high quality feed in tropical animal production systems, and their role is likely to expand as the demand for re-afforestation and sustainable use of degraded grazing lands increases. There is an extensive literature on a few tropical fodder trees suited to tropical environments (*leucaena*, *calliandra*, *gliricidia*, *albizia*, *sesbania* spp.), and the effects of tannins on palatability, nutritive value and production are now well documented (Shelton et al. 1995; Evans 1996; Stewart et al. 1996; Shelton et al. 1998).

These relationships have been best described in a comprehensive study of all leucaena species (Dalzell et al. 1998) and Table 2 shows some selected values from these studies. As CT increased, there was a progressive reduction in in vitro digestibility of dry matter (DM), but much larger decrease in in vivo OM digestibility, when CT content exceeded 2.7% DM or 11 g crude protein/g CT. The effects on N

Table 1. Some values for the concentrations (g/kg dry matter) of crude protein, total phenolics and tannin content and in vitro digestibility of leaf from tropical forage legumes (from Lowry et al. 1992).

Species	Crude protein	Total phenolics	Condensed tannins[1]	In vitro DMD%[2]
Contains tannins				
Aeschynomene americana	210	16	8	70, *64*[4]
Clitorea laurifolia	150–180	84	20–60	—
Desmodium heterophyllum	130–140	34–39	17–26	—
Desmodium intortum	110–245	nm	32–34[3]	36–45, *64*
Desmodium ovalifolium	153–230	nm	83–194	51
Indigofera spicata	170–210	12–26	6–10	—
Mimosa pigra	210–230	90	80	40
Peuraria phaseoloides	160–190	9	3	—
Vigna hosei	190–240	7	4	—
No tannins				
Calopogonium mucinoides	150–210	5	nd	63
Centrosema pubescens	120–300	nm	nd	54
Chamaecrista rotundifolia	80–140	nm	nd	*56–64*
Desmodium triflorus	150–180	nd	nd	—

nm = not measured, nd = not detected. [1]tannins measured by pepsin precipitation using tannic acid standards (Hagerman and Butler 1978). [2]DMD = dry matter digestibility. [3]tannins estimated with butanol/HCL (Perez-Maldonado and Norton 1996a). [4]values in italics are in vivo DMD (%).

Table 2. Values for the range of concentrations in bound and total condensed tannins (TCT), ratios of crude protein to TCT (g/g), in vitro digestibilities of dry matter (DMD) and the in vivo digestibilities of organic matter and nitrogen in sheep given different leucaena species and accessions (from Dalzell et al. 1998; McNeill et al. 1998).

Leucaena species/hybrid	Condensed tannins g/kg DM		Crude protein/ TCT g/g	In vitro DMD%	In vivo digestibility (%)	
	Bound	Total (TCT)			Organic matter	Nitrogen
L. collinsii var. collinsii	0	1–1	370	68.9	58.8	80.5
L. leucocephala var. leucocephala	5–12	17–37	11.2	63.3	60.1	66.4
L. pallida × *L. leucocephala* (KX2)	5–12	30–73	6.0	61.9	—	—
L. diversifolia × *L. leucocephala* (KX3)	6–18	42–91	5.2	61.9	—	—
L. trichandra	2–31	4–226	2.5	61.8	42.3	37.8
L. pallida	8–16	50–171	3.4	59.9	48.2	37.5
L. diversifolia	11–38	57–185	2.2	58.8	—	—

digestibility were even more marked, with digestibility falling progressively as CT increased from the lowest level in *L. collinsii*. It may be concluded that care needs to be taken interpreting the effects of tannins from in vitro digestibilities since they significantly underestimate the real (in vivo) effects.

Acacia species are widespread through the tropical and sub-tropical areas of the world, and are particularly important sources of fodder in low rainfall areas of Australia, Africa, Central and South America. Many Australia acacias (and eucalypyts) have proved to be useful introductions to forestry programs in developing countries, but few of these programs have considered how these trees might fulfil a multi-purpose role as a source of feed as well as wood. Table 3 shows values from the literature for the chemical composition (protein, phenolics, tannins) and nutritive value (digestibility) for some tropical fodder trees with and without tannins. Acacias form a large proportion of the fodder trees used as feed, and are almost all characterised by high contents of condensed tannins often associated with low nutritive values. When compared with the amount of research which has been completed on the high quality tropical species (leucaena, gliricidia, calliandra, sesbania), there is now an urgent need to develop the significant potential of the acacia species as sources of feed for grazing animals. An important aspect of this research must be some definition of the opportunities to improve the nutritive value of these species by manipulation of tannin content and metabolism by the animal.

The practical implications of the effects of tannins in tropical legumes is best demonstrated by recent grazing trials with cattle grazing different accessions/species of leucaena (Jones et al. 1998). These trials were conducted at four sites, Lansdown, North Queensland (NQ), Kununurra, Western Australia (WA), Munum, Papua-New Guinea (PNG) and Masbate, Philippines, and used six different accessions/species of leucaena. Live weight gains were not related to edible forage yield or to damage by psyllids, nor were they clearly related to the varying tannin contents of the different species.

The beneficial effects of tannins

Tannins in plants are thought to have a major role in plant defence against invasion and herbivory. This hypothesis has been recently explored by Mullen et al. 1998 who investigated the relationship between the tannin content and the resistance of 116 different leucaena accessions to damage by the leucaena psyllid, *Heteropsylla cubana*. These workers found that while variation between accessions in condensed tannin content did account for 28% ($r^2 = 0.28$) of the variation found in resistance to psyllids, high tannin varieties were not necessarily the most resistant (*L. pulverulenta* CT = 159 g/kg DM, resistance score moderately susceptible) nor were low tannin plants the most susceptible (*L. collinsii* subsp *collinsii*, lowest CT of all accessions (1 g/kg DM) but highly resistant to psyllid damage). In this case, tannins formed only part of the plant response to insect attack, and tannin content alone is probably not a useful measure of resistance to insect attack.

A major benefit of tannins in feed has been thought to be the protection of plant proteins from digestion in the rumen and their subsequent release as protein available for digestion and utilisation by the ruminant. Studies with *Lotus* spp of varying CT content (2.2% and 5.5%) have confirmed that tannins do protect dietary proteins from digestion in the rumen, increase the flux of essential amino acids (EAA) to small intestine, and at low CT concentrations, increase the apparent absorption of EAA in the intestines (Waghorn 1990). However, at high CT

Table 3. Mean values and ranges for the concentrations of crude protein, total phenolics and condensed tannins and for in vivo (and in vitro in italics) digestibilities of dry matter from a selection of tannin-containing and tannin-free fodder tree legumes. Sources of data are from Lowry et al. 1992 and those referenced in table footnote.

Species	Crude protein (N × 6.25)	Total phenolics	Condensed tannins			in vivo DMD%*	Comments
			Pepsin Pcptn[1]	vanillin-HCl[2]	Butanol-HCl[3]		
Contains tannins							
Acacia aneura	92–203	86	—	31–44, 96	11–14	44–63	+oxalates
Acacia angustissima	210–230	161	—	59–66	nd[4]	—	—
Acacia auriculiformis	110–170	80–130	11–83	—	—	*40*	—
Acacia cyanophylla	112–212	nm[5]	40–70	—	—	51–53	—
Acacia nilotica	112–167	?	79–90	—	—	69	—
Acacia senegal	141–336	nm	4	—	—	nm	—
Acacia seyal	111–293	nm	2–4	—	—	nm	—
Acacia sieberiana	123–158	nm	37	—	—	54	+HT
Acacia tortilis	103–210	nm	40–61	—	—	54	—
Acacia villosa	220–280	120–130	6	—	—	nm	—
Albizia chinensis	151–263	7–68	10–22	24–33	12–15	38	—
Albizia falcataria	230	50–60	22	—	—	nm	—
Calliandra calothyrsus	173–212	30–90	40–90	79–111	15–21	*35–48*	+HT?
Codariocalyx gyroides	128–198	82–120	—	42–71	26–28	*39–44*	—
Flemingia macrophylla	175	34–118	130–190	155	—	*9–36*	—
Gliricidia sepium	200–280	24–46	0	0–30[6]	0–17[6]	*68–74*	coumarins
Leucaena spp	174–380	9–92	7–40	37–43	1–262[7]	63–68	mimosine
Prosopis juliflora	142–222	nm	—	—	—	nm	—
Prosopis cineraria	119–154	nm	—	—	105[8]	39	—
Prosopis tamarugo	90–357	nm	105	—	—	32	+HT
Ziziphus nummularia	141	nm	—	—	130	41–46	—
No tannins							
Albizia lebbek	181–240	22–24	0	nd	nd	43–64	—
Enterolobium cyclocarpum	168–250	0–10	0	nd	nd	*69*	—
Samanea saman	240	16	0	nd	nd	*65*	—
Sesbania grandiflora	206–348	9	0	—	—	63,*67*	cyanogens
Sesbania sesban	152–263	25–30	—	nd	nd	65,*68*	saponins
Tipuana tipu	200–260	22–198	—	0–42	nd	62–64	—

1. Hagerman and Butler 1978 — tannic acid standards. 2. Broadhurst and Jones 1978 — vanillin-HCL — catechin standards. 3. Bate-Smith 1981 — Butanol-HCl — tannic acid standard, tannic acid equivalents (g)/kg DM. 4. nd = not detected 5. nm = not measured. 6. No tannins detected in dried samples. 7. Dalzell et al. 1998 — *L. pallida* CT as standard. 8. CT standard.

concentrations, the efficiency of EAA absorption was significantly decreased from 78% to 63%, and it is not clear whether the presence of tannins in either diet resulted in a net gain in N retained or improved animal productivity. Norton and Ahn (1997) have shown that while the tannins of *Calliandra calothyrsus* (2.5% to 3.7% CT) do also protect proteins from digestion in the rumen, and increase the flow of N to the small intestine, there was no net gain in N retained. In this case, the efficiency of N absorption from the small intestine was decreased from 55% to 50% when polyethylene glycol (PEG) was added to the diet, suggesting that tannins interfered in some way with absorption and possibly utilisation. Perez-Maldonado and Norton (1996a) have reported studies using *Centrosema pubescens*, *Desmodium intortum* and *Calliandra calothyrsus* as supplements to low quality pangola grass (*Digitaria decumbens*), and found that while tannins (0% to 2.3% in diet) did decrease feed protein digestion in the rumen and increase flow to the small intestines, again there was no net gain in N retained (0.29–0.33 gN retained/g N intake. In this case, there was no significant effect of CT on post-ruminal N digestion which was more efficient (69%) than that found for high CT calliandra. McNeill et al. 1998 have recently reviewed similar evidence, and their conclusions support the above findings that, contrary to popular opinion, there is little evidence which supports a view that tannins, even at low concentrations, improve the

nutritive value of tropical legume forages and fodder trees. At best, there is no effect of CT on nutritive value, at worst a decreased intake and efficiency of feed utilisation.

Apart from the direct effects of tannins on feed intake and utilisation, other benefits have been ascribed to presence of tannins in tropical legumes. For example, sorghum is one of the few crops which has tannin in the grain, and high tannin varieties have been promoted in Africa as bird-resistant sorghum. It has also been shown that cattle grazing or supplemented with CT-containing temperate forages do not suffer bloat, because tannins complex the soluble plant proteins which were responsible for the formation of the stable bloat foam (Jones and Mangan 1977). Since bloat is seldom recorded on tropical pastures, this benefit is rarely realised in the tropics.

The detrimental effects of tannins

Tannins and toxicity

While hydrolysable and condensed tannins both form reversible insoluble complexes with proteins, CT are more widespread in plants, are more stable and less susceptible to hydrolysis than HT. HT are usually highly toxic to non-ruminants, but less toxic to ruminants because they may be degraded by either acid or enzymatic hydrolysis in the rumen, and absorbed phenolics excreted in urine as glucuronides. HT toxicity is usually associated with rates of ingestion which exceed the rumen capacity for degradation, and absorbed HT may cause liver and kidney necrosis, jaundice, photo-sensitisation and death in severe cases. The toxic effects of CT are less well understood, but generally binding to plant proteins and cell wall carbohydrates (Van Soest et al. 1986) decreases the digestibility of usually protein and sometimes fibre.

Tannins and palatability

The astringent nature of tannins has encouraged a view that some animals deem high tannin plants unpalatable which then discourages grazing and favours plant survival. This hypothesis has been recently challenged by Foley et al. 1999 and it seems that astringency alone is not sufficient to explain palatability and selectivity by grazing animals (Provenza et al. 1990). Faint et al. (1998) have reported studies which related the palatability of leucaena accessions to plant composition. These workers could find no relationship between palatability and condensed tannin contents (or any other component) of some 27 different leucaena accessions, although some accessions (*L. leucocephala*, and hybrids with *L. pallida*) were clearly more palatable than others (*L. pallida*). It was also noted that palatability rankings varied with site, for example, *L. diversifolia* appeared to be relative unpalatable when grown in Queensland and the Philippines but highly palatable when grown in Honduras.

Tannins decrease digestibility

In studies with tropical forages, increasing levels of CT in the diet (0%–2.3%) decreased N digestibility but had no significant effect on either feed intake, organic matter (OM) or neutral detergent fibre (NDF) digestibility (Perez-Maldonado and Norton 1996a). Norton and Ahn (1997) have shown that drying calliandra significantly decreased CT content (3.7% to 2.5%), and when provided as a supplement (30% diet) to pangola grass hay for sheep, fresh (frozen) calliandra depressed the voluntary intake of hay, decreased N, OM, NDF and acid detergent fibre (ADF) digestibility. Similarly, increasing CT contents of leucaena leaf (2%–14%) decrease in vitro dry matter digestibility (IVDMD) of leaf material (Wheeler et al. 1994), and decreases in vivo OM, N digestibility and N retention in sheep given diets of varying CT content (0.6%–6.5% CT).

Tannins probably have their greatest effect on the nutrition of animals in arid and semi-arid environments where Acacia species are a significant source of supplemental and reserve feed. Mulga (*Acacia aneura*) contains such high levels of tannins that the availability of N and S from protein digestion in the rumen is so restricted that sheep suffer N and S deficiencies in the rumen, which limits feed intake and productivity (Hoey et al. 1976; Pritchard et al. 1992). While some relief from these effects can be afforded by additional supplements of N, P and S, the daily application of 24 g PEG alone increased DM intake by 78%, converted live-weight loss (−64 g/d) to gain (36 g/d) and resulted in an almost 3-fold increase in the volumetric growth of wool. It is of interest that while PEG supplementation increased N digestibility (36.6% to 58.4%) there was no effect on DM digestibility (49.7% to 48.8%) which suggests that the tannins of mulga are having a specific effect on intake, unrelated to the rates of feed digestion and removal from the rumen. Similar effects of tannins have been reported for a wide range of tropical forages (Kumar 1992).

Tannins and digestive enzymes

Tannins are also known to inhibit intestinal enzymes in pigs, poultry and rats, and to also reduce the in vitro activity of ruminal cellulase (Kumar and D'Mello 1995) and urease (Benoit and Starkey 1968). Inhibition of celluloytic activity by tannins

may explain the decrease in OM, NDF and ADF digestibilities in the rumen of sheep given diets of different CT content (Perez-Maldonado and Norton 1996a). Tannins also inhibit the degradation of dietary protein in the rumen and decrease ruminal ammonia concentrations, which may suggest an inhibition of proteolytic enzymes in the rumen (Norton and Ahn 1997). However, it is also possible that tannins bound to plant proteins and fibre was the primary cause of depressed protein and fibre digestion in these sheep. There appears to no effect of ingested tannins on the amounts or efficiency of microbial synthesis in the rumen. There has also been some reports that tannins, particularly tannic acid (HT), causing gastro-enteritis and damage to the gut wall in non-ruminants (Salunkhe et al. 1990) and increasing endogenous losses of protein by either reducing reabsorption or causing hyper-secretion (Jansman et al. 1993). Although these specific effects have not yet been reported in ruminants, observations of increased faecal N excretion may be related to increased endogenous losses.

The presence of tannins in tropical legumes is also probably important in alley cropping systems where legumes are applied as a soil amendment. Under these conditions, tannin containing tree legumes have a slower and less effective short-term release of plant nitrogen for crop growth than plants without tannins (Gutteridge 1990). However, such slow release characteristics may be beneficial for long duration crops and pastures.

Techniques for modifying the deleterious effects of tannins

Physiological mechanisms of adaptation to tannin

The previous studies all suggest that tannins generally act as toxins and/or inhibitors of intake, digestion and utilisation of feeds by animals and food by humans. Although the relationship between aversion to food, palatability and tannin content is not clear, there are both physiological mechanisms in animals and management techniques which may modify the detrimental effects of tannins. Proline-rich proteins (PRP) with a high affinity for CT have been found in the saliva of deer, rodents, some marsupials and humans. It has been suggested that these proteins protect these animals from the toxic effects of tannins (Mehanso et al. 1987). However, PRP are not found in the saliva of cattle, sheep or goats (Perez-Maldonado et al. 1995), although there are suggestions that goats produce an active tannase enzyme (Begovic et al. 1978) and have a tannin-resistant *Streptococcus caprinus* in their rumen (Brooker et al. 1994). There is also recent evidence that more than 60% of ingested CT is degraded (lost) during transit through the digestive tract, but it not clear what proportion of this loss is through microbial action (Perez-Maldonado and Norton 1996b). The microbial degradation of HT and CT tannins, and the possibility that animals might be innoculated with tannin metabolising micro-organisms to offset toxicity, will be explored more fully by other papers in these Proceedings. However, despite these apparent microbial adaptations, there appear to be few differences between sheep and goats in their metabolism and utilisation of high tannin tropical feeds (Perez-Maldonado and Norton 1996a).

Supplements and feed processing

A number of techniques have been used to ameliorate the effects of tannins in legume and sorghum grains and pods, with heating, drying, soaking in water, acid, alkali (sodium hydroxide), oxidising, urea or formaldehyde solutions, and the application of selective binding agents such as polyvinyl-pyrrolidine (PVP), PEG and ferric salts, being varingly successful. However, techniques relevant to the feeding of high tannin forages are more limited, and have been restricted to supplementation with PEG, which is expensive, and with urea. Although it seems unlikely that urea supplements are needed as an additional N source for ruminants fed tropical legumes and fodder tree leaves, there has, nevertheless, been responses found to urea supplemention in animals given these diets (Karda et al. 1998). It has been proposed that urea deactivates tannins (Russell and Lolley 1989), and this method needs further study as a possibly cheap and effective method of overcoming the deleterious effects of tannins in feeds. The drying of tropical forages also decreases the apparent content and activity of CT tannins (Ahn et al. 1989), and increases the digestibility of organic matter, fibre and N and NB of sheep fed diets supplemented with *Calliandra calothyrsus* leaf (Norton and Ahn 1997).

An alternative approach has been to restrict the intake of high tannin forages offered, and to supplement with other grasses and legumes. This has the effect of providing some dilution of the effects of tannins and possible saturation of feed tannins with proteins from the other feeds. Similarly, high tannin tropical legumes can also be used in silages made from tropical grasses, and is an effective way to improve the quality of feed available to animals in these areas (Tandraatmadja et al. 1993). It is also clear from the now substantial collections of tropical forage and fodder tree legumes that there is some considerable variation in tannin content between species and accessions (Dalzell et al. 1998) providing the opportunity to select and breed highly

productive low tannin varieties of high nutritive value for future introduction into tropical farming systems.

Speculations and Directions for Future Research

There is an urgent need for a better characterisation of chemical nature and biological activity of phenolic compounds in plants with particular reference to the tannins. Some of the difficulties now being encountered in the interpretation of the effects of tannins on animal metabolism is associated with a lack of understanding of how the chemical structure of tannins relates to biological activity. Firstly, there must be a clear differentiation between hydrolysable and condensed tannins and their action, and all tannin containing plants should be analysed for both. It now recognised that the activity of CT depends on whether it is in a free form, or bound to protein and/or fibre, and that CT may occur in a number of molecular forms of varying size and flavanol composition. It is possible that better understanding of the tannin structure may help explain how some high tannin feeds stimulate the secretion of growth-hormone and have anthelminthic effects in animals. There is also a need to more thoroughly explore compounds like PEG which deactivate tannins and promote better utilisation as a high quality feed. In this context, a study of simple complexing agents such as cheap analogues of PEG, ferric salts, clays and urea as feed additives which might act with saliva during eating and mastication to better inactivate ingested tannins. There may also be significant opportunities for developing microbial inoculates which might modify, inhibit or destroy tannin action in the rumen. The selection of forage legumes and fodder trees for low tannin and high nutritive value is another obvious course for future action.

One of the greatest opportunities arising from a better understanding of how tannins limit nutritive value is the prospect that we may be able to make better use of some forage resources which are now only of limited value to livestock farmers. For example, mulga (*Acacia aneura*) is well adapted to the arid zone of Australia, but it is little better than a maintenance feed for sheep. The recognition that tannins are limiting the quality of mulga as a feed, and that there are techniques by which these limits can be overcome, now provides a means by which these scarce resources might be more efficienctly used as a source of high quality feed for livestock. In many arid areas, Acacia and Propopis species are significant weeds of rangelands mainly because they are not normally eaten by grazing animals. In Australia, *Acacia nilotica* is rapidly becoming one of the worst woody weeds in the arid zone, and in the USA, mesquite (*Prosopis glandulosa*) dominates large areas of rangeland in the south. If these species were able to be rendered palatable by limiting the effects of tannins, then a solution to both the feed shortage and weed problem would be found. Many Australian acacias have shown promise as new forestry species in developing countries, and it is possible that with some application of the accumulating knowledge on tannins, that these trees may also have value as sources of both wood and fodder.

References

Ahn, J.H., Robertson, B.M., Elliott, R., Gutteridge, R.C. and Ford, C.W. 1989. Quality assessment of tropical browse legumes: tannin content and protein degradation. Animal Feed Science and Technology, 27: 147–156.

Bate-Smith, E.C. 1954. Leucoanthocyanidins. I. Detection and indentification of anthocyanidins formed from leucoanthocyanidins in plant tissues. Biochemical Journal, 58: 122–125.

Bate-Smith, E.C. 1981. Astringent tannins of leaves of Geranium species. Phytochemistry, 20: 211–216.

Begovic, S., Duzic, E., Sacirbrebegovic, A. and Tafro, A. 1978. Examination of variations of tannase activity in ruminal content and mucosa of goats on oak leaf diet and during the intraruminal administration of 3–10% tannic acid. Veterinaria (Sarajevo), 27: 459–465.

Benoit, R.E. and Starkey, R.L. 1968. Enzyme inactivation as a factor in the inhibition of decomposition of organic matter by tannins. Soil Science, 105 (4): 203–208.

Broadhurst, R.B. and Jones, W.T. 1978. Analysis of condensed tannins using acidified vanillin. Journal of the Science of Food and Technology, 29: 788–794.

Brooker, J.D., O'Donovan, L.A., Skene, L., Clarke, K., Blackall, L. and Muslera, P. 1994. *Streptococcus caprinus* sp. Nov., a tannin resistant ruminal bacterium from feral goats. Letters in Applied Microbiology, 18: 313–318.

Dalzell, S.A., Stewart, J.L., Tolera, A. and McNeill, D.M. 1998. Chemical composition of Leucaena and implications for forage quality. In: Shelton, H.M., Gutteridge, R.C., Mullen, B.F. and Bray, R.A. ed. Leucaena — Adaptation, Quality and Farming Systems. ACIAR Proceedings No. 86, Canberra, Australia, 227–246.

Evans, D.O. 1996. ed. Forest, Farm and Community Tree Research, Special Issue. Proceedings of an International Workshop on the Genus Calliandra, 23–27 January, 1996, Bogor, Indonesia, Winrock International, 234 p.

Faint, M.A., McNeill, D.M., Stewart, J.L., Castillo, A.C., Acasio, R.N. and Lynch, J.J. 1998. Palatability of Leucaena to ruminants. In: Shelton, H.M., Gutteridge, R.C., Mullen, B.F. and Bray, R.A. ed. Leucaena — Adaptation, Quality and Farming Systems. ACIAR Proceedings No. 86, Canberra, Australia, 215–227.

Foley, W.J., Iason, G.R. and McArthur, C. 1999. Role of plant secondary metabolites in the nutritional ecology of

mammalian herbivores: how far have we come in 25 years? In: Jung, H-J.G. and Fahey Jr., G.C. ed. Nutritional Ecology of Herbivores. Proceedings of the Fifth International Symposium on the Nutrition of Herbivores, April 11–17, 1999 in San Antonio, Texas, USA. 130–209, American Society of Animal Science, Savoy, Illinois USA.

Foo, L.Y., Jones, W.T., Porter, L.J. and Williams, K.M. 1982. Proanthocyanidin polymers of fodder legumes. Phytochemistry, 21: 933–938.

Gutteridge, R.G. 1990. The effects of three fodder tree species as sources of nitrogen on alley cropping systems. Experimental Agriculture, 43: 231–237.

Hagerman, A.E. and Butler, L.G. 1978. Protein precipitation method for the quantitative determination of tannins. Journal of Agriculture and Food Chemistry, 26: 809–812.

Hagerman, A.E. and Butler, L.G. 1989. Choosing appropriate methods and standards for assaying tannin. Journal of Chemical Ecology, 15: 1795–1810.

Hoey, W.A., Norton, B.W. and Entwistle, K.W. 1976. Preliminary investigations with molasses and sulphur supplementation of sheep fed mulga (*Acacia aneura*). Proceedings of the Australian Society of Animal Production, 11: 377–380.

Jansman, A.J.M., Vestegen, M.W.A. and Huisman, J. 1993. Effects of dietary inclusion of hulls of Faba beans (*Vicia faba* L.) with low and high content of condensed tannins on some physiological parameters in piglets. Animal Feed Science and Technology, 43: 239–257.

Jones, W.T., Broadhurst, R.B., and Lyttleton, J.W. 1976. The condensed tannins of pasture legume species. Phytochemistry, 15: 1407–1409.

Jones, W.T and Mangan, J.L. 1977. Complexes of the condensed tannins of sainfoin (*Onobrychis viciifolia* Scop) with Fraction 1 Leaf Protein and with submaxillary mucoprotein and their reversal by polyethylene glycol and pH. Journal of the Science of Food and Agriculture, 28: 126–136.

Jones, R.J., Galgal, K.K., Castillo, A.C., Palmer, B., Deocareza, A. and Bolam, M. 1998. Animal production from five species of Leucaena. In: Shelton, H.M., Gutteridge, R.C., Mullen, B.F. and Bray, R.A. ed. Leucaena – Adaptation, Quality and Farming Systems. ACIAR Proceedings No. 86, Canberra, Australia, 247–252.

Karda, I.W., Dryden, G. McL. and Gutteridge, R.G. 1998. In: Shelton, H.M., Gutteridge, R.C., Mullen, B.F. and Bray, R.A. ed. Leucaena – Adaptation, Quality and Farming Systems. ACIAR Proceedings No. 86, Canberra, Australia, 280–281.

Kumar, R. 1992. Anti-nutritional factors, the potential risks of toxicity and methods to alleviate them. In: Speedy, A. and Pugliese, P-L. ed. Legume trees and other fodder trees as protein sources for livestock. FAO Animal Production and Health Paper 102. Food and Agriculture Organisation of the United Nations, Rome.

Kumar, R. and D'Mello, J.P.F. 1995. Anti-nutritional factors in forage legumes. In: D'Mello, J.P.F. and Devendra, C. ed. Tropical Legumes in Animal Nutrition, 95–134, CAB International, Wallingford, Oxon., UK.

Lowry, J.B., Petheram, J.R and Tangendjaja, B. 1992. Plant Fed to Village Ruminants in Indonesia. ACIAR Technical Reports 22, Australian Centre for International Agricultural Research, Canberra, Australia, 60 p.

Lowry, J.B., McSweeney, C.S. and Palmer, B. 1996. Changing perceptions of the effect of plant phenolics on nutrient supply in the ruminant. Australian Journal of Agricultural Research, 47: 829–842.

McLeod, M.N. 1974. Plant-tannins. Their role in forage quality. Nutrition Abstracts and Reviews, 11: 803–815.

McNeill, D.M., Osborne, N., Komolong, M.K. and Nankervis, D. 1998. Condensed tannins in the Genus Leucaena and their nutritional significance for ruminants. In: Shelton, H.M., Gutteridge, R.C., Mullen, B.F. and Bray, R.A. ed. 1998, Leucaena – Adaptation, Quality and Farming Systems. ACIAR Proceedings No. 86, Canberra, Australia, 205–214.

Mehanso, H., Butler, L.G., and Carlson, D.M. 1987. Dietary tannins and salivary proline-rich proteins: Interactions, induction and defense mechanisms. Annual Review of Nutrition, 7: 423–440.

Minson, D.J. 1990. Forage in Ruminant Nutrition. Academic Press, London, 483 p.

Mullen, B.F., Gabunada, F., Shelton, H.M., Stur, W.W. and Napompeth, B. 1998. Psyllid Resistance in Leucaena. In: Shelton, H.M., Gutteridge, R.C., Mullen, B.F. and Bray, R.A. ed. Leucaena – Adaptation, Quality and Farming Systems. ACIAR Proceedings No. 86, Canberra, Australia, 51–60.

Norton, B.W. 1994. Anti-nutritive and toxic factors in forage tree legumes. In: Gutteridge, R.G. and Shelton, H.M. ed. Forage Tree Legumes in Tropical Agriculture, 202–215. CAB International, Wallingford, Oxon., UK.

Norton, B.W. and Ahn, J.H. 1997. A comparison of fresh and dried *Calliandra calothyrsus* supplements for sheep given a basal diet of barley straw. Journal of Agricultural Science, Cambridge, 129: 485–494.

Perez-Maldonado, R.A., Norton, B.W. and Kerven, G.L. 1995. Factors affecting in vitro formation of tannin-protein complexes. Journal of the Science of Food and Agriculture, 69: 291–298.

Perez-Maldonado, R.A. and Norton, B.W. 1996a. The effects of condensed tannins from *Desmodium intortum* and *Calliandra calothyrsus* on protein and carbohydrate digestion in sheep and goats. British Journal of Nutrition, 76: 515–533.

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

Pritchard, D.A., Martin, P.R. and O'Rourke, P.K. 1992. The role of condensed tannins in the nutritional value of mulga (*Acacia aneura*) for sheep. Australian Journal of Agricultural Research, 43: 1739–1746.

Provenza, F.D., Burritt, E.A., Clausen, T.P., Bryant, J.P., Reichardt, P.B. and Distel, R.A. 1990. Conditioned flavour aversion: A mechanism for goats to avoid condensed tannin in black brush. American Naturalist, 136: 810–828.

Reed, J.D. 1986. Relationship among soluble phenolics, insoluble proanthocyanidins and fibre in East African browse species. Journal of Range Management, 39: 5–7.

Rittner, U. and Reed, J.D. 1992. Phenolics and in vitro degradability of protein and fibre in West African browse. Journal of the Science of Food and Agriculture, 58: 21–28.

Russell, R.W. and Lolley, J.R. 1989. Deactivation of tannin in high tannin milo by treatment with urea. Journal of Dairy Science, 72: 2427–2430.

Salunkhe, D.K., Chavan, J.K. and Kadam, S.S. 1990. Dietary tannins: Consequences and remedies. CRC Press Inc. Florida, 310 p.

Shelton, H.M., Piggin, C.M. and Brewbaker, J.L. ed. 1995. Leucaena: Opportunities and Limitations. ACIAR Proceedings No. 57, Canberra, Australia, 241 p.

Shelton, H.M., Gutteridge, R.C., Mullen, B.F. and Bray, R.A. ed. 1998. Leucaena – Adapation, Quality and Farming Systems. ACIAR Proceedings No. 86, Canberra, Australia, 358 p.

Skerman, P.J., Cameron, D.G. and Riveros, F. 1988. Tropical Forage Legumes. Food and Agriculture Organisation of the United Nations, Rome, 257 p.

Stewart, J.L., Allison, G.E. and Simons, A.J. ed. 1996. *Gliricidia sepium*: Genetic Resources for Farmers. Tropical Forestry Papers 33, Oxford Forestry Institute, Department of Plant Sciences, University of Oxford, 125 p.

Tandraatmadja, M., Macrae, I.C. and Norton, B.W. 1993. Effect of the inclusion of tropical tree legumes, *Gliricidia sepium* and *Leucaena leucocephala*, on the nutritive value of silages prepared from tropical grasses. Journal of Agricultural Science, Cambridge, 120: 397–406.

Tanner, J.C., Reed, J.D. and Owen, E. 1990. The nutritive value of fruits (pods with seeds) from four *Acacia* spp. Compared with noug (*Guizotia abyssinica*) meal as supplements to maize stover for Ethiopian highland sheep. Animal Production, 51: 127–133.

Van Soest, P.J., Conklin, N.L. and Horvath, P.J. 1986. Tannins in foods and feeds. Proceedings of the Cornell Nutrition Conference for Feed Manufacturers, 115–122.

Waghorn, G.C. 1990. Effects of condensed tannin on protein digestion and nutritive value of fresh herbage. Proceedings of the Australian Society of Animal Production, 18: 412–415.

Waghorn, G.C., Jones, W.T., Shelton, I.D. and McNabb, W.C. 1990. Condensed tannins and the nutritive value of herbage. Proceedings of the New Zealand Grassland Association, 51: 171–176.

Wheeler, R.A., Norton, B.W. and Shelton, H.M. 1994. Condensed tannins in Leucaena species and hybrids and implications for nutritive value. In: Shelton, H.M., Piggin, C.M. and Brewbaker, J.L. eds. Leucaena: Opportunities and Limitations. ACIAR Proceedings No. 57, Canberra, Australia, 112–118.

Wilson, J.R. 1993. Organisation of forage plant tissues. In: Jung, H.G., Buxton, D.R., Hatfield, R.D. and Ralph, J. ed. Forage Cell Wall Structure and Digestibility. American Society of Agronomy, Madison, 1–32.

Tannins in Feedstuffs used in the Diets of Pigs and Poultry in Australia

D.J. Farrell[1] and R.A. Perez-Maldonado[1]

Abstract

Grain sorghum has historically been high in condensed tannins. This has often restricted its inclusion in the diets of pigs and poultry. Current Australian-grown cultivars are almost tannin free. Some grain legumes, particularly faba beans and field peas, contain significant concentrations of tannins but cultivars used in Australia, although high in tannins, do not appear to reduce the performance of pigs and poultry. However, research in France found that with pigs the digestible energy of Australian-grown peas was 1.7 MJ/kg less than a French cultivar. Cottonseed meal, a potentially useful source of protein for livestock, is included in only very small amounts in diets of non-ruminants because of high tannin (32 g/kg) levels in hulls and a phenolic compound, gossypol. Rapeseed (or canola meal) contains significant levels of tannins (8.8 g/kg) and other phenolic compounds such as sinapine. Some of these can be converted to trimethylamine which discolours the yolk and taints brown-shelled eggs. But tannins in rapeseed meal do not seem to be responsible for sub-optimum production of pigs and poultry observed when used in high amounts. The mode of action of tannins is reviewed briefly. These may damage the mucosal lining of the digestive tract, particularly of poultry. Tannins reduce the digestibility of proteins by complexing with them as well as with some digestive enzymes, and to a lesser extent starch. Chickens appear to be affected to a greater extent than pigs. It is suggested that they may be less sensitive to the astringent effect of tannins because they have much fewer taste buds in their oral cavity.

THE AIM of this paper is to identify feedstuffs that may contain tannins and are used in the diets of pigs and poultry in Australia. These are mainly grain sorghum and some grain legume seeds, but also rapeseed meal and cottonseed meal. A major difficulty in any review of this subject is (i) terminology and (ii) the variety of methods used to extract and then to analyse tannins (Perez-Maldonado 1994). Although there are two classes of tannins, hydrolysable tannins and condensed tannins, the latter are far more important in feedstuffs for pigs and poultry and are the only ones considered here. Because of their adverse effects in animal production, tannins may be classified as an anti-nutritional factor (ANF).

The poor acceptability of tannins by livestock has been attributed to the astringent taste when tannins bind with saliva proteins and mucous epithelium in the mouth. Differences between pigs and poultry in their tolerance for feedstuffs rich in tannins may be due to the very few taste buds (24) in the mouth of chickens compared to the high number (15 000) in the mouth of pigs (Moran 1982).

[1] School of Land and Food, The University of Queensland, Brisbane Qld 4072 Australia

Feedstuffs

Grain sorghum

In Australia, unlike in the United States, sorghum is an important feed grain for pigs and poultry. About 1.3 million metric tonnes are used each year to feed all livestock in Australia (Farrell 1997). Unlike yellow maize, which contains xanthophyll (cryptoxanthin and casotene), grain sorghum, wheat and barley do not contain a pigment which produces a yellow skin and yellow fat in poultry. There is therefore little interest in sorghum in those many countries which produce pigs and poultry on 'corn/soybean' diets. In these countries, particularly in the US, there has been much less attention given to

KEYWORDS: Tannins, Pigs, Poultry, Feedstuffs, Rapeseed meal, Grain sorghum, Anti-nutritional factors (ANF)

sorghum breeding. It is well known that low-tannin sorghums (white seeds) of less than 2.6 g tannin/kg (Pour-Reza and Edriss 1997) are of similar nutritive value to maize but they do vary substantially in their protein content (Nyachoti et al. 1997). The function of tannins in sorghum, as in many other seeds, is thought to be to protect the seed from wild birds, insects and against bacterial and fungal attack. Tannins are deposited just underneath the seedcoat; because birds may cause very high losses of seed, the presence of tannins is seen as a way of protecting the crop from bird predation and this is, up to a point, desirable. Consequently, livestock do not also accept the grain readily. However, it should be recognised that the stringent taste and reduced acceptability associated with high levels of tannin will be diluted, depending on the level of inclusion of sorghum in the formulated diet.

In Australia, plant breeding programs have paid considerable attention to low-tannin cultivars. This has been successful, and, without compromising yield or grain quality, grain sorghum poses no problems in the feeding of livestock (Willis 1992).

Grain legumes

Several of these seeds are used widely in pig and poultry feeding in Australia (Pettersen and Mackintosh 1994). Many contain tannins usually in the seed coat (Table 1) and several other ANFs. Sweet lupin is the most widely used grain legume with an estimated 1.4 million metric tonnes (mt) in 1998–1999 but contains relatively small concentrations of tannins, averaging 3–4 g total tannin/kg. Because of other ANFs associated with lupins which will depend on variety, their inclusion in pig and poultry diets is limited to not more than 100–300 g/kg (King 1990).

There is a large discrepancy between tannin values shown in Tables 1 and those reported in a survey by Pettersen and Mackintosh (1994) in that much higher values are shown for all three legume seeds in Table 1 (Perez-Maldonado et al. 1999). Cultivars of faba beans and field peas which have coloured flowers are usually high in tannins; those with white flowers contain very little.

Rapeseed (canola) meal

Rapeseed is a rapidly expanding oil seed grown in Australia. Annual production is expected to increase by 93% in 1998–1999 to 1.7 million mt. There has been little research on cultivars that are grown here. Blair et al. (1983) gave tannin contents of several Canadian canola (rapeseed low in ANF) seed and rapeseed meal cultivars using a modified vanillin method of analysis (Jansman 1993). Values ranged from 1.7 to 4.3 g tannin/kg rapeseed meal to 2.3–6.2 g/kg canola meal. This analysis gave a value of 8.8 g/kg (Table 1). The authors concluded that tannins in these meals were of minor nutritional significance in livestock production. Vapar and Clandinin (1972) came to the same conclusion in earlier studies with chickens. However, sinapine, found in rapeseed meal, is a phenolic compound and

Table 1. Tannin fractions of cottonseed and rapeseed meals and of commercial cultivars of chick peas, field peas and faba beans grown in Australia (g/kg dry matter) (Perez-Maldonado et al. 1999).

	Chick peas (cv. Amethyst)	Field peas (cv. Glenroy)	Faba beans (cv. Fiord)	Cottonseed meal[1]	Rapeseed meal[1]
Free-tannins	5.1	16.9	11.7	6.4	2.0
Protein-bound	2.9	2.5	3.7	25.7	6.8
Fibre-bound	0.70	0.82	0.67	—	—
Total	8.6	20.2	16.0	32.1	8.8

[1] Unknown cultivar

Table 2. Results of measurements made over 40 weeks on groups of 50 cages (2 birds/cage) on diets containing 250 g grain legume/kg (Perez-Maldonado et al. 1999).

Treatments	Hen-day egg production (%)	Egg weight (g)	Egg mass (g/d)	Food intake (g/d)	Food conversion ratio
Chick peas	82.4 $^{ab[1]}$	56.9 ^{a}	46.7 ^{a}	115.4 ^{bc}	2.60 ^{a}
Field peas	84.1 ^{a}	55.9 ^{b}	46.9 ^{a}	116.3 ^{b}	2.56 ^{a}
Sweet lupins	84.0 ^{a}	55.9 ^{b}	46.8 ^{a}	118.9 ^{a}	2.64 ^{a}
Faba beans	80.7 ^{b}	53.7 ^{c}	43.2 ^{b}	113.3 ^{c}	2.80 ^{b}
LSD (P = 0.05)	2.68	0.86	1.65	2.57	0.148

[1] Values with different superscript are significantly different (P<0.05)

Table 3. The effect of grain legumes and pelleting temperature on liveweight gain, food intake, food conversion ratio (FCR), intestinal viscosity, intestinal length, liver and pancreas weight, and excreta score in chickens (0–21d) of age (Farrell et al. 1999).

Treatment	Weight gain (g)	Food intake (g)	FCR[1]	Viscosity (cP)	Intestinal length (cm/100 gW)[2]	Liver (g/100 gW)	Pancreas (g/100 gW)	Excreta score
Field peas	673 [a2]	831 [a]	1.24 [b]	3.3 [c]	14.7 [b]	2.79 [ab]	0.33 [b]	0.57 [c]
Faba beans	664 [a]	816 [ab]	1.24 [b]	4.0 [b]	15.0 [b]	2.79 [ab]	0.33 [b]	0.77 [c]
Lupins	645 [b]	828 [a]	1.29 [a]	8.6 [a]	15.9 [a]	2.87 [a]	0.34 [b]	2.67 [a]
Chick peas	630 c	812 [b]	1.30 [a]	4.1 [b]	15.7 [a]	2.74 [b]	0.42 [a]	1.60 [b]
LSD (P = 0.05)	14.8	15.5	0.022	0.64	0.57	0.116	0.016	0.302
Hot (90 °C)	660 [a2]	829 [a]	1.27	5.1	15.2	2.81	0.358	1.46
Cold (65 °C)	646 [b]	815 [b]	1.26	4.9	15.5	2.79	0.355	1.34
LSD (P<0.05)	10.4	10.9	0.016	0.46	0.405	0.082	0.011	0.581

[1] Corrected to dry matter
[2] Body weight

although not a tannin it could be included as one in some assay procedures. Sinapine can be hydrolysed to trimethylamine by entereal bacterial enzymes; this can discolour the yolk and cause off-flavours in eggs from certain strains of laying hens, particularly those producing brown-shelled eggs (Blair et al. 1983; Brettschneider et al. 1995). These strains have lost the ability to convert trimethylamine (TMA) to the N-oxide. Fenwick et al. (1981) concluded that tannins also play a key role in the inhibition of TMA oxidase in hens fed rapeseed meal leading to off-flavours in their eggs.

Cottonseed meal

Cottonseed meal has been the most abundant oilseed meal produced in Australia. However, its nutritive value is much lower than expected on the basis of chemical analyses (Batterham 1993). Chemical composition varies widely because of different type of process, processing conditions and cultivars. Australian-produced cottonseed meal exceeds 0.5 million mt/year and contains up to 32 g total tannin/kg (Table 1). Most of these are found in the hulls which comprise 130–300 g/kg meal; they contain 32–65 g tannins/kg; of these over 50% are protein bound (Yu et al. 1993). Cottonseed kernel contains no tannins.

Livestock Performance

Grain sorghum

Many years ago, McClymont and Duncan (1952) demonstrated in Australia the toxic effects of grain sorghum when fed to poultry. Connor et al. (1969) showed depressed performance in crossbred cockerels on diets with high (25 g/kg) compared to low (1 g/kg) tannin sorghums. This depression was alleviated in part by the addition of choline and methionine. Their explanation was that the major metabolite in urine was 4-0-methyl gallic acid and that methionine and choline were methyly donors for the 0-methylation. True digestibility of several critical amino acids in high-tannin sorghums is about 20 percentage points lower than in low-tannin sorghums (Anon. 1989).

Grain legumes

Faba beans have received more attention than any other grain legume seed in relation to tannins. Despite the high tannin level, values for the true digestibility of critical amino acids in adult cockerels was around 85% (Perez-Maldonado et al. 1999). Thacker (1990a) summarised research undertaken with starter pigs, growing-finishing pigs and breeding stock. The high tannin cultivars adversely affected palatability and hence intake, particularly in young pigs. Upper levels of inclusion of faba beans were 150 g, 200 g and 100 g/kg for starter, growing-finishing and breeding sows respectively. For the latter, faba beans at 170 g/kg reduced pigs born alive, milk yield and composition (Thacker 1990a). Because faba beans contain various ANFs such as lectins and haemaglutinins, it is difficult to identify the actual cause of negative effects on animal production.

Despite their high tannin content (Table 1), field peas have been used successfully in pig and poultry diets, particularly in South Australia, for several years. The early work of Davies (1984a, b) demonstrated no adverse effects of Australian-grown peas (cv. Early Dun) in the diets of growing finishing pigs at 300 g/kg, although an upper limit of 150 g/kg is recommended for starter pigs (Castell 1990).

The authors' recent work (Perez-Maldonado et al. 1999; Farrell et al. 1999) with poultry has

demonstrated the usefulness of commercial cultivars of faba beans and field peas in the diets of poultry. The results of the layer experiment showed that when four grain legumes were each included at 250 g/kg diet, only faba beans gave reduced hen-day egg production, egg mass and egg size (Table 2). The latter effects may be due to the presence of vicine and convicine and not to tannins (Wiseman and Cole 1988). Field peas, on the other hand, gave excellent production.

When these same grain legumes were included in boiler poultry diets at 120–360 g/kg and birds grew to 21 days of age, mean weight gain and some other parameters on diets with either field peas or faba beans were superior to chick peas and sometimes to sweet lupins (Table 3). However, steam pelleting improved ($P<0.05$) growth rate and feed conversion ratio (FCR) for all four grain legumes compared to cold pelleting. Interestingly, in the finisher period (21–42 days), field peas did not give growth rates or FCR as good as faba beans. There are several examples in the literature of different European cultivars of both field peas and faba beans with a wide range of tannin and nutrient concentrations which can depress livestock performance (Wiseman and Cole 1988).

Carré and Conan (1989) reported high levels of trysin inhibitor (TI) activity in peas which was reduced by heat treatment. The authors concluded that TI was not a major factor that could explain variability in protein digestibility in poultry.

Rapeseed meal

There is limited research in Australia on rapeseed meal and most of the research has been undertaken in North America on canola meal cultivars which are low in glucosinolotes and erucic acid. Starter pigs given a choice discriminated against a diet with only 50 g canola meal/kg (Thacker 1990b). Aherne and Baidoo (1991) presented data showing that young pigs (12–20 kg) tended to have reduced feed intake and growth rate at levels of 85 g canola meal/kg and a significant depression at 170 g/kg. In older pigs (50–100 kg), canola meal can replace completely soybean meal in the diet and canola meal can be used without restriction in the diets of breeding sows (Thacker 1990b).

Recent studies in Thailand (Tangtaweeipat et al. 1998) showed that bodyweight gain was reduced in broilers when canola meal was substituted for 75% soybean meal in the diet. Egg production and egg size were reduced in layers on diets with 12% canola meal. It would seem therefore that poultry can tolerate reasonable levels of recent cultivars of rapeseed meal before performance is affected.

Cottonseed meal

In addition to tannins, cottonseed meal contains gossypol. Some of this is in the free-form which is toxic to pigs and poultry. Iron salts are an effective way of binding free gossypol. Lipids extracted using an expeller (screw press) process normally gives meals low in free gossypol. When fed to broiler chickens, up to 300 g cottonseed meal/kg can be used, provided the correct apparent metabolisable energy (AME) and digestible amino acid coefficients are assigned to the meal and the free gossypol is neutralised (Watkins et al. 1993, 1994).

Batterham (1993) gave a range of values for availability of lysine of only 0.27–0.30 with some other important amino acids being poorly available in growing pigs. Ileal digestibility of lysine was high (0.58–0.72) indicating that much of the lysine was absorbed but in an unavailable form (Batterham 1993). Lysine may have complexed with tannins or with the phenolic compound, gossypol in the cottonseed meal, or with both. Lysine digestibility of cottonseed meal is in only 0.55 in growing chickens (Ravindran et al. 1998).

In diets for pigs, provided similar precautions are taken, grower and finisher pigs can grow well on diets limited to 80–111 g cottonseed meal/kg. For breeding stock, around 70 g/kg gives reasonable production (Tanskley 1990). For laying hens most nutritionists are reluctant to include cottonseed meal in formulations because gossypol can be transferred to the yolk which may discolour it.

Mode of Action of Tannins

Tannins form soluble and insoluble and sometimes irreversible complexes with proteins, digestive enzymes and possibly starch in the digestive tract of pigs and poultry. Sorghum tannins may bind and precipitate at least 12 times their own weight of protein (Jansman 1993). Formation of these complexes increases with molecular size of the tannins and inhibit enzymatic breakdown of protein and can increase endogenous amino acid loss. Results of in vitro enzyme assays with tannins do not necessarily mimic reactions in the digestive tract because of the special conditions in vivo (Butler 1992). Tannins can increase the size of the parotid glands, and damage the mucosal lining of the gastro intestinal tract of chickens, but to a lesser extent in the laboratory rat (Oritz et al. 1994) and with much less evidence for pigs.

Studies on the effects of condensed tannins have given equivocal results. For example, Lacassagne et al. (1988) showed with poultry that starch digestibility was much lower in faba bean cultivars low in tannins than those high in tannins. Flores et al.

(1994) concluded that there was a negative effect of tannins on starch digestibility in 3-week old chickens but the extent of the depression depended on the quantity of tannin ingested. Jansman et al. (1993) found with young pigs no difference in starch digestibility on diets with faba beans of high and low condensed tannin content. The authors concluded that condensed tannins have a preference to complex with proteins rather than carbohydrates. In chickens, protein digestibility of tannin-free cultivars of faba beans was more digestible (0.83) than in tannin-containing (0.68) cultivars (Lacassagne et al. 1988). Studies in France on imported Australian peas containing high levels of tannins (3.6–3.9 g/kg) gave 1.7 MJ DE less than a low tannin French cultivar when measured in pigs (Grosjean et al. 1991).

Yu et al. (1996a, b) demonstrated that the addition of polyethylene glycol (PEG), which binds strongly with tannins, to diets of pigs and rats containing cottonseed hulls and casein, reversed with few exceptions the depression in ileal amino acid digestibility observed in diets with cottonseed hulls alone.

Butler (1992) suggested that increased faecal protein observed on diets rich in tannins is due largely to endogenous protein from the lining and secretions of the digestive tract. However, Jansman et al. (1994) have observed with pigs on diets high in tannins a reduced activity of trypsin in ileal digesta which was probably responsible for the lower digestibility of protein (0.61 vs 0.73).

Conclusion

The cultivars of grain legumes currently used in the Australian feed industry do not appear to have anti-nutritional effects on livestock performance although some cultivars are high in tannins (Table 1). Grain sorghum, a widely used grain, poses no problems since cultivars used here are low in tannins. Tannins in rapeseed meal appear to be of minor importance although there is little information on tannin content of cultivars grown in Australia. The phenolic compound sinapine, may be a problem in some layer strains. High levels of cottonseed hulls in cottonseed meal makes this feedstuff unattractive to the feed industry although there is evidence that adding a source of iron salts increases its usefulness. Clearly reducing the hull content of the meal makes it more attractive to the feed manufacturer.

References

Aherne, F. and Baidoo, S. 1991. An evaluation of the nutritive value of canola meal for young pigs. In: Farrell, D.J. ed. Recent Advances in Animal Nutrition in Australia, p. 24A. (University of New England, Armidale).

Anon. 1989. Nutrition Guide. Feed Formulation with Digestible Amino Acids. 34 p. (Rhone-Poulenc Animal Nutrition, Antony, France).

Batterham, E.S. 1993 Availability of amino acids in feeds. In: Batterham, E.S. ed. Manipulating Pig Production IV, 197–203. (Australasian Pig Science Association, Attwood, Vic.).

Blair, R., Mitaru, B.N. and Reichert, R.D. 1983. Carbohydrate and phenolic constituents in rapeseed and their nutritional significance for poultry. In: Robards, G.E. and Packham, R.G. ed. Feed Information and Animal Production, 407–410. (CAB, Oxon).

Brettschneider, J., Danicke, S. and Jeroch, H. 1995. The influence of graded levels of rapeseed in laying hen diets in egg quality with special consideration of hydrothermal treatment of rapeseed. In: Briz, R.C. ed. Eggs and Egg Products Quality, 227–232. (University of Zaragoza, Spain).

Butler, L.G. 1992. Anti-nutritional effects of condensed and hydrolysable tannins. In: Hemingway. R.W. and Labs. P.E. ed. Plant Polyphenols, 693–698. (Plenum Press, New York).

Carré, B. and Conan, L. 1989. Relationship between trypsin-inhibitor content of pea seeds and pea protein digestibility in poultry. In: Huisman, J., van der Poel, T.F.B. and Liener, I.E. ed. Recent Advances of Research in Anti-Nutritional Factors in Legume Seeds, 103–106. (Pudoc, Wageningein, 1989).

Castell, A.G. 1990. Field Peas. In: Thacker, P.A. and Kirkwood, R.N. ed. Non Traditional Feed Sources for Use in Swine Production, 185–196. (Butterworths, Stoneham, MA).

Connor, J.K., Hurwood, I.S., Burlin, H.W. and Fuelling, D.E. 1969. Some nutritional aspects of feeding sorghum grain of high tannin content to growing chickens. Australian Journal of Experimental Agriculture and Animal Husbandry, 9: 497–501.

Davies, R.L. 1984a. Field peas (*Pisum sativum*) as a feed for growing and finishing pigs. 1. Nutrient level in commercial crops. Australian Journal of Experimental Agriculture and Animal Husbandry, 24: 350–353.

Davies, R.L. 1984b. Field peas (*Pisum sativum*) as a feed for growing and finishing pigs. 2. Effects of substituting peas for meatmeal or fishmeal in conventional diets. Australian Journal of Experimental Agriculture and Animal Husbandry, 24: 507–511.

Farrell, D.J. 1997. Where in the world will we find the ingredients to feed our livestock by the year 2007? In: Corbett, J.L., Choct, M., Nolan, J.V. and Rowe, J. ed. Recent Advances in Animal Nutrition in Australia 1997, 136–145. (University of New England, Armidale).

Farrell, D.J., Perez-Maldonado, R.A. and Mannim, P.F. 1999. The optimum inclusion of field peas, faba beans, chick peas and sweet lupins in poultry diets. II. Broiler experiments. British Poultry Science (in press).

Fenwick, G.R., Pearson, A.W., Greenwood, N.M. and Butler, E.J. 1981. Rapeseed meal tannins and egg taint. Animal Feed Science and Technology, 6: 421–431.

Flores, M.P., Castanon, J.I.R. and McNab, J.M. 1994. Effect of tannins on starch digestibility and TME_n, of triticale and semipurified starches from triticale and field beans. British Poultry Science, 35: 281–286.

Grosjean, F. Bourdon, D., Kiener, T., Castaing, J. and Gatel, F. 1991. Nutritive value of French and imported peas for pigs. Journees de la Recherche Porcine en France, 23: 53–60.

Jansman, A.J.M., Huisman, J. and van der Poel, A.F.B. 1993. Ileal and faecal digestibility in piglets of field beans (*Vicia faba* L.) varying in tannin content. Animal Feed Science and Technology, 42: 83–96.

Jansman, A.J.M. 1993. Tannins in feedstuffs of simple stomached animals. Nutrition Research Reviews 1993, 6: 209–236.

Jansman, A.J.M., Enting, H., Verstegen, M.W.A. and Huisman, J. 1994. Effect of condensed tannins in hulls of faba beans (*Vicia faba* L.) in the activities of trypsin (EC2.4. 21.4) and chymotrypsin (EC2.4.21.1) in digesta collected from the small intestine of pigs. British Journal of Nutrition, 71: 627–641.

King, R.H. 1990. Lupins. In: Thacker, P.A. and Kirkwood, R.N. ed. Non-traditional Feed Sources for Use in Swine Production, 237–246. (Butterworths, Stoneham, MA).

Lacassagne, L., Franchesch, M., Carré, B. and Melicon, J.P. 1988. Utilisation of tannin-containing and tannin-free faba beans (*Vicia faba*) by young chicks: effects of pelleting feeds on energy, protein and starch digestibility. Animal Feed Science and Technology, 20: 59–68.

McClymont, G. and Duncan, D.C. 1952. Studies on the nutrition of poultry. 3 Toxicity of grain sorghum for chickens. Australian Veterinary Journal, 28: 229–233.

Moran, E.T. 1982. Comparative Nutrition of Fowl and Swine. The Gastrointestinal Systems, 253 p. (University of Guelph, Guelph, Ontario).

Nyachoti, C.M., Atkinson, J.L. and Leeson, S. 1997. Sorghum tannins: a review. World's Poultry Science, 53: 5–21.

Oritz, L.T., Alzueta, C., Trevino, J. and Castano, M. 1994. Effects of faba bean tannins on the growth and histological structures of the intestinal tract and liver of chicks and rats. British Poultry Science, 35: 743–754.

Petterson, D.S. and Mackintosh, J.B. 1994. The Chemical Composition and Nutritive Value of Australian Grain Legumes, 68 p. (Grains Research and Development Corporation, Canberra).

Perez-Maldonado, R.A. 1994. The Chemical Nature and Biological Activity of Tannins in Forage Legumes Fed to Sheep and Goats. PhD Thesis. The University of Queensland, St Lucia.

Perez-Maldonado, R.A., Mannion, P.F. and Farrell, D.J. 1999. The optimum inclusion of field peas, faba beans, chick peas and sweet lupins in poultry diets. I. Chemical composition and layer experiments. British Poultry Science (in press).

Pour-Reza, I. and Edriss, M.A. 1997. Effects of dietary sorghum of different tannin concentrations and tallow supplementation on the performance of broiler chicks. British Poultry Science 38: 512–517.

Ravindran, V., Hew, L.I. and Bryden, W.L. 1998. Digestible Amino Acids in Poultry Feedstuffs. (RIRDC Publication No 98/9, Kingston, ACT).

Tangtaweeipat, S., Boonlom, C-I. and Paspisanu, P. 1998. The use of rapeseed meal in poultry diets. Proceedings Sixth Asian Pacific Poultry Congress, 82–87, Nagoya June 4–7, 1998. Japan Poultry Science Association.

Tanskley, T.D. 1990. Cottonseed Meal. In: Thacker, P.A. and Kirkwood, R.N. ed. Non-traditional Feed Sources for Use in Swine Production, 139–151. (Butterworths, Stoneham, MA).

Thacker, P.A. 1990a. Faba beans. In: Thacker, P.A. and Kirkwood, R.N. ed. Non-traditional Feed Sources for Use in Swine Production, 175–184. (Butterworths, Stoneham, MA).

Thacker, P.A. 1990b. Canola Meal. In: Thacker, P.A. and Kirkwood, R.N. ed. Non-traditional Feed Sources for Use in Swine Production, 69–79. (Butterworths, Stoneham, MA).

Vapar, Z. and Clandinin, D.R. 1972. Effect of tannins in rapeseed meal in its nutritional value for chicks. Poultry Science, 51: 222–228.

Watkins, S.E., Skinner, J.T., Adams, M.H. and Waldsoup, P.W. 1993. An evaluation of low-gossypol cottonseed meal in diets for broiler chickens. I: Effect of cottonseed meal level and lysine supplementation. Journal of Applied Poultry Research, 2: 221–226.

Watkins, S.E., Skinner, J.T., Adams, M.H. and Waldsoup, P.W. 1994. An evaluation of low-gossypol cottonseed meal in diets for broiler chickens. II: Influence of assigned metabolisable energy values and supplementation with essential amino acids on performance. Journal of Applied Poultry Research, 2: 7–16.

Willis, S. 1992. Utilisation of sorghum in the pig and poultry industries. In: Foale, M.A., Henzell, R.G. and Vance. P.N. ed. Proceedings of the Second Australian Sorghum Conference, 442–449. (Australian Institute of Agricultural Science, Publication No. 62).

Wiseman, J. and Cole, D.J.A. 1988. European legumes in diets for non-ruminants. In: Haresign, W. and Cole, D.J.A. ed. Recent Advances in Animal Nutrition, 13–17. (Butterworths, London).

Yu, F., Barry, T.N., Moughan, P.J. and Wilson, G.F. 1993. Condensed tannins and gossypol concentrations in processed cottonseed meal. Journal of the Science of Food and Agriculture, 63: 7–15.

Yu, F., Moughan, P.J. and Barry, T.N. 1996a. The effect of cottonseed condensed tannins in the ileal digestibility of amino acids in casein and cottonseed kernel. British Journal of Nutrition, 75: 683–698.

Yu, F., Moughan, P.J., Barry, T.N. and McNabb, W.C. 1996b. The effect of condensed tannin from heated and unheated cottonseed on the ileal digestibility of amino acids of growing pigs. British Journal of Nutrition, 76: 359–371.

The Effect of Condensed Tannins in Temperate Forages on Animal Nutrition and Productivity

T.N. Barry[1] and W.C. McNabb[2]

Abstract

Condensed tannins (CT) react with forage proteins in a pH-reversible manner, with reactivity determined by the concentration, structure and molecular weight of the CT. Increasing concentrations of CT in *Lotus corniculatus* and *Lotus pedunculatus* reduce the rates of solubilisation and degradation of Fraction 1 leaf protein in the rumen and increase duodenal NAN flow. Action of medium concentrations of total CT in *L. corniculatus* (30–40g/kg DM) increased the absorption of EAA from the small intestine, increased wool growth and increased reproductive rate in grazing sheep without affecting VFI, thus improving the efficiency of food conversion. High concentrations of CT in *L. pedunculatus* (75–100 g/kg DM) depressed VFI and rumen carbohydrate digestion and depressed rates of body and wool growth in grazing sheep, in line with these concentrations acting as a plant defense. The minimum concentration of CT to prevent rumen frothy bloat in cattle is defined as 5 g/kg DM. Defined concentrations of forage CT can be used to increase the efficiencies of protein digestion and animal productivity in ruminants fed temperate forages. Plant breeding and molecular techniques should be examined for increasing CT content of the common temperate grasses and legumes from approx 1 g/k DM to 5 g CT/kg DM or greater.

TEMPERATE forages grazed in the leafy vegetative state contain high concentrations of metabolisable energy (ME) 11.5 MJ/kg dry matter (DM) and total nitrogen (N) 30 g/kg DM. However, duodenal flow of non-ammonia nitrogen (NAN) is only about 65% of the N eaten (MacRae and Ulyatt 1974). This paper reviews condensed tannins (CT) for reducing the degradation of proteins in the rumen and increasing essential amino acid (EAA) absorption in ruminants fed fresh forages.

Condensed tannin concentration, structure and reactivity

The concentration of CT in a range of temperate forages is shown in Table 1. Most animal nutrition work in New Zealand (NZ) has been done with the two *Lotus* species. Average molecular weight (MW) for *Lotus pedunculatus* is 2200, while it is 1900 for *Lotus corniculatus*. CT structure also differs in that prodelphinedin type sub-units predominate in *L. pedunculatus*, whereas pro-cyanidin type sub-units predominate in *L. corniculatus* CT (Foo et al. 1996; 1997).

Condensed tannins bind strongly to proteins. Jones and Mangan (1977) first showed that reactivity between CT and forage protein was pH-dependant, with stable complexes being formed at pH 3.5–7.5, but the complexes dissociating and releasing protein at pH <3.5. Much research with animals then followed, examining this reactivity as the basis for increasing UDP and EAA absorption in ruminants fed sole diets of fresh forages. Effects of CT have been deduced by comparing unsupplemented sheep (CT acting) with a group of sheep supplemented with polyethylene glycol (PEG; MW 3350), as PEG specifically binds and inactivates CT (Jones and Mangan 1977; Barry and Manley 1986).

Voluntary feed intake

High CT concentrations in *L. pedunculatus* (63 and 106 g/kg DM) substantially depressed VFI in sheep (–27%), in line with plant CT production being a defence against consumption by herbivores (Barry and Duncan 1984). Lower depressions in VFI (–12%)

[1] Institute for Food, Nutrition and Human Health, Massey University, Palmerston North, New Zealand
[2] Nutrition Group, Agresearch, Grasslands Research Centre, Palmerston North, New Zealand

KEYWORDS: Condensed tannins, Temperate forages, Animal nutrition, Animal productivity

Table 1. The extractable and bound condensed tannin content of legumes, grasses and herbs fed to ruminants in temperate grazing systems, measured by the butanol-HCL method.

Forage		Condensed tannin (g/kg DM)			
		Extractable	Protein-bound	Fibre-bound	Total
	Legumes				
Big trefoil	*L. pedunculatus*	61	14	1	77
Birdsfoot trefoil	*L. corniculatus*	36	9	2	47
Sulla	*Hedysarum coronarium*	33	9	3	45
Sainfoin	*Onobrychis vicifolia*	29			
Red Clover	*Trifolium pratense*	0.4	0.6	0.7	1.7
Lucerne	*Medicago sativa*	0.0	0.5	0.0	0.5
	Grasses				
Perennial ryegrass	*Lolium perenne*	0.8	0.5	0.5	1.8
	Herbs				
Chicory	*Chicorium intybus*	1.4	2.6	0.2	4.2
Sheeps burnet	*Sanguisorba minor*	1.0	1.4	1.0	3.4

From Terrill et al. (1992b); Jackson et al. (1996).

were produced by 55 g CT/kg DM in *L. pedunculatus* (Waghorn et al. 1994). However, medium CT concentrations in sulla (45 g/kg DM) and in *L. corniculatus* (34 and 44 g/kg DM) had no effect upon VFI (Terrill et al. 1992a; Wang et al. 1996 a,b).

Digestion of nitrogen and carbohydrate

With perennial ryegrass, short rotation ryegrass and white clover, which contain only traces of CT, duodenal NAN flow is only about 0.75 of N intake (Figure 1), illustrating extensive absorption of ammonia from the rumen. However, with *Lotus* sp., duodenal NAN flow increased linearly with increasing CT concentration and equalled N intake at a CT concentration of approximately 40 g/kg DM (Figure 1).

Effects of CT on apparent absorption of EAA from the small intestine (Table 2) differed between *L. corniculatus* (22 g extractable CT/kg DM; Waghorn et al. 1987) and *L. pedunculatus* (55 g extractable CT/kg DM; Waghorn et al. 1994). When expressed as a proportion of N intake, action of CT in *L. corniculatus* increased both abomasal flow (+53%) and the net absorption of EAA from the

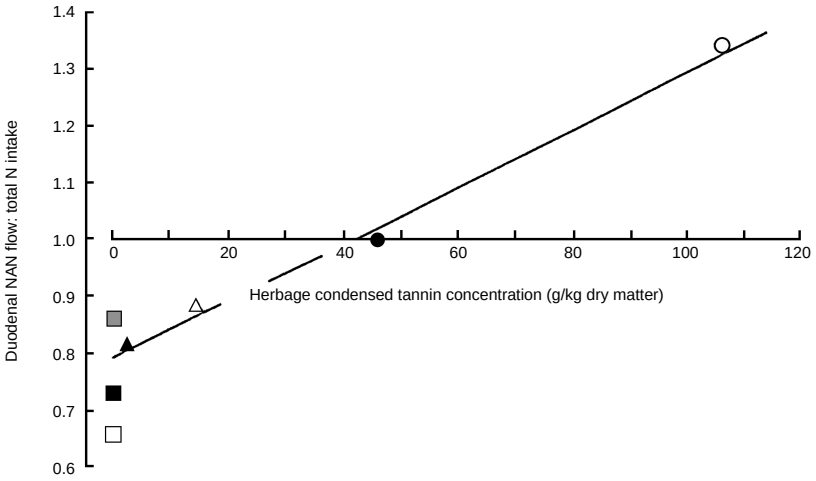


Figure 1. Duodenal non-ammonia nitrogen (NAN) flow per unit N intake as a function of herbage condensed tannin concentration in sheep fed on Lotus species. (○) high — and (●) low-tannin *L. pedunculatus*. (△) high- and (▲) low tannin *L. corniculatus*. (▩) short rotation ryegrass, (□) perennial ryegrass, and (■) white clover. From Barry and Manley (1984).

small intestine (+59%), with no effect on apparent digestibility (proportion abomasal flow) in the small intestine. However, while action of CT increased abomasal flow in sheep fed *L. pedunculatus* (+30%), this was counteracted by reduced apparent digestibility in the small intestine, with there being only a small increase in apparent absorption of EAA from the small intestine (+10%).

High concentrations of CT in *L. pedunculatus* (95 and 106 g/kg DM) depressed rumen digestion of readily fermentable carbohydrate (soluble sugar + pectin) and hemicellulose, but this was counteracted by increased post-ruminal digestion (Barry and Manley 1984; Barry et al. 1986). Carbohydrate digestion in sheep fed *L. corniculatus* (25–35 g CT/kg DM) has not been affected by CT (Waghorn et al. 1987).

Effects of CT on rumen fermentation of carbohydrate and protein can be explained by the concept of 'free tannin', defined as the CT not precipitated in high speed centrifugation of plant macerates (Barry and Manley 1986; Figure 2). Up to a total CT concentration of approximately 90 g/kg DM, 90% of the CT in *Lotus* sp. was precipitated with plant constituents (i.e. protein) and 10% was free in solution, whereas increments in total CT concentration above 90 g/kg DM were all released as 'free tannin'. Thus, for *Lotus* sp. almost all the CT reacted with proteins in the host plant until the binding capacity of this system had been saturated (at about 90 g CT/kg DM). It is proposed (i.e. suggested) that insoluble CT functions through reducing plant protein degradation in the rumen, while free CT can react with and inactive microbial enzymes, explaining why high levels of free CT reduce rumen carbohydrate digestion (Figure 3). This concept also explains why mixing CT-containing and non CT-containing temperate forages may not produce a beneficial outcome in some circumstances (Beever and Siddons 1986), as the CT will preferably react with proteins in the forage of the CT-containing plant. It is suggested that beneficial effects of forage mixing can only be expected if the CT content is high and the protein content relatively low in the CT-containing plant, thus releasing some 'free' CT to bind with proteins in the non CT-containing plant. These conditions occur with some tropical legume forages and legume shrubs, especially if grown under low soil fertility conditions.

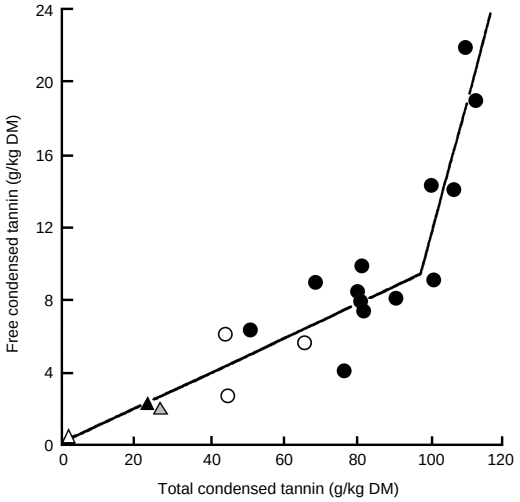


Figure 2. Free condensed tannin concentration as a function of total condensed tannin concentration in macerates of fresh legumes. *L. pedunculatus* (cv. Grasslands Maku) grown under ○ high and ● low soil fertility conditions; *Lotus corniculatus* cultivars △ Winnar, ▲ El Boyero and ◭ Granger grown under low soil fertility conditions. From Barry and Manley (1986).

Table 2. Effect of condensed tannin (CT) upon the intake and absorption of essential amino acids from the small intestine of sheep fed fresh *L. corniculatus* and *L. pedunculatus*, containing respectively 22 and 55 g extractable CT/kg DM.

	L. corniculatus		*L. pedunculatus*	
	CT Acting	PEG Supplemented	CT Acting	PEG Supplemented
Rumen ammonia (mg N/l)	367	504	175	458
Intake (g/d)	98.9	98.9	103.2	116.8
Abomasal flow:				
g/d	84.7	55.5	121.1	105.6
Proportion intake	0.86	0.56	1.17	0.90
Apparent absorption from small intestine:				
g/d	58.8	36.2	81.4	83.5
Proportion abomasal flow	0.67	0.67	0.66	0.79
Proportion intake	0.59	0.37	0.79	0.72

From: Waghorn et al. (1987, 1994)
[1]excluding arginine [2]including arginine

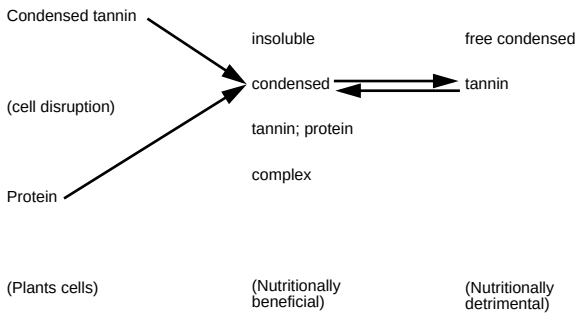


Figure 3. Proposed mechanism of condensed tannin reaction with plant proteins and free tannin formation during cell disruption, and the suggested roles of insoluble and free condensed tannin in ruminant nutrition. From Barry and Manley (1986).

Effects of condensed tannins on forage feeding values

The effects of CT on forage feeding value can be regarded as the sum of its effects on VFI, on the digestive process and on the metabolism of absorbed nutrients. In growing lambs (initial liveweight 22.4 kg) grazing *L. corniculatus* for four months during summer and autumn (Experiment 1), action of CT (i.e. unsupplemented sheep — PEG supplemented sheep) increased wool growth by 12% without affecting rate of body growth or VFI (Wang et al. 1996a; Table 3), while there was no response to PEG supplementation in comparable sheep grazing lucerne, containing only traces of CT (0.3 g/kg DM). Action of CT in dry ewes (initial liveweight 54 kg) restricted to maintenance feeding for four months during summer (Experiment 2) increased wool growth by 19% without affecting VFI or LWG (Table 3).

A review of many years' data implicated a role for protein nutrition in the ovulation rate of ewes (Smith 1991), and this was illustrated by an increase in ewes showing multiple ovulations in response to abomasal infusions of lactalbumin and soy protein isolate (73% vs. 55%; Cruickshank et al. 1988). Grazing trials were then carried out for 6–12 weeks, with ewes grazing perennial ryegrass/white clover pasture and *L. corniculatus*, containing 1 and 23 g CT/kg DM (Experiments 3 and 4; Table 4), to study if this effect on ovulation rate could be induced by CT. In both years, grazing *L. corniculatus* consistently increased the proportion of multiple ovulations and increased wool growth by an average of 15%. As

Table 3. Voluntary feed intake, liveweight gain, carcass gain and wool growth in lambs (Experiment 1) and dry ewes (Experiment 2) grazing the forage legumes *L. corniculatus* (27–34 g CT/kg DM) and lucerne (0.3 g total CT/kg/ DM) during summer.

	Lotus		Lucerne		SE
	CT acting	PEG supplemented	CT acting	PEG supplemented	
			Experiment 1 (1992–93)		
Rumen ammonia (mg N/l)	255	370	555	535	
VFI (kg OM/day)	1.19	1.20	1.32	1.34	0.056
LWG (g/d)	203	188	185	178	5.8
Carcass gain (g/d)	79	75	68	63	2.9
Wool growth (g/d)	12.1	10.9	10.8	10.2	0.39
			Experiment 2 (1995–96)		
Rumen ammonia (mg N/l)	221	278	ND	ND	8.5
VFI (kg OM/day)	1.23	1.20	ND	ND	0.051
LWG (g/d)	54	67	ND	ND	9.3
Wool growth (g/d)	13.2	11.1	ND	ND	0.66

From Wang et al. (1996a); Min et al. (1998)
ND; not determined

judged by responses to PEG supplementation, part of the *Lotus* response in multiple ovulation can be explained by action of CT in Experiment 1 but not Experiment 2. The difference may be due to the lower protein requirements of the heavier ewes used in Experiment 2. Approximately 6 weeks of grazing on *L. corniculatus* was required to produce the maximum increase in multiple ovulations.

In contrast to the increased productivity obtained from CT in *L. corniculatus*, action of CT in *L. pedunculatus* containing 76–90 g CT/kg DM markedly depressed rates of both body growth and wool growth (Barry 1985), further illustrating the ecological role of high CT concentrations as a chemical defence.

Conclusions

When used under defined conditions, CT in temperate forages can be used to improve the efficiency of N digestion and to increase the productivity of grazing animals. New methodology has shown the presence of trace amounts of CT in most of the common grasses and legumes grazed in temperate agriculture (1–2 g/kg DM). This is too low to reduce protein solubility and degradation in the rumen (Min et al. 1999a) and a minimum concentration of 5 g/kg DM or greater is suggested to both increase wool production (Montossi et al. 1997) and to prevent rumen bloat in cattle (Li et al. 1996). Evaluation of traditional plant selection techniques and also molecular techniques for increasing CT concentration in common legumes such as white clover, red clover and lucerne offers exciting future possibilities.

References

Barry, T.N. 1985. The role of condensed tannins in the nutritional value of *L. pedunculatus* for sheep. 3. Rates of body and wool growth. British Journal of Nutrition, 54: 211–217.

Barry, T.N. and Duncan, S.J. 1984. The role of condensed tannins in the nutritional value of *L. pedunculatus* for sheep. 1, Voluntary intake. British Journal of Nutrition, 51: 485–491.

Barry, T.N. and Manley, T.R. 1984. The role of condensed tannins in the nutritional value of *L. pedunculatus* for sheep. 2. Quantitative digestion of carbohydrates and proteins. British Journal of Nutrition, 51: 493–504.

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

Barry, T.N., Manley, T.R. and Duncan, S.J. 1986. The role of condensed tannins in the nutritional value of *L. pedunculatus* for sheep. 4 sites of carbohydrate and protein digestion. British Journal of Nutrition, 55: 123–137.

Beever, D.E. and Siddons, R.C. 1986. Digestion and metabolism in the grazing ruminant. In: Milligan, L.P., Grovum, W.L. and Dobson, A. ed. Control of Digestion and Metabolism in Ruminants. 479–497. Englewood Cliffs, New Jersey, Prentice-Hall.

Cruickshank, G.J., Smith, J.F. and Frazer, D.G. 1988. The influence of abomasal infusion of protein or energy on ovulation rates in ewes. Proceedings of the New Zealand Society of Animal Production, 48: 77–79.

Foo, L.Y., Newman, R., Waghorn, G.C., McNabb, W.C. and Ulyatt, M.J. 1996. The proanthocyanidins of *L. corniculatus*. Phytochemistry, 41: 617–624.

Table 4. Voluntary feed intake, liveweight gain, wool production and reproduction in ewes grazing *L. corniculatus* (17–24 g total CT/kg DM) and perennial ryegrass/white clover pasture (1 g total CT/kg DM) during autumn.

	Lotus		Pasture		SE
	CT acting	PEG supplemented	CT acting	PEG supplemented	
	Experiment 1 (1997; initial liveweight 54 kg)				
VFI (kg OM/ewe/d)	1.70	1.85	1.83	1.98	0.087
LWG (g/d)	40	34	19	5	6.9
Clean fleece (kg)	1.35	1.31	1.09	1.14	0.027
Multiple ovulation (%)	69.4	59.2	30.6	33.4	0.60
	Experiment 2 (1998; initial liveweight 60kg)				
VFI (kg OM/ewe/d)	1.96	1.86	1.78	ND	0.09
LWG (g/d)	−20	−25	−12	ND	6.7
Clean fleece (kg)	1.73	1.69	1.54	ND	0.029
Multiple ovulation (%)	63.5	61.5	47.4	ND	0.074

ND; not determined
From Min et al. (1999b); Luque et al. (1999)

Foo, L.Y., Lu, Y., McNabb, W.C., Waghorn, G.C. and Ulyatt, M.J. 1997. Proanthocyanidins from *L. pedunculatus*. Phytochemistry, 45: 1689–1696.

Jackson, F.S., McNabb, W.C., Barry, T.N., Foo, Y.L. and Peters, J.S. 1996. The condensed tannin content of a range of subtropical and temperate forages and the reactivity of condensed tannin with Ribulose-1,5-bisphosphate carboxylase (Rubisco) protein. Journal of the Science of Food and Agriculture, 72: 483–492.

Jones, W.T. and Mangan, J.L. 1977. Complexes of the condensed tannins of sainfoin with fraction 1 leaf protein and with submaxillary mucoprotein, and their reversal by polyethylene glycol and pH. Journal of the Science of Food and Agriculture, 28: 126–136.

Li, Y.G., Tanner, G. and Larkin, P. 1996. The DMCA-HCl protocol and the threshold proanthocyanidin content for bloat safety in forage legumes. Journal of the Science of Food and Agriculture, 70: 89–101.

Luque, A., Barry, T.N., McNabb, W.C., Kemp, P.D. and McDonald, M.F. 1999. The effect of length feeding on *L. corniculatus* during late summer/autumn upon reproductive efficiency and wool production in ewes. Australian Journal of Agricultural Research (submitted).

MacRae, T.C. and Ulyatt, M.J. 1974. Quantitative digestion of fresh herbage by sheep. 2 The sites of digestion of some nitrogenous constituents. Journal of Agricultural Science, Cambridge, 82: 309–319.

Min, B.R., Barry, T.N., McNabb, W.C. and Kemp, P.D. 1998. The effects of condensed tannins on the production of wool and on its processing characteristics in sheep grazing *L. corniculatus*. Australian Journal of Agricultural Research, 49: 597–605.

Min, B.R., McNabb, W.C., Barry, T.N. and Peters, J.S. 1999a. Solubilisation and degradation of protein from white clover and *L. corniculatus* by rumen microorganisms and the effect of condensed tannins in these processes. Journal of Agricultural Science, Cambridge. (In press).

Min, B.R., McNabb, W.C., Barry, T.N., Kemp, P.D., Waghorn, G.C. and McDonald, M.F. 1999b. The effect of condensed tannins in *L. corniculatus* upon reproductive efficiency and wool production in sheep during late summer and autumn. Journal of Agricultural Science, Cambridge (In press).

Montossi, F., Liu, F., Hodgson, J., Morris, S.T., Barry, T.N. and Risso, D.F. 1997. Influence of low-level condensed tannins concentration in temperate forages on sheep performance. Proceedings Seventeenth International Grasslands Congress. (In press).

Smith, J.F. 1991. A review of recent developments on the effect of nutrition on ovulation rate in ewes. Proceedings of the New Zealand Society of Animal Production, 51: 15–23.

Terrill, T.H., Douglas, G.B., Foote, A.G., Purchas, R.W., Wilson, G.F. and Barry, T.N. 1992a. Effect of condensed tannins upon body growth, wool growth and rumen metabolism in sheep grazing sulla and perennial pasture. Journal of Agricultural Science, Cambridge, 119: 265–273.

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

Waghorn, G.C., Shelton, I.D., McNabb, W.C. and McCutcheon, S.H. 1994. Effects of condensed tannins in *L. pedunculatus* on its nutritive value for sheep. 2 Nitrogenous aspects. Journal of Agricultural Science, Cambridge, 123: 109–119.

Waghorn, G.C., Ulyatt, M.J., Johns, A. and Fisher, M.T. 1987. The effect of condensed tannins on the site of digestion of amino acids and other nutrients in sheep feed *L. corniculatus*. British Journal of Nutrition, 57: 115–126.

Wang, Y., Douglas, G.B., Waghorn, G.C., Barry, T.N., Foote, A.G. and Purchas, R.W. 1996a. Effect of condensed tannins upon the performance of lambs grazing *L. corniculatus* and lucerne. Journal of Agricultural Science, Cambridge, 126: 87–98.

Wang, Y., Douglas, G.B., Waghorn, G.C., Barry T.N. and Foote, A.G. 1996b. Effect of condensed tannins upon the performance of lambs grazing *L. corniculatus* and lucerne. Journal of Agricultural Science, Cambridge. 126: 87–98.

Tannins in *Calliandra calothyrsus*: Effect of Polyethylene Glycol (PEG) and an Evaluation of 19 Accessions

B. Palmer[1] and C.S. McSweeney[2]

Abstract

Of the tropical shrub legumes currently available for assessment as animal feed, *Calliandra calothyrsus* showed most promise for development. Nineteen accessions of *C. calothyrsus* were evaluated for a range of attributes including nutritional, agronomic and chemical characteristics. The relationships between these attributes are reported, the lack of association between the estimates of tannin and the nutritional characteristics demonstrates the inappropriate measures that are commonly taken. The role of tannin as an anti-nutrient is demonstrated and the minimising of this effect by either feed supplements or manipulation of the rumen microflora is discussed.

INCREASES in livestock production to satisfy the growing demand for animal protein are constrained by sufficient supply of high quality feed throughout the year. Tropical grasses are of such low quality that they cannot sustain high productivity.

One approach to overcoming the lack of high quality forage is to grow shrub legumes. Shrub and tree legumes can grow rapidly, produce high protein leaf, and often retain this through the dry season (Gray 1970). Furthermore, most legumes can fix atmospheric nitrogen, eliminating the need for expensive nitrogenous fertilisers.

Since man's domestication of animals, shrub and tree legumes have played an important role in providing fodder. In addition, they provide fuel, fencing and shade and promote soil stabilisation and improvement. Leucaena (*Leucaena leucocephala*) has been widely grown to fulfil these roles. Although Leucaena is known for producing high yields of good quality feed, it is poorly adapted to acid soils (NAS 1984). This poor adaptation precludes the use of Leucaena on inherently acid soils that predominate in Southeast Asia. Recent evidence of its susceptibility to psyllid infestation has also restricted its usefulness in high rainfall areas (NFTA 1987). There is thus a need to find suitable shrub legumes either as a replacement for, or to be complementary to, Leucaena.

In a study of 21 shrub legume accessions grown at two sites in Indonesia and two in Australia (Bray et al. 1997), some species did not persist more than one year, but produced relatively high yields. These included *Cajanus cajan*, *Codariocalyx gyroides* and *Sesbania sesban*. *L. leucocephala* did not grow well at the two most infertile sites, and was badly affected by the Leucaena psyllid at both Australian sites. Overall, the highest yielding species were *Calliandra calothyrsus*, *Acacia angustissima*, *Gliricidia sepium*, *L. diversifolia*, and *L. pallida*.

Anecdotal information has suggested that there are problems with palatability and fodder quality with *C. calothyrsus*. It is commonly inferred that any nutritional constraint is due to its tannin profile; no toxic substances have been found but high concentrations of condensed tannins (up to 11%) have been reported

Work by Palmer and Schlink (1992) has shown that if fed fresh, the forage value of *C. calothyrsus* (CPI 115690) is high whereas if dried (wilted) the voluntary intake was markedly reduced. They also reported that the higher level of voluntary intake was associated with a higher nylon bag in vitro digestibility of fresh compared with oven-dried, wilted or freeze-dried material.

To maximise the feed value of *C. calothyrsus*, it should be fed fresh, but in a cut-and-carry system, it will be extremely difficult to do this as the reduction

[1] CSIRO, Tropical Agriculture, Davies Laboratory, PMB Post Office, Aitkenvale, Qld 4814 Australia
[2] CSIRO, Tropical Agriculture, Indooroopilly, Qld 4076 Australia

KEYWORDS: Tannins, *Calliandra calothyrsus*, Anti-nutrient, Polyethylene glycol (PEG), Rumen microflora

in digestibility can be up to 50% after six hours (Palmer and Schlink 1992). There are three possible approaches to overcome this lack in nutrient value: use feed supplements to ameliorate the tannin effect; select or develop lines without the deleterious drying effect; manipulate the rumen microflora to overcome the anti-nutritive effects of tannins.

Results and Discussion

Feed additives

In a feeding trial with sheep, 30% *C. calothyrsus*, both with and without polyethylene glycol (PEG) addition (40 g), was fed wilted and fresh on a basal diet of hay. The improvement in dry matter and nitrogen digestibility with PEG addition (Figure 1) strongly supports the view that the major anti-nutrient factor in *C. calothyrsus* is tannin. The dry matter and nitrogen digestibilities of wilted *C. calothyrsus* were not significantly different to fresh, after the addition of PEG.

Wool production can be taken as an indicator of production and has the added advantage in that it reflects the supply of by-pass protein to the animal. Figure 2 shows the wool production where PEG has been added to the calliandra/hay diets. After PEG addition, wool production after supplementing with 30% wilted and fresh material was not significantly different. Using the commercially available product BrowsePlus at an application rate of 10 g/day gave the same increase in production as 40 g of PEG administered intra-ruminally. The economics of these strategies are unknown but are probably prohibitive.

Forty grams/day of PEG infused into the abomasum supported a higher wool production than the same amount of PEG added to the rumen, either infused or directly as a single dose in animals fed hay plus 30% wilted calliandra. These responses suggest the importance of tannin post-ruminally, where it may act on gut cells and enzymes.

Choice of accession

The genus Calliandra has its centre of diversity in South America. *C. calothyrsus* is the most well-known species, but is rarely utilised in its centre of origin. Nineteen accessions of *C. calothyrsus* have been assembled by the Oxford Forestry Institute and are being evaluated more widely to select and breed adapted material for different agricultural uses.

The requirements for an adequate shrub legume can be summarised as follows:

It should be

- High yielding;
- Highly digestible;
- High in protein;
- Eaten by livestock;
- Perennial;
- Tolerant to predation;
- Tolerant to soil acidity;
- Low acid forming.

These nineteen accessions were evaluated in North Queensland for a range of nutritional, chemical and agronomic attributes. When these data were subjected to pattern analysis, they separated into six groups. This grouping has been the basis of selecting representatives of the species for detailed study. The groupings are shown in Figure 3.

C. calothyrsus Cisarua (CPI 115690) was agronomically superior, was highly palatable, highly digestible but had one of the highest estimates of tannin measured. This may of course reflect the inappropriateness of our measures of tannin.

Data in Table 1 show the poor relationship between measures of digestibility and measures of tannin content. The acceptability to cattle is, however, related to extractable tannin measures in Butanol-HCl and to PEG binding (Silanikove et al. 1996). Dry matter and nitrogen digestibility were also negatively correlated to PEG binding.

There were no significant relationships between dry matter digestibility or nitrogen digestibility estimates between fresh, oven-dried and freeze-dried

Table 1. Correlation coefficients on 19 *Calliandra calothyrsus* accessions (OD 65 °C) between PEG binding (PEG_ bd), Acceptability to Cattle (Accept), Extactable tannin determined by Butanol- HCL and Protein Precipitation (Ext. Tan_BUHCl and Ext. Tan_Pppt) and IVDMD on OD sample.

	PEG_bd	Accept.	Ext.Tan._BuHCl	Ext.Tan._Pppt	DMD_OD
Accept.	−0.603**				
Ext. TanBu-HCl	0.664**	−0.687**			
Ext. Tan Pppte	0.533*	−0.433	−0.571*		
DMD_OD	−0.532*	0.434	−0.199	−0.216	
NDIG_OD	−0.493*	0.527*	−0.212	−0.151	0.963*

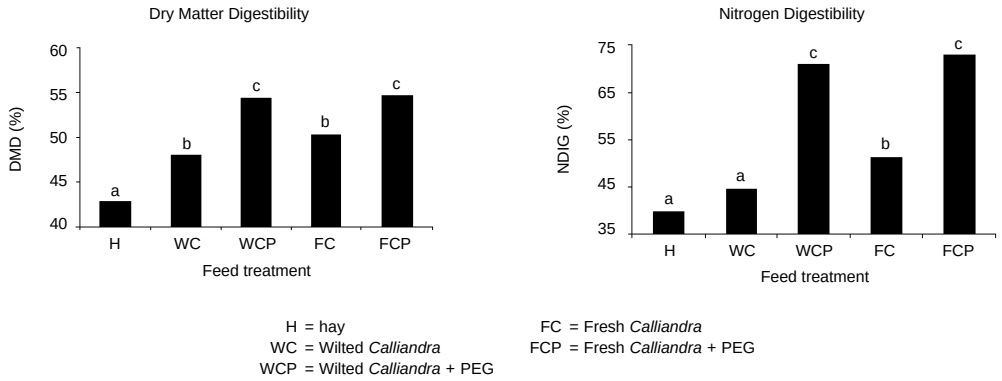


Figure 1. Dry matter and nitrogen digestibility.

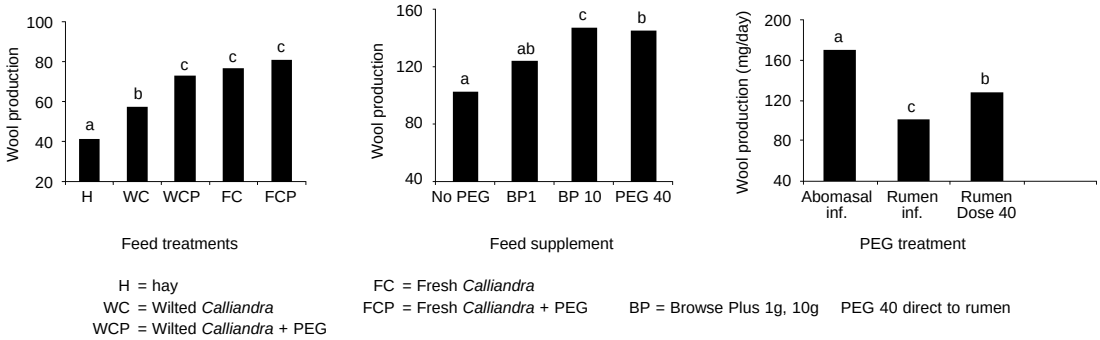


Figure 2. Wool production mg/100 cm^2/day.

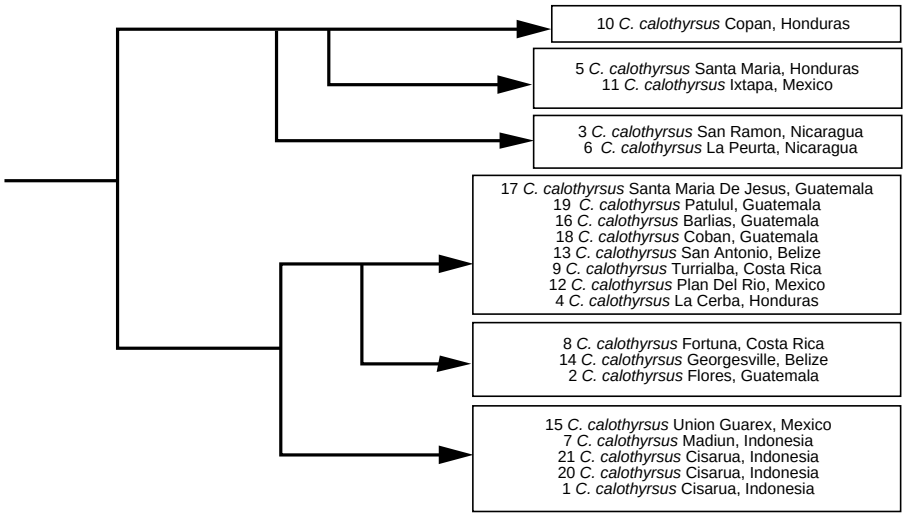


Figure 3. Pattern analysis of 21 accessions (l = 20 = 21) reduced to six groups.

samples when measured intra-ruminally in a nylon bag.

The selection and introduction of appropriate germplasm will be difficult and, as the species predominantly out-crosses, is only suitable for areas where *C. calothyrsus* is not already grown. The variation in the species is large and so selection and breeding of new lines is feasible.

Manipulation of rumen microflora

The manipulation of rumen microflora appears to be the most promising approach if exotic organisms can be isolated and do persist in the rumen. In the world-wide search for micro-organisms capable of degrading tannin, rumen liquor from exotic sources are evaluated for their ability to digest tannin containing feeds. Samples are digested both with and without PEG addition to measure the microbial tolerance to tannin as well as the effect of tannin on irreversibly complexing components of the feed, such as protein. Digestibility could be underestimated because of PEG absorbed onto the residue. The response to PEG addition can be used both to identify regions/animal species where micro-organisms can be sought and also target areas where suitable micro-organisms could be used. Where the response is low, micro-organisms could be present that are tolerant to or degrade tannin. Where the response is high a need is identified for introducing exotic micro-organisms.

Conclusions

There is a need to develop techniques to assess the activity of tannin in the forages that are related to the nutritional value of the feed. The most appropriate approach to overcoming the deleterious effect of condensed tannin is likely to be through the manipulation of rumen microflora.

References

Ahn, J.H., Robertson, B.M., Elliott, R., Gutteridge, R.C. and Ford, C.W. 1989. Quality assessment of tropical browse legumes: Tannin content and protein degradation. Animal Feed Science and Technology, 27: 147–156.

Bray, R.A., Palmer, B. and Ibrahim, T. 1997. The performance of shrub legumes at four sites in Indonesia and Australia. Tropical Grasslands, 31: 31–39.

Gray, S.G. 1970. The place of trees and shrubs as sources of forage in tropical and subtropical pastures. Tropical Grasslands, 4: 57–62.

NAS 1984. Leucaena: Promising Forage and Tree Crop for the Tropics. National Academy Press. Washington DC.

NFTA 1987. Proceedings of a Workshop on Biological and Genetic Control Strategies for the Leucaena Psyllid. 7(2). NFTA, Honolulu, HI.

Palmer, B. and Schlink, A.C. 1992. The effect of drying on the intake and rate of digestion of the shrub legume *Calliandra calothyrsus*. Tropical Grasslands, 26: 89–93.

Silanikove, N., Shinder, D., Gilboa, N., Eyal, M. and Nitsan, Z. 1996. Journal of Agricultural Food Chemistry, 44: 3230–3234

Tannins and Ruminant Production in Indonesia

B. Tangendjaja[1] and E. Wina[1]

Abstract

Ruminant livestock in Indonesia are fed almost exclusively on forages, by cut-and-carry systems or grazing sub systems, although in the past five years, feedlot operations have been developed for fattening cattle fed with industrial by-products as supplements and forages. A very wide range of plant species was fed to ruminants depending upon areas, season, altitude and animal species. Currently, more than 50 species of plants are fed, including grasses, leguminous trees, herbs and leaves. Tannins in these plants were detected by protein precipitation in low (0–1%), medium (1–4%) and high (4–10%) levels. Low tannin content was detected in many grasses and legumes, medium content in leucaena and cassava and high content in calliandra, acacia and mango leaves. Depending on season, the grasses (*Pennisetum*, *Panicum*, *Setaria* etc.) in the ration may reach up to 95% during the wet season and 40% in the dry season. The highest amount of tannin ingested by animals (goats) was found in Kaligesing (Central Java), derived from calliandra and acacia, but the content was less than 1%. Total phenolics content was detected in several species, the highest in calliandra (9%), leucaena (7.8%), acacia (8.3%) and mango leaves (7.3%). In cut-and-carry feeding systems, many forages were wilted which may decrease its nutritive value. There was no report on acute toxicity due to tannin containing materials. This low tannin in feed material may have a beneficial effect on animals. In general, sheep and goats were able to grow at 20–60 g/day.

INDONESIA, Java and Bali, in particular, have complex and productive agricultural systems, supporting some of the highest human population densities in the world (1 and 10 persons/ha in fertile areas). Agriculture in Indonesia is oriented toward food crops, but animal production is important within the system for various reasons (Knipscheer and Levine 1984). Large ruminants are widely kept, mainly for draught power, and account for the greater proportion (85%) of livestock unit. However, in 1997, the number of small ruminants, (±22 million; 14 million goats and 8 million sheep) were higher than large ruminant (±5 millions cattle, 12 million buffalo, 3 million and 0.3 million dairy). The reason for keeping animals is not for supplying meat but mainly for saving, in case a cash money is needed and farmers sell animals for their income.

Unlike modern chickens or pigs which can be produced efficiently, similar to world production standard, ruminant productivity under village conditions is relatively low. It is frequently claimed that one of the limitations on ruminant production in Indonesia is lack of good quanlity feed in sufficient quantity. Lack of knowledge on feed composition and lack of understanding by farmers on feed animals also contributes to this low productivity. Despite the nutrient composition of forages, the poor quality of forages in Indonesia or in the tropics in general relative to temperate areas, relates to the toxicant content detected naturally in plants. One of the important toxicants is a phenolic compound in terms of condensed tannin.

This paper reports on the forage composition fed to animals in Indonesia and the importance of condensed tannin that may influence ruminant productivity.

[1] Research Institute for Animal Production, PO Box 221, Bogor, Indonesia 16002

Results

Production system

Land ownership by farmers is small. On average, each farmer has less than 0.3 ha, so animals are reared by people with little or no land. Therefore, animals are fed with the forages available in villages. There are at least three production systems for ruminants:

KEYWORDS: Tannins, Forages, Ruminants, Legumes

1. Animals are reared post harvest, grazing on cropped fields;
2. Animals are tethered;
3. Animals are kept in cages and fed by cut-and-carry systems of forages under cultivation, or wayside grasess or trees.

Most of ruminants are reared under a labour-intensive system in which the father or children spends hours to cut grasses/forages for animal feed. Surveys on village animal production have shown growth rates in general to be low (Robinson 1977). Small ruminants may grow only 20 g/day while large ruminants may grow only 100 g/day. Under experimental conditions, local breeds such as Javanese sheep might grow up to 80–100 g/day and Madura cattle could grow up to 300–400 g/day, but these animals were supplemented with high quality rations, either legume leaves or rice bran or concentrated feed. Thus, it has usualy been assumed that village feedstuffs were of low nutritional quality (Lowry et al. 1992).

Forage for ruminants

The type of forages fed to ruminants varied, depending on the areas, altitude, season, etc. Wide varieties of plant species were found. No single forage was fed to animals. The majority of farmers fed native grasses and small portions of legume/leaves or agricultural waste such on corn stover or soybean/peanut waste (Table 1).

Table 1. Percentage of farmers feeding various forages.

Forage	Lowland	Upland
Native grass	86	100
Corn stovers	2	70
Legume straw	1	32
Rice straw	0	13
Sesbania sp	20	0
Artocarpus sp	0	10
Banana leaf	0	72
Cassava leaf	3	22

Nitis et al. 1982

In lowland areas, *Sesbania grandiflora* was used while in upland areas, agriculture waste and leaves such or cassava leaves, banana leaves, etc. were commonly fed. There was little difference between goat and sheep diets and native grasses were the main forage (Lubis 1989). Depending upon the area, in Madura, many agricultural wastes such as corn stover and rice straw were used for feeding, while in Bali many leaf materials such or *Hisbiscus* sp, leucaena, banana leaves or jack fruit leaves were used a feed (Table 2).

Table 2. Forages fed to cattle in Madura and Bali coastal area.

Common name		Madura* % Diet	Bali** % Diet	***		
				Tannin	NDF	Phenolic
Corn stover	*Zea mays*	48.3		—	50	—
Rice straw	*Oryzae sativa*	17.0				
Soybean straw	*Glycine max*	1.2				
Native grass		9.8		—	65–69	0.3–0.6
Elephant grass	*Penisetum purpureum*	1.2		0.2	69	0.6
Mango leaves	*Mangifera indira*	2.6		6.0	41	7.2
Bambo leaves	*Bambusa vulgaris*	3.8				
Jackfruit leaves	*Artocarpus heterophyllus*	3.3	9.8			
Banana leaves	*Musa sapientum*	2.4	8.2	0	52	2.4
Jambu leaves	*Eugena densiflora*	1.1				
Acacia leaves	*Acacia auriculiformis*	1.7		8.3	49	8.3
Turi	*Sesbania grandiflora*	0.3	6.0	0	25	1.6
Leucaena	*Leucaena leucocephala*	0.3	26.2	1.2	34	7.8
Waru	*Hibiscus tiliaceous*		24.2	—	47	–
Gamal	*Gliricidia sepium*		2.9	0	21	0.1
Dadap	*Erythrina subumbrans*		3.0	0	45	2.2
Kaliandra	*Calliandra calothyrsus*			9	34	9
Cassava leaves	*Manihot esculenta*			2.3	19	6.0
Setaria	*Setaria spacelata*			0.1	74	1.2
Kayu santen			8.0			

* Hermanto et al. 1993; ** Nitis et al. (1982); *** Lowry et al. (1992).

The highest percentage of legume leaves given to animal was in Kaligesing, Central Java, where a well-known breed of goat is raised. The diet consisted of 50% native grasses, 34% legume leaves comprising calliandra, gliricidia, leucaena and erythrina and 15% of cassava and artocarpus leaves. It is a typical high altitude area and many legume trees have been planted (Table 3). In the areas where improved varieties of grasses has been introduced, elephant grasses or Setaria spacelata became major grasses. However, in many areas, farmers rely on the native grasses which consist of Axnonopus, Paspalum, Cynodon, Themeda, etc. These grasses grow alongside roads or in any suitable area and hardly any need to be planted.

Table 3. Forages fed to Etawah goat in Kaligesing.

Forages	Per cent of diet	
Native grass	50.6	
Calliandra calothyrsus	11.7	
Gliricidia sepium	7.9	34.2
Leucaena leucocephala	5.0	
Erythrina subumbrans	8.8	
Cassava leaves	3.5	15.2
Artocarpus leaves	11.7	

Martawidjaja et al. (1994).

Tannin and phenolic content and protein

Data collected by the Research Institute for Animal Production (Lowry et al. 1992) indicated a wide range of tannins and phenolic content of the forage species fed to ruminants in Indonesia (Table 2). The high tannin contents were found in calliandra, *Acacia auriculiformis* and mango leaves (4–10%) while cassava leaves and leucaena contained a medium level (1–4%) of condensed tannin, and other plant species contained low tannin (0–<1%). Total phenolics measured by Folin reagent also varied among the species. There seems to be a relation between high content of tannin with high content of phenolics. However, few plant species such or leucaena and cassava contained high levels of phenolic (>6%) but contained medium levels of tannin.

Among the grasses fed to ruminants, the majority contained low levels of tannin (<1%) except for *Imperata cylindrica* which contained 2.5% tannin (Table 4). Total phenolic content of grasses was also low or medium. Most grasses contained low levels of crude protein (7–14%) but contained high levels of neutral detergent fibre (>60%), as expected.

Table 4. Crude protein (CP), neutral detergent fibre (NDF), total phenolics (TP) and tannin content of grasses fed to ruminants in Indonesia.

	CP	NDF	Phenolics	Tannin
Axonopus compressus	11	69	0.5	0
Chrysopogon accularis	13	68	1.1	0.2
Cynodon dactilon	14	65	—	—
Eleusine indica	7	75	0.8	0
Imperata cylindrica	9	76	2	2.5
Paspalum conjugatum	8	65	2.3	0
Paspalum notatum	10	68	—	—
Panicum maximum	7	69	0.6	—
Panicum repens	12	74	1.0	0
Themeda arguens	9	64	—	—

Lowry et al. 1992.

Legume leaves contained high level of protein (>20%) and low level of NDF (<40%). Considering the amount of forages fed to animals and the level of tannins in the plant, it seems that high levels of tannin intake was found in Kaligesing goat but the total tannin content in the diet was only 1.2%. When animals are fed with a majority of grasses and agricultural waste such as corn stover, tannin intake will be very small. There is no report of tannin toxicity to animals in Indonesia. On the contrary, small amounts of tannin may be benificial to animals. The Kaligesing goat has been well known as a productive goat. However, it is not known whether the productivity is related to high consumption of legumes or medium levels ot tannin intake or specific bacteria in the rumen that tolerate tannin.

Calliandra feeding experiment

Comparison between feeding fresh calliandra and leucaena or gliricidia as a supplement to sheep indicated no difference in growth rate or feed intake, although the digestibility of fresh calliandra, both for dry matter and protein, was less than that of gliricidia and leucaena (Table 5).

Table 5. Comparison of fresh calliandra, gliricidia and leucaena as supplements for sheep.

Parameter	Calliandra	Gliricidia	Leucaena
Growth rate (g/day)	54.4	56.0	47.7
Feed intake (g/day)	609.5	650.5	627.9
Feed/gain	11.2	11.6	13.2
Digestibility (%):			
Dry matter	43.3a	49.8a	59.6b
Protein	56.3p	67.3q	77.1c

Wina (1992).

Other feeding trials indicated that dried calliandra produced less growth in sheep than fresh calliandra. When dried legumes leaves were incorporated in a pelleted diet, cattle fed calliandra leaves resulted in a smaller growth rate than leucaena and gliricidia, which probably related to lower digestible dry matter and protein (Table 6).

Table 6. Comparison of calliandra, gliricidia and leucaena in a pelleted diet for growing cattle.

	Calliandra	Gliricidia	Leucaena
Growth rate (g/day)	136	505	394
Feed intake (kg/day)	3.47	3.35	3.12
Digestibility (%):			
Dry matter	47.3	62.7	59
Protein	40.7	61.9	57.4

Diet composed 48–56% legume, 12–25% rice straw and cassava 24–33%.

Manurung (1996).

A long-term feeding trial with fresh calliandra (more than 6 months) proved benificial to pregnant ewes. Table 7 shows that the performance of ewes (liveweight) and lambs is remarkably better when fresh calliandra is used as a supplement.

Table 7. Performance of ewes fed calliandra.

	Grass	Grass + calliandra (7 + 3)
Live weight ewes (kg):		
At start	22.6	22.3
At lambing	27.3	29.1
At weaning	24.5	27.1
Feed intake (gram/day):		
During pregnancy	974.9	1015.3
During lactation	1016.6	1287.4
Lambs:		
Birth weight (kg)	1.8	1.8
Growth rate (g/day)	53.7a	89.2b
Weaning weight (kg)	6.7p	9.1q
Mortality (%)	24.7	19.9

Sutama et al. (1994).

Conclusions

Ruminants in Indonesia are fed a mixture of a wide variety of forages in a labour-intensive feeding system. Depending on the season, altitude and area, grasses still account for the majority of forages, plus other leaf materials derived from legumes and trees.

Tannin content of forages varied widely from 0–9%. A few plants, such as calliandra, acacia and *Mangifera* sp leaves, contained high (6–9%) levels of condensed tannin and high amounts of phenolic compounds.

Considering the forage composition in the diet, the total tannin content in the diet was relatively low with the highest in Kaligesing goats (1.2%). There was no toxicity due to tannins, and experimental feeding showed that the fresh calliandra gave equal performance compared to other legumes but not in the dried form.

References

Hermanto, Mashudi, Hendrawan, S., Soebarnoto, Soeharjono, Hanief, E.S and Herni, S. 1993. Observation on botanical composition and nutritional value of forages for Madura cattle at village breeding centre, Pamekasan district. Proc. Pertemuan Ilmiah Hasil Penelitian Peternakan and Pengembangan Sapi Madura. Sub Balai Penelitian Ternak Grati, 132–140.

Knipscheer, H. and Levine, J. 1984. The status of ruminants in Indonesia, with special reference to Research needs. Small Ruminant Cooperative Research Program. Balai Penelitian Ternak – USAID, Bogor, Indonesia.

Lowry, J.B., Petheram, R.J. and Tangendjaja, B. 1992. Plants fed to village ruminants in Indonesia. Technical Reports No. 22. ACIAR. Canberra.

Lubis, D. 1989. Nutritional factors restricting production systems of sheep and goats in Jrantunseluna watershed. Risalah Diskusi Ilmiah Hasil Penelitian Litbang, 166–171.

Manurung, 1996. Utilisation of tree legume forages as protein sources of beef cattle ration. J. Ilmu Ternak and Veteriner, 1(3): 143–148.

Martawidjaja, M., Setiadi, B. and Triwulaningsih, E. 1994. Feed and feeding pattern of Etawah crossing goat in Kaligesing, Purworejo district, Central Java. Proc. Pertemuan Ilmiah Hasil Penelitian Peternakan Lahan Kering. Sub Balai Penelitian Ternak Grati, 373–378.

Nitis, I.M., Lana., K., Sudana, I.B and Suji, N. 1982. Area classification for botanical composition of forages fed to goats in Bali during the dry season. Proc. Seminar Penelitian Peternakan. Puslitbangnak Bogor, 125–134.

Robinson, D.W. 1977. Livestock in Indonesia. Centre for Animal Research and Development. Centre Report No. 1 Ciawi, Indonesia.

Sutama, K., Muhamad, A. and Wina., E. 1994. The effect of supplementation of calliandra leaves on reproductive performance of Javanese fat-tailed sheep. Ilmu dan Peternakan, 7(2): 13–16.

Wina, E. 1992. Nutritive value of calliandra, gliricidia and leucaena as supplement for sheep fed elephant grass Proc. Pengolahan and Komunikasi Hasil Penelitian Teknologi and Tanaman Pakan Balitnak: 13–19.

New Perspectives on the Degradation of Plant Biomass in the Rumen in the Absence and Presence of Condensed Tannins

M.K. Theodorou[1], R. Barahona[1,3], A. Kingston-Smith[1], S. Sanchez[1], C. Lascano[2], E. Owen[3] and P. Morris[1]

Abstract

This paper considers some positive and negative aspects of tannins on the degradation characteristics and nutritive quality of tanniniferous forages intended for use as feeds for ruminants. The paper presents some new ideas and evidence for the role of plant proteases in the degradation of plant proteins in grazing ruminants. Consideration is also given to the possible interaction between proteases, proteins and condensed tannins in plants during their ingestion and digestion in the rumen.

THE ABILITY of condensed tannins to form complexes with protein, carbohydrate and other molecules has traditionally been regarded as the means by which tannins inhibit digestion and reduce plant preference (Rhoades and Cates 1976; Swain 1979). Condensed tannins can complex and render inactive mammalian digestive enzymes (Swain 1979) and precipitate dietary protein (Feeney 1969). Moreover, the formation of complexes between dietary constituents and condensed tannins might result in nutrients becoming unavailable to rumen micro-organisms and/or to their digestive enzymes. This could be mediated by the masking of potential binding sites of the enzymes (Martin and Martin 1983). Although many reports in the literature suggest that dietary condensed tannins are detrimental to livestock production, there are other reports suggesting that at the right concentration, some condensed tannins are beneficial, permitting protection and sparing of plant proteins in the rumen and their subsequent beneficial utilisation in the abomasum.

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

Great diversity has been reported on the molecular structure of condensed tannins. Such variation includes factors such as molecular weight, stereochemistry (*cis-trans* ratio) and monomeric composition (Foo et al. 1982; Williams et al. 1983; Eberhardt and Young 1994). As with the case of condensed tannin molecular weight, there is evidence that both stereochemistry and monomeric

[1] Institute of Grassland and Environmental Research, Plas Gogerddan, Aberystwyth, SY23 3EB, UK
[2] Centro Internacional de Agricultura Tropical (CIAT), Cali, Colombia
[3] Department of Agriculture, University of Reading, Early Gate, PO Box 236, Reading RG6 6AT UK
Corresponding author: Prof. M.K. Theodorou, Institute of Grassland and Environmental Research, Plas Gogerddan, Aberystwyth, SY23 3EB, UK, E-mail: mike.theodorou @bbsrc.ac.uk

KEYWORDS: Condensed tannin, Tannin, Rumen, Protease, Proteolysis, Plant, Micro-organism

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

Substantial effort has been directed to identifying high-quality tropical forage legumes adapted to acid soils. During that process, the need to fully comprehend how the presence of condensed tannins affects the overall quality of tropical forage legumes has become evident. This task is difficult to accomplish as condensed tannins are not a uniform chemical entity. While we accept that continued consideration should be given to the chemistry of condensed tannins, particularly in relation to their interaction with other plant cell constituents, it is considered, as is indicated below, that the mechanisms associated with the biology of digestion of tanniniferous forages may be fundamentally flawed and in need of substantial re-evaluation.

Questioning Old Dogma and Introducing New Hypotheses: How Are Proteins Degraded in Grazing Ruminants?

Grazing cattle can ingest 100 kg of fresh forage as several meals over the course of a day. The forage is excised from pasture, cut into relatively long lengths depending on sward height, rolled into a ball by the actions of tongue and teeth which is then swallowed (Orr et al. 1997). Thus, in pasture-fed ruminants, the majority of the plant cells entering the rumen are intact as evident by the presence of a raft and stratified rumen. Under these conditions, the plant cells entering the rumen are able to respond as biological entities to the imposition of stress in their changed environment.

The majority of the soluble plant protein is in the form of ribulose-1,5-bisphosphate carboxylase/ oxygenase (Rubisco) but this is located within the chloroplast. Therefore, for successful microbial access to plant proteins, the cell wall, plasma membrane and the chloroplastic double envelope must be breached. This would require attachment by micro-organisms to be rapid and cellulase activity to be extremely high to account for the observed rates of degradation of protein in the rumen of animals fed fresh forage. However, although micro-organisms are reported to be responsible for the degradation of plant proteins in the rumen, the contribution made by the ingested plant biomass to its own digestion has been largely ignored.

According to current dogma, protein breakdown in the rumen in the presence or absence of condensed tannins is generally regarded as a two-stage process whereby extra-cellular proteases produced by the microbial population convert plant proteins into lower molecular weight peptides which are then deaminated to ammonia by microbial deaminases (Figure 1a). However, the proteolytic activity of rumen micro-organisms is only moderate when compared with that of other proteolytic micro-organisms and the animal's own gut secretions (Asoa et al. 1993). Moreover, the major cellulolytic species in the rumen (i.e. those attached to plant biomass fragments) are generally not proteolytic (Theodorou and France 1993). Having questioned the assertion that proteolysis in grazing ruminants is a process mediated by microbial enzymes (Theodorou et al. 1996; Kingston-Smith and Theodorou 1999), the authors assert that proteases of plant origin are responsible for the breakdown of plant proteins; the microbial population contributing downstream at the deamination stage of the process (Figure 1b). If it is correct, this plant protease hypothesis will have a profound impact on our view of protein transactions in the rumen. Moreover, because proteases and condensed tannins are located in plant cell vacuoles, understanding the influence of condensed tannins on protease activity may also alter our view of the mode of action of condensed tannins in freshly ingested tanniniferous forages.

Proteins in living cells are in a continual process of turnover, with the balance between synthesis and degradation resulting in a net protein content. In plants, proteolysis is fundamental to the onset and progression of seed germination and senescence. The latter is widely believed to be a form of controlled cell death involving activation of existing proteases and *de novo* synthesis of new proteases (Callis 1995). This facilitates the catabolism of complex proteins to amines and amino acids in order to translocate organic nitrogen to the actively growing parts of the plant (Huffacker 1990; Thomas and Feller 1993; Callis 1995; Morris et al. 1996). Although the majority of the protease activity is located within the vacuole, proteases also exist to remove damaged proteins or to process those proteins synthesised in the nucleus but imported into organelles (Corpas et al. 1993; Adam 1996; Distefano et al. 1997). Thus for the plant to survive, plant proteolysis must be highly regulated, either biochemically or by compartmentation, to allow proteases, proteins and condensed tannins, where applicable, to co-exist within the same cell.

Senescence-related processes are often studied in excised, darkened, leaves (Thomas and Stoddart 1980; Morris et al. 1996). This is a situation similar

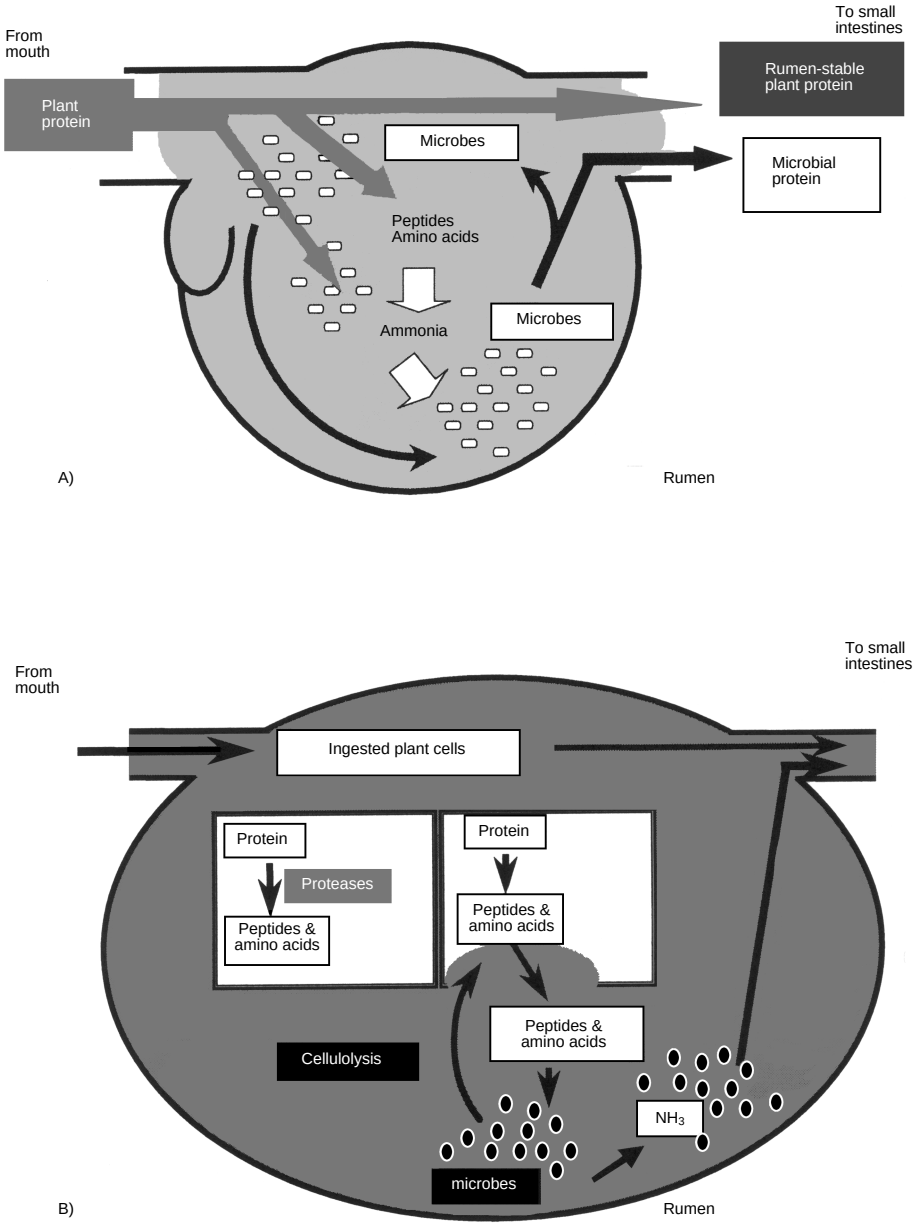


Figure 1. Diagrammatic representation of the traditional (A) and more novel (B) view of protein degradation in the rumen. In the traditional scheme, the microbial population is purported to be responsible for the entire degradation process of plant proteins in the rumen. In the novel scheme, plant proteases are responsible for the initial degradation of plant proteins (modified from Kingston-Smith and Theodorou, submitted).

to that arising after grazing or cutting of herbage for ensiling. In the plant cell death processes occurring during production of grass silage, plant proteases are known to have a role in converting plant proteins to lower molecular weight peptides and amino acids. These products of proteolysis are subsequently degraded by the developing silage microflora. In the production of grass silage, the pH of the herbage quickly falls to 4.0 or less, below the operational range of many plant enzymes, but consistent with the pH optima of many of the acid proteases. These enzymes have broad-spectrum pH optima and would retain some activity in the rumen where the pH is closer to neutral. Moreover, the rate of reaction of these enzymes is also likely to be significantly enhanced at 39 ºC, the temperature within the rumen.

Although protein availability in the rumen is generally not a problem in developed, temperate agriculture, the rapid breakdown of herbage proteins and their subsequent inefficiency of use by the rumen microbial population represents one of the largest causes of nitrogen loss and pollution in pasture-based production systems. In tropical agriculture, where protein is a somewhat scarcer commodity, tanniniferous plants are used to prevent protein degradation and smuggle intact proteins to the abomasum and lower tract where they are used more efficiently for incorporation into meat and milk. Hence, a detailed knowledge of the mechanism of protein degradation in both temperate and tropical systems is likely to confer future advantages through the development of plant-oriented, pasture-based approaches to modifying the rate of protein degradation, and hence nitrogen supply in grazing ruminants.

Can Condensed Tannins Reduce Plant and Microbial Enzyme Activities in the Rumen?

Plants are sessile organisms and therefore have evolved a multitude of responses to adverse conditions in order to survive. Environmental conditions often encountered are extremes of temperature, water status or nutrient supply. Plant material is often used as a feed for insects and larger herbivores. Much research effort in the past has concentrated on resource allocation in the plant parts remaining after herbivory and so we are well aware of localised response involving the toxic effects of cell decompartmentation and systemic damage responses involving chemical signalling to induce new DNA and protein synthesis. To our knowledge, little if anything is known about the enzyme activity of excised plant parts at elevated (39 ºC) temperature prior to and in the early stages of digestion. Thus, we are forced to extrapolate from observed responses of the effects of condensed tannins on microbial enzymes to those involving plant enzymes. From what follows, we do not wish to imply that condensed tannins only affect plant enzymes. We do, however, wish to raise the possibility that condensed tannins may influence the activity of enzymes of both plant and microbial origin and that their association with plant proteases, for example, may be of consequence in considering the role of tannins in the rumen.

The initial response of living plant cells entering the rumen is to try to adapt to the prevailing hostile conditions of constant darkness, a temperature of 39 °C and anaerobiosis; these stresses have all been shown to affect protein turnover in intact plants. In studies involving excised plant leaves held at 39 °C under anaerobic conditions, we have shown that plant proteins are rapidly and extensively degraded to amino acids in the absence of a rumen microbial population, (Beha et al. 1997; Theodorou et al. 1996; Zhu et al. 1999). Thus in tanniniferous forages, loss of structural and functional integrity of plant cells will result in the ‘mixing’ of proteins, proteases and condensed tannins. Although this provides plant proteases with an opportunity to encounter their substrates, it could also facilitate an interaction between proteases and condensed tannins, thereby reducing protease activity and preserving proteins in the plant in an otherwise hostile environment.

The overall impact of decompartmentation of cells in tanniniferous forages would depend on several factors. For instance, the newly liberated condensed tannins could specifically bind to plant proteases, cytoplasmic proteins or even non-protein, cell wall constituents. Given that tannin-protein binding can be quite specific for both the protein and the tannin (Butler 1989), it is difficult to predict which of these interactions would take precedence. In the event that condensed tannin bind specifically to plant protease, it is feasible to assume that enzyme activity will be reduced to some extent. Although it is not possible to speculate on the magnitude of this effect, the extent of inhibition is likely to be related to the structure of both the condensed tannin and the protease. This was demonstrated by the results of an analogous study where the activity of several microbial enzymes in the presence of different concentrations of purified condensed tannins from several tropical legumes was estimated (Figure 2a, b).

In this Figure, the impact of tannin concentration on enzyme activity is clearly evident. The experiment measured CMCase and xylanase activities in culture filtrates of the anaerobic fungi, *Neocallimastix hurleyensis*, on the absence and presence of purified condensed tannins from a range of tropical forage legumes. In each case, condensed tannins from the different legumes differed in their ability to

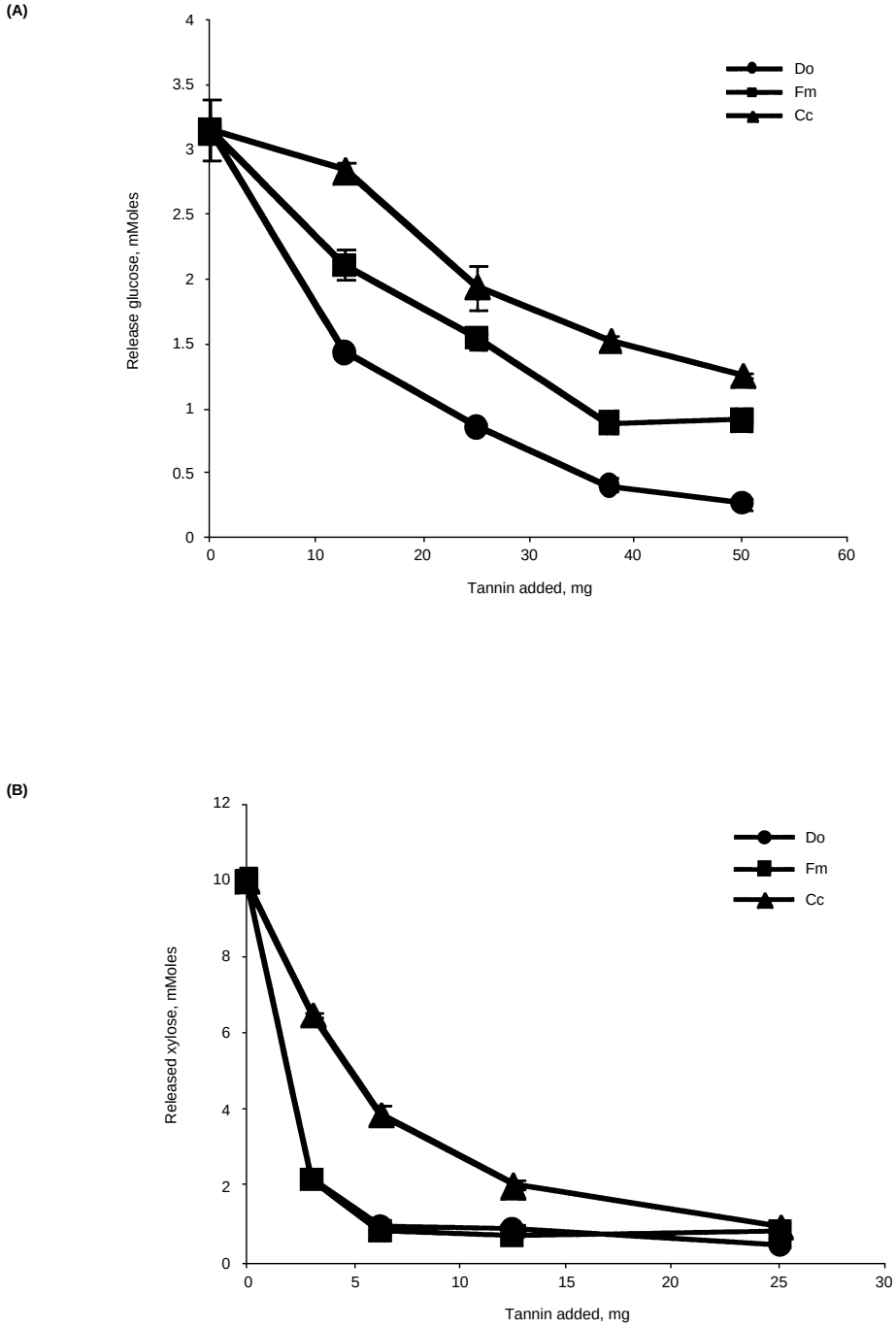


Figure 2. *Activity of Neocallimastix hurleyensis* CMCase (A) and xylanase (B) in the presence of different concentrations of condensed tannin extracted from mature leaves of three tropical legumes. Do = *Desmodium ovalifolium*, ●; Fm = *Flemingia macrophylla*, ■; Cc = *Calliandra calothyrsus*, ▲. Portrayed curves are the average of two independent assays. Error bars represent SEM in each figure where n = 4.

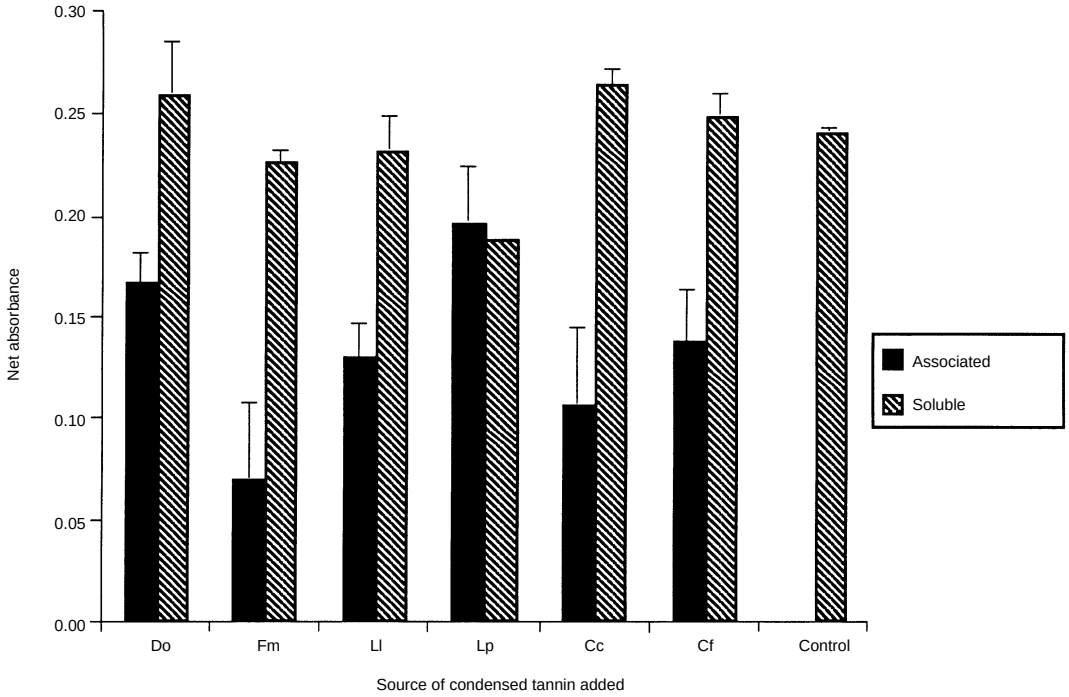


Figure 3. Net release of reducing sugars from the primary plant cell walls of *Festuca arundinacea* by the action of fibrolytic enzymes from *Neocallimastix hurleyensis* in the presence of free-soluble and substrate-associated condensed tannins (30 g/kg of cell walls). Do = *Desmodium ovalifolium*, Fm = *Flemingia macrophylla*, Ll = *Leucaena leucocephala*, Lp = *Leucaena pallida*, Cc = *Calliandra calothyrsus* and Cf = *Clitoria fairchildiana*. Error bars represent SEM in each figure where n = 4.

reduce the activity of the enzymes studied. Subsequently, we found that a significant proportion of the variability was due to structural difference among the various tannins. For example, tannin molecular weights ranged from 2300 to 4900 (estimated by gel permeation chromatography) and an increase in molecular weight was correlated with a negative impact on enzyme activity. Comparisons also indicate that the enzymes themselves influenced their susceptibility to tannin inhibition, the susceptibility of xylanase to inhibition by condensed tannin being significantly greater than that observed for CMCase. This was in agreement with observations of Salawu et al. (1998) that the xylanase activity from a cell-free preparation of the rumen fungus *N. frontalis* was more affected by a *Calliandra calothyrsus* leaf extract than the corresponding CMCase activity. This may partly explain why, among the different non-starch polysaccharide constituents in tannin-containing tropical legumes, xylose was found to be the least digestible fraction (Longland et al. 1995).

Can Condensed Tannins Protect Plant Biomass Constituents by Binding to Substrates Other Than Proteins?

If, instead of binding to proteases, the newly-released condensed tannins were to bind to their protein substrates, this to would result in reduced proteolytic activity. This possibility was illustrated for an analogous situation (Figure 3) which reports the results from an experiment where the activity of fibrolytic microbial enzymes was determined in the presence of condensed tannins, either soluble in the liquid phase, or bound to the cell-wall substrate. The results show the substrate-associated tannins to be more effective at inhibiting the degradation of primary plant cell walls of *Festuca arundinacea* than tannins in the freely soluble form. This is probably due to a dilution effect, condensed tannins in the liquid phase being too diffuse to influence the degradation of a dietary entity. On the other hand, by associating the condensed tannin with the enzyme substrate, enzyme activity was effectively inhibited.

This scenario is in agreement with the earlier suggestions that condensed tannins in the liquid phase would act mainly by interacting directly with the rumen microbes 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 (Martin and Martin 1983) or the enzymes of plant and microbial origin.

Conclusion

Despite the fact that ruminants often graze fresh tanniniferous forages, many laboratory studies designed to consider the nutritional implications of tannin-protein interactions use forages which have been oven-dried and ground through a mill. Similarly, in investigations concerned with proteolysis and protein degradation in the rumen, studies often use conserved (silage) and oven-dried feeds.

Thus, in these systems, as enzyme activities are destroyed by the processing of the substrate, it is not surprising that the importance of plant-enzyme mediated proteolysis and the role of plant cell death in rumen function has been overlooked until comparatively recently.

We now have sufficient evidence to show that plant proteases are involved in the breakdown of plant proteins in the rumen. Moreover, the rate at which plant cells die in the rumen may have a profound impact on rumen function, affecting the efficiency of nitrogen incorporation by the microbial population in the rumen.

Nevertheless, the effect of condensed tannins on plant protease activity in grazing ruminants and the nature of their protective mechanism during decompartmentation of plant cells remains completely unknown. What is certain, however, is that we need a detailed understanding of the mechanism(s) associated with protein breakdown in the presence and absence of condensed tannins if we are to derive maximum benefit from the manipulation of protein status of the ruminant diet using tanniniferous forages.

References

Adam, Z. 1996. Protein stability and degradation in chloroplasts. Plant and Molecular Biology, 32: 773–783.

Asoa, N., Ushida, K. and Kojima, Y. 1993. Proteolytic activity of rumen fungi belonging to the genera *Neocallimastix* and *Piromyces*. Letters in Applied Microbiology, 16: 247–250.

Ayres, M.P., Clausen, M.P., McLean, Jr., E.F., Redman, A.M. and Reichardt, P.B. 1997. Diversity of structure and antihervibore activity in condensed tannins. Ecology, 78: 1696–1712.

Beha, E.M., Kingston-Smith, A.H. and Theodorou, M.K. 1997. Death of plant cells in the rumen, a hostile, anaerobic, hot (39 °C) and dark environment. Journal of Experimental Botany, 49: 68.

Butler, L.G. 1989. New perspectives on the antinutritional effects of tannins. In: Kinsella, J.E. and Soucie, W.B. ed. Food Products. American Oil Chemist Society. Champaign, Illinois, USA, 402–409.

Callis, J. 1995. Regulation of protein degradation. Plant Cell. 7: 845–857.

Clausen, T.P., Provenza, F.D., Burritt, E.A., Reichardt, P.B. and Bryant, J.P. 1990. Ecological implications of condensed tannin structure: a case study. Journal of Chemical Ecology, 16: 2381–2392.

Corpas, F.J., Palma, J.M. and del Rio, L.A. 1993. Evidence for the presence of proteolytic activity in peroxisomes. Euopean Journal of Cell Biology, 61: 81–85.

Distefano, S., Palma, J.M., Gomez, M. and del Rio, L.A. 1997. Characterisation of endoproteases from plant peroxisomes. Biochemical Journal, 327: 399–405.

Eberhardt, T.L. and Young, R.A. 1994. Conifer seed cone proanthocyanidin polymers: characterisation by 13C NMR spectroscopy and determination of antifungal activities. Journal of Agriculture and Food Chemistry, 42: 1704–1708.

Feeney, P.P. 1969. Inhibitory effect of oak leaf tannin on the hydrolysis of protein by trypsin. Phytochemistry, 8: 2119–2126.

Foo, L.Y., Jones, W.T., Porter, L.J. and Williams, V.M. 1982. Proanthocyanidin polymers of fodder legumes. Phytochemistry, 21: 933–935.

Jones, W.T., Broadhurst, R.B. and Lyttleton, J.W. 1976. The condensed tannins of pasture legume species. Phytochemistry, 15: 1407–1409.

Kingston-Smith, A.H. and Theodorou, M.K. 1999. Plant cell death and ruminant nutrition. Plant Physiology (in press).

Longland, A.C., Theodorou, M.K., Sanderson, R., Lister, S.J., Powell, C.J. and Morris, P. 1995. Non-starch polysaccharide composition and in vitro fermentability of tropical forage legumes varying in phenolic content. Animal Feed Science and Technology, 55: 161–177.

Martin, J.S. and Martin, M.M. 1983. Tannin assays in ecological studies: Precipitation of ribulose-1,5-bisphosphate carboxylase/oxygenase by tannic acid, quebracho, and oak foliage extracts. Journal of Chemical Ecology, 9: 285–294.

McLeod, M.N. 1974. Plant tannins — their role in forage quality. Nutrition Abstracts Reviews, 44: 803–815.

Mehansho, H., Hagerman, A., Clements, S., Butler, L., Rogler, J. and Carlson, D.M. 1983. Modulation of proline-rich protein biosynthesis in rat parotid glands by sorghums with high tannin levels. Proceedings of the National Academy of Sciences, USA, 80: 3948–3952.

Morris, K., Thomas, H. and Rodgers, L. 1996. Endopeptidases during the development and senescence of *Lolium temulentum* leaves. Phytochemistry, 41: 377–384.

Orr, R.J., Penning, P.D., Harvey, A. and Champion, R.A. 1997. Diurnal patterns of intake rate by sheep grazing monocultures of ryegrass or white clover. Applied Animal Behaviour Science, 52: 65–77.

Rhoades, D.F. and Cates, R.G. 1976. Towards a general theory of plant antiherbivore chemistry. In: Wallace, J.W. and Mansell, R.L. ed. Recent Advances in Phytochemistry, Vol. 10. Biochemical Interaction between Plants and Insects. Plenum Press, New York, USA, 168–213.

Salawu, M.B., Acamovic, T. and Stewart, C.S. 1998. *Calliandra calothyrsus* leaf extracts, effects on microbial growth and enzyme activities. In: Garland T. and Barr, C. ed. Toxic Plants and Other Natural Toxicants. CAB International, Wallingford, UK.

Spencer, C.M., Cai, Y., Martin, R., Gaffney, S.H., Goulding, P.N., Magnolato, D., Lilley, T.H. and Haslam, E. 1988. Polyphenol complexation — some thoughts and observations. Phytochemistry, 27: 2397–2409.

Swain, T. 1979. Tannins and lignins. In: Rosenthal, G.A. and Janzen, D.H. ed. Herbivores: Their Interaction with Plant Metabolites. Academic Press, New York, USA, 657–682.

Theodorou, M.K. and France, J. 1993. Rumen microorganisms and their interactions. In: Forbes, J.M. and France, J. ed. Quantitative aspects of ruminant digestion and metabolism. CAB International, 145–162.

Theodorou, M.K., Merry, R.J. and Thomas, H. 1996. Is proteolysis in the rumen of grazing animals mediated by plant enzymes? British Journal of Nutrition, 75: 507–510.

Thomas, H. and Feller, U. 1993. Leaf development in *Lolium temulentum* — Differential susceptibility of transaminase isoenzymes to proteolysis. Journal of Plant Physiology, 142: 37–42.

Thomas, H. and Stoddart, J.L. 1980. Separation of chlorophyll degradation from other senescence processes in leaves of a mutant genotype of medow fescue (*Festuca pratensis*). Plant Physiology, 56: 438–441.

Williams, V.M., Porter, L.J. and Hemingway, R.W. 1983. Molecular weight profiles of proanthocyanidins polymers. Phytochemistry, 22: 569–572.

Zhu, W-Y., Kingston-Smith, A.H., Troncoso, D., Merry, R.J., Davies, D.R., Pichard, G., Thomas, H. and Theodorou, M.K. 1999. Evidence of a role for plant proteases in the degradation of herbage proteins in the rumen of grazing cattle. Journal of Dairy Science, (in press).

Biosynthesis of Proanthocyanidins (Condensed Tannins)

G.J. Tanner[1], S. Abrahams[1] and P.J. Larkin[1]

Abstract

A capacity to manipulate tannin levels in forage legumes for grazing animals has prompted research efforts on proanthocyanidin biosynthesis. There are two biosynthetically distinct classes of tannins, the hydrolysable tannins, which are esters of gallic or ellagic acid and glucose, and the condensed tannins (proanthocyanidins). Hydrolysable tannins are generally anti-nutritional, although condensed tannins at moderate concentrations can be beneficial to grazing ruminants. But elucidation of proanthocyanidin enzymology lags behind the structural determination of the various proanthocyanidins. An understanding of the properties of purified biosynthetic activities is expected to lead to a fuller understanding of the complexity of proanthocyanidin biosynthesis, and eventually allow manipulation of the functional properties of the proanthocyanidin polymer. This review will concentrate on proanthocyanidin biosynthesis beyond the level of 2,3-*cis*-leucocyanidin in barley and the legumes *L. corniculatus* and sainfoin. Enzyme data will be reviewed to draw general conclusions which illustrate the similarity between anthocyanin and proanthocyanidin biosynthesis. Finally, several speculative schemes for the, as yet, unknown mechanism of intervacuolar transport and polymerisation of the monomers of proanthocyanidins will be reviewed.

TANNINS derive their main biochemical properties from an ability to precipitate protein at neutral pH. There are two biosynthetically distinct classes of tannins: the hydrolysable tannins which are esters of gallic or ellagic acid and glucose; and the condensed tannins (proanthocyanidins), the subject of this review. Hydrolysable tannins are generally anti-nutritional. However, condensed tannins at moderate concentrations can be beneficial to grazing ruminants. It is from a desire to manipulate tannin levels in forage legumes that we began our work on proanthocyanidin biosynthesis.

The term proanthocyanidin is a misnomer, originally arising from the observation that when heated in mineral acid, condensed tannins give rise to anthocyanin pigments. It was wrongly deduced that proanthocyanidins were the biosynthetic precursors to anthocyanidins.

Phlobaphenes are similar to proanthocyanidins, and the term is confusingly used in the literature to refer to either a heterogeneous polymer of predominantly 3-deoxy flavan-4-ols (Grotewold et al. 1994) extracted from plants and soluble in alcohol but water insoluble, or a reddish water insoluble pigment (tannin reds) formed after acid treatment of proanthocyanidins in the tanning industry (Foo and Karchesy 1989). It is likely that tannin reds are oxidation products formed during hydrolysis of proanthocyanidins.

The sequence of enzymatic reactions leading to the biosynthesis of proanthocyanidins is largely common with the pathway which leads to the biosynthesis of anthocyanins (Figure 1). Anthocyanin biosynthesis has been reviewed by Forkman 1993; Heller and Forkman 1993; Martin and Gerats 1993; Holten and Cornish 1995; and Mol et al. 1998; proanthocyanidin biosynthesis has been reviewed by Porter 1993; Hergert 1989; and Stafford 1990.

The enzymology of the anthocyanin pathway has been demonstrated to anthocyanin (Figure 1; Marrs et al. 1995; Saito et al. 1999). Proanthocyanidin enzymology has been established to the level of catechin for *Hordeum vulgare* (barley) testa (Kristiansen 1984, 1986) and *Onobrychis viciifolia* (sainfoin) leaves (Tanner and Kristiansen 1993).

Most of the genes which code for the catalytic enzymes and regulatory proteins of the anthocyanin pathway have been cloned, originally from maize

[1]CSIRO Division of Plant Industry, GPO Box 1600, Canberra, ACT, 2601

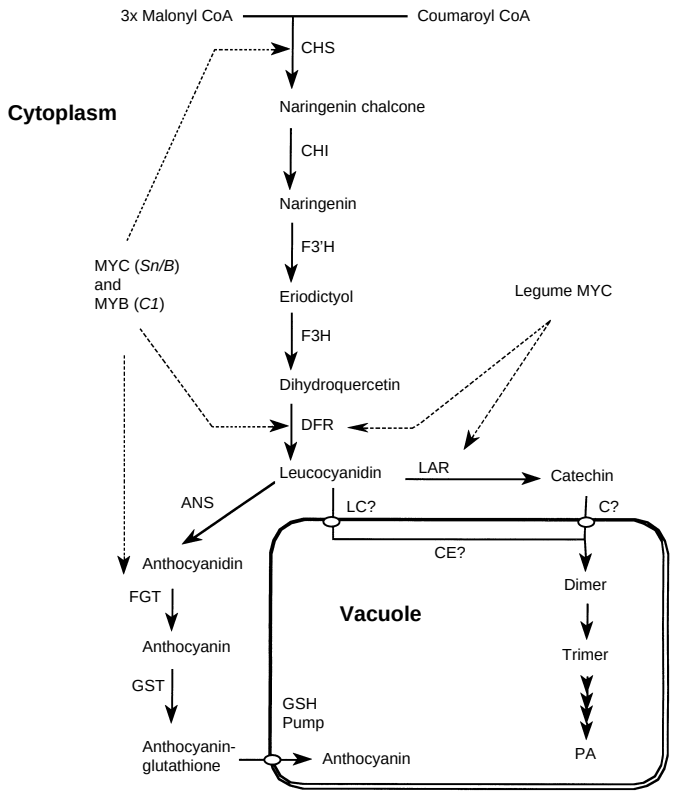


Figure 1. Subcellular compartmentalisation and control of (pro)anthocyanidin biosynthesis. The intracellular localisation of the intermediates of anthocyanidin and proanthocyanidin biosynthesis are shown with the known enzymes: chalcone synthase (CHS), chalcone isomerase (CHI), flavanoid-3'-hydroxylase (F3'H), flavanone-3-hydroxylase (F3H), dihydroflavanol reductase (DFR), leucoanthocyanidin reductase (LAR), anthocyanin synthase (ANS, also known as LDOX), flavanol-UDP-glucosyl transferase (FGT), glutathione-S-transferase (GST), glutathione transmembrane pump (GSH pump) and the putative condensing enzyme (CE?), and intervacuolar flavan-3,4-diol (LC?) and flavan-3-ol (C?) transporters. The interaction between the regulatory proteins and genes (*italics*) and structural genes of maize anthocyanin synthesis is shown by dotted lines on the left of the diagram; the proposed interaction between a legume MYC protein and the *DFR* and *LAR* genes is similarly shown on the right of the diagram.

kernel and dicot petal tissues by transposon mutagenesis and later from other tissues by homology with the known sequences (Holten and Cornish 1995).

Several flavonoid biosynthetic genes have been cloned from tissues that accumulate proanthocyanidins. cDNA's coding for CHS, F3H and DFR were cloned from barley by Rohde et al. 1987; Meldgard 1992; and Kristiansen and Rohde 1991; respectively. cDNA's coding for most of the anthocyanidin biosynthetic enzymes were cloned from *Vitis vinifera* (grape) by Sparvoli et al. 1994; cDNA's coding for CHS and DFR were isolated from sainfoin by Joseph et al. 1998. Bavage and Robbins (1994) cloned a gene fragment coding for DFR from *Lotus corniculatus*.

This review will concentrate on proanthocyanidin biosynthesis beyond the level of 2,3-*cis*-leucocyanidin (Figure 3) in barley and the legumes *L. corniculatus* and sainfoin. The biosynthesis of epicatechin (Figure 2B) has not yet been demonstrated. Enzyme data will be reviewed to draw general conclusions which illustrate the similarity between anthocyanin and proanthocyanidin biosynthesis. Finally, several speculative schemes for the, as yet, unknown mechanism of intervacuolar transport and polymerisation of the monomers of proanthocyanidins will be reviewed.

Structure and Analysis

Proanthocyanidins vary widely in composition and some idea of the complexity of the biosynthesis and the limitations of the known enzymology rapidly become apparent when the diverse structures of a few representative species are considered.

The simplest proanthocyanidins are oligomers of catechin and gallocatechin (Figure 2B) as found in barley testa. It is thought that the polymerisation is initiated by a nucleophillic attack of catechin on 3,4-*cis*-leucocyanidin to form a dimer (Figure 3). The dimer then attacks another molecule of 3,4-*cis*-leucocyanidin to form a trimer. By repeating this process, a polymer may be formed extending as a linear chain from the initiating group, catechin (Stafford 1990; Figure 2A). The stereochemistry about the 2,3-bond in the barley oligomers, is *trans* and neighbouring flavan-4-ol subunits are linked through C4-C8 to form a linear polymer (Figures 2, 3), The 4-8 linkage is the dominant linkage encountered in most proanthocyanidins. Other less commonly encountered linkages of proanthocyanidins, including 4-6 (leading to branched polymers, Figure 2A) and double linkages C4-C8 and C4-C6, will not be considered.

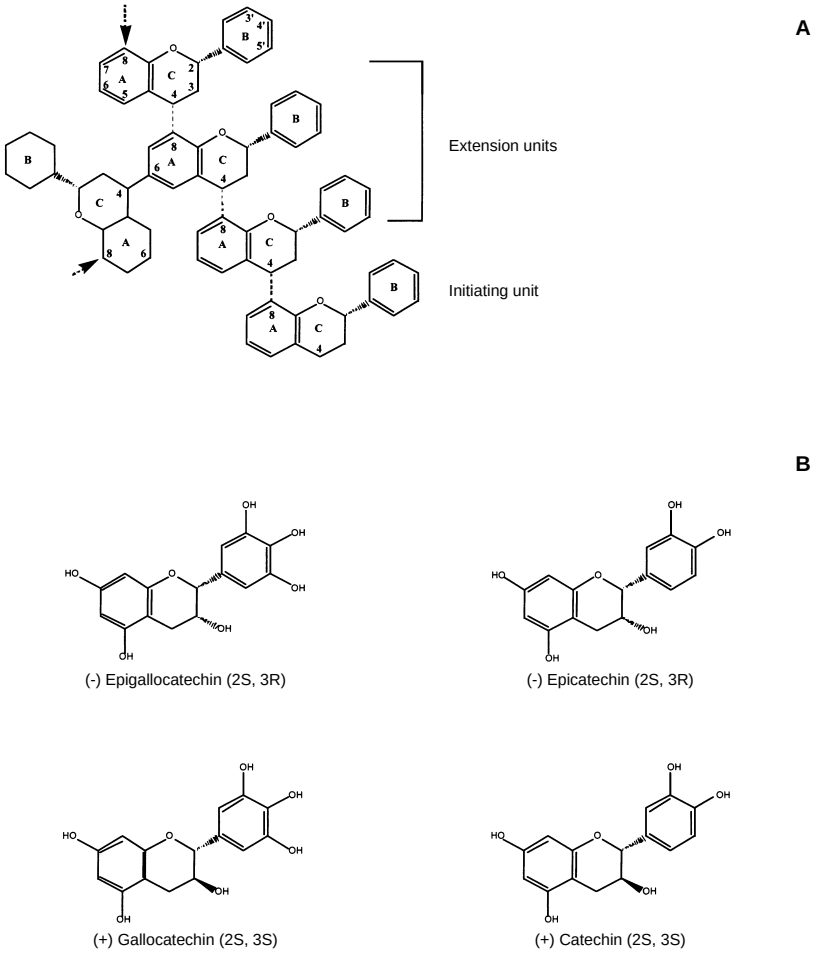


Figure 2A. Model proanthocyanidin polymer (after Stafford 1990) showing the position and carbon numbering of the A, B, and C rings, and 4–8 and 4–6 inter-flavonoid linkages. For clarity hydroxyl groups are not shown. The arrow shows where the chains may be extended by addition of another flavan-3,4-diol unit. **Figure 2B.** Common flavan-3-ols, with di- and tri-hydroxylated B rings and either 2,3-*trans* stereochemistry (catechin and gallocatechin); or 2,3-*cis* stereochemistry (epicatechin and epigalocatechin) are shown with the absolute configuration about C2 and C3.

The stereochemistry observed with barley proanthocyanidin is not typical of the proanthocyanidins commonly encountered in other plant species, where the predominant extension units are the 2,3-cis compounds, epicatechin and epigallocatechin (Figure 2B; Foo and Porter 1981). The 2,3-trans compound, catechin, however, often acts as a chain initiator in these 'mixed' proanthocyanidins (Koupai-Abayzani et al. 1993a, 1993b; Foo et al. 1996).

A proanthocyanidin molecule contains many units linked by single bonds which potentially embody the polymer with a high degree of flexibility. However, due to restricted rotation about the interflavonoid bond, the polymers tend to form a random coil rather than a true helix (Haslam 1977). It is thought that a polymer of catechin units tends to form a right-handed helix whereas a polymer of epicatechin units may form a left-handed helix (Haslam 1977). It is likely that the tertiary structure and hence the protein binding properties of the polymer may be affected by minor changes to the chemical structure of the subunits. The most convincing evidence of this concerns the different biological properties of proanthocyanidins purified from *L. corniculatus* (rich in epicatechin extension units, Figure 2B) compared to those from *L. pedunculatus* (rich in epigallocatechin

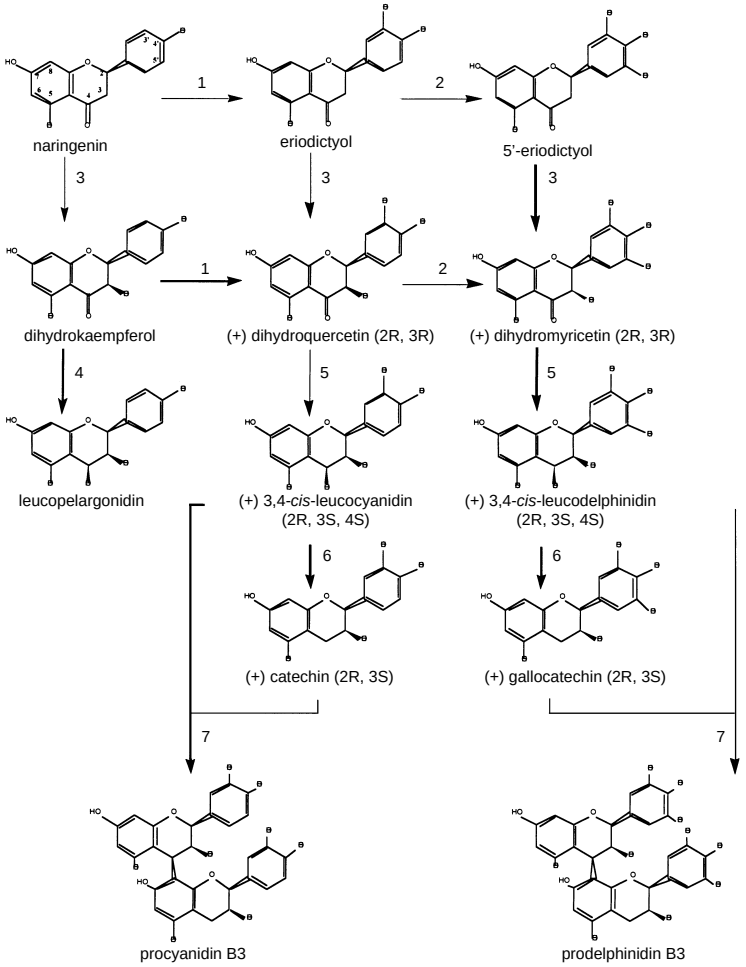


Figure 3. The intermediates of proanthocyanidin biosynthesis. The intermediates from naringenin to the 2,3- *trans* proanthocyanidin dimers are shown with the known enzymes: flavonoid-3'-hydroxylase (F3'H, 1), flavonoid-3',5'-hydroxylase (F3',5'H, 2), flavanone-3-hydroxylase (F3H, 3); dihydroflavonol reductase (DFR, 4 & 5); leucoanthocyanidin reductase (LAR, 6 & 7). Intervacuolar transport and condensation may be associated with step 7. Barley testa accumulates the 2,3-*trans* proanthocyanidins, however, other plant tissues such as the leaves of *Onobrychis viciifolia*, and *Lotus* species also synthesise the 2,3-*cis* compounds epicatechin, epigallocatechin and 2,3-*cis* proanthocyanidins (not shown).

extension units, Figure 2B) presented in this volume by McNabb.

Mild acid hydrolysis of proanthocyanidins releases the initiating flavanol unit (Figure 2A), and converts the extension units into the corresponding anthocyanidin. Gallocatechin and epigallocatechin extension units are converted into delphinidin, while catechin and epicatechin extension units are converted into cyanidin. The average proportion of tri- and di-hydroxylated B rings (Table 1; PD:PC ratio) may be estimated by quantifying delphinidin and cyanidin following TLC or HPLC. The stereochemistry at the carbon 4 linkage may be conserved if mild hydrolysis is conducted in the presence of phloroglucinol or benzene α–thiol. The corresponding flavanol-phenolic adduct is formed and the average proportion of 2,3-*trans* to 2,3-*cis* units may be calculated following separation of the flavanol-adducts by HPLC (Table 1; *cis*: *trans*). The average degree of polymerisation (Table 1; DP) may be calculated from the ratio of extension units to initiating units. DP data may be obtained directly by NMR of the purified polymer (Foo and Porter 1981). NMR also enables the calculation of the average ratio of 2,3-*cis*: 2,3-*trans* units in the proanthocyanidin polymer.

Comparison of Proanthocyanidins in Representative Plant Tissues

Various grasses

With the notable exceptions of barley and *Sorghum vulgare* (sorghum), the grasses do not contain appreciable levels of proanthocyanidins.

Trace levels of catechin, procyanidin and prodelphinidin dimers, and higher oligomers have been reported in *Triticum aestivum* (wheat) bran up to a total concentration of 0.004% (McCallum and Walker 1990).

Oryza sativa (rice) leaves have been reported to contain trace levels of proanthocyanidins (Reddy et al. 1995) as have other tropical grasses (Du Toit et al. 1991; Chesselet et al. 1992). The grasses, *Holcus lanatus* (Yorkshire Fog), and *Lolium perenne* (perennial ryegrass) have been reported to contain low levels of proanthocyanidin of 0.11% dwt and 0.16% dwt respectively (Terrill et al. 1992). *Eleusine coracana* (finger millet) has been reported to contain between 0.06% and 2.0% dwt (Salunke et al. 1990).

Hordeum vulgare

The composition, biosynthesis, genetics and molecular biology of barley proanthocyanidin has been widely studied (Outtrup and Schaumburg 1981; Kristiansen 1984, 1986; Jende-Strid 1993). Barley contains a low level of proanthocyanidins (0.1% dwt) composed of 2–3 units of catechin and gallocatechin with the initiating unit being catechin (Table 1). Catechin accumulates in the developing testa/pericarp with maximum levels at about 18 days after flowering. After 18 days, catechin levels decline and there is a coincident accumulation of catechin dimers and trimers (Kristiansen 1984). Mixed dimers and trimers of catechin and gallocatechin have been isolated from the mature grain (Outtrup and Schaumburg 1981). The enzymes dihydroflavanol reductase and leucocyanidin reductase are also found in extracts of these tissues at approximately 14 days after flowering. (Figure 1; Kristiansen 1986; Tanner and Kristiansen 1993).

Sorghum vulgare

Mature seeds of sorghum contain up to 5% dwt of proanthocyanidins consisting mostly of epicatechin extension units with a degree of polymerisation of up to nine (Table 1). The degree of polymerisation increases slightly as maturity progresses (Butler 1982).

Table 1. Composition of some representative proanthocyanidins.

Plant tissue	DP[1]	PD:PC[1]	2,3-stereochemistry[1] (% 2,3-*cis*)	Dominant extension unit
H. vulgare testa [2,3]	3	60:40	0 %	gallocatechin
S. vulgare testa[2]	8–9	10:90	80%	epicatechin
O. viciifolia leaf[4]	7–9	77:23	90%	epigallocatechin
L. corniculatus leaf[5]	6–7	30:70	97%	epicatechin

[1] See 'Structure and analysis'.
[2] Brandon et al. 1982.
[3] McMurrough and McDowell 1978.
[4] Changes as leaves mature; see Foo et al. 1992 and Koupai-Abyazani et al. 1993b.
[5] Foo et al. 1996.

Lotus corniculatus

Leaves of the forage legume *L. corniculatus* contain approximately 5% dwt of proanthocyanidins and consist of a majority of epicatechin extension units with a minority of epigallocatechin units, and a degree of polymerisation of 6–7 (Table 1). There are indications that the composition and level of proanthocyanidin is affected strongly by environmental factors such as soil fertility and growth season and growth stage (Lowether et al. 1987; Kelman and Tanner 1990; Foo et al. 1992; Carron et al. 1992).

The manipulation of proanthocyanidin levels and composition in tissue cultures and whole plants of *L. corniculatus* by environment, growth stage, growth regulators, and genetic transformation has been extensively studied (Morris and Robbins 1997).

Onobrychis viciifolia

Sainfoin leaves contain up to 10% dwt proanthocyanidins with degree of polymerisation 7–9. The composition changes as leaves age with the proportion of 2,3-*cis* units decreasing from 83% to 48%, and the PD:PC ratio increasing from 60% to 90% (Table 1). It has been suggested that this may be due to *de novo* synthesis of gallocatechin polymer in older leaves, and that this may occur in specific leaf cells (Koupai-Abyazani et al. 1993b; Lees et al. 1995). The level of proanthocyanidin is also sensitive to environmental conditions such as light intensity (Tanner, unpublished)

Enzymology of 2,3-*trans*-Proanthocyanidin Biosynthesis

The 'hydroxylation grid'

From naringenin the biosynthesis of proanthocyanidins proceeds through a series of hydroxylations at the 3' and 5' carbons of the B ring (Figure 3; 1, 2) and a stereospecific β-hydroxylation of the 3 carbon of the C ring (Figure 3; 3). No fixed hydroxylation order has been described and it is possible that a 'metabolic grid' exists in vivo, where a number of enzymes (Figure 3; 1, 2, 3) compete for the same substrates to produce the same end-products via different paths (Stafford 1990). For example, hydroxylation at the 3'- or the 3',5'- positions of the B-ring are catalysed by two enzymes, flavanoid-3'-hydroxylase (Figure 3; 1) and flavanoid-3',5'-hydroxylsase (Figure 3; 2) hydroxylating both naringen and dihydrokaempferol in the relevant positions 3' and 3',5', respectively. These enzymes are P450 microsomal enzymes and require NADPH and O_2 (Heller and Forkman 1993). On the other hand, flavanone-3-hydroxylase (Figure 3; 3) purified from *Petunia* converts naringenin to dihydrokaempferol and eriodictyol to dihydroquercetin (Britsch and Grisebach 1986). The *Petunia* enzyme did not act on 5'-eriodictyol (Figure 3). However, enzyme extracts from *Verbena* acted on all three flavanones (Britsch and Grisebach 1986). Flavanone-3-hydroxylase is a soluble dioxygenase and requires 0_2, 2-oxoglutarate, Fe^{2+} and ascorbate.

It is not known if a similar grid exists in tissues which synthesise proanthocyanidins.

From dihydroflavanol to catechin

The carbonyl group on the 4C of the dihydroflavanols, dihydro-kaempferol, -quercetin, or -myricetin is reduced by a NADP dehydrogenase, dihydroflavanol reductase (Figure 3; 4, 5) to 3,4-*cis*-leuco-pelargonidin, -cyanidin or -delphinidin respectively. The dihydroflavanol reductases involved in anthocyanin biosynthesis display variation in substrate specificity, e.g. the maize dihydroflavanol reductase acts preferentially on dihydrokaempferol and dihydroquercetin; while the *Petunia* reductase preferentially converts dihydromyricetin (Stafford 1990).

No dihydroflavanol reductase involved in proanthocyanidin biosynthesis has yet been substantially purified. However, a partially purified *Cryptomeria japonica* dihydroflavanol reductase preferentially reduced dihydroquercetin compared to dihydromyricetin (Ishikura et al. 1988). Barley dihydroflavanol reductase reduces dihydroquercetin and is inhibited by the product, 3,4-*cis*-leucocyanidin; however, the substrate specificity has not been established (Kristiansen 1986). Transgenic *L. corniculatus* plants carrying antisense dihydroflavanol reductase constructs showed significantly different chemistry where the proportion of dihydroxylated B-rings was increased (Carron et al. 1994). Inhibition of only one of several stereospecific dihydroflavanol reductases may be responsible for the shift in chemistry of the final proanthocyanidin.

The 4-hydroxyl of the flavan-3-4-*cis*-diols is removed in a single step by leucoanthocyanidin reductase (Figure 3; 6, 7), an NADP dehydrogenase. This enzyme has been demonstrated in crude extracts of barley, and sainfoin (Tanner and Kristiansen 1993), and in Douglas fir and *Ginko biloba* suspension cultures (Stafford 1990). The sainfoin enzyme is unstable, but has been extensively purified and reduces both 3,4-*cis*-leucocyanidin and 3,4-*cis*-leucodelphinidin but not the 3,4-*trans*-isomers (Tanner, unpublished). The sainfoin enzyme transfers a hydride ion from the 4-*pro*-R position of NADPH to the product catechin (Abrahams, unpublished). The dihydroflavanol and leucoanthocyanidin

reductase activities in sainfoin extracts may exist as a multi-enzyme complex which effectively channels intermediates directly between the enzymes without exchange with the external media (Singh et al. 1997).

Inter-vacuolar transport and polymerisation

The flavan-3-4-diol proanthocyanidin extension units, leucocyanidin and leucodelphinidin, and the flavan-3-ol initiating units, catechin or gallocatechin (Figure 3), must enter the vacuole since the proanthocyanidins accumulate inside the vacuole (Lees et al. 1995; Tanner, unpublished). The flavan-3-4-diols and the flavan-3-ols are soluble in organic solvents and may passively diffuse across the membrane; or they may be transported through the action of specific transporters (Figure 1; LC? and C?). There may be a transportor for each substrate or one general transporter. It is also possible this flavanol transporter may be similar to the membrane glutathione pump believed to be involved in anthocyanin transport—in this case, an additional step would be required, analgous to the glutathione-S-transferase step in anthocyanin biosynthesis (Figure 1; GST).

Once inside the vacuole, the flavan-3-4-diols are attacked by the flavan-3-ols to form a dimer. The dimer then attacks another flavan-3-4-diol molecule to form a trimer. This process is repeated until a polymer is formed (Figures 1 and 3). This polymerisation may occur non-enzymatically or be directed by a condensing enzyme. Stafford (1990) has proposed that the transport and polymerisation occur simultaneously on the surface of a trans-membrane multi-enzyme complex.

Successful biomimetic syntheses of proanthocyanidin oligomers at pH conditions expected in plant vacuoles have been demonstrated (Delcour et al. 1983). No experimental evidence has yet been obtained for specific transporters or condensing enzymes. However, it is likely that such evidence would require a functional whole cell or protoplast membrane system.

Regulation of the Anthocyanin Biosynthesis

There are similarities between the regulation of the anthocyanin and proanthocyanin biosynthesis. The level and tissue specificity of anthocyanin pigmentation is controlled by a number of regulatory genes (Holton and Cornish 1995; Mol et al. 1998). These regulatory genes have homology to mammalian proto-oncogenes of either the MYC or MYB families of proteins. MYC proteins carry a basic helix-loop-helix motif (Ludwig and Wessler 1990). The presence of a member from each of the two families of regulatory proteins is required for the coordinate expression of at least three genes, coding for chalcone synthase, dihydroflavanol reductase, and flavanol-UDP-glucosyl transferase of the anthocyanin pathway in maize (Figure 1). The situation in dicots appears to be different with the 'upper' and 'lower' parts of the anthocyanin pathway regulated by different sets of genes (Mol et al. 1998). The coding regions of the various regulators are functionally conserved among plant species and have distinct sets of target genes. The different tissue specificity observed with these regulatory proteins appears to result from divergence of the promoters of the genes with which they interact (Quattrocchio et al. 1993, 1998).

Regulation of Proanthocyanidin Biosynthesis

Lotus corniculatus

Transgenic *L. corniculatus* plants carrying the maize *Sn* gene showed modified proanthocyanidin phenotypes compared to untransformed controls (Damiani et al. 1999). Six of seven transgenic plants exhibited increased proanthocyanidin levels in the roots when compared to controls. *L. corniculatus* does not normally synthesise proanthocyanidin in the root tip. Conversely, four transgenic plants also exhibited dramatically decreased proanthocyanidin levels in leaf tissues. The level of the enzymes, dihydroflavanol reductase and leucoanthocyanidin reductase (Figure 1), were dramatically decreased in those leaves where proanthocyanidins were decreased. Conversely, the level of chalcone synthase protein (Figure 1) was not reduced in transgenics where proanthocyanidin was reduced. All transgenic plants were able to initiate synthesis of *Sn* message. However, mature *Sn* message did not accumulate in the plants where proanthocyanidin levels were reduced. It was proposed that the *Sn* transgene interacted with the endogenous *Myc* gene whose protein product was necessary for synthesis of dihydroflavanol reductase and leucoanthocyanidin reductase (Figure 1). If this is correct, it is the first evidence that the same regulator which controls dihydroflavanol reductase also controls leucoanthocyanidin reductase, the first committed enzyme of proanthocyanidin biosynthesis. It appears that the 'upper' part of the proanthocyanidin pathway is regulated by separate genes as occurs for other dicots (Mol et al. 1998).

Hordeum vulgare

Approximately 500 proanthocyanidin free barley mutants have been isolated and localised to 10 complementation groups or *Ant* genes involved with

proanthocyanidin biosynthesis (Jende-Strid 1993). All of the mutations are recessive and monofactorial. The most important of these mutants synthesise anthocyanins and therefore appear to carry a metabolic lesion below the level of leucocyanidin, specific to the proanthocyanidin pathway (Figure 1). By enzyme and end-product analysis, a number of these lesions have been localised to specific enzymes (Table 2).

Mutations in the *Ant* 25 gene result in the loss of both dihydroflavanol reductase and leucoanthocyanidin reductase activities (Table 2). This gene may code for a transcriptional regulator whose expression is required for synthesis of these enzymes. *Ant* 25 may be similar to the unknown endogenous *L. corniculatus* gene down-regulated by transformation with *Sn* (Figure 1).

Grains carrying a mutation in *Ant* 19, and grown in soil, accumulate approximately 10% wild type proanthocyanidin; dihydroflavanol reductase and particularly leucoanthocyanidin reductase activities are also reduced dramatically in these grains (Table 2; -N). Grains carrying a mutation in *Ant* 19 are typically shrunken with proanthocyanidin only accumulating in the testa close to the dorsal vein. When grown with supplemental nutrients the normal plump appearance of the grain is restored; proanthocyanidin accumulation occurs more evenly around the testa and the levels of both dihydroflavanol reductase and leucoanthocyanidin reductase activities are significantly increased (Table 2; +N). It was initially thought that *Ant* 19 coded for leucoanthocyanidin reductase; however, it now seems likely that this gene is involved in nutrient supply to the developing grain.

Grains carrying mutations in *Ant* 26 or *Ant* 27 have essentially wild type levels of both dihydroflavanol reductase and leucoanthocyanidin reductase activities. However, *Ant* 26 mutants accumulate wild type levels of catechin whereas *Ant* 27 mutants only accumulate trace levels of catechin. It is proposed that *Ant* 26 is involved in transport of leucocyanidin into the vacuole (Figure 1; LC?). Interference with this function would still allow accumulation of catechin in the vacuole but no polymer would be formed. *Ant* 27 may be involved with transport of catechin into the vacuole (Figure 1; C?). Loss of this function would cause a build-up of cytosolic catechin, leading to feedback inhibition of leucocyanidin reductase and subsequently dihydroflavanol reductase activities, ultimately limiting the accumulation of both leucocyanidin and catechin (Tanner, unpublished; Kristiansen 1986).

Mutation of either *Ant* 28 or *Ant* 29 blocks the accumulation of proanthocyanidin and catechin but not anthocyanidin. The function of these genes remains unknown.

Some Final Questions

There are two major shortfalls in knowledge of proanthocyanidin biosynthesis:

1: *What controls the different subunit composition observed in various proanthocyanidins?*

Is the polymerisation controlled and directed by a condensing enzyme, or is the polymerisation non-enzymatic similar to the various biomimetic syntheses where the composition of the final polymer is specified by the availability and reactivity of suitable extension and initiating units? The availability of substrates for the polymerisation may be dictated by the specificity and degree of competition between the enzymes of the 'metabolic grid' which may exist above the level of 3,4-*cis*-leucocyanidin (Figure 3). Substrate availability may also be affected by the manner of transport of the 'monomers' into the vacuole.

2. *How are epicatechin and 2,3-cis-proanthocyanidins synthesised?*

We can explain the synthesis of catechin; but catechin only accounts for about 10% of most proanthocyanidins. How is the bulk of the material used for proanthocyanidin synthesis made?

Table 2. Proanthocyanidin free, *Hordeum vulgarie* mutants which accumulate anthocyanidin[1].

Mutation	DFR (%)	LAR (%)	Catechin	PA	Function
Ant 19 (+N)	55	23	Trace	Med	Nutrition
(–N)	45	8	Trace	Low	
Ant 25	0	0	None	None	Regulator
Ant 26	86–96	65–82	+	None	Transport LC?[2]
Ant 27	32–41	48–51	Trace	None	Transport C?[2]

[1] Tanner et al. 1992.
[2] See Figure 1.

Conclusion

The elucidation of proanthocyanidin enzymology lags far behind the structural determination of the various proanthocyanidins. However, it is hoped that an understanding of the properties of purified biosynthetic activities can lead to a fuller understanding of the complexity of proanthocyanidin biosynthesis, and eventually allow manipulation of the functional properties of the proanthocyanidin polymer.

References

Bavage, A.D. and Robbins, M. 1994. Dihydroflavanol reductase a *Lotus corniculatus* L. tannin biosynthetic gene: isolation of a partial gene clone by partial PCR. Lotus Newsletter, 25: 37–40.

Brandon, M.J., Foo, L.Y., Porter, L.J. and Meridith, P. 1982. Proanthocyanidins of barley and sorghum. Composition as function of maturity of barley ears. Phytochem., 21: 2953–2957.

Britsch, L. and Grisebach, H. 1986. Purification and characterisation of (2S)-flavanone 3-hydroxylase from *Petunia hybrida*. European J. Biochem., 156: 569–577.

Butler, L.G. 1982. Relative degree of polymerisation of sorghum tannin during seed development and maturation. J. Agric. Food Chem., 30: 1090–1094.

Carron, T.R., Morris, P., Evans, T.E. and Robbins, M.P. 1992. Condensed tannin levels in different tissues and different developmental stages of transformed and non-transformed *Lotus corniculatus*. Lotus Newsletter, 23: 49–52.

Carron, T.R., Robbins, M.P. and Morris, P. 1994. Genetic modification of condensed tannin biosynthesis in *Lotus corniculatus*. 1. Heterologous antisense dihydroflavonol reductase down-regulates tannin accumulation in 'hairy root' cultures. Theor. Appl. Genet., 84: 1006–1015.

Chesselet, P., Wolfson, M.M. and Ellis, R.P. 1992. A comparative histochemical study of plant polyphenols in southern African grasses. J. Grasslands Soc. South Afr., 9: 119–125.

Damiani, F., Paolocci, F., Cluster, P.D., Arcioni, S., Tanner, G.J., Joseph, R.G., Li, Y-G., de Majnik, J. and Larkin, P.J. 1999. The maize transcription factor *Sn* alters proanthocyanidin synthesis in transgenic *Lotus corniculatus* plants. Aust. J. Pl. Physiol., 26: 159–169.

Delcour, J.A., Ferreira, D. and Roux, D.G. 1983. Synthesis of condensed tannins. IX. The condensation sequence of leucocyanidin with (+)-catechin and with the resultant procyanidins. J. Chem.Soc. Perkin Trans., 1: 1711–1717.

Du Toit, E.W., Wolfson, M.M. and Ellis, R.P. 1991. The presence of condensed tannin in the leaves of Eulalia villosa. Tydskrif Weidingsveren. S.A., 8: 74–76.

Foo, L.Y. and Porter, L.J. 1981. The structure of tannins in some edible fruits. J. Sci. Food Agric., 32: 711–716.

Foo, L.Y., Jones, W.T., Porter, L.J. and Williams, V.M. 1992. Proanthocyanidin polymers of fodder legumes. Phytochemistry, 21: 933–935.

Foo, L.Y. and Karchesy, J.J. 1989. Chemical nature of phlobaphenes. In: Hemingway, R. and Karchesy, W. ed. Chemistry and significance of condensed tannins. New York, Plenum, 109–118.

Foo, L.Y., Newman, R., Waghorn, G., McNabb, W.C. and Ulyatt, M.J. 1996. Proanthocyanidins from *Lotus corniculatus*. Phytochemistry, 41: 617–624.

Forkman, G. 1993. Genetics of flavonoids. In: Harbourne, J.B. ed. The Flavonoids: Advances in Research Since 1986. London, Chapman and Hall, 537–564.

Grotewold, E., Drummond, B.J., Bowen, B. and Peterson, T. 1994. The *myb*-homologous *P* gene controls phlobaphene pigmentation in maize floral organs by directly activating a flavonoid biosynthetic gene subset. Cell, 76: 543–553.

Haslam, E. 1977. Symmetry and promiscuity in procyanidin biochemistry. Phytochemistry, 16: 1625–1640.

Heller, W. and Forkman, G. 1993. Biosynthesis of flavonoids. In: Harbourne, J.B. ed. The Flavonoids: Advances in Research Since 1986. London, Chapman and Hall, 499–536.

Hergert, H.L. 1989. Biogenesis of condensed tannins: An overview. In: Hemingway, R. and Karchesy, W. ed. Chemistry and Significance of Condensed Tannins. New York, Plenum, 71–82.

Holten, T.A. and Cornish, E.C. 1995. Genetics and biochemistry of anthocyanin biosynthesis. Plant Cell, 7: 1071–1083.

Ishikura, N., Murakami, H. and Fuji, Y. 1988. Conversion of (+)dihydroquercetin to 3,4-*cis*-leucocyanidin by a reductase from cell suspension cultures of *Cryptomeria japonica*. Plant Cell Physiol., 29: 795–799.

Jende-Strid, J. 1993. Genetic control of flavonoid biosynthesis in barley. Hereditas, 119: 187–204.

Joseph, R.G., Tanner, G.J. and Larkin, P.J. 1998. Proanthocyanidin synthesis in the forage legume *Onobrychis viciifolia*. A study of chalcone synthase, dihydroflavonol 4-reductase and leucoanthocyanidin 4-reductase in developing leaves. Aust. J. Plant Physiol., 25: 271–278.

Kelman, W.M. and Tanner, G.J. 1990. Foliar condensed tannin levels in lotus species growing on limed and unlimed soils in South-Eastern Australia. Proc. New Zealand Grasslands Assoc., 52: 51–54.

Koupai-Abyazani, M.R., McCallum, J., Muir, A., Lees, G., Bohm, B.A., Towers, G.H.N. and Gruber, M.Y. 1993a. Purification and characterisation of a proanthocyanidin polymer from seed of alfalfa (*Medicago sativa* cv Beaver). J. Agric. and Food Chem., 41: 565–569.

Koupai-Abyazani, M.R., McCallum, J., Muir, A., Bohm, B.A., Towers, G.H.N. and Gruber, M.Y. 1993b. Developmental changes in the composition of proanthocyanidins from leaves of sainfoin (*Onobrychis viciifolia* Scop.) as determined by HPLC analysis. J.Agric. Food Chem., 41: 1066–1070.

Kristiansen, K.N. 1984. Biosynthesis of proanthocyanidins in barley: Genetic control of the conversion of dihydroquercetin to catechin and procyanidins. Carlsberg Res. Commun., 49: 503–524.

Kristiansen, K.N. 1986. Conversion of (+)-dihydroquercetin to (+)-2,3-*trans*-3,4-*cis*-leucocyanidin and (+)-catechin with an enzyme extract from maturing grains of barley. Carlsberg Res. Commun., 51: 51–60.

Kristiansen, K.N. and Rohde, W. 1991. Structure of the *Hordeum vulgare* gene encoding dihydroflavanol-4-reductase

and molecular analysis of *Ant* 18 mutants blocked in flavanoid synthesis. Mol. Gen. Genet., 230: 49–59.

Lees, G.L., Gruber, M.Y. and Suttill, N.H. 1995. Condensed tannins in sainfoin. II. Occurrence and changes during leaf development. Can. J. Bot., 73: 1540–1547.

Lowether, W.L., Manley, T.R. and Barry, T.N. 1987. Condensed tannin concentrations in *Lotus corniculatus* and *L. peducnulatus* cultivars grown under low soil fertility conditions. New Zealand J. Ag. Research, 30: 23–25.

Ludwig, S.R. and Wessler, S.R. 1990. Maize R gene family: tissue specific helix-loop-helix proteins. Cell, 62: 849–851.

Marrs, K.A., Alfenito, M.R., Lloyd, A.M. and Walbot, V. 1995. A glutathione S-transferase involved in vacuolar transfer encoded by the maize gene *Bronze-2*. Nature, 375: 397–400.

Martin, C. and Gerats, T. 1993. The control of flower coloration. In: Jordan, B.R. ed. The Molecular Biology of Flowering. Wallingford, CAB International, 219–255.

McCallum, J.A. and Walker, J.R.L. 1990. Proanthocyanidins in wheat bran. Cereal Chemistry, 67: 282–285.

McMurrough, I. and McDowell, J. 1978. Chromatographic separation and automated analysis of flavanols. Anal. Biochem., 91: 92–100.

Meldgard, M. 1992. Expression of chalcone synthase, dihydroflavanol reductase and flavanone-3-hydroxylase in mutants of barley deficient in anthocyanin and proanthocyanidin biosynthesis. Theor. Appl. Genet., 83: 695–706.

Mol, J., Grotwold, E. and Koes, R. 1998. How genes paint flowers and seeds. Trends in Plant Science, 3: 212–217.

Morris, P. and Robbins, M.P. 1997. Manipulating condensed tannins in forage legumes. In: McKersie, B.D. and Brown, D.C.W. ed. Biotechnology and The Improvement of Forage Legumes. Wallingford, CAB International, 147–173.

Outtrup, H. and Schaumburg, K. 1981. Structure elucidation of some proanthocyanidins in barley by ^{1}H 270 Hz NMR spectroscopy. Carlsberg Res. Commun., 46: 43–52.

Porter, L.J. 1993. Flavans and proanthocyanidins. In: Harbourne, J.B. ed. The Flavonoids: Advances in Research Since 1986. London, Chapman and Hall, 23–56.

Quattrocchio, F., Wing, J.F., Leppen, H.T.C., Mol, J.N.M. and Koes, R.E. 1993. Regulatory genes controlling anthocyanin pigmentation are functionally conserved among plant species and have distinct sets of target genes. Plant Cell, 5: 1497–1512.

Quattrocchio, F., Wing, J.F., Vanderwoude, K., Mol, J.N. M. and Koes, R. 1998. Analysis of Bhlh and Myb domain proteins—species-specific regulatory differences are caused by divergent evolution of target anthocyanin genes. Plant Journal, 13: 475– 488.

Reddy, V.S., Dash, S. and Reddy, A.R. 1995. Anthocyanin pathway in rice (*Oryza sativa* L): Identification of a mutant showing dominant inhibition of anthocyanins in leaf and accumulation of proanthocyanidins in pericarp. TAG, 91: 301–321.

Rohde, W., Barzen, E., Marocco, A., Schwarz-Sommer, Z., Saedler, H. and Salamini, F. 1987. Isolation of genes that could serve as traps for transposable elements in *H. vulgare*. Barley Genet., V: 533–541.

Saito, K., Kobayashi, M., Gong, Z., Tanaka, Y. and Yamazaki, M. 1999. Direct evidence for anthocyanidin synthase as a 2-oxoglutarate-dependent oxygenase: molecular cloning and functional expression of cDNA from a red forma of *Perilla frutescens*. The Plant Journal, 17: 181–189.

Salunke, D.A., Chavan, J.K. and Kadam, S.S. 1990. Dietary Tannins: Consequences and Remedies. Boca Raton, Fl., CRC Press, 200 p.

Singh, S., McCallum, J., Gruber, M.Y., Towers, G.H.N., Muir, A., Bohm, B.A., Koupai-Abyazani, M.R. and Glass, A.D.M. 1997. Biosynthesis of flavan-3-ols by leaf extracts of *Onobrychis viciifolia*. Phytochem., 44: 425–432.

Sparvoli, F., Martin, C., Scienza, A., Gavazi, G. and Tonelli, C. 1994. Cloning and molecular analysis of structural genes involved in flavonoid and stilbene biosynthesis in grapes (*Vitis vinifera*). Plant Mol. Biol., 24: 743–755.

Stafford, H.A. 1990. Flavonoid Metabolism. Boca Raton, Fl., CRC Press, 298 p.

Tanner, G.J., Kristiansen K.N. and Jende-Strid B. 1992. Biosynthesis of proanthocyanidins (condensed tannins) in barley. Proceedings of Groupe Polyphenols, 16: 170–173.

Tanner, G.J. and Kristiansen, K.N. 1993. Synthesis of 3,4-cis-[^{3}H]-leucocyanidin and enzymatic reduction to catechin. Analytical Biochemistry, 209: 274–277.

Terrill, T.H., Rowan, A.M., Douglas, G.B. and Barry, T.N. 1992. Determination of extractable and bound condensed tannin concentrations in forage plants, protein concentrate meals and cereal grains. J. Food Sci. Agric., 58: 321–329.

Evaluation and Enhancement of Feeding Value of Tanniniferous Feeds

H.P.S. Makkar[1]

Abstract

Various chemical and protein precipitation tannin assays are used for characterisation of tannin-containing feeds. Each type of tannin responds differently in each of these assays. This variability in response makes it impossible to use any single method. Use of a battery of methods is suggested. Tannin assay based on the in vitro rumen fermentation system coupled with inactivation of tannins by polyethylene glycol, PEG (MW 6000) was developed. This method can be complementary to other tannin assays in evaluating the nutritional quality of tanniniferous feeds. Two approaches, based on measurement of feed degraded or products formed (gases, short-chain fatty acids, and microbial mass), were considered in this assay. The determination of feed degraded was distorted by the presence of tannin-protein complexes present in the truly undigested matter determined using neutral detergent solution. The second approach, though more difficult to perform, can successfully be used. This approach also enables study of: 1) nutritional significance of both extractable and unextractable (bound) tannins; 2) partitioning of nutrients to microbial protein and short-chain fatty acids or gases; and 3) efficiency of rumen microbial protein synthesis. A limitation of this technique is that the role of post-rumen sections of the gastrointestinal tract cannot be determined. In vivo studies on nutritional evaluation of tanniniferous feeds using sheep and goats showed that caution should be exercised in interpreting results on balance of nutrients due to the presence of tannins, proteins or tannin-protein complexes in faecal samples. For browses, the poor correlation ($r = 0.19$; $n = 18$; $P > 0.05$) between percent increase in in vitro rumen degradable nitrogen on addition of PEG and intestinal degradable nitrogen observed using the mobile bag technique indicated that the proteins protected from microbial degradation do not seem to be available post-ruminally from these browses. Detaninification approaches to enhance feeding value have been attempted keeping in mind their applicability at farmers (use of wood ash, storage in the presence and absence of urea, drying) and small-scale industry (use of organic solvents, oxidising agents, heat treatments, biodegradation) levels. Storage in the presence of urea and the use of oxidising agents in the presence of alkalis were very effective (up to 95% decrease in tannins and tannin activity). Use of PEG is an attractive method for enhancing the feeding value. The use of PEG increased the organic matter and nitrogen degradability but decreased the efficiency of microbial protein synthesis. An approach to enhance the efficiency of microbial protein synthesis and to decrease the amount of PEG, with the aim of increasing the benefit-to-cost ratio and decrease methane production, are presented.

TANNINIFEROUS trees and shrubs are of importance in animal production because they can provide significant protein supplements but unfortunately the amounts of tannins that they contain vary widely and unpredictably. Their effects on animals range from beneficial to toxic, including death. The toxic or anti-nutritional effects may be exacerbated in times of stress when a very large proportion of the diet is tanniniferous. With a better understanding of tannin properties, the mechanism of tannin action and proper management of forages, browses could

[1] Institute for Animal Production in the Tropics and Subtropics (480), University of Hohenheim, D-70599 Stuttgart, Germany
(Present address: Animal Production and Health Section, International Atomic Energy Agency, Vienna, Austria. E-mail: h.makkar@iaea.org)

KEYWORDS: Tannins, Feeding value, Tannin assay, Rumen, Fermentation, Polyethylene glycol (PEG), Nutrition

become an invaluable source of protein for strategic supplementation. As the demand for food rises, tanniniferous plants and agro-industrial by-products must play an increasingly important part in the diet of animals. It is therefore critical that proper techniques be developed and used to evaluate and enhance feeding value of tannin-containing forages and by-products.

Evaluation of Feeding Value

In the process of evaluating the feeding value of tanniniferous feeds, an important element is the quantification of tannins. Several methods are available for quantification of tannins. These methods are generally divided into two categories:

1. Chemical assays, based on chemical properties of tannins;
2. Protein precipitation assays, based on protein binding property of tannins.

These methods will not be discussed here since a number of good reviews are available on these methods (Makkar 1989; Waterman and Mole 1994; Hagerman et al. 1997). A tannin bioassay is presented here, which attempts to quantify tannins in terms of rumen fermentation parameters. This assay is based on in vitro rumen fermentation system coupled with the use of a tannin-complexing agent, polyethylene glycol (PEG).

Tannin bioassay

The in vitro rumen fermentation system is based on Hohenheim gas method (Menke et al. 1979) with some modifications (Makkar et al. 1995a). In brief, feed samples (500 mg) are incubated in the presence and absence of PEG (MW 6000) in graduated glass syringes of 100 mL capacity. Incubation is started by injecting 40 mL medium containing rumen liquor and bicarbonate buffer and then transferring the syringes in a water bath adjusted at 39 ˚C. PEG binds to tannins and make them inert. PEG also has the capability to release protein from the already formed tannin-protein complexes. The difference in the parameters observed in the presence and absence of PEG can be attributed to the biological effects of tannins on rumen fermentation.

The feed degraded in the in vitro system leads to the production of fermentative gases, mainly methane and carbon dioxide, short chain fatty acids (SCFA) and microbial mass. The gas measured in the syringe is a sum of the direct gas (fermentative gases) and the indirect gas released from the bicarbonate buffer as a result of neutralisation of SCFA produced during fermentation. One mole of SCFA produced, on neutralisation, leads to the production of one mole of carbon dioxide. Hence, the gas production in the syringe represents the production of fermentative gases and SCFA (Blümmel et al. 1997; Getachew et al. 1998).

Two approaches were investigated for quantification of tannins. These were based on the determination of: 1) the substrate (feed) degraded, and 2) the products formed, in the presence and absence of PEG.

Quantification of substrate degraded

On incubation of *Dichostachys cinerea* and *Acioa barteri* leaves (rich in tannins), apparent digestibility was 8.9% and the true digestibility 43.8% in the absence of PEG, whereas in the presence of PEG, these values were lower. On the other hand, SCFA production was much higher in the presence of PEG, suggesting higher dry matter digestibility in the presence of PEG. Similar results were obtained for *A. barteri*. For wheat straw which is devoid of tannins, PEG had no affect (Table 1). Tannin content in the truly undigested residue was approximately 340% and 150% higher for *D. cineria* and *A. barteri* respectively, in the presence of PEG. PEG was also detected in these residues. These observations suggest that tannin-PEG complexes are present as artefacts in the truly undigested residue, which lead to overestimation of apparent and true digestibility values (Makkar et al. 1995a). Therefore, the first approach to quantifying the amount of feed degraded cannot be used for evaluation of tannin effects. The measurement of true digestibility for tannin-rich samples, even in the absence of PEG, is not free of error (Makkar et al. 1997a, 1998).

Table 1. Effect of addition of PEG on some in vitro rumen fermentation parameters.

	Apparent digestibility (%)	True digestibility (%)	SCFA production (µmol/ syringe)
Dichostachys cinerea			
Control	8.9	43.8	475
With PEG	0.1	35.5	687
Acioa barteri			
Control	11.5	31.7	308
With PEG	2.7	32.1	388
Wheat straw			
Control	—	42.8	—
With PEG	—	42.2	—

Quantification of products

The measurement of gas in the Hohenheim gas method is very easy. The correlation between increase in gas production from tannin-containing

feeds as a result of PEG addition and tannins measured as protein precipitation capacity of samples was highest (Makkar et al, 1995a; Makkar and Becker 1996a).

Equations have been derived for calculation of organic matter digestibility (OMD) and metabolisable energy (ME) of a feed from the volume of gas production at 24 h fermentation and crude protein content of the feed (Menke and Steingass 1988). Using these equations OMD and ME can be calculated for a feed in the presence and absence of PEG. Because of higher gas production in the presence of PEG, OMD and ME values are higher in the presence of PEG. For *Acacia saligna* leaves, the OMD was 51.4% in absence and 60.2% in the presence of PEG, suggesting that tannins present in the leaves have the capability to decrease OMD by 60.2–51.4 = 8.8% units, and similarly tannins have the potential to decrease ME by 1.34 MJ/kg DM. From the gas volume, SCFA can be determined using an equation (Makkar et al. 1995b). For *A. saligna* leaf, tannins have the potential to decrease the production of SCFA by 47%. Therefore, using this method, tannin effects can be quantified in terms of decrease in OMD, ME or SCFA production (Makkar and Becker 1996a).

Measurement of gas only does not give a true picture since the relationship between production of gas or SCFA and microbial mass is not constant when expressed per unit feed degraded. This relationship could vary widely from no relationship to negative (Beever 1993; Leng 1993; Makkar et al. 1995b; Blümmel et al. 1997). Determination of the other product, microbial mass, needs to be taken into account. This approach also enables determination of the efficiency of microbial protein synthesis. *D. cinerea*, a tannin-rich browse, was incubated in the presence and absence of PEG. The gas production increased from 30 to 73.5 mL, i.e. an increase of 2.4-fold by PEG. Microbial mass in terms of DAPA increased by only 1.3-fold, and similarly increase in ^{15}N incorporation was 1.2-fold in the presence of PEG. This increase of microbial mass was 50% that of gas or SCFA production. These results suggest that in the presence of PEG, although the degradability of substrate and microbial mass production were higher, the efficiency of microbial protein synthesis was lower (Makkar et al. 1998).

This in vitro assay also measures nutritional implications of bound or unextracted tannins. Neutral detergent fibre (NDF) was prepared from tannin-rich browses, and this NDF contained condensed tannins. Addition of PEG in the incubation of tannin-rich NDF led to an increase in gas production, suggesting that tannins released as a result of NDF degradation by rumen microbes are biologically active and have the potential to influence rumen fermentation (Makkar et al. 1997b). Similar results were obtained on incubation of tannin-rich browses made free of extractable tannins by repeated use of 70% aqueous acetone. The increase in gas production was from 2% to 80% on addition of PEG (Table 2).

Table 2. Increase in gas production from extractable tannin-free browses on addition of PEG.

Browses	Increase in gas at 24 h (%)
Bauhinia	02
Acacia	25
Erica	80
Azadirachta	19

In Vivo Evaluation of Tannin-Rich Feeds

A. saligna leaves were fed to sheep and goats. The OMD was 34% and 43% in sheep and goats and N-balance was negative. An interesting point to note is the negative digestibility of NDF, acid detergent fibre (ADF) and acid detergent lignin (ADL). The reason for these negative digestibility values were established to be the presence of tannins and/or tannin-protein complexes as artefacts in the fibre residues (Degen et al. 1995). The output of extractable tannins and condensed tannins was almost nil, i.e. almost 100% of the extractable tannins could not be recovered in the faeces. Condensed tannins are neither metabolised nor absorbed from the gastrointestinal tract (GIT) (Terrill et al. 1994; Makkar et al. 1995b). It appears that extractable tannins become bound to proteins in vivo. Tannins in these tannin-protein complexes present in the faeces cannot be measured by the assays used (total phenol assay coupled with the use of insoluble polyvinly pyrrolidone, and the butanol-HCl reagent). The use of the sequential approach, i.e. measurement of ADF from NDF and of ADL from ADF also did not overcome the problem of negative fibre digestibility (Makkar et al. 1995c).

Both in vivo and in vitro, tannins bind to proteins and these tannin-protein complexes are not removed by detergent systems of fibre analysis leading to misleading values of fibre. The origin of proteins could be microbes, feed or GIT. Therefore, caution is required in interpretation of results obtained from in vivo or in vitro experiments on evaluation of tannin-containing feeds using the detergent system of fibre analysis (Van Soest et al. 1991).

Hanley et al. (1992) proposed equations for predicting the digestibility of protein and dry matter of

tannin-containing feeds in ruminants. The equations are based on the contents of crude protein, lignin, cutin, neutral detergent fibre and biogenic silica, and protein precipitation capacity of the feed. Based on these equations, the predicted protein and dry matter digestibility were 34.4% and 45.5% respectively for both sheep and goats since the equations do not take into account the animal species. The measured dry matter digestibility for goats (40.8%) was closer to the predicted value than that of sheep (31.9%) but still the difference was substantial (Degen et al. 1995). There was a big difference between predicted and measured protein digestibility for both species (Degen et al. 1995) which could be attributed to the presence of proline-rich proteins in the saliva of deer, animals used to develop the predictive equations (Hanley et al. 1992), but were absent in goats and sheep (see Makkar and Becker 1998). The predictive equations proposed by Hanley et al. (1992) cannot therefore be applied to sheep and goats.

Enhancement of Feeding Value

Various studies aimed at detannification of tannin-rich feeds were conducted. Two types of approaches were developed, one for farmers and the other for small-scale industries. Promising approaches are mentioned below.

Storage. Whole and chopped oak leaves containing 40% moisture were stored (Makkar and Singh 1993). The rate of inactivation of tannins was higher for the chopped leaves. A reduction of approximately 50% tannins was obtained in 4 days of storage. These leaves were also stored in the presence of 4% urea. Storage in the presence of urea led to 50% tannin reduction in only 2 days.

Oxidising agents. Treatment with potassium permanganate and potassium dichromate, and hydrogen peroxide under alkaline conditions led to decrease in tannin content and tannin activity by about 90% in agro-industrial by-products (Makkar and Singh 1992; Makkar and Becker 1996b).

White-rot fungi. Solid-state fermentation of browses using *Sporotricum pulverulentum*, *Ceriporiopsis subvermispora* and *Cyathus steroreus* decreased tannin content by about 60% in 10 to 20 days of fermentation (Makkar et al. 1994; Gamble et al. 1996).

Use of PEG. Addition of PEG to tannin-rich diets is another attractive option to enhance the feeding value of such diets. On perusal of the literature, it is evident that addition of PEG is advantageous when tannin content of the feed is high and is deleterious when tannin content is low. We studied the effect of manner of application of PEG (MW 6000) on some fermentation parameters at 24 h of incubation of tannin-rich feeds in the in vitro rumen fermentation system (mentioned above). PEG was applied as a single dose (51 mg) or in split manner (7 times, each time 7.3 mg at 0 h and then after every 2 h interval) in incubations containing *Calliandra* leaves (protein precipitation capacity: 0.45 mg BSA pptd./mg feed). The gas and SCFA production increased substantially on addition of PEG. Purine as a marker for microbial mass was similar for control and single application of PEG but with split application the microbial mass was higher. Efficiency of microbial protein production was also higher with split application of PEG (Getachew et al. 1999). The implications of these results are that PEG when given to ruminants in the slow release form will be more beneficial than the highly soluble form. Under the auspices of the IAEA, projects are now being planned for improving the utilisation of tanniniferous forages by feeding PEG-molasses-multi-nutrient blocks. The licking of these blocks will release PEG slowly, which is expected to supply higher microbial protein post-ruminally as a result of better efficiency of microbial protein synthesis mediated probably via better synchronisation of ATP production and release of nutrients.

In conclusion, the in vitro rumen fermentation method based on measurement of products, i.e. gases, SCFA and microbial mass could be complimentary to other tannin assays. It enables study of the partitioning of nutrients to gases, SCFA and microbial mass. This information can be used to determine degradability of feeds, efficiency of microbial protein synthesis and production of gases. This method also determines nutritional significance of bound or unextractable tannins. A disadvantage of this method is that it does not give information on the post-rumen effects of tannins. The detergent system of fibre analysis should be used with caution for characterising tannin-rich feeds. Enhancement of feeding value of tannin-rich feeds can be achieved by anaerobic storage in the presence or absence of urea or by the use of oxidising agents, white rot fungi or PEG in the slow release form.

References

Beever, D.E. 1993. Ruminant animal production from forages — present position and future opportunities. In: Baker, J.M. ed. Grassland for our World. 158–164. SIR Publishing, Wellington.

Blümmel, M., Makkar, H.P.S. and Becker, K. 1997. In vitro gas production: a technique revisited. J. Anim. Physiol. Anim. Nutr., 77: 24–34.

Degen, A.A., Becker, K., Makkar, H.P.S. and Borowy, N. 1995. *Acacia saligna* as a fodder tree for desert livestock and the interaction of its tannins with fibre fractions. J. Sci. Food Agric., 68: 65–71.

Gamble, G.R., Akin, D.E., Makkar, H.P.S. and Becker, K. 1996. Biological degradation of tannins in *Sericea lespedeza* by the white rot fungi *Ceriporiopsis subvermispora* and *Cyathus stercoreus* analysed by solid state ^{13}C NMR spectroscopy. Appl. Environ. Microbiol., 62: 3600–3604.

Getachew, G. 1999. Tannins in tropical multipurpose tree species: Localisation and quantification of tannins using histochemical approaches and the effect of tannins in invitro rumen fermentation. PhD thesis, University of Hohenheim, Stuttgart, Germany.

Getachew, G., Blümmel, M., Makkar, H.P.S. and Becker, K. 1998. In vitro gas measuring techniques for assessment of nutritional quality of feeds: a review. Anim. Feed Sci. Technol. 72: 261–281.

Hagerman, A.E., Zhao, Y. and Johnson, S. 1997. Methods for determination of condensed and hydrolysable tannins. In: Shahidi, F. ed. Antinutrients and Phytochemicals in Foods, American Chemical Society (ACS Symposium Series 662), 209–222.

Hanley, T.A., Robbins, C.T., Hagerman, A.E. and McArthur, C. 1992. Predicting digestible protein and digestible dry matter in tannin-containing forages consumed by ruminants. Ecology, 73: 573–541.

Leng, R.A. 1993. Quantitative ruminant nutrition — a green science. Aust. J. Agric. Res., 44: 363–380.

Makkar, H.P.S. 1989. Protein precipitation methods for quantitation of tannins: a review. J. Agric. Food Chem., 37: 1197–1202.

Makkar, H.P.S., Blümmel, M. and Becker, K. 1995a. Formation of complexes between polyvinyl pyrrolidone and polyethylene glycol with tannins and their implications in gas production and true digestibility in in vitro techniques. British Journal of Nutrition, 73: 897–913.

Makkar, H.P.S., Blümmel, M. and Becker, K. 1995b. In vitro effects and interactions of tannins and saponins and fate of tannins in rumen. J. Sci. Food Agric., 69: 481–493.

Makkar, H.P.S., Borowy, N.K., Becker, K. and Degen, A. 1995c. Some problems in fibre determination of a tannin-rich forage (*Acacia saligna* leaves) and their implications in in vivo studies. Animal Feed Science and Technology, 55: 67–76.

Makkar, H.P.S. and Becker, K. 1996a. A bioassay for polyphenols (tannins). Polyphenol Communications 96, Bordeaux (France), 197–198.

Makkar, H.P.S. and Becker, K. 1996b. Effect of pH, temperature, and time on inactivation of tannins and possible implications in detannification studies. J. Agric. Food Chem., 44: 1291–1295.

Makkar, H.P.S., Blümmel, M. and Becker, K. 1997a. In vitro rumen apparent and true digestibility of tannin-rich forages. Animal Feed Science and Technology, 67: 245–251.

Makkar, H.P.S., Becker, K. and Younan, M. 1997b. Nutritional implications of bound proanthocyanidins. Proc. Eighteenth International Grassland Congress, June 8–12, Winnipeg, Canada.

Makkar, H.P.S. and Becker, K. 1998. Adaptation of cattle to tannins: role of proline-rich proteins in oak-fed cattle. Anim. Sci., 67: 277–281.

Makkar, H.P.S., Blümmel, M. and Becker, K. 1998. Application of an in vitro gas method to understand the effects of natural plant products on availability and partitioning of nutrients. In: Deaville, E.R., Owen, E., Adesogan, A.T., Rymer, C., Huntington, J.A. and Lawrence, T.L.J. ed. In vitro Techniques for Measuring Nutrient Supply to Ruminants. Edinburgh: BSAS No. 22. 147–150.

Makkar, H.P.S. and Singh, B. 1992. Detannification of oak leaves: treatments and their optimisation. Animal Feed Science and Technology, 36, 113–127.

Makkar, H.P.S. and Singh, B. 1993. Effect of storage and urea addition on detannification and in sacco dry matter digestibility of mature oak (*Quercus incana*) leaves. Animal Feed Science and Technology, 41: 247–259.

Makkar, H.P.S., Singh, B. and Kamra, D.N. 1994. Biodegradation of tannins in oak (*Quercus incana*) leaves by *Sporotricum pulverulentum*. Letters Appl. Microbiol., 18: 39–41.

Menke, K.H., Raab, L., Salewski, A., Steingass, H., Fritz, D. and Schneider, W. 1979. The estimation of the digestibility and metabolisable energy content of ruminant feedstuffs from the gas production when they are incubated with rumen liquor in vitro. J. Agric. Sci., (Cambridge) 92: 217–222.

Menke, K.H. and Steingass, H. 1988. Estimation of the energetic feed value obtained from chemical analysis and in vitro gas production using rumen fluid. Anim. Res. Develop., 28: 7–55.

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

VanSoest, P.J., Robertson, J.B. and Lewis, B.A. 1991. Methods for dietary fibre, neutral detergent fibre, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci., 74: 3583–3597.

Waterman, P.G. and Mole, S. 1994. Analysis of Phenolic Plant Metabolites. Blackwell Scientific Publications, Oxford, UK.

Influence of Dietary Condensed Tannin on Microbial Crude Protein Supply in Sheep

D.M. McNeill,[1] M.K. Komolong,[1] N. Gobius[1] and D. Barber[1]

Abstract

Excessive intakes of condensed tannin (CT) are associated with a reduction in N retention in ruminants. It is commonly presumed that part of this reduction is explained by an inhibitory effect of CT on the production of microbial crude protein (MCP). Recent data from our group questions this presumption. Neutralisation of CT in Leucaena foliage, by supplementing sheep fed Leucaena with the tannin-neutralising agent polyethylene glycol (PEG), induced no change in MCP production. Similarly, MCP production remained unchanged when sheep fed lucerne hay were supplemented with increasing amounts of Quebracho CT. Some decline was observed when MCP production was compared in sheep fed Leucaena genotypes differing in CT content. The less than expected effects of CT at the level of the rumen lend support to the hypothesis that CT exert their major effects post-ruminally.

CONDENSED TANNINS (CTs) interfere with the digestion of protein via their ability to form reversible complexes with protein (Mangan 1988; Spencer et al. 1988; Perez-Maldonado et al. 1995). Our interest is in defining the points along the ruminant digestive tract at which CTs exert their greatest effects (McNeill et al. 1998). In the rumen, CTs are expected to bind strongly to protein and protect them from degradation by rumen microbes. Excessive intakes of CT should therefore starve the rumen microbes of rumen degradable nitrogen (RDN) such that the production of microbial crude protein (MCP) is depressed (Mangan 1988; Barry et al. 1986; Perez-Maldonado et al. 1995). This effect could help to explain why high CT diets reduce whole tract apparent digestibility of nitrogen and N retention in ruminants (Mangan 1988; Kumar and Vaithiyanathan 1990; McNeill et al. 1998; Foley et al. 1999). The aim of the following experiments was to quantify the effect of CT intake on MCP production.

[1] School of Land and Food, University of Queensland, Australia 4072

Materials and Methods

Four N balance experiments are described in which the influence of CT on the production of MCP by sheep was measured. MCP productions in each were estimated by monitoring total urinary excretion of purines derivatives over the urine collection periods given below (Chen et al. 1990). CT contents were determined using the butanol/HCl method, with modification as described by Dalzell and Kerven (1998), and in each assay the same batch of purified *Leucaena pallida* CT was used as a standard.

In experiments 1 and 2, the CT neutralising agent polyethylene glycol (PEG, MW = 4000) was added to 100% diets of either *L. leucocephala* (K636) or the *L. leucocephala* × *L. pallida hybrid* (KX2) foliage.

1. *L. leucocephala* was fed fresh, with or without PEG, to weaner lambs. A further group was fed a 100% diet of lucerne chaff as a positive control (n = 5/treatment, mean LW = 35 kg). The foliage was harvested daily and fed at a rate of approximately 1 kg DM/sheep/day by automatic feeder in equal portions every 2 hours for 20 hours/day. Either PEG in water (50 g in 100 mL) or water was applied as a foliar spray to each sheep's ration at the beginning of each day. A 5-day

KEYWORDS: Condensed tannins, Microbial crude protein (MCP), Polyethylene glycol (PEG), Rumen degradable nitrogen (RDN)

acclimatisation period was followed by 7 days total collection of faecal and urine outputs for the determination of apparent digestibility of N, N retention, and purine derivative excretion.

2. Eighteen wethers were randomly allocated into 3 treatments: (1) lucerne chaff; (2) KX2 chaff – PEG; and (3) KX2 chaff + PEG. Wethers (n = 6/ treatment, mean LW = 31.5 kg) were fed their treatment diets for 12 days, with urine and faeces collected over the final 6 days.

In experiment 3, 100% diets of 4 species of Leucaena that differed dramatically in CT content were compared. *L. collinsii* (OFI 51/88), *L. leucocephala* (K636), *L. pallida* (CQ 3439) and *L. trichandra* (OFI 53/88) were fed to lambs (mean LW = 24 kg, n = 6/treatment) at a rate of 0.75 kg DM/ day. They were given 7 days to acclimatise to the diets and total urine and faecal collections made for 7 days thereafter.

In experiment 4, the impact of the addition of increasing amounts of a commercial CT extract from Quebracho (*Schinopsis* spp.) (Unitan, Superior ATO grade, 73% w/w CT by sephadex LH20) to weaner sheep (mean LW = 34 kg) fed 1 kg of lucerne chaff/ day was assessed. The Quebracho extract was fed at 0, 20, 40 or 60 g/day, with 3 sheep per feeding level. The lucerne chaff was offered by automatic feeder in equal portions every 2 hours for 20 hours/day. The Quebracho extract was offered by mixing the daily dose/sheep in 1 L of warm water and orally dosing each sheep their 1 L/day in 4 equal aliqouts over each 24 hour period, approximately every 6 hours. The sheep were acclimatised to their diets for 10 days, followed by total urine and faecal collections for the next 7 days.

Results and Discussion

In each experiment, an increase in the amount of dietary CT was associated with a reduction in whole-tract apparent digestibility of nitrogen and an increase in faecal N (Table 1, Figure 1). These endpoints are consistent with those generally reported in the literature (see reviews by McNeill et al. 1998; Foley et al. 1999), and show that in our experiments the CTs were active. Yet, across experiments, we were unable to demonstrate an unambiguous reduction in MCP production due to an increased intake of active CT.

Two of the experiments provide the most reliable evidence that CT per se do not reduce MCP production (i.e. *L. leucocephala* +/– PEG, Leucaena KX2 +/– PEG experiments; Table 1). The tannin neutralising agent PEG was used in each which means the diets compared differed only in their contents of active CT. This lack of effect is particularly convincing given the much higher dietary concentrations of CT used (7.3–12.9% DM) as compared to those normally reported in the literature (Waghorn et al. 1994; Perez-Maldonado and Norton 1996; McSweeney et al., these Proceedings).

In vitro, CT commonly depress gas production by rumen microbes (Makkar et al. 1995a; Nelson et al. 1997; McSweeney et al. and Theodorou et al., these Proceedings). Based on this, we were surprised to find no effect of CT on MCP production in vivo. Yet, the work of Makkar and colleagues highlights the dangers of using gas production in vitro as indicative of changes in MCP production. Their data consistently show CT depress gas production. However, the effect of CT on MCP production is unpredictable. For example, Makkar et al. (1995b) noted Quebracho CT stimulated up to a 39% increase in the incorporation of ^{15}N into apparent digested residue. In contrast, ^{15}N incorporation into the apparently digested residue of *Dichostachis cinerea* was 16% less when its CT were active compared to when they were deactivated with PEG (Makkar et al. 1998). Getachew et al. (1998) noted either a nil or positive effect of active CT in Calliandra leaves on MCP production, by incubating them with or without PEG. A clear conclusion from these in vitro studies is that CT improve the efficiency of MCP production, defined as weight of MCP per unit of gas produced or short chain fatty acid (SCFA). This increase in efficiency raises the possibility that in situations where MCP remains unaffected by CT, as we have observed in vivo, CT could limit the performance of ruminants by reducing their supply of metabolisable energy.

The lack of an in vivo effect of CT on MCP production in our experiments involving PEG could be the result of the high levels of crude protein in the Leucaenas tested (approximately 19–24% DM). There may well have been enough protein for the CT to bind protein to its maximal extent, leaving sufficient RDN for the needs of the rumen microbes. Additionally, microbes in vivo may be advantaged by a steadier supply of RDN compared to those in vitro, via nitrogen recycling to the rumen. In vivo, a significant proportion of feed protein bypasses the rumen through complexation with CT, but is then released at the level of the abomasum for digestion. The absorbed nitrogen not used for tissue accretion would be converted to plasma urea and potentially diffuse back into the rumen. CT in the rumen would not be expected to interfere with the availability of N in the form of urea for MCP synthesis.

The data of Waghorn et al. (1994), Perez-Maldonado and Norton (1996), and McSweeney et al. (these Proceedings) support our observations of minimal if any effects of CT on MCP in vivo. Sheep

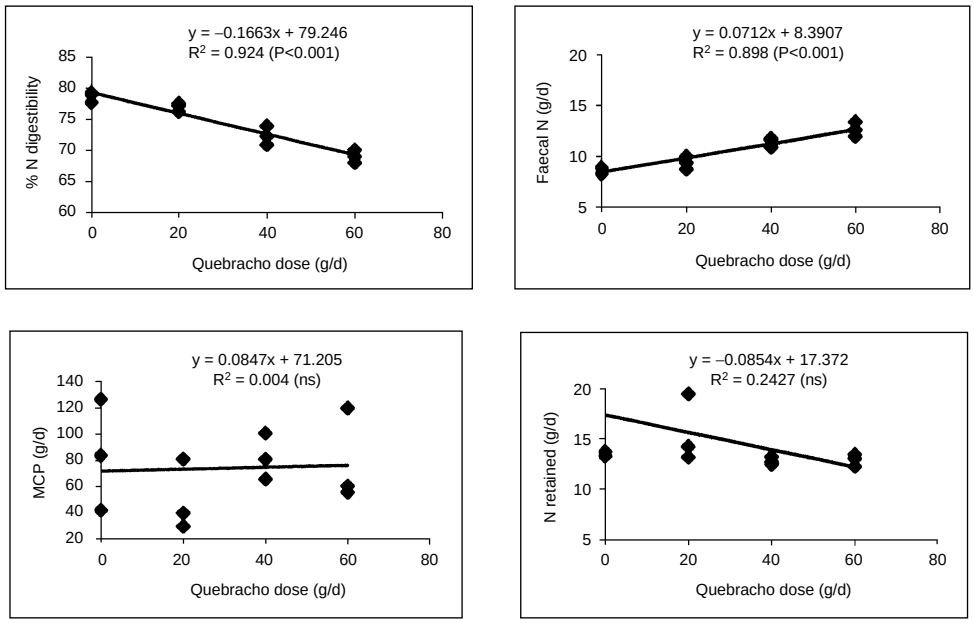


Figure 1. Nitrogen responses and microbial crude protein production (MCP) in sheep supplemented with increasing amounts of CT rich Quebracho extract against a basal diet of lucerne chaff. Individual points represent individual sheep.

Table 1. Nitrogen utilisation and microbial crude protein (MCP) responses relative to digestible organic matter intake (DOMI) in sheep fed diets differing in the content of active condensed tannin (CT).

Treatment	N digestibility (%)	Faecal N (g/d)	N retention (g/d)	DOMI (g/d)	MCP (g/d)
L. leucocephala (CT = 7.3 %):					
CT active	64.2a	11.3a	7.21	570	110.0a
CT neutralised	77.5b	7.5b	10.37	603	115.6ab
Lucerne chaff	77.8b	7.6b	12.6	611	135.6b
sem	1.2	0.3	1.37	27	7.4
Leucaena KX2 (CT = 12.9 %):					
CT active	43.2a	15.9a	3.01a	444a	60.9a
CT neutralised	71.4b	8.1b	4.96b	520b	65.6a
Lucerne chaff	76.5b	6.9c	5.51b	536b	80.3b
sem	0.8	0.2	0.29	6.2	3.8
L. collinsii (CT = 0.6 %)	80.5a	4.3a	4.70a	309a	64.8a
L. leucocephala (CT = 3.8 %)	66.4b	6.0b	2.46ab	310a	61.6a
L. pallida (CT = 5.8 %)	37.5c	10.9d	−0.01bc	253b	42.5b
L. trichandra (CT = 6.5 %)	37.8c	9.7c	−0.53c	199c	50.5ab
sem	2.2	0.4	0.94	15	6.0

CT content expressed as % of forage DM offered, and determined on freeze-dried feed for the *L. leucocephala* +/− PEG experiment, or on oven-dried feed for the other two experiments.
Within a column, for each experiment, means lacking a common script differ (P <0.05).

fed 100% diets of fresh *Lotus pedunculatus* (CT = 5.5% DM) had similar microbial protein fluxes to the abomasum as those on the same diet but with CT inactivated by intraruminal infusions of PEG. Perez-Maldonado and Norton (1996) detected no difference in MCP production between sheep fed pangola hay-based diets supplemented (at 30% of total dry matter intake) with either Centrosema (no detectable CT in whole diet), Desmodium (CT in total diet = 9.5 g/kg DM), or Calliandra (CT in total diet = 22.5 g/kg DM). Similarly, McSweeney et al. showed no change in the MCP produced/kg of digestible organic matter in sheep fed tropical grass hay supplemented at 30% of ad libitum intake with Calliandra (CT in total diet 2–3% DM).

When the highest CT containing Leucaena was fed (Leucaena KX2, CT = 12.9%) with or without PEG, N rentention was reduced by 39% (Table 1). Yet MCP production remained unaltered. Only in the experiment where the 4 Leucaena genotypes were compared was a drop in N retention related to a drop in MCP production. Yet, as PEG was not used in the 4 genotype comparison, we cannot be sure the decline in MCP was due to differences between the genotypes in CT content. Declines in MCP may also have been due to declines in DOMI and N intake. We conclude that a reduction in MCP is not a major mechanism of action of CTs in sheep fed high nitrogen legumes such as Leucaena and lucerne. Our focus now remains on 2 other potential mechanisms of CT action in ruminants. These are 1) that observed reductions in N balance due to high CT diets are a function of an exacerbated obligatory loss of endogenous protein post-ruminally, and 2) that CT vary in their ability to release protein in the acidic environment of the abomasum. These mechanisms are discussed in more detail by McNeill et al. (1998).

Our data also conflict with the hypothesis that small amounts of CT improve animal performance (Barry et al. 1986; Mangan 1988). Barry and McNabb (these Proceedings) review several studies in which CT from Lotus spp. improve milk and wool production, and reproductive rates in grazing ruminants. The proposed mechanism behind the hypothesis is that small amounts of CT promote the escape of protein from the rumen without reducing MCP production such that the post-ruminal delivery of protein is enhanced. Yet in our experiments we were unable to demonstrate any improvements in N balance due to low contents of CT. *L. leucocephala* was no better than the virtually CT free *L. collinsii* (Table 1). Neither was an increase in N balance detectable when small amounts of Quebracho CT were added to a basal diet of lucerne hay (Figure 1). Others have also found no positive effects of low to moderate dietary intakes of CT on animal performance (Nunez-Hernandez et al. 1991; Waghorn et al. 1994; Waghorn and Shelton 1995; Mashudi et al. 1997; Douglas et al. 1999). The lack of positive effect in these studies may be a consequence of a lack of metabolisable energy in the diets to match any improvement in the delivery of metabolisable protein (McNabb et al., these Proceedings). Such a hypothesis is consistent with the in vitro observations of Makkar et al. (1998), that CT have a disproportionately negative effect on VFA relative to MCP production per unit of feed digested. We also speculate that the difficulty in defining a positive effect of CT on N retention in the whole animal may be a consequence of CT advantaging only specific tissues; a function of the ability of CT to change the profile of absorbed amino acids protein (McNabb et al., these Proceedings), or that some tissues are more responsive to an improvement in protein to energy supply than others (e.g. wool v. liveweight gain, Black and Reis 1979).

It is concluded that CT from Leucaena spp. and Quebracho consistently reduce N digestibility and enhance the excretion of faecal N. Leucaena CT can reduce N retention in sheep, but it is questionable whether such reductions are due to effects of CT on MCP production in the rumen.

References

Barry, T.N., Manley, T.R. and Duncan, S.J. 1986. The role of condensed tannins in the nutritional value of *Lotus pedunculatus* for sheep. 4. Sites of carbohydrate and protein digestion as influenced by dietary reactive tannin concentration. British Journal of Nutrition, 55: 123–137.

Black, J.L. and Reis, P.J. 1979. Speculation on the control of nutrient partition between wool growth and other body functions. In: Black, J.L. and Reis, P.J. ed, Physiological and Environmetal Limitations to Wool Growth. University of New England Publishing Unit, Armidale NSW, 269–294.

Chen, X.B., Hovell, F.D.DeB., Orskov, E.R. and Brown, D.S. 1990. Excretion of purine derivatives by ruminants: effect of exogenous nucleic acid supply on purine derivative excretion by sheep. British Journal of Nutrition, 63: 131–142.

Dalzell, S.A., and Kerven, G.L. 1998. A rapid method for the measurement of Leucaena spp proanthocyanidins by the proanthocyanidin (Butanol/HCl) assay. Journal of the Science of Food in Agriculture, 78: 405–416.

Douglas, G.B., Stienezen, M., Waghorn, G.C., Foote, A.G. and Purchas, R.W. 1999. Effect of condensed tannins in birdsfoot trefoil (*Lotus corniculatis*) and sulla (*Hedysarum coronarium*) on body weight, carcass fat depth, and wool growth of lambs in New Zealand. New Zealand Journal of Agricultural Research, 42: 55–64.

Foley, W.J., Iason, G.R. and McArthur, C. 1999. Role of plant secondary metabolites in the nutritional ecology of the mammalian herbivores: how far have we come in 25 years? In: Jung, H.J. and Fahey, Jr. G.C. ed. Nutritional Ecology of Herbivores. Proceedings of the Fifth International Symposium on the Nutrition of Herbivores. American Society of Animal Production, Illinois, USA. 130–209.

Getachew, G., Blümmel, M., Makkar, H.P.S. and Becker, K. 1998. In vitro gas measuring techniques for assessment of nutritional quality of feeds: a review. Animal Feed Science and Technology, 72: 261–281.

Kumar, R. and Vaithiyanathan, S. 1990. Occurrence, nutritional significance and effect on animal productivity of tannins in tree leaves. Animal Feed Science and Technology, 30: 21–38.

McNeill, D.M., Osborne, N.J., Komolong, M. and Nankervis, D. 1998. Condensed tannins in the genus Leucaena and their nutritional significance for ruminants. In: Shelton, H.M., Gutteridge, R.C. and Mullin, B. ed. Proceedings of a Workshop on Leucaena — Adaptation, Quality and Farming systems. ACIAR: Canberra. 205–214.

Makkar, H.P.S., Blümmel, M. and Becker, K. 1995a. Formation of complexes between polyvinyl pyrrolidonines or polyethylene glycols and tannins, and their implication in gas production and true digestibility in in vitro techniques. British Journal of Nutrition, 73: 897–913.

Makkar, H.P.S., Blümmel, M. and Becker, K. 1995b. In vitro effects of and interactions between tannins and saponins and fate of tannins in the rumen. Journal of the Science of Food and Agriculture, 69: 481–493.

Makkar, H.P.S., Blümmel, M. and Becker, K. 1998. Application of an in vitro gas method to understand the effects of natural plant products on availability and partitioning of nutrients. In: Deaville, E.R., Owen, E., Adesogan, A.T., Rymer, C., Huntington, J.A. and Lawrence, T.L.J. ed. In vitro Techniques for Measuring Nutrient Supply to Ruminants. Edinburgh: BSAS No. 22. 147–150.

Mangan, J.L. 1988. Nutritional effects of tannins in animal feeds. Nutrition Research Reviews, 1: 209–31.

Mashudi, Brookes, I.M., Holmes, C.W. and Wilson, G.F. 1997. Effects of Mimosa bark extract containing condensed tannins on rumen metabolism in sheep and milk production by grazing cows. Proceedings of the New Zealand Society of Animal Production, 57: 126–129.

Nelson, K.E., Pell, A.N., Doane, P.H., Giner-Chavez, B.I. and Schofield, P. 1997. Chemical and biological assays to evaluate bacterial inhibition by tannins. Journal of Chemical Ecology, 23: 1175–1194.

Nunez-Hernandez, G., Wallace, J.D., Holechek, J.L., Galyean, M.L. and Cardenas, M. 1991. Condensed tannins and nutrient utilisation by lambs and goats fed low-quality diets. Journal of Animal Science, 69: 1167–1177.

Perez-Maldonado, R.A., Norton, B.W. and Kerven, G.L. 1995. Factors affecting in vitro formation of tannin-protein complexes. Journal of the Science of Food and Agriculture, 69: 291–298.

Perez-Maldonado, R.S. and Norton, B.W. 1996. The effects of condensed tannins from *Desmodium intortum* and *Calliandra calothyrsus* on protein and carbohydrate digestion in sheep and goats. British Journal of Nutrition, 76: 515–533.

Spencer, C.M., Cai, Y., Martin, R., Gaffney, S.H., Goulding, P.N., Magnolato, D., Lilley, T.H. and Haslam, E. 1988. Polyphenol complexation — some thoughts and observations. Phytochemistry, 27: 2397–2409.

Waghorn, G.C., Shelton, I.D., McNabb, W.C. and McCutcheon, S.N. 1994. Effects of condensed tannins in *Lotus pedunculatus* on its nutritive value for sheep. 2. Nitrogenous aspects. Journal of Agricultural Science, Cambridge, 123: 109–119.

Waghorn, G.C. and Shelton, I.D. 1995. Effect of condensed tannins in *Lotus pedunculatus* on the nutritive value of ryegrass (*Lolium perenne*) fed to sheep. Journal of Agricultural Science, Cambridge, 125: 291–297.

Implications of Feeding Condensed Tannin-Containing Forages for Amino Acid and Protein Metabolism in the Lactating Ruminant

W.C. McNabb[1]*, S.L. Woodward[2], G.C. Waghorn[1], T.N. Barry[3] and N. Roy[1]

Abstract

The New Zealand dairy industry relies on year-round utilisation of fresh temperate forages, but conventional pastures are constraining milk production. A major problem is that the rapid and extensive microbial degradation of forage protein that occurs during digestion prevents sufficient essential amino acids (EAA) from reaching the duodenum to meet animal requirements. This has focused attention on forages containing condensed tannins (CT; proanthocyanidins) because the CT in *Lotus corniculatus* has been shown to increase milk yield and milk protein concentration when fed to dairy cows in late lactation. However, while the CT in *L. corniculatus* has increased the apparent absorption of EAA from the small intestine of sheep, the CT in *Lotus pedunculatus* did not improve this absorption. Furthermore, the CT in a mixed diet consisting of *L. pedunculatus* and ryegrass (1:2 ratio) reduced nitrogen (N) digestion by a similar amount as CT in *L. pedunculatus* fed as a sole diet. We do not fully understand how CT affects amino acid absorption from the small intestine. The concentration of CT in the diet is important, but more recent research suggests that the chemical structure and source of the CT may be equally important when evaluating the merit of specific forages for use in grazing systems. The current research focus of the Nutrition Group at AgResearch is to measure in vivo the effect of CT on amino acid transport across the small intestine in lactating ewes fed both *L. corniculatus* and *L. pedunculatus*. Consequences of changes in EAA absorption for net flux (and transport rate and intracellular kinetics) of amino acid(s) across the mammary gland and their incorporation into specific milk proteins will also be studied.

WORLD-WIDE consumption of milk protein, both as food products and as isolated compounds, is increasing. Attention has been focused on strategies capable of increasing both yield and efficiency of milk protein production. The New Zealand dairy industry relies on year-round utilisation of fresh temperate forages. However, feeding pasture imposes several limitations. In particular, there is extensive rumen fermentation of dietary protein to peptides, amino acids and ammonia, some of which is incorporated into microbial protein. However, the rapid release of ammonia from forage protein often exceeds ammonia incorporation into microbial protein, resulting in 20–35% of N being lost as ammonia absorbed from the rumen (MacRae and Ulyatt 1974).

When ruminants fed on fresh forages have been given abomasal infusions of protein or fed supplementary protein protected from rumen degradation, milk production has been increased in dairy cattle (Rogers et al. 1980) and sheep (Penning et al. 1988). This suggests that productivity is limited when insufficient EAA are absorbed from the small

[1] Nutrition Group, AgResearch, Grasslands Research Centre, Private Bag 11008, Palmerston North, New Zealand
[2] Dairying Research Corporation, Private Bag 3123, Hamilton, New Zealand
[3] Institute of Food, Nutrition and Human Health, Massey University, Private Bag 11222, Palmerston North, New Zealand
*Corresponding author: Phone +64-6-356 8019; Fax +64-6-351 8003; E-mail mcnabbw@agresearch.cri.nz

KEYWORDS: Condensed tannins, Protein metabolism, Lactating ruminant, Amino acid absorption, Essential amino acids (EAA), Proanthocyanidins

intestine relative to energy. Reduced availability of amino acids for peripheral tissues is due, in part to substantial losses of dietary protein from the rumen as ammonia and also to the catabolism of absorbed amino acids for use in ureagenesis in the liver (Lobley et al. 1995).

Several studies have shown that low concentrations of condensed tannins (CT; proanthocyanidins) in the diet increase non-ammonia-nitrogen (NAN) flow to the intestine, reduce degradation of protein in the rumen, can increase apparent EAA absorption from the small intestine and can increase sheep productivity (for review, see Barry and McNabb 1999). Such results have caused a resurgence of interest in forages containing CT and suggest these forages may have potential as feeds for dairy cattle production systems.

Effect of Condensed Tannins on Lactation

Wang et al. (1996) conducted a grazing experiment to study the effects of CT in *L. corniculatus* on lactation performance of ewes rearing twin lambs. In that study, effects of CT were elucidated by comparing ewes orally supplemented with polyethylene glycol (PEG), with ewes which had not received PEG. Polyethylene glycol preferentially binds and inactivates CT. The milk yield and composition were similar for control (CT acting) and PEG-supplemented (CT not acting) ewes at peak lactation. However, as the lactation progressed, control ewes experienced a slower decline in milk production. In mid and late lactation, control ewes were producing more milk (21%) and more milk protein (14%) than the comparable PEG-supplemented ewes.

Woodward et al. (1999) reported that CT in *L. corniculatus* fed to dairy cows in late lactation also increased milk and milk protein yield, and milk protein concentration. In that study, CT was responsible for 57% of the increase in the milk protein concentration because cows fed *L. corniculatus* had a higher milk protein concentration (3.61%) than comparable cows fed *L. corniculatus* supplemented with PEG (3.44%), ryegrass (3.31%) or ryegrass supplemented with PEG (3.30%). Changes in lactation performance were not a consequence of changes in intake because CT did not affect this parameter in either study. These results suggest that CT-containing legumes like *L. corniculatus* have potential as forages for dairy cows. However, difficulties with establishment, low competitive ability, poor winter growth and other agronomic problems (Waghorn et al. 1998) currently limit their widespread use in temperate grazing systems.

Effect of Condensed Tannins on the Digestion of Amino Acids

Waghorn et al. (1987) demonstrated that CT in *L. corniculatus* (22 g/kg DM) fed to sheep increased abomasal flux of EAA by 50%. This change was associated with increased (63%) apparent absorption of EAA from the small intestine (Waghorn et al. 1987). Not all EAA were affected equally, apparent absorption of valine (89%), isoleucine (94%), phenylalanine (93%) and histidine (90%) were increased to a much a greater extent than other EAA. While the abomasal flux of non-essential amino acids (NEAA) was also increased (14%) by the CT, a significant reduction (20%) in the digestibility of NEAA in the small intestine resulted in the apparent absorption of NEAA being similar in control and PEG-supplemented sheep (see Table 1). The ratio of EAA:NEAA absorbed from the small intestine was 1.57 and 0.87 for control and PEG-supplemented sheep, respectively. The ratio of EAA:NEAA in the *L. corniculatus* was 1.14 and in rumen bacteria it was about 1.08, so that the value of 1.57 for control sheep could only arise from selective absorption of EAA.

Table 1. The effect of condensed tannins (CT) on amino acid digestion in the small intestine of sheep fed *Lotus corniculatus* (22 g CT/kg DM) or *Lotus pedunculatus* (55 g CT/kg DM) with (−CT) or without (+CT) a continuous intraruminal infusion of polyethylene glycol (PEG; MW 3500).

	Lotus corniculatus[1]		*Lotus pedunculatus*[2]	
	+CT	−CT	+CT	−CT
N Intake (g N/d)	37.8	37.8	42.4	47.6
N Digestibility	0.70	0.78	0.67	0.81
Abomasal NAN Flux	29.5	25.8	34.0	31.3
Abomasal EAA Flux	95.6	63.9	121.1	105.6
Apparent EAA Absorption	58.8	36.1	81.4	83.5
EAA Digestibility in SI	0.69	0.65	0.66	0.79
Abomasal NEAA Flux	68.5	60.0	84.3	77.7
Apparent NEAA Absorption	37.4	41.3	50.8	57.2
NEAA Digestibility in SI	0.55	0.69	0.59	0.73

[1] Waghorn et al. (1997)

[2] Waghorn et al. (1994)

N, nitrogen; NAN, non-ammonia-nitrogen; EAA, essential amino acids; SI, small intestine, NEAA, nonessential amino acids

We have yet to fully understand how CT affects amino acid absorption from the small intestine. While concentration in the diet is important, more recent research suggests that other factors like the chemical structure and source of the CT may be equally important. The CT in *L. pedunculatus* (55 g/kg DM) also increased (by 15%) the flux of EAA through the abomasum (Waghorn et al. 1994).

However, unlike *L. corniculatus*, this CT reduced apparent digestibility of EAA in the small intestine by 13 percentage units. The net effect of these changes was that apparent absorption of EAA from the small intestine was unaffected by CT (see Table 1). In that experiment, the CT in *L. pedunculatus* reduced N digestibility by 12 percentage units and voluntary intake by 12%. In sheep fed a mixed diet consisting of *L. pedunculatus* and ryegrass (*Lolium perenne*) with a final CT concentration in the mixed diet of 18 g CT/kg DM, N digestibility was reduced by 13 percentage units (Waghorn and Shelton 1995) and the effects of CT were similar to *L. pedunculatus* fed as a sole diet.

Chemical Structure and Reactivity of Condensed Tannins

Variation in nutritional responses to CT from different forages is affected by the concentration and the chemical structure of the CT (for review, see Barry and McNabb 1999). The chemistry of CT is complex. Differences occur in the hydroxylation of the B-ring of the constitutive flavan-3-ol units. The stereochemistry of the heterocyclic C-rings takes the form of 2,3-cis or 2,3-trans and this dictates how flavan-3-ol sub-units are attached relative to one another. Constitutive flavan-3-ol units are linked by either C4/C8 or C4/C6 interflavanoid bonds, and this affects the final shape of the polymer. The number of constitutive flavan-3-ol units also varies. These differences produce an infinite number of chemical structures, which, in turn, affect the reactivity of the CT during digestion.

The CT from *L. corniculatus* and *L. pedunculatus* differ considerably in their chemical structures (Foo et al. 1996, 1997). The average molecular weight (MW) of CT in *L. pedunculatus* is 2200, while in *L. corniculatus* it is 1900. The CT in *L. pedunculatus* contains, predominantly prodelphinidin subunits with epigallocatechin (64%) dominating this CT. The CT from *L. corniculatus* is predominantly procyanidin with epicatechin (67%) dominating this CT.

The CT from *L. pedunculatus* was more effective at reducing the degradation of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) protein by rumen micro-organisms than the CT from *L. corniculatus* (Aerts et al. 1999). This suggests that the extent to which CT effect protein degradation may be responsive to differences in the chemical structure of the CT. Clearly, concentration of CT and the chemical structure of that CT will need to be considered when assessing the merit of particular forages for use in any grazing system.

Effects of Condensed Tannins on Protein and Amino Acid Metabolism: Future Research

Future research will improve our understanding of the mechanisms by which CT effect amino acid absorption from the small intestine and the consequences for transport of EAA from the lumen of the small intestine to the mesenteric-drained circulation and the flux of EAA to the mammary gland (and other tissues). These complex physiological functions can be addressed by preparing sheep with arterio-venous catheters across the small intestine, liver and mammary gland (or other tissues). The Nutrition Group has developed surgical preparations that enable amino acid fluxes to be partitioned across organs and tissues. Fluxes are interpreted using a mathematical model (Biolo et al. 1995) to obtain a quantitative understanding of the amino acid exchanges between tissues and their metabolic fates within tissues. This approach can be applied to any tissue(s) including the small intestine, liver, mammary gland or the muscles where the catheterisation of blood supply to and from the tissue(s) is surgically feasible.

Roy et al. (1999) has demonstrated the power of combining this surgical preparation with the interpretative model in a study of glutamine supplementation on the partitioning of amino acids within the hind-limbs of undernourished sheep. Their measurement of arterio-venous balance of circulating and labelled amino acids ([d5]-phenylalanine) together with free phenylalanine isotopic enrichment in muscle, enabled the fluxes between arterial supply to the intracellular pool (inward transport), and from the intracellular pool to the venous drainage (outward transport) to be calculated. The technique also enabled a more precise calculation of protein synthesis because the actual flux of phenylalanine into and out of cells was estimated. This was not possible when the calculations were based on the more conventional arterio-venous (A-V) technique.

The current focus of the Nutrition Group is to modify the Biolo approach to measure the transport of amino acids from both the lumen of the small intestine and the arterial supply to the mesenteric-drained circulation. Transport rates will be estimated using an infusion of labelled amino acid (in this case [3,4-3H]-valine) into the vascular compartment (including measuring blood flow) and an infusion of a second label of the same amino acid (in this case [1-13C]-valine) into the small intestine (via the abomasum, together with measurement of digesta flow). *Lotus corniculatus* and *L. pedunculatus* are ideal forages for comparison with lactating ewes because these forages contain CT which have quite different effects on the apparent absorption of EAA

from the small intestine. This approach will enable us to make significant progress towards understanding the mechanisms behind how CT effect the transport of amino acids from the lumen of the small intestine to the mesenteric-drained circulation and the consequence of this for the synthesis of milk protein by the lactating mammary gland.

References

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

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

Biolo, G., Declan Fleming, R.Y., Maggi, S.P. and Wolfe, R.R. 1995. Transmembrane transport and intracellular kinetics of amino acids in human skeletal muscle. American Journal of Physiology, 268 (Endocrinology Metababolism 31): E75–E84.

Foo, L.Y., Newman, R., Waghorn, G., McNabb, W.C. and Ulyatt, M.J. 1996. Proanthocyanidins from *L. corniculatus*. Phytochemistry, 41: 617–624.

Foo, L.Y., Li, Y., McNabb, W.C., Waghorn, G.C. and Ulyatt, M.J. 1997. The proanthyocyanidins of *L. pedunculatus*. Phytochemistry, 45: 1689–1695.

Lobley, G.E., Connell, A., Lomax, M.A., Brown, D.S., Milne, E., Calder, A.G. and Farningham, D.A.H. 1995. Hepatic detoxification of ammonia in the ovine liver: possible consequences for amino acid catabolism. British Journal of Nutrition, 73: 667–685.

MacRae, J.C. and Ulyatt, M.J. 1974. Quantitative digestion of fresh herbages by sheep. II. The sites of digestion of some nitrogenous constituents. Journal of Agricultural Science, Cambridge, 82: 309–319.

Penning, P.D., Orr, J.R. and Treacher, T.T. 1988. Responses of lactating ewes, offered fresh herbage indoors and when grazing, to supplements containing differing protein concentrations. Animal Production, 46: 403–415.

Rogers, G.L., Porter, R.H.D., Clark, T. and Stewart, J.A. 1980. Effect of protected casein supplements on pasture intake, milk yield and composition of cows in early lactation. Australian Journal of Agricultural Research, 31: 1146–1152.

Roy, N., Holtrop, G. and Lobley, G.E. 1999. Amino acid metabolism across the hind-quarters of undernourished sheep supplemented with glutamine. Proceedings of the 8th International Symposium on Protein Metabolism and Nutrition, Aberdeen, Scotland (in press).

Waghorn, G.C., Douglas, G.B., Niezen, J.H., McNabb, W.C. and Foote, A.G. 1998. Forages with condensed tannins — their management and nutritive value for ruminants. Proceedings of the New Zealand Grasslands Association (in press).

Waghorn, G.C. and Shelton, I.D. 1995. Effect of condensed tannins in *L. pedunculatus* on the nutritive value of ryegrass (*Lolium perenne*) fed to sheep. Journal of Agricultural Science, Cambridge, 125: 291–297.

Waghorn, G.C., Shelton, I.D., McNabb, W.C. and McCutcheon, S.N. 1994. Effects of condensed tannins in *L. pedunculatus* on its nutritive value for sheep. 2. Nitrogenous aspects. Journal of Agricultural Science, Cambridge, 123: 109–119.

Waghorn, G.C., Ulyatt, M.J., John, A. and Fisher, M.T. 1987. The effect of condensed tannins on the site of digestion of amino acids and other nutrients in sheep fed on *L. corniculatus*. British Journal of Nutrition, 57: 115–126.

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

Woodward, S.L., Auldist, M.J., Laboyrie, P.J. and Jansen, E.B.L. 1999. Effect of *L. corniculatus* and condensed tannins on milk yield and milk composition of dairy cows. Proceedings of the New Zealand Society of Animal Production (in press).

Tannins in Livestock Feeds in China

Diao Qiyu and Qi Guanghai

Abstract

Sorghum is one of the most important crops in China. It is used mainly as a livestock feed, although it has some use as human food, as well as for brewing. There are about 7000 varieties and hybrids including native and introduced species being cultivated in the country. The quality and quantity of the food supply in China is getting more and more serious as the Chinese population increases. Therefore, it is urgent to explore the use of alternate feeds such as sorghum. The effect of dietary tannins in sorghum on the performance of animals has been the subject of study for many years in China. Three poultry studies, a feeding trial with broiler chicks, a balancing trial with adult leghorn cocks, and a duck study on the effects of digestive enzymes, are reviewed in this paper. The main results are summarised as follows: 1. Broiler chicks could tolerate up to 0.48% and 0.64% dietary tannin in sorghum in the first four weeks and last four weeks of age respectively without any adverse effects on weight gain, feed efficiency, dressing percentage, total serum lipid content, serum cholesterol content and glutamate-pyruvate transaminase levels. However, feeding chicks with sorghum may reduce leg and skin pigmentation. 2. In the balancing trial with adult leghorn cocks, it was observed that as dietary tannin content increased, protein retention, dry matter (DM) digestibility, and metabolic rate of gross energy decreased. However, there was no significant difference between 0% and 0.64% tannin diets on digestibility of DM and the metabolic rate of gross energy. 3. When commercial tannin was added to duck diets at a level of 1.5% DM, it was shown that tannin could significantly reduce total proteinase, trypsin and α-amylase activities in the small intestine of Shaoxing duck.

SORGHUM, sometimes referred to as the great millet, has been grown in China, according to archaeological evidence, for 5000 years. It is one of the most important crops in China and is popular worldwide due to its great range of adaptation, and its multiple functions as feed for livestock and humans, a component of brewing and many other uses. Sorghum straws are often used to make brooms. Prior to the 1960s, it was the staple diet of millions of Chinese in northern China. In the animal industries, as a feed, it is involved in the production of meat, milk, and eggs, and for animal husbandry. As a principal source of an alcoholic beverage, sorghum is used in brewing.

Sorghum is grown in most areas of China, the most important being Liaoning and Shanxi provinces where most of the sorghum research is carried out. At present, there are about 7000 varieties and hybrids including native and introduced species being cultivated in various experimental stations around the country. In 1996, the production of sorghum grain was about 5.67 million metric tonnes which was produced on 1.29 million hectares. The outside colours of the grain are usually white, yellow, red or solemn pink and brown-seeded. Chinese sorghum normally contains 85.2%–95.3% of dry matter, 7.5%–15% of crude protein, 1.4%–4.6% of crude fat, 1.3%–2.6% of crude fibre, and 1.5%–5.0% of crude ash. Of the common cereals, sorghum ranks fifth after wheat, rice, maize and barley, based on their annual output and areas under cultivation (Anon. 1986, 1997).

Feed Research Institute, Chinese Academy of Agricultural Sciences, Beijing 100081, P.R. China

Studies of Tannin in Livestock Feeds in China

Sorghum is widely planted in the tropical and subtropical areas because of its greater resistance to drought and disease than maize or wheat. In China, sorghum is grown mainly in northern areas where

KEYWORDS: Tannins, Sorghum, Digestive enzymes, Livestock feed, Poultry studies, Digestibility

rainfall is marginal. In northern China, sorghum is used as an energy source for livestock. Considerable research has been conducted on the feeding value of sorghum for livestock. Many studies suggest that tannin-containing sorghum is poorly utilised by livestock, especially poultry, compared with no-tannin containing sorghum or maize. One negative characteristic of sorghum is the presence of tannins which lower its nutritional value for non-ruminant animals by reducing retention of protein, digestibility of dry matter and metabolic rate of gross energy (Cao et al. 1985; Diao et al. 1990; Guo 1995). Inhibition of digestive enzyme activity has also been reported (Zhou 1990; Li 1998). The potential to replace maize by sorghum in poultry diets has been frequently investigated in past years. However, those studies were difficult to compare because sorghum cultivars used in different experiments varied significantly in tannin content and methods used for tannin determination. The amount of maize that could be replaced by sorghum depended on the tannin content and other conditions of the livestock.

Investigations of feeding value of sorghum were studied mainly in poultry and pigs in China. Poultry are more sensitive to tannin than other animals. The objectives of this paper were to review the studies on broilers fed tannin containing diets, a balancing trial with adult leghorn cocks and the influence of tannins on digestive enzymes of duck.

Effects of Different Sorghum Tannin Concentrations on the Performance of Broiler Chicks

Materials and methods

Three sorghums (SCS, JLW, JSS), which contained 0.49%, 0.913% and 1.35% of tannin respectively were used. With these sorghums, five diets were formulated to reach tannin levels 0.16%, 0.24%, 0.32%, 0.48% and 0.64% respectively. An additional diet, Diet 1, was a control of corn-soybean meal. All six diets were formulated to contain approximately equal amounts of protein and energy adjusted with soybean oil. They were designed to meet the nutritional requirements of starting and finishing broiler chicks (Table 1). Diets were offered ab libitum in mash form.

In this study, 432 day-old broiler chicks from a commercial hatchery were randomly assigned to 6 dietary treatments of 72 chicks each with four replicates. They were then put into individual cage batteries. The room temperature was maintained at 32 °C from 0 to 7 days, then gradually reduced according to standard brooding practices. Chicks received 24-hour lighting throughout the trial. Water was supplied ad libitum. Feed intake, weight gain, and feed efficiency (F/G) were measured. At the end of trial, 10 birds from each treatment were sacrificed to get carcass data and to collect tissue samples for analysis of physiological parameters. At the end of trial, 30 birds from each dietary treatment were used to score leg and beak pigmentation using the Roche Yolk Colour Fan.

Results and discussion

Body weight and feed efficiency

The performance data from the feeding trial are shown in Tables 2–5. There was no significant effect of sorghum tannin on weight gain over the entire eight weeks (Table 2). Although weight gain for the diet containing 0.64% tannin was significantly lower ($p < 0.05$) during the period of 0–4 weeks, this difference was not evident from 5 to 8 weeks or over the entire period. No significant differences ($p > 05$) attributable to dietary treatment occurred in feed efficiency (Table 2), suggesting that chicks were able to utilise the diets as well as they did the maize control.

Carcass

Slaughter test results indicated that there were no significant differences on carcass production ($p > .05$) among treatments (Table 3) although abdominal fat content was increased with dietary tannin level. This was not due to sorghum tannin because diets were supplemented with increasing soybean oil as the sorghum content changed.

Table 1. Sorghum, tannin and nutrient content of experimental diets in the trial.

Treatment	1	2	3	4	5	6
Sorghum (%)		17.70	26.60	35.50	53.30	57.04
Tannin (%)		0.16	0.24	0.32	0.48	0.64
ME (MJ/kg)	13.05	13.05	13.05	13.05	13.05	13.05
CP (%)	22.05	22.04	22.04	21.95	22.08	22.08
Crude Fat (%)	6.35	7.28	7.69	8.18	9.03	9.23

Table 2. Weight gain and feed efficiency of broiler chicks diet differing in tannin levels*.

Parameter	Period (week)	Treatment (tannin in %)						SEM
		Control	0.16	0.24	0.32	0.48	0.64	
Weight gain (g/bird)	0–4	802[ab]	821[ab]	817[ab]	836[ab]	851[a]	790[b]	72.22
	5–8	1080 [a]	1027[a]	1023[a]	1051[a]	1167[a]	1160[a]	113.20
	0–8	1834[a]	1809[a]	1800[a]	1847[a]	1979[a]	1911[a]	204.12
Feed:Gain	0–4	1.58[a]	1.55[a]	1.55[a]	1.58[a]	1.53[a]	1.64[a]	0.09
	5–8	2.69[a]	2.87[a]	2.81[a]	2.75[a]	2.74[a]	2.61[a]	0.15
	0–8	2.20[a]	2.24[a]	2.23[a]	2.22[a]	2.20[a]	2.20[a]	0.11

[a–b] Means within columns with no common superscript differ significantly ($p < 0.05$).

Table 3. Performance of meat production on broiler chicks with different tannin level at 8 weeks.

Treatment (tannin %)	Control	0.16	0.24	0.32	0.48	0.64
Dressing percentage (%)	90.74[a]	90.67[a]	90.32[a]	91.16[a]	90.30[a]	91.22[a]
Half JT percentage (%)	92.30[a]	91.76[a]	92.73[a]	91.45[a]	93.38[a]	93.63[a]
All JT percentage (%)	75.95[a]	75.55[a]	76.03[a]	75.19[a]	76.85[a]	77.18[a]
Abdominal fat (%)	2.10b[c]	1.80[c]	1.93b[c]	2.38[ab]	2.77[a]	2.68[a]

[a–c] Means within columns with no common superscript differ significantly ($p < 0.05$).
Dressing percentage = (body weight − blood − feathers)/live weight.
Half JT percentage = (Carcass + heart + lung + liver + stomach (without content) + kidney)/(body weight − blood − feathers).
All JT percentage = Carcass/(body weight − blood − feathers).
No significant differences ($p > .05$).

Table 4. Some physiological parameters of broiler chicks with different tannin level at 8 weeks.

Treatment (tannin %)	Control	0.16	0.24	0.32	0.48	0.64
TSL (mg/100 mL)	116.43	133.33	116.44	133.63	117.23	110.96
CHOL (mg/100 mL)	42.37	53.50	38.13	38.75	37.00	35.13
GTP (unit/100 mL)	12.28	15.50	22.25	24.00	13.50	17.00

TSL: total serum lipid; CHOL: cholesterol; GPT: glutamate-pyruvate transaminase.

Table 5. Pigmentation on leg and beak of broiler chicks with different tannin levels at day 56.

Treatment (tannin %)	Control	0.16	0.24	0.32	0.48	0.64
Beak	1.60[a]	1.20	1.15[c]	1.05[d]	1.05[d]	1.00[d]
Leg	4.34[a]	3.42[c]	3.10[d]	2.00[e]	1.66[f]	1.20[g]

[a–g] Means within columns with no common superscript differ significantly ($p < 0.05$).

Physiological parameters

There were no significant differences in total serum lipid content, cholesterol level and glutamate-pyruvate transaminase activity among different dietary treatments ($p > .05$, Table 4). This indicates that sorghum tannin did not affect those parameters analysed in the trials.

To sum up, based on performance, carcass and physiological parameters, dietary maize can be replaced by sorghum grain provided the tannin content does not exceed 0.64%.

Pigment of skin

Pigment in control treatments (corn-soybean diet) was yellow; treatment 6 was near white. Because sorghum has no yellow pigment, higher sorghum-containing diets resulted in light outward pigmentation of broilers. This trait has a negative effect on certain markets.

Nitrogen and Energy Utilisations

Materials and methods

In the balancing trial, 48 adult leghorn cocks were divided into six groups, each consisting of eight animals and were individually housed in metabolic cages. A total collection method was used. The pre-treatment period was 7 days, and during the following 5 days excreta were collected. Excreta were freeze-dried to constant weight and finely

ground for subsequent analysis. The gross energy (GE) content of both diets and excreta were determined by oxygen bomb calorimetry (IKA-Calorimeter, Adiabatisch c 400), while nitrogen content was determined by the macro-Kjeldahl procedure. They were used to investigate the effect of sorghum tannin on the utilisation of nitrogen and dry matter and the metabolic rate of gross energy. In the balancing trial, the diets were the same as those used in the preceding feeding trial.

Results and discussion

Table 6 shows the effect of sorghum tannin on the utilisation of nitrogen and dry matter, and the metabolic rate of gross energy. The dietary tannin content had a significant effect on the retention of nitrogen ($p < .05$) which was decreased with increasing dietary sorghum tannin content. The reason for this phenomenon could be due to formation of insoluble compounds from tannin binding protein excreted in the faeces.

The metabolic rate of gross energy and the digestibility of dry matter were decreased with increasing dietary tannin (Table 6) although the differences among six treatments were not significant ($p > .05$). The reduction in AME could be explained by a reduction in dry matter digestibility (DMD). It was reported that diets containing sorghum tannin could reduce both DMD and digestive enzyme activity. The reduced DMD could be due to tannins forming complex compounds with the carbohydrate components of diets (Nyachoti 1996).

In general, it is evident that the tannin present in the diet may have only limited effects on feeding value for poultry.

Effects of Tannin on Activities of Digestive Enzymes in Ducks (Zhou)

Description of the trial

In this experiment, Shaoxing ducks were used to study the effects of tannins on activities of digestive enzymes. Eighty eight one-day-old Shaoxing ducks were divided into two groups of 44 each and were housed in floor pens with wood shavings as litter. In the experiment, two diets were assigned, one a control diet, the other with the addition of commercial tannin (Table 7).

Table 7. Composition of diets in experiment with duck.

Composition	Control diet	Tannin diet
Dry matter %	90.50	90.50
Protein %	17.7	17.6
Lysine %	0.83	0.83
Met + Cys %	0.52	0.52
Commercial tannin %	—	1.5

The experiment began at day 6 after hatching, feed and water were offered ab libitum. On days 27, 47 and 67 respectively, 12 ducks from each group were weighed and sacrificed. The abdomens of these ducks were then opened and the small intestine was removed. The intestine was divided into 2 sections, the upper section (duodenum and jejunum) and the lower section (ileum). Mucus from the intestine was removed and then trypsin, α-amylase and total proteinase activities were determined by the method of Erlanger and Bernfeld (Zhou 1990).

Results and discussion

Dietary tannin had significant effects on trypsin, α-amylase and total proteinase activities ($p < .01$) (Table 8). The commercial tannin content of 1.5% DM reduced the activities of these three digestive enzymes. Such effects could reduce the absorptive capacity of the tract, thus contributing to poor animal performance which is often observed when high tannin-containing diets are fed.

Table 8. Effects of tannin on activities of digestive enzymes in ducks.

Enzyme	Part of intestine	Day	Control	Tannin (1.5%)
Trypsin	Above part	27	35.05[a]	29.55[b]
		47	34.95[a]	29.10[b]
		67	35.10[a]	30.40[b]
α-amylase	Above part	27	4.98[a]	4.82[b]
		47	4.96[a]	4.78[b]
		67	4.88[a]	4.76[b]
Total proteinase	Above part	27	4000[a]	2900[b]
		47	3800[a]	2500[b]
		67	3450[a]	2300[b]

[a–b] Means within columns with no common superscript differ significantly ($p < 0.05$).

Table 6. Effects of sorghum tannin on retention of nutrients.

Tannin content (%)	Control	0.16	0.24	0.32	0.48	0.64
Retention of protein (%)	86.86[a]	86.55[ab]	85.77[ab]	85.41[ab]	85.12[ab]	84.34[b]
Percentage GE metabolised (%)	79.06[a]	79.56[a]	79.42[a]	78.41[a]	78.40[a]	78.39[a]
Retention of dry matter (%)	72.11[a]	72.06[a]	71.58[a]	70.68[a]	70.50[a]	70.40[a]

[a–g] Means within columns with no common superscript differ significantly ($p < 0.05$).

Conclusion and Applications

1. Broiler chicks in their first 4 weeks could tolerate up to 0.48% sorghum tannin in the diets and in the later 4 weeks could tolerate up to 0.64% sorghum tannin without any adverse effect on weight gain, feed efficiency, dressing percentage, total serum lipid, cholesterol and glutamate-pyruvate transaminase levels.

2. Feeding a sorghum diet to chicks could reduce the yellow pigment on their beaks and legs.

3. Retention of protein, dry matter digestibility (DMD) and the metabolic rate of gross energy decreased in Leghorn cocks as the dietary tannin content increased.

4. Commercial tannins added to the diet at a level of 1.5% DM reduced the activities of total proteinase, trypsin and α-amylase in the small intestine of Shaoxing ducks.

References

Anon. 1986. Ministry of Agriculture of PRC. In: Statistical Data of China Agriculture, Beijing.

Anon. 1997. Ministry of Agriculture of PRC. In: Statistical Data of China Agriculture, Beijing.

Cao Zhenchuan; Jiang Yun Xia et al. 1985. Comparison of using high and low tannin content sorghum as broiler feed. Acta Veterinaria et Zootechnica Sinica, 16 (2): 89–91.

Guo Rongfu and Chen Kelin 1995. Tannin in the diets of monogastric animals. China Feed, (19): 18–20.

Li Youqian and Zhang Yanyun 1998. The effects of sorghum tannin on utilisation of nutrients. Chinese Animal Magazine, 34 (4): 24–25.

Nyachoti, J.L., Atkinson and Leeson, S. 1996. Response of broiler chicks fed a high-tannin sorghum diets. Poultry Research, 5: 329–245.

Q.Y. Diao; Yang Zhong Yuan and Huang Jun Chun 1990. The effects of sorghum tannin on digestion of nutrients in leghorn cocks. Chinese Animal Magazine, 26 (2): 30–32.

Zhou, W. and SH. CH. Ni 1990. Effects of tannin on the activities of digestive enzymes and utilisation of amino acids. Feed Panorama, 2: 7–10.

Cassava Hay: an Important On-Farm Feed for Ruminants

M. Wanapat, O. Pimpa, W. Sripuek, T. Puramongkol, A. Petlum, U. Boontao, C. Wachirapakorn and K. Sommart

Abstract

Cassava (*Manihot esculenta* Crantz), an annual tropical/sub-tropical tuber crop, was nutritionally evaluated as a foliage for ruminants especially for dairy cattle. Cultivation of cassava biomass to produce hay at three months after planting and followed every one–two months thereafter until one year, produced a collective DM yield of 11 786 kg/ha. Intercropping of leguminous fodder in rows like *Leucaena leucocephala* would enrich soil fertility and provide additional fodder. Cassava hay contained 24.9% CP and with very minimal HCN content (0.348 mg%). Feeding trials with cattle revealed high levels of DM intake (11.2 kg/hd/d, 3.2% BW) and DM digestibility (71%). Ruminal protein degradation of cassava hay was relatively low (48.8%) since it contained tannin-protein complexes which would act as by-pass protein in the small intestine. Therefore, supplementation with cassava hay at 2–3 kg/hd/d or provision as a sole source of roughage in dairy cattle could remarkably reduce concentrate supplementation and increase the fat and protein content of milk. Moreover, cassava hay supplementation in dairy cattle will significantly increase milk thiocyanate which could possibly enhance milk quality and milk storage especially in small holder-dairy farming. Cassava hay was therefore an excellent source of supplemental protein (by-pass protein) for dairy cattle especially during the long dry season and has the potential to increase productivity and profitability.

CASSAVA or tapioca (*Manihot esculenta* Crantz) is an annual tuber crop grown widely in tropical and sub-tropical areas. It can easily thrive in sandy-loam soil with low organic matter, receiving low rainfall and high temperatures. It is therefore a cash crop cultivated by small-holder farmers within the existing farming systems in many countries.

Cassava tubers contain high levels of energy and minimal levels of crude protein and have been used as readily fermentable energy in ruminant rations. Cassava leaves have been used as a protein source when collected at tuber harvesting time. However, the intake and digestibility was low due to the high level of condensed tannins (Reed et al. 1982; Onwuka 1992). Harvesting of cassava at an early growth stage (3 months) to make hay could reduce the condensed tannin content and increase protein content (25% DM) resulting in a higher nutritive value (Wanapat et al. 1997).

Department of Animal Science, Faculty of Agriculture, Khon Kaen University, Khon Kaen, Thailand 40002. Email: metha@kku1.kku.ac.th

The objectives of these studies were to explore the feasibility of using the whole cassava crop as hay when harvested in the dry season after 3 months of cultivation, and subsequently every 2 months throughout the year to assess yield, hay-making, feeding value, supplementation and its effect on milk thiocyanate as a milk preservative.

Materials and Methods

Experiment 1. Cassava cultivation and hay-making

A cassava crop was planted in rows using stems with 30 × 30 cm. spacing in October and harvested in December (3 months) by cutting the whole crop at 15 cm above the ground. Leucaena was also densely planted as strips (1 m.) to help fix N in the soil. Fresh yield was measured immediately and was left in the field to be dried for 1–3 days before being collected.

The dried cassava biomass can either be stored in bundles or made into baled-hay for storage and later feeding. Cassava hay (CH) can easily be prepared in

KEYWORDS: Cassava, Digestibility, Protein degradation

a simple square, wooden box to obtain a 15 kg bale. It is recommended that the finished bale be further sun-dried to secure dry matter of at least 85–90% and to reduce hydro-cyanic acid and/or sprinkled with 0.5% urea solution to prevent mould growth.

Cassava hay feeding value studies

Cassava hay was fed ad libitum to four rumen fistulated Holstein-Friesian steers, weighing about 350 kg using CRD, for two consecutive weeks before a metabolism trial was imposed for 5 days to measure voluntary intake, digestibility, rumen fluid for pH and volatile fatty acids (VFA). In addition, various parts of cassava hay, leaf, branch, stem and whole crop, were inculated to measure rumen degradation at 0, 24, 48 and 72 h using the nylon-bag technique. Samples of feed and faeces were chemically analysed for DM, CP, NDF, ADF and ADL.

Experiment 2

Twenty-five first lactation Holstein Friesian dairy cattle in late lactation (6–7 months) were randomly assigned according to a RCBD to receive the following five dietary treatments:

T1 = 3 kg concentrate + 0 kg cassava hay(CH)/hd/d;
T2 = 3kg concentrate + 1 kg CH/hd/d;
T3 = 1.8 kg concentrate + 2 kg CH/hd/d;
T4 = 1.5 kg concentrate + 3 kg CH/hd/d;
T5 = 2.0 kg concentrate + CH ad libitum.

All cows were individually housed for 60 days and received urea-treated rice straw on an ad libitum basis. Feeds were given twice a day during milking time. Milk yields were recorded daily and samples of feed, rumen fluid, urine and faeces were collected during the last two days prior to termination of the experiment. Feed and milk samples were analysed for chemical composition and statistically analysed using Proc. GLM.

Experiment 3

Two farms were selected to test the effect of CH supplementation on residual milk thiocyanate concentration in 10 (6 and 4) multiparous milking Holstein Friesian cows. Animals were divided into two groups (non- and CH supplemented groups) and fed for one week before milk samples were collected and analysed for milk thiocyanate as a milk preservative, as reported by Claesson (1994).

Results

Experiment 1 (Tables 1, 2 and 3)

The cassava crop (whole) grew very well after three months and yielded 12 131 kg fresh/ha or 3302 kg DM/ha and the combined yield of subsequent cuttings every two months were 50 087 fresh or 11 786 kg/ha dried of CH. CH contained a high content of crude protein (24.9%) and intermediate

Table 1. Yield and chemical compositions of cassava hay.

Cutting time	Yield (kg/ha)		Cassava	DM	Ash	CP	NDF % DM	ADF	ADL	Condensed tannin mg%	HCN mg%
	Fresh	Dried									
First cut 3 months after planting	12131	3300	Leaf	95.3	5.7	32.3	25.7	25.2	6.5	0.126	
1 m after first cut (4 m)	5469	981	Branch	93.2	5.0	8.9	49.3	47.3	8.1	0.086	
2 m after first cut (5 m)	6000	1200	Stem	82.7	5.3	14.6	38.8	32.5	5.7	0.083	
3 m after first cut (6 m)	7731	1712	Whole (hay)	93.4	6.6	24.9	34.4	27.0	5.8	0.134	.348
6 m after first cut (9 m)	9069	2162									
9 m after first cut (12 m)	9687	2431									
Total	50087	11786									

Table 2. Voluntary cassava hay intake, digestibility and ruminal protein degradability when fed to Holstein Friesian steers.

Item	Cassava hay		Leaf	Branch	Stem	Whole crop hay
DM intake, kg/d	11.2 ± 0.06	Protein degradability				
% BW	3.2 ± 0.48	a, %	30.0	22.2	55.2	28.4
g/kg W.75	138.0 ± 3.09	b, %	70.0	77.8	44.8	47.9
Ruminal pH		c/%/h	1.6	0.4	0.2	3.7
0, h-post feeding	7.11 ± 0.16	ED, %	47.0	28.0	56.9	48.8
2,	7.05 ± 0.21					
4,	6.25 ± 0.29					
DM digestibility, %						

a = soluble fraction, b = potential degradation, c = degradation rate, ED = effective degradation.

levels of fibre fraction. Ruminal protein degradation was relatively low which may have provided a higher content of rumen by-pass protein. DM intakes of CH fed solely were 3.2% BW and demonstrated high DM digestibility of 71.0%. HCN content of CH was minimal (0.348 mg%) after sun-drying. Total VFA concentration was intermediate resulting in relatively higher levels of acetate (C_2) and propionate (C_3) (Table 3).

Experiment 2 (Table 4)

Supplementation with CH could reduce the amount of concentrate used, especially at 3 kg/hd/d. Cows fed CH on an ad libitum basis produced a similar milk yield and higher milk fat and protein content while SNF and total solids were similar.

Experiment 3 (Table 5)

Feeding CH to lactating cows significantly increased milk thiocyanate concentrations from 3.4 to 5.6 ppm on farm A and from 7.8 to 19.5 ppm on farm B. This thiocyanate in milk may activate the lactoperoxidase system (LPS) to preserve milk quality as reported by Claesson (1994).

Conclusions

Experiment 1. The results showed that a cassava crop could be used successfully as CH when grown densely and stripped with leucaena. CH was well consumed by cattle and contained high protein. The formation of tannin-protein complexes (mainly from proanthocyanidins) could render higher by-pass protein in the lower gut. CH was demonstrated to be an excellent foliage for the dry season as earlier reported by Wanapat et al. (1997).

Experiment 2. Higher levels of CH supplementation reduced the level of concentrate supplement required and improved milk yield. CH supplementation, especially at 2–3 kg/hd/d could improve efficiency, reduce production costs and increase profits.

Experiment 3. The level of milk thiocyanate increased as an effect of supplementation. CH could be used as a natural preservative for milk storage and milk quality. However, more detailed studies need to be conducted.

Table 3. Ruminal volatile fatty acids (VFA) in dairy steers fed on cassava hay.

	TVFAs	C2	C3	i-C4	C4	i-C5	C5	C2:C3
	mM/L ←				mol/100 mol →			
h-post feeding								
0	40.0	72.8	16.2	1.5	6.2	2.6	0.7	4.5
2	43.2	71.1	17.3	1.2	7.3	2.1	0.9	4.1
4	50.4	70.6	17.6	1.2	7.8	1.8	1.1	4.0
Mean	44.5	72.0	17.0	1.3	7.0	2.1	0.9	4.2

Table 4. Effect of CH supplementation for late lactating dairy cows fed on urea-treated rice straw.

	T1	T2	T3	T4	T5	SEM
Milk yield, kg/d*	6.3	6.1	5.4	6.1	5.4	.24
3.5% FCM	6.46	5.96	5.50	6.32	5.99	.13
Fat, %	4.0	3.6	3.6	3.7	4.2	.11
Protein, %	4.4 [ab]	4.0 [b]	3.8 [b]	4.1 [b]	5.3 [a]	.17
SNF, %	8.6	8.8	8.4	8.6	8.4	.12
Total solids, %	12.6	12.3	12.0	12.2	12.6	.18

T1 = 0 kg CH + 3.0 kg conc., T2 = 1 kg CH + 3.0 kg conc., T3 = 2 kg CH + 1.8 kg conc., T4 = 3 kg CH + 1.5 kg conc., T5 = ad lib. CH + 2.0 kg conc.

Table 5. Effect of CH supplementation (2–3 kg/hd/d) on residual milk thiocyanate in lactating Holstein Friesian cows.

	Milk thiocyanate, ppm		SE
	Non-suppl.	CH suppl.	
Farm A (6 head)	3.4	5.6	1.37
Farm B (4 head)	7.8 a	19.5 b	4.1

Conclusions and Recommendations

Based on the previous findings, cassava whole crop could be grown as an on-farm feed resource especially for the long dry season feeding. Its high concentration of condensed tannins could be manipulated by harvesting at an early stage. The remaining tannins appeared to be beneficial as they may remain as tannin-protein complexes in the rumen and be available post-ruminally. It is, therefore, imperative to investigate further the role of condensed tannins of cassava hay for use on-farm for dairy cattle as a high protein roughage in the dry season.

Acknowledgments

The authors are grateful for the collaborations of the Dairy Farming Organisation of Thailand, the University of Newcastle upon Tyne, UK, Dr Peter Rowlinson and Dr David Parker; the technical supervision of Prof. Leng and Mr T. Siirtola, and the financial support of FAO for research and development activities.

References

Claesson, O. 1994. The use of the lactoperoxidase system. In: Proc. Regional Workshop on Raw Milk Handling and Preservation in the Near East Region. FAO, Rome, Italy.

Onwuka, C.F.I. 1992. Tannin and saponin contents of some tropical browse species fed to goats. Trop. Agric. (Trinidad) 69-142-1440.

Reed, J.D., McDowell, E., Van Soest, P.J. and Horvath, P.J. 1982. Condensed tannins: A factor limiting the use of cassava forage. J. Sci. Food Agric., 33: 213–220.

Wanapat, M., Pimpa, O., Petlum, A. and Boontao, U. 1997. Cassava hay: A new strategic feed for ruminants during the dry season. Livestock Research for Rural Development, 9(2): IRRD Home Page.

The Role of Polyphenols in Human Health

R.A. King[1]

Abstract

The polyphenols are one of the most widely distributed groups of non-nutritive phytochemicals. They range from simple phenolics to highly polymerised tannins with molecular weights greater than 30 000 Da and are generally present in plants as glycoside conjugates. Dietary sources of polyphenols include cereals, legumes, fruit, vegetables and some beverages, notably tea and wine. However, concentrations vary markedly within and between these groups. The daily intake of polyphenols from a Western diet has been estimated to be as much as one gram. However, only relatively recently have studies examined the extent to which ingested polyphenols are absorbed from the gastrointestinal tract in humans. Polyphenols, particularly tannins, have traditionally been considered as anti-nutrients because of their ability to reduce the digestibility of proteins. However, over the past one to two decades, there has been a growing appreciation of the potentially important role of polyphenols in protection against common Western chronic diseases. This has been based on studies in animal models and in vitro systems as well as on epidemiological and clinical studies in humans. A major class of polyphenols is the flavonoids of which more than 4000 have been identified in nature. Epidemiological studies have suggested that the flavonoids from soy foods may protect against cardiovascular disease and some forms of cancer; clinical studies have supported a role in the prevention of osteoporosis and alleviation of menopausal symptoms in women; animal and in vitro studies have provided further evidence of their protective role. Similarly, there is evidence that flavonoids from other dietary sources may protect against cardiovascular disease. Dietary polyphenols are thought to be partly responsible for the 'French Paradox', a lower incidence of cardiovascular disease in some regions of Europe than would be predicted from the high intake of saturated fats and the high levels of plasma cholesterol. Similarly, the polyphenols in green and black tea are thought to contribute to their beneficial health properties. This paper reviews studies related to the role of polyphenols in human health, particularly cancer and cardiovascular disease.

ALTHOUGH not precisely defined, the polyphenols are an extremely broad class of substances ranging from simple phenolic acids to highly polymerised condensed tannins (Bravo 1999). Many thousands have been identified in plants and foods. Gut bacteria and the host organism can also transform these substances to a range of metabolites. It is not surprising therefore that the polyphenols, as a class, display a diverse range of properties that may be of potential importance to human health. Because of this diversity it is not possible, within the limitations of this paper, to discuss the role of this entire class in all aspects of human health, rather it will focus on the flavonoids and the tannins, with emphasis on their role in protection against the two most important Western diseases — cancer and cardiovascular disease. This topic has been recently reviewed (see for example (Bravo 1999; Chung et al. 1998).

Classes of Polyphenols

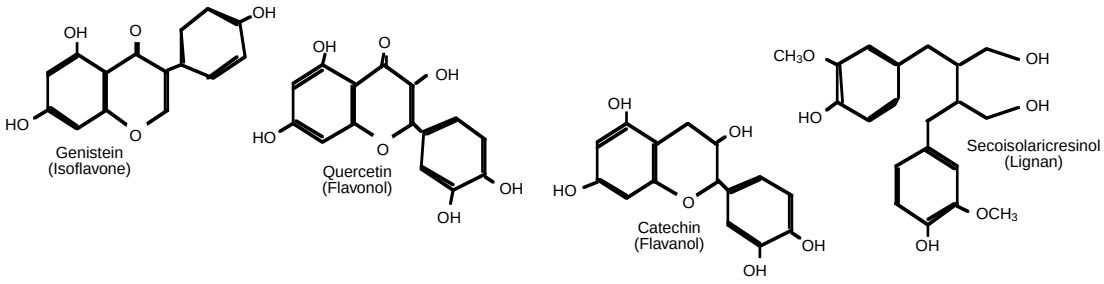
It is beyond the scope of this paper to discuss all the many classes of plant phenolics which are present in food in detail. However, some classes and an example of each are shown on the next page.

Dietary Sources and Intake of Polyphenols

Estimation of the intake of polyphenols is clearly an important element in the assessment of their

[1] CSIRO Human Nutrition, Kintore Avenue, Adelaide, 5000, South Australia

KEYWORDS: Polyphenols, Flavonoids, French paradox, Cardiovascular disease, Cancer



potential importance to human health. The polyphenols are widely distributed in foods where, except for the catechins, they are generally present as glycosides. Because methods for their measurement have often been imprecise or non-specific and because levels are influenced substantially by plant genetics, state of maturity and environmental factors, reported values for content of polyphenols in foods vary substantially. In addition, diet and cooking methods differ between countries and therefore the nature and amount of polyphenols consumed can vary substantially. For example, soy foods, which are a uniquely rich source of isoflavones, are consumed mainly in Asian countries. Some indicative values for polyphenol content of foods are summarised in Table 1 and illustrate the large differences in polyphenol content between foods.

Partly reflecting the problems noted above, estimates of intake of polyphenols have varied widely. For example, Kanhau (1976) estimated that the daily intake of flavonoids in the USA in 1971 was approximately 500 mg (expressed as aglycones), of which approximately 100 mg was 4-oxo-flavonoids. In Dutch (Hertog et al. 1993) and Finnish (Knekt 1996) studies, intakes of flavones and flavonols (the major 4-oxo-flavonoids) were recently estimated to be approximately 26 mg and 3.4 mg respectively. The development of comprehensive computer-based databases of the phytochemical content of foods is commencing (Pillow et al. 1999) and this, together with more accurate analytical methods, will assist in more accurately estimating dietary intake of polyphenols and hence their role in human health.

Metabolism and Bioavailability of Polyphenols

Polyphenols may potentially influence health by effects within the gastrointestinal tract without the need for absorption, either directly, for example, as antioxidants (Hagerman et al. 1998), or indirectly via effects on bacterial populations (Bravo et al. 1994). However, information about the extent to which ingested polyphenols are absorbed from the gastrointestinal tract into the circulation, their concentrations in the blood and their residence times in the body is clearly critically important in assessing their role in human health. Until recently, there had been few such studies. However, with the growth in interest in the role in human health of phyto-

Table 1. Polyphenol content of foods (Bravo 1999; Thompson et al. 1997).

Food	Polyphenol content mg/100 g	Food	Polyphenol content mg/100 g or mg/100 mL
Rice	~10	Apple	27–298
Millet	590–1060	Blackcurrent	140–1200
Barley	1200–1500	Cherry	60–90
Sorghum	170–10 260	Orange	50–100
Flaxseed	33–107	Peach	10–150
Chick peas	78–230	Tomato	85–130
Common beans	34–280	Apple juice	37–710
Soybeans	100–300	Orange juice	66–100
Betel nuts	26 000–33 000	Tea leaves (green)	20 000–35 000
Peanuts	40	Tea leaves (black)	22 000–33 000
Cabbage	25	Black tea	75–105
Leek	20–40	Coffee	130–370
Onion	100–2025	Red wine	100–400
		White wine	20–30

chemicals in general and polyphenols in particular, this is rapidly changing. For example, studies have recently been reported for the catechins from tea (van het Hof et al. 1999) and chocolate (Richelle et al. 1999), polyphenols from wine (Serafini et al. 1998), quercetin from onions (Hollman et al. 1997a; McAnlis et al. 1999; Hollman et al. 1996a) and from tea (Hollman et al. 1997b) and for the isoflavones daidzein and genistein from soy (King and Bursill 1998). Reported bioavailabilities of the flavonoids range from about 10% to 50% (King and Bursill 1998; Hollman et al. 1995; Xu et al. 1994). The bioavailability of tannins does not appear to have been studied in humans, but, based on their size and chemistry and on animal studies, it is unlikely that they are absorbed significantly per se. It is possible, however, that there may be some absorption of the constituent polyphenols or their bacterial metabolic products (Buchanan et al. 1996).

As discussed earlier, polyphenols are generally present in foods as glycosidic conjugates. Until recently, it was accepted that polyphenols must be hydrolysed to their aglycones by bacterial enzymes in the gut before they could be effectively transported across the gut wall (Kuhnau 1976). Recent studies, however, have questioned this tenet and, at least for some flavonoids, it appears that transport as the glycoside occurs, possibly by the glucose carrier (Hollman et al. 1995). Thus, it may not only be the nature of the polyphenol that influences its bioavailability, but also the nature and extent of its glycosidic conjugation (Hollman et al. 1995).

Once the polyphenols reach the large intestine, they may be degraded by bacterial action (Kuhnau 1976). This degradation will decrease the bioavailability of ingested polyphenols and it may generate metabolites with different bioactivities or biopotencies compared to the precursor polyphenol. In addition, for flavonoids, and probably for most polyphenols, glucuronide conjugates, which have different biopotencies to the aglycones, are formed by the host organism. Flavonoids which carry meta-hydroxy groups (e.g. quercetin) can also be methylated in the liver by the action of catechol-O-methyltransferase. Thus, metabolism of polyphenols by gut bacteria and by the host organism is likely to influence the way in which they impact on human health.

Human Health Implications of Polyphenols

Tannins

The role of tannins in human health has been recently reviewed (Chung et al. 1998) and the reader is referred to that review for a more detailed discussion of the topic.

Tannins can be divided into two classes — hydrolysable and condensed. Hydrolysable tannins such as tannic acid are present only in low concentrations in commonly consumed foods and therefore most of the focus has been on the condensed tannins (also called proanthocyanidins). Based largely on animal studies, tannins have been considered as antinutrients due to a range of adverse effects including reduced feed conversion, reduced micronutrient bioavailability, liver damage and reduced growth (Chung et al. 1998). While, as Chung has pointed out (Chung et al. 1998), ingestion of tannins may not be a problem for those whose diet includes animal protein and cereals, it may have consequences for people living in countries where diets are based largely on high-tannin grains such as sorghum and pulses. Based on animal and in vitro studies, there is evidence that tannins may be either procarcinogenic or anti-carcinogenic and either mutagenic or antimutagenic. However, except for extreme cases such as the chewing of betel nuts (which contain up to about 25% tannins by weight), there appears to be no evidence that tannins are procarcinogenic in humans (Chung et al. 1998). While not directly related to human health, the important contribution of the tannins to the organoleptic properties of foods should not be overlooked.

Flavonoids and Cardiovascular Disease

Four cohort studies and one cross-cultural study (the Seven Countries Study) have examined the relationship between estimated flavonoid intake and coronary heart disease (CHD). After correction for other known risk factors such as smoking and saturated fat intake, one cohort study showed a strong protective effect (Hertog et al. 1993), two showed no effect (Knekt et al. 1996; Rimm et al. 1996), and one a statistically non-significant positive association (Hertog et al. 1997) between flavonoid intake and CHD risk. The Seven Countries Study found a significant inverse relationship (Hertog et al. 1995) between flavonoid intake and CHD risk. There is also some evidence for an inverse association between flavonoid intake and stroke (Keli et al. 1996). Although suggestive of some protection by flavonoids, the results of epidemiological studies are therefore inconclusive. Recent reviews (Tijburg et al. 1997; Hollman et al. 1996a) provide more detailed discussion.

Clinical studies have focused on the effects of consumption of polyphenol-rich foods such as onions, green and black tea, wine and soyfoods on known risk factors for cardiovascular disease (CVD), usually the levels of cholesterol and low density lipoproteins (LDL) in plasma, as well as the ability

of LDL to withstand oxidation ex vivo, since oxidation of LDL is recognised as an important process in the initiation of atherosclerosis. For example, McAnlis et al. (1999) showed an approximate 9-fold rise in plasma quercetin levels to 0.73 μM following consumption of a single meal of onions, but there was no decrease in the susceptibility of LDL to oxidation following isolation from plasma. Consumption of onions also had no effect on ex vivo platelet aggregation (Janssen et al. 1998) a mediator of cardiovascular risk.

Black and green teas are both good sources of catechin-related polyphenols, although the detailed chemical composition of the polyphenols differs between the two. Consumption of black tea for four weeks was shown in one study to increase the resistance of LDL to oxidation (Ishikawa et al. 1997). However, in another study, consumption of green tea or black tea for the same period had no effect on LDL levels nor on the resistance of LDL to oxidation ex vivo (van het Hof et al. 1997). Similarly, consumption of three cups of green or black tea daily for three days increased plasma catechin levels to approximately 0.3 μM and 1.0 μM respectively but had no effect on the ability of isolated LDL to resist oxidation. Other studies have also failed to show an effect of tea consumption on LDL oxidation (van het Hof et al. 1997; McAnlis et al. 1998) or plasma cholesterol levels (Tijburg et al. 1997).

The so-called 'French Paradox' (Constant 1997) has sparked interest in the possible role of wine and wine polyphenols in protection against cardiovascular disease. However, clinical studies of the relationship between red wine consumption and CVD risk have also been equivocal. For example, consumption of a red wine phenolics extract has been shown to increase the antioxidant capacity of plasma but it did not change the resistance of LDL to oxidation (Carbonneau et al. 1997), whereas consumption of red wine did increase the resistance of LDL to oxidation (Serafini et al. 1998; Miyagi et al. 1997).

A group of polyphenols in which there has been great recent interest in relation to protection against cardiovascular disease is the isoflavones, which are present in uniquely high concentrations in soyfoods. Consumption of soyfoods reduces plasma cholesterol concentrations, particularly in individuals with high initial levels (Anderson et al. 1995), although the evidence that the isoflavones play a major role is not strong (Sirtori et al. 1997). However, consumption of soy for two weeks increased the resistance of LDL to oxidation ex vivo (Takkanen et al. 1998) and genistein, one of the main soy isoflavones, strongly inhibits oxidation of LDL in vitro (Kerry and Abbey 1998). Beneficial effects on arterial compliance in humans following consumption of an isoflavone extract from soy have also been demonstrated (Nestel et al. 1997).

While the studies discussed above do not suggest a strong influence of polyphenols on CVD in populations overall, animal studies have provided evidence for improvement of cardiovascular risk factors (Tebib et al. 1994; Hayek et al. 1997) and in vitro studies have consistently shown antioxidant properties of polyphenols including inhibition of LDL oxidation (Frankel et al. 1993; Rifici et al. 1999; Kerry and Abbey 1999). There are a number of possible reasons for differences between the results of the epidemiological and clinical studies and the experimental studies. Firstly, it is possible that genetic heterogeneity masks effects in human studies. For example, only when the results were analysed for individual apolipoprotein-E genoptypes was an effect of black tea consumption on the blood coagulating factor PAl-1, a known cardiovascular risk factor, detected (Loktionov et al. 1998). Secondly, the influence of confounders in epidemiological studies, the ability to more effectively control animal experiments compared to human studies and the use of higher doses of flavonoids in animal studies compared to human studies should also be considered. Thirdly, in vitro studies typically use flavonoids in their aglycone forms, whereas they are present in vivo predominantly as glucuronide and sulphate conjugates which may have lower biopotencies (Manach et al. 1996). More studies are needed to clarify the role of polyphenols in cardiovascular disease.

Flavonoids and Cancer

There is overwhelming evidence that regular consumption of fruit and vegetables protects against cancer (Steinmetz and Potter 1996). However, the extent to which this protection can be attributed to their flavonoid content is not clear. Two epidemiological studies that demonstrated an inverse association between intake of flavonoids and coronary heart disease were unable to show any reduction in total cancer risk (Hertog et al. 1994, 1995). A third study, which involved a 20-year follow-up of 10 000 men and women in Finland, did however show a strong inverse association with the risk of lung cancer (Knekt et al. 1997), although other cancer sites showed non-significant associations.

There is stronger evidence for a protective effect of the soy isoflavones against some cancers. Intake of soyfoods, particularly tofu, has been shown to be inversely associated with the incidence of cancers of the breast (Lee et al. 1991; Wu et al. 1997; Witte et al. 1997), stomach (Nagai et al. 1982; Lee et al.

1995) and prostate (Sanderson et al. 1999), although one study was unable to demonstrate any association with breast cancer (Yuan et al. 1995). Animal and in vitro studies (Sanderson et al. 1999; Record et al. 1997) suggest that the isoflavones in soy, particularly genistein, are likely to be one group of mediators of this protection.

The epidemiological evidence of a relationship between black tea consumption and cancer is not strong (Kohlmeier et al. 1997). For example, per capita intake of tea varies at least 30-fold between countries, but there is no correlation with total cancer incidence or cancers at individual sites, with the possible exception of stomach and bladder (Blot et al. 1997). Case-control and cohort studies suggest that there may be a modest reduction associated with tea consumption for some cancers, but again the relationships are not strong (Blot et al. 1997). However, many of these studies were not specifically designed to test the effect of tea and so control was sometimes poor resulting in confounding by other lifestyle variables. The possible influence of genetic factors also needs to be considered in light of the findings in relation to cardiovascular disease noted earlier (Loktionov et al. 1998).

In contrast to the uncertainty of human studies, animal studies have generally shown protective effects of black tea, green tea and the polyphenols from these beverages, such as the gallocatechins and flavonols, against a wide range of cancer types. Many in vitro studies have also demonstrated anticarcinogenic and antimutagenic properties of tea and tea flavonoids. Two recent reviews (Dreosti 1996; Dreosti et al. 1997) have discussed the role of tea in cancer prevention. The possible reasons for the differences between the human studies and the experimental studies discussed in relation to cardiovascular disease could also apply here.

Conclusion

In summary, epidemiological studies to date have not generally established a strong relationship between dietary flavonoid intake and the incidences of cardiovascular disease and cancer in humans. However, experimental studies continue to provide evidence of protection and of possible mechanisms.

In a recent position statement, the American Dietetic Association (Bloch and Thomson 1995) concluded that 'specific substances in foods (e.g. phytochemicals as naturally occurring components) may have a beneficial role in health as part of a varied diet' and that 'The Association supports research regarding the health benefits and risks of these substances.' In the case of the polyphenols, further studies are clearly needed to clarify their role in human health. These studies should focus on the need for:

- more accurate and comprehensive values for content of the entire spectrum of polyphenols in foods;
- comprehensive databases of the polyphenol content of foods;
- more information on absorption and metabolism of polyphenols in humans;
- more biomarkers of cancer and cardiovascular disease for use in human studies; and
- more studies in which the responses of individual genotypes rather than entire populations are examined.

References

Anderson, J.W., Johnstone, B.M. and Cook-Newell, M.E. 1995. Meta-analysis of the effects of soy protein intake on serum lipids. New England Journal of Medicine, 333: 276–282.

Bloch, A. and Thomson, C.A. 1995. Position of the American Dietetic Association: Phytochemicals and functional foods. Journal of the American Dietetic Association, 95: 493–496.

Blot, W.J., McLaughlin, J.K. and Chow, W. 1997. Cancer rates among drinkers of black tea. Critical Reviews in Food Science Nutrition, 37:7 39–760.

Bravo, L., Abia, R., Eastwood, M.A. and Saura-Calixto, F. 1994. Degradation of polyphenols (catechin and tannic acid) in the rat intestinal tract. Effect on colonic fermentation and faecal output. British Journal of Nutrition, 71: 933–946.

Bravo, L. 1999. Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance. Nutrition Review, 56: 317–333.

Buchanan, C.J., Wallace, E., Fry, S.C. 1996. In vivo release of ^{14}C-labelled phenolic groups from intact dietary spinach cell walls during passage through the rat intestine. J. Sci. Food Agric.; 71: 459–469.

Carbonneau, M.A., Leger, C.L., Monnier, L., et al. 1997. Supplementation with wine phenolic compounds increases the antioxidant capacity of plasma and vitamin E of low-density lipoprotein without changing the lipoprotein Cu^{2+}-oxidisability — possible explanation by phenolic location. European Journal of Clinical Nutrition, 51: 682–690.

Chung, K., Wong, T.Y., Wei, C., Huang, Y. and Lin, Y. 1998. Tannins and human health. Critical Reviews in Food Science and Nutrition, 38: 421–464.

Constant, J. 1997. Alcohol, ischemic heart disease, and the French paradox. Clinical Cardiology, 20: 420–424.

Dreosti, I.E., Wargovich, M.J. and Yang, C.S. 1997. Inhibition of carcinogenesis by tea: The evidence from experimental studies. Critical Reviews in Food Science and Nutrition, 37: 761–770.

Dreosti, I.E. 1996. Bioactive ingredients: Antioxidants and polyphenols in tea. Nutrition Reviews, 54: S51–S58.

Frankel, E.N., Kanner, J., German, J.B., Parks, E. and Kinsella, J.E. 1993. Inhibition of the oxidation of low-density lipoprotein by phenolic substances in red wine. Lancet, 341: 454–457.

Hagerman, A.E., Riedl, K.M., Jones, G.A. et al. 1998. High molecular weight plant polyphenols (tannins) as biological antioxidants. Journal of Agriculture and Food Chemistry, 46: 1887–1892.

Hayek, T., Fuhrman, B., Vaya, J. et al. 1997. Reduced progression of atherosclerosis in apolipoprotein E-deficient mice following consumption of red wine, or its polyphenols quercetin or catechin, is associated with reduced susceptibility of LDL to oxidation and aggregation. Aterioscler. Thromb. Vasc. Biol., 17: 2744–2752.

Hertog, M.G.L., Feskens, E.J., Hollman, P.C.H., Katan, M.B. and Kromhout, D. 1993. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen elderly study. Lancet, 342: 1007–1011.

Hertog, M.G.L., Feskens, E.J.M., Hollman, P.C.H., Katan, M.B. and Kromhout, D. 1994. Dietary flavonoids and cancer risk in the Zutphen elderly study. Nutr. Cancer, 22: 175–184.

Hertog, M.G.L., Kromhout, D., Aravanis, C. et al. 1995. Flavonoid intake and long-term risk of coronary heart disease and cancer in the seven countries study. Arch. Intern. Med., 155: 381–386.

Hertog, M.G.L., Sweetnam, P.M., Fehily, A.M., Elwood, P.C. and Kromhout, D. 1997. Antioxidant flavonols and ischemic heart disease in a Welsh population of men — the Caerphilly study. American Journal of Clinical Nutrition, 65: 1489–1494.

Hollman, P.C.H., De Vries, J.H.M., van Leeuwen, S.D. and Mengelers, M.J.B. 1995. Absorption of dietary quercetin glycosides and quercetin in healthy ileostomy volunteers. American Journal of Clinical Nutrition, 62: 1276–1282.

Hollman, P.C.H., Van Trijp, J.M.P., Buysman, M.N.C.P. et al. 1997a. Relative bioavailability of the antioxidant flavonoid quercetin from various foods in man. FEBS Letters, 418: 152–156.

Hollman, P.C.H., Tijburg, L.B.M. and Yang, C.S. 1997b. Bioavailability of flavonoids from tea. Crit. Rev. Food Sci. Nutr., 37: 719–738.

Hollman, P.C.H., Van der Gaag, M., Mengelers, M.J.B., Van Trijp, J.M.P., de Vries, J.H. and Katan, M.B. 1996a. Absorption and disposition kinetics of the dietary antioxidant quercetin in man. Free Radic. Biol. Med., 21: 703–707.

Hollman, P.C.H., Hertog, M.G.L. and Katan, M.B. 1996b. Role of dietary flavonoids in protection against cancer and coronary heart disease. Biochem. Soc. Trans., 24: 785–789.

Ishikawa, T., Suzukawa, M., Ito, T. et al. 1997. Effect of tea flavonoid supplementation on the susceptibility of low-density lipoprotein to oxidative modification. American Journal of Clinical Nutrition, 66: 261–266.

Janssen, P.L.T.M.K., Mensink, R.P., Cox, F.J.J. et al. 1998. Effects of the flavonoids quercetin and apigenin on hemostasis in healthy volunteers: results from an in vitro and a dietary supplement study. American Journal of Clinical Nutrition, 67: 255–262.

Keli, S.O., Hertog, M.G.L., Feskens, E.J.M., Kromhout, D. 1996. Dietary flavonoids, antioxidant vitamins, and incidence of stroke — the Zutphen study. Arch. Intern. Med., 156: 637–642.

Kerry, N. and Abbey, M. 1998. The isoflavone genistein inhibits copper and peroxyl radical mediated low density lipoprotein oxidation in vitro. Atherosclerosis, 140: 341–347.

Kerry, N.L. and Abbey, M. 1999. Red wine and fractionated phenolic compounds prepared from red wine inhibit low density lipoprotein oxidation in vitro. Atherosclerosis, 135: 93–102.

King, R.A. and Bursill, D.B. 1998. Plasma and urinary kinetics of the isoflavones daidzein and genistein after a single soy meal in humans. American Journal of Clinical Nutrition, 67: 867–872.

Knekt, P., Jarvinen, R., Seppanen, R. et al. 1997. Dietary flavonoids and the risk of lung cancer and other malignant neoplasms. American Journal of Epidemiology, 146: 223–230.

Knekt, P., Jarvinen, R., Reunanen, A. and Maatela, J. 1996. Flavonoid intake and coronary mortality in Finland — A cohort study. British Medical Journal, 312: 478–481.

Kohlmeier, L., Weterings, K.G.C., Steck, S. and Kok, F.J. 1997. Tea and cancer prevention: An evaluation of the epidemiolic literature. Nutr. Cancer, 27: 1–13.

Kuhnau, J. 1976. The flavonoids. A class of semi-essential food components: Their role in human nutrition. In: Bigwood, E.J., Cuthbertson, D.P., Ganguly, J. et al. ed. World review of nutrition and dietetics, 24: 117–191.

Lee, H.P., Gourley, L., Duffy, S.W., Esteve, J., Lee, J. and Day, N.E. 1991. Dietary effects on breast-cancer risk in Singapore. Lancet, 337: 1197–1200.

Lee, J.K., Park, B.J., Yoo, K.Y. and Ahn, Y.O. 1995. Dietary factors and stomach cancer — a case-control study in Korea. International Journal of Epidemiology, 24: 33–41.

Loktionov, A., Bingham, S.A., Vorster, H., Jerling, J.C., Runswick, S.A. and Cummings, J.H. 1998. Apolipoprotein E genotype modulates the effect of black tea drinking on blood lipids and blood coagulation factors: a pilot study. British Journal of Nutrition, 79: 133–139.

Manach, C., Texier, O., Regerat, F., Agullo, G., Demigne, C. and Remesy, C. 1996. Dietary quercetin is recovered in rat plasma as conjugated derivatives of isorhamnetin and quercetin. J. Nutr. Biochem., 7: 375–380.

McAnlis, G.T., McAneny, J., Pearce, J. and Young, I.S. 1998. Black tea consumption does not protect low density lipoprotein from oxidative modification. European Journal of Clinical Nutrition, 52: 202–206.

McAnlis, G.T., McEneny, J., Pearce, J. and Young, I.S. 1999. Absorption and antioxidant effects of quercetin from onions in man. European Journal of Clinical Nutrition, 53: 92–96.

Miyagi, Y., Miwa, K. and Inoue, H. 1997. Inhibition of human low-density lipoprotein oxidation by flavonoids in red wine and grape juice. American Journal of Cardiology, 80: 1627

Nagai, M., Hashimoto, T., Yanagawa, H., Yokoyama, H. and Minowa, M. 1982. Relationship of diet to the incidence of esophageal and stomach cancer in Japan. Nutr. Cancer, 3: 257–268.

Nestel, P.J., Yamashita, T., Sasahara, T. et al. 1997. Soy isoflavones improve systemic arterial compliance but not plasma lipids in menopausal and perimenopausal women. Aterioscler. Thromb. Vasc. Biol., 17: 3392–3398.

Pillow, P.C., Duphorne, C.M., Chang, S. et al. 1999. Development of a database for assessing dietary phytoestrogen intake. Nutr. Cancer, 33: 3–19.

Record, I.R., Broadbent, J.L., King, R.A., Dreosti, I.E., Head, R.J. and Tonkin, A.L. 1997. Genistein inhibits growth of B16 melanoma cells in vivo and in vitro and promotes differentiation in vitro. Int. J. Cancer, 72: 860–864.

Richelle, M., Tavazzi, I., Enslen, M., Offord, E.A. 1999. Plasma kinetics in man of epicatechin from black chocolate. European Journal of Clinical Nutrition, 53: 22–26.

Rifici, V.A., Stephan, E.M., Schneider, S.H. and Khachadurian, A.K. 1999. Red wine inhibits the cell-mediated oxidation of LDL and HDL. J. Am. Coll. Nutr., 18: 137–143.

Rimm, E.B., Katan, M.B., Ascherio, A., Stampfer, M.J. and Willett, W.C. 1996. Relation between intake of flavonoids and risk for coronary heart disease in male health professionals. Ann. Int. Med., 125: 384–389.

Sanderson, J., Mclauchlan, W.R. and Williamson, G. 1999. Quercetin inhibits hydrogen peroxide-induced oxidation of the rat lens. Free Radic. Biol. Med., 26: 639–645.

Serafini,. M, Maiani, G. and Ferro-Luzzi, A. 1998. Alcohol-free red wine enhances plasma antioxidant capacity in humans. Journal of Nutrition, 128: 1003–1007.

Sirtori, C.R., Gianazza, E., Manzoni, C., Lovati, M.R. and Murphy, P.A. 1997. Role of isoflavones in the cholesterol reduction by soy proteins in the clinic. American Journal of Clinical Nutrition, 65: 166–167.

Steinmetz, K.A. and Potter, J.D. 1996. Vegetables, fruit, and cancer prevention: A review. Journal of the American Dietetic Association, 96: 1027–1039.

Tebib, K., Bitri, L., Besancon, P. and Rouanet, J. 1994. Polymeric grape seed tannins prevent plasma cholesterol changes in high-cholesterol-fed rats. Food Chemistry, 49: 403–406.

Thompson, L.U., Rickard, S.E., Cheung, F., Kenaschuk, E.O., Obermeyer, W.R. 1997. Variability in anticancer lignan levels in flaxseed. Nutr. Cancer, 27: 26–30.

Tijburg, L.B.M., Mattern, T., Folts, J.D., Weisgerber, U.M. and Katan, M.B. 1997. Tea flavonoids and cardiovascular diseases — a review. Crit. Rev. Food Sci. Nutr., 37: 771–785.

Tikkanen, M.J., Wahala, K., Ojala, S., Vihma, V. and Adlercreutz, H. 1998. Effect of soybean phytoestrogen intake on low density lipoprotein oxidation resistance. Proceedings of the National Academy of Sciences, USA, 95: 3106–3110.

van het Hof, K.H., Wiseman, S.A., Yang, C.S. and Tijburg, L.B.M. 1999. Plasma and lipoprotein levels of tea catechins following repeated tea consumption. Proc. Soc. Exp. Biol. Med., 220: 203–209.

van het Hof, K.H., de Boer, H.S.M., Wiseman, S.A., Lien, N., Weststrate, J.A. and Tijburg, L.B.M. 1997. Consumption of green or black tea does not increase resistance of low-density lipoprotein to oxidation in humans. American Journal of Clinical Nutrition, 66: 1125–1132.

Xu, X., Wang, H.-J., Murphy, P.A., Cook, L. and Hendrich, S. 1994. Daidzein is a more bioavailable soymilk isoflavone than is genistein in adult women. Journal of Nutrition, 124: 825–832.

Wu, A.H., Ziegler, R.G., Hornross, P.L. et al. 1996. Tofu and risk of breast cancer in Asian-Americans. Cancer Epidemiol. Biomarkers Prev., 5: 901–906.

Witte, J.S., Ursin, G., Siemiatycki, J., Thompson, W.D., Paganini-Hill, A. and Haile, R.W. 1997. Diet and premenopausal bilateral breast cancer — a case-control study. Breast Cancer Res. Treat., 42: 243–251.

Yuan, J.M., Wang, Q.S., Ross, R.K., Henderson, B.E. and Yu, M.C. 1995. Diet and breast cancer in Shanghai and Tianjin, China. Br. J. Cancer, 71: 1353–1358.

Free and Bound Tannin Analysis in Legume Forage

E. Wina[1], B. Tangendjaja[1] and B. Palmer[2]

Abstract

Many methods have been developed to analyse tannins. One of the methods that use a tannin biological character is a protein precipitation method using bovine serum albumin. The method, so far, only measured the extractable tannin. It was modified so that it measured not only extractable or free tannin but also tannin that bound to protein or fibre. The free tannin was analysed from acetone fraction and bound tannin was extracted by heating the residue with phenol to dissolve protein and released protein-bound tannin and further heating with 0.1 N HCl released fibre-bound tannin. Those tannins were then analysed by protein precipitation using bovine serum albumin. A recovery test of this method found that only 80% of protein-bound tannin was recovered, but 100% of fibre-bound tannin was recovered. Comparison of the modified protein-precipitation method to the Butanol-HCl method to determine tannin content in some legumes is discussed.

THERE ARE many methods of analysis for tannins and the results vary considerably due to the diversity of the chemical structures of these compounds. Several methods are based on the ability of tannins to form coloured complexes with some cations or other entities which are then quantified by colorimetry, i.e. Vanillin-HCl (Price et al. 1978), Folin-Ciocalteau (Singleton and Rossi 1965), n-Butanol HCl (Porter et al. 1986; Terrill et al. 1992). Other methods are based on the ability of tannin to complex with protein, e.g. the precipitation method using bovine serum albumin (BSA) (Hagerman and Butler 1978) or precipitation methods using haemoglobin have all been commonly used. It is very difficult to compare the value of tannin measured by these different methods because of the different reaction processes.

A modified method of Butanol-HCl developed by Terrill et al. (1992) described a fractionation procedure to separate not only free tannin but also bound tannin into either protein or fibre. The final determination was carried out colorimetrically using Butanol HCl (Bu-HCL). The potential usefulness of this fractionation method was quickly recognised and followed by many scientists. However, since a protein precipitation method is based on the ability of tannin to precipitate protein, which is more related to its biological value, the development of a procedure using this method combined with a fractionation procedure was identified as an objective in this paper.

[1] Research Institute for Animal Production, PO Box 221, Bogor, Indonesia
[2] Tropical Agriculture, CSIRO, Davies Laboratories, Aitkenvale, Qld, Australia

Materials and Methods

Materials

1. Tannin was isolated from freeze-dried *Calliandra calothyrsus* according to Hagerman and Butler (1980) and used as a standard tannin.
2. Calliandra samples and other legumes were provided by B. Palmer (CSIRO Townsville, Australia).

Methods

Tannin analysis was conducted:

1. By the Butanol-HCl method (= Bu-HCl; Terrill et al. 1992): tannin was extracted by 70% acetone containing 0.1% acetone followed by diethyl ether extraction after acetone was evaporated. The rest of the procedure was the same as that of Terrill et al. (1992).
2. By a modified protein-precipitation method: the tannin extraction procedure was modified so that it could measure free, protein-bound and fibre-bound tannin. The precipitation using BSA and the colouring reaction using $FeCl_3$ followed the method of Hagerman and Butler (1978).

KEYWORDS: Tannin, Analysis, Legume

Extraction of free, protein-bound and fibre-bound tannin (modified protein-precipitation method = P-P)

Duplicate 500 mg of dried-milled samples were extracted with 20 mL of 70% acetone containing 0.1% ascorbic acid in a McCartney bottle by rotating the bottle for 150 minutes. After centrifugation at 3000 r/min for 10 minutes, the supernatant was separated and the solid residue was dried in the oven at 60 ^{o}C for two days. Ten mL of diethyl ether was added to the supernatant (3 ×) to remove pigments. The upper layer was removed and the lower layer (aqueous solution) was rotary evaporated at 40 ^{o}C to remove traces of solvent. The solution was centrifuged to remove non-tannin debris and the supernatant was taken and made up to a volume of 10 mL with distilled water and contained 'free tannin'.

One hundred (100) mg of dried solid residue after acetone extraction was put in a test tube. Water (5 mL) and liquid phenol (3 mL) were mixed with the solid residue by vortex mixer. The tube was covered with a marble and place in a water bath at 95 ^{o}C for two hours. After extraction, the tube was centrifuged at 3000 r/min for 10 minutes. The aqueous (top) layer was separated, leaving the phenol layer and the residue. Diethyl ether (5 mL) was added to the aqueous layer to get rid of phenol (2 ×). The aqueous layer was rotary evaporated to remove the solvent and this solution contained 'protein-bound tannin'.

The phenol layer was removed from the residue and the residue left in the test tube was washed with diethyl ether (2 × 5 mL). The residue was left in the fume cupboard until the smell of ether could not be detected. Ten mL of 0.1 N HCl was added to the residue. The tube was covered with marble and placed in a water bath at 95 ^{o}C for four hours. After extraction and cooling, the tube was centrifuged at 3000 r/min for 10 minutes. The supernatant contained 'fibre-bound tannin'.

One mL of each solution containing free or protein-bound or fibre-bound tannin was precipitated by one mL of BSA (2 mg/mL) followed the method of Hagerman and Butler (1978).

Results and Discussion

The modification of the protein precipitation method was in the extraction procedure. The extraction procedure was extended to extract not only free tannin but also the protein-bound and fibre-bound tannin. Extraction of protein-bound tannin used phenol as it has been suggested by Hagerman and Butler (1980) that phenol could dissolve the protein so that the binding of tannin-protein was broken. The temperature and time of extraction with phenol were previously tested so that 95 ^{o}C and two hours were the optimum conditions for extraction.

A recovery test was carried out on this modified method. Tannin-BSA was used as the standard of protein-tannin complex while tannin-xylan was used the standard of fibre-tannin complex. Xylan was found to be reactive to tannin compared to other carbohydrate (Wina, unpublished).

Table 1. Recovery test of the modified tannin analysis.

Tannin isolate = TI (3 mg/mL)	Treatment	Measured tannin, mg	Percentage recovery
TI	Heated in phenol	3.04	101
TI+BSA	Heated in phenol	2.37	79
TI	Heated in 0.1 N HCl	1.01	33
TI+xylan	Heated in 0.1 N HCl	3.28	109

Table 1 shows that there was no degradation of tannin when tannin in the free form was heated in phenol (the recovery of tannin reached 100%). However, when protein (BSA)-tannin complex was heated in phenol, the recovery of tannin reduced to 79%. This indicates that tannin itself could not be destroyed in heated phenol and some protein-tannin complex could not be degraded by heated phenol.

Extraction of fibre-bound tannin used 0.1 N HCl as Terrill et al. (1992) previously used the same acid to release bound-tannin from the residue. Table 1 also shows that there was a big loss when tannin isolate was heated in 0.1 N HCl (33% recovered). The same phenomena occurred when other phenolic acids were heated in 1N HCl (stronger concentration) for 30 minutes, that the losses varied from 15.1% to 91.7% (Krygier et al. 1982). Interestingly, when fibre (xylan)-tannin heated in 0.1 N HCl, a complete recovery of tannin was obtained. It seems that once tannin bound with fibre, it was more stable in heated acid than when it is alone in the acid.

The modified protein precipitation method (P-P) was then compared to Butanol-HCl (Bu-HCl), a slight modification of the Terrill et al. (1992) method to measure free, protein-bound and fibre-bound tannins in legumes (Table 2). The result shows that P-P gave much lower values in free, bound or total tannin compared to the Bu-HCl method. The principle of the reaction of each method may explain the different values. The P-P method is based on the ability of tannin to bind to protein; therefore, it depends on the number of binding positions in the polymer of the tannin. The Bu-HCl method is based on the formation of cyanidin from the depolymerisation of the tannin molecule (Haslam 1981); therefore, it depends on the number of monomers that are released from the tannin polymer to react with H^{+} ions to form cyanidin (pink colour). Therefore, the

positions or places for binding the protein in the polymer tannin would be limited whereas the formation of cyanidin would be much more abundant.

Most of the tannins that precipitated protein were free or extractable tannins. This can be seen from the higher values of free tannins. Actually, Jackson et al. (1996) also found the same phenomena with the Bu-HCl method, that 70–95% of tannins were in the free or extractable form, except for those from Flemingia and Gliricidia forages. Therefore, the significant effect of the tannin will be certainly due to the amount of extractable tannin in the forage.

The difference between these two methods was evident when analysing the protein and fibre-bound tannin of Gliricidia (Table 2). Protein-bound and fibre-bound tannins measured by Bu-HCl were extremely high compared to those measured by P-P (15.4%, 10.3% vs 0.3%, 0.7%, respectively). The explanation might be that Bu-HCl might measure other compounds which have lost their ability to precipitate protein. Using the Bu-HCl method, Jackson et al. (1996) also reported that there was tannins in Gliricidia and all of them were protein-bound (3.7%). A much lower value was obtained when a different standard used. This analysis used tannin isolated from *Calliandra calothyrsus*, while Jackson et al. (1996) used tannin isolated from *Lotus pedunculatus* as a standard.

The implication is that one must be careful interpreting the tannin values or comparing tannin values from various laboratories because of the different methods or standards used.

Perez-Maldonado (1994) suggested that as the Butanol-HCl method could not determine tannin in biological matrices (such as urine, rumen content etc.), developing methodologies to do it was a necessity. The modified precipitation method is considered appropriate for further studies.

Palmer (1997) reported that free or extractable tannin values of 26 provenances of Calliandra measured by the P-P method had a higher correlation between freeze-dried and oven-dried (0.87**) compared to those of Bu-HCl (0.83**). However, with dry matter digestibility of fresh material, free tannin values measured by P-P showed a significantly high negative correlation (–0.80**) but not with those measured by Bu-HCl (–0.53*). This indicates that tannin values by the P-P method can describe nutritive values related to digestibility of forage better than those by the Bu-HCl method.

When the two methods were conducted in the same laboratory for the same Calliandra dried aerobically at different temperature, Wina et al. (these proceedings) found a high correlation of tannin values between P-P and Bu-HCl methods (r = 0.98, 0.88, 0.90 and 0.76 for free, protein-bound, fibre-bound and total tannin, respectively). The result shows that either the P-P or the Bu-HCl method can be used to analyse tannin in Calliandra (Table 3). Perhaps this is due to the tannins in Calliandra consisting mostly of condensed tannins. The correlation might be different if analysing other forages containing high hydrolysable tannin.

Table 2. Free and bound tannin contents (% DM) in legume measured by protein precipitation and Bu-HCl methods.

	Tannin in Calliandra				Tannin in Gliricidia				Tannin in *L. diversifolia*			
	Free	Protein bound	Fibre bound	Total	Free	Protein bound	Fibre bound	Total	Free	Protein bound	Fibre bound	Total
PP*	7.1	1.5	1.0	9.6	0	0.3	0.7	1.0	2.3	0.3	0.6	3.2
Bu-H	13.4	2.8	5.2	21.4	1.6	15.4	10.3	27.3	13.2	4.8	10.4	28.4

* PP = protein precipitation method, Bu-H = Butanol-HCl method.

Table 3. The tannin values (%) in Calliandra dried at different temperatures measured by two methods.

Temperature (°C)	Free tannin		Protein-bound		Fibre-bound		Total tannin	
	P-P	Bu-HCl	P-P	Bu-HCl	P-P	Bu-HCl	P-P	Bu-HCl
25	13.01	23.36	0.65	3.29	1.44	2.76	15.10	29.41
45	11.53	21.54	0.76	3.44	1.91	3.10	14.20	28.08
65	9.85	18.56	1.14	3.93	2.35	3.40	13.34	25.89
85	8.91	18.32	1.12	4.86	2.99	5.05	13.02	28.23
105	7.43	14.59	1.58	4.99	3.06	6.62	12.07	26.20
R	0.98		0.88		0.90		0.76	

Conclusion

The modified tannin analysis using the protein precipitation method (P-P) can be applied for forage analysis. Compared to the Butanol-HCl method (Bu-HCl), tannin values measured by P-P were always lower. A high correlation was obtained between Bu-HCl and P-P when analysing the same Calliandra leaves dried at a series of temperatures. But the major considerations to use when choosing an appropriate tannin analysis are the simplicity and accuracy of the method, the costs of the analyses and the relationship between the tannin result and biological value of the forage.

Acknowledgments

These experiments were supported by the ACIAR Project No ASI/1993/018. The grateful appreciation of the authors is extended to Farida Hartati, Sri Rahayu and Ade Hesti for their help.

References

Hagerman, A.E. and Butler, L.G. 1978. Protein precipitation method for the quantitative determination of tannins. J. Agric. Food and Chem., 26: 809–812.

Hagerman, A.E. and Butler, L.G. 1980. Condensed tannin purification and characterisation of tannin associated proteins. J. Agric. Food and Chem., 28: 947–952.

Haslam, E. 1981. Vegetable tannins. In: Stumpf, P.K. and Conn, E.E. ed. The Biochemistry of Plants, a Comprehensive Treatise Vol. 7, Chapter 18: 527–556, Academy Press.

Jackson, F.S., Barry, T.N., Lascano, C. and Palmer, B. 1996. The extractable and bound condensed tannin content of leaves from tropical tree, shrub and forage legumes. J. Sci. Food Agric., 71: 103–110.

Krygier, K, Sosulski, F. and Hogge, L. 1982. Free, esterified and insoluble-bound phenolic acids. 1. Extraction and purification procedure. J. Agric. Food Chem., 30: 330–334.

Palmer, B. 1997. Improved ruminant production through the efficient use of tannin containing shrub legumes. ACIAR Annual Report 1996–1997.

Perez-Maldonado, R.A. 1994. The chemical nature and biological activity of tannins in forage legumes fed to sheep and goats. PhD Thesis, Department of Agriculture, The University of Queensland, 1994.

Porter, L.J., Horstich, L.N. and Ghan, B.G. 1986. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. Phytochemistry, 25: 223–230.

Price, M.L., Scoyoc, S.C. and Butler, L.G. 1978. A critical evaluation of the vanillin reaction as an assay for tannin in sorghum grain. J. Agric. & Food Chem., 26: 1214–1218.

Singleton, V.L. and Rossi Jr., J.A. 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid and reagents. American Journal of Enology and Viticulture, 16: 144–143.

Terrill, T.H., Rowan, A.M., Douglas, G.B. and Barry, T.N. 1992. Determination of extractable and bound condensed tannin concentrations in forage plants, protein concentrate meals and cereal grains. J. Sci. Food Agric., 58: 321–329.

Analysis of Free, Soluble and Insoluble Bound Gallic Acid in *Calliandra calothyrsus*

E. Wina[1], B. Tangendjaja[1] and B. Palmer[2]

Abstract

The presence of condensed tannin in legumes was commonly reported but not with hydrolysable tannin. Gallic acid which was a monomer of hydrolysable tannin was determined by HPLC from three fractions of Calliandra leaves. Those were ethyl acetate fraction containing free gallic acid, acid hydrolysis of aqueous fraction containing soluble-bound gallic acid and acid hydrolysis of residue fraction containing insoluble-bound tannin. The content of free gallic acid varied (less than 2700 ppm). The presence of soluble bound gallic acid in the aqueous fraction indicated the presence of hydrolysable tannin in Calliandra but the amount of it varied (0-0.28%). Insoluble bound gallic acid in the residue was hardly found in Calliandra. The significance of low hydrolysable tannin content in Calliandra must be low compared to condensed tannin in affecting the nutritive value of Calliandra.

METHODS to measure the hydrolysable tannins in legume leaves are limited. Inoune and Hagerman (1988) reported measurement of hydrolysable tannin using a colour reaction with Rhodamine. Gallic acid is recognised as a monomer of hydrolysable tannin, so the presence of free and bound gallic acid would indirectly indicate the presence of hydrolysable tannin. Calliandra has been reported to have very high tannin content, varying between 8% and 20% (Wina and Tangendjaja these Proceedings) but the presence of hydrolysable tannin in Calliandra has never been reported. Hydrolysable tannin was reported to be toxic to animals.

Murdiati et al. (1990) reported that there was necrotic damage to the liver of sheep fed with *Climedia hirta* containing very high hydrolysable tannin. A method of fractionation and measuring free and soluble bound and insoluble bound phenolic acids followed by HPLC identification and measurement had been developed by Wina (1988). A modification of this method was developed and is the objective of this paper. In addition, the analysis of rumen liquor, faeces and urine from each goat fed Calliandra using this method was also carried out.

[1] Research Institute for Animal Production, PO Box 221, Bogor, Indonesia
[2] Tropical Agriculture, CSIRO, Davies Laboratories, Aitkenvale, Qld, Australia

Materials and Methods

Materials

Calliandra samples in dried milled form were provided by Palmer (CSIRO, Townsville, Australia). Rumen liquor, faeces and urine from each goat fed grass and Calliandra were obtained from the ruminant complex at Research Institute for Animal Production, Bogor, Indonesia.

Methods

1. Gallic acid analysis was carried out by the HPLC method (Wina 1988); the extraction procedure was modified so that it could measure free, soluble-bound and insoluble-bound gallic acid. Each fraction was then injected following the method of Wina (1988).
2. Total phenol analysis was carried out by the method of Titto (1985) to measure acid-hydrolysed aqueous fraction (soluble-bound phenol) and acid-hydrolysed insoluble fraction (insoluble-bound phenol).

Extraction of free, soluble-bound, insoluble-bound gallic acid

Duplicate 500 mg of dried-milled samples were extracted 20 mL of 70% acetone containing 0.1% ascorbic acid in a McCartney's bottle by rotating the

KEYWORDS: Gallic acid, HPLC method, *Calliandra calothyrsus*

bottle for 150 minutes. After centrifugation at 3000 r/min for 10 minutes, the supernatant was separated and the residue was dried in the oven at 60 °C for two days. The supernatant was extracted with 10 mL of ethyl acetate (3 ×). The upper layer (ethyl acetate) was separated and combined. The ethyl acetate was evaporated to dryness by rotary evaporator and dissolved in 5 mL of 80% methanol. This solution contained 'free gallic acid'.

The lower (aqueous) layer was made up into 10 mL of distilled water. A portion of aqueous solution (3 mL) was hydrolysed by 10 mL of 0.1 N HCl in a water bath at 90 °C for 4 hours (the tube was covered with marble). This aqueous solution was extracted with 10 mL of ethyl acetate (3 ×). The combined ethyl acetate was evaporated into dryness and dissolved in 5 mL of 80% methanol. This solution contained 'soluble-bound gallic acid'.

The residue after acetone extraction (0.3 g) was weighed into a test tube and hydrolysed with 10 mL of 0.1 N HCl. The tube was covered with marble and put in a water bath at 90 °C for 4 hours. After hydrolysis, the tube was centrifuged at 3000 r/min for 10 minutes. The supernatant was separated and extracted with 10 mL of ethyl acetate (3 ×). The combined ethyl acetate was evaporated into dryness and dissolved in 5 mL of 80% methanol. This solution contained 'insoluble-bound gallic acid'.

All free, soluble-bound and insoluble-bound gallic acid was analysed by the HPLC method. The solution was filtered through a Millpore filter 0.45 um and injected to Water HPLC machine (20 uL) with a eluent containing 15% methanol in 2% acetic acid solution with a flow rate of 1.0 mL/min. A UV detector was used at 280 nm to detect the gallic acid peak. The gallic acid peak comes very early in the chromatogram.

Results and Discussion

As hydrolysable tannins are considered important in toxicity studies both for the animal and for micro-organisms, a study of the gallic acid contents of the various provenances was initiated. In the first instance, the free gallic acid was determined to be followed by the gallic determinations on the hydrolysed fraction. Table 1 shows the free gallic acid content of a selection of the Calliandra provenances.

Free gallic acid content in Calliandra varied and ranged from 719 to 1415 ppm in freeze-dried material and from 566 to 2456 ppm in the oven-dried samples (Table1). Some freeze-dried Calliandra had higher tannin values but some had lower tannin values than oven dried Calliandra. It seems that the drying process did not cause a reduction on free gallic acid content. Free gallic acid contents in different species of Calliandra was also similar with that in *Calliandra calothyrsus*. Besides gallic acid, there are several unidentified peaks in the chromatogram. These peaks must represent other phenolic acid compounds. Para-coumaric and ferulic acids are common phenolic compounds found in forages, especially those which have high fibre content like grasses/straw, but the amount of these phenolics in the free form are also very small (Wina 1988). The content of free gallic acid in Calliandra is considered too small to give a significant effect to the animal. Phenolic acids standard with concentration of 6% significantly reduced in vitro dry matter digestibility

Table 1. Gallic acid content in several provenances and species of Calliandra (freeze and oven dried).

Species	Source location	Code	Gallic acid content (ppm)	
			Freeze dried	Oven dried
C. calothyrsus	Union Juarez, Mexico	50/92	796	612
	Turrialba, Costa Rica	20/91	798	975
	Madiun, Indonesia	147/91	962	651
	San Ramon, Nicaragua	11/91	1067	2066
	La Peurta, Nicaragua	134/91	1147	1889
	Flores, Guatemala	10/91	1181	1028
	Cisarua, Indonesia	115690	1202	
	Georgeville, Belize	48/92	1303	2305
	Santa Maria, Honduras	13/91	1415	1779
C. juzepczukii	Cintalapa, Mexico	55/92	894	865
C. acupulcensis	Guerrero, Mexico	85832	895	949
C. acupulcensis	El Mezquite, Mexico	64/92	1036	1292
C. cal.X houst.	Meambar, Mexico	108458	1116	1687
C. cal.X houst.	Meambar, Mexico	109988	1250	1654
C. acupulcensis	El Mezquite, Mexico	35/92	1313	2456

of cellulose but this concentration was substantially higher than those found in forages (Jung 1985).

Table 2 shows gallic acid content as a product of acid-hydrolysis of aqueous phase after 70% acetone extraction (soluble gallic acid) and of acid-hydrolysis of residue (insoluble gallic acid).

The soluble-bound gallic acid varied from traces to 2862 ppm. This soluble-bound gallic acid came from a compound that was soluble in the aqueous phase. This compound must be hydrolysable tannin that also can precipitate tannin. Therefore, free tannins in Calliandra that were measured by the protein precipitation method (Wina and Tangendjaja, these Proceedings) consisted not only condensed tannin but also hydrolysable tannin. However, the presence of soluble-bound gallic acid was only limited (max. 0.28%) compared to tannin content by protein precipitation (max. 20%; Wina and Tangendjaja, these Proceedings). Therefore, nutritive value of Calliandra may not be affected by hydrolysable tannin.

Tripathy et al. (1984) reported that when high levels of tannic acid (an hydrolysable tannin, 22.68 g/day/head) was fed to goats, it caused degenerative changes in the heart, intestine and spleen. However, Silanikove et al. (1996) reported that certain goats (Mamber goat) did not exhibit any toxic syndrome when consuming 10–23 g/kg of tannin-containing leaves such as oak, carob and pistacia or consuming 1.1–2.7 g/kg BW of condensed tannins and 0.4–0.9 g/kg soluble phenolics.

Most Calliandra contained less insoluble-bound gallic acid contents and some provenances of Calliandra did not contain the insoluble-bound gallic acid. There are very limited reports on gallic acid as insoluble-bound gallic acid. Other phenolics, p-coumaric and ferulic acids, were frequently found in ester soluble form or ester-linked to polysaccharide (Chesson et al. 1982). In rice plants, p-coumaric and ferulic acids were also found in ether soluble form and ether-links to the insoluble fraction. (Wina 1988). In soybean and flax cell wall fraction, ferulic acid in ester and ether-linked was released by cold and hot 1 M alkali (Lozovaya et al. 1999). Some unidentified peaks in this fraction must consist of p-coumaric or ferulic acid. Table 3 shows that total phenol in acid-hydrolysed aqueous fraction (soluble-bound phenol) and acid-hydrolysed insoluble fraction (insoluble-bound phenol) were much higher than soluble-bound or insoluble-bound gallic acid (Table 2). The effect of drying the sample would give lower total phenol content compared to those in freeze-dried samples. This is a similar phenomenon to tannin measured by the protein-precipitation method. It seems that drying caused a structural change to phenolic compounds and less reactive to Folin reaction or protein precipitation.

Analysis of gallic acid in rumen liquor, urine and faeces from goats fed with 100% Calliandra is shown in Table 4. The liquid fraction of the rumen liquor and urine was separated after centrifugation and this fraction was extracted and hydrolysed for gallic acid analysis. Surprisingly, there were only trace amounts of free and soluble-bound gallic acids. It seems that gallic acid might disappear easily in the rumen or change to another compound or bind to the feed matrix. Chesson et al. (1982) reported that when sheep were fed with grasses that contained 0.51% phenolic acids, only trace amounts of ferulic and p-coumaric acids were detected in rumen liquor and the major phenolic acid identified was phenylpropionic acid. There was also only trace amounts or small amounts of free and soluble-bound gallic acid in urine. Only in faeces was there higher gallic acid than in rumen liquor or urine, but very high of total phenol content in hydrolysed soluble fractions of faeces.

Table 2. Soluble and insoluble-bound gallic acid content in several provenances and species of Calliandra (freeze and oven dried).

Species	Source location	Soluble-bound gallic acid		Insoluble-bound gallic acid	
C. calothyrsus		FD*	OD	FD	OD
44/92	Plan del Rio, Mexico	99	Tr	20	tr
147/91	Madiun, Indonesia	117	60	nd	212
48/92	Georgeville, Belize	tr	1837	tr	tr
45/92	San Antonio, Belize	1181	2346	153	733
50/92	Union Juarez, Mexico	1257	107	1181	tr
53/92	SM deJesus, Guatemala	1897	1171	785	1436
40/92	Ixtapa, Mexico	2661	885	321	778
51/92	Barillas, Guatemala	2862	1518	tr	544
C. juzepczukii, 55/92	Cintalapa, Mexico	tr	41	tr	tr
C. houstoniana, 58/92	Palenque, Mexico	tr	254	198	99
C. grandiflora		583	205	tr	42

*FD = freeze dried, OD = oven dried.

Table 3. Soluble-bound and insoluble-bound total phenol content (ppm) in several provenances of Calliandra.

Species, code	Source location	Soluble-bound total phenol		Insoluble-bound total phenol	
C. calothyrsus		FD	OD	FD	OD
147/91	Madiun, Indonesia	3576	3490	2208	Nd
53/92	SM deJesus, Guatemala	9251	6534	1977	1821
50/92	Union Juarez, Mexico	9653	14362	5081	3015
45/92	San Antonio, Belize	11828	11116	3189	2703
51/92	Barillas, Guatemala	11265	6216	2823	2044
48/92	Georgeville, Belize	14572	10101	2627	1676
44/92	Plan del Rio, Mexico	14793	6292	2623	2343
40/92	Ixtapa, Mexico	18320	4580	3988	2070
C. grandiflora		16013	8169	3609	2370
C. juzepczukii 55/92	Cintalapa, Mexico	17413	6683	2617	1542
C. houstoniana 58/92	Palenque, Mexico	34101	6941	3479	1800

FD = freeze dried, OD = oven dried, nd = not determined.

Table 4. Gallic acid and total phenol content (ppm) in rumen liquor, urine and feces of goat fed 100% Calliandra.

	Total gallic acid (ppm)			Total phenol (ppm)		
	Free	Soluble bound	Insoluble bound	Free	Soluble bound	Insoluble bound
Rumen liquor	Tr	Tr	*	182	704	*
Urine	Tr	130	*	241	141	*
Faeces	158	453	traces	4376	25 493	2297

*not determined. Tr = traces.

Conclusion

Using the present method, gallic acid in Calliandra was identified as a free, soluble-bound and insoluble-bound form. The amount of free and soluble-bound gallic acids were higher than the insoluble-bound one. The presence of soluble-bound gallic acid indicates the presence of hydrolysable tannin in Calliandra. Since the level of soluble-bound gallic acid was only 0.28% compared to condensed tannins (20%), its effect on the nutritive value of Calliandra is likely to be negligible.

References

Chesson, A., Stewart, C.S. and Wallace, R.J. 1982. Influence of plant phenolic acids on growth and cellulolytic activity of rumen bacteria. Appl. Environ. Microbiol., 44: 597–603.

Inoune, K.H. and Hagerman, A. 1988. Determination of gallotannin with rhodamine. Anal. Biochem., 169: 363–369.

Jung, H-J.G. 1985. Inhibition of structural carbohydrate fermentation of forage phenolics. J. Sci. Food Agric., 36: 74–80.

Lozovaya, W., Gorshkova, T.A., Yablokova, E.V., Rumyantseva, N., Valieva, A., Ulanov, A. and Widholm, J.M. 1999. Cold alkali can extract phenolic acids that are ether linked to cell wall components in dicotyledonous plants. Phytochemistry, 50: 395–400.

Murdiati, T.B., McSweeney, C.S., Campbell, R.S.F. and Stoltz, D.S. 1990. Prevention of hydrolysable tannin toxicity in goats fed *Clidemia hirta* by calcium hydroxide supplementation. J. Appl. Toxicol., 10: 325–332.

Silanikove, N., Gilboa, N., Perevolotsky, A. and Nitsan, Z. 1996. Goats fed tannin-containing leaves do not exhibit toxic syndromes. Small Ruminant Res., 21: 195–201.

Titto, R.J. 1985. Phenolic constituents in the leaves of northern willows: Methods for the analysis of certain phenolics. J. Agric. Food Chem., 33: 213–217.

Wina, E. 1988. Chemistry of phenolic compounds in rice plants. MSc Thesis, Biochemistry Dept, Univ. of La Trobe, Melbourne, Australia.

Tannins in Grape and Grape Products

V. Cheynier[1]

Abstract

Grape and wine polyphenols show a great diversity of structures, from rather simple molecules (monomers, oligomers) to polymers. The latter, usually designated by the term 'tannins' referring to their ability to interact with proteins, are classically divided into condensed tannins and hydrolysable tannins. Only the former are present in grapes but wine may also contains hydrolysable tannins of exogenous origin (wood barrels, enological tannins). Condensed tannins from grapes and wines have been analysed by thiolysis followed by reversed-phase HPLC and by LC-MS. Grape seed tannins consist of partly galloylated procyanidins whereas grape skins and stems also contain prodelphinidins. Tannin average molecular weight is larger in skins than in seeds and stems. Wine tannin composition depends on that of the grape from which the wine is made and on the wine-making conditions, which influence extraction of tannins from the solid parts of the cluster and their subsequent reactions. Thus, prodelphinidins diffuse faster than procyanidins whereas galloylated and larger molecular weight tannins are extracted slower. Besides, tannin-like structures are formed from tannin and non-tannin precursors by various mechanisms, including in particular enzymatic oxidation and aldehyde mediated condensation reactions. These reactions have been studied in model systems and some of the resulting products detected in grape extracts and wines. The structures and major properties of these molecules are reviewed.

GRAPE and wine polyphenols show a great diversity of structures and properties and are responsible for major organoleptic properties of wines, including, in particular, colour and astringency. They are also attracting considerable interest with respect to their potential implication in beneficial effects of wine on human health, known as the 'French paradox'. This is in particular attributed to rather complex polyphenolic structures, which are usually designated by the term tannins, referring to their ability to interact with proteins, permitting their use in the production of leather from hide.

Tannins are classically divided in two groups: hydrolysable tannins, deriving from gallic acid and ellagic acid, and condensed tannins, which are oligomers and polymers of flavanols. Only members of the latter class occur in grapes but wine may also contain hydrolysable tannins extracted from oak cooperage in the course of barrel ageing, or added in the form of enological tannins. Besides, transformation products of the original phenolic compounds may bind to proteins, and thus be regarded as tannins. The occurrence of specific tannins arising from oxidation (i.e. thearubigins and theaflavins) is well documented in black tea, which is also frequently mentionned as a health promoting food. As well, grape phenolics are known to proceed to polymeric pigments during wine making and ageing (Somers 1971; Haslam 1980; Ribéreau-Gayon 1982). Although various products have been obtained in model solutions either by oxidation or by tannin-anthocyanin complexation, the reactions actually taking place in wine as well as the structure and properties of the resulting products are still largely unknown. The present paper summarises the recent findings about the structure of tannins and related compounds in grapes and wines as well as some results concerning structure-activity relationships.

[1] Research Unit Biopolymers and Aromas. INRA-IPV-ISVVM, 2, place Viala, 34060 Montpellier cedex. France. Email : cheynier@ensam.inra.fr

Structural Determination of Proanthocyanidins (Condensed Tannins)

As mentioned above, grape tannins are condensed tannins, i.e. oligomers and polymers of flavan-3-ols, also called proanthocyanidins, because they release

KEYWORDS: Tannins, Proanthocyanidins, Structure, Reactivity, Grape, Vine

red anthocyanidin pigments when heated in acidic medium.

Proanthocyanidins comprise a great diversity of structures, which may show different properties and reactivities, due to the occurrence of:

- numerous constitutive units, which differ by the hydroxylation pattern on the general flavanol skeleton (e.g. catechin, gallocatechin), 2,3 stereochemistry (e.g. catechin, epicatechin) and the presence of substituents (e.g. epicatechin 3-gallate);
- different linkage positions (C4-C6 or C4-C8 bonds in the case of B-type proanthocyanidins, additional ether linkages for A-type ones);
- variable number of units in the molecule.

The general structure of grape proanthocyanidins is shown, as an example, in Figure 1.

Formal identification of proanthocyanidins, including the determination of the C-C bond position, requires sophisticated NMR techniques as described by Balas and Vercauteren (1994). However, a procedure based on partial thiolysis followed by reversed-phase HPLC analysis gives access to the nature and sequence of constitutive monomers of isolated oligomeric proanthocyanidins (Rigaud et al. 1991). The principle of this methods is as follows: Breakage of the interflavanic C-C bonds under mild acidic conditions releases the terminal unit as the corresponding flavanols and the upper and intermediate units as carbocations which react with toluene-α-thiol to form stable benzylthioethers. It also enables formal identification of trimers and larger oligomers, provided that the linkage position in the released dimeric fragments can be unambiguously established. Application of these identification methods is, however, restricted to pure compounds which become increasingly difficult to obtain as their molecular weight increases, owing to the larger number of possible isomers, smaller amounts of each individual compound, and poorer resolution of the chromatographic profiles. This is especially true in the case of grape products which contain a large diversity of tannin structures, based on several monomers, whereas some other plants synthesize essentially one series, e.g. (-)-epicatechin derivatives in the case of cacao.

Several methods have thus been proposed to analyse oligomeric and polymeric proanthocyanidins in heterogeneous solutions. Among them, various chromatography procedures aim to separate tannins

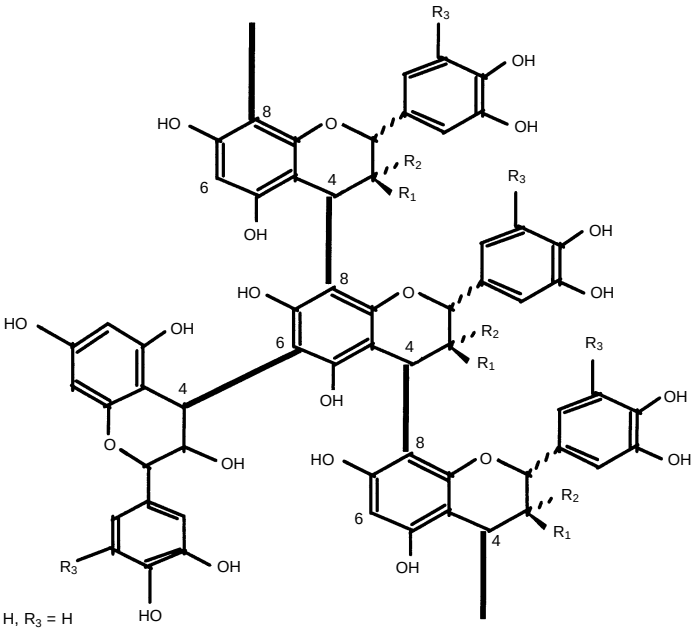


Figure 1. General structure of grape proanthocyanidins.

on a molecular weight basis. Other methods relying upon acid-catalysed degradation in the presence of nucleophilic agents such as toluene-α-thiol, as described above, followed by HPLC analysis of the released units, allow one to determine the monomeric composition and mean degree of polymerisation of tannin extracts or fractions. However, they only give access to average compositional data and provide no information on polymer size distribution. In contrast, mass spectrometry with mild ionisation sources, such as electrospray ionisation (ESI) enables individual detection of oligomers and polymers within a mixture. Besides, it can be coupled to LC and serve as a highly selective detector, providing on-line important structural information (molecular weight, nature and number of constitutive moieties and substituents) which can be used for rapid characterisation of individual constituents (Cheynier et al. 1997).

Structure and Distribution of Proanthocyanidins in Grapes

About 20 dimeric and trimeric proanthocyanidins have been identified in grape seeds (Ricardo da Silva et al. 1991a) and found in skins (Escribano-Bailon et al. 1994). All of them are B-type procyanidins, consisting of (+)-catechin, (−)-epicatechin and (−)-epicatechin-3-O-gallate units. Small amounts of dimers and trimers containing 4-6 linkages occur along with the most common 4-8 linked oligomers. Besides, oligomeric compounds containing both (epi)catechin and (epi)gallocatechin units, sometimes substituted by galloyl groups, have been detected by LC-MS in wine (Fulcrand et al. 1999), but they have not been formally identified.

Monomers and oligomers (dimers and trimers) usually account for less than 10% of the total flavanol content in grapes, which are thus mostly represented by polymeric species.

Application of thiolysis to proanthocyanidins extracted from the different parts of the cluster showed that grape seed tannins consist of partly galloylated procyanidins, based on (+)-catechin, (−)-epicatechin and (−)-epicatechin 3-O-gallate units (Prieur et al. 1994) whereas skin and stem tannins also contain prodelphinidins (Souquet et al. 1996). The mean degrees of polymerisation (mDP) are around 10 in seed and stem tannins, with chain length varying from two to about 30 units. They are to close to 30, with tannin chains containing up to 80 units, in skin tannins. Tannins from skins show a much lower proportion of galloylated units than those from seeds and stem tannins have an intermediate composition.

The content and cluster distribution of tannins as well as the proportions of constitutive units and mDP in each compartment vary with both the variety and the vine-growing conditions. Flavanol levels decrease slightly during grape maturation but their composition does not change qualitatively.

Structure and Origin of Wine Tannins

Wine phenolic composition depends not only on that of the grape used as the raw material but also on the respective diffusion rates of grape constituents into the fermenting must. Since tannins are mostly confined to the solid parts of the cluster, their extraction into the must and wine requires a maceration step, which is most commonly performed in red wine making. Monitoring of phenolic compounds throughout red-wine fermentation indicated that phenolic acids and anthocyanins diffuse faster than flavanols. Among proanthocyanidins, prodelphinidins are extracted earlier than procyanidins and especially galloylated structures, due either to their localisation in skins (as opposed to seeds) or to their higher water solubility. Higher molecular weight tannins also diffuse slower than oligomers.

Once extracted, all phenolic compounds undergo various types of reactions, themselves depending on the presence of other wine components as well as on the storage conditions.

Two major types of reactions lead to tannin-like species in the course of wine-making. The first one is enzymatic oxidation, also called enzymatic browning, catalyzed by polyphenoloxidase. It takes place in the early vinification stages, that is when the grape is crushed or pressed, and yields *o*-quinones which react with nucleophilic compounds, including various polyphenols such as flavanols, to form tannin-like adducts. Several of these have been obtained from non-tannin precursors in model solutions and characterised. In particular, enzymatic oxidation of catechin generates catechin dimers which differ from their procyanidin isomers by the nature and position of interflavanic linkages (Guyot et al. 1996a).

The second reaction, usually referred to as anthocyanin-tannin condensation in the enological literature, has been thoroughly studied since it is responsible for the colour and taste changes occuring during wine ageing. Two mechanisms have been postulated: direct nucleophilic addition of flavanols onto the electrophilic anthocyanin, generating orange xanthylium salts, and acetaldehyde-mediated condensation, yielding purple pigments (Somers 1971; Timberlake and Bridle 1976).

While the occurrence of the former reaction has never been confirmed, products of the latter

(i.e. ethyl-linked anthocyanin-catechin adducts) have been detected in wine (Cheynier et al. 1997; Saucier et al. 1998). However, the anthocyanin can be replaced in this process by another flavanol molecule, and the ethyl-linked catechin dimer thus formed can polymerise further, by the same mechanism. Besides, acetaldehyde can also be replaced by another aldehyde. Thus, reaction of catechin with glyoxylic acid (CHO-COOH), resulting from tartaric acid oxidation, generates ethanoic-linked dimers (Fulcrand et al. 1997), which proceed to xanthylium salts (Es-Safi et al. 1999). All these reactions have been shown to occur concomittantly in wine, their relative importance depending on the wine-making conditions. In particular, the anthocyanin to tannin ratio after racking determines the respective levels of tannin-anthocyanin adducts and tanin-derived polymers in the wine. Air exposure, as well as the presence of oxidation catalysts such as metal ions, are the major limiting factors for aldehyde-induced reactions, although some acetaldehyde also arises from yeast metabolism.

The various tannin-like structures identified so far in grape derived products and related model solutions are presented in Figure 2.

Structure-activity relationships

Free radical scavenging capacity, as well as the ability to complex with proteins (tanning capacity), which are the major properties of phenolic compounds incriminated in their potential health effects depend on the number and accessibility of interaction sites and thus are strongly related to structure. In particular, oxygen scavenging capacity is influenced by galloylation, position of galloyl substituent and to a lesser extent by the chain length (Ricardo da Silva et al. 1991b).

The affinity of procyanidins for proteins also increases with both the DP and the extent of galloylation (Ricardo da Silva et al. 1991c; Bacon et al. 1998; Sarni-Manchado et al., 1999a,b) and varies with the position of interflavanic bonds (Ricardo da Silva et al., 1991c; Bacon et al. 1998). Prodelphinidin units do not seem to co-precipitate with salivary (Sarni-Manchado et al. 1999a) or fining (Sarni-Manchado et al. 1999b) proteins, but epigallocatechin gallate bind strongly to salivary proteins (Bacon et al. 1998). Derived tannins such as dimers arising from catechin oxidation showed tanning effects similar to that of their procyanidin isomers

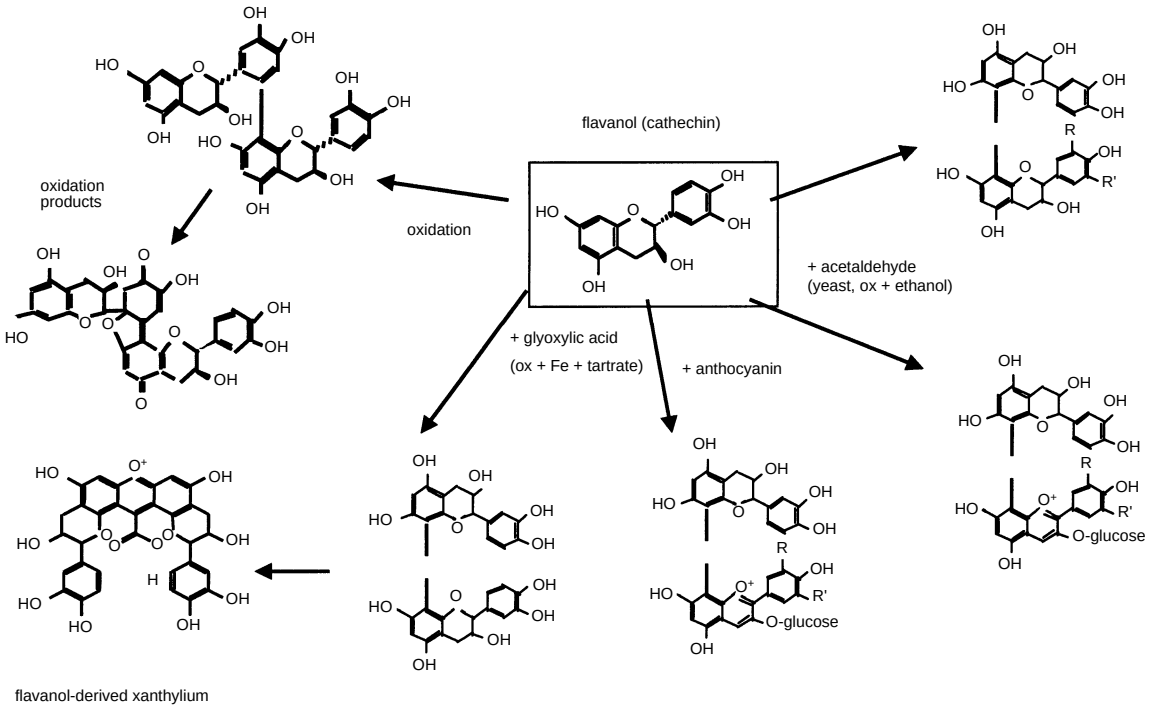


Figure 2. Major pathways yielding tannin-like structures from catechin in grape products.

whereas yellow products obtained by another oxidation step were more active (Guyot et al. 1996b). Catechin itself was inhibitory to some enzymes but this effect disappeared after purification, although the original commercial sample was chromatographically pure, meaning that this effect can be attributed to contaminants in trace amounts — presumably oxidation products. Its is not known whether other tannin-like molecules such as ethyl- or ethanoic-linked adducts and xanthylium salts also behave like tannins but it seems that those containing anthocyanin moieties (derived pigments) are actually less astringent than flavanol derivatives.

In conclusion, grape products contain a great diversity of phenolic constituents, due to the number of precursors present in grape and to the occurrence of various competing reactions during processing. Since the properties of these molecules are strongly related to their structures, accurate knowledge of the composition of polyphenol-enriched foods or extracts is a prerequisite to undergo studies on their health related effects. In fact, commercially available compounds are often rather poor models, since they usually represent only a small proportion of natural food constituents. Besides, one must be aware that contaminants in trace amounts can exert powerful effects which may be wrongly attributed to the major product tested.

References

Bacon, J., Rhodes, M.J.C., Santos-Buelga, C. and Vivar-Quintana, A. 1998. Evaluation of the binding of dietary condensed tannins by proline-rich proteins in human saliva. Polyphenol Communications, 98: 85–86.

Balas, L. and Vercauteren, J. 1994. Extensive high-resolution reverse 2D NMR analysis for the structural elucidation of procyanidin oligomers. Magn. Res. Chem., 32: 386–393.

Cheynier, V., Doco, T., Fulcrand, H., Guyot, S., Le Roux E., Souquet, J.M., Rigaud J. and Moutounet, M. 1997. ESI-MS analysis of polyphenolic oligomers and polymers. Analusis, 25 (8): M32–M37.

Escribano-Bailon, M.T., Guerra, M.T., Rivas-Gonzalo, J. and Santos-Buelga, C. 1994. Procyanidin composition in skin and seed from grapes. JIEP, 94: 225–227.

Es-Safi, N., Le Guernevé, C., Labarbe, B., Fulcrand, H., Cheynier, V. and Moutounet, M. 1999. Identification of new xanthylium compounds formed through interaction between (+)-catechin and glyoxylic acid. In: Chèze, C. and Vercauteren, J. ed. Polyphenols, Wine and Health Communications, Bordeaux, 15–17 April 1999, 31–32.

Fulcrand, H., Cheynier, V., Oszmianski, J. and Moutounet, M. 1997. An oxidised tartaric acid residue as a new bridge potentially competing with acetaldehyde in flavan-3-ol condensation. Phytochemistry, 46 (7): 223–227.

Fulcrand, H., Remy, S., Souquet, J.M., Cheynier, V. and Moutounet, M. 1999. Identification of wine tannin oligomers by on-line liquid chromatography electrospray ionisation mass spectrometry. J. Agric. Food. Chem., 47: 1023–1028.

Guyot, S., Vercauteren, J. and Cheynier, V. 1996a. Colourless and yellow dimers resulting from (+)-catechin oxidative coupling catalysed by grape polyphenol-oxidase. Phytochemistry, 42: 1279–1288.

Guyot, S., Pellerin, P., Brillouet, J.M.and Cheynier, V. 1996b. Inhibition of ?-glucosidase (Amygdalae dulces) by (+)-catechin oxidation products and procyanidin dimers. Biosci. Biotech. Biochem., 60: 1131–1135.

Haslam, E. 1980. In vino veritas: oligomeric procyanidins and the ageing of red wines. Phytochemistry, 19: 2577–2582.

Prieur, C., Rigaud, J., Cheynier, V. and Moutounet, M. 1994. Oligomeric and polymeric procyanidins from grape seeds (*Vitis vinifera*). Phytochemistry, 36 (3): 781–784.

Ribéreau-Gayon, P. 1982. The anthocyanins of grapes and wines. In: Markakis, P. Anthocyanins as Food Colors. New York, Academic Press, 209–244.

Ricardo da Silva, J.M., Rigaud, J., Cheynier, V., Cheminat, A. and Moutounet, M. 1991a. Procyanidin dimers and trimers from grape seeds. Phytochemistry, 30 (4): 1259–1264 .

Ricardo-da-Silva, J.M., Darmon, N., Fernandez, Y. and Mitjavila, S. 1991b. Oxygen free radical scavenger capacity in aqueous models of different procyanidins from grape seeds. J. Agric. Food Chem., 39(9): 1549–1552.

Ricardo daSilva, J.M., Cheynier, V., Souquet, J.-M., Moutounet, M., Cabanis, C. and Bourzeix, M. 1991c. Interaction of grape seed procyanidins with various proteins in relation to wine fining. J. Sci. Food Agric., 57: 111–125.

Rigaud, J., Perez-Ilzarbe, X., Ricardo da Silva, J. and Cheynier, V. 1991. Micro method for the identification of proanthocyanidin using thiolysis monitored by high-performance liquid chromatography. J. Chromatogr., 540: 401–405.

Sarni-Manchado, P., Cheynier, V. and Moutounet, M. 1999a. Interactions of grape seed tannins with salivary proteins. J. Agric. Food Chem., 47: 42–47.

Sarni-Manchado, P., Deleris, A., Avalonne, S., Cheynier, V. and Moutounet, M. 1999b. Analysis and characterisation of wine condensed tannins pecipitated by proteins used as fining agents. Am. J. Enol. Vitic. 50: 81–86.

Saucier, C., Little, D. and Glories, Y. 1998. First evidence of acetaldehyde-flavanol condensation products in red wine. Am. J. Enol. Vitic., 48 (3): 370–373.

Somers, T.C. 1971. The polymeric nature of wine pigments. Phytochemistry, 10: 2175–2186.

Souquet, J.M., Cheynier, V., Brossaud, F. and Moutounet, M. 1996. Polymeric proanthocyanidins from grape skins. Phytochemistry, 43: 509–512.

Timberlake, C.F. and Bridle, P. 1976. Interactions between anthocyanins, phenolic compounds, and acetaldehyde and their significance in red wines. Am. J. Enol. Vitic. 27: 97–105.

The Effect of Level of PEG Addition on In Vitro Dry Matter Digestibility (IVDMD), In Vitro Nitrogen Digestibility (IVND) and PEG Binding by *Calliandra calothyrsus* and *Leucaena leucocephala* Leaves

B. Palmer[1] and R. Jones[1]

Objective

TO ASSESS the level of polyethylene glycol (PEG) addition on IVDMD and IVND using a modified Tilley and Terry (1963) in vitro technique and using ^{14}C-labelled PEG to correct IVDMD for PEG binding (CIVDMD).

Treatments

Samples were the terminal 5 fully expanded leaves from actively growing shoots of the tropical shrub legumes *Calliandra calothyrsus* (CPI 115690) and *Leucaena leucocephala* cv. Cunningham.

Duplicate samples of 0.4g DW were spiked with ^{14}C-labelled PEG 4000 before digestion in an anaerobic chamber using a modified Tilley and Terry (1963) in vitro technique. Supernatant after Stage 1 — rumen fluid/buffer (72 h) and Stage 2 — acid pepsin (24 h) digestion was counted on a scintillation counter to measure absorption or release of PEG.

Ten levels of PEG addition (0–1200 mg/g sample) were added to *C. calothyrsus* and five levels to *L. leucocephala* samples.

Statistical Analyses

Data for IVDMD, CIVDMD and IVND were analysed using ANOVA to test for differences between individual levels of PEG addition and species × PEG addition interactions. Curves were then fitted to treatment means using an iterative non-linear regression model of the form:

$$Y = b_0 + b_1(1 - \exp.b_2X)$$

Results

L. leucocephala had significantly higher IVDMD and CIVDMD than *C. calothyrsus* at all rates of PEG addition (Figures 1 and 2). With *C. calothyrsus*, the more marked difference between IVDMD and CIVDMD reflects the higher activity of tannin.

For IVND Leucaena had a higher level than Calliandra at zero PEG. However, above 80 mg there were no differences between species. Maximum IVND with *L. leucocephala* was achieved with 40 and for *C. calothyrsus* between 80 and 100 mg of PEG/g DM. Maximum IVDMD and CIVDMD were achieved at slightly higher levels than these.

The amount of PEG absorbed by *C. calothyrsus* was approximately three times that of *L. leucocephala* (103 and 33 mg/g respectively) (Figure 3). This is in line with the levels to support maximum IVND. This difference is not reflected in the levels of tannin commonly reported for these species.

[1] CSIRO, Tropical Agriculture, Davies Laboratory, PMB Post Office, Aitkenvale, Qld. 4814

KEYWORDS: *Leucaena leucocephala*, *Calliandra calothyrsus*, Polyethylene glycol (PEG), In vitro dry matter digestibility (IVDMD), In vitro nitrogen digestibility (IVND)

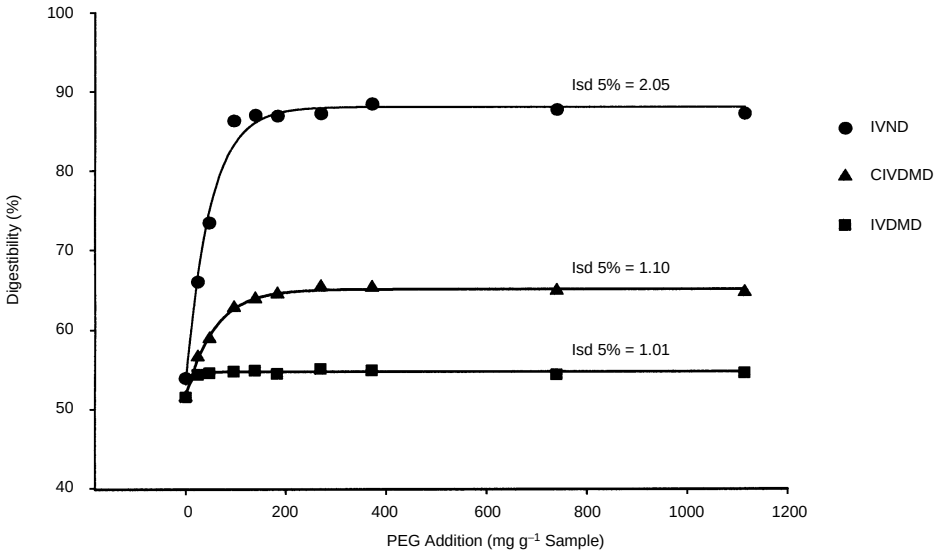


Figure 1. PEG Addition vs Digestibility — *C. calothyrsus*.

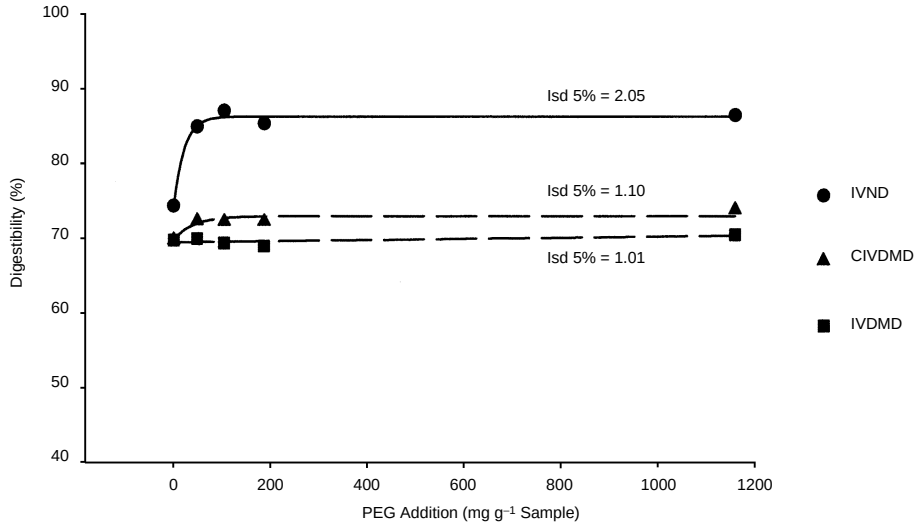


Figure 2. PEG Addition vs Digestibility — *L. leucocephala*.

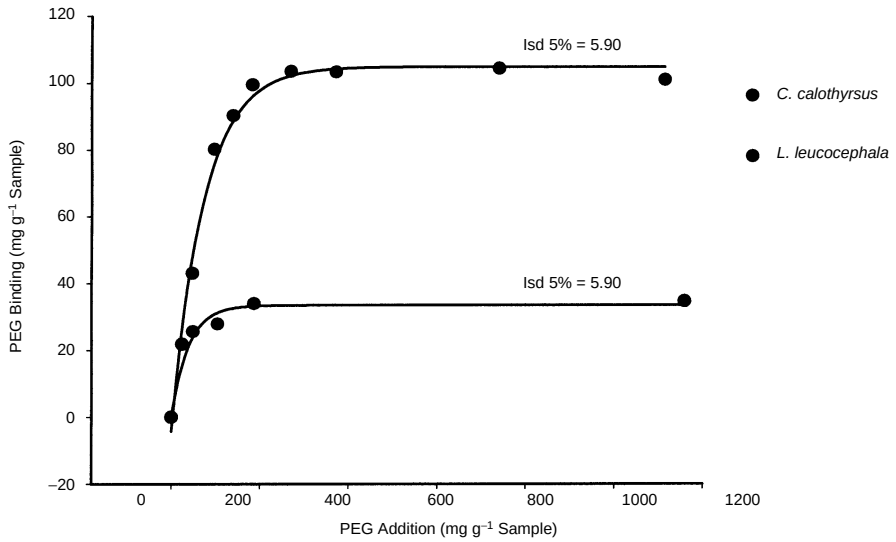


Figure 3. PEG Addition vs PEG Binding.

Conclusions

For both species, the use of 160 mg PEG/g DM was sufficient to maximise estimates of digestibility. The improvement due to PEG at this level was far greater with Calliandra than with Leucaena. This improvement is associated with the higher reported tannin levels in Calliandra.

Reference

Tilley, J.M.A. and Terry, R.A., 1963. J. Br. Grassl. Soc., 18: 104–111.

Estimation of the 'Tannin Effect' by In Vitro Digestion With and Without PEG

R.J. Jones[1], J.H.F. Meyer[2], F.M. Bechaz[2] and M.A. Stoltz[2]

Background

SCREENING potential forage plants containing condensed tannins (CT) is not easy. There is no guarantee that the CT concentration measured by any particular method reflects the activity of the CT in affecting nutritive value.

The authors studied the in vitro digestibility of leaves of six shrub legumes with potential as forage species in the presence and absence of polyethylene glycol (PEG) in the medium, and then related the improvement in digestibility (the PEG effect) to various chemical estimates of CT.

Methods

The tropical leguminous shrubs used were: *A. boliviana* (A.b); *Calliandra calothyrsus* (C.c); *Gliricidia sepium* (G.s); *Leucaena diversifolia* (L.d); *L. leucocephala* (L.l) and *L. pallida* (L.p).

The 5 terminal, fully expanded leaves from actively growing shoots were freeze-dried and ground (1 mm screen). In vitro dry matter digestibility (IVDMD) and in vitro nitrogen digestibility (IVND) were determined by a modified Tilley and Terry (1963) method. N was measured on the samples and the residues to estimate IVND. Samples received either 2 mL distilled water or 2 mL PEG solution to provide 80 mg PEG 4000 per tube to nullify the effects of tannin.

Samples were analysed for tannins by the vanillin and butanol/HCl methods (Jackson et al. 1996). Samples were also equilibrated for 24 hours in tris buffer and PEG spiked with ^{14}C PEG, centrifuged and the supernatant counted in a scintillation counter. PEG binding was then calculated and expressed as mg/g DM (Silanikove et al. 1996).

The results for the tannin estimations were regressed on the differences in IVND between the + and − PEG treatments for the 6 species.

Results

There were large differences between accessions in tannin levels and IVND in the absence of PEG ($p<0.01$). The range in IVND was much reduced, from 47.7%–80.2% in the absence of PEG and from 79.8%–86.2% in the presence of PEG.

The PEG effect was very poorly related to extractable CT by the butanol/HCl method, moderately related to the vanillin CT and well related to the PEG absorption measures (Figure 1). For the bound tannins and the total butanol/HCl tannins, the regressions were negative (Table 1).

Table 1. Relation between the PEG effect (y) and the level of CT (x) in the various fractions measured by the butanol/HCl method.

CT Fraction	a	b	r^2
Protein-bound	26.2	−0.619	0.485
Fibre-bound	31.9	−6.413	0.566
Total	28.3	−0.181	0.147

[1] CSIRO Tropical Agriculture, Davies Laboratory, PMB Post Office, Aitkenvale, Queensland, 4814, Australia
[2] ARC-RFI, Private Bag X05, Lynn East, 0039, Republic of South Africa

KEYWORDS: Tannin effect, Condensed tannins (CT), In vitro dry matter digestibility (IVDMD), In vitro nitrogen digestibility (IVND)

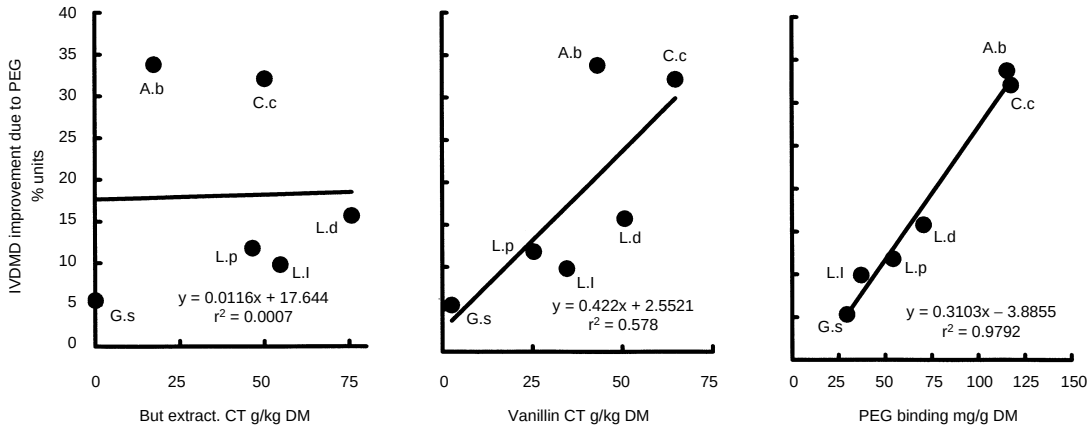


Figure 1.

Conclusions

For screening of new accessions for CT using a standard reference tannin, neither the vanillin or butanol/HCl methods appear appropriate.

Estimation of the PEG effect ranked accessions appropriately and provided other useful information on digestibility. It is simple and needs no special equipment.

The PEG binding method has real promise for this and other purposes.

References

Jackson, F.S., Barry, T.N., Lascano, C. and Palmer, B. 1996. The extractable and bound condensed tannin content of leaves from tropical tree, shrub and forage legumes. Journal of the Science of Food and Agriculture, 71: 103–110.

Silanikove, N., Shinder, D., Gilboa, N., Eyal, M. and Nitsan, Z. 1996. Binding of poly (ethylene glycol) to samples of forage plants as an assay of tannins and their negative effects on ruminal degradation. Journal of the Science of Food and Agriculture, 44: 3230–3234.

Tilley, J.M.A., and Terry, R.A., 1963. A two-stage technique for the in vitro digestion of forage crops. Journal British Grassland Society, 18: 104–111.

The Effect of PEG Addition on In Vitro Digestibility after Stage 1 and Stage 2 Digestion of *Calliandra calothyrsus* and *Leucaena leucocephala* Leaves

B. Palmer[1] and R. Jones[1]

Objective

TO ASSESS the level of polyethylene glycol (PEG) addition on dry matter digestibility (IVDMD), nitrogen digestibility (IVND) and corrected dry matter digestibility (CIVDMD) after Stage 1 and Stage 2 digestion.

Treatments

Samples were the terminal 5 fully expanded leaves from actively growing shoots of the tropical shrub legumes *Calliandra calothyrsus* (CPI 115690) and *Leucaena leucocephala* cv. Cunningham.

Duplicate samples of 0.4g DW were either spiked with ^{14}C-labelled PEG 4000 or an equivalent volume of water before digestion in an anaerobic chamber using a modified Tilley and Terry (1963) in vitro technique. Digestion was terminated after Stage 1 — rumen fluid/buffer (72 h) or Stage 2 — acid pepsin (24 h) digestion (Table 1).

Table 1. Treatment sequences for in vitro digestibility.

	Stage 1	Stage 2
	(Rumen fluid/buffer)	(Acid pepsin)
w	No PEG	
p	PEG	
w + w	No PEG	No PEG
w + p	No PEG	PEG
p + w	PEG	No PEG

Results

There were no differences in IVDMD between Stage 1 and Stage 2 digestion for Leucaena, except where PEG was added to Stage 1 of a two-stage digestion. With Calliandra, there was a significant increase after PEG was added at Stage 1, and digestion after Stage 2 was always higher than after Stage 1 (Figure 1). The results were similar with CIVDMD (Figure 2).

With Leucaena, IVND increased with both the second stage and with PEG addition the highest digestibility occurred when PEG was added at Stage 1 in a two-stage digestion. With Calliandra the effects were similar (Figure 3) except the addition of PEG resulted in a dramatic increase in IVND.

Where no PEG was added the IVND was not significantly different from 0 after Stage 1.

With the addition of PEG, the concentration of ammonium N remaining in the supernatant was high and not significantly different between species. Where no PEG was added, species differences were large and the concentration with Calliandra was not significantly different from 0 (Figure 4).

[1] CSIRO, Tropical Agriculture, Davies Laboratory, PMB Post Office, Aitkenvale, Qld. 4814

KEYWORDS: *Leucaena leucocephala*, *Calliandra calothyrsus*, Polyethylene glycol (PEG), In vitro dry matter digestibility (IVDMD), In vitro nitrogen digestibility (IVND)

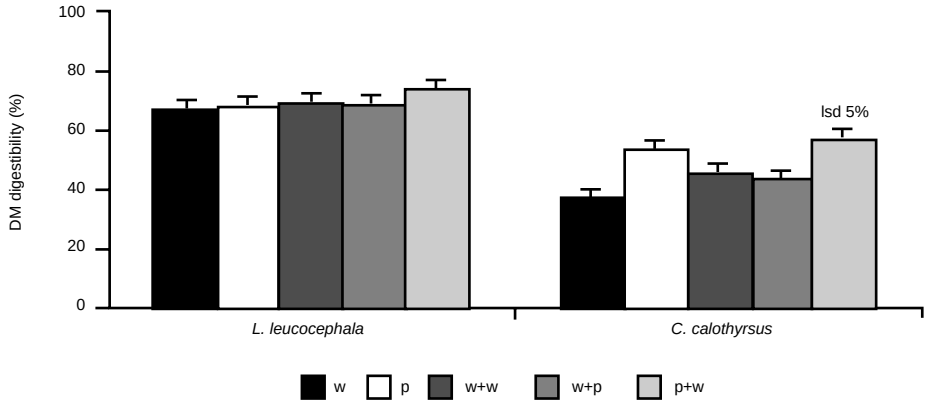


Figure 1. IVDMD.

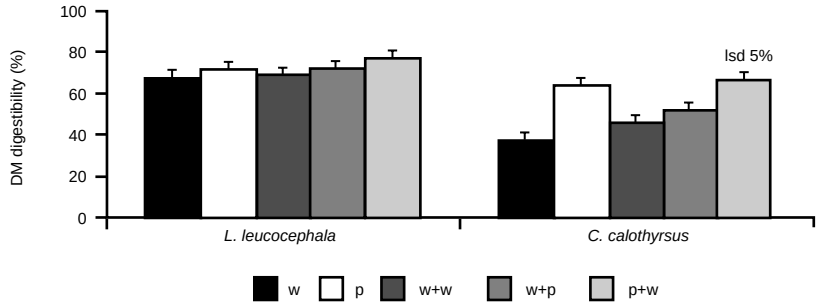


Figure 2. CIVDMD.

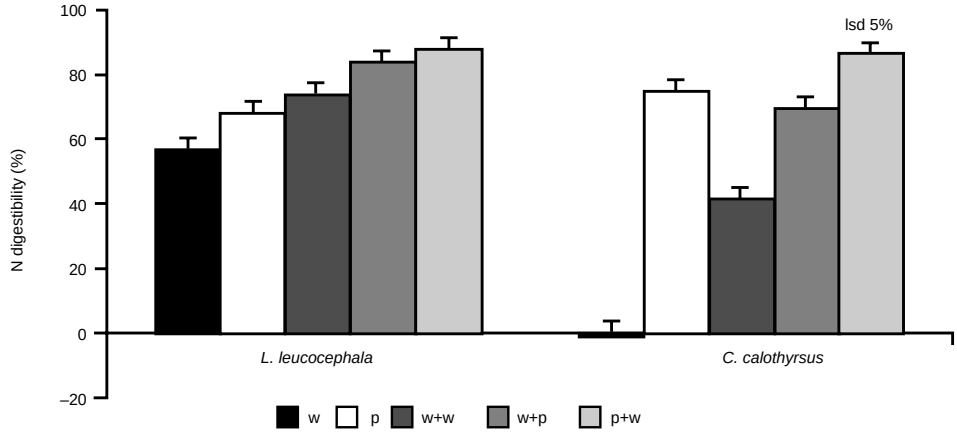


Figure 3. IVND.

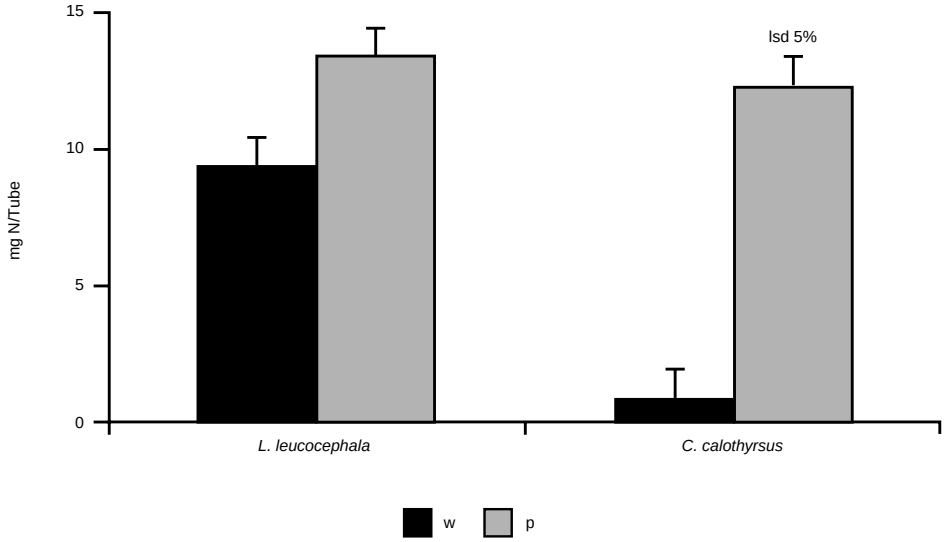


Figure 4. Ammonium N in supernatant.

Conclusions

With Leucaena (low tannin), the effects of adding Stage 2 or PEG were relatively small. With Calliandra (high tannin) the effects of adding Stage 2, and/or PEG were large. The binding of protein to tannin dramatically reduced ammonia N in Stage 1 and could have markedly affected microbial growth.

Reference

Tilley, J.M.A., and Terry, R.A., 1963. A two-stage technique for the in vitro digestion of forage crops. Journal British Grassland Society, 18: 104–111.

The Effect of Drying Conditions on Condensed Tannin Estimates (CT) in *Calliandra calothyrsus*

E. Wina[1], B. Tangedjaja[1] and B. Palmer[2]

Objective

TO STUDY the effect of temperature of drying and aerobic/anaerobic conditions on the estimation of CT fractions in *Calliandra calothyrsus* leaves.

Treatments

Samples were the terminal 5 fully expanded leaves from actively growing shoots of the tropical shrub legume *C. calothyrsus* (CPI115690). Samples were collected from the field and transported chilled in an atmosphere of nitrogen. The samples were dried for 48 h in an flow of dry nitrogen (anaerobic) or dry air (aerobic). The temperatures of drying were 25 ^{o}C, 45 ^{o}C, 65 ^{o}C, 85 ^{o}C and 105 ^{o}C, an additional sample was freeze dried. The plant material was ground to pass a 1 mm sieve.

Plant material was extracted following the method of Terrill et al. (1992). The tannin was fractionated as free, protein bound, fibre bound and total.

Tannin content was determined by both butanol/HCl and protein-precipitation techniques using purified *C. calothyrsus* condensed tannin as the standard.

Data were analysed by ANOVA and then linear trends were estimated using a multi-regression technique with dummy variables for aerobic/anaerobic condition (A) and for its interaction with temperature.

The model used was

$$Y = b_0 + b_1 T + b_2 A + b_3 A*T$$

where A is a dummy variable having the value 1 for aerobic and 0 for anaerobic treatment, the level of significance and standard errors are estimated for each coefficient.

ACD is the adjusted coefficient of determination (R^2 adjusted for degrees of freedom).

Conclusions

The trends in the estimates of tannin in the various fractions are similar. The estimates using butanol/HCl are higher than with protein precipitation.

Free tannin

There was a significant interaction between temperature and aerobic condition with a marked reduction in free tannin in the aerobic samples.

Protein bound

There was a significant increase with temperature in the aerobic samples and a decrease in the anaerobic.

Fibre bound

There were significant increases with temperature; however, the increase is greater when dried under aerobic conditions.

Total

There was a small decrease under aerobic conditions with a minor increase under anaerobic conditions.

Of note is the marked relative increase in protein-bound tannins in the presence of oxygen, whereas PEG absorption was unaffected by condition of drying.

[1] Balai Penelitian Ternak, Ciawi, Indonesia
[2] CSIRO, Tropical Agriculture, Davies Laboratory, PMB Post Office, Aitkenvale, Qld. 4814

KEYWORDS: Condensed tannins (CT), *Calliandra calothyrsus*

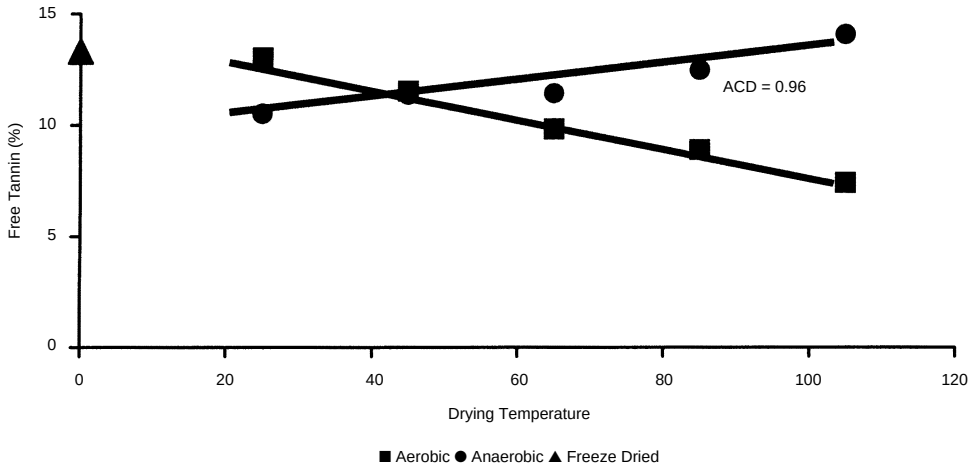


Figure 1. Free tannin vs drying temperature.

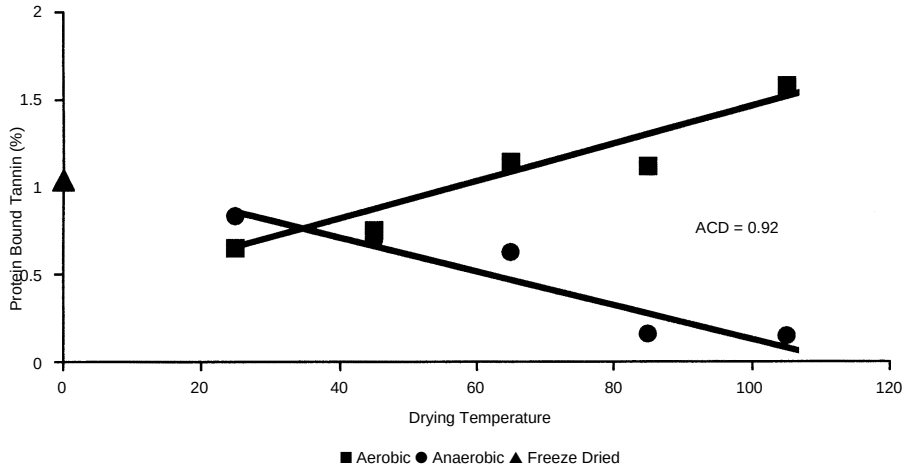


Figure 2. Protein bound tannins vs drying temperature.

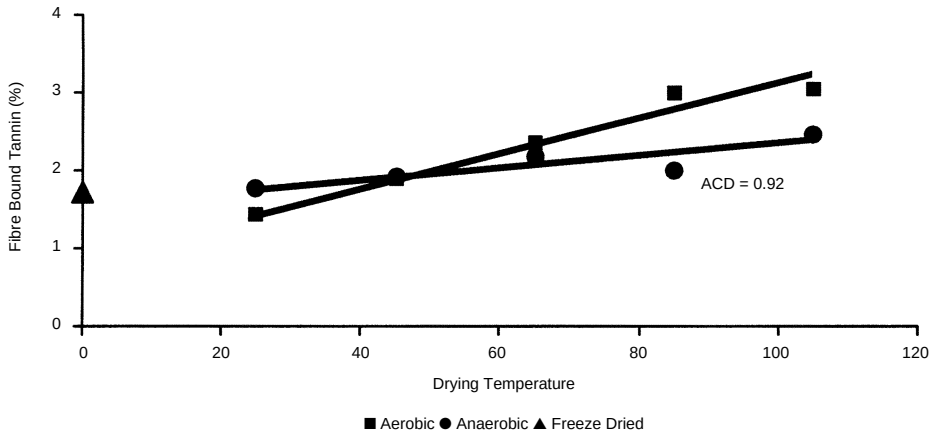


Figure 3. Fibre bound tannin vs drying temperature.

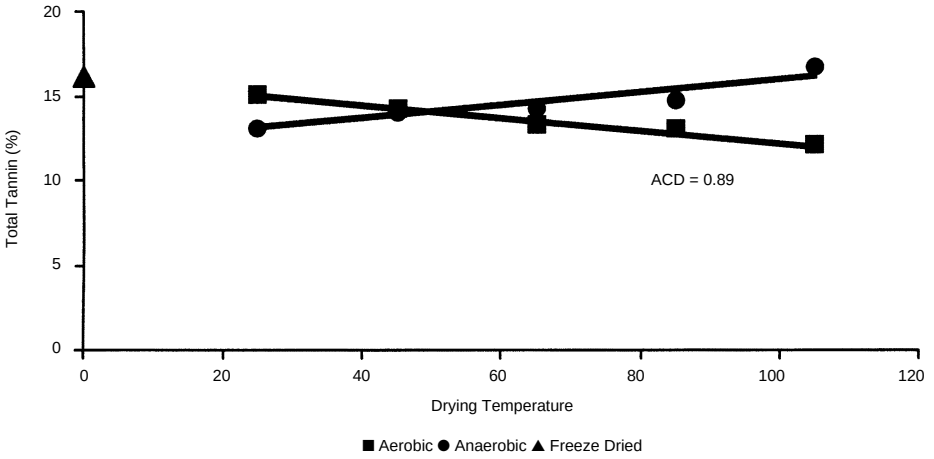


Figure 4. Total tannin vs drying temperature.

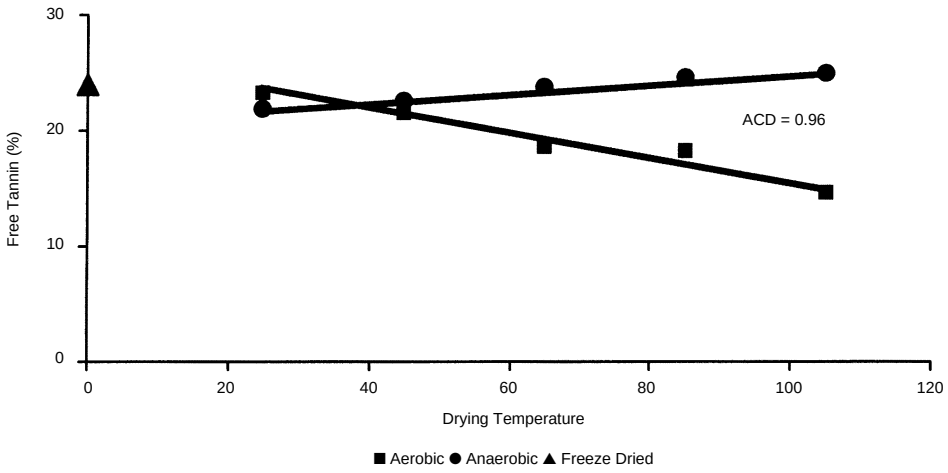


Figure 5. Free tannin vs drying temperature.

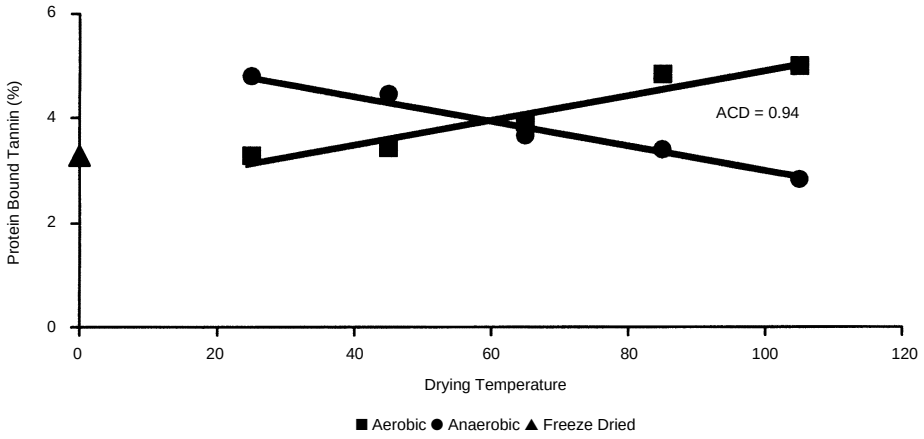


Figure 6. Protein bound tannin vs drying temperature.

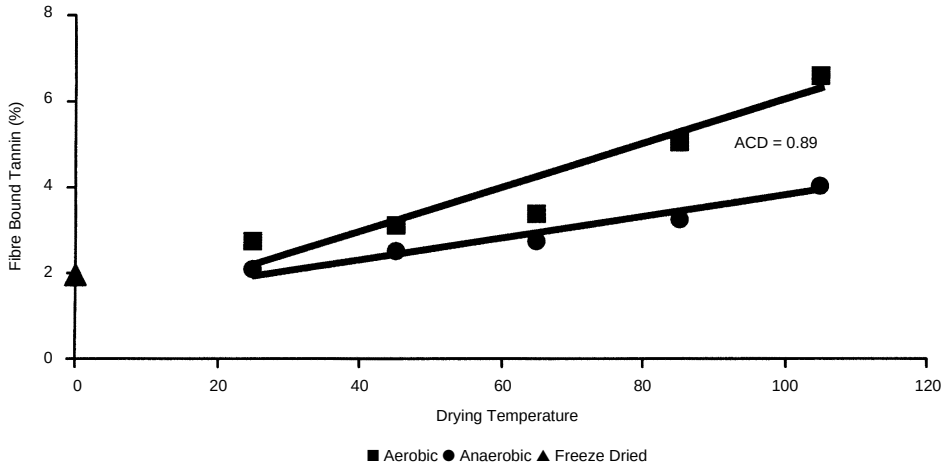


Figure 7. Fibre bound tannin vs drying temperature.

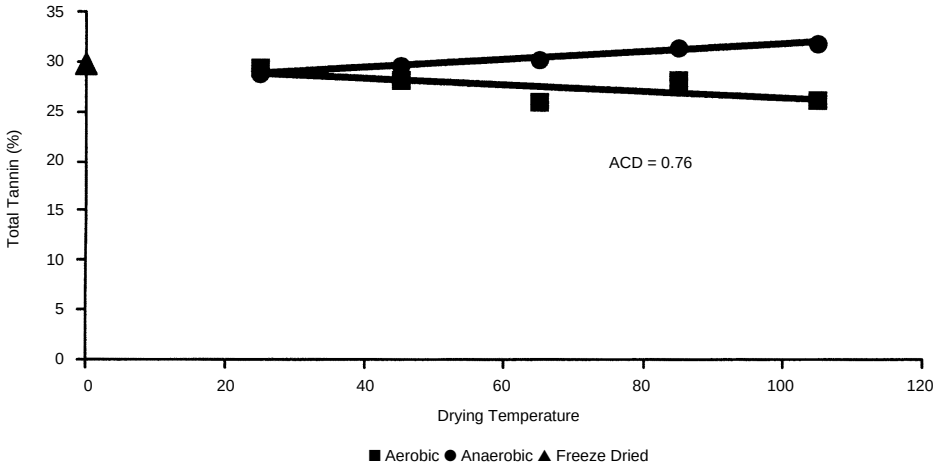


Figure 8. Total tannin vs drying temperature.

Reference

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

Tannins: Biological Activity and Bacterial Tolerance

A.N. Pell,[1] T.K. Woolston,[1] K.E. Nelson[1] and P. Schofield[1]

Abstract

Two areas of tannin-related research will be discussed: 1) interrelationships, assessed by comparing 16S rDNA sequences, among tannin-tolerant bacteria (TTB) isolated from different animal species and locations; and 2) methods to assess the biological activity of tannins that do not rely on precipitation as an end-point. The ability of gastrointestinal microbes to tolerate and detoxify tannins may explain why some animals can tolerate much higher dietary levels of proanthocyanidins than others. To determine how TTB from different species and locations are related, faecal, crop or ruminal samples were obtained from the following locations and species: Venezuelan hoatzin (*Opisthocomus hoazin*), Ugandan mountain gorilla (*Gorilla gorilla berengei*), Malagasy red-bellied lemur (*Eulemur rubriventer*), New York white-tail deer (*Odocoileus virginianus*), Rocky Mountain elk (*Cervus elaphus nelsoni*) from Oregon, Sardinian sheep (*Ovis aries*), Colombian and Honduran goats (*Capra hircus*) and non-lactating New York cows (*Bos taurus*). Bacteria able to tolerate at least 0.2% (w/v) of tannic acid or 0.1% (w/v) purified quebracho were isolated from all samples except from those from cows at Cornell University. Closely related members of the *Streptococcus* genus were isolated from gorilla and lemur faeces as well as from ruminal samples of elk, white-tail deer, goats and sheep. In addition, TTB representing *Enterobacteriaceae*, and taxon subcluster XIVa were isolated. Tannin tolerance is not limited by either species or geographical barriers. Failure to isolate TTB from cows fed grass hay suggests that diet affects the presence of TTB. The authors have developed a new colorimetric assay for tannin biological activity based on tannin inhibition of the lysozyme-catalysed hydrolysis of dye-labelled *Micrococcus luteus* cells.

TWO UNRESOLVED issues in the study of the antinutritional effects of tannins are: 1) the role of gastrointestinal micro-organisms in tannin tolerance and detoxification; and 2) prediction of the biological activity of tannins. Tannin-tolerant bacteria (TTB) have been isolated from the gastrointestinal tracts of animals consuming high tannin diets including koalas, dogs, possums, cattle, kangaroos, guinea pigs and humans (Nemoto et al. 1995), goats (Brooker et al. 1994) and other species (Nelson et al. 1998). Are these TTB from different host species closely related and do they employ similar mechanisms to protect themselves against tannins? What roles do they play in tannin tolerance of the host animals?

Animals respond differently to dietary tannins in part because of the variation in the biological activity of the tannins themselves (Hagerman et al. 1992; Reed 1995). Most biological assays for tannins have relied on protein precipitation (Hagerman and Butler 1978; Asquith and Butler 1985) despite the fact that both soluble and insoluble tannin-protein complexes are formed (Mueller-Harvey and McAllan 1992). One explanation of why the correlations between tannin content and biological activity are low (Giner-Chavez 1996) is that the assays for biological activity are based on an unrealistic end point, precipitation. Part of this research program has involved development of methods to measure the biological activity of tannins without using precipitation as the end point.

[1] Department of Animal Science, Cornell University, Ithaca, NY 14853, USA

Relatedness of Tannin-Tolerant Bacteria

The ability of gastrointestinal microbes to tolerate and detoxify tannins may explain why some animals can tolerate much higher dietary levels of tannins than others. It is generally accepted that ruminants can tolerate higher levels of many antinutritional

KEYWORDS: Tannins, Biological activity, Bacterial tolerance

factors than monogastrics because ruminal bacteria can metabolise many toxic compounds. The goal was to determine how TTB from different ruminant and non-ruminant species and locations are related.

Fresh faecal samples were collected from the mountain gorilla (*Gorilla gorilla berengei*) in the Bwindi Impenetrable Forest in Uganda and from the red-bellied lemur (*Eulemur rubriventer*) in the Ranomafana National Park in Madagascar. These primates were selected because they both consume diets high in secondary compounds, are simple-stomached animals and represent diverse geographical locations. Samples of rumen contents were taken from white-tail deer (*Odocoileus virginianus*) in New York, Rocky Mountain elk (*Cervus elaphus nelsoni*) in Oregon, Sardinian sheep (*Ovis aries*), Colombian and Honduran goats (*Capra hircus*), and nonlactating New York cows (*Bos taurus*). The deer samples were collected after the animals were killed during hunting season. Samples from the Cornell cows were obtained through ruminal fistulas and all other samples were obtained by stomach tube. All of the ruminants from which samples were collected except for the cows at Cornell shared one characteristic, consumption of diets containing tannins. In addition, crop samples from the hoatzin (*Opisthocomus hoazin*) were collected either by Pasteur pipette or from sacrificed birds. The hoatzin, the only avian folivore that can fly (Strahl and Schmitz 1990), relies on foregut fermentation for provision of essential amino acids and vitamins and inhabits the basins of the Amazon and Orinoco Rivers (Grajal 1995; Thomas 1996). Preliminary data suggest that the diet of the hoatzin contains relatively low levels of condensed tannins (T.K. Woolston, unpublished data) but that saponins and other antinutritional factors are present in their diets. In addition to isolating samples from both young and mature birds, we received cultures courtesy of M.-G. Dominguez-Bello (IVIC, Venezuela) which had been enriched for saponin tolerance. We were interested in the hoatzin samples to determine whether bacteria tolerant of one secondary compound, saponins, were able to tolerate tannins as well.

Bacteria able to tolerate at least 0.2% (w/v) of tannic acid or 0.1% (w/v) purified quebracho were isolated from all samples except from those from Cornell cows. We evaluated the ability of the isolated bacteria to tolerate hydrolysable (tannic acid) and condensed (quebracho) tannins as well as p-coumaric acid, ferulic acid and catechin. Genomic DNA was extracted from broth cultures of the organisms using a phenol: chloroform:isoamyl alcohol protocol and was amplified by PCR. After bands on agarose gels that corresponded to the anticipated size of the PCR product were excised, the DNA was extracted and sequenced on an automated DNA sequencer. After alignment, these sequences were compared to known sequences from the Ribosomal Database Project (Maidak et al. 1997).

There are three important observations from this analysis: 1) TTB were isolated from all of the samples except for those from the cows receiving low tannin diets at Cornell; 2) a cluster of bacteria of the *Streptococcus* genus were isolated from the faeces of the gorilla and the lemur as well as from ruminal samples of elk, goats, gorilla and sheep. The gorilla isolate was a very close relative of *Streptococcus caprinus* (Sly et al. 1997); and 3) TTB representing *Enterobacteriaceae*, and taxon subcluster XIVa were isolated (Collins et al. 1994). Unfortunately, we did not obtain the sequence for *Eubacterium oxidoreducens*, a bacterium known to metabolise phloroglucinol and gallate (Krumholz et al. 1987). One of the TTB isolated from elk not only could tolerate high levels of both condensed and hydrolysable tannins, but it was also weakly cellulolytic and a strict anaerobe (Nelson et al. 1998). The conclusions from these analyses are that tannin-tolerance is not limited by either species or geographical barriers. Tannin-tolerant bacteria were found in primates, ruminants and birds located on four continents, North America, South America, Africa and Europe. Others have isolated TTBs from Australia and Asia (Nemoto et al. 1995; Sly et al. 1997). Figure 1 shows locations from which TTBs have been isolated both by others and in our laboratory.

Repeated failures to isolate TTB from cows fed mixed grass hay suggests that diet affects the presence of TTB. Some, but not all, of the saponin-tolerant bacteria from the hoatzin crop were able to tolerate both tannins and saponins. Because many gastrointestinal microbes are as yet uncultured and because of bias during enrichment, the bacteria obtained by enrichment may not be the most important bacteria involved in tannin tolerance. The *Streptococcus* subcluster can provide useful information on tannin tolerance because *Streptococcus bovis* JB1, a close relative of this group of TTBs, was among the least tannin tolerant of the bacteria that we have worked with (Nelson et al. 1997).

Observations on Mechanisms of Tannin Tolerance

From a nutritionist's viewpoint, tannins in animal feeds are a double-edged sword. Potential benefits include protein sparing in the rumen and potential detriments include microbial inhibition and decreased microbial yield. Thus a clear understanding of the mechanisms used by bacteria to achieve tannin tolerance may help us to use this sword productively.

Figure 1. Stars indicate the locations from which samples were collected that resulted in the successful isolation of tannin-tolerant bacteria.

Consider, first, some possible mechanisms by which tannins act as cell toxins. There is little, if any, evidence that tannins exert their effects intracellularly. They must therefore act on either the cell wall or cell membrane, or on extracellular elements such as cell-secreted enzymes. The cell membrane is extensively involved in the transport of small molecules (e.g. sugars, amino acids) into the cell and it is clear that these transport processes are vital to the cellular economy. Such processes are potential targets for tannin action. Tannins often cause aggregation of bacterial cells, but it is difficult to see how aggregation per se would have a pronounced effect on transport events. Aggregation might, however, have more serious consequences for cell division.

There are at least three possible means of defence for a tannin-tolerant bacterium. The first, a 'diversionary' tactic, is for the cell to provide alternative and biologically inexpensive targets for tannin binding such as capsule. The second, a more 'active' approach, is to elaborate tannin-resistant enzymes to attack the tannin structure; for example, by hydrolysis of galloyl esters or gallotannins (Deschamps 1989) or by methylation of phenolic hyroxyl groups. The third tactic is to protect key membrane proteins from tannins by strategic deployment of lipids (Horigome et al. 1988). All three tactics require some energy investment by the cell, a cost that has been verified experimentally (Nelson et al. 1997).

We must recognise that toxicity in vivo is moderated by the tendency of tannins to 'stick to everything'. In practice, vital cell targets are defended by a huge 'infantry' of alternative and non-essential targets or alternative binding sites akin to the role that proline-rich salivary proteins play in some mammals (Mole et al. 1990). In vitro experiments have shown that while only a few tannin molecules may completely inhibit a given enzyme (Schofield and Pell, unpublished data), this enzyme can be almost completely protected by the simultaneous presence of either a different protein or of other strongly tannin-binding polymers such as PEG or PVP (Firenzuoli et al. 1969).

A New Tannin Assay Based on Enzyme Inhibition

Many of the methods currently used to measure tannin levels in plant extracts rely on the ability of tannins to precipitate proteins from aqueous solution (Waterman and Mole 1994). This 'defining property'

(Hagerman et al. 1998) may not be the only cause of tannin biological activity. Other mechanisms may include the sequestration of metal ions (McDonald et al. 1996) and enzyme inhibition (Haslam 1996; McAllister et al. 1994). These other mechanisms can operate in aqueous solution and at lower tannin and protein concentrations than are needed to produce a precipitate.

To investigate enzyme inhibition, we have devised an assay using lysozyme. This enzyme is pre-incubated with known amounts of a tannin preparation and then assayed using a suspension of *Micrococcus luteus* cells dyed with Remazol Brilliant Blue (Ito et al. 1992). Only microgram amounts of enzyme and tannin are needed.

To explore this assay, aqueous acetone extracts from various tropical legumes were compared quantitatively by calculating the amount of dry matter of each extract required to reduce lysozyme activity to 50% of the control, or by measuring the slope of the inhibition plot. Assays for total phenolics and proanthocyanidins were compared with the lysozyme inhibition results.

Materials and methods

Plant Acetone Extracts: We thank Carlos Lascano (CIAT, Columbia) for ground and lyophilised samples of the following legumes: *Calliandra calothyrsus*, *Cratylia argentea*, *Desmodium ovalifolium*, *Flemingia macrophylla* (leaf and stem samples were treated separately). Each tissue sample (2 g) was extracted by sonication (15 min) with 2 by 20 mL of 70% aqueous acetone. After removal of the acetone, the aqueous residue was filtered and then extracted with petroleum ether and ethyl acetate to remove lipids, chlorophyll and simple phenolics.

Lysozyme Assay: This procedure was modified from that described by Ito et al. (1992) as follows:

1. Pre-incubation: lysozyme (Hen egg white, Sigma L6876, 2 μg), 0.2 M NaCl, and 0.05 M phosphate buffer, pH 7.0 is incubated for 1 h at 37 °C with tannin in a total volume of 0.5 mL. in a 1.2 mL Eppendorf centrifuge tube. A set of assays, using different tannin dilutions, were first done to determine the appropriate amounts of tannin required to give approximately 50% inhibition.
2. Digestion: a suspension of dyed *M. luteus* cells (~ 350 μg) in 0.2 M NaCl, 0.05 M phosphate pH 7 (total volume 500 μL) is then added and incubation at 37 °C is terminated after 1 h by adding 50 μL 1 N NaOH.
3. Reading: after centrifugation for 10 min at 5000 × g, the absorbance of the supernatant is read at 600 nm. The readings from the blanks typically are <0.04.

Other Assays: Total phenolics (TP) (Graham 1992) and proanthocyanidins (butanol HCl–BuHCl) (Porter et al. 1986) as modified by Hagerman (1998) were measured.

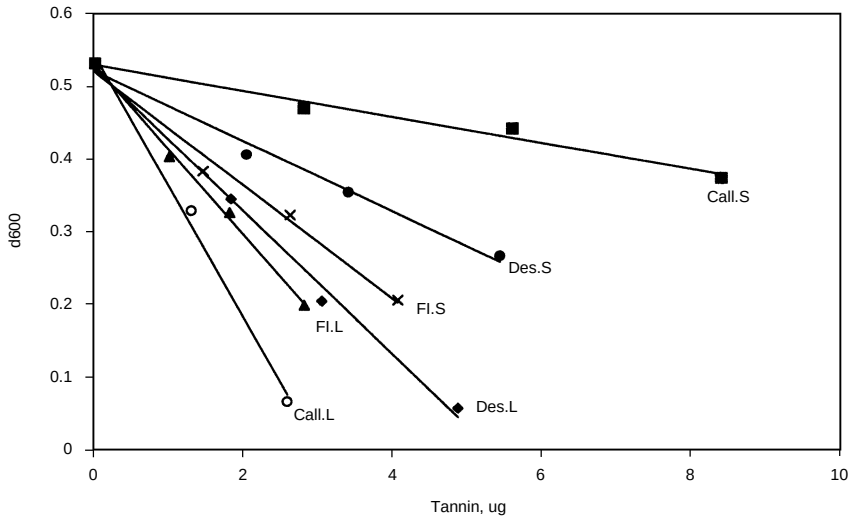


Figure 2. Raw data from tannin inhibition assays using lysozyme. Call = *Calliandra calothyrsus*, D = *Desmodium ovalifolium*, Fl = *Flemingia macrophylla*, L = leaf, S = stem.

Results and discussion

Figure 2 shows results from the lysozyme inhibition assays. The most active tannin extract was that from *C. calothyrsus* leaf, and the least active was from *C. calothyrsus* stem. *Flemingia macrophylla* and *D. ovalifolium* tannins had intermediate activity.

To compare data from different assays, all are reported on a per microgram basis, with the most active component in each assay assigned a value of 1.0 and the activities of all other components (e.g. slopes of the lines in Figure 2) were expressed as a fraction of this value. Figure 3 displays these comparisons.

From Figure 3, we see that:

1. The measured tannin relative activity depends on the assay used. The activity of calliandra leaf was highest for both TP and lysozyme inhibition (LI). That of desmodium leaf was highest for the BuHCl assay.
2. The total phenolics assay correlated well (r^2 = 0.92) with LI, less well with BuHCl (R^2 = 0.57). LI correlated poorly with BuHCl (R^2 = 0.15).
3. Cratylia appears to contain only a low level and activity of these polyphenolic compounds.

There is a need for a biologically-based alternative to the standard chemical assays like the proanthocyanidin and total phenolics methods. These latter assays, together with protein precipitation, measure quite different tannin properties none of which are clearly linked to tannin:protein interactions in solution. Enzyme inhibition assays may provide an insight into these interactions. If the biological activity of tannins can be well characterised, it should be easier to learn how bacteria tolerate and detoxify tannins.

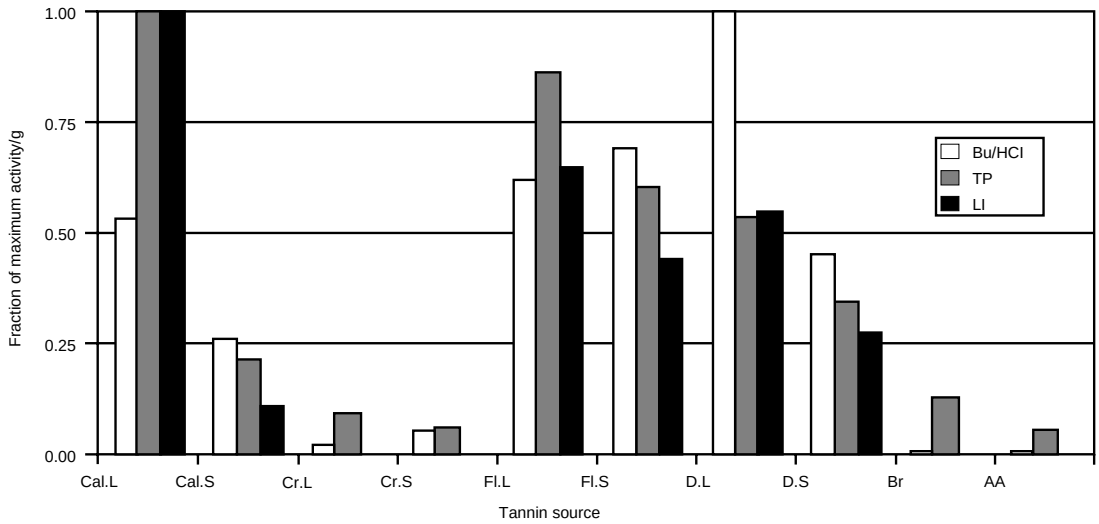


Figure 3. Comparison among proanthocyanidin (BuHCl), total phenolics (TP) and lysozyme inhibition (LI) assays. All results expressed as a fraction of the maximum response. Cal = *Calliandra calothyrsus*, Cr = *Cratylia argentea*, D = *Desmodium ovalifolium*, Fl = *Flemingia macrophylla*, Br = *Bromus inermis*, AA = *Medicago sativa*, L = leaf, S = stem.

References

Asquith, T.N. and Butler, L.G. 1985. Use of dye-labeled protein as spectrophotometric assay for protein precipitants such as tannin. Journal of Chemical Ecology, 11: 1535–1544.

Brooker, J.D., O'Donovan, L.A., Skene, I., Clarke, K., Blackall, L. and Muslera, P. 1994. *Streptococcus caprinus* sp. nov., a tannin-resistant ruminal bacterium from feral goats. Letters in Applied Microbiology, 18: 313–318.

Collins, M.D., Lawson, P.A., Willems, A., Cordoba, J.J., Fernandez-Garayzabal, J., Garcia, P., Cai, J., Hippe, H. and Farrow, J.A.E. 1994. The phylogeny of the genus *Clostridium*: Proposal of five new genera and eleven new species combinations. International Journal of Systematic Bacteriology, 44: 812–826.

Deschamps, A.M. 1989. Microbial degradation of tannins and related compounds. In: Lewis, N.G. and Paice, M.G. ed. Plant Cell Wall Polymers. American Chemistry Society, Washington, D.C., 559–567.

Firenzuoli, A.M., Vanni, P. and Mastronuzzi, E. 1969. The effect of some aromatic compounds on pure enzymes and their subsequent reactivation by PVP and Tween 80. Phytochemistry, 8: 61–64.

Giner-Chavez, B. 1996. Condensed Tannins in Tropical Forages. Ph. D. Dissertation. Cornell University, Ithaca, NY. 1–196.

Graham, H.D. 1992. Stabilisation of the Prussian blue color in the determination of polyphenols. Journal of Agriculture and Food Chemistry, 40: 801–805.

Grajal, A. 1995. Digestive efficiency of the Hoatzin, *Opisthocomus hoazin*: a folivorous bird with foregut fermentation. IBIS, 137: 383–388.

Hagerman, A.E. 1998. Tannin Analysis. Laboratory Notes/ Web Site http://miavx1.edu/~hagermae/.

Hagerman, A.E. and Butler, L.G. 1978. Protein precipitation method for the quantitative determination of tannins. Journal of Agriculture and Food Chemistry, 26: 809–812.

Hagerman, A.E., Rice, M.E. and Ritchard, N.T. 1998. Mechanisms of protein precipitation for two tannins, pentagalloyl glucose and epicatechin$_{16}$ (4>) catechin (procyanidin). Journal of Agriculture and Food Chemistry, 46: 2590–2595.

Hagerman, A.E., Robbins, C.T., Weerasuriya, Y., Wilson, T.C. and McArthur, C. 1992. Tannin chemistry in relation to digestion. Journal of Range Management, 45: 57–62.

Haslam, E. 1996. Natural polyphenols (vegetable tannins) as drugs: Possible modes of action. Journal of Natural Products, 59: 205–215.

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

Ito, Y., Yamada, H. and Imoto, T. 1992. Colorimetric assay for lysozyme using *Micrococcus luteus* labeled with a blue dye, Remazol Brilliant blue R, as a substrate. Chemical and Pharmaceutical Bulletin (Tokyo), 40: 1523–1526.

Krumholz, L.R., Crawford, R.L., Hemling, M.E. and Bryant, M.P. 1987. Metabolism of gallate and phloroglucinol in *Eubacterium oxidoreducens* via 3-hydroxy-5-oxohexanoate. Journal of Bacteriology, 169: 1886–1890.

Maidak, B.L., Olsen, G.J., Larsen, N., Overbeek, R., McCaughey, M.J. and Woese, C.R. 1997. The RDP (ribosomal database project). Nucleic Acids Research, 24: 109–111.

McAllister, T.A., Bae, H.D., Yanke, L.J. and Cheng, K.-J. 1994. Effect of condensed tannins from birdsfoot trefoil on endoglucanase activity and the digestion of cellulose filter paper by ruminal fungi. Canadian Journal of Microbiology, 40: 298–305.

McDonald, M., Mila, I. and Scalbert, A. 1996. Precipitation of metal ions by plant polyphenols: Optimal conditions and origin of precipitation. Journal of Agriculture and Food Chemistry, 44: 599–606.

Mole, S., Butler, L.G. and Iason, G. 1990. Defense against dietary tannin in herbivores: a survey for proline rich salivary proteins in mammals. Biochemical Systematics and Ecology, 18: 287–293.

Mueller-Harvey, I. and McAllan, A.B. 1992. Tannins: Their biochemistry and nutritional properties. Advances in Plant Cell Biochemistry and Biotechnology, 1: 151–217.

Nelson, K.E., Pell, A.N., Doane, P.H., Giner-Chavez, B. and Schofield, P. 1997. Chemical and biological assays to evaluate bacterial inhibition by tannins. Journal of Chemical Ecology, 23: 1175–1194.

Nelson, K.E., Thonney, M.L., Woolston, T.K., Zinder, S.H. and Pell, A.N. 1998. Phenotypic and phylogenetic characterisation of ruminal tannin-tolerant bacteria. Applied and Environmental Microbiology, 64: 3824–3830.

Nemoto, K., Osawa, R., Hirota, K., Ono, T. and Miyake, Y. 1995. An investigation of gram-negative tannin-protein complex degrading protein in faecal flora of various mammals. Journal of Veterinary Medical Science, 57: 921–926.

Porter, L.J., Hrstich, L.N. and Chan, B.G. 1986. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. Phytochemistry, 1: 223–230.

Reed, J.D. 1995. Nutritional toxicology of tannins and related polyphenols in forage legumes. Journal of Animal Science, 73: 1516–1528.

Sly, L.I., Cahill, M.M., Osawa, R. and Fujisawa, T. 1997. The tannin-degrading species *Streptococcus gallolyticus* and *Streptococcus caprinus* are subjective synonyms. International Journal of Systematic Bacteriology, 47: 893–894.

Strahl, S.D. and Schmitz, A. 1990. Hoatzins: cooperative breeding in a folivorous neotropical bird. In: Stacey, P.B. and Koenig, W.D. ed. Cooperative Breeding in Birds: Long Term Studies of Ecology and Behaviour. Cambridge University Press. Cambridge, 133–155.

Thomas, B.T. 1996. Family *Opisthocomidae* (Hoatzin). In: del Oyo, J., Elliott, A. and Sargatal, J. ed. Birds of the World, Volume 3. Hoatzin to Auks. Lynx Edicions. Barcelona, 24–32.

Waterman, P.G. and Mole, S. 1994. Analysis of Phenolic Plant Metabolites. Blackwell Scientific Publications, London. 1–238.

Mechanisms of Tannin Resistance and Detoxification in the Rumen

J.D. Brooker[1], L. O'Donovan[1], I. Skene[1] and G. Sellick[1]

Abstract

Tannins reduce the nutritive value of forage through inhibitory effects on ruminal and intestinal functions. Some animals have adapted to tannins through the synthesis of tannin-binding salivary proteins, the presence of tannin-resistant or tannin-degrading ruminal/intestinal micro-organisms, or other potential adaptations in the lower intestinal tract. *Streptococcus caprinus/gallolyticus* is found ubiquitously in the rumen of many animals browsing tannin-rich forage legumes. Biochemical studies have shown that this bacterium metabolises gallic acid to pyrogallol, although it does not metabolise pyrogallol, and produces extracellular polysaccharide (EPS) in response to tannins in the growth medium. Induction of EPS appears to be a bacterial defence mechanism that permits the bacterium to maintain its population when related species are dying. *Selenomonas ruminantium* K2 grows in the presence of hydrolysable or condensed tannins as a sole carbon source and secretes a tannin-inducible tannin acylhydrolase. This enzyme has been isolated and characterised. A number of other tannin-resistant bacterial species, including *Lactobacillus*, *Butyrivibrio* and *Enterobacteriacae* have been isolated, although their mechanisms of tannin resistance are not known. In addition to effects of tannins on microbial function, intestinal function studies have revealed that tannins inhibit nutrient metabolism and uptake in the abomasum and small intestine of ruminants. Alkaline phosphatase and aminopeptidase-N activities are inhibited, intestinal microvilli structure is disturbed and signs of tissue fragility are evident. These studies indicate that the protein-complexing action of tannins may effect intestinal as well as rumen function and that microbial interactions in the rumen may reduce but not eliminate tannin toxicity.

PHENOLIC secondary plant products, mainly comprising tannins, are ubiquitous in plants ranging in concentrations from <2% to more than 20% of dry weight and pose a worldwide problem for grazing livestock because they often prevent effective utilisation of forage (Haslam 1979). Tannins characteristically bind with proteins, carbohydrates and minerals and dramatically inhibit digestive and absorptive processes in the rumen of grazing ruminants (Kumar and Singh 1984). Studies on intestinal structure suggest that post-fermentative changes to digestive functions may also occur in ruminant and monogastric animals (unpublished). Livestock consuming tannin-rich diets (>5% w/v tannin) usually develop a negative nitrogen balance and lose weight and body condition unless supplemented with non-protein nitrogen, carbohydrate and minerals. Phenolic compounds also interact with salivary and mucosa-associated proteins producing astringency which is reflected in reduced feed intakes in grazing animals.

Studies of feral ruminants (goats, camels) in Australia have demonstrated that these animals exhibit resistance to tannins, possibly mediated through rumen microbial populations that may modify or degrade these compounds (Brooker et al. 1994; Skene and Brooker 1995). However, it is not clear how this occurs, nor what role these tannin-resistant organisms play in the overall ecology of the rumen. The studies described here report on possible mechanisms of tannin tolerance in two ruminal isolates, *Streptococcus caprinus* (*gallolyticus*) and *Selenomonas ruminantium* K2, and also on changes in intestinal function as a result of tannin interactions.

[1] Animal Science Department, University of Adelaide, Waite Campus. Glen Osmond 5064, Australia. Email: jbrooker@waite.adelaide.edu.au

KEYWORDS: Tannins, Bacterial resistance, Selenomonas, Streptococcus, Tannin acylhydrolase

Materials and Methods

Bacterial isolation

All bacteria used in this study were isolated from crude rumen fluid obtained from feral goats or camels browsing *Acacia aneura* (Mulga) after selection on Brain Heart Infusion (BHI) medium containing varying concentrations (up to 5% w/v) of tannic acid or condensed tannin (Brooker et al. 1994). Isolates were colony purified and identified by metabolic, biochemical characteristics and 16 S rDNA mapping.

Condensed tannin isolation

Condensed tannin (CT) was isolated from *Acacia* and *C. Calliandra* leaves by 70% acetone extraction followed by fractionation on Sephadex LH-20. The CT fraction was freeze-dried and stored in the dark under anaerobic conditions. Tannin content was analysed by the butanol-HCL method (Waterman 1994).

Tannin acylhydrolase

Methods for assay and purification of tannin acylhydrolase (TAH) were as described (Skene and Brooker 1995).

Animal feeding experiments

Five experimental diets comprised oaten hay chaff (OHC) (ad libitum), oaten hay chaff (800 g/day), Mulga, oaten hay chaff plus PEG-4000, and Mulga plus PEG-4000. Twenty animals on OHC or OHC + PEG diets were pair fed with corresponding sheep fed Mulga or Mulga + PEG respectively.

Enzyme assays

Alkaline phosphatase

Sections were prefixed in formal calcium (1% w/v $CaCl_2$, 8.75% v/v formalin in water) for 10 min, washed in 125 mM Tris-HCl (pH 9.2) at 39 ºC, and incubated in AP substrate (3.1 mg naphthol AS-BI phosphate (Sigma), 10 mg Fast Red (BDH Chemicals, UK), 60 uL dimethylformamide in 10 mL 125 mM Tris-HCl pH 9.2) for 21 minutes. The reaction was stopped by immersing sections in ice-cold 125 mM Tris-HCl pH 7.5, fixed in 4% v/v formaldehyde at room temperature and mounted in warm glycerin jelly. Samples were stored in the dark at 4 ºC until analysis.

Aminopeptidase-N

Samples were fixed in formal calcium (as above) at 4 ºC for 10 min, rinsed in 0.85% w/v saline solution and incubated in 0.1 M $CuSO_4$ for 2 min. Treated samples were incubated at 39 ºC in a substrate solution comprising 2 mg of L-alanine 4-methoxy-β-napthylamide dissolved in 0.05 mL ethanol, 0.45 mL distilled water, 5 mL of 0.1 M sodium acetate buffer pH 6.5, 4 mL of 0.5% w/v saline, 0.5 mL of 13% w/v KCN and 5 mg of Fast Blue B (BDH Chemicals, UK). Assayed samples were stored at 4 ºC until analysis.

For each enzyme assay, precipitated reaction product was measured on a calibrated (density, brightness, perimeter) image analysis program. Measurements were made along the crypt-villous axis, recorded at 4 pixel intervals using a 492 nm polarised filter on an Olympus BH-2 microscope.

Statistical analyses

Measurements were analysed in a repeated-measures ANOVA, including 'within sheep' factors of replication and region.

Results

Streptococcus caprinus

This organism grew in the presence of up to 5% w/v tannic acid or condensed tannins isolated from *Acacia*, but it did not utilise the tannins as a carbon source. Significant differences in the pre-exponential phase lag period were observed as the concentration of tannic acid or condensed tannin in the medium was increased. For *S. caprinus*, lag periods were 3, 5, 7, 16 and 23 h for concentrations of tannic acid in the medium of 0.5, 1.0, 2.0, 3.0 and 5.0% w/v (Figure 1). The presence of 0.5, 1.0 or 2.0% w/v condensed tannin caused a lag time for *S. caprinus* of 5, 8 and 11 h respectively.

GLC analysis of spent medium revealed the presence of bacterium produced in a time dependent manner when *S. caprinus* was incubated in the presence of tannic acid or gallic acid (Figure 2). The bacterium could not utilise pyrogallol for growth. Pyrogallol was not detected following anaerobic incubation of cell-free extracts prepared from *S. caprinus* with either tannic acid or gallic acid. However, incubation of gallic acid and tannic acid with washed whole *S. caprinus* cells resulted in the production of pyrogallol.

The specific activity of gallate decarboxylase increased 4-fold when the bacteria were grown in gallic acid compared with cells grown in mBHI medium containing no phenolic acid. After growth in the presence of tannic acid, the specific activity of gallate decarboxylase was increased 2.5 fold. However, gallate decarboxylase activity did not appear to be significantly up-regulated after growth in the

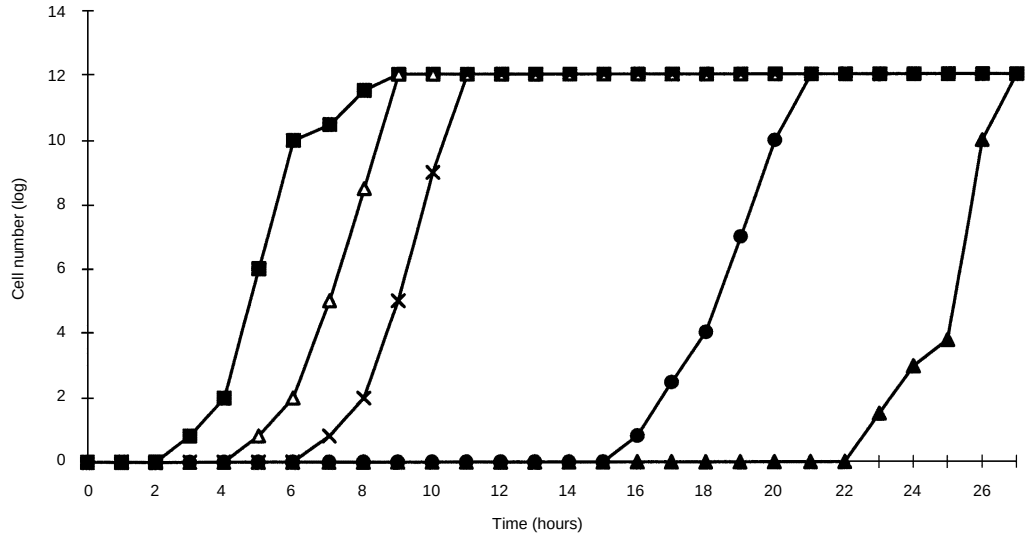


Figure 1. The effect of tannic acid on the growth lag period of *S. caprinus*. Cultures containing increasing amounts of tannic acid were inoculated with *S. caprinus* and growth was measured in samples by serial dilution, plating on nutrient agar (without tannic acid) and viable cell count. ■, 0; □, 0.5; Δ, 1.0; X, 2.0; ●, 3.0; ▲, 5.0% (w/v) tannic acid. Counts are expressed as the log of cell number.

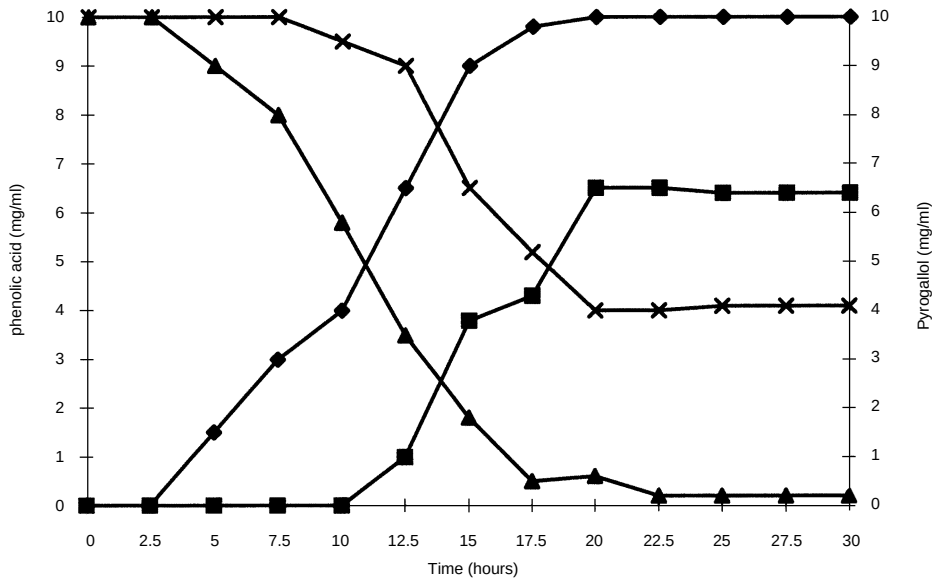


Figure 2. Time-dependent production of pyrogallol by *S. caprinus* incubated with tannic acid or gallic acid. Cultures were incubated with 1% (w/v) tannic acid or gallic acid and samples of medium were extracted with ethyl acetate, derivatised with TMS and analysed by GLC for the production of pyrogallol. Data points represent the mean of triplicate assays. Growth in; ▲, gallic acid; X, tannic acid. Pyrogallol production from; ◆ gallic acid; ■, tannic acid.

presence of condensed tannin or other phenolic acids including protocatechuic, vanillic, syringic or hydroxybenzoic acids.

When *S. caprinus*, grown in the absence of tannin, was observed under field emission scanning electron microscopy (FESEM), extracellular material surrounding the cells appeared globular in structure and were present in patches on the surface of the bacterium. Under the same growth conditions, no extracellular material was evident on *S. bovis*. With the addition of 0.2–0.5% w/v tannic acid to the growth medium, the amount of extracellular material surrounding both *S. caprinus* and *S. bovis* increased. However, at concentrations of tannic acid greater than 2% w/v, the extracellular material completely encased *S. caprinus* whereas growth of *S. bovis* ceased. A time course of EPS synthesis showed that little EPS accumulated during early logarithmic growth of *S. caprinus*, but increasing amounts were produced as the culture moved from late log into stationary phase (Figure 3).

Characterisation of extracellular material

The average yield of crude extracellular material isolated from S. caprinus was approximately 0.95 ± 0.12 mg/mg cells (dry weight), the material had a molecular weight equal to or greater than blue dextran (2×10^6) and was principally associated with the bacterial cell surface. Analysis of the alditol acetate derivatives of hydrolysates by GLC and GLC-MS indicated that the neutral sugar composition was primarily glucose with trace amounts of mannose (glucose:mannose = 1:0.2). Acyl and N-acyl residues were also detected. No uronic acids or hexosamines were present when *S. caprinus* was grown in mBHI media in the absence of tannic acid, but increased amounts of uronic acids were detected after growth in tannic acid-containing medium. Extracellular material of a similar molecular weight was isolated from cultures of *S. bovis* (0.8 ± 0.06 mg/mg cells dry weight) grown in the absence of tannic acid. However, its composition differed, comprising mannose, glucose and galactose in the ratio of 1: 0.7: 0.2, as well as a larger amount of acyl and N-acyl groups, and some uronic acids. *S. bovis* did not grow (and therefore did not produce extracellular material) in the presence of tannic acid.

Selenomonas ruminantium K2

Isolated on BHI plates containing up to 5% w/v tannic acid, this organism was able to grow on either tannic acid or condensed tannin as a sole carbon source and was shown to produce gallic acid from tannic acid. Tannin acylhydrolase activity was demonstrated using gallic acid methyl ester (GAME) as an artificial substrate and activity was shown to

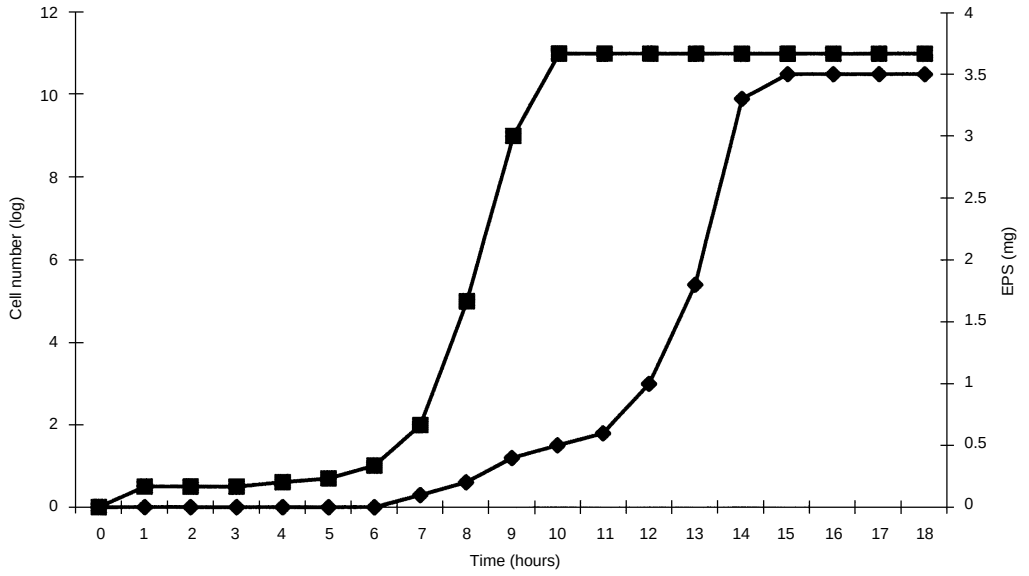


Figure 3. Production of extracellular polysaccharide by *S. caprinus*. Cells were incubated in medium containing 1% (w/v) tannic acid and samples were removed at various times for viable cell counts. EPS was determined by colorimetric assay and expressed as glucose equivalents. Values represent the average of triplicate assays. ■, log viable cell count; ◆, EPS (mg).

increase by up to 35 fold when K2 was grown in the presence of tannic acid or GAME (Table 1), but not monomeric phenols. The enzyme was demonstrated by zymogram to have a molecular weight of 59 kilodaltons and was purified by 2-dimensional gel electrophoresis and isoelectric focussing. Purified enzyme was sequenced at the N terminus by automated Edman degradation, and at an internal site following endo-lysC degradation.

Table 1. Effect of phenolic compounds on specific activity of TAH in vivo. *Sel. ruminantium* K2 cells were grown in the presence or absence of phenolic compounds and cell-free extracts were assayed for TAH activity.

Growth medium	TAH activity
mBHI	0.13 ± 0.02
mBHI + 0.2% tannic acid	4.52 ± 1.10
mBHI + 0.2% ferulic acid	0.12 ± 0.03
mBHI + 0.2% catechin	0.18 ± 0.04
mBHI + 0.1% GAME	2.10 ± 0.20
mBHI + 0.2% gallic acid	0.42 ± 0.09

Histochemistry of intestinal brush border enzymes

To determine whether tannins had effects downstream from the rumen, the authors examined enzyme profiles from regions of the intestine of sheep fed *Acacia aneura* (Mulga).

Alkaline phosphatase (AP) activity was measured in the duodenum, jejunum and ileum of sheep in five different groups. The results (Table 2) show that there was no significant difference in AP activity across the various regions of the intestine within treatment groups but between groups, activity in the Mulga-fed sheep was 50–60% lower than oaten hay chaff (OHC), OHC + polyethylene glycol (PEG) or Mulga + PEG fed sheep.

Aminopeptidase-N (AP-N) specific activity was approximately 3 fold greater than AP activity, but the regional distribution was similar (Table 2). OHC, OHC + PEG and Mulga + PEG expressed similar AP-N activity, whereas activity in the duodenum, jejunum and ileum of Mulga-fed sheep was approximately 25% of the other treatment groups. The addition of PEG to the Mulga diet restored AP-N activity and regional distribution was demonstrated in the epithelial cells of the duodenum, and the ileum.

Discussion

The results with *S. caprinus* demonstrate that this bacterium reacts with several adaptive responses to the presence of tannins. The pre-exponential growth lag period increases, the activity of gallate decarboxylase is elevated and the synthesis of EPS is induced. However, the extent to which each of these contributes to the overall tolerance of *S. caprinus* to high concentrations of tannic acid or condensed tannins in the growth medium is unclear. The increased lag period suggests that some prior adaptation such as the synthesis of an enzyme or production of a glycocalyx may be a necessary requirement for continued growth.

The fact that at low concentrations of tannin, a similar lag period occurs with *S. bovis* (result not shown), but at higher tannin concentrations the bacterium does not continue to grow, suggests that this bacterium is not able to develop the necessary protective strategy. The production of pyrogallol from gallate is another possible mechanism of resistance, but this is only relevant where tannic acid or catachin-gallates are present. Where condensed tannins are predominant, it is possible that the production of a protective extracellular coat is the best strategy for *S. caprinus*.

Table 2. Effect of diet on AP and AP-N activities in the intestinal tract of sheep fed *Acacia aneura*.

Enzyme and location	Enzyme activities versus diet				
	Ad lib.	OHC	Mulga	OHC + PEG	Mulga + PEG
Alkaline phosphatase*					
Duodenum	0.25 ± 0.04	0.28 ± 0.05	0.17 ± 0.02	0.25 ± 0.02	0.22 ± 0.02
Jejunum	0.33 ± 0.02	0.26 ± 0.02	0.16 ± 0.03	0.31 ± 0.02	0.24 ± 0.03
Ileum	0.26 ± 0.03	0.21 ± 0.03	0.17 ± 0.04	0.32 ± 0.03	0.31 ± 0.03
Aminopeptidase N*					
Duodenum	0.79 ± 0.02	0.81 ± 0.02	0.15 ± 0.01	0.51 ± 0.11	0.59 ± 0.02
Jejunum	0.55 ± 0.08	0.80 ± 0.02	0.10 ± 0.01	0.77 ± 0.03	0.88 ± 0.03
Ileum	0.85 ± 0.03	0.81 ± 0.02	0.20 ± 0.02	0.65 ± 0.06	0.75 ± 0.02

* Activities are expressed as mean absorbance/μm^2 of microvillus membrane.

For *Sel. ruminantium* K2, the only strategy appears to be the synthesis of tannin acylhydrolase. A 35-fold induction of the enzyme results in significant cell-associated activity that cleaves the glucose moiety from hydrolysable tannins, and presumably allows the bacterium to ferment the glucose. It is not known how this organism develops tolerance to condensed tannins since it does not appear to secrete large quantities of EPS as does *S. caprinus*. The tannin acylhydrolase enzyme is not structurally identical to other acylhydrolases isolated from fungi and the partial gene sequence from TAH1 does not reveal any striking homologies with other genes. This is perhaps not surprising since there are very few genes sequenced from any *Selenomonad* and so direct comparisons are not possible. However, confirmation that TAH1 does indeed contain the correct gene awaits expression studies. The Southern data suggest that there are at least two similar genes but it is possible that only one of those is expressed.

The results of histochemical studies clearly demonstrate inhibitory effects on abomasal and intestinal function which are separate from effects on bacterial populations. Reduced activity of AP and AP-N were evident, and this was restored by the inclusion of PEG in the diet.

The simplest explanation of this effect is that enzyme activity was inhibited by the protein-binding action of tannins and this was alleviated by pre-binding the tannins with PEG. However, an alternate explanation is that the tannins inhibited enzyme secretion by forming a lining on the intestinal mucosa, thus preventing the export of proteins from the intestinal epithelial cells. This second explanation is supported by additional data (not shown) which demonstrates that tannins induce histological changes in the intestinal mucosa including abnormal villous structure and disruption of cellular networks of communication.

Therefore, these results indicate that tannins may inhibit several different processes including microbial and digestive tract functions, and that these effects may have an impact upon animal production over a range of grazing and browse feeds. However, recognition of the effects may be more problematic since the extent and duration of inhibition will depend upon a number of factors including diversity of forage available, intake, age of plant and other environmental influences. Resistance to tannins may therefore occur at several levels: microbial tolerance, tannin degradation and intestinal tract adaptation.

Acknowledgments

This work was supported by the Australian Centre for International Agricultural Research (ACIAR), the Australian Wool Corporation, and the Australian Research Council.

References

Brooker, J.D., O'Donovan, L.A., Skene, I., Blackall, L. and Muslera, P. 1994. *Streptococcus caprinus* sp. Nov., a tannin-resistant ruminal bacterium from feral goats. Letters in Applied Microbiology, 18: 313–318.

Haslam, E. 1979. Vegetable tannins. In: Swain, T., Harborne, J.B. and Van Sumere, C.F. ed. Biochemistry of Plant Phenolics (Recent Advances in Phytochemistry). Plenum Press, New York, 475–523.

Kumar, R. and Singh, M. 1984. Tannins, their adverse role in ruminant nutrition. Journal of Agriculture and Food Chemistry, 32: 377–453.

Skene, I.K. and Brooker, J.D. 1995. Characterisation of tannin acylhydrolase activity in the ruminal bacterium, *Selenomonas ruminantium*. Anaerobe, 1: 321-327.

Waterman, P.G. 1994. Analysis of phenolic plant metabolites. Waterman, P. and Mole, S. eds. Blackwell Scientific Publishers, Oxford.

Tannin Degrading Bacteria from Indonesian Ruminants

K.G. Wiryawan[1, 2], B. Tangendjaja[3] and Suryahadi [2]

Abstract

Five tannins degrading bacteria were isolated from goats naturally fed or adapted to calliandra diet. The bacteria could grow on Brain Heart Infusion medium containing 3% tannic acid or 1% condensed tannin. These bacteria were able to reduce 52% of tannic acid concentration (from 1% to 0.48%) in defined medium in 12 hours. Meanwhile, condensed tannins concentration was reduced by 48% (from 0.5% to 0.26%) in 72 hours in vitro. Inoculation of 3×10^{11} cfu of the bacteria could significantly increase goat body weight gain compared to that of untreated animals. Based on the experimental results, it can be concluded that Indonesian goats posses bacteria which are able to degrade condensed and hydrolysable tannins and its inoculation into goats unadapted to calliandra could improve digestibility (in vitro) and body weight gain.

CALLIANDRA (*Calliandra calothyrsus*) is a potential shrub legume for ruminant feed because it contains high protein (> 20% of dry weight) (Tangendjaja et al. 1992). However, its utilisation is limited due to its high tannins content (±10% of dry weight). This phenolic compound can bind protein, carbohydrate and minerals (Makkar 1991; White 1957) causing the nutrients to be not degradable in the digestive tract.

Some Indonesian ruminants such as goats have long been fed with fresh calliandra and do not show any symptoms of nutrient deficiency. This evidence indicates that the Indonesian goats may posses certain microbes in the digestive tract that could tolerate or degrade tannin compounds in calliandra.

The objective of this study was to investigate the existence of tannins tolerant/degrading bacteria of Indonesian goats and the possibility of using the isolated bacteria for cross-inoculation into animals sensitive to calliandra feeding.

Materials and Methods

Rumen microbial changes during adaptation to calliandra feeding

Eight fistulated goats previously fed with all grasses were adapted for 3 months with 50% calliandra diet and another three months with 100% calliandra. At the end of each feeding regime, tannin tolerant-bacterial populations were counted by culturing the rumen liquor in Brain Heart Infusion (BHI) agar medium containing 1% tannic acid.

Digestibility trial (in vitro) (Tilley and Terry 1963)

Digestibility of dry matter and crude protein of calliandra was compared in three different rumen liquors obtained from goats fed with 100% grasses, goats adapted to 100% calliandra and goats naturally fed with calliandra (Kaligesing goats). Into each ferment tube was added 24 mL McDougall solution (pH 6.9, temperature 39 °C), 6 mL rumen liquor and 0.5 g calliandra powder. To maintain anaerobic conditions, CO_2 was flushed continuously. Incubation was carried out for 2×48 hours (two-stage fermentation method).

Bacterial isolation

Tannin-tolerant bacteria were isolated through an enrichment process. Rumen liquor (0.5 mL) of goats fed with 100% calliandra or goats naturally fed with calliandra was transferred into 10 mL liquid BHI medium containing 0.4% tannic acid (TA). Cultures were then incubated for 24 hours. The following day, 0.5 mL of culture was transferred into other tubes containing 0.8% TA and incubated for 24 hours. This process was repeated for tubes containing 1.2,

[1] Life Sciences Inter University Center, Bogor Agricultural University, Indonesia
[2] Faculty of Animal Science, Bogor Agricultural University, Indonesia
[3] Animal Research Center, Ciawi — Bogor, Indonesia

KEYWORDS: Tannins, Bacteria, Goat, Calliandra

1.6, 2.0, 2.4% and finally 3% TA concentration. At each level of TA, different colonies of bacteria were investigated by growing the bacteria in agar media using a roll tube method.

Nine different colonies (six of adapted goats and three of Kaligesing goats) were isolated, but only five colonies (three of adapted goats and two of Kaligesing goats) showed clearing activity on tannic acid containing medium and grew on 1% condensed tannin. These bacteria were further identified biochemically.

Bacterial selection for inoculum

To investigate the most effective bacteria in degrading calliandra, three bacteria isolated from adapted goats were used as inocula, either single or mixture in in vitro digestibility trial. Each inoculum consisted of 3×10^9 cfu. Rumen fluid of goats that had never been fed with calliandra was used as medium and 0.5 g of powdered calliandra was the only substrate added. Incubation was carried out for 48 hours (fermentative stage).

Effective populations of inoculum

Dry matter digestibility of calliandra was used as an indicator for studying the effective populations required for inoculation. Three different population levels (3×10^8; 3×10^9; 3×10^{10}) of the most effective bacteria in the previous experiment were studied in in vitro trials.

Tannin degradation

The ability of the three isolates obtained from adapted goats was tested in degrading tannic acid and condensed tannins in 10 mL defined medium (Nili and Brooker 1993). One per cent (1%) (w/v) tannic acid or 0.5% (w/v) condensed tannins was added into the medium. Tannic acid and condensed tannin disappearances were measured for 48 hours and 72 hours respectively. As control, a defined medium containing the same amount of tannins minus bacteria was used. Tannin concentration was measured using protein precipitation method.

In vivo inoculation

Ten goats with the average body weight 22.4 ± 2.3 kg and previously fed with grasses were used in this experiment. At the time of inoculation, feed was abruptly changed from all grasses to all calliandra. The number of inoculum used was 10^8 cfu/mL final population (3×10^{11} cfu) for treated goats and no bacteria for control. Body weight of control and inoculated goats were recorded for 40 days after the inoculation.

Results and Discussion

The existence of tannin-tolerant bacteria in calliandra fed goats

Rumen bacterial population is influenced by some factors such as animal breed, geography and feeds. Different feeds may stimulate different bacteria in the rumen. Data in Table 1 show that calliandra feeding increased tannic acid tolerant bacterial population. The increase in bacterial population might be due to higher availability of tannins in the rumen or might be related to the increase in protein intake as all isolates showed proteolytic activity when grown on casein containing medium.

Table 1. Tannic acid tolerant bacterial population of goats fed different rations.

Diet	Population (cfu/mL)
100% grass	4×10^2
50% grass/50% calliandra	1.1×10^5
100% calliandra	2.0×10^6

To reconfirm the existence of tannin degrading bacteria in calliandra-fed goats, its rumen fluid was collected and used in in vitro assessment of calliandra digestibility. The result is shown in Table 2. Data indicated that rumen fluid of goats adapted to 100% calliandra and goats naturally fed calliandra had significantly ($P<0.05$) higher dry matter and protein digestibility compared with rumen fluid of grass-fed goats. The increase in dry matter and protein digestibility of calliandra-fed goats might be due to the increase in tannin tolerant bacterial populations in the rumen.

Table 2. Dry matter and crude protein digestibility of calliandra.

Sources of rumen fluid	Dry matter (%)	Crude protein (%)
100% grass	26.97^a	23.86^a
100% calliandra	34.13^b	28.36^a
Kaligesing	43.09^c	41.27^b

Different superscripts in the same column mean significantly different ($P<0.05$).

Based on the signal indicated above, isolation and purification of tannin-tolerant bacteria were carried out. Six different colonies of bacteria were isolated and purified, but only three isolates showed clearing zones on tannic acid and grew on 1% condensed tannin containing BHI medium. The three isolates were later used as inoculum in vitro to obtain the most effective bacterium in degrading calliandra.

Results indicated that isolate 3 was the most effective bacterium in degrading calliandra as shown by significant (P<0.05) improvement of dry matter digestibility of calliandra compared to the other treatments. Its VFA production was also higher compared to the other treatments, although statistically was not different (Table 3).

Table 3. The effect of bacterial inoculation on calliandra degradation.

Parameter	Control	U3	U4	U5	U3,4,5
DM digestibility (%)	26.80	30.72*	28.80	28.40	30.07
Total VFA (mM)	94.85	140.44	94.50	95.10	109.80

* Different superscripts in the same row mean significantly different (P<0.05).

Isolate 3 was further studied to find out the appropriate level of inoculum required to improve calliandra digestibility. Data in Table 4 show that at least 10^8 cfu/mL (final population) was required to significantly (P<0.05) increased dry matter digestibility of calliandra. However, a higher inoculum level (10^9 cfu/mL final population) would not improve calliandra digestibility.

Table 4. The effect of inoculum level on calliandra digestibility.

Parameter	Control	U3 (10^7)	U3 (10^8)	U3 (10^9)
DM digestibility (%)	27.34	27.89	30.70*	30.40*

* means significantly different (P<0.05).

Tannic acid and condensed tannin disappearance

During the tannins disappearance assay, the three isolates were able to reduce the concentration of tannic acid and condensed tannins in defined medium. Tannic acid concentration was reduced up to 52% (from 1% to 0.48%) in 48 hours and condensed tannin reduction was ±48% (from 0.5% to 26%) in 72 hours (Figures 1 and 2). However, the mechanism of tannin reduction is not clearly understood. It might be degraded by the bacteria or chemically modified so that modified tannins could not react with BSA used for tannin analysis. Further investigation is required to understand the bacterial mode of action.

Bacterial inoculation in vivo

Based on the above experimental results, isolate 3 was then used as an inoculum at the population of 3×10^{11} cfu on goats never been fed with calliandra. The results indicated that in the first twenty days both control and inoculated animals had body weight reduction. This could be due to the abrupt changed over of diet from all grasses to all calliandra diet. However, during the period of 21 to 40 days after inoculation, body weight gain of inoculated goats was significantly (P<0.05) higher than that of control. This means that inoculated goats could adapt calliandra faster than uninoculated goats. This might be the result of tannin deactivation by the bacteria so that the availability of free nutrient for rumen microbial degradation increased.

Conclusion

Indonesian ruminants especially goats posses tannin degrading bacteria and its population can be increased by feeding the animals with tannin containing ration. The bacteria could reduce tannic acid and condensed tannin concentration, therefore improving calliandra degradation. Moreover, in vivo inoculation of 3×10^{11} cfu bacteria improved the animals' ability to adapt calliandra feeding.

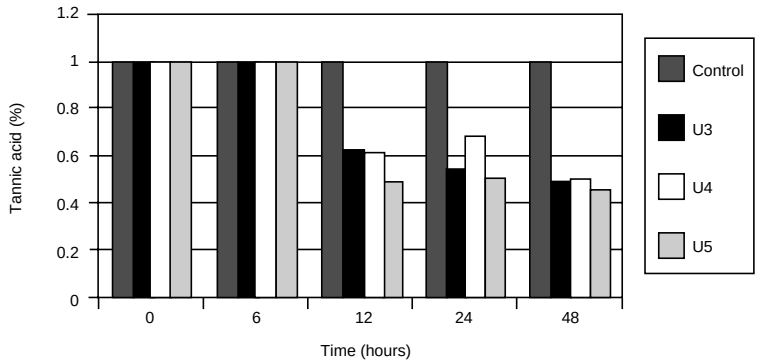


Figure 1. Tannic acid disappearance.

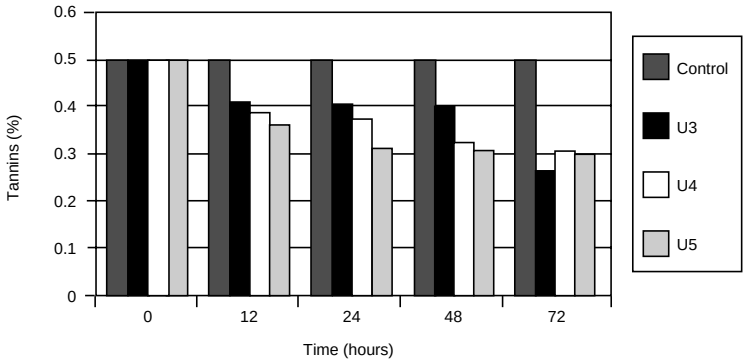


Figure 2. Condensed tannins disappearance.

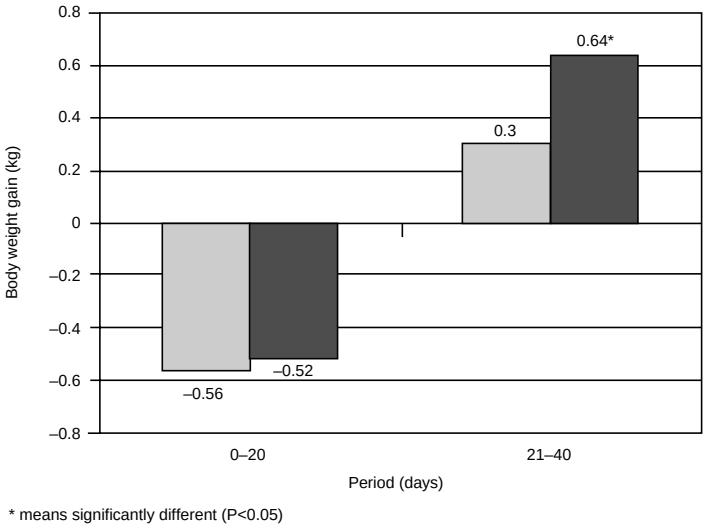


Figure 3. The effect of bacterial inoculation on body weight gain.

Acknowledgments

The authors thank the Australian Centre for International Agricultural Research (ACIAR) for funding this research through Project PN 9318.

References

Makkar, H.P.S. 1991. Antinutritional factors in animals' feedstuffs mode of actions. International Journal of Animal Science, 2(2): 127–140.

Nili, N. and Brooker, J.D. 1995. A defined medium for rumen bacteria and identification of strains impaired in *de novo* biosynthesis of certain amino acids. Letters in Applied Microbiology, 21: 69–74.

Tangendjaja, B., Wina, E. and Ibrahim, T. In: Palmer, B. 1992. Kaliandra (*Calliandra calothyrsus*) dan Pemanfaatannya. Balai Penelitian Ternak dan The Australian Centre for International Agricultural Research.

Tilley, J.M.A., and Terry, R.A., 1963. A two-stage technique for the in vitro digestion of forage crops. Journal British Grassland Society, 18: 104–111.

White, T. 1957. Tannins: their occurrences and significance. Journal of the Science of Food and Agriculture, 8: 377–384.

Plant Phenolic Compounds and Gastrointestinal Micro-Organisms

T. Acamovic[1] and C.S. Stewart[2]

Abstract

The effects of tannins and other polyphenolics on microbial function and the potential mechanisms by which they exert their effects are presented. It is suggested that although some micro-organisms in the gastrointestinal tract (GIT) may be resistant to the presence of tannins, it is unlikely that microbial degradation of the complex compounds classed as the proanthocyanidins will occur within the gastrointestinal tract. Creating conditions in which micro-organisms can function effectively within the GIT may be a realistic aim. The effects of some polyphenolic compounds on some, especially pathogenic, micro-organisms found in the GIT may be of benefit to man and other animals.

THE NUMBER of plant phenolic compounds which has been identified is estimated to be around 5000 (Bors et al. 1996). These compounds vary widely in complexity from simple single aromatic rings of relative molecular masses (RMMs) ranging from about 150 to much more complex oligomers and polymers with RMM up to about 40 000 (Waterman and Mole 1994; Bors et al. 1996; Waterman, these Proceedings; Chenyier, these Proceedings; Tanner et al., these Proceedings). The most commonly studied are the less complex compounds with RMMs less than 3000–4000.

The common characteristic of all of these compounds is that they are readily oxidised and undergo phenolic reactions (Waterman and Mole 1994; Bors et al. 1996). Because of the large variation in molecular mass, complexity and chemical lability, they have been difficult to characterise, quantify and study. The larger molecules are likely to exert their effects on surface-located microbial enzyme complexes and uptake systems while the smaller molecules, or degradation products of the larger compounds, are also likely to have a major effect within the micro-organisms, inhibiting the activity of cell enzymes (Bors et al. 1996).

The factor which has the greatest influence on microbial species profiles in the gastrointestinal tract of farmed species is the presence or absence of dietary antibiotics. The removal of these from monogastric diets in Europe will considerably influence microbial populations and some polyphenolic compounds may be beneficially used to control the growth of undesirable micro-organisms in such circumstances.

The effects of different polyphenolic compounds on different micro-organisms found in the GIT of ruminants and monogastrics are presented and discussed. Information on possible mechanisms of action of the different compounds is discussed, including interaction with surface proteins and interference in trace element metabolism. We will speculate on the consequences of the effects, possible methods of alleviation of adverse effects and promotion of beneficial effects in monogastrics and ruminants.

Polyphenol complexity

The complexity in molecular structure of polyphenols makes ready quantitative determination of these essentially impossible in most dietary plant species. The response of various purified proanthocyanidins to butanol-HCl has been shown to be different for a

[1] Department of Biochemistry and Nutrition, Scottish Agricultural College, Auchincruive, Ayr, KA6 5HW, Scotland, UK. (t.acamovic@au.sac.ac.uk)
[2] Rowett Research Institute, Bucksburn, Aberdeen, AB21 9SB, Scotland, UK. (css@rri.sari.ac.uk)

KEYWORDS: Polyphenols, Tannins, Microbes, Gastrointestinal tract, Monogastrics, Ruminants

series (e.g. *Lotus corniculatus*, *Lotus pedunculatus*, sorghum) of these (Acamovic and McNabb, unpublished). Chromatographic analyses of various purified tannins also demonstrated that more than a single compound was present, perhaps because of a lack of original purity or because of degeneration after purification (Benzie and Acamovic, unpublished). Such uncertainties in purity and quantity thus make it extremely difficult to assess the relative effects of numerous polyphenolic compounds on microbes, (and vice versa) especially when these compounds are present in dietary ingredients. The complexity and variability of tannins in feedstuffs, especially the proanthocyanidins, militate against finding a universal microbe that will degrade these compounds within the gastro-intestinal tract of animals.

Possible mechanisms

It is well demonstrated that tannins and other polyphenolic compounds increase endogenous losses and alter digestibility and the site of digestibility of nutrients, including minerals, protein and amino acids, in monogastrics and ruminants (Terrill et al. 1994; Wang et al. 1994; Tanner et al. 1994; Jansman et al. 1994; Yu et al. 1995; Reed 1995; Yu et al. 1996; Hewitt et al. 1997; Mansoori and Acamovic 1998a, b; Salawu et al. 1999). It thus seems feasible that tannins and other polyphenolics may also affect micro-organisms by adversely influencing their physiological functions, interfering with the uptake of minerals and other nutrients and with secretion, as has been demonstrated in animal and in vitro (Scalbert 1991; Makkar et al. 1995; Bors et al. 1996; Kainja et al. 1998). The sequestration of minerals such as Cu, Co, and Fe (McDonald et al. 1996; Kainja et al. 1998) may influence the metabolic capability of micro-organisms as well as their ability to attach to their various substrates and secrete appropriately active enzyme complexes. The ability of polyethylene glycol (PEG) to reduce and alleviate the adverse effects on microbial function may be due to PEG:protein:tannin interactions (Makkar et al. 1995; Salawu et al. 1997) as well as to reversal of the chelation of essential mineral elements (Kainja et al. 1998).

Effects of tannins on micro-organisms

There is abundant evidence that tannins exert inhibitory effects on micro-organisms from many different ecosystems, including the rumen. For example, Jones et al. (1994) reported that condensed tannins of the legume sainfoin (*Onobrychis vicifolia*) inhibited proteolytic activity and growth of several species of rumen bacteria. The inhibition of attachment of *Fibrobacter* to cellulose was reported by Bae et al. (1993). The effects of plant metabolites on gut microbial ecosystems are inevitably complex, and are likely to depend on the diversity of the species present. Some experiments in which tannins have been fed to ruminants have shown marked effects of these compounds on rumen protozoa and other micro-organisms (Salawu et al. 1998; Salawu et al. 1999). Muhammed et al. (1995) and Muhammed (1997) investigated the effects of the gallotannin tannic acid and lower RMM components of hydrolysable and condensed tannins in different experimental systems, including those containing mixed rumen micro-organisms, and pure cultures.

In a continuous batch culture system inoculated with mixed rumen organisms, tannic acid exerted a more marked inhibitory effect than was obtained with gallate, ellagic acid, catechin or epicatechin. Interestingly, gallate initially reduced cellulolysis, but after several culture transfers, appeared to enhance cellulose breakdown. Gallate can be degraded by some rumen bacteria, and the stimulatory effect on cellulolysis in mixed culture may have resulted from the supply of vitamins or other growth factors to cellulolytic bacteria in the incubations.

All of the compounds tested inhibited cellulolysis and zoospore attachment to cellulose by pure cultures of the rumen anaerobic fungus *Neocallimastix frontalis* strain RE1. However, gallic acid, ellagic acid and catechin were all more inhibitory to cellulolysis than was tannic acid, and ellagic acid was most inhibitory to zoospore attachment, perhaps indicating the involvement of different cell-surface receptors in these processes.

The activity of rumen fungal and bacterial xylanases was inhibited by tannic acid, and the enzyme assay system was used to compare the effectiveness of binding agents in protection against tannin binding. PEG 8000 was the most effective of the binding agents tested in the xylanase-tannic acid system, and the commercial product Browse Plus, which contained PEG 4000, was also shown to exert a protective effect.

Further work with pure cultures and cloned enzymes of rumen microbes could lead to a mechanistic understanding of the effects of phenolic compounds on the rumen fermentation, facilitating the development of new approaches to alleviating their deleterious effects.

Although there has been little work to compare rumen and colonic micro-organisms, recent work with ferulates suggest that there may be significant differences between the secondary metabolite transforming properties of these different microbial populations (Chesson et al. 1999).

More work is needed if the effects of these compounds on monogastric digestive processes are to be understood.

Finally, in view of the finding that some coumarin aglycones reduce the survival of the pathogenic bacterium *Escherichia coli* O157 under experimental conditions (Duncan et al. 1998) further work with polyphenolic metabolites may reveal other compounds with potentially protective effects.

Such revelations of the effectiveness of polyphenolic compounds including the tannins may be extremely beneficial as alternatives to the dietary antimicrobial growth promoters, the use of which is currently banned within the EU.

References

Bae, H.D., McAllister, T.A., Tanke, J., Cheng, K.J. and Muir, A.D. 1993. Effect of condensed tannins on endoglucanase activity and filter paper digestion by Fibrobacter succinogenes S85. Applied and Environmental Microbiology, 59: 2132–2138.

Bors, W., Heller, W., Michel, C. and Stettmaier, K. 1996. Flavonoids and polyphenols: Chemistry and Biology. In: Cardenas, E and Packer, L. ed. Handbook of Antioxidants, New York, Marcel Dekker, 409–466.

Chesson, A., Provan, G.J., Russell, W.R., Scobbie, L., Richardson, A.J. and Stewart, C.S. 1999. Hydroxycinnamic acids in the digestive tract of livestock and humans. Journal of the Science of Food and Agriculture, 79: 373–378.

Duncan, S.H., Flint, H.J. and Stewart, C.S. 1998. Inhibitory activity of gut bacteria against *Escherichia coli* 0157 mediated by dietary plant metabolites FEMS Microbiol. Lett., 164: 283–288.

Hewitt, D.G., Lafon, N.W. and Kirkpatrick, R.L. 1997. Effect of tannins on galliform cecal partitioning. Physiol. Zool., 70: 175–180.

Jansman, A.J.M., Enting, H., Verstegen, M.W.A. and Huisman, J. 1994. Effect of condensed tannins in hulls of faba beans (*Vicia faba* L.) on the activities of trypsin (EC 2.4.21.4) and chymotrypsin (EC 2.4.21.1) in digesta collected from the small intestine of pigs. J. Nutr., 71: 627–641.

Jones, G.A., McAllister, T.A., Muir, A.D. and Cheng, K.J. 1994. Effects of asainfoin (*Onobrychis vicifolia* Scop.) condensed tannins on growth and proteolysis by four strains of ruminal bacteria. Applied and Environmental Microbiology, 60: 1374–137.

Kainja, C., Bates, L. and Acamovic, T. 1998. The chelation of trace elements by tannins. In: Garland, T. and Barr A.C. ed. Toxic Plants and Other Natural Toxicants, CAB Intl., Wallingford, UK, 111–114.

Makkar, H.P.S., Blümmel, M. and Becker, K. 1995. Formation of complexes between polyvinylpyrrolidones or polyethylene glycol and tannins, and their implications in gas production and true digestibility in in vivo techniques. British J. Nutr., 73: 897–913.

Mansoori, B. and Acamovic, T. 1998a. The excretion of minerals from broilers fed tannic acid, polyethylene glycol and protein. In: Garland, T. and Barr, A.C. ed. Toxic Plants and Other Natural Toxicants, CAB Intl., Wallingford, UK, 101–105.

Mansoori, B. and Acamovic, T. 1998b. The influence of tannic acid on amino acid deigesibility in broilers. In: Garland, T. and Barr, A.C. ed.Toxic Plants and Other Natural Toxicants, CAB Intl., Wallingford, UK, 106–110.

McDonald, M., Mila, I. and Scalbert, A. 1996. Precipitation of metal ions by plant polyphenols: optimal conditions and origin of precipitation. Journal of Agric. and Food Chem., 44: 599–606.

Muhammed, S., Stewart, C.S. and Acamovic, T. 1995. Effects of tannic acid, ellagic acid, gallic acid and catchin on cellulose degradation by the rumen fungus *Neocalimastix frontalis* strain RE1. Animal Science, 60: 550A.

Muhammed, S.A. 1997. Anti-nutrient effects of plant polyphenolic compounds. PhD Thesis, University of Aberdeen.

Reed, J.D. 1995. Nutritional toxicology of tannins and related polyphenols in forage legumes. Journal of Animal Science, 73: 1516–1528.

Salawu, M.B., Acamovic, T., Stewart, C.S. and Hovell, F.D.DeB. 1997. Quebracho tannins with or without Browse Plus (a commercial preparation of polyethylene glycol) in sheep diets: effect on digestibility of nutrients in vivo and degradation of grass hay in sacco and in vivo. Animal Feed Science and Technology, 67: 67–78.

Salawu, M.B., Acamovic, T. and Stewart, C.S. 1998. Inhibition of microbial growth and enzyme activities by *Calliandra calothyrsus* leaf extracts. In: Garland, T. and Barr, A.C. ed.Toxic Plants and Other Natural Toxicants, CAB Intl., Wallingford, UK, 111–114.

Salawu, M.B., Acamovic, T., Stewart, C.S. and Hovell, F.D.DeB. 1999. Effects of feeding Quebracho tannin diets, with or without a dietary modifier, on rumen function in sheep. Animal Science, 69: (accepted and in press).

Scalbert, A. 1991. Anti-microbial properties of tannins. Review article No 63. Phytochemistry, 30: 3875–3883.

Tanner, G.J., Moore, A.E. and Larkin, P.J. 1994. Proanthocyanidins inhibit hydrolysis of leaf proteins by rumen microflora in vitro. British Journal of Nutrition, 71: 947–958.

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

Wang, Y., Waghorn, G.C., Barry, T.N. and Shelton, I.D. 1994. The effect of condensed tannins in *Lotus corniculatus* on plasma metabolism of methionine, cystine and inorganic sulphate by sheep. British Journal of Nutrition, 72: 923–935.

Waterman, P.G. and Mole, S. 1994. In Analysis of Phenolic Plant Metabolites. Publ; Blackwell Scientific Publications, Oxford, UK.

Yu, F., McNabb, W.C., Barry, T.N. and Waghorn, G.C. 1995. Effect of condensed tannin in cottonseed hulls upon the in vitro degradation of cottonseed kernel proteins by rumen micro-organisms. Journal of the Science of Food and Agriculture, 69: 223–234.

Yu, F., Moughan, P.J. and Barry, T.N. 1996. The effect of cottonseed condensed tannins on ileal digestibility of amino acids in casein and cottonseed kernel. British Journal of Nutrition, 75: 683–698.

Tannins with Anthelmintic Properties

L.P. Kahn[1]* and A. Diaz-Hernandez[1]

Abstract

Condensed tannins (CT) are a part of the polyphenols present in plants and are found at greatest concentration in dicotyledons such as leguminous plants. Consumption of plants containing CT may affect resistance and resilience of ruminant livestock to parasitic gastrointestinal (GI) nematodes in either indirect or direct ways. Condensed tannins of various temperate herbaceous species have the potential to increase the supply and absorption of digestible protein which will indirectly improve host resistance and resilience to GI nematodes Condensed tannin-containing forages and CT extracts may also directly assist in reducing infections of GI nematodes. For example, consumption of the CT-containing perennial Mediterranean legume *Hedysarum coronarium* is associated with reduced abomasal and intestinal nematode numbers and this association does not appear to be mediated through digestible protein supply. In addition, CT extracted from a number of woody plants has been shown to reduce in vitro nematode viability. Condensed tannins have the potential to offer a viable alternative to the use of chemotherapy to control infections of parasitic GI nematodes but further research is required.

CONTROL OF gastrointestinal (GI) nematodes for the past 30 years has relied heavily on the use of anthelmintics. These compounds have been very successful but the development of anthelmintic resistance in GI nematodes in a number of countries (Jackson 1993; Sanyal 1996; Rolfe 1997; van Wyk et al. 1997; Waller 1997) gives a clear indication that control programs based exclusively on their use are not sustainable. Even when anthelmintics are effective at controlling GI parasites, rates of live weight gain may still be inferior to that of unparasitised controls (Coop et al. 1982). The development of integrated programs to control GI nematodes is vital, but such control programs require viable alternatives to the use of anthelmintics (Waller 1999). One such alternative may be the use of plants containing condensed tannins (CT).

Condensed tannins are a part of the polyphenols present in plants and are found at greatest concentration in dicotyledons such as leguminous plants (Bate-Smith 1962). It seems plausible that consumption of plants containing CT may affect GI nematode numbers and animal performance in a number of ways that involve direct and indirect mechanisms. In this review, the authors discuss the evidence that exists to support the notion that tannins have anthelmintic properties and consider whether these properties are likely to be mediated indirectly through effects on protein and mineral metabolism or directly through effects on nematode viability.

Impact of Nematode Infection on Nitrogen and Mineral Metabolism in the Host

Subclinical infections of GI nematodes such as *Trichostrongylus colubriformis*, *Ostertagia circumcincta* and *Haemonchus contortus*, can severely depress appetite (Poppi et al. 1985; Kimambo et al. 1988; Kyriazakis et al. 1996) and also increase the protein requirement of the animal (Poppi et al. 1986; Kimambo et al. 1988; Bown et al. 1991). The increased protein requirement arises from loss of endogenous nitrogen (blood, plasma, mucin and sloughed cells from intestinal epithelium) into the gut, diversion of amino acids away from peripheral tissues towards GI tract tissue proteins and secretory products (Yu et al. 1998) and the requirements to repair damaged tissue associated with the establishment of adult worms. Yu et al. (1998) reported that

[1] Animal Science, School of Rural Science and Natural Resources, University of New England, Armidale, 2351, NSW, Australia
* Corresponding author

KEYWORDS: Tannins, Anthelmintic properties, Polyphenols, Gastrointestinal mematodes (GI)

lambs infected with *T. colubriformis* had amino acid requirements of the GI tract increased by 33% at the expense of peripheral tissue such as muscle. Diversion of amino acids away from tissue towards the GI tract is consistent with the commonly observed occurrence of lower growth rates and less body protein (Coop et al. 1982) in lambs infected with GI nematodes.

The extra loss of endogenous nitrogen is largely reabsorbed but this process is not complete (Bown et al. 1984) lowering net absorption of non-ammonia nitrogen (NAN) and may result in specific amino acid deficiencies (Macrae 1993; Coop et al. 1997). Amino acid deficiencies may arise as a consequence of various elements of the immune response which involve secretion of amino acid-containing products into the GI tract. For example, intestinal secretions of mucus are rich in threonine (Neutra and Forstner 1987) and leukotrienes rich in cysteine (Lewis and Austin 1981) and reabsorption of these amino acids is unlikely to be complete. With infections of *T. colubriformis*, the losses of endogenous nitrogen are greatest in the later stages of infection, typically at weeks 10–12 post infection (Poppi et al. 1986; Kimambo et al. 1988). The extra protein flow at the ileum in sheep infected with *T. colubriformis* has been calculated to be 20–125g crude protein per day depending on the stage of infection (Poppi et al. 1986).

Intestinal nematodes reduce apparent absorption of phosphorus (P) from the small intestine (Poppi et al. 1985) leading to reduced plasma P concentrations, reduced salivary P secretions (Coop and Field 1983; Poppi et al. 1985), reduced P concentration in rumen fluid and digesta and lower growth rate (Coop and Field 1983). Increasing dietary intake of P is associated with reduced burdens of the intestinal nematode *Trichostrongylus vitrinus* and greater rates of growth (Coop and Field 1983). Abomasal infections of *O. circumcincta* result in an increase in abomasal pH and reduce copper (Cu) solubility and apparent absorption (Bang et al. 1990a). Increasing Cu solubility by administration of copper oxide wire particles reduces the establishment of *H. contortus* and *O. circumcincta* (Bang et al. 1990b)

Increasing Protein Supply Improves Resistance and Resilience to GI Nematodes

There are a number of reports that suggest that an increase in the supply of digestible protein (DP) will improve the resilience and resistance of sheep to GI nematodes (Coop and Holmes 1996; van Houtert and Sykes 1996; Donaldson et al. 1997). Resilience is the ability of a parasitised animal to perform (i.e. grow and produce milk and fibre) to a level similar to an unparasitised control (Albers et al. 1987). Resistance is the ability of an animal to resist parasite establishment and to impair the development of and/or expel previously established parasites. Resistance to nematodes is acquired by the development of the host's immunity and is manifested in three sequential stages which are: decreased establishment rate of incoming larvae and an increase in the arrested development at the third larval stage; decreased egg production by established females; and eventually rejection of established adult worms (Dobson et al. 1990a-c).

Increasing DP supply appears to have little effect on the acquisition of host resistance in the early stages of nematode infections. There are a number of studies which indicate that DP supply has no effect on the establishment of nematode larvae (Abbott et al. 1985; van Houtert et al. 1995) and the effect on nematode fecundity appears to differ between nematode species with a reduction in the fecundity of *H. contortus* reported by Wallace et al. (1995).

Increasing DP supply appears to be most effective in enhancing particular immune responses associated with the latter stages in the acquisition of host resistance. In support of this, it has been demonstrated that an increase in the DP supply to nematode-naïve lambs infected with *T. colubriformis* results in lower nematode burdens but this effect only becomes apparent from about 10 weeks post infection (Bown et al. 1991; van Houtert et al. 1995). The temporal importance of DP supply on nematode burden is not surprising considering that rejection of established *T. colubriformis* in previously nematode-naïve Merino sheep commences at about 7–10 weeks post infection (Dobson et al. 1990c).

Condensed Tannins and Resistance and Resilience to GI Nematode Infection

When considering the likely effect of CT on the resistance and resilience of animals to GI nematode infections it seemed reasonable to us to start from the premise that CT may have effects via indirect or direct mechanisms. Indirect effects on resistance and resilience could be mediated by changes in the supply of DP, changes in amino acid supply, particularly that of threonine and methionine/cystine, changes in mineral absorption, and interactions with intestinal mucosal epithelia. Direct effects would need to be mediated through tannin-nematode interactions which reduce nematode viability.

The authors have previously discussed the role of DP supply on resilience and acquisition of resistance to GI nematode infections. The experiments that have contributed to understanding in this area have relied on either abomasal infusions or the use of

protein meals that contain significant quantities of rumen undegradable protein. It seemed to us that there should be no particular reason that other measures which may increase DP supply, such as CT, would not also influence resistance and resilience in a similar fashion. Discussion about the effects of CT on resistance and resilience to GI nematode infections is restricted to those plant species that have been investigated for anthelmintic properties.

Indirect Effects on Resistance and Resilience Mediated by Changes to Mineral Metabolism

Condensed tannins of *L. pedunculatus* do not effect P concentration throughout the GI tract (Waghorn et al. 1994) but it has been reported (Waghorn et al. 1994) that apparent absorption of P is greater (22%) in animals not given polyethylene glycol (PEG) indicating a beneficial role of CT for P absorption. In contrast, the CT of *L. corniculatus* do not appear to effect apparent absorption of P (Waghorn et al. 1987). Based on these results, CT of *L. pedunculatus* may influence resistance to intestinal nematodes through changes to P absorption but this has not yet been experimentally verified. It has also been shown that the concentration of Cu in rumen fluid and abomasal digesta is reduced by the CT of *L. pedunculatus* (Waghorn et al. 1994) suggesting that CT, at least from *L. pedunculatus*, may increase susceptibility to abomasal nematode infections but this has not been confirmed.

Indirect Effects on Resistance and Resilience by Interactions with Intestinal Mucosal Epithelium

Condensed tannins may have the potential to interact with endogenous proteins such as proteins of the intestinal epithelium. As a consequence, the intestinal environment may be altered in such a way that may affect the success of GI nematode establishment. Brooker et al. (these Proceedings) has reported damage to the structure of intestinal villi in animals fed a pure diet of Mulga (*Acacia aneura*) for a prolonged period. The authors are unaware of other studies with ruminants that have examined the effects of CT on characteristics of the intestinal mucosal epithelia. There is evidence that CT at concentrations greater than would be expected physiologically may damage the mucosal lining of the intestinal tract in chickens (Farrell and Perez-Maldonado, these Proceedings). In contrast, it has been demonstrated that inclusion of tannins in the diet of rats and pigs has no effects on the morphological characteristics of the small intestine mucosa (Van Leeuwen et al. 1995; Sell et al. 1985). Further work is required before we could anticipate the likely indirect effects on resistance and resilience to GI nematodes by interactions of CT with intestinal mucosal epithelium.

Indirect Effects on Resistance and Resilience by Changes to Nitrogen Metabolism

The effect of CT on nitrogen metabolism has been discussed in detail elsewhere in these Proceedings and only a brief summary, pertinent to effects on resistance and resilience to GI nematodes, will be presented here. Condensed tannins have been reported to increase abomasal and duodenal flows of N and NAN. A greater post-ruminal flow of N and NAN has been reported for animals fed the temperate herbaceous legumes *L. pedunculatus* (Barry and Manley 1984; Barry et al. 1986) and L. corniculatus (Waghorn et al. 1987) and the tropical shrub, *Calliandra calothyrsus* (Perez-Maldonado and Norton 1996; Norton and Ahn 1997). However, the greater post-ruminal flow of NAN in animals consuming CT may be negated by lower rates of N absorption from the small intestine as demonstrated for *L. pedunculatus* (Barry et al. 1986), *L. corniculatus* (Waghorn et al. 1987) and *C. calothyrsus* (Norton and Ahn 1997) resulting in a greater loss of faecal N for these species (Barry and Manley 1984; Barry et al. 1986; Terrill et al. 1992; Perez-Maldonado and Norton 1996) and sulla (*Hedysarum coronarium*) (Stienzen et al. 1996). Recent evidence from pigs fed Quebracho tannin suggests that a significant fraction of the N lost in faeces is endogenous in origin (Steendam et al. 1998). This is yet to be resolved in ruminants.

The effects of CT on intestinal absorption of NAN and specific amino acids differ between plant species and only for *L. corniculatus* has increased apparent absorption of amino acids been demonstrated (Waghorn et al. 1987). Further, CT of *L. corniculatus* are known to increase the entry of cystine but not methionine into blood plasma (Wang et al. 1994). In contrast, the CT of *L. pedunculatus* increase post-ruminal flow of essential amino acids but do not result in greater apparent absorption from the small intestine (Waghorn et al. 1994). On the basis of metabolic studies, the authors predict that *L. corniculatus* is the most likely candidate to indirectly improve resilience and resistance to GI nematode infections.

There is now a number of reports in the literature which allowed exploration of the effect of CT on resistance and resilience in these species. However,

many of these experiments were of a short duration, making it difficult to assess the significance of any DP-mediated effects on resistance since such effects in nematode-naïve animals generally become apparent from about 10 weeks post infection.

Effect of CT-Containing Forages on Resistance and Resilience to Nematode Infections

Niezen et al. (1998) compared growth and resistance of nematode-naïve lambs, fed either maku lotus (*Lotus pedunculatus*; 3.2% N; 5.6% CT) or perennial ryegrass (*Lolium perenne*; 1.8% N), for 5 weeks following an artificial single infection of 10 000 *O. circumcincta* and 10 000 *T. colubriformis*. To examine the effect of CT, PEG, known to reduce protein binding by CT, was given to about half of the lambs on each feed. Live weight gain was greatest for lambs fed maku lotus indicating greater resilience to the effects of GI nematode infection. Live weight gain of lambs fed maku lotus was unaffected by PEG indicating that the increased resilience of lambs fed maku lotus was independent of CT content and probably arose from a greater supply of DP and ME. In support of this, the N content and feed intake of maku lotus were × 1.77 and × 1.60 that of the ryegrass respectively.

Faecal egg counts (FEC; 4 weeks post infection) were greater from lambs fed ryegrass (3043 epg) than for maku lotus (1552 epg) and were unaffected by PEG. However, when total daily egg output was calculated on the basis of estimated faecal output, Niezen et al. (1998) concluded that lambs fed maku lotus excreted the greater number of eggs (× 1.24). Total nematode burdens in lambs did not differ between ryegrass and maku lotus but were greatest in lambs fed ryegrass and given PEG, suggesting that PEG may have some unspecified effect on GI nematode numbers. Numbers of *O. circumcincta* and *T. circumcincta* in the absence of PEG did not differ between feeds and within those lambs fed maku lotus, were unaffected by PEG. Considered together, these results indicate that acquisition of resistance during the first 5 weeks of infection was unaffected by forage type and CT content and presumably was independent of estimated DP supply. These results are consistent with the temporal dependence of DP-mediated effects on acquisition of resistance (Coop and Holmes 1996; van Houtert and Sykes 1996).

Robertson et al. (1995) compared growth and resistance of lambs with existing GI nematode infections (mean FEC 1403 epg) which subsequently grazed on various forage species for 6 weeks. Animals were further subdivided to create two groups, one of which received no anthelmintic treatment (undrenched) and the other drenched at 2 week intervals. Growth rate, FEC and nematode burdens are reproduced in Table 1. Growth rate of drenched lambs was unaffected by probable CT content of the forage. When undrenched, lambs which grazed maku lotus and sulla had the greatest growth rates but those which grazed goldie lotus (*L. corniculatus*) the least. We calculated the ratio of growth rate of lambs when undrenched and drenched and used this as an indication of the degree of resilience conferred by each forage type. Greatest resilience was conferred by sulla and maku lotus and least by goldie lotus, lucerne and the ryegrass/white clover mix. The inferior resilience of lambs grazing goldie lotus as compared to maku lotus is difficult to explain as metabolic trials indicate that goldie lotus is most likely to increase apparent absorption of essential amino acids (see earlier discussion).

Table 1. Growth rate and resistance to gastrointestinal (GI) nematodes in lambs with naturally acquired GI nematode infections and either drenched at 2 week intervals or undrenched.

Forage	Live weight gain (g/d)			Faecal egg count	Abomasal nematodes	Intestinal nematodes	Total nematodes
	D	UD	UD : D	(epg)			
Rg/wc	166^{a}	88^{ab}	0.53	2109^{ab}	3094^{a}	12712^{ab}	15806^{ab}
Lucerne	243^{b}	121^{b}	0.50	2199^{b}	2984^{a}	15100^{ab}	18084^{ab}
Maku	232^{b}	160^{c}	0.69	2854^{c}	3286^{a}	20378^{b}	23665^{b}
Goldie	208^{b}	86^{a}	0.41	2571^{bc}	5256^{b}	17734^{b}	22990^{b}
Sulla	226^{b}	175^{c}	0.77	1538^{a}	2278^{a}	10812^{a}	13090^{a}

Source: Robertson et al. (1995).
Rg/wc: ryegrass/white clover mix; lucerne: *Medicago sativa* cv Otaio; maku: *Lotus pedunculatus* cv Grasslands maku; goldie: *Lotus corniculatus* cv Grasslands goldie; sulla: *Hedysarum coronarium* cv Aokau.
D: drenched at 2 week intervals; UD: undrenched.

In accord with the effect on resilience, FEC and total nematode burdens were numerically least for sulla but lambs which grazed either of the lotus species had the greatest FEC and total nematode burdens although the latter was not statistically different from that of the ryegrass/white clover mix or lucerne. Based on the superior growth rate of undrenched lambs grazing maku lotus and sulla, it can be speculated that DP supply was greatest from these forages but the greater DP supply from maku lotus was ineffective in improving resistance to GI nematodes over the 6 weeks of the trial. This supports the observations of Niezen et al. (1998) that maku lotus is ineffective at increasing resistance to GI nematodes over the short term.

Niezen et al. (1994) compared growth and resistance of lambs (previously managed to minimise parasite exposure) which were artificially infected 3 times per week with 3000 *O. circumcincta* and 3000 *T. colubriformis* and grazed for 6 weeks on various forage types. Growth rate of infected lambs was not consistently greater for animals which grazed forages containing CT. Growth rate was least for lambs grazing the ryegrass/white clover mix, lucerne and sulla (mean growth rate on these forages was 121 g/d), intermediate for chicory and two varieties of red clover (mean growth rate was 172 g/d) and greatest for maku lotus (286 g/d). This lends further support to the notion that maku lotus supports high rates of growth in lambs carrying nematode infections but the poor growth rate of lambs grazing sulla is in contrast to that reported by Niezen et al. (1994) and Robertson et al. (1995).

At 6 weeks post infection, FEC was greatest in lambs grazing the ryegrass/white clover mix (10650 epg) but was not different between the other forages. Nematode burdens were determined (6 weeks post infection) in lambs which grazed the ryegrass/white clover mix and sulla. *O. circumcincta* numbers were reduced by nearly 90% in lambs which grazed sulla (550 and 59 for the ryegrass/white clover mix and sulla respectively). Intestinal nematode numbers were reduced by 45% (2952 and 1622 for ryegrass/white clover mix and sulla respectively) but the difference was not statistically significant.

Improved resistance to GI nematodes in lambs grazing sulla is interesting because the growth rate of these lambs, although greater than that of lambs which grazed the ryegrass/white clover mix (141 and 84 g/d respectively) was not significantly increased (Niezen et al. 1994). Even if the supply of DP was greater in the lambs which grazed sulla, it is difficult to reconcile this with such a large effect on resistance, particularly to *O. circumcincta*, during the first 6 weeks of infection, a period that is known to be relatively insensitive to DP supply. It seems possible that sulla increased resistance through non-protein-mediated effects.

Gastrointestinal nematode parasitism is also a major problem of grazing livestock in tropical countries but access to anthelmintics can be limited (Hammond et al. 1997) making plants with anthelmintic properties an attractive alternative. Numerous plants from a number of countries have been listed as having anthelmintic activity and many are currently used as part of traditional veterinary practices but few have been scientifically tested (Hammond et al. 1997). Following anecdotal reports from Indonesia of the medicinal properties of *C. calothyrsus* in ruminants, Parker and Palmer (1991) fed C. calothyrsus for 1 week to 8 month-old Merino wethers carrying naturally acquired *H. contortus* and *Trichostrongylus* spp infections. After 1 week of feeding, FEC and the relative importance of *H. contortus* and *Trichostrongylus* spp from larval differentials had not changed, leading those authors to conclude that *C. calothyrsus* was ineffective at reducing GI nematode populations. While this may be evidence that *C. calothyrsus* lacks any direct nematacidal activity, the period of supplementation was insufficient to make any conclusions about the potential for *C. calothyrsus* to indirectly reduce GI nematode burdens.

Effect of CT Extracts on Resistance and Resilience to Nematode Infections

Recently, the effect of Quebracho tannin on the growth rate of lambs and resistance to GI nematode infection has been investigated (Butter et al. 1998). Quebracho is a commercial product extracted from several species of South American evergreen trees and is a mixture of phenolic compounds in which CT represent about 50% of the material (Degen et al. 1998). Butter et al. (1998) fed parasite-free lambs that subsequently received subclinical infections of *T. colubriformis* feeds that differed in crude protein (CP) content (10% and 22% CP for LP and HP respectively) with or without Quebracho tannin (QT; unspecified CT content) included at 5% of the feed for 10 weeks (Table 2).

At 10 weeks post infection, live weight was unaffected by the addition of QT to animals fed the LP and HP feed indicating that at an inclusion rate of 5%, QT had no anti-nutritional effects and did not affect resilience to nematode infection. Addition of QT to the LP feed reduced mean FEC (averaged over the 10 week trial) by 56% to levels not different from that for animals fed the HP feed. Addition of QT to the HP feed did not affect mean FEC implying that QT does not have any direct effect on resistance. Increased resistance but not live weight (and the

apparent absence of any direct effects) caused by the addition of QT to the LP feed lends support to the notion that QT-mediated increases in DP supply were prioritised for tissue repair and immune response rather than for furthering growth. It can be speculated that this effect was absent when QT was added to the HP feed because DP supply was already beyond the increased DP requirements caused by GI nematode infections. Further, mean FEC was unaffected by the inclusion of QT prior to day 23 post infection, indicating that QT does not effect nematode establishment which is in agreement with DP-mediated effects on immune response to GI nematode infections.

Table 2. Change in live weight and mean faecal egg counts (FEC) of lambs fed low or high protein feeds with (+QT) or without (−QT) the addition of Quebracho tannin and either infected with *T. colubriformis* (+P) or uninfected controls (−P) for 10 weeks.

	Low protein				Low – high protein[A]	
	+P			−P	−P	
	−QT	+QT	−QT/ +QTB	−QT	−QT	+QT
Weight change (kg)	7.6[b]	8.3[b]	9.5[bc]	17.6[ac]	15.6[ac]	12.7[ac]
FEC (epg)	5083[a]	2227[bd]	1779[b]	0[c]	2639[bd]	3050[d]

Source: Butter et al. (1998).
[A] Feeds changed from low to high protein at day 23 post infection.
[B] Quebracho tannin added after day 23 post infection.

Effects of Sulla on Resistance and Resilience to Nematode Infections

Of the CT-containing plants that have been discussed here, improvements in resistance to GI nematodes has been demonstrated for sulla and QT. We suggest that QT-mediated improvements in resistance result from increases in DP supply. However, the effect of sulla on resistance to GI nematodes appears to be different. Niezen et al. (1995) grazed lambs which carried naturally acquired nematode burdens (mean FEC 220 at day 0) on sulla (3.3% N and 10% CT) or lucerne (4.7% N and 0.2% CT) for 6 weeks. On day zero, 60% of the lambs received an artificial infection of 20 000 *T. colubriformis*. The remaining lambs (40%) were drenched at day 0 and thereafter at 2-week intervals. Live weight gain and wool growth of drenched lambs did not differ between sulla and lucerne (mean 192 g/d and 1.5 mg/cm^2/d) but when lambs were not drenched were greatest for lambs which grazed sulla (129 and –39 g/d and 1.4 and 1.0 mg/cm^2/d for sulla and lucerne respectively). The degree of resilience conferred by each forage type was calculated to be 0.65 and –0.21 for sulla and lucerne respectively.

Following the artificial infection with *T. colubriformis*, FEC of lambs which grazed on lucerne increased to reach a maximum at week 3 post infection and declined thereafter. In contrast, FEC of lambs which grazed sulla were unaffected by infection and did not differ during the 6 weeks of the trial (Figure 1). These results indicate that few, if any, of the infective larvae given to animals which subsequently grazed sulla established. This effect is unlikely to have been mediated by possible CT-induced increases in DP supply (see earlier discussion). Total nematode burdens, 6 weeks post infection, were less in animals which grazed sulla (8016 and 19 268 for sulla and lucerne respectively) with 85% of the difference accounted for by differences in numbers of *T. colubriformis*: numbers of *O. circumcincta* were unaffected by forage type.

In some ways, these results contrast with the findings of Niezen et al. (1994) where lambs which grazed sulla had significantly lower burdens of *O. circumcincta* than those which grazed a ryegrass/ white clover mix. However, in that trial, animals were artificially infected with *O. circumcincta* whereas in the trial of Niezen et al. (1995) artificial infection was exclusively *T. colubriformis*. It seems plausible that animals which graze sulla are able to successfully inhibit nematode establishment but effects against pre-existing established nematodes are less dramatic. Although the specific mechanisms by which sulla increases host resistance to GI nematodes has not yet been elucidated it appears that these mechanisms are not mediated through effects on DP supply.

Direct Effects of CT on the Viability of Nematode Parasites of Sheep

It has been speculated (Niezen et al. 1995) that direct effects of CT on GI nematodes may account for reduced nematode burdens in lambs which graze sulla. Some evidence in support of the anthelmintic activity of CT was provided by Lorimer et al. (1996) when they demonstrated that the inhibitory effects on migration of exsheathed *T. colubriformis* L_3 larvae by a plant extract known to contain polyphenolics were greatly reduced after the polyphenolics were removed. Further evidence for an anthelmintic effect of CT were reported from a preliminary project (Duncan 1996) which evaluated the potential of CT to inhibit the viability of sheep nematode parasites. Various bioassays were used including larval

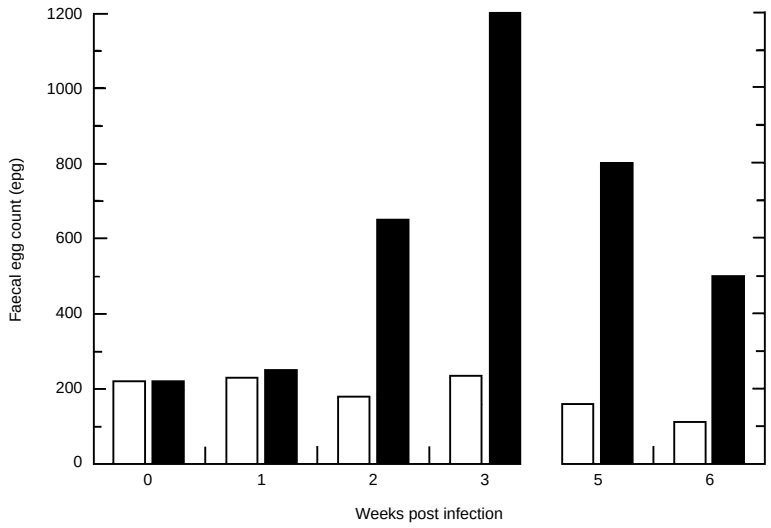


Figure 1. Faecal egg counts of lambs carrying naturally acquired gastrointestinal nematode infections and artificially infected with 20 000 *T. colubriformis* on day 0. Following the artificial infection, lambs were allocated to graze either sulla (*Hedysarum coronarium*; unfilled columns) or lucerne (*Medicago sativa*; shaded columns). Source: Niezen et al. (1995).

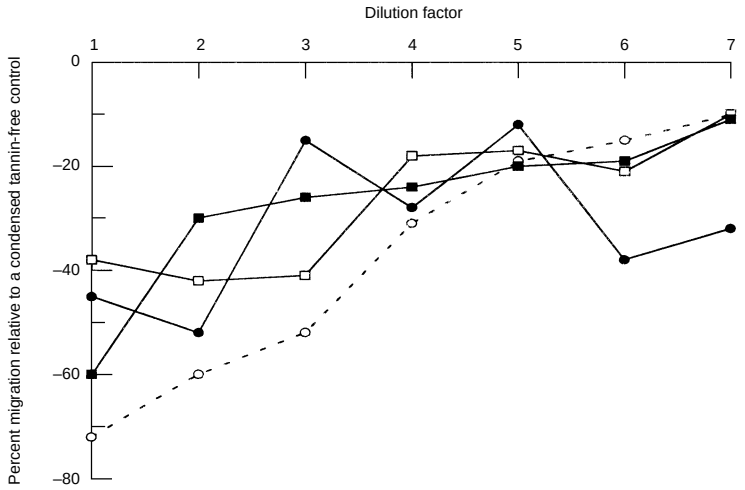


Figure 2. Percent migration of sheep parasitic gastrointestinal nematode exsheathed L_3 (unspecified) in the presence of various concentrations of the anthelmintic, Levamisole (open circles and dashed line) and purified condensed tannins extracted from *L. leucocephala* (filled squares), *A. aneura* (filled circles) and *A. saligna* (open squares) in relation to a condensed tannin-free control. Concentration of condensed tannin solution at dilution factor of 1 was 24 mg/mL. Source: Duncan (1996).

migration through screening sieves and larval development where development of nematode eggs was quantified after exposure to CT.

Migration of sheathed and exsheathed L_3 nematode larvae (unspecified species) was determined after exposure to various concentrations of CT (stock solution in water of 24 mg/mL) extracted from *Acacia aneura* (mulga), *Acacia saligna*, *Leucaena leucocephala*, and *Acacia harpophylla* (brigalow). Condensed tannins were subsequently diluted with a salt solution containing 12% fatty acids and 0.5% sodium carbonate. Relative to a CT-free control, migration of sheathed larvae was unaffected but migration of exsheathed larvae was reduced by CT. Condensed tannin from *L. leucocephala* was most effective at reducing migration of exsheathed larvae (–60%) at high CT concentrations but at concentrations of CT from 6–12 mg/mL the CT from *A. aneura* and *A. saligna* were most effective (Figure 2). Development of nematode eggs to the third larval stage was found to be reduced in the presence of CT from all test species with reductions of the magnitude −70% to −40% recorded for CT from *A. aneura* and *A. saligna*. Possible mechanisms through which CT may reduce larval migration and development remain to be elucidated but may be mediated through ingestion of CT or interactions of CT with the external surface of larvae.

Conclusion

Studies which have examined the effects of CT extracts and CT-containing forages on resistance and resilience to GI nematodes indicate that forages that confer a greater degree of resilience do not necessarily enhance resistance. Conclusions about the ability of CT-containing forages to improve resistance via enhanced DP supply are difficult to make because of the short duration of many of the trials. Increased resistance to GI nematodes has been demonstrated for sulla and QT but we conclude that these effects appear to be mediated through 2 distinct mechanisms. Quebracho tannin may enhance resistance through increases in DP supply which are prioritised for tissue repair and immune response. Sulla has been demonstrated to greatly reduce establishment of *O. circumcincta* and *T. colubriformis* which is unlikely to be due to changes to DP supply and may or may not be associated with CT. Condensed tannins appear to have direct anthelmintic properties and reduce larval migration and development. Further work to confirm and identify the anthelmintic properties of CT in general, and from sulla specifically, would be of much interest.

References

Abbott, E.M., Parkins, J.J. and Holmes, P.H. 1985 . Influence of dietary protein on parasite establishment and pathogenesis in Finn Dorset and Scottish Blackface lambs given a single moderate infection of *Haemonchus contortus*. Research in Veterinary Science, 38: 6–13.

Albers, G.A.A., Gray, G.D., Piper, L.R., Barker, J.S.F., Le Jambre, L.F. and Barger, I.A. 1987. The genetics of resistance and resilience to *Haemonchus contortus* infections in young Merino sheep. International Journal for Parasitology, 17: 1355–1363.

Bang, K.S., Familton, A.S. and Sykes, A.R. 1990a. Effect of ostertagiasis on copper status in sheep: a study involving use of copper oxide wire particles. Research in Veterinary Science, 49: 306–314.

Bang, K.S., Familton, A.S. and Sykes, A.R. 1990b. Effect of copper oxide wire particle treatment on establishment of major gastrointestinal nematodes in lambs. Research in Veterinary Science, 49: 132–137.

Barry, T.N. and Manley, T.R. 1984. The role of condensed tannins in the nutritional value of *Lotus pedunculatus* for sheep. 2. Quantitative digestion of carbohydrates and proteins. British Journal of Nutrition, 51: 493–504.

Barry, T.N., Manley, T.R. and Duncan, S.J. 1986. The role of condensed tannins in the nutritional value of *Lotus pedunculatus* for sheep. 4. Sites of carbohydrate and protein digestion as influenced by the dietary reactive tannin concentration. British Journal of Nutrition, 55: 123–137.

Bate-Smith, E.C. 1962. The phenolic constituents of plants and their taxonomic significance. I. Dicotyledons. Journal of the Linnean Society Bot., 58: 95–173.

Bown, M.D., Poppi, D.P. and Sykes, A.R. 1984. The effect of a mixed nematode infection on the site of plasma protein absorption in the small intestine. Canadian Journal of Animal Science, Supplement, 197–198.

Bown, M.D., Poppi, D.P. and Sykes, A.R. 1991. The effect of post-ruminal infusion of protein or energy on the pathophysiology of *Trichostrongylus colubriformis* infection and body composition in lambs. Australian Journal of Agricultural Research, 42: 253–267.

Butter, N.L., Dawson, J.M., Wakelin, D., and Buttery, P.J. 1998. Effect of dietary tannin and protein level on the susceptibility of sheep to parasitic infection. Proceedings of the British Society of Animal Science, 97 p.

Coop, R.L., Sykes, A.R. and Angus, K.W. 1982. The effect of three levels of intake of *Ostertagia circumcincta* larvae on growth rate, food intake and body composition of growing lambs. Journal of Agricultural Science, Cambridge, 98: 247–255.

Coop, R.L. and Field, A.C. 1983. Effect of phosphorus intake on growth rate, food intake and quality of the skeleton of growing lambs infected with the intestinal nematode *Trichostrongylus vitrinus*. Research in Veterinary Science, 35: 175–181.

Coop, R.L. and Holmes, P.H. 1996. Nutrition and parasite interaction. International Journal for Parasitology, 26: 951–962.

Coop, R.L., Kyriazakis, I., Huntley, J.F., Jackson, E. and Jackson, F. 1997. The influence of protein and amino acid on the resilience of sheep to intestinal parasitism. Proceedings of the Fourth International Congress for Sheep Veterinarians, Armidale, Australia, February 1997, 196–198.

Degen et al. 1998 **Author to supply**

Dobson, R.J., Waller, P.J. and Donald, A.D. 1990a. Population dynamics of *Trichostrongylus colubriformis* in sheep: the effect of host age on the establishment of infective larvae. International Journal for Parasitology, 20: 353–357.

Dobson, R.J., Waller, P.J. and Donald, A.D. 1990b. Population dynamics of *Trichostrongylus colubriformis* in sheep: the effect of infection rate on the establishment of infective larvae and parasite fecundity. International Journal for Parasitology, 20: 347–352.

Dobson, R.J., Waller, P.J. and Donald, A.D. 1990c. Population dynamics of *Trichostrongylus colubriformis* in sheep: the effect of infection rate on loss of adult parasites. International Journal for Parasitology, 20: 359–363.

Donaldson, J., van Houtert, M.F.J. and Sykes, A.R. 1997. The effect of protein supply on the periparturient parasite status of the mature ewe. Proceedings of the New Zealand Society of Animal Production, 186–189.

Duncan, R.A. 1996. An evaluation of suitable bioassays to measure the potential of condensed tannins to inhibit the viability of sheep nematode parasites. Bachelor of Rural Science, Honours Thesis, Animal Science, University of New England, Armidale, NSW, Australia.

Hammond, J.A., Fielding, D. and Bishop, S.C. 1997. Prospects for plant anthelmintics in tropical veterinary medicine. Veterinary Research Communications, 21: 213–228.

Jackson, F. 1993. Anthelmintic resistance — the state of play. British Veterinary Journal, 149: 123–138.

Kimambo, A.E., MacRae, J.C., Walker, A., Watt, C.F. and Coop, R.L. 1988. Effect of prolonged subclinical infection with *Trichostrongylus colubriformis* on the performance and nitrogen metabolism of growing lambs. Veterinary Parasitology, 28: 191–203.

Kyriazakis, I., Anderson, D.H., Oldham, J.D., Coop, R.L. and Jackson, F. 1996. Long-term subclinical infection with *Trichostrongylus colubriformis*: effects on food intake, diet selection and performance of growing lambs. Veterinary Parasitology, 61: 297–313.

Lewis, R.A. and Austen, K.F. 1981. Mediation of local homeostasis and inflammation by leukotrienes and other master cell-dependent compounds. Nature, 293: 103–108.

Lorimer, S.D., Perry, N.B., Foster, L.M. and Burgess, E.J. 1996. A nematode larval motility inhibition assay for screening plant extracts and natural products. Journal of Agricultural and Food Chemistry, 44: 2842–2845.

Macrae, J.C. 1993. Metabolic consequences of intestinal parasitism. Proceedings of the Nutrition Society, 52: 121–130.

Neutra, M.R and Forstner, J.M. 1987. Gastrointestinal mucus: synthesis, secretion and function. In: Johnson, L.R. ed. Physiology of the Gastrointestinal Tract, Vol. 2, Second Edition, Raven Press, New York, 975–1009.

Niezen, J.H., Waghorn, T.S., Raufaut, K., Robertson, H.A. and McFarlane, R.G. 1994. Lamb weight gain and faecal egg count when grazing one of seven herbages and dosed with larvae for six weeks. Proceedings of the New Zealand Society of Animal Production, 54: 15–18.

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

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

Norton, B.W. and Ahn, J.H. 1997. A comparison of fresh and dried *Calliandra calothyrsus* supplements for sheep given a basal diet of barley straw. Journal of Agricultural Science, Cambridge, 129: 485–494.

Parker, R.J. and Palmer, B. 1991. Lack of anthelmintic effect of *Calliandra calothyrsus* in sheep. Australian Veterinary Journal, 68: p 309.

Perez-Maldonado, R.A. and Norton, B.W. 1996. The effects of condensed tannins from *Desmodium intortum* and *Calliandra calothyrsus* on protein and carbohydrate digestion in sheep and goats. British Journal of Nutrition, 76: 515–533.

Poppi, D.P., MacRae, J.C., Brewer, A., Dewey, P.J.S. and Walker, A. 1985. Calcium and phosphorus absorption in lambs exposed to *Trichostrongylus colubriformis*. Journal of Comparative Pathology, 95: 453–464.

Poppi, D.P., MacRae, J.C., Brewer, A. and Coop, R.L. 1986. Nitrogen transactions in the digestive tract of lambs exposed to the intestinal parasite, *Trichostrongylus* colubriformis. British Journal of Nutrition, 55: 593–602.

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

Rolfe, P.F. 1997. Anthelmintic resistance in Australia, its development and management. Proceedings of the Fourth International Congress for Sheep Veterinarians, Armidale, Australia, February 1997, 51–58.

Sanyal, P.K. 1996. Gastrointestinal parasites and small ruminant production in India. In: LeJambre, L.F. and Knox, M.R. ed. Sustainable Parasite Control in Small Ruminants. Proceedings of a workshop Bogor, Indonesia, 22–25 April 1996, ACIAR Proceedings No. 74: 109–112.

Sell, D.R., Reed, W.M., Chrisman, C.L. and Rogler, J.C. 1985. Mucin excretion and morphology of the intestinal tract as influenced by sorghum tannins. Nutrition Reports International, 6: 1269–1274.

Steendam, C.A., de Jong, E.J., Mattuzzi, S. and Visser, G.H. 1998. Comparison of three methods for the measurement of the endogenous N-flow at the terminal ileum of pigs, as affected by dietary quebracho extract. In: Jansman, A.J.M., Hill, G.D., Huisman, J. and VanderPoel, A.F.B. ed. Recent Advances of Research in Anti-nutritional Factors in Legume Seeds and Rapeseed, 93: 335–340.

Stienezen, M., Waghorn, G.C. and Douglas, G.B. 1996. Digestibility and effects of condensed tannins on digestion of sulla (*Hedysarum coronarium*) when fed to sheep. New Zealand Journal of Agricultural Research, 39: 215–221.

Terrill, T.H., Douglas, G.B., Foote, A.G., Purchas, R.W., Wilson, G.F. and Barry, T.N. 1992. Effect of condensed tannins upon growth, wool growth and rumen metabolism in sheep grazing sulla (*Hedysarum coronarium*). Journal of Agricultural Science, Cambridge, 119: 265–273.

van Houtert, M.F.J., Barger, I.A., Steel, J.W., Windon, R.G. and Emery, D.L. 1995. Effects of dietary protein intake on responses of young sheep to infection with *Trichostrongylus colubriformis*. Veterinary Parasitology, 56: 163–180.

van Houtert, M.F.J. and Sykes, A.R. 1996. Implications of nutrition for the ability of ruminants to withstand gastrointestinal nematode infections. International Journal for Parasitology, 26: 1151–1167.

Van Leeuwen, P., Jansman, A.J.M., Wiebenga, J., Koninkx, J.F.J.G. and Mouwen, J.M.V.M. 1995. Dietary effects of faba-bean (*Vicia faba* L.) tannins on the morphology and function of the small-intestinal mucosa of weaned pigs. British Journal of Nutrition, 73: 31–39.

van Wyk, J.A., Malan, F.S. and Randles, J.L. 1997. How long before resistance makes it impossible to control some field strains of *Haemonchus contortus* in South Africa with any of the modern anthelmintics? Veterinary Parasitology, 70: 111–122.

Waghorn, G.C., Ulyatt, M.J., John, A. and Fisher, M.T. 1987. The effect of condensed tannins on the site of digestion of amino acids and other nutrients in sheep fed on *Lotus corniculatus* L. British Journal of Nutrition, 57: 115–126.

Waghorn, G.C., Shelton, I.D., McNabb, W.C. and McCutcheon, S.N. 1994. Effects of condensed tannins in *Lotus pedunculatus* on its nutritive value for sheep. 2. Nitrogenous aspects. Journal of Agricultural Science, Cambridge, 123: 109–119.

Wallace, D.S., Bairden, K., Duncan, J.L., Fishwick, G., Holmes, P.H., McKellar, Q.A., Murray, M., Parkins, J.J. and Stear, M.J. 1995. Influence of supplementation with dietary soyabean meal on resistance to haemonchosis in Hampshire down lambs. Research in Veterinary Science, 58: 232–237.

Waller, P.J. 1997. The global perspective of anthelmintic resistance in nematode parasites of sheep — excluding Australasia. Proceedings of the Fourth International Congress for Sheep Veterinarians. Armidale, Australia, February 1997, 59–63.

Waller, P.J. 1999. International approaches to the concept of integrated control of nematode parasites of livestock. International Journal for Parasitology, 29: 155–164.

Wang, Y., Waghorn, G.C., Douglas, G.B., Barry, T.N. and Wilson, G.F. 1994. The effects of condensed tannin in *Lotus corniculatus* upon nutrient metabolism and upon body and wool growth in grazing sheep. Proceedings of the New Zealand Society of Animal Production, 54: 219–222.

Yu. F, Bruce, L.A., Coop, R.L. and MacRae, J.C. 1998. Leucine metabolism across the gastrointestinal tract of sheep infected with *Trichostrongylus colubriformis*. Proceedings of the British Society of Animal Science, page 1.

Rumen Microbial Ecology and Physiology in Sheep and Goats Fed a Tannin-Containing Diet

C.S. McSweeney[1], B. Palmer[2] and D.O. Krause[1]

Abstract

Tannins reduce the digestion of plant protein and carbohydrate but the effects of these polyphenolics on rumen micro-organisms are not well understood. The predominant rumen bacteria are inhibited by tannins when grown as pure cultures in vitro although some strains of these bacteria are tolerant of tannins. The current research on rumen microbial ecology of animals supplemented with the tannin-rich forage *Calliandra calothyrsus* showed that populations of fibre degrading *Ruminococcus* spp and *Fibrobacter* spp were reduced by tannins. However, fungi, protozoa and proteolytic bacteria appeared to be less affected but further experiments are required to determine the impact of tannins on these micro-organisms in experiments where polyethylene glycol is used to neutralise the tannins. Even though tannins affected some bacteria, the rumen microbial population appeared to adapt, and the efficiency of microbial protein synthesis (g microbial protein/kg organic matter apparently digested in the rumen) in the rumen was unaffected.

TANNINS complex with dietary protein and carbohydrate and reduce nitrogen supply to the ruminant animal but the effects of these polyphenolics on rumen micro-organisms and efficiency of digestion are not well understood. Inhibition of growth of predominant rumen bacteria by polyphenolics has been demonstrated in vitro with pure cultures but their effects on micro-organisms in the rumen have not been quantified (Bae et al. 1993; Jones et al.1994). Also, it appears that closely related bacterial strains of the same species can differ markedly in their tolerance of tannins (Nelson et al. 1998; Brooker et al. 1994). The significance of tannin tolerance to efficiency of digestion is yet to be determined.

In this paper, the authors present their current research findings on (1) the interaction between tannins and rumen micro-organisms and (2) rumen microbial ecology of animals fed the tannin-rich forage *Calliandra calothyrsus* CPI 115690. The shrub legume *Calliandra calothyrsus* (calliandra) is used as a protein supplement for ruminants fed roughage diets in the humid tropics (Palmer and Schlink 1992). However, the concentration of condensed tannin (6–10%) reduces the nutritive value of the plant (Ahn et al. 1989).

CSIRO Tropical Agriculture:
[1] Long Pocket Laboratories, Private Bag No. 3 PO, Indooroopilly, 4068 Qld, Australia
[2] Davies Laboratory, Private Mail Bag, Aitkenvale Townsville, 4814 Qld, Australia

Effect of Calliandra Tannins on Rumen Microbial Function

Materials and methods

A series of experiments were conducted with rumen cannulated sheep that were held indoors in individual metabolism crates. Legume supplements of calliandra (*Calliandra calothyrsus*) and lucerne (*Medicago sativa*) were fed a sole diet or provided as a proportion (3:7) of the ad libitum intake of sheep fed either brachiaria grass (*Brachiaria humidicola*) or rhodes grass (*Chloris gayana*) as a basal diet of roughage hay. Sheep were adapted to the diets for 10 days before any measurements were made. Rumen samples for analysis of microbial populations were collected 4 hours prior to feeding unless otherwise described. Diets were made isonitrogenous by providing nitrogen in the form of urea to animals fed a grass diet without supplementary legume. Sheep were also dosed with polyethylene glycol (PEG) (40 g/day; PEG, M.W. 4000) to counteract the effect of tannins

KEYWORDS: Tannins, Rumen microbial ecology, Polyphenolics

(Jones and Mangan 1977). Efficiency of microbial protein synthesis in the rumen was estimated by the urinary purine method (Chen et al. 1990).

A combination of conventional and modern molecular microbial ecology techniques were used to compare and contrast the effect of condensed tannins on populations of rumen micro-organisms. Total counts of protozoa were made in a counting chamber (McSweeney et al. 1983); fungal colony units were determined in both roll tubes (Joblin 1981) and liquid medium with a rhodes grass substrate (Theodorou et al. 1990); and numbers of xylanolytic, pectinolytic and proteolytic bacteria were estimated on selective media agar plates (Mackie and Wilkins 1988). Numbers of cellulolytic bacteria were determined in selective broth medium (cellulosic filter paper or cotton thread substrate) using the most probable numbers (MPN) technique (Dehority et al. 1989). Populations of fungi, *Ruminococcus* spp., *Fibrobacter* spp., *Prevotella* spp. and *Bacteroides* spp. were also estimated as a percentage of the total rumen microbial population using 16S rDNA probes (Dore et al. 1998; Faichney et al. 1997; Krause et al. 1999; Lin et al. 1994). Quantitation of microbial populations based on relative abundance of extracted ribosomal RNA was performed using the techniques described by Krause and Russell (1996).

Results and discussion

Fungal numbers were significantly lower in sheep fed 100% calliandra compared with animals fed brachiaria grass or brachiaria supplemented with 30% calliandra (Table 1). Tannins were probably responsible for the small reduction in the fungal population because PEG supplementation was associated with a significant increase ($P<0.05$) in fungal colony forming units in sheep fed 30% wilted calliandra. Total protozoal numbers, and the bacterial groups grown on selective agar media plates were not significantly affected by the presence of callianda in the diet (Table 1). However when a MPN method based on cellulose degradation was employed for enumerating cellulolytic bacteria, it was observed that sheep fed varying levels of calliandra had significantly fewer ($P<0.05$) cellulolytic bacteria compared with animals fed brachiaria grass (Table 1). This was confirmed in a second experiment in which sheep fed a roughage diet of rhodes grass supplemented with 30% wilted calliandra had significantly fewer bacteria (6.8 v 8.2 and 8.3; $\log_{10}$ g/digesta) which degraded cellulose than those supplemented with 30% calliandra plus PEG or 30% lucerne respectively. Using genus specific 16S rDNA probes, the *Fibrobacter* and *Ruminococcus* populations respectively (expressed as a percentage of total rumen micro-organisms) were significantly lower in calliandra supplemented sheep (3.3% and 5.8%) compared with lucerne supplemented controls (10.6% and 11.5%). In the same experiment, tannins did not appear to affect the number of cellulolytic fungi (3.4 v 3.7; $\log_{10}$ g/digesta) in the calliandra and lucerne supplemented groups. Also, a 16S rDNA probe for *Bacteroides-Porphyromonas-Prevotella* group showed that the population of these bacteria (which contains a large proportion of the protoelytic rumen bacteria) was not significantly different

Table 1. Comparisons of the number of fungi, protozoa and functional groups of bacteria in rumen samples from sheep fed varying amounts of calliandra and brachiaria grass hay.

Number of organisms ($\log_{10}$/g digesta)	Diet				
	Brachiaria grass	Calliandra + Brachiaria grass (3:7)		Calliandra	
		– PEG	+ PEG		s.e.m.
Fungi	4.00[b]	3.92[b]	4.51[c]	3.18[a]	0.13
Protozoa	4.80	4.79	4.54	4.91	0.06
Non-cellulolytic bacteria					
Xylanolytics	8.68	8.80	8.66	8.68	0.03
Pectinolytics	8.39	8.40	8.51	8.45	0.03
Proteolytics	8.11	8.29	8.24	8.21	0.06
Cellulolytic bacteria					
Cotton thread substrate	5.95[b]	4.58[a]	—	5.25[b]	0.21
Cellulose disc substrate	8.36[c]	6.36[b]	—	5.60[a]	0.40

Mean values in rows which do not have a common superscript letter are significantly different ($P<0.05$).
Enumeration methods were as follows: fungi, roll tubes; protozoa, light microscopy; non-cellulolytic bacteria, substrate selective agar plates; cellulolytic bacteria, MPN analysis of cellulose degradation in liquid medium.

between groups supplemented with either calliandra or lucerne (12. 9% v 11.6%).

It appears therefore that tanniniferous diets inhibit the cellulolytic bacteria in the rumen. This is in accord with previous studies which demonstrate that structural carbohydrate digestion in the rumen and whole tract is reduced in animals fed *Lotus pedunculatus* (9.5% condensed tannin) or the tropical legume *Calliandra calothyrsus* (6% condensed tannin) (Barry et al. 1986; Waghorn et al. 1987; Perez-Maldonado and Norton 1996; Palmer, unpublished data). However, this effect of tannins on the rumen microbial population does not significantly affect the efficiency of digestion (g microbial N synthesised/g organic matter digested) in the rumen. Supplementation of roughage fed sheep with either calliandra or lucerne resulted in a significant increase in digestible organic matter intake (DOMI, Table 2) and microbial N (g/d) reaching the intestines was higher ($P<0.05$) in sheep supplemented with lucerne (with or without PEG) compared with the unsupplemented group. Although there were differences in DOMI between treatments, microbial N flowing to the intestines per kg DOMR was not significantly different among the three treatment groups (Table 2). Addition of PEG to the calliandra or lucerne supplement did not affect the efficiency of microbial protein synthesis in the rumen (McSweeney et al. 1998).

Table 2. Effect of 30% legume supplements (calliandra or lucerne) on the efficiency of rumen microbial protein synthesis in sheep fed a roughage diet.

Treatment		Microbial N	
	DOMI[a] (g/d)	g N/d	g N/kg DOMR[b]
Grass	327[a]	6.37[a]	29.3
Grass + calliandra	387[b]	7.0[ab]	28.0
Grass + lucerne	394[b]	7.99[b]	31.5
s.e.m.	11	0.25	0.9

[a] DOMI (digestible organic matter intake).

[b] DOMR (organic matter apparently fermented in the rumen) was taken as 0.65 DOMI.

Mean values in columns which do not have a common superscript are significantly different ($P<0.05$).

Identification and Physiology of Proteolytic Ruminal Bacteria from Calliandra Fed Animals

Some micro-organisms appear to be tolerant of tannins and therefore strains of bacteria may proliferate in response to tannin-rich diets such as calliandra. Ruminal bacteria that ferment protein or peptides in the presence of tannins could benefit digestion of these diets since tannins reduce the availability of protein for micro-organisms. We examined whether proteolytic bacteria, which are present as significant populations in the rumina of animals fed a sole diet of calliandra, are able to ferment amino acids in the presence of tannins, or hydrolyse protein that is complexed with tannin.

Materials and methods

A full description of these experiments is given by McSweeney and co-workers (1999). Rumen digesta was taken from four sheep and four goats which were fed 100% fresh calliandra in metabolism crates for 2–3 weeks and used to isolate bacteria capable of degrading tannin-protein complexes (TPC) on nutrient media containing precipitated TPC (Osawa 1990).

The number of proteolytic bacteria in sheep was estimated on selective media plates as described previously in this paper. Growth, ammonia production and protease activity were determined for individual isolates grown on peptide medium (PM) with and without carbohydrates. Ability of selected isolates to ferment protein in calliandra or grow on purified protein (1.5% casein or 3% fraction 1 leaf protein) was also examined. Bacterial isolates were further screened for an ability to degrade protein that was complexed with tannin by growing them in a medium in which the substrate protein was present in the tannin complexed form. The evolution of ammonia was used as an indicator of fermentation of protein.

The genotypic diversity and phylogeny of the isolates was determined using restriction fragment length polymorphisms (RFLP) and sequencing of 16S rDNA amplified by the polymerase chain reaction (Krause et al. 1997; Moyer et al. 1994).

Results and discusssion

Thirteen distinct genotypes were isolated and all strains were proteolytic and fermented peptides to a varying extent (McSweeney et al. 1999). The 16S rDNA gene of six of these isolates (Lp1265, Lp1275, Lp1276, Lp1283B, Lp1284, and Lp1311) were sequenced and their phylogenetic relationship to other tannin tolerant bacteria is shown (Figure 1). These bacteria were both proteolytic and peptidolytic when grown on peptide medium with and without carbohydrates (McSweeney et al. 1999). The isolates represent a diverse group of bacteria belonging to the genera *Streptococcus* (Lp1276), *Enterococcus* (Lp1275), *Butyrivibrio* (Lp1265), *Clostridium* (Lp1284), *Actinomyces* (Lp1283B) and *Propionibacterium* (Lp1311). Prior to this study the only

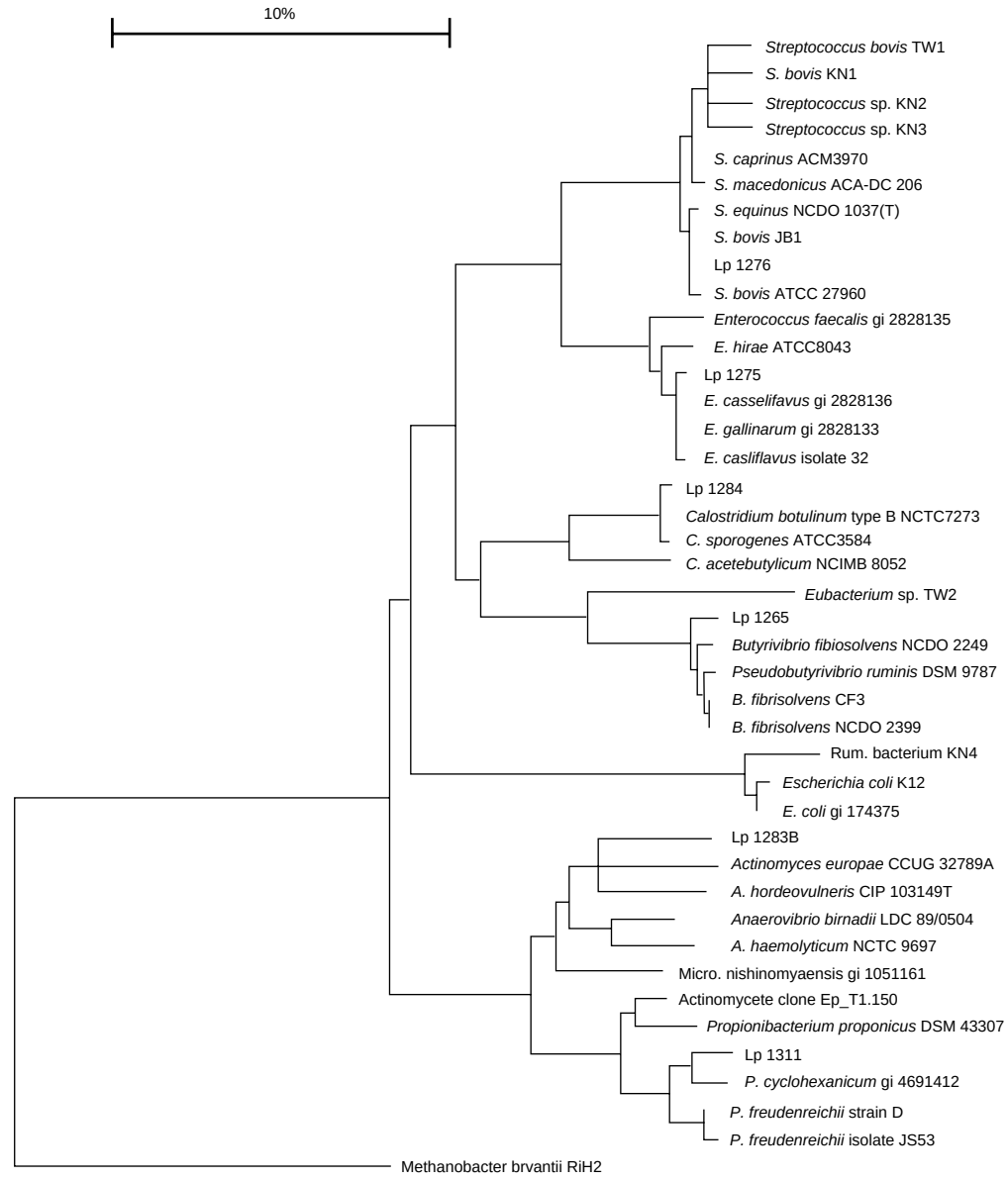


Figure 1. Phylogenetic tree of Lp1265, Lp1275, Lp1276, Lp1283B, Lp1284 and Lp1311 and related organisms based on 16S rDNA sequences. The scale bar represents a 10% difference in nucleotide sequence.

tannin tolerant bacteria isolated from ruminants were *Streptococcus* sp. *Clostridium* sp., and a gram-negative rod which belongs to the class *Proteobacteria* (Nelson et al. 1998, Brooker et al. 1994). Although these bacteria are proteolytic, peptidolytic and present at relatively high numbers in the rumina of animals fed a tannin-rich diet, they were unable to ferment protein complexed with tannin in calliandra (McSweeney et al. 1999).

Conclusion

These studies demonstrate that calliandra tannins reduce the population of ruminal cellulolytic bacteria but the proteolytic community seems to be less affected even though protein is not readily available. The fact that high numbers of proteolytic bacteria were present in the rumen under these circumstances supports previous observations that availability of carbohydrate rather than protein determines the abundance of the proteolytic population in the rumen on tropical forages. Future studies should concentrate on whether some cellulolytic and proteolytic bacteria are better adapted to tannin containing diets and as a consortia are more efficient at digesting structural carbohydrate and protein in those forages. Tannin tolerant micro-organisms are more likely to be found in geographical regions where tannin rich plants are a natural component of the ruminant diet.

Acknowledgments

This work was partly supported by the Australian Centre for International Agricultural Research (ACIAR).

References

Ahn, J.H., Robertson, B.M., Elliott, R.R., Gutteridge, C. and Ford, C.W. 1989. Quality assessment of tropical browse legumes: tannin content and protein degradation. Animal Feed Science Technology, 27: 147–156.

Bae, H.D., McAllister, T.A., Yanke, J., Cheng, K.J., and Muir, A.D. 1993. Effect of condensed tannins on endoglucanase activity and filter paper digestion by *Fibrobacter succinogenes* S85. Applied and Environmental Microbiology, 59: 2132–2138.

Barry, T.N., Manley, T.R. and Duncan, S.J. 1986. The role of condensed tannins in the nutritional value of *Lotus pedunculatus* for sheep. 4. Sites of carbohydrate and protein digestion as influenced by dietary reactive tannin concentration. British Journal of Nutrition, 55: 123–137.

Brooker, J.D., O'Donovan, L.A., Skene, I., Clarke, K., Blackall, L. and Muslera, P. 1994. *Streptococcus caprinus* sp. Nov, a tannin-resistant ruminal bacterium from feral goats. Letters in Applied Microbiology, 18: 313–318.

Chen, X.B., Hovell, F.D.DeB., Orskov, E.R. and Brown, D.S. 1990. Excretion of purine derivatives by ruminants: effect of exogenous nucleic acid supply on purine derivative excretion by sheep. British Journal of Nutrition, 63: 131–142.

Dehority, B.A., Tirabasso, P.A. and Grifo, A.P. 1989. Most-probable-number procedures for enumerating ruminal bacteria, including the simultaneous estimation of total and cellulolytic numbers in one medium. Applied and Environmental Microbiology, 55: 2789–2792.

Dore, J., Sghir, A., Hannequart-Gramet, G., Corthier, G. and Pochart, P. 1998. Design and evaluation of a 16S rRNA-targeted oligonucleotide probe for specific detection and quantitation of human faecal *Bacteroides* populations. Systematic and Applied Microbiology, 21: 65–71.

Faichney, G.J., Poncet, C., Lassalas, B., Jouany, J.P., Millet, L., Dore J. and Brownlee, A.G. 1997. Effect of concentrates in a hay diet on the contribution of anaerobic fungi, protozoa and bacteria to nitrogen in rumen and duodenal digesta in sheep. Animal Feed Science and Technology, 64: 193–213.

Joblin, K.N. 1981. Isolation, enumeration, and maintenance of rumen anaerobic fungi in roll tubes. Applied and Environmental Microbiology, 42: 1119–1122.

Jones, W.T. and Mangan, J.L. 1977. Complexes of the condensed tannins of sainfoin (*Onobrychis viciifolia* Scop.) with fraction 1 leaf protein and with submaxillary-mucoprotein, and their reversal by polyethylene glycol and pH. Journal Science Food Agriculture, 28: 126–136.

Jones, G.A., McAllister, T.A., Cheng, K-J. and Muir, A.D. 1994. Effect of sainfoin (*Onobrychris viciifolia* Scop) condensed tannins on growth and proteolysis by four strains of rumen bacteria. Applied and Environmental Microbiology, 60: 1374–1378.

Krause, D.O. and Russell, J.B. 1996. An rRNA approach for assessing the role of obligate amino acid-fermenting bacteria in ruminal amino acid deamination. Applied and Environmental Microbiology, 62: 815–821.

Krause, D.O., White, B.A. and Mackie, R.I. 1997. Ribotyping of adherent *Lactobacillus* from weaning pigs: a basis for probiotic selection based on diet and gut compartment. Anaerobe, 3: 317–325.

Krause, D.O., Dalrymple, B.P., Smith, W.J. and McSweeney, C.S. 1999. 16S rDNA sequencing of *Ruminococcus albus* and *Ruminococcus flavefaciens*: design of a signature probe and application in adult sheep. Microbiology (in press).

Lin, C., Flesher, B., Capman, W.C., Amann, R.I. and Stahl, D.A. 1994. Taxon specific hybridisation probes for fibre-digesting bacteria suggest novel gut-associated *Fibrobacter*. Systematic and Applied Microbiology, 17: 418–424.

Mackie, R.I. and Wilkins, C.A. 1988. Enumeration of anaerobic bacterial microflora of the equine gastrointestinal tract. Applied and Environmental Microbiology, 54: 2155–2160.

McSweeney, C.S., Pass, M.A. and Henry, P. 1983. Changes in rumen contents associated with lantana poisoning of sheep. Comparative Biochemistry and Physiology, 75C: 361–367.

McSweeney, C.S., Palmer, B., Kennedy, P.M. and Krause, D.O. 1998. Effect of calliandra tannins on rumen microbial function. Proceedings Australian Society for Animal Production, 22: 289.

McSweeney, C.S., Palmer, B., Bunch, R. and Krause, D.O. 1999. Isolation and characterisation of proteolytic ruminal bacteria from sheep and goats fed the tannin-containing shrub legume *Calliandra calothyrsus*. Applied and Environmental Microbiology (in press).

Moyer, C.L., Dobbs, F.C. and Karl, D.M. 1994. Estimation of diversity and community structure through restriction fragment polymorphism distribution analysis of bacterial 16S rRNA genes from a microbial mat at an active, hydrothermal vent system, Loihi Seamount, Hawaii. Applied and Environmental Microbiology, 60: 871–879.

Nelson, K.E., Thonney, M.L., Woolston, T.K., Zinder, S.H. and Pell, A.N. 1998. Phenotypic and phylogenetic characterisation of ruminal tannin-tolerant bacteria Applied and Environmental Microbiology, 64: 3824–3830.

Osawa, R.O. 1990. Formation of a clear zone on tannin-treated brain heart infusion agar by a *Streptococcus* sp. isolated from faeces of koalas. Applied and Environmental Microbiology, 56: 829–831.

Perez-Maldonado, R. A., and Norton, B.W. 1996. The effects of condensed tannins from *Desmodium intortum* and *Calliandra calothyrsus* on protein and carbohydrate digestion in sheep and goats. British Journal Nutrition, 76: 515–533.

Palmer, B. and Schlink, A.C. 1992. The effect of drying on the intake and rate of digestion of the shrub legume *Calliandra calothyrsus*. Tropical Grasslands, 26: 89–93.

Theodorou, M.K., Gill, M., King-Spooner, C. and Beever, D.E. 1990. Enumeration of anaerobic chytridiomycetes as thallus-forming units: novel method for quantification of fibrolytic fungal populations from the digestive tract ecosystem. Applied and Environmental Microbiology, 56: 1073–1078.

Waghorn, G.C., Ulyatt, M.J., John, A. and Fisher, M.T. 1987. The effects of condensed tannins on the site of digestion of amino acids and other nutrients in sheep fed on *Lotus coniculatus* L. British Journal of Nutrition, 57: 115–126.

Rumen Bacterial Diversity With and Without Mulga (*Acacia aneura*) Tannins

J.J. Plumb[1], L.L. Blackall[1] and A.V. Klieve[2]*

Abstract

Feral goats are able to survive in many semi-arid areas of Australia. Under drought conditions, the only form of available feed is often mulga, which has a very high content of condensed tannins (5–24% dry weight). While feral goats apparently thrive on this diet, sheep do very poorly and lose liveweight rapidly. It has been shown that the transfer of rumen contents from feral goats to sheep can significantly improve mulga digestion, suggesting that the ruminal microflora of feral goats may contain tannin tolerant or degrading bacteria. To identify likely communities or associations of bacteria that may undertake this task, a comparative study of the bacterial ecology of the rumens of feral goats fed mulga and sheep fed either mulga or grass was undertaken. This study used the culture independent techniques of generation of 16S rDNA clone libraries and fluorescence in situ hybridisation (FISH) probing. From the clone libraries, bacteria were mainly (>90%) within the divisions *Cytophaga-Flexibacter-Bacteroides* (CFB) and low mol% G+C Gram positive bacteria (LGCGPB). In animals fed mulga, the CFB predominated (goat – 82% CFB and 11% LGCGPB; sheep – 78% CFB and 21% LGCGPB) whereas in sheep fed grass, the LGCGPB predominated (25% CFB vs 74% LGCGPB). In all clone libraries, few bacterial species were closely related to previously cultured bacteria, making it difficult to assign phenotypic traits. FISH probing of mulga fed –rumen (feral goats and sheep) or –fermentor samples demonstrated a predominance of CFB and gamma proteobacteria. This first molecular ecological study of tannin associated microbial communities suggested that bacteria from these two groups may be either more tolerant to tannins or able to degrade tannins. Further work will be required to elucidate the important members of these groups and to obtain them in culture.

DOCUMENTATION of the use of mulga (*Acacia aneura*) as a source of feed for sheep in southwest Queensland dates back to the late 1800s. During this time, mulga has enabled the sheep industry to survive and prosper in the area to the point where the region is responsible for more than 40% of the state's wool production. Mulga-based pasture associations extend over 150 million hectares of the continent, including also South Australia, Western Australia and New South Wales where its presence is also of value to the wool industry.

[1] The Department of Microbiology, The University of Queensland, Brisbane, Qld, 4072
[2] Sheep and Wool Institute, Animal Research Institute, Queensland Department of Primary industries, Locked Mail Bag No. 4, Moorooka, Qld, 4105
* Corresponding author

The high frequency of drought in these regions requires that livestock rely heavily on mulga for survival. Supplementation of stock with nitrogen, phosphorous and sulphur is necessary to overcome the nutrient deficiencies induced by the high concentration of condensed tannins (CTs: 5–24% dry weight) in the mulga leaf. The action of CTs, now widely recognised as a plant chemical defence against herbivory, is one of reducing plant protein digestibility. In high concentrations (greater than 3 g/kg) tannins may act as anti-feedants because they:

1. complex with food protein;
2. bind microbial enzymes, reducing fermentation and degradation of fibrous tissue;
3. bind digestive enzymes in general, reducing their activity; and
4. have an astringent taste.

KEYWORDS: Rumen, Mulga, Condensed tannins, Bacterial diversity

The failure of these compounds to be broken down during passage through the digestive tract results in a reduction of the nutritional value of mulga.

While sheep do very poorly and lose liveweight rapidly when fed a diet comprising predominantly mulga, without supplementation, feral goats apparently thrive on this diet. It has been demonstrated that both feral goat rumen fluid and a fermentor-enriched consortium of micro-organisms from the feral goat rumen improve the nutritional value of mulga when administered to mulga-fed sheep (Miller et al. 1995, 1997) Therefore, it appears likely that micro-organisms present in the feral goat rumen are able to tolerate the presence of high levels of mulga tannins and also digest this harsh fodder. Inoculation with pure cultures of *Streptococcus caprinus*, a bacterium isolated from feral goat rumen fluid (Brooker et al. 1994) and capable of disrupting tannic acid-protein complexes in vitro, does not enhance protein digestion, suggesting that a consortium of micro-organisms may be involved in enhancing digestion (Miller et al. 1996). On this basis and assuming that at least some, and maybe the majority, of organisms in the consortium are not culturable, a comparative study of the bacterial ecology of the rumen of feral goats fed mulga and sheep fed either mulga or grass was undertaken. This study used the culture independent techniques of generation or construction of 16S rDNA clone libraries and fluorescent in situ hybridisation (FISH) probing.

Materials and Methods

Construction of clone libraries

Rumen samples were collected from a feral goat browsing on mulga (Charleville region, Western Queensland), a sheep being fed mulga in a feeding trial (Charleville) and grass fed sheep (Brisbane). Samples were stored frozen, with glycerol, until required for DNA extraction.

Community DNA was extracted by standard methods (Maniatis et al. 1982) and purified by electrophoresing through a 0.8% low melting point agarose gel. From the purified DNA, 16S rDNA was amplified by PCR using primers to conserved regions. PCR products were purified again using low melting point agarose gel electrophoresis.

16S rRNA gene libraries were constructed either by ligating purified 16S rDNA amplicons into the plasmid vector and transforming into competent cells using the TA Cloning Kit (Invitrogen) or constructed in a like manner using the pGem-T plasmid vector (Promega) and competent cells (Stratagene). Clones with full sized inserts were detected by either: 1) plasmid extractions, alkaline lysis followed by PEG precipitation; or 2) direct lysis PCR using the plasmid specific primers. With the latter method, clones containing 16S rDNA inserts were determined based on the size of the PCR products obtained.

The DNA sequence of cloned 16S rDNA inserts were determined using automated sequencing and the data phylogenetically analysed using methods from Blackall et al. 1994.

Fluorescent in situ hybridisation (FISH)

Rumen samples were collected from a feral goat browsing on mulga (Charleville), a sheep being fed mulga in a feeding trial (Charleville) and grass fed sheep (Brisbane). Samples were fixed according to previously described methods, using both ethanol and ethanol/paraformaldehyde as cell fixatives (Wagner et al. 1994). Samples were then stored at −20 °C until used for whole cell probing.

The probes used for FISH probing of rRNA in the microbial cells in fixed rumen samples were: EUB338 (domain Bacteria), ALF1b (alpha proteobacteria), BET42a (beta proteobacteria), GAM42a (gamma proteobacteria), CF319a (*Cytophaga-Flavobacterium*), BAC303 (*Bacteroides-Prevotella*) and HGC69a (high mol% G+C Gram positive bacteria). These probes were chosen as they cover some of the major lines of descent within the domain Bacteria. The BAC303 probe was also chosen due to the abundance of clones in the CFB phylum, observed previously (see results). Oligonucleotides were synthesised with a 5'-C6-TFA aminolinker and labelled with tetramethylrhodamine-5-isothiocyanate and 5(6)-carboxyfluorescein-N-hydroxysuccinimide-ester (Amann et al. 1990).

Hybridisation of fixed rumen samples was performed as previously described (Erhart et al. 1997). Probed samples were mounted with Citifluor and visualised using a Nikon Microphot-FXA microscope with filter blocks V-2B, B-2A and G-2A and a Leitz Wetzlar NPL FLUOTAR 100x fluorescence oil immersion objective. Cell counts were taken by viewing at least 20 different fields and counting usually between 1500 and 2000 cells.

Results

Clone libraries

Evolutionary distance trees constructed to compare clone sequences with representatives from the domain *Bacteria*, revealed the presence of bacteria belonging to several different phyla. In the feral goat

library, 82% (69/84) of clone sequences were affiliated with the *Cytophaga-Flexibacter-Bacteroides* (CFB) phylum. Sequences affiliated with the low mol% G+C Gram positive bacteria (LGCGPB) comprised 11% (9/84) of the feral goat library, while the remaining 7% (6/84) were not affiliated with any phylum in the domain *Bacteria*. The vast majority of the clone sequences in the mulga-fed sheep and the grass-fed sheep belonged to two phyla, the CFB and the LGCGPB. In the grass-fed sheep library, members of the LGCGPB phylum comprised the majority of phylotypes (74.2%), whereas in the mulga-fed sheep library, 78% of clones were in the CFB phylum. Two out of 193 sequences analysed did not fall within these two phyla. The clone sequences affiliated with the CFB phylum were generally positioned in 4 different places associated with the bacteroides subgroup. Cluster I formed an outlying part of the subgroup. Cluster II is more closely affiliated with the true *Bacteroides* such as *Bacteroides fragilis*. Cluster III and cluster IV are more closely affiliated with species of *Prevotella* (Figure 1).

Novel bacterial diversity was assessed by comparing clone sequences with reference sequence data. Using >97% sequence similarity as an arbitrary criterion for defining a 'species' (Bond et al. 1995), only four clones from the feral goat library were determined to be closely related to previously described rumen species [*Prevotella ruminicola* (3 clones) and *Selenomonas ruminantium*].

Figure 1 is presented as an example of the genetic diversity of clones from the feral goat library in the CFB phylum and how closely these relate to described bacterial species.

In the grass and mulga fed sheep libraries, about 22% (42/193) of clones were determined to be affiliated with reference sequences. Only 5 of these 42 clones were affiliated to previously described rumen bacteria (*Ruminococcus bromii* and *Butyrivibrio fibrisolvens*). The majority of clones in these libraries were dissimilar to reference sequences with 49.7% (96/193) ranging between 90% and 97% similarity and the remaining 28.5% (55/193) less than 90% similar.

Fluorescent in situ hybridisation (FISH)

Greater than 75% of DAPI stained cells was detected using the EUB338 probe. In the three rumen samples, numbers of bacteria within the CFB phylum were higher than for any of the other bacterial groups detected by the probes employed. Almost half of all bacterial cells in the grass-fed sheep sample that hybridised with EUB338 also hybridised with BAC303 and, when combined with data for the CF319a probe, 76.4% of EUB338 probing cells were detected as being members of the CFB phylum. In the feral goat sample, 52.7% of bacteria were detected as being members of the CFB phylum. In all samples, bacteria belonging to different subclasses of the proteobacteria were also detected with alpha and gamma subclasses generally more numerous than the beta proteobacteria. Members of the high mol% G+C Gram positive bacteria phylum were not detected in significant numbers in any of the samples studied. At the time the study was undertaken, a probe was not available for bacteria characterised as low mol% G+C Gram positive bacteria.

Discussion.

The research undertaken to compare mulga-degrading communities in feral goats and sheep indicates that bacteria belonging to the CFB phylum and gamma subclass of the proteobacteria are likely to be important in the digestion of mulga and therefore could be the main reservoir of tannin tolerance or possibly degradation, in feral goats. According to FISH probing, members of these two phyla comprise the majority of bacteria present in the feral goat rumen. However, bacteria belonging to these phyla are also numerically dominant in rumen bacterial populations of grass-fed sheep. It is not known whether these species of CFB are identical to the CFB members in the feral goat rumen, nor whether they possess similar phenotypes in relation to mulga tannin tolerance or digestion. Lower numbers of gamma proteobacteria were present in the rumen of grass-fed sheep compared with the feral goat rumen, indicating a possible role for this group in mulga digestion. As many of the clone sequences retrieved from the ecosystems studied represent novel species, it is difficult to infer phenotypic traits to these bacteria. Of interest, however, is a report of the resistance to the negative effects of condensed tannins by a species of *Prevotella* (Jones et al. 1994) which may indicate an ability of closely related species within the CFB phylum to tolerate tannin compounds.

While this first molecular ecological study of tannin associated microbial communities in the rumen suggests that bacteria from these two groups may be either more tolerant to tannins or able to degrade tannins, further work will be required to elucidate the important members of these groups and their role in tannin metabolism.

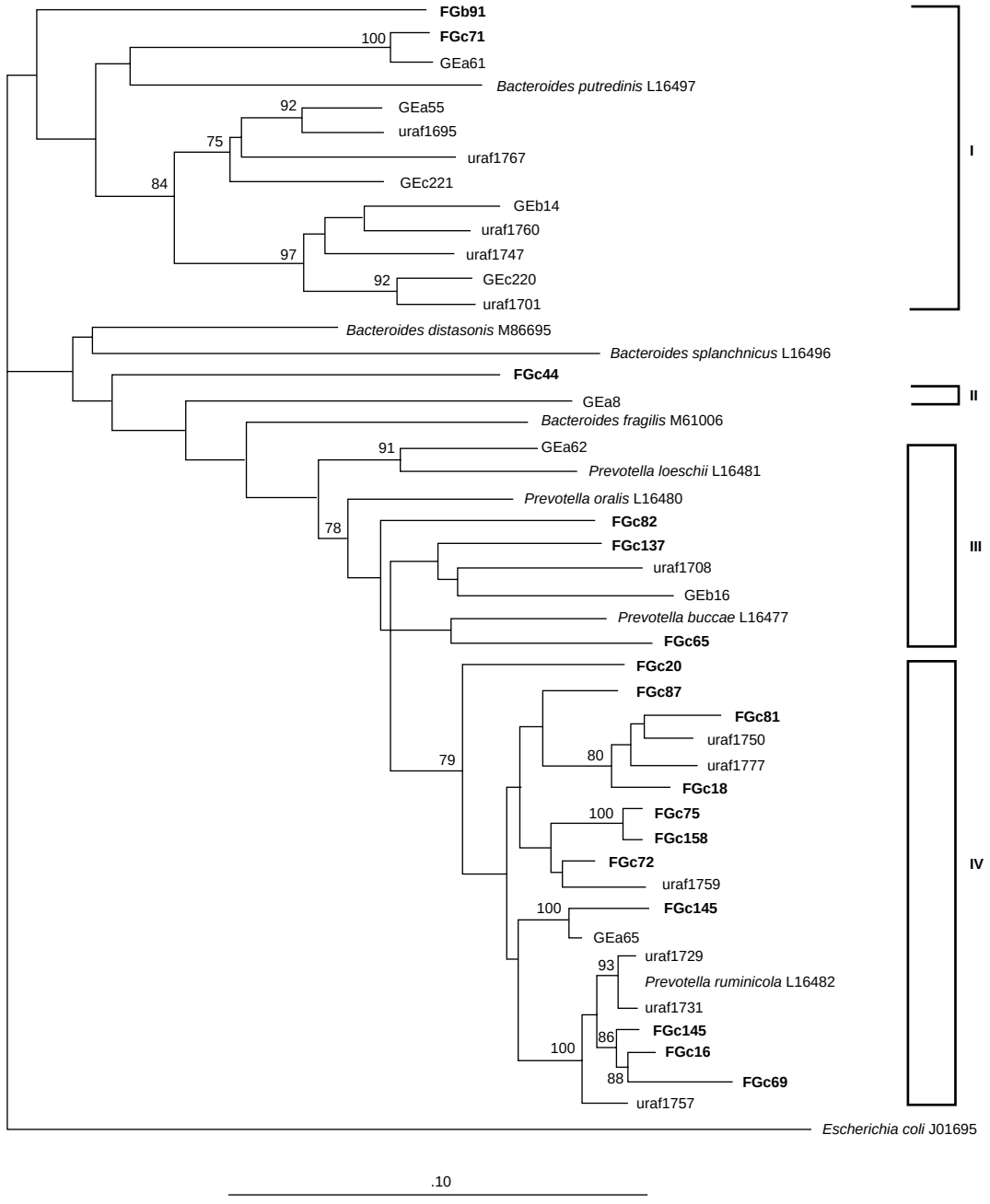


Figure 1. Evolutionary distance tree based on partial 16S rDNA sequence data (361 nucleotides) showing the phylogenetic position of representative clones within the CFB phylum. Clones derived from feral goat rumen contents are in bold type and have a FG designation. Designates beginning GE or uraf are other clonal sequences.

As future directions, we suggest the following may help further elucidate important tannin resistant or degrading bacteria:

(a) the design of new probes for members of the CFB phylum and other novel species which may be used to help clarify their role;
(b) a focused effort to isolate in culture CFB species present in the feral goat rumen that are currently uncultivable so that phenotype and tannin resistance/degradation can be investigated; and
(c) FISH probing of fixed sectioned mulga leaf retrieved from the rumen to identify attached bacteria.

References.

Amann, R.I., Krumholz, L. and Stahl, D.A. 1990. Fluorescent oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. Journal of Bacteriology, 172: 762–770.

Blackall, L.L., Seviour, E.M., Cunningham, M.A., Seviour, R.J. and Hugenholtz, P. 1994. *Microthrix parvicella* is a novel, deep branching member of the actinomycetes subphylum. Systematic and Applied Microbiology, 17: 513–518.

Bond, P.L., Hugenholtz, P., Keller, J. and Blackall, L.L. 1995. Bacterial community structures of phosphate-removing and non-phosphate-removing activated sludges from sequencing batch reactors. Applied and Environmental Microbiology, 61: 1910–1916.

Brooker, J.D., O'Donovan, L.A., Skene, I., Clarke, K., Blackall, L. and Muslera, P. 1994. *Streptococcus caprinus* sp. nov., a tannin-resistant ruminal bacterium from feral goats. Letters in Applied Microbiology, 18: 313–318.

Erhart, R., Bradford, D, Seviour, R.J., Amann, R.I. and Blackall, L.L. 1997. Development and use of fluorescent in situ hybridisation probes for the detection and identification of *Microthrix parvicella* in activated sludge. Systematic and Applied Microbiology, 20: 310–318.

Jones, G.A., McAllister, T.A., Muir, A.D. and Cheng, K.-J. 1994. Effects of sainfoin (*Onobrychus viciifolia* Scop.) condensed tannins on growth and proteolysis by four strains of ruminal bacteria. Applied and Environmental Microbiology, 60: 1374–1378.

Maniatis, T., Fritsch, E.F. and Sambrook, J. 1982. Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, New York.

Miller, S.M., Brooker, J.D. and Blackall, L.L. 1995. A feral goat rumen fluid inoculum improves nitrogen retention in sheep consuming a mulga (*Acacia aneura*) diet. Australian Journal of Agricultural Research, 46: 1545–1553.

Miller, S.M., Brooker, J.D., Phillips, A. and Blackall, L.L. 1996. *Streptococcus caprinus* is ineffective as a rumen inoculum to improve digestion of mulga (*Acacia aneura*) by sheep. Australian Journal of Agricultural Research, 47: 1323–1331.

Miller, S.M., Klieve, A.V., Plumb, J.J., Aisthorpe, R. and Blackall, L.L. 1997. An in vitro cultured rumen inoculum improves nitrogen digestion in mulga-fed sheep. Australian Journal of Agricultural Research, 48: 403–409.

Wagner, M., Erhart, R., Manz, W., Amann, R., Lemmer, H., Wedi, D. and Schleifer, K.-H. 1994. Development of an rRNA-targeted oligonucleotide probe specific for the genus *Acinetobacter* and its application for in situ monitoring in activated sludge. Applied and Environmental Microbiology, 60: 792–800.

Characterisation of Tannin-Resistant Bacteria from the Rumen Fluid of Feral Goats and Camels with Restriction Analysis of Amplified 16S rDNA

A.S. Tjakradidjaja[1], J.D. Brooker[1] and C.D.K. Bottema[1]

Abstract

Acacia sp. and *Caliandra callothyrsus* contain tannin at concentration of 8–11% DM. These legumes can only be digested by ruminants that naturally adapt to feed with high tannin content. This capability is due to the presence of tannin-resistant bacteria in their rumen, such as *Streptococcus caprinus* and *Selenomonas ruminantium* K2, which were isolated from the rumen fluid of feral goats browsing Acacia. Other tannin-resistant bacteria also exist in the rumen. These bacteria have been isolated recently from the rumen fluid of feral goats and camels in enrichment experiments using tannic acid or tannin extracts from Acacia and Caliandra leaves as limiting substrates. These tannin-resistant bacteria were grouped morphologically into Gram-positive streptococci (6 isolates), Gram-positive cocci/rods (3 isolates), Gram-negative coccus (1 isolate), Gram-negative curved rods (6 isolates), and Gram-negative slender rods (4 isolates). These isolates have been identified by physiological and biochemical tests, as well as API test, and the possible genera are *Streptococcus* sp., *Leuconostoc* sp. or *Lactobacillus* sp., *Megasphaera* sp. or *Prevotella* sp., *Selenomonas* sp., *Butyrivibrio* sp. or *Clostridium* sp. Confirmation of these genera is still necessary, for example, by the use of a molecular approach. The present study was conducted to characterise tannin-resistant bacteria using 16S rDNA restriction fragment length polymorphism (RFLP) analysis. This method was capable of identifying isolates that belong to *Streptococcus* sp. and *Selenomonas* sp. The other two isolates appeared to be *Lactobacillus* sp. and *Butyrivibrio* sp. However, restriction analysis of amplified 16S rDNA did not characterise Gram-negative coccus to be the same genus as identified phenotypically. This bacterium could be characterised by sequencing its amplified 16S rDNA as *Escherichia coli*. This method also confirmed the identification of the other two isolates to be *Lactobacillus* sp. and *Butyrivibrio* sp. This study indicated that restriction analysis of amplified 16S rDNA followed by sequencing of 16S rDNA are useful for characterisation of tannin-resistant bacteria. This characterisation is important to study the role of tannin-resistant bacteria to digest legume leaves that contained tannin at high concentration.

ACACIA sp. and *Caliandra callothyrsus* contain tannin at concentration of 8–11% dry matter (DM) which limits their utilisation as animal feeds (Soebarinoto 1986; Elliott and McMeniman 1987). However, ruminants that naturally adapt to feed with high tannin content were able to digest those legumes. This capability is due to the presence of tannin-resistant bacteria in their rumen.

Two tannin-resistant bacteria, *Streptococcus caprinus* and *Selenomonas ruminantium* K2, had been isolated from the rumen fluid of feral goats browsing *Acacia* sp. (Brooker et al. 1994; Skene and Brooker 1995). However, these species are not the only bacteria tolerating tannin. Other tannin-resistant bacteria also exist in the rumen. Twenty bacteria that are resistant to tannin have been isolated recently from the rumen fluid of feral goats and camels in enrichment experiments. In these experiments, tannic acid or tannin extracts from Acacia and calliandra were used as limiting substrates (Tjakradidjaja et al. 1997; unpublished data).

These isolates were grouped based on their morphology and identified phenotypically with

[1] Department of Animal Science, The University of Adelaide, Glen Osmond, Adelaide SA 5064 Australia

KEYWORDS: Tannnins, *Acacia* sp. *Caliandra callothysus*, Tannin-resistant bacteria; Rumen fluid; Ruminants, Amplified 16S rDNA

physiological tests, biochemical reactions and API tests (Table 1). However, the metabolic identification techniques were not sufficient to distinguish the organisms that were similar (Johnson 1985). This conventional approach should be integrated with molecular approach, i.e. studying nucleic acids of bacteria to confirm the identification of bacteria (Johnson 1985; Staley 1996; Tiedje and Zhou 1996).

An example of molecular techniques for bacterial identification is amplification of DNA using polymerase chain reaction (PCR) which is usually employed together with other methods, such as restriction fragment length polymorphism (RFLP), random amplified polymorphism DNA (RAPD) or PCR ribotyping (Randles et al. 1996; Momol et al. 1997). In the present study, tannin-resistant bacteria were characterised with RFLP analysis of PCR-amplified 16S rDNA.

Materials and Methods

Bacteria

All isolates were characterised in this experiment (Table 1). To confirm this identification, several ruminal and non-ruminal bacteria were also included as references. These bacteria were *Streptococcus* (S) *bovis* WJ-1, *S. caprinus* 2.2, *S. gallolyticus* (*S. bovis* biotype I), *Selenomonas* (Sel.) *ruminantium* HD4, *Sel. ruminantium* K2, *Butyrivibrio* (B.) *fibrisolvens* E14, *Prevotella* (P.) *ruminicola*, *Ruminococcus* (R.) *albus*, *Clostridium* (C.) *perfringens*, *Lactobacillus* (L.) *plantarum*, *Megasphaera* (M.) *elsdeni*, *Enterococcus* (Ent.) *faecalis*, *Bacillus* (Bac.) *fragilis* and *Escherichia* (E.) *coli* ED8299.

DNA extraction and amplification

Extraction of DNA was carried out by the method of Ausubel et al. (1989). The purified DNAs of tannin-resistant and reference bacteria were used as templates in PCR. The 16S rDNA genes were amplified with PCR using two universal ribosomal DNA primers: fD1 (5'GAA TTC GTC GAC AGA GTT TGA TCC TGG CTC AG3') and rP2 (5'AAG CTT GGA TCC ACG GCT ACC TTG TTA CGA CTT3').

The PCR mixture contained : 5 µL 10x PCR buffer (Gibco BRL), 0.4 mM dNTP consisting of dATP, dCTP, dGTP and dTTP (Boehringer Mannheim), 30 pmol of each primer, 1.5 mM $MgCl_2$ (Gibco BRL), 2.5 unit Taq polymerase enzyme (Gibco BRL), 50 ng purified DNA and sterilised water to make up 50 µL. The amplification of DNA was performed using a Perkin Elmer Cetus DNA Thermal Cycler (Norwalk, Conn.). The reaction was

Table 1. Tannin-resistant bacteria isolated from the rumen fluid of feral goats and camels.

Group number	Isolates	Tannin sources in enrichment experiments	Bacterial groups based on Gram-staining morphology	Possible genera as identified phenotypically
I	K1T – goat C1T – camel	Tannic acid	Gram-positive streptococci	*Streptococcus* sp.
	C13A – camel	Acacia		
	G13C – goat G23C – goat C13C – camel	Caliandra		
II	G33A – goat C23A – camel	Acacia	Gram-positive cocci/rods	*Leuconostoc* sp. or *Lactobacillus* sp.
	G43C – goat	Calliandra		
III	C43C – camel	Calliandra	Gram-negative coccus	*Megasphaera* sp. or *Prevotella* sp.
IV	K2T – goat C2T – camel	Tannic acid	Gram-negative curved rods	*Selenomonas* sp.
	G13A – goat	Acacia		
	G33C – goat C23C – camel C53C – camel	Calliandra		
V	G23A – goat G53C – goat G63C – goat C33C – camel	Acacia Calliandra	Gram-negative slender rods	*Butyrivibrio* sp. or *Clostridium* sp.

hot-started by denaturation at 95 °C for 4 min, followed by one cycle of 94 °C 5 min; 57 °C 2 min; 72 °C 2 min; and then 30 cycles of 94 °C 2 min; 57 °C 2 min; 72 °C 2 min, one cycle of 94 °C 2 min; 57 °C 2 min; 72 °C 10 min. The reaction was then held at 4 °C until analysis of products.

The PCR products were then electrophoresed on a 1% (w/v) agarose gel (Sigma Co. Mo.) in 1x TAE buffer. The agarose gel was stained with ethidium bromide (50 µL.1000/mL) and visualised under UV light using a molecular analysis program (Biorad) in GelDoc to view the PCR product.

Restriction digestion of PCR products (16S rDNA RFLP)

The PCR products were precipitated with cold ethanol and salt, and purified with 70% ethanol. These precipitated DNAs dissolved in 25 µL sterilised water after vacuum-drying. The amplified DNAs (5 µL) were digested with 10 unit of restriction enzymes (AluI, HaeIII, TaqI - Promega, and MspI – Boehringer Mannheim) in 2 µL of their appropriate buffers (Promega, Boehringer Mannheim); sterilised water was added to make up the volume to 20 µL. The digestion was conducted overnight at 37 °C for AluI, HaeIII and MspI, and at 65 °C for TaqI.

The restriction products (16S rDNA RFLP) were electrophoresed on a 10% preformed polyacrylamide gel (CleanGels 10% 48S – Pharmacia Biotech) which was stained with silver staining following a printed procedure (Pharmacia Biotech). The 16S rDNA RFLP profile was recorded by scanning the gel using the Biorad GelDoc and its Molecular Analysis Program.

Sequence analysis

The identification of tannin-resistant bacteria was also carried out by sequence analysis of 16S rDNA. However, there were only three isolates which were selected from the following groups: Gram-positive cocci/rods (G43C), Gram-negative coccus (C43C) and Gram-negative slender rods (G53C). Sequence analysis of their PCR products was conducted and the results were compared to published sequences in database.

Results

One fragment of 1500 bp in size was obtained by PCR with all isolates and reference bacteria. *E. coli* was used as a general reference in the present experiment. Digestion its 16S rDNA with restriction endonucleases such as HaeIII, TaqI, AluI and MspI, produced different RFLP profiles. However, there was several samples of *E. coli* that were not digested by HaeIII, TaqI and MspI as indicated by the presence of DNA fragment at 1500 bp.

Digestion of PCR products of all isolates and reference bacteria of Gram-positive streptococci with HaeIII and TaqI produced RFLP profiles demonstrating that they were *Streptococcus* sp. These results were also confirmed by digestion with AluI and MspI. However, several isolates (C1T, C13A and G13C) had a slightly different RFLP profiles from the reference bacteria when their amplified 16S rDNA was digested with AluI. *S. caprinus* was also found to have a slightly different RFLP pattern when its 16S rDNA was digested with MspI.

It was difficult to determine the identification of Gram-positive rods/cocci because their RFLP patterns were not clear. All reference bacteria in this group could be differentiated from their RFLP profiles. However, digestion with TaqI appeared to produce similar RFLP patterns in *R. albus*, *Ent. faecalis* and *L. plantarum*. These similarities were also observed among tannin-resistant isolates of Gram-positive rods/cocci. A further observation on RFLP profiles produced from MspI digestion showed that they might be *Lactobacillus* sp. However, fragmentation of their PCR products with HaeIII and AluI did not support those observations.

Gram-negative coccus did not have RFLP profiles that were similar to those of reference bacteria. As a result, this bacterium could not be identified as *Megasphaera* sp., *Prevotella* sp. or *E. coli*.

RFLP profiles of *Sel. ruminantium* HD4 appeared to be different from those of *Sel. ruminantium* K2. Most isolates of Gram-negative curved rods had the same RFLP profiles as those of *Sel. ruminantium* K2. However, the RFLP patterns of C53C isolate did not show any similarities to the other isolates. C23C appeared to have a slightly different RFLP pattern when its PCR product was digested with AluI.

Although the RFLP profiles of *B. fibrisolvens* were not clear, it could still be seen on the gel. The bacteria used as reference for identification of Gram-negative slender rods could be differentiated by their RFLP profiles. None of the isolates could be characterised as one of the reference bacteria when their RFLP profiles of HaeIII digestion were compared. There was an indication that G23A might be *Butyrivibrio* sp. based on RFLP patterns of TaqI digestion. The other isolates could not be identified as *Butyrivibrio* sp., but those isolates were identical among themselves. AluI and MspI digestions produced RFLP profiles showing that the isolates were identical and appeared to be similar to *Butyrivibrio* sp. or *Clostridium* sp. However, G23A had slightly different RFLP pattern produced from MspI digestion which could distinguish this bacterium from the other isolates. G53C and G63C were identical in all their RFLP profiles.

C33C basically had the same RFLP profiles as those of G53C and G63C, but several DNA fragments could be used to differentiate them.

Since several isolates could not be identified exactly, a further analysis was carried out using 16S rDNA sequence analysis. The results which were indicated as sequence identity of 500 bp at each end of 16S rDNA showed that C43C (Gram-negative coccus) was 95–96% similar to *E. coli*. 16S rDNA sequence analysis also indicated that Gram-positive rod/coccus (G43C) to be identical to *Lactobacillus* sp. with similarity values about 94–99%. The other isolate (G53C-Gram-negative slender rod) has been characterised to be similar to *Butyrivibrio* sp. (93–95%).

Discussion

The 16S rDNA could be amplified from tannin-resistant and reference bacteria. These amplified DNA could also be digested by restriction endonucleases used in the present experiment producing different RFLP profiles. This result demonstrated that restriction sites for the enzymes were available in sequence of amplified DNA of all bacteria (Ward et al. 1998). The occurrence of undigested PCR product did not relate to the unavailability of restriction sites in 16S rDNA sequence, but this was due to technical problems such as sample preparations for restriction digests.

Restriction analysis of 16S rDNA could be used to characterised tannin-resistant bacteria that belong to *Streptococcus* sp. and *Sel. ruminantium* K2. The restriction endonucleases used in the present experiment could produce RFLP patterns that showed similarities between the isolates and reference bacteria. Several isolates had slightly different RFLP patterns which could be used to distinguish them. However, those RFLP profiles did not indicate heterogeneity among those isolates. Discrimination between species with similar characteristics could also be carried out with RFLP analysis, but it depended on the number of restriction enzymes used (Jayarao et al. 1991, 1992).

The present experiment showed that RFLP profiles of Gram-positive rods/cocci and Gram-negative slender rods could not strongly identify them, respectively, as *Lactobacillus* sp. and *Butyrivibrio* sp. These might be due to the numbers of restriction enzymes used in this experiment were not sufficient to show similarities among bacterial species with high diversity such as *Lactobacillus* sp. (Zhong et al. 1998) and *Butyrivibrio fibrisolvens* (Mannarelli et al. 1990; Forster et al. 1996). Similar RFLP patterns among bacteria with high heterogeneity could be obtained by inclusion varieties of strains or species which was combined with the use of several types of restriction enzymes to digest their 16S rDNAs.

16S rDNA sequence analysis was also useful to confirm the identification of bacteria and its utilisation could be limited to those that could not be characterised with RFLP analysis (Lyra et al. 1997). This analysis confirmed the identification of *Lactobacillus* sp. and *Butyrivibrio* sp. Gram-negative coccus could also be identified as *E. coli*, but this result was not supported by their RFLP patterns. A further study was necessary to compare this bacterium with other tannin-resistant *Enterobacteria* (Osawa 1992; Nelson et al. 1998).

In the present experiment, restriction analysis of amplified 16S rDNA followed by sequencing 16S rDNA are useful to characterise tannin-resistant bacteria. This identification is important to study the role of tannin-resistant bacteria to digest legume leaves that contain tannin at high concentration.

Acknowledgments

Thanks are due to the Australian Government for assistance through the Australian Agency for International Development (AusAID), the Institut Pertanian Bogor (IPB-Bogor), ACIAR and the University of Adelaide (Adelaide) for the opportunity, scholarship and research support to study in Australia. Mrs. Jane McCarthy is acknowledged for conducting the 16S rDNA sequence analysis.

References

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. 1989. Current protocols in molecular biology. John Wiley and Sons Inc. New York, USA.

Brooker, J.D., O'Donovan, L.A., Skene, I.K., Clarke, K., Blackall, L. and Muslera, P. 1994. *Streptococcus caprinus* sp. *nov*., a tannin-resistant ruminal bacterium from feral goats. Letters in Applied Microbiology, 18: 313–318.

Elliot, R.E. and McMeniman, N.P. 1987. Forages as supplements. In: Hacker, J.B. and Ternouth, J.H. ed. The Nutrition of Herbivores. Academic Press, Sydney, Australia, 409–428.

Forster, R.J., Teather, R.M., Gong, J., and Deng, S.J. 1996. 16S rDNA analysis of *Butyrivibrio fibrisolvens*: phylogenetic position and relation to butyrate-producing anaerobic bacteria from the rumen of white-tailed deer. Letters in Applied Microbiology, 23: 218–222.

Jayarao, B.M., Dore Jr., J.J.E., Baumbach, G.A., Matthews, K.R. and Oliver, S.P. 1991. Differentiation of *Streptococcus uberis* from *Streptococcus parauberis* by polymerase chain reaction and restriction fragment length polymorphism analysis of 16S ribosomal DNA. Journal of Clinical Microbiology, 29: 2774–2778.

Jayarao, B.M., Dore Jr., J.J.E. and Oliver, S.P. 1992. Restriction fragment length polymorphism analysis of 16S ribosomal DNA of Streptococcus and Enterococcus species of bovine origin. Journal of Clinical Microbiology, 30: 2235–2240.

Johnson, J.L. 1985. Determination of DNA base composition. In: Gottschalk, G. ed. Methods in Microbiology 18. Academic Press, London, UK, 1–32.

Lyra, C., Hantula, J., Vainio, E., Rapala, J., Rouchiainen, L. and Sivonen, K. 1997. Characterisation of *Cyanobacteria* by SDS-PAGE of whole-cell proteins and PCR/RFLP of the 16S rRNA gene. Archives Microbiology, 168: 176–184.

Mannarelli, B.M., Stack, R.J., Lee, D. and Ericsson, L. 1990. Taxonomic relatedness of *Butyrivibrio*, *Lachnospira*, *Roseburia*, and *Eubacterium* species as determined by DNA hybridisation and extracellular-polysaccharide analysis. International Journal of Systematic Bacteriology, 40: 370–378.

Momol, M.T., Momol, E.A., Lamboy, W.F., Norelli, J.L., Beer, S.V. and Aldwinckle, H.S. 1997. Characterisation of *Erwinia amylovora* strains using random amplified polymorphic DNA fragments (RAPDs). Journal of Applied Microbiology, 82: 389–398.

Nelson, K.E., Thonney, M.L., Woolston, T.K., Zinder, S.H., and Pell, A.N. 1998. Phenotypic and phylogenetic characterisation of ruminal tannin-tolerant bacteria. Applied and Environmental Microbiology, 64: 3824–3830.

Osawa, R. 1992. Tannin-protein complex-degrading *Enterobacteria* isolated from the alimentary tracts of koalas and a selective medium for their enumeration. Applied and Environmental Microbiology, 58: 1754–1759.

Randles, J.W., Hodgson, R.A.J. and Wefels, E. 1996. The rapid and sensitive detection of plant pathogens by molecular methods. Australasian Plant Pathogen, 25: 71–85.

Skene, I.K. and Brooker, J.D. 1995. Characterisation of tannin acylhydrolase from the anaerobic rumen bacterium *Selenomonas ruminantium*. Proceedings of the Australian Society for Biochemistry and Molecular Biology, 27: Pos. 1–2.

Soebarinoto 1986. Evaluasi beberapa hijauan leguminosa pohon sebagai sumber protein untuk ternak. Disertasi Doktor. Fakultas Pasca Sarjana, Institut Pertanian Bogor, Bogor-Indonesia.

Staley, J.T. 1996. Heterotrophic bactria: The cultivation approach. In: Hall, G.S. ed. Methods for the examination of organismal diversity in soils and sediments. IUBS, CAB International Oxon., UK, 1–10.

Tiedje, J.M. and Zhou, J. 1996. Analysis of non-culturable bacteria. In: Hall, G.S. ed. Methods for the examination of organismal diversity in soils and sediments. IUBS, CAB International Oxon., UK, 53–66.

Tjakradidjaja, A.S., Brooker, J.D. and Bottema, C.D.K. 1997. Are *Streptococcus caprinus* and *Selenomonas ruminantium* K2 the only tannin-resistant bacteria in feral ruminants? Microbiology Australia, 18: A114(PO4.5).

Ward, L.J.H., Brown, J.C.S. and Davey, G.P. 1998. Two methods for the genetic differentiation of *Lactococcus lactis* ssp. *lactis* and *cremoris* based on differences in the 16S rRNA gene sequence. FEMS Microbiology Letters, 166: 15–20.

Zhong, W., Millsap., K., Bialkowska-Hobrzanska, H. and Reid, G. 1998. Differentiation of *Lactobacillus* species by molecular typing. Applied and Environmental Microbiology, 64: 2418–2423.

The Nutritive Value of Tree Fodder: Assessments made by Nepalese Smallholder Farmers and by Laboratory Techniques

P.J. Thorne[1], D.B. Subba[2], D.H. Walker[3], B. Thapa[4], C.D. Wood[1*] and F.L. Sinclair[5]

Abstract

Tree fodders are important components of ruminant diets in many less developed countries and tannins are an important factor in determining their nutritive value. This paper explores an indigenous knowledge system that relates to the quality of tree fodder used by farmers in Nepal. Our results suggested that the knowledge of tree fodder quality possessed by farmers is quite consistent with the information that may be generated from laboratory analyses. Of the two distinct indigenous quality scales used, one (*obanopan*) appeared to relate to digestibility of tree fodder (as predicted by an in vitro test) and the other (*posilopan*), that was perceived as an indicator of general nutritional quality, may relate to the ability of a tree fodder to promote the supply of protein at the duodenum. However, the relationship between *obanopan* and in vitro digestibility indicated that Nepalese farmers preferred to feed less digestible fodder. Nepalese farmers keep animals for a range of objectives, not just milk and meat production. Manure production and the need to fill animals at times of feed shortage are also important. This observation highlights the importance of interpreting nutritional information against farmers' objectives for a given set of circumstances.

TREE FODDERS are important livestock feeds in many tropical and sub-tropical regions. They are often used in the dry season or other times of feed shortage when there may be a general shortage of livestock feed. Tree fodders are fed mainly to ruminant livestock. They may be browsed particularly in more extensive grazing systems, lopped to facilitate browsing or lopped for carrying to the homestead in cut and carry feeding systems. Most tree fodders contain tannins, which are an important factor in determining their nutritive value.

Nepalese smallholder farmers use lopped tree fodder to supplement crop residues, notably rice straw, during the November to June dry season when feeds are scarce (Panday 1982). Rice production is a major activity, with livestock being kept for draught power and as providers of manure as well as for the production of meat and milk. Farmers collect tree fodder from communal forests and from trees grown on their own land. They have an extensive practical knowledge about the effects the available tree fodders have when fed to their livestock.

A previous investigation of farmers' knowledge (Thapa et al. 1997) had identified two local classification systems used by farmers in this region to describe tree fodder quality: *posilopan* and *obanopan*. The Nepalese term *posilo* may be literally translated as 'nutritious'. *Posilo* fodder is said by farmers to promote milk and butter fat production in lactating animals, rapid live weight gain and animal health.

[1] Natural Resources Institute, University of Greenwich, Central Avenue, Chatham Maritime, Kent ME4 4TB, United Kingdom. E-mail c.d.wood@gre.ac.uk
[2] Pakhribas Agricultural Centre, British Aid Project Support Office, c/o British Embassy, PO Box 106, Lainchaur, Lazimpat, Kathmandu, Nepal
[3] CSIRO Tropical Agriculture, Davies Laboratory, PMB PO, Aitkenvale Qld 4814, Australia. E-mail daniel.walker @tvadmin.tvl.tcp.csiro.au
[4] CARE International, PO Box 1661, Kathmandu, Nepal
[5] School of Agricultural and Forest Sciences, University of Wales, Bangor, Gwynedd, LL57 2UW, United Kingdom. E-mail f.l.sinclair@bangor.ac.uk
* corresponding author

KEYWORDS: Nutritive value, Digestibility, Tree fodders, Nepal, Tannins

They are also palatable and satisfy appetite (Thapa et al. 1997). *Obano* fodder is also valued by farmers. The term may be translated as 'dry and warm'. Rusten and Gold (1991) suggested that the term refers to the consistency of dung produced. However, farmers also state that *obano* fodder 'fills the animal', is highly palatable particularly during colder months, and is eaten voraciously, although causing constipation if fed in excess. The two classification systems are, generally, applied consistently among farmers and are demonstrably independent of each other (Thapa et al. 1997; Walker et al. in review). This study seeks to derive a biological interpretation of the farmers' knowledge classifications used in Nepal. The implications to future work on tannins of the tree fodder quality characteristics apparently preferred by Nepalese farmers are discussed.

Materials and Methods

The data and fodder samples used in the study were collected on farms in Solma Village Development Committee Area, Terathum District, situated in the middle hills of eastern Nepal. Over 90 species, subspecies and landraces of trees, shrubs and bamboos provide farmers in Solma with fodder (Thapa et al. 1997). Eight of these were selected for study. These represented five species (*Albizia julibrissin*, *Ficus nemoralis*, *Ficus roxburghii*, *Ficus semicordata* and *Prunus cerasoides*), two botanically-differentiated subspecies of *F. semicordata* (var. *Montana* and var. *Semicordata*) and two landraces (distinguished by farmers but considered to be the same botanical species) of *F. nemoralis* (*sano pate dudhilo* [SPD] and *thulo pate dudhilo* [TPD]) and of *F. roxburghii* (*chillo pate nebharo* [CPN] and *khasre pate nebharo* [KPN]).

Sixty farmers ranked the eight tree fodders according to the *obanopan* and *posilopan* characteristics. Tree leaf samples were taken at three sampling times and their major chemical components (crude protein, fibre (ADF, NDF and ADIN), lignin and ash), tannins (Total phenols by the Prussian Blue method, extractable and non-extractable condensed tannins by acid butanol, protein precipitation by radial diffusion), in vitro digestibility (by neutral cellulase and by in vitro gas production) determined. The methodologies have been described in more detail by Walker et al. (in review).

A set of correlation analyses was carried out in order to:

- interpret the *obanopan* and *posilopan* criteria, on which farmers' assessments of fodder quality are based, in terms of the laboratory indicators used by scientists for the same purpose;
- consider the extent to which the perceptions of nutritive value that nutritionists derive from laboratory analyses are adequate for supporting improvements in the use of feed resources in developing countries. This analysis was based on a set of rankings of the relative quality of the different types of tree fodder generated by a group of expert nutritionists presented with a nutritional profile summarising the laboratory parameters.

A set of protein supply indices, which might be expected to indicate duodenal protein supplies, were also derived from laboratory parameters for each fodder sample. Correlation coefficients (Rs) were determined for each of these protein supply indices on farmers' rankings for *obano* and *posilo* status. These analyses are described in detail by Thorne et al. (in press).

Results

The results of correlating ranks based on individual laboratory indicators of nutritive value with farmers' rankings for *obano* and *posilo* status are presented in Table 1. Farmers' rankings for *posilo* status did not

Table 1. Rank correlations coefficients (Rs) of individual laboratory assessment parameters on *obano* and *posilo* status.

Laboratory parameter (g/kg DM unless otherwise stated)	Rank correlation coefficient	
	Obanopan	*Posilopan*
Dry matter	−0.64	−0.45
Crude protein	0.43	0.12
Acid detergent fibre	−0.52	0.40
Neutral detergent fibre	0.24	0.14
Lignin	−0.52	0.26
Acid detergent insoluble nitrogen (g/kg total N)	0.14	−0.12
Total phenols (g gallic acid eq/kg)	−0.38	−0.07
Non–extractable condensed tannin (arbitrary units)	0.50	−0.52
Neutral cellulase digestibility	0.79*	0.60
Gas produced during 72 h in vitro fermentation (mL)	0.76*	0.40
DM loss after 72 h in vitro fermentation (g/kg substrate)	0.81*	0.02

* $P<0.05$

correlate significantly (P>0.05) with any of the rankings by individual laboratory parameters, although *posilo* feeds tended to have lower tannin contents. *Obano* status was significantly correlated (P< 0.05) with the volume of gas produced after a 72 h in vitro fermentation in rumen fluid using the method of Theodorou et al. (1994) and the overall loss of dry matter during this fermentation. The positive value of Rs for both of these relationships indicated that *obano* fodder, which was highly valued by farmers, might be expected to be relatively undegradable in the rumen.

Values of Rs for simple protein supply indices, based on the aggregated effects of crude protein content and estimates of degradability or digestibility in vitro, were not significant. However, the introduction of the term representing condensed tannins resulted in significant correlation with *posilo* status (P<0.05). The protein supply index (PSI), defined in equation 1 below, had the strongest correlation.

$$PSI = CP/\text{mean } CP + DMD_{70}/\text{mean } DMD_{70} + CT/\text{mean}CT \quad \text{(equation 1)}$$

where DMD_{70} = dry matter digestibility after 70 hours incubation in an in vitro gas production system; CP = crude protein; NCD = neutral cellulase digestibility; CT = non-extractable condensed tannin.

The mean ranks of the group of nutritionists for the relative nutritive values of the eight types of tree fodder were weighted heavily on in vitro digestibility. Table 2 summarises the correlations achieved between PSI, expert rankings and farmers' *obano* and *posilo* rankings. Farmers were able to discriminate tree fodder types effectively using the *obanopan* classification system for all pairwise comparisons with the exception of that between *Ficus nemoralis* [SPD] and *F. nemoralis* [TPD]. However, this pair could be distinguished by the in vitro neutral cellulase digestibility technique (NCD). Conversely, NCD was not as effective as *obanopan* in discriminating the sub-species of *F. semicordata* and the landraces of *F. roxburghii*. *Albizia julibrissin* and *Prunus cerasoides* were effectively distinguished from each other and from the Ficus species by both NCD and *obanopan* rankings. A similar degree of complementarity was observed between assessments based on PSI and the *posilopan* classification system.

Table 2. Correlations between farmers' rankings and rankings derived from laboratory data.

	Obano ranking	*Posilo* ranking
Protein supply index (PSI)	−0.34	0.80*
Expert ranking	−0.87*	0.25

* (P<0.05)

Discussion

A more detailed investigation, beyond the simple correlations presented here, is clearly required to interpret fully the biology of *obanopan*. However, the observation that farmers' prefer relatively poorly degradable (*obano*) tree fodders was contrary to expectations. This finding is consistent with farmers' observations that *obano* fodder 'fills the animal' (Thapa et al. 1997). The study reported by Rusten and Gold (1991) also conducted in the Nepal, confirms this characteristic of *obano* fodder and a similar knowledge system has been observed in Himachal Pradesh (Louise Garde, pers. comm.). Indeed, a perception among farmers of this desirable attribute of tree fodder may be widespread. Roothaert et al. (1997) interviewing farmers in Embu, Kenya, reported that 48% of respondents in an agro-ecological zone in which serious, seasonal restrictions to feed supplies are common, expressed a need for tree fodder which could induce 'satisfaction of the animal'.

None of the existing laboratory parameters tested offered a satisfactory interpretation of *posilopan*. The PSI used in our interpretation of *posilopan* has not been validated. However, the approach does suggest that this criterion is associated with the ability to improve the supply of protein at the duodenum. This might also be inferred from farmers' descriptions of the characteristics of *posilo* fodder and its role in their production system. To be effective in predicting the impacts of tree fodder on duodenal protein supplies, a laboratory technique would need to embrace both ruminal and post-ruminal effects and should allow for the effects of tannins.

Our observation suggests that there is significant complementarity between farmers' assessments of tree fodder feeding values and relative assessments derived from laboratory information hence laboratory techniques may also prove valuable in investigating the potential for genetic improvement of indigenous species and selecting superior types. Increasing knowledge on the effects of tannins on nutritive value, possibly together with developments in the field of genetic engineering, could facilitate future developments in the selection of improved fodder trees. However, this potential will only benefit poor smallholder farmers in less developed countries if the complex objectives and preferences of these farmers are taken into account. In Nepal, tree fodders that are good protein sources appear to be preferred, but there is also evidence that farmers prefer tree fodders of low dry matter digestibility for the purposes of their farming system.

References

Panday, K.K. 1982. Fodder trees and tree fodder in Nepal, Berne, Switzerland, Swiss Development Cooperation. 107 p.

Roothaert, R.L., Armini, H.K. and Kamau, E.N. 1997. Improving Indigenous Fodder Trees and Shrubs in Kenya: Starting with Farmers' Knowledge, AFRENA Report No. 111, 27 p.

Rusten, E.P. and Gold M.A. 1991. Understanding an indigenous knowledge system for tree fodder via a multi-method on-farm research approach. Agroforestry Systems, 15: 139–165.

Thapa, B., Walker, D.H. and Sinclair, F.L. 1997. Indigenous knowledge of the feeding value of tree fodder. Animal Feed Science and Technology, 67: 97–114.

Theodorou, M.K., Williams, B.A., Dhanoa, M.S., McAllan, A.B. and France, J. 1994. A new gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. Animal Feed Science and Technology, 48: 185–197.

Thorne, P.J., Subba, D.B., Walker, D.H., Thapa, B., Wood, C.D. and Sinclair, F.L. 1999. The basis of indigenous knowledge of tree fodder quality and its implications for improving the use of tree fodder in developing countries. Animal Feed Science and Technology, 81: 119–131.

Walker, D.H., Thorne, P.J., Sinclair, F.L., Thapa, B., Wood, C.D. and Subba, D.B. 1999. A systems approach to comparing indigenous and scientific knowledge: consistency and discriminatory power of indigenous and laboratory assessment of the nutritive value of tree fodder. Agricultural Systems, 62: 87–103.

A Review of Tannins and Other Secondary Metabolites in the Fodder Shrub Tagasaste (*Chamaecytisus proliferus*)

N.J. Edwards[1]

Abstract

The principal phenolic metabolites in tagasaste (*Chamaecytisus proliferus*) belong to the flavone group, although low concentrations of condensed tannins have also been detected in some samples. The flavones have been identified as the *Aglycones apigenin* (3', 5, 7-trihydroxyflavone) and luteolin (3', 4', 5, 7-tetrahydroxyflavone). In the plant, they occur in glycosidic form, possibly the *c*-glycosides Vitexin (Apigenin 8-*c*-glucoside) and iso-Vitexin (Luteolin 8-*c*-glucoside), although more work is required to confirm this and identify the sugars. The biological activity of flavones in tagasaste is still unclear, as both crude and purified extracts of tagasaste containing these compounds have stimulatory effects on in vitro rumen fermentation. This activity is consistent with rumen micro-organisms utilising the glucoside component of these compounds. The total phenolic concentration of tagasaste, as measured by a modified Prussian-Blue assay, shows a strong inverse relationship with animal performance. Although not equivocal, current data also indicates an inverse relationship between the concentration of phenolic compounds in tagasaste and its palatability. Fluctuating intake, possibly due to these changes in palatability, appears to be the major cause of a marked seasonality in liveweight performance of livestock grazing tagasaste. The isoflavonoid Diadzein has also been detected in some tagasaste samples; however, no flavonols have been detected. Of the other groups of secondary metabolites, only the alkaloids sparteine and cytosine have been detected in tagasaste, albeit at relatively low concentrations. Study of the chemistry of tagasaste has not been exhaustive and there remains an urgent need to screen tagasaste from a range of growth conditions for all classes of secondary metabolites. Identification of these compounds will help an understanding of the seasonal constraints to livestock production associated with this important fodder shrub.

TAGASASTE (*Chamaecytisus proliferus*) is established as a profitable and sustainable addition to annual pasture systems in areas with deep sandy soils in southern Australia (Oldham 1994; Lefroy et al. 1997). Introducing this fodder shrub to the traditional annual pasture system in such areas results in a 5-fold increase in livestock carrying capacity (Oldham 1994), with cattle now generally the preferred grazing animal.

Despite much well placed optimism and enthusiasm for tagasaste, particularly in Western Australia, there remain some unresolved productivity issues with this relatively new farming system. For example, production responses are extremely seasonal, being excellent at 1.0–1.5 kg/head/day with cattle in winter and spring but disappointing (maintenance only) during summer/autumn (Edwards et al. 1997a) despite ample feed of apparently adequate quality invariably being available (Tudor et al. 1997).

Sheep grazing tagasaste over the summer/autumn period will grow slowly and produce twice as much wool as flockmates grazing dry pasture and slowly losing weight (Oldham 1994). However, this also is far below production estimates suggested by the chemical analysis of the available tagasaste.

While supplementary feeding strategies are being developed to increase cattle growth rates over the summer/autumn period, the underlying reasons for the seasonality of animal performance are poorly understood.

[1] Faculty of Agriculture (Animal Science), The University of Western Australia, Nedlands, WA 6907, Australia
Current Address: CSIRO Animal Production, Private Bag, PO Wembley, WA 6014, Australia

KEYWORDS: Tannins, Tagasaste, *Chamaecytisus proliferus*, Secondary metabolites, Phenolics, Flavones

This paper summarises a series of investigations into secondary metabolites in tagasaste. Their identification may help explain the seasonality of livestock performance on tagasaste, including a potential link between their presence and fluctuating liveweight gains observed in animals grazing this fodder shrub. This work was published in part at the XVIII International Grassland Congress (Edwards et al. 1997b).

Secondary Metabolites in Tagasaste

Tagasaste contains both phenolics and tannins, the concentrations of which change with superphosphate application and time of the year. The concentration of phenolic compounds in edible leaf and stem material of tagasaste routinely varies from 0.5% to 5% in the cool, wet winter/spring growth period and up to 10% to 12% in the hot, dry late summer/ autumn (measured by the Folin-Dennis assay with tannic acid as a standard). Values as high as 25% have been recorded in response to locust attack, while 17% phenolics has been associated with rejection of tagasaste by grazing sheep (Oldham 1994). The relationship between the seasonality of animal performance and fluctuations in the concentration of phenolic compounds in the edible fraction of tagasaste is more clearly demonstrated by Figure 1.

Intake of tagasaste by livestock appears to be a major factor explaining the animal production in the late summer/autumn period in Australia, as well as in Africa (Varvikko and Khalili 1993). A field study of intake and digestibility of tagasaste using n-alkane dosed cattle indicated that a 2-fold fluctuation occurs in intake through the year (N.J. Edwards, unpublished data). In sacco digestibility results from this study showed that digestibility of tagasaste material, while seasonally variable, remained relatively high throughout the year and should not limit animal liveweight gain (Edwards et al. 1997b).

The relationship in Figure 1 initially led to the assumption that the phenolic component comprised largely of classic protein binding tannins. Borens and Poppi (1990), who were unable to detect condensed tannins in tagasaste leaves in New Zealand, did not support this view. However, analysis of edible leaf and stem material from Western Australia by near infrared spectrophotometry (NIR) showed an anomaly in the spectra that exactly mirrored that of *Sericea lespedeza* (Oldham 1994), a species known to have low 'feeding value' caused by excessive binding of proteins by tannins (Windham et al. 1988).

More recently, low concentrations of condensed tannin (0.7% to 5.9%) were detected using a combination of the Vanillin-HCl (for protein and non-protein bound tannin – also used by Borens and Poppi 1990) and Butanol-HCl methods (for fibre-bound tannin). Tannin concentrations decrease in all

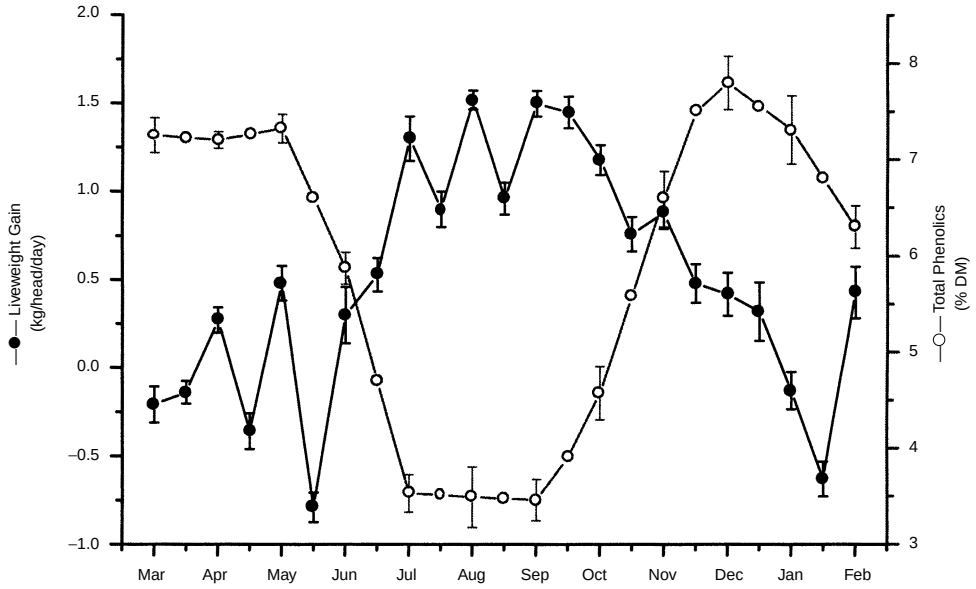


Figure 1. Seasonal fluctuations in the concentration of phenolic compounds in hand picked portions of the edible leaf and stem material of tagasaste (○) and liveweight performance of cattle grazing that material (●) in 1994–1995. Values are means +/– s.e.m.

of the fractions (Table 1) with an increased rate of superphosphate application as well as when the plant material is oven versus freeze dried (C.S. McSweeney, unpublished data).

Table 1. Condensed tannin concentrations (%) in tagasaste grown under 2 fertiliser regimes and freeze (F/D) or oven (O/D) dried (analysis by Vanillin and Butanol methods; C.S. McSweeney, unpublished data).

Annual superphosphate history	Vanillin Method		Butanol method	Total
	Non-protein bound	Protein bound	Fibre bound	
0 kg/ha-F/D	0.67	1.57	3.67	5.91
0 kg/ha-O/D	0.11	1.00	3.36	4.47
240 kg/ha-F/D	0.08	0.52	2.17	2.78
240 kg/ha-O/D	0.07	0.26	2.09	2.42

McNeill et al. (1996), in experiments to determine whether the tannin-resistant bacterium *Streptococcus caprinus* could improve the performance of ruminants grazing tagasaste, found that the presence of bacteria that hydrolyse tannic acid-protein complexes in vitro in the rumen of cattle grazing tagasaste did not improve liveweight gains. This is evidence of the possible low importance of tannins in tagasaste.

Analysis of tagasaste leaf and edible stem using HPLC methods identified the flavones, apigenin (3', 5, 7-trihydroxyflavone) and luteolin (3', 4', 5, 7-tetrahydroxyflavone), as the major components, comprising greater than 70% of the phenolic fraction (Edwards et al. 1997b). Although initially thought to occur at a relatively fixed ratio of 4–5 : 1 apigenin : luteolin (J.B. Lowry, pers. comm. 1994), analysis of more samples has indicated a marked change in the ratio through the year (Table 2).

As was the case above for the tannin fractions, the concentration of these compounds is higher in unfertilised tagasaste than tagasaste receiving annual applications of 240 kg superphosphate/ha. These two flavones occur as glycosides of undetermined nature in the plant, although W. Best (pers. comm. 1996) suggests they may be the c-glycosides, Vitexin (Apigenin 8-*c*-glucoside) and iso-Vitexin (Luteolin 8-*c*-glucoside). More work is, however required to confirm this and identify the sugars.

Flavones typically have anti-microbial, anti-oxidant and enzyme inhibition effects on biological systems (Harborne 1991). However, the effects of these compounds at their relatively high concentrations in tagasaste (up to 15% of the dry matter) are unknown.

None of the common flavonols have been detected in tagasaste, nor oestrogenic isoflavones (formononetin, genistein and biochanin A), but the isoflavone Daidzein was found (Edwards et al. 1997b). Similarly, the alkaloid sparteine has been identified in tagasaste grown in New Zealand (White 1943, 1951), as well as in a number of tagasaste subspecies grown in Spain (Muzquiz et al. 1996) where it was also reported to comprise over 80% of the total alkaloids.

Furthermore, Perez and Sagot (1898) reported the alkaloid cytosine in tagasaste. A preliminary study of Australian tagasaste did not detect cytosine (C.S. McSweeney, pers. comm., 1999) and the presence of many other classes of secondary metabolites has yet to be determined.

Effects on Rumen Fermentation

Phenolics in tagasaste do not appear to have significant effects on rumen fermentation. Studies to understand the effects of tagasaste phenolic compounds on rumen fermentation were performed using two slightly different in vitro techniques, but with very similar results. J.B. Lowry and P.M. Kennedy (unpublished report), measuring gas pressure, residual cell wall and total microbes (by purine content), found that there were no inhibitory effects on rumen fermentation with a crude (70% ethanol)

Table 2. Concentration of apigenin and luteolin (%) in tagasaste grown under 2 fertiliser regimes (C.S. McSweeney, unpublished data).

Annual superphosphate history	February		June		September	
	Apigenin	Luteolin	Apigenin	Luteolin	Apigenin	Luteolin
0 kg/ha (Pdk 1)	2.90	6.99	4.60	1.48	9.02	3.84
0 kg/ha (Pdk 2)	3.60	6.90	4.76	4.35	7.61	5.32
240 kg/ha (Pdk 1)	1.65	4.11	3.85	2.21	9.12	3.31
240 kg/ha (Pdk 2)	2.30	5.82	3.37	2.94	10.88	4.25

extract of tagasaste. In fact, all three parameters increased when spear grass or lucerne were incubated in the presence of the extract.

Similarly, Thyer (1996) concluded that the rate and quantity of gas produced by mixed rumen microbial cultures when ground oaten chaff was fermented increased significantly with a crude ethanol/water (50:50) extract of tagasaste containing phenolic compounds. This was also the case when a purified mixture of apigenin and luteolin glucosides was used. Furthermore, he demonstrated that the presence of the flavone aglycones (i.e. apigenin and luteolin – no glucoside attached) had no effect on similar cultures. Both findings are consistent with the addition of soluble, readily fermentable material to the in vitro system, which in this case contributed up to 25% of the potentially fermentable substrate.

Palatability of Tagasaste

Palatability of tagasaste can be defined as the relative preference by a grazing animal between plants. Under Australian grazing conditions, sheep and cattle consume tagasaste with no apparent ill effects, but differential grazing pressure on individual plants suggests an array of palatabilities, probably due to genetic and environmental variation.

Mailey (1994) observed that there was no change in preference or potential intake rate (PIR) for fresh tagasaste as phenolic concentration decreased from 6 to 4%. However, preference increased from 18% to 40%[1] as phenolic concentration decreased from 4% to 2.75% within wilted (overnight at 60 °C) treatments, but PIR did not change. Furthermore, both preference (30% v 11%; $P<0.05$) and PIR (50 g DM/min v 31 g DM/min; $P<0.05$) were greater for wilted than fresh tagasaste.

While these results partly support the hypothesis that palatability of tagasaste increases as phenolic content decreases, the situation is unclear, since both preference and PIR were expected to increase as phenolic concentration decreased. The inconsistency of these results may be due to the relatively low phenolic concentrations in the test materials or the fact that phenolic concentration was not the only factor that differed between the test materials. For example, crude protein was 19.0% (DM basis) in low phenolic tagasaste and 13.9% in high phenolic tagasaste, dry matter digestibility was 77.8% and 75.7%, respectively and percent of leaf in the sample was 80 and 74%.

Conclusions

While tagasaste contains significant amounts of phenolic compounds, in particular the flavones apigenin and luteolin, they do not have any detrimental effects on rumen fermentation. Nevertheless, the mechanism(s) of action of phenolic compounds in general, and apigenin and luteolin in particular, in the chemical defence of tagasaste from grazing is unclear. This broad group of secondary metabolites is, however, well known for their effects on palatability, intake, diet selection and digestibility as chemical means of defense for plants (Harborne 1991; Provenza 1996). While no cause and effect relationship has been established for these compounds in tagasaste, indications are that the seasonal peak in their concentration corresponding to a trough in cattle liveweight gain is more than coincidental. Current evidence suggests that seasonal productivity of animals grazing tagasaste is largely due to changes in feed intake through the year, mediated by the concentration of phenolic compounds in its edible leaf and stem material. There is an urgent need to identify and to quantify the full range of secondary metabolites present in tagasaste under a variety of environmental and management conditions and to determine their effects on voluntary food intake.

Acknowledgment

The author thanks The University of Western Australia, the Meat Research Corporation and Martindale Pty Ltd for funding this research. Thanks also to Greg Allen for his tireless help and Wayne Best, Brian Lowry, Juliet Mailey, Chris McSweeney and Matt Thyer for the use of their data.

References

Borens, F.M.P. and Poppi, D.P. 1990. The nutritive value for ruminants of tagasaste (*Chamaecytisus palmensis*), a leguminous tree. Animal Feed Science and Technology, 28: 275–292.

Edwards, N.J., Allen, G.M., McNeill, D.M. and Oldham, C.M. 1997a. Grazing management of tagasaste (*Chamaecytisus proliferus*) for sheep and cattle production in southern Australia. In: Proceedings of the XVIII International Grassland Congress, June 8–19, 1997, Winnipeg and Saskatoon, Canada.

Edwards, N.J., Mailey, J.C., McNeill, D.M., Lowry, J.B., McSweeney, C.S., Henry, D. and Oldham, C.M. 1997b. The effect on intake, palatability and digestibility of phenolic compounds in tagasaste (*Chamaecytisus proliferus*). In: Proceedings of the XVIII International Grassland Congress, June 8–19, 1997, Winnipeg and Saskatoon, Canada.

[1] Expressed as the percentage of the total feed eaten which is test material when test and standard material are offered simultaneously for 30 seconds twice daily.

Harborne, J.B. 1991. The chemical basis of plant defence. In: Palo, R.T. and Robbins, C.T. ed. Plant Defences Against Mammalian Herbivory. Boca Raton, Florida, CRC Press, 45–59.

Lefroy, E.C., Abadi Ghadim, A.K., Edwards, N.J. and Ewing, M.A. 1997. The role of tagasaste (*Chamaecytisus proliferus*) in farming systems of southern Australia. In: Proceedings of the XVIII International Grassland Congress, June 8–19, 1997, Winnipeg and Saskatoon, Canada.

Mailey, J.C. 1994. Does phenolic content of tagasaste affect its palatability? Honours Thesis, Faculty of Agriculture, University of Western Australia, Nedlands, W.A., Australia.

McNeill, D.M., Allen, G.M., Brooker, J.D. and Oldham, C.M. 1996. Presence of ruminal bacteria that hydrolyse tannic acid-protein complexes does not improve the liveweight gain of cattle grazing tagasaste. Animal Production in Australia, 21: 412.

Muzquiz, M., Robredo, L.M., Burbano, C., Cuadrado, C., Ayet, G. and Mendez, P. 1996. Variation in the alkaloid content of different subspecies of *Chamaecytisus proliferus* from the Canary Islands. Journal of Chromatography A, 719: 237–243.

Oldham, C.M. 1994. Tagasaste (*Chamaecytisus palmensis*) – A fodder shrub and alternative forage. In: Michalk, D.L., Craig, A.D. and Collins, W.J. ed. Alternative Pasture Legumes 1993, Primary Industries South Australia, Technical Report 219, 118–138.

Perez, V. and Sagot, P. 1898. Le Tagasaste – *Cytisus proliferus varietas* – fourrage important. In: Perez, V. ed. Imprimerie de la Semaine Medicale.

Provenza, F.D. 1996. Familiarity and novelty in animal diets: Implications for management. Animal Production in Australia, 21: 12–16.

Thyer, M.Q. 1996. Do low molecular weight phenolics reduce the liveweight gain of cattle grazing tagasaste (*Chamaecytisus palmensis*) during summer and autumn? Honours Thesis, Faculty of Agriculture, University of Western Australia, Nedlands, W.A., Australia.

Tudor, G.T., Costa, N., Standing, W. and Leadbetter, E. 1997. Supplementary feeding of cattle and sheep grazing tagasaste. In: Lefroy, E.C., Oldham, C.M. and Costa, N.J. ed. 'Tagasaste' *Chamaecytisus proliferus*. The University of Western Australia, CLIMA Occasional Publication 19.

Varvikko, T. and Khalili, H. 1993. Wilted tagasaste (*Chamaecytisus palmensis*) forage as a replacement for a concentrate supplement for lactating crossbred Friesian × Zebu (Boran) dairy cows fed low-quality native hay. Animal Feed Science and Technology, 40: 239–250.

White, E.P. 1943. Alkaloids of the leguminosae. Part III – Alkaloids of 'tree lucerne,' *Cytisus proliferus* L. New Zealand Journal of Science and Technology, 25: 103–105.

White, E.P. 1951. Alkaloids of the leguminosae. Part XIX – Identification of *d*- and *l*-sparteine in *Cytisus proliferus* L. New Zealand Journal of Science and Technology, 33: 44–47.

Windham, W.R., Fales, S.L. and Hoveland, C.S. 1988. Analysis for tannin concentration in *Sericea lespedeza* by Near Infrared Reflectance Spectroscopy. Crop Science, 28: 705–708.

HPLC Profiles of Phenolic Compounds in the Accessions of Calliandra (*Calliandra calothyrsus*)

S.I.W. Rakhmani[1], J.D. Brooker[1] and G.P. Jones[1]

Abstract

The phenolic profiles of 7 accessions of calliandra (*Calliandra calothyrsus*) have been examined using high performance liquid chromatography (HPLC). The phenolic compounds were fractionated through SepPak C_{18} cartridges yielding 4 fractions: phenolic acids (Fraction 1), monomers (Fraction 2), flavonols/flavonol glycosides (Fraction 3) and polymers/condensed tannin (Fraction 4). A gradient system of water, 10% acetic acid-methanol, was used to examine the phenolic fractions by HPLC using a Spherisorb S5 ODS-2 column (25 cm × 4.6 mm). Detection at 280 nm (most of the phenolics), 320 nm (for hydroxycinnamic derivatives) and 350 nm for flavonols/flavonol-glycosides were carried out. Most of the accessions showed a similar pattern of phenolic acids except for accession No. 45/92 that had a high content of gallic acid (RT 9.8 min). The hydroxycinnamic derivatives were very low in all seven accessions. Catechin (RT 13.7 min) is distributed in all accessions. However, accession Nos. 8/91 and 9/91 are high in epicatechin (RT 14.5 min) and accession No. 53/92 contains both catechin and epicatechin. Flavonols and polymeric species were detected in all seven accessions in fractions 3 and 4 respectively.

THE TROPICAL shrub legume *Calliandra calothyrsus* (calliandra) is one of many legumes that can be used as forage in tropical and sub-tropic regions (Palmer et al. 1994; Shelton et al. 1996) as a potential nitrogen source for ruminants. It became popular among small farmers in Indonesia for feeding their animals (especially goats) as fresh cuttings. Calliandra contains high levels of condensed tannin (5%–15% dry matter, DM), a secondary metabolite that can be a limiting factor for animals to utilise calliandra protein. Tannins are naturally occurring, water soluble polyphenolic compounds that have a molecular weight of between 500 and 3000 and demonstrate phenolic reactions such as the ability to precipitate alkaloids, gelatine and other proteins (Swain and Bate-Smith 1962). Tannins can also bind with other macromolecules such as simple and complex carbohydrates, can act as a ligand that binds with minerals (Haslam 1993).

Tannins are classified into two broad groups: hydrolysable tannins (HT) and condensed tannins (CT). HTs are esters of the phenolic acids (e.g. gallic or ellagic acids) with glucose as a nucleus. This type of tannin can be easily hydrolysed by enzymes, acids or alkalis. The second group, CTs, are polymers of flavanols (flavan-3-ols, catechin and epicatechin) and/or flavan-3,4-diols (leucoanthocyanidin) which are bound together to form a polymeric chain through the C4-C8 or C4-C6 inter-flavanoid bond. In contrast to HTs, CTs do not have a polyol nucleus and cannot be hydrolysed by acids, enzymes or alkalis but acid can break down the interflavanoid bond to form anthocyanins. The formation of anthocyanins from acid degradation is used as a basic reaction to quantify CTs using acid-butanol assay (Porter et al. 1986). CTs are also known as condensed proanthocyanidins (Haslam 1989).

Several methods had been developed to quantify tannins such as Folin-Denis/Ciocalteu (Singleton and Rossi 1965; Waterman and Mole 1994) that determine all phenolic compounds, acid-vanillin (Price et al. 1978; Broadhurst and Jones 1978) or acid-butanol

[1] Department of Animal Science, The University of Adelaide, Waite Institute, Glen Osmond SA 5064

KEYWORDS: Condensed Tannins, HPLC, Digestibility, Flavonols, Proanthocyanidins

(Porter et al. 1986; Waterman and Mole 1994) assay that are specific for condensed tannins. Protein binding methods that represent the biological activity of tannins had been developed (Hagerman and Butler 1978, 1980, 1981; Martin and Martin 1982; Hagerman et al. 1998). Recently, HPLC has also been used for analysing CTs using either an isocratic or gradient systems (Mueller-Harvey et al. 1987; Bartolome et al. 1993). Heering et al. (1996) reported that the HPLC profile of phenolic compounds in *Sesbania* sp. were used to differentiate and for grouping among *Sesbania* accessions. However, the profiles of phenolic compounds among calliandra accessions have not been examined. The phenolic profile of seven calliandra accessions have been fractionated through SepPak C 18 cartridges and the fractions examined using HPLC.

Materials and Methods

Materials

Calliandra accessions 15690 (1-Cisarua Indonesia), 18/91 (8-Fortuna Costa Rica), 45/92 (13-San Antonio-Belize), 48/92 (14-Georgesville Belize), 53/92 (17-Santa Maria de Jesus Guatemala), 8/91 (18-Coban Guatemala), 9/91 (19-Patulul Guatemala) were supplied by Dr Brian Palmer (CSIRO, Townsville). SepPak plus C18 cartridges (1 gram) from Waters were used to fractionate tannin compounds. Catechin (C1251 SIGMA), epicatechin (E1753 SIGMA) and gallic acid (G7384 SIGMA) were used as external HPLC standards. Extraction solvents (methanol, acetone, acetonitrile, ethyl acetate and diethyl ether) and other chemicals such as sodium hydroxide, ascorbic acid, butanol and hydrochloric acid were pro-analysis grade. Methanol was HPLC grade.

Fractionation of phenolic compounds

Fractionation of phenolic compounds was done by a modification of Oszmianski et al. (1988). Briefly, ground leaf (ca. 500 mg) was extracted with 70% aqueous-acetone containing 0.1% ascorbic acid. Acetone was removed by vacuum-rotary evaporation below 40 °C and the aqueous-residue was washed with diethylether. The neutral aqueous fraction was loaded on a pre-conditioning neutral SepPak cartridge. The cartridge was flushed with water and 0.01 M HCl, the eluate was collected and passed through into another cartridge that was pre-conditioned with 0.01 M HCl. The eluate from acidic SepPak that contained phenolic acids was collected (Fraction 1). From the neutral SepPak, 3 fractions were collected (see diagram). Each Fraction (0.5–1 mL) was dried and redissolved in 250 mL with 70% methanol and readied for HPLC analysis). The total polymer in Fraction 4 was determined using acid butanol assay as described by Waterman and Mole (1994).

HPLC analysis

HPLC from Hewlett Packard HP1100 with a quartenary pump and multi-wavelength diode array detector was used to analyse the phenolic fractions. HPChem Station for LC systems is the system controller for retention time, peak width, peak area, peak height and % peak area measurements. Detection was performed at 280 nm (for most phenolics), 320 (for hydroxycinnamic derivatives), and 350 (flavonol-glycosides). Analytical column of Spherisorb S5 ODS2 from Activon Gold Pack was used to analyse all fractions and the gradient system consists of water (A)-methanol (B)-10% acetic acid (D) was applied as follow:

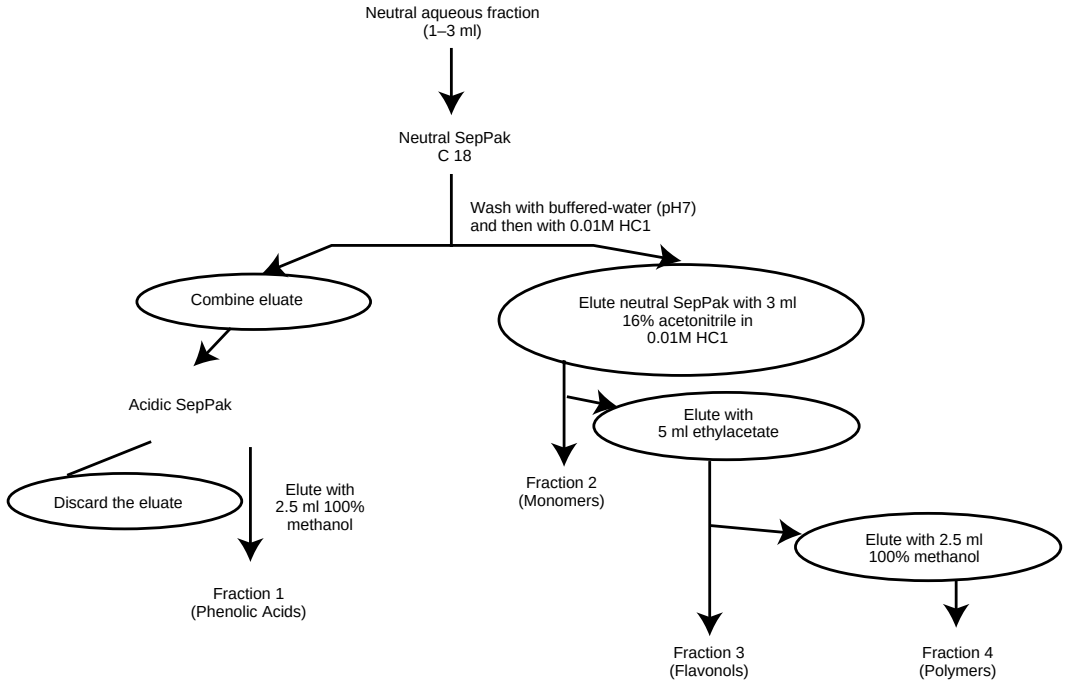
Time (minute):	0	10	20	30	35	40	45
%A	75	0	5	5	30	30	75
%B	0	50	70	70	70	70	0
%D	25	50	25	25	0	0	25

Results and Discussion

HPLC has been used to characterise phenolic compounds either with or without SepPak C18 pre-separation. These SepPak cartridges were successfully used to separate phenolic compounds in grape and grapeseed (Ozmianski et al. 1988) yielding 4 fractions: Fraction 1 (phenolic acids), Fraction 2 (monomers, flavanols, dimers), Fraction 3 (flavonols, flavonol glycosides) and Fraction 4 (polymers). HPLC analysis using a gradient system for each fraction and detection with a multi-wavelength diode array detector may be used to study different phenolic compounds of calliandra at the same time.

Most of the calliandra accessions showed similar patterns of phenolic acids (F1) at 280 nm (Figure 1A), except for accession 45/92 (Figure 1B) which has a high content of gallic acid (RT 9.85 min). The hydroxycinnamic derivatives were detected at 320 nm (Guyot 1998) and were very low in all seven calliandra accessions.

Catechin (RT 13.7 min) and epicatechin (RT 14.5 min) are monomers that can be detected in Fraction 2 of calliandra phenolics. Accessions 15690 (Figure 2A), 18/91, 45/92 and 48/92 contain catechin. However, accession 8/91 and 9/91 (Figure 2C and 2D) contain epicatechin. Both catechin and epicatechin were detected in accession 53/92 (Figure 2B). Catechin and epicatechin were not detected at 320 nm or 350 nm. Calliandra, therefore, contains



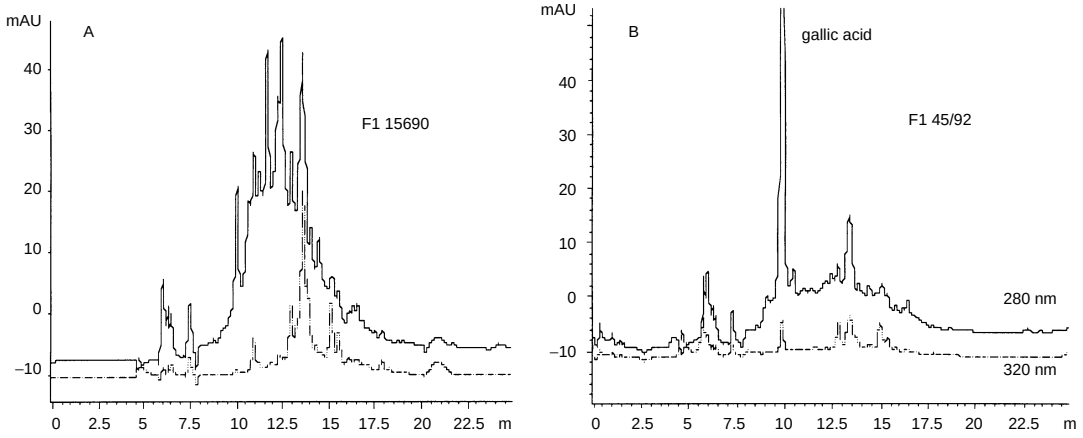


Figure 1. Phenolic acids in calliandra accessions. (A) accession No. 15690 (1-Cisarua Indonesia) at 280 nm (high absorption) and 320 nm (low absorption) and (B) accession No. 45/92 that has a high content of gallic acid.

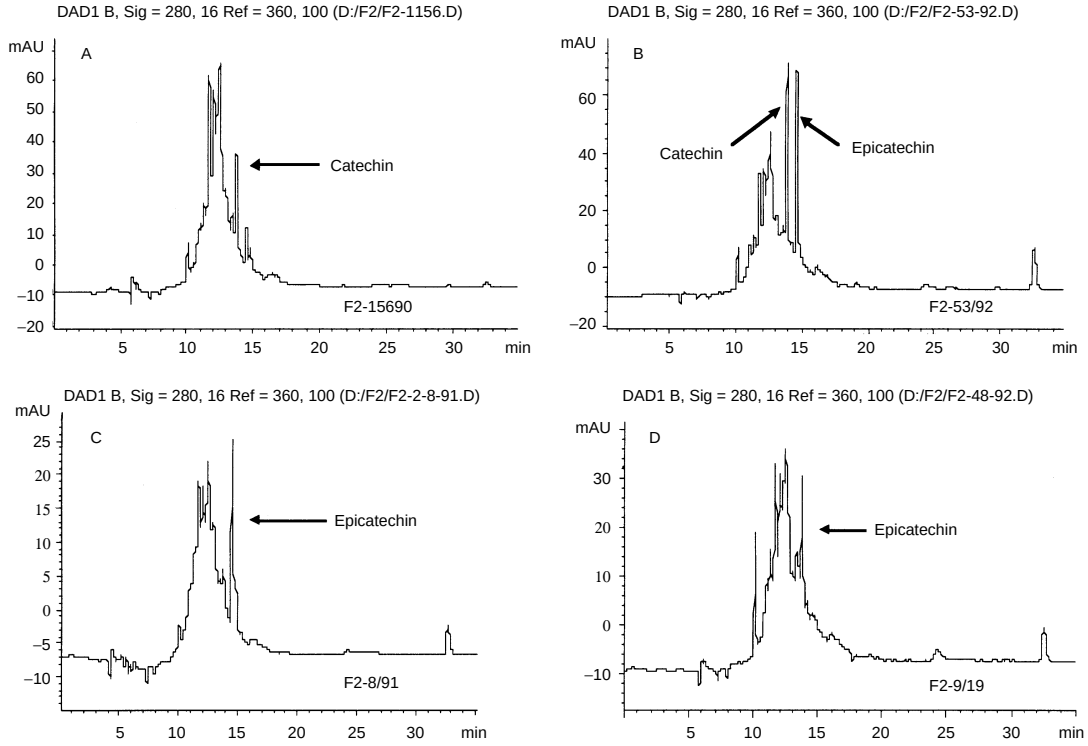


Figure 2. Monomer fraction (F2) of calliandra phenolic.

free catechin and/or epicatechin that can be separated from other phenolic fractions using SepPak C18 cartridges.

Fraction 3 of calliandra phenolics at 280 nm (Figure 3A) showed a wide peak between 10 and 15 minutes. All seven calliandra accessions showed a similar pattern for Fraction 3 at 280 nm. However, at 350 nm (Figure 3B), there were eight peaks that represented flavonol glycosides with the exception of 48/92 which only showed four peaks when compared with 15690 (data not shown). The wide peak that is shown at 280 nm was not detected at 350 nm. The 350 nm wavelength is specific for flavonols as described in a previous finding (Guyot 1998). In a previous report (Mueller-Harvey et al. 1987), this wide peak was also shown for phenolic compounds of Ethiopian browse at the same retention time, followed by several small peaks of lower molecular weight phenolics. Pre-separation of phenolic extracts using SepPak C 18 cartridges allowed separation of this wide peak as Fraction 4 (polymer).

Figure 3C shows Fraction 4 of calliandra phenolics (Figure 3C) with a clear wide peak at 280 nm between 10 and 15 minutes. No signals were found at 320 and 350 nm, suggesting that Fraction 4 is a polymer of procyanidins/condensed tannin.

Figure 4 shows a comparison of procyanidin content using 3 methods (protein precipitation method, acid butanol and HPLC). Accession 15690 had the highest procyanidin content assayed by the acid butanol method. A previous study (ACIAR report, unpublished) also showed that accession 15690 (1-Cisarua) had the highest content of condensed tannin but also had a high in vitro digestibility (Figure 4). In this Figure, it is clearly shown that the result from the acid-butanol assay is not well correlated with the digestibility value of calliandra accessions. Some that have a lower level of condensed tannin than accession 15690 have a lower digestibility value. The HPLC fractionation result showed a closer relationship between condensed tannin content and digestibility. Accession 15690 had a lower peak area value ([a] low level of condensed tannin) than accessions 18/91, 48/92, 45/92, 53/92, correlating with a high digestibility value. These two methods (acid-butanol and HPLC) were then applied to the Fraction 4

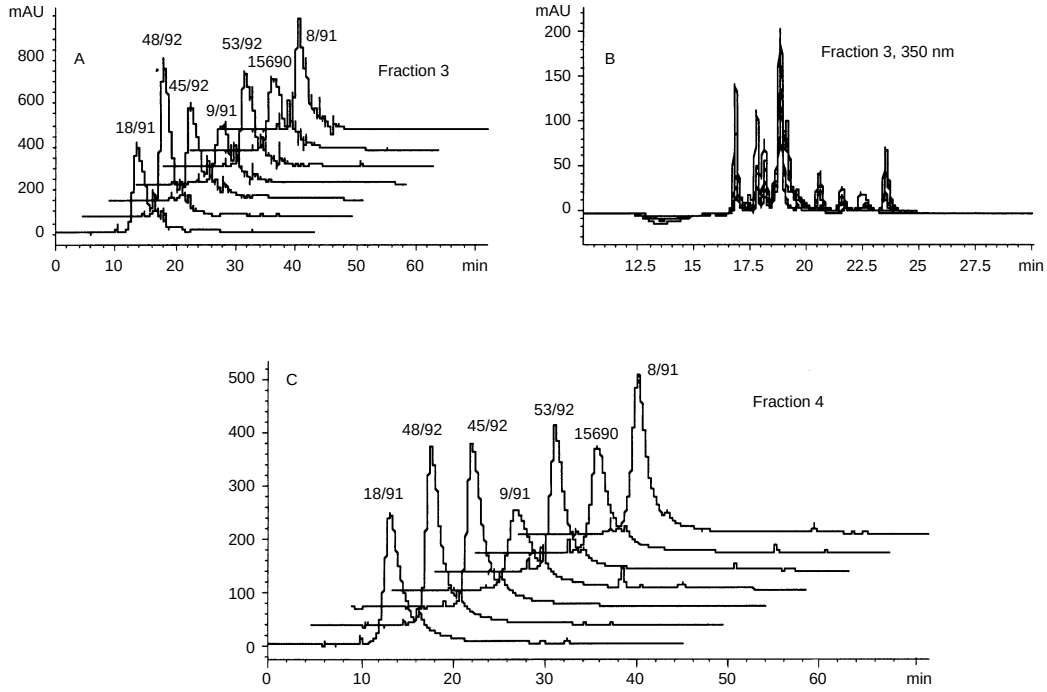


Figure 3. (A) Fraction 3; 3-dimensional overlay of seven calliandra accessions at 280 nm; (B) Fraction 3 of all seven calliandra accessions at 350 nm; and (C) Fraction 4, 3-dimensional overlay of seven calliandra accessions.

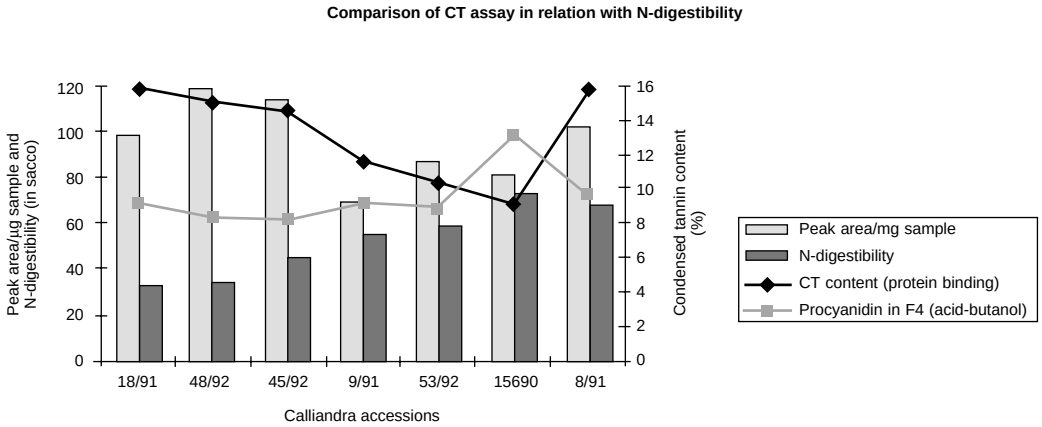


Figure 4. Total procyanidin content in calliandra leaf (protein precipitation method) and in Fraction 4 (acid-butanol and HPLC).

collected from pre-separation with SepPak. The HPLC result was more reliable and more closely correlated with the in vitro digestibility value than the acid-butanol analysis.

These results show that the acid-butanol analysis is not suitable for correlating condensed tannin content in raw extracts of leaf material with in vitro digestibility, without further separation/purification (Waterman and Mole 1994).

Conclusions

The phenolic acids profile in seven calliandra accessions are similar with the exception of accession 45/92 that has a high level of gallic acid. The hydroxycinnamic derivatives were very low in all seven accessions. Catechin was distributed in all accessions. However, accessions 8/91 and 9/91 were high in epicatechin and accession 53/92 contained both catechin and epicatechin. Flavonols were detected in all seven accessions and the polymers were clearly shown in Fraction 4 at 280 nm without any peak of acids, monomers or flavonols. Measurement of condensed tannins using HPLC after preseparation with SepPak gives a result more closely correlated with in vitro digestibility than the acid-butanol analysis.

Acknowledgments

This project was funded by ACIAR under the Project PN 9318.

References

Bartolome, B., Bengoechea, M.L., Galvez, M.C., Perez-Ilzarbe, F.J., Hernandez, T., Estrela, I. and Gomez-Cordovez, R. 1993. Photodiodearray detection for elucidation of the structure of phenolic compounds. Journal of Chromatography, 655: 119–125.

Broadhurst, R.B. and Jones, W.T. 1978. Analysis of condensed tannins using acidified vanillin. Journal of the Science of Food Agriculture, 29: 788–794.

Guyot, S. 1998. Reversed-phase HPLC following thiolysis for quatitative estimation and characterisation of the four main classes of phenolic compounds in different tissue zones of a french cider apple variety (*Malus domestica* var. Kermerrien). Journal of Agricultural and Food Chemistry, 46: 1698–1705.

Hagerman, A.E. and Butler, L.G. 1980. Determination of protein in tannin-protein. Journal of Agricultural and Food Chemistry, 28: 944–947.

Hagerman, A.E. and Butler, L.G. 1978. Protein precipitation method for the quantitative determination of tannin. Journal of Agricultural and Food Chemistry, 26: 809–812.

Hagerman, A.E. and Butler, L.G. 1981. Specificity of proanthocyanidin-protein interaction. Journal of Biological Chemistry, 256: 4494–4497.

Hagerman, A.E., Rice, M.E. and Ritchard, N.T. 1998. Mechanisms of protein precipitation of two tannins, pentagalloyl glucose and Epicatechin$_{16}$ (4→8)-catechin (procyanidin). Journal of Agricultural and Food Chemistry, 46: 2590–2595.

Haslam, E. 1989. Plant polyphenol, vegetable tannins. Revisited. Cambridge University Press, Cambridge, UK.

Haslam, E. 1993. Polyphenol complexation. In: Scalbert, A. ed. Polyphenolic phenomena. INRA edition, Paris, 23 p.

Heering, H., Reed, J.D. and Hanson, J. 1996. Differences in *Sesbania sesban* accessions in relation to their phenolic concentration and high performance fingerprints. The Journal of the Science of Food and Agriculture, 71: 92–98.

Martin, J.S. and Martin, M.M. 1983. Tannin assays in ecological studies: Precipitation of ribulose-1,5-bisphosphate carboxylase/oxygenase by tannic acid in quebracho and oak foliage extracts. Journal of Chemical Ecology, 11: 1323–1332.

Mueller-Harvey, I., Reed, J.D. and Hartley, R.D. 1987. Characterisation of phenolic compounds, including flavonoids and tannins, of ten Ethiopian browse. Journal of the Science of Food and Agriculture, 39: 1–14.

Oszmainski, J., Rammos, T. and Bourzeik, M. 1988. Fractionation of phenolic compounds in red wine. American Journal of Enology and Viticulture, 39: 259–262.

Palmer, B., McQueen, D.J. and Gutteridge, R.C. 1994. *Calliandra calothyrsus* a multipurpose tree legume for humid locations. In: Gutteridge, R.C. and Shelton, H.M. ed. Forage Tree Legumes in Tropical Agriculture. CAB International. Wallingford, UK, 65–74.

Porter, L.J., Hrstich, L.N. and Chan, B.C. 1986. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. Phytochemistry, 25: 223–230.

Price, M.L., VanScoyoc, S. and Butler, L.G. 1978. A critical evaluation of the vanillin reaction as an assay for tannin in sorghum grain. Journal of Agricultural and Food Chemistry, 26: 1214–1218.

Shelton, H.M., Norton, B.W., Mullen, B.F., Gutteridge, R.C. and Dart, P.J. 1996. Utilisation and nutritive value of *Calliandra calothyrsus* for forage. Proc. of Intl. Workshop on the Genus Calliandra. Bogor, Indonesia, 210–221. Morrilton Arkansas, Winrock International.

Singleton, V.L. and Rossi, J.A. 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. American Journal of Enology and Viticulture, 16: 144–158.

Swain, T. and Bate-Smith, E.C. 1962. Flavonoid compounds. In: Florkin, A.M. and Mason, H.S. ed. Comparative Biochemistry, Vol. III. Academic Press, NY, 764 p.

Waterman, P.G. and Mole, S. 1994. Analysis of phenolic plant metabolites. Oxford-Blackwell Publishers, London.